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# Industria y Comercio

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División de Nuevas Creaciones

PCT

SOLICITUD

# PATENTE DE INVENCIÓN

21. **EXPEDIENTE No.** 05-79319

54. **TÍTULO** Formulaciones Parenterales de un péptido para el tratamiento de lupus sistemático eritematoso

51. **CLASIFICACIÓN INTERNACIONAL** A61K 39/395; A61P 37/00

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**DOMICILIO** Petatch. Tikva. Israel

74. **APODERADO** Dilia María Rodríguez D'Aleman

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# FORMULARIO ÚNICO DE SOLICITUD DE PATENTE 14-08-05

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**1) SOLICITUD DE:**

☒ Patente de invención ☐ Patent

☒ Capitulo I. ☐ Capitulo II.

| | | |
|---|---|---|
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**6)Título (54) FORMULACIONES PARENTERALES DE UN PÉPTIDO PARA EL TRATAMIENTO DE LUPUS SISTEMÁTICO ERITEMATOSO**

**7) Clasificación Internacional: A 61 K** 39/395 // A61P 37/00

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Instrucciones para el diligenciamiento del presente formulario. Ver reverso de esta pagina

**10) ANEXOS**

- [x] Comprobante de pago de la tasa de presentación de la solicitud
- [ ] Comprobante de pago por reivindicación de prioridad
- [ ] Documento que acredita la existencia y representación legal cuando el solicitante sea persona Jurídica
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- [x] Otros
  1. Reporte internacional de búsqueda
  2. Opinión escrita de la búsqueda internacional
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  4. Transmisión del reporte de búsqueda
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  8. Patente 5 646 131
  9. Patente US2004/0127408 A1
  10. Patente US2004/0127408 A1
  11. Patente US5 646 131
  12. Patente US4 656 158

**11) FIGURA CARACTERÍSTICA**

**12) Solicitud concesión de la patente,**

**NOMBRE** DILIA RODRÍGUEZ D ALEMAN

**FIRMA**

C C 41 654 882 de Bogota TP 20824 CSJ

**Instrucciones para el diligenciamiento del presente formulario Ver reverso de esta pagina**

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(54) Title: PARENTERAL FORMULATIONS OF A PEPTIDE FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

(57) Abstract: The subject invention provides a pharmaceutical composition comprising an aqueous carrier, from 0.1 mg/ml to 20 mg/ml of the composition of a pharmaceutically acceptable salt of a peptide having the structural formula $NH_2$-Gly Tyr Tyr Tip Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooH; and a substituted 13-cyclodextrin in an amount effective to dissolve the peptide in the aqueous carrier, wherein the composition has a pH between 4 and 9, a process for preparation, and a method of alleviating symptoms of systemic lupus erythematosus (SLE) in a human subject comprising administering to the human subject the pharmaceutical composition.

WO 2004/064788 A2

# PARENTERAL FORMULATIONS OF A PEPTIDE FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

This application claims the benefit of U.S. Provisional Application No. 60/439,950, filed January 14, 2003, the entire contents of which are hereby incorporated by reference.

Throughout this application, various publications are referenced by full citations. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

## Background of the Invention

Systemic lupus erythematosus (SLE), or lupus, is a debilitating autoimmune disease characterized by the presence of an array of autoantibodies, including antibodies to dsDNA, to nuclear antigens and to ribonucleoproteins. SLE affects approximately 1 in 2000 individuals (U.S. 1 in 700 women). The disease primarily affects young women, with a female-to male ratio of approximately 9:1.

Systemic lupus can affect almost any organ or system of the body. Systemic lupus may include periods in which few, if any, symptoms are evident ("remission") and other times when the disease becomes more active ("flare"). Most often when people mention "lupus," they are referring to the systemic form of the disease.

Corticosteroids are the mainstay in treating systemic autoimmune disorders. Life threatening, severely disabling manifestations of SLE are treated with high doses of

glucocorticoids (1-2 mg/kg/day). Undesirable effects of chronic glucocorticoids include an array of prominent adverse effects such as cushingoid habitus, central obesity, hypertension, infection, capillary fragility, hirsutism, accelerated osteoporosis, cataracts, diabetes mellitus, myopathy and psychosis. In addition to corticosteroid toxicity, patient compliance to a dosage regimen also poses a serious problem.

Cytotoxic agents are also used for controlling active disease, reducing the rate of disease flares, and reducing steroid requirements. Undesirable side effects of the latter include bone marrow depression, increased infections with opportunistic organisms, irreversible ovarian failure, alopecia and increased risk of malignancy.

SLE is an inflammatory disease for which to date there is no definitive treatment or cure. The disease results in acute and chronic complications. The only treatments available are palliative, aimed at relieving acute symptoms and preventing chronic complications, often with profound side effects. There is therefore an unmet need in this field, and both physicians and patients would welcome new treatments which could potentially eliminate or reduce the unwanted manifestations of the disease.

Peptides based on the complementarity-determining region of the human monoclonal anti-DNA 16/6Id antibody capable of immunomodulating SLE associated responses have been disclosed in PCT International Publication No. WO 02/067848 A2, the entire contents of which are hereby incorporated by reference. In particular, region CDR1 was found to inhibit the proliferative response of peripheral blood lymphocytes (PBL) of SLE patients to the human anti-DNA 16/6Id mAB, and to

ameliorate disease manifestations of mice afflicted with spontaneous or experimental SLE.

Human CDR1, Compound 1, shown in Figure 1, is a synthetic peptide of 19 amino acids based on the complementarity-determining region 1 (CDR1) of the human anti-dsDNA mAb denoted 16/6 Id (Waisman, A., et al. "Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of pathogenic anti-DNA monoclonal antibodies." *Proc. Natl. Acad. Sci. U.S.A.* (1997), 94(4): 4620-4625).

In experimental SLE models - Balb/c mice and SLE-prone mice, i.e. (NZBxNZW)F1 mice - treatment with either mCDR based-peptides or Compound 1 significantly reduced the SLE related findings, notably immune complex deposits (ICD) in the kidney, proteinuria and leukopenia. The treatment had no effect on the 16/6 Id specific antibody response (Waisman, A., et al. "Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of pathogenic anti -DNA monoclonal antibodies." *Proc. Natl. Acad. Sci. U.S.A.* (1997), 94(4): 620; Eilat, E., et al., "Prevention of systemic lupus erythematosus-like disease in (NZBxNZW)F1 mice by treating with CDR1- and CDR3- based peptides of pathogenic autoantibody" *J. Clin. Immunol.* (2000), 20: 268; Eilat, E., et al., "The mechanism by which a peptide based on complementarity determining region-1 of pathogenic anti-DNA antibody ameliorates experimental SLE" (2001), *Proc.Natl.Acad. Sci. U.S.A.* 98: 1148).

These peptides, like many peptides, are not very soluble. Therefore, formulations that improve the solubility of the peptides are desired.

**Summary of Invention**

The subject invention provides a pharmaceutical composition comprising

an aqueous carrier;

from 0.1 mg/ml to 20 mg/ml of the composition of a pharmaceutically acceptable salt of a peptide having the structural formula

$NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1); and

a substituted β-cyclodextrin in an amount effective to dissolve the peptide in the aqueous carrier,

wherein the composition has a pH between 4 and 9.

The subject invention also provides a pharmaceutical composition comprising

an aqueous carrier;

from 0.1 mg/ml to 20 mg/ml of the composition of an acetate salt of a peptide having the structural formula

$NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1);

and

from 70 mg/ml to 170 mg/ml of the composition of hepta-(sulfobutyl ether)-β-cyclodextrin,

wherein the peptide and the hepta-(sulfobutyl ether)-β-cyclodextrin are dissolved in the aqueous carrier; and

wherein the solution has a pH between 6.5 and 8.5.

The subject invention also provides a method of alleviating symptoms of systemic lupus erythematosus (SLE) in a human subject comprising administering to the human subject any of the above pharmaceutical compositions in an amount effective to alleviate the symptoms of SLE in the human subject.

The subject invention also provides a process for manufacturing the above pharmaceutical composition comprising the steps of:

a) preparing a solution of a substituted β-cyclodextrin in an aqueous carrier at a predetermined concentration;
b) adding predetermined amount of a pharmaceutically acceptable salt of the peptide $NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1) to the solution of step a);
c) adjusting the pH of the solution of step b) until the peptide dissolves in the solution; and
d) if necessary, adjusting the pH of the solution of step c) to a pH of 4-9, thereby manufacturing the pharmaceutical composition.

The subject invention also provides a process of lyophilizing the above pharmaceutical composition, comprising the steps of:

a) lowering the temperature of the pharmaceutical composition to -40°C;
b) holding the temperature at -40°C for a predetermined time;
c) raising the temperature of the solution to 20°C;
d) holding the temperature at 20°C for a predetermined time; and
e) reducing the pressure to 10μbar, thereby lyophilizing the pharmaceutical composition.

The subject invention also provides a process of lyophilizing the above pharmaceutical composition, comprising the steps of:

a) lowering the temperature of the pharmaceutical composition to -45°C;
b) holding the temperature at -45°C for a predetermined time;
c) raising the temperature of the solution to -20°C;

d) raising the temperature of the solution to 25°C; and

e) holding the temperature at 25°C for a predetermined time, thereby lyophilizing the pharmaceutical composition.

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**Brief Description of Figures**

**Figure 1.** Human CDR1 (Compound 1) as acetate salt - showing the molecular and structural formulas of hCDR1, the amino acid sequence, and physical parameters

**Figure 2.** IL-2 Secretion from cells taken from mice treated with Compound 1 and Captisol® solution after the cells were subsequently activated with a solution of Compound 1 in PBS.

-■- Compound 1 (RS) 50µg/mouse
-▲- Compound 1 (RS) 200µg/mouse
-□- DP 50µg/mouse
-Δ- DP 200µg/mouse
-o- 12% Captisol® ampulized

**Figure 3.** IFN-γ Secretion from cells taken from mice treated with Compound 1 solution after the cells were subsequently activated with a solution of compound 1 in EM-1 ($2.5 \times 10^6$ cells/well).

-♦- Placebo
-●- Compound 1 50 µg/mouse (treatment dose)
-Δ- Compound 1 100 µg/mouse (treatment dose)
-X- Compound 1 200 µg/mouse (treatment dose)

**Figure 4.** IFN-γ Secretion from cells taken from mice treated with Compound 1 solution after the cells were subsequently activated with a solution of compound 1 in EM-1 ($5 \times 10^6$ cells/well).

-◊- Placebo
-□- Compound 1 25 µg/mouse
-Δ- Compound 1 50 µg/mouse
-X- Compound 1 100 µg/mouse
-*- Compound 1 200 µg/mouse

**Figure 5.** Anti-dsDNA antibodies in (NZBxNZW)F1 mice after 10 injections with Compound 1 in Captisol® [OD=Optical Density; Compound 1 (C)= Compound 1 dissolved in Captisol®]

-□- Placebo

-◊- Compound 1 50 µg/mouse

-o- Compound 1 25 µg/mouse

**Figure 6.** Kidney sections from (NZBxNZW)F1 mice showing intensity of Immune Complex Deposits. The top row sections are from a Captisol®-treated mouse, the mid-row sections are from a mouse treated with 50 µg/mouse Compound 1 and the bottom row sections are from a mouse treated with 25 µg/mouse Compound 1. *Magnification*: Left: x100, Right: x400. FITC immunohistology.

**Detailed description**

The subject invention provides a pharmaceutical composition comprising

an aqueous carrier;

from 0.1 mg/ml to 20 mg/ml of the composition of a pharmaceutically acceptable salt of a peptide having the structural formula

$NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1); and

a substituted β-cyclodextrin in an amount effective to dissolve the peptide in the aqueous carrier,

wherein the composition has a pH between 4 and 9.

In one embodiment, the concentration of the acetate salt of the peptide is at least 0.5 mg/ml.

In one embodiment, the concentration of the salt of the peptide is from 0.5 mg/ml to 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 0.5 mg/ml to 2.5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 2.5 mg/ml to 5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 5 mg/ml to 7 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 7 mg/ml to 8.5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 8.5 mg/ml to 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 9 mg/ml to 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 10 mg/ml to 15 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 15 mg/ml to 20 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 1.0 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 2.5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 15 mg/ml.

In another embodiment, the concentration of the salt is from 0.1 mg/ml to 0.5 mg/ml.

In another embodiment, the concentration of the salt is from 0.1 mg/ml to 0.2 mg/ml.

In another embodiment, the concentration of the salt is from 0.2 mg/ml to 0.3 mg/ml.

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In another embodiment, the concentration of the salt is from 0.3 mg/ml to 0.4 mg/ml.

In another embodiment, the concentration of the salt is from 0.4 mg/ml to 0.5 mg/ml.

In a further embodiment, the composition has a pH between 6.5 and 8.5.

In a further embodiment, the composition has a pH between 7.5 and 8.5.

In a further embodiment, the composition has a pH between 4 and 5.

In a further embodiment, the composition has a pH between 5 and 6.

In a further embodiment, the composition has a pH between 6 and 7.

In a further embodiment, the composition has a pH between 7 and 8.

In a further embodiment, the composition has a pH between 8 and 9.

In another embodiment, the pharmaceutically acceptable salt is an acetate salt.

In another embodiment, the substituted β-cyclodextrin is a hydroxypropyl, a sulfobutyl ether, or a sulfopropyl ether substituted β-cyclodextrin.

In a further embodiment, the substituted β-cyclodextrin is a

sulfobutyl ether substituted β-cyclodextrin.

In a further embodiment, the pharmaceutically acceptable salt is an acetate salt, and the substituted β-cyclodextrin is hepta-(sulfobutyl ether)-β-cyclodextrin.

In another embodiment, the composition further comprises a pharmaceutically acceptable buffer in an amount and of a type suitable to make the pH of the pharmaceutical composition in the range of 4-9. The buffer may be acetate buffer, citrate buffer, or sodium carbonate.

The subject invention also provides a pharmaceutical composition comprising

an aqueous carrier;

from 0.1 mg/ml to 20 mg/ml of the composition of an acetate salt of a peptide having the structural formula $NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1);

and

from 70 mg/ml to 170 mg/ml of the composition of hepta-(sulfobutyl ether)-β-cyclodextrin,

wherein the peptide and the hepta-(sulfobutyl ether)-β-cyclodextrin are dissolved in the aqueous carrier; and

wherein the composition has a pH between 6.5 and 8.5.

In one embodiment, the concentration of the acetate salt of the peptide is at least 0.5 mg/ml.

In one embodiment, the concentration of the acetate salt of the peptide is from 0.5 mg/ml to 10 mg/ml.

In a further embodiment, the concentration of the acetate salt of the peptide is from 0.5 mg/ml to 2.5 mg/ml.

In another embodiment, the concentration of the salt is from 0.1 mg/ml to 0.5 mg/ml.

In another embodiment, the concentration of the salt is from 0.1 mg/ml to 0.2 mg/ml.

In another embodiment, the concentration of the salt is from 0.2 mg/ml to 0.3 mg/ml.

In another embodiment, the concentration of the salt is from 0.3 mg/ml to 0.4 mg/ml.

In another embodiment, the concentration of the salt is from 0.4 mg/ml to 0.5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 5 mg/ml to 7 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 7 mg/ml to 8.5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 8.5 mg/ml to 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 9 mg/ml to 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 10 mg/ml to 15 mg/ml.

In another embodiment, the concentration of the salt of the peptide is from 15 mg/ml to 20 mg/ml.

In a further embodiment, the concentration of acetate salt of the peptide is 1.0 mg/ml.

In a further embodiment, the concentration of acetate salt of the peptide is 2.5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 5 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 10 mg/ml.

In another embodiment, the concentration of the salt of the peptide is 15 mg/ml.

In another embodiment, the concentration of hepta-(sulfobutyl ether)-β-cyclodextrin is 120 mg/ml and the pH of the composition is between 7.5 and 8.5.

The subject invention also provides a method of alleviating symptoms of systemic lupus erythematosus (SLE) in a human subject comprising administering to the human subject any of the above pharmaceutical compositions in an amount effective to alleviate the symptoms of SLE in the human subject.

The subject invention also provides the above pharmaceutical compositions for use in treating SLE in a human subject.

The subject invention also provides a process for manufacturing any of the above pharmaceutical compositions comprising the steps of:

a) preparing a solution of a substituted β-cyclodextrin in an aqueous carrier at a predetermined concentration;

b) adding predetermined amount of a pharmaceutically acceptable salt of the peptide $NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1) to the solution of step a);

c) adjusting the pH of the solution of step b) until the peptide dissolves in the solution; and

d) if necessary, adjusting the pH of the solution of step c) to a pH of 4-9, thereby manufacturing the pharmaceutical composition.

In one embodiment of the process, the resulting final concentration of the substituted β-cyclodextrin in the pharmaceutical composition is from 70 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 80 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 90 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 100 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 110 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 120 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 130 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 140 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 150 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 160 mg/ml to 170 mg/ml.

In one embodiment of the process, the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition is 120 mg/ml.

In another embodiment, the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition is at least 0.1 mg/ml.

In another embodiment, the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition is at least 0.5 mg/ml.

In another embodiment, the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition is 2.5 mg/ml, 2.0mg/ml, 1.0mg/ml, 0.5 mg/ml or 0.1 mg/ml.

In another embodiment, the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition is 5 mg/ml, 10 mg/ml or 15 mg/ml.

In another embodiment of the process, step b) further comprises mixing the solution for 1 hour.

In another embodiment, in step c) the pH is adjusted using HCl or NaOH 1.0N.

In another embodiment, the process further comprises filtering the solution of step d) through a cellulose acetate filter.

In another embodiment of the above process,

the predetermined concentration of the substituted β-cyclodextrin is such which results in a final

concentration of substituted β-cyclodextrin in the pharmaceutical composition is 120 mg/ml;

the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition is 2.5 mg/ml, 2.0mg/ml, 1.0mg/ml, 0.5 mg/ml or 0.1 mg/ml;

step b) further comprises mixing the solution for 1 hour; and

in step c) the pH is adjusted using HCl or NaOH 1.0N, and the process further comprises filtering the solution of step d) through a cellulose acetate filter.

The subject invention also provides a composition prepared by the above process.

The subject invention also provides a process of lyophilizing the above pharmaceutical composition, comprising the steps of:

a) lowering the temperature of the pharmaceutical composition to -40°C;

b) holding the temperature at -40°C for a predetermined time;

c) raising the temperature of the solution to 20°C;

d) holding the temperature at 20°C for a predetermined time; and

e) holding the temperature at 25°C for a predetermined time, thereby lyophilizing the pharmaceutical composition.

In one embodiment of the process, step a) is performed within 2 hours.

In another embodiment, step b) is performed within 3 hours.

In a further embodiment, step c) is performed over 13 hours.

In a further embodiment, step c) is performed at a pressure of 110µbar.

In a further embodiment, step d) is performed over 13 hours.

In a further embodiment, step d) is performed at a pressure of 110µbar.

In a further embodiment, in step e) the pressure is reduced to 10µbar.

In a further embodiment, step e) is performed over 5 hours.

In another embodiment of the process,

step a) is performed within 2 hours;

step b) is performed within 3 hours;

step c) is performed over 13 hours and at a pressure of 110µbar;

step d) is performed over 13 hours and at a pressure of 110µbar; and

step e) is performed over 5 hours and the pressure is reduced to 10µbar.

The subject invention also provides a lyophilized pharmaceutical composition prepared by the above process.

The subject invention also provides a process of lyophilizing the above pharmaceutical composition, comprising the steps of:

a) lowering the temperature of the pharmaceutical composition to -45°C;

b) holding the temperature at -45°C for a predetermined time;

c) raising the temperature of the solution to -20°C;

d) raising the temperature of the solution to 25°C; and

e) holding the temperature at 25°C for a predetermined time, thereby lyophilizing the pharmaceutical composition.

In one embodiment, step a) is performed within 6 hours.

In another embodiment, step b) is performed within 3 hours.

In another embodiment, step c) is performed over 19 hours.

In another embodiment, step c) is performed at a pressure of 150μbar.

In another embodiment, step d) is performed over 13 hours.

In another embodiment, step d) is performed at a pressure of 150μbar.

In another embodiment, step e) is performed over 8 hours.

In another embodiment, step e) is performed at a pressure of 150μbar.

In another embodiment of the process,

step a) is performed within 6 hours;

step b) is performed within 3 hours;

step c) is performed over 19 hours and at a pressure of 150μbar;

step d) is performed over 13 hours and at a pressure of 150μbar; and

step e) is performed over 8 hours and at a pressure of 150μbar.

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The subject invention also provides a lyophilized pharmaceutical composition prepared by any of the above processes.

In one embodiment of the above lyophilized pharmaceutical composition, the water content of the composition is less than 5%.

In another embodiment, the water content of the composition is less than 4.0%.

In another embodiment, the water content of the composition is less then 3.5%.

The subject invention also provides a lyophilized pharmaceutical composition comprising

a pharmaceutically acceptable salt of a peptide having the structural formula

$NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1); and

a substituted β-cyclodextrin.

The subject invention also provides a packaged pharmaceutical composition comprised of:

a packaging material; and

a predetermined amount of the above lyophilized pharmaceutical composition.

The preparations of the present invention may be given parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered by injection, inhalation, ointment, suppository, etc. administration by injection, infusion or

inhalation; topical by lotion or ointment; and rectal by suppositories.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systematically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

Details of general formulation procedures and information on additional excipients may be found in Remington: The Science and Practice of Pharmacy, 20th Edition.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

**Experimental Details**

**Example 1: Formulation Development for Compound 1**

The human hCDR1 peptide (Compound 1) is described in PCT International Publication No WO 02/067848, published September 6, 2002, and can be prepared by methods well known in the art, (see, for example, Peptides: Synthesis, Structure and Applications, ed. by B. Gutte, Academic Press, 1995; Peptide Synthesis Protocols, ed. By M. Pennington and B. Dunn, Humana Press, 1994; Schnolzer, M. et al., "In situ neutralization in Boc-chemistry solid phase synthesis. Rapid, High yield assembly of difficult sequences." *Int. J. Pept. Protein Res.* (1992) 40: 180-193).

Compound 1 is a synthetic polypeptide composed of 19 amino acids. It is provided as an acetate salt. The aqueous solubility of the peptide has been determined to be less than 0.5 mg/ml. Figure 1 shows compound 1 as an acetate salt.

In order to develop a formulation with peptide concentration exceeding 2 mg/ml, preferably up to 10 mg/ml, experiments with several solubility enhancers were performed. The preliminary experiments indicated that a concentration of 2 mg/ml cannot be easily attained. In order to develop a formulation for sub-cutaneous injection, it is also desirable that the pH be in the range of 4 to 9 and that the solution be iso-osmotic.

Based on an extensive literature survey, a few principal approaches were adopted in order to produce a formulation with maximal solubility. The factors considered were:

- pH adjustment and buffers
- Solvents
- Co-solvents

- Solubilizing agents

**Methods**

Compound 1 was dissolved in the chosen solubility enhancer solution either separately or in combination with other excipients and the solutions were stirred for at least an hour. The pH was adjusted if needed. The solutions were visually examined to estimate the solubility and sent for analytical assay determination. For a few chosen formulations, biological activity was also tested.

**Results**

Table 1 presents the type of solubility enhancers used for the formulation development. Tables 2 and 3 summarize the experiments that were performed with the various solubility enhancers. Table 2 summarizes the initial screening performed with peptide concentrations in the range of 5 to 10 mg/ml. The experimental work that was performed with higher peptide concentration was then repeated with the lower doses (see table 3)

Initial tests indicated that Compound 1 was more soluble at the limits of the desired pH levels, both acidic and basic, but was less stable at the basic pH range. Thus, several buffers and pH adjustment agents were tested, including acetate buffer, citrate buffer and sodium carbonate. None of the initially tested buffers achieved the desired peptide solubility level. Only above pH 9.2 and below pH 3.0 were solubility levels of 2 mg/ml observed. Nevertheless, at the initial stage, formulations with acetate buffer and citrate buffer (with Mannitol as a tonicity agent) were selected for initial toxicology studies. These formulations were tested for biological activity and proven active.

Non aqueous solvents (see table 1) such as Ethanol, Glycerin, Propylene glycol, Chremophore and their combinations were tested but did not increase the solubility of Compound 1. A solution of 30% DMA (dimethyl-acetamide) yielded solubility in the desired ranges (5 to 9 mg/ml), but was not suitable for a pharmaceutical formulation due to its toxicity profile. Improved solubility was also observed using 30% (w/w) PEG 400 (5 to 9 mg/ml). This latter formulation was chosen for the toxicology studies, but it has proved to be both inactive in the biological assay, and may have been the cause of some adverse effects in a mouse toxicity study. Thus, it was decided not to further pursue this formulation. In view of the preliminary experiments non-aqueous solvents were not used in the subject formulations.

Several amino acids (see table 1) including L-Arginine, L-Glutamic acid, L-Glycine and L-Lysine were tested to improve the protein solubility. The solubility of the peptide in L-Arginine was at the desired level but the resulting pH was above 9. An attempt to decrease the pH or use an Arginine HCl salt resulted in precipitation of the peptide. Human Serum Albumin was also tested and improved the solubility of the peptide at low peptide concentrations (1 mg/ml) (see table 3). However, due to its potential immunogenicity and the low peptide solubility, it was not utilized in further experiments.

Bulking agents (see table 1) including Mannitol, Sorbitol and Dextran were tested alone and in combination with other excipients, but did not improve the solubility of the peptide in solution.

Co-solvents (see table 1), including Polysorbate 20 and Polysorbate 80 were tested alone and in combination with other excipients. While lower concentrations of Polysorbates (up to 6%) did not improve the solubility of the peptide, higher concentrations (up to 10% - see table 2) improved the solubility of the peptide up to 2 mg/ml. However, such high concentrations of Polysorbates were deemed unsuitable for pharmaceutical formulations.

Two types of cyclodextrins, both approved for use in marketed parenteral products, were also tested: Hydroxypropyl-β-cyclodextrin and Sulfobutylether-β-cyclodextrin (Captisol®). Both markedly increased the solubility of the peptide (concentrations in the levels of 10 mg/ml for Hydroxypropyl-β-cyclodextrin and 2.5 for Captisol®). The biological activity of the two cyclodextrin formulations was tested and was found to be equal to the activity of the peptide alone.

CAPTISOL® is a commercially available polyanionic β-cyclodextrin derivative with a sodium sulfonate salt separated from the hydrophobic cavity by a butyl ether spacer group, or sulfobutylether (SBE). CAPTISOL® is the trade name for CyDex Inc.'s hepta-substituted sulfobutylether β-cyclodextrin (SBE7-β-CD) preparation (www.captisol.com). The structure of CAPTISOL® allows drug molecules to fit in the hydrophobic cavity, thereby isolating the drug molecule from the aqueous solvent. Because the outer surface of CAPTISOL® is hydrophilic, the solubility of the complexed drug molecule is thereby enhanced. The use of cyclodextrins to enhance the solubility of drug molecules is disclosed in U.S. Patent Nos. 5,134,127 and 5,376,645, the entire contents of which are hereby incorporated by reference.

According to the literature of CyDex Inc., CAPTISOL® is safe when administered parenterally and does not exhibit the nephrotoxicity associated with beta-cyclodextrin. Relative to beta-cyclodextrin, CAPTISOL® provides comparable or higher complexation characteristics and superior water solubility in excess of 90 grams/100ml - a 50-fold improvement.

**Conclusions**

Several solubility enhancers were found to match the desired solubility range: DMA, PEG-400, dimethyl-acetamide, polyethylene glycol, polyoxylated castor oil, N-methyl-2-pyrrolidinone, 1-ethenyl-2-pyrrolidinone, Polysorbate 20, Polysorbate 80, Hydroxypropyl-β-cyclodextrin and Sulfobutylether-β-cycldextrin (Captisol®). Of these solubility enhancers both cyclodextrins have proven to be superior with respect to solubility, biological activity and stability. Thus, it was decided to select Captisol® as the solubility enhancer for use in Example 5 formulations and to further study both cyclodextrin formulations. The final formulation for the Example 5 clinical studies consists of: 120 mg/ml of Captisol® in water with the desired amount of peptide (0.5, 1.0 or 2.5 mg/ml), and HCl and NaOH for pH adjustment.

**Table 1: Solubility enhancers used for Compound 1 formulation development**

| Solubility enhancer classification | Solubility Enhancers |
|---|---|
| Solvents | Cremophor EL, CMC, Ethanol, DMA, Gycerin, Propylene Glycol, PEG 400, Monotioglycerol |
| Co-solvents | Polysorbate 20, Ploysorbate 80 |
| Solubilizing agents | Argenine, HSA, Glycine, Creatinine, Glutamic acid, Lysine (acetate salt and free base), Captisol®, Hydroxypropyl-β-cyclodextrin, |
| Bulking agents | Mannitol, Sorbitol, Dextrose, Lactose Dextran |
| pH Adjustment Agents | Citrate buffer, Acetate buffer, Sodium Carbonate |

**Table 2 List of Cosolvents and Stabilizers evaluated in Compound 1 Peptide Formulations**

| Solubility Enhancer * | % Used | % of Standard amount from the literature | Amount of peptide added (mg/ml) | Assay,% | pH of formulation | Remarks |
|---|---|---|---|---|---|---|
| Albumin (HSA),<br>Dextrose | 1.5<br>1.5 | 0.4-5.0 | 5 | | 6.0<br>Adjust. to 4.1 | Insoluble |
| Albumin (HSA),<br>Polysorbate 80,<br>Glycine | 1.0<br>0.6<br>2.0 | 0.4-5.0<br>0.8-4.0<br>0.2-2.1 | 5 | | 5.8<br>Adjust. to 4.1 | Insoluble |
| Arginine | 1.5 | 0.8-1.6 | 15 | 93 | 9.8 | Clear solution |
| Arginine HCl | 2.0 | 0.8-1.6 | 5 | | 3.5 | Insoluble |
| Arginine<br>Lactose | 1.5<br>1.5 | 0.8-1.6 | 15 | 93 | 9.8 | When the pH was lowered below 8.5 the peptide precipitated and the solution turned into gel |
| Captisol® | 10.0<br>20.0 | Up to 30.0 | 10 | 86<br>89 | 4.9<br>Adjust. to 4.4 | Turbid solution |
| CMC (carboxy methyl cellulose) in acetate | 0.2<br>0.05M | | 5 | 90 | 5.0 | For toxicology studies |
| Creatanine | 0.8 | Up to 0.6 | 5 | | 6.1<br>Adjust. to 4.1 | Insoluble |
| Cremophor EL<br>Ethanol<br>Dimethylacetamide | 15.0<br>10.0<br>6.0 | – 10.0<br>0.6-32.9<br>0.012-6.0 | 5 | | 4.0 | Very turbid |
| Dextran | 4.0 to 15.0 | 3.0-30.0 | 5 | | 3.9 | Insoluble |
| Dimethylacetamide (DMA) | 6.0-20.0 | 0.012-6.0 | 5 | | 4.6 | Insoluble |

| Solubility Enhancer | *% Used* | *% of Standard amount* | *Amount of peptide added (mg/ml)* | *Assay,%* | *pH of formulation* | *Remarks* |
|---|---|---|---|---|---|---|
| Dimethylacetamide (DMA) | 25 0 | 0.012-6.0 | 5 | 87 | 5 1 | Clear solution |
| Dimethylacetamide (DMA) | 30 0 | 0.012-6.0 | 10 | 93 | 5 1 | Clear solution |
| Ethanol | 10.0 | 0.6-32 9 | 5 | | | Insoluble |
| Gluramic acid | 2 0 | | 5 | | 3 7 | When the pH was increased above 4 the peptide precipitated and the solution turned into gel |
| Glycerin | 1 5 | 1 6-32.5 | 5 | 37 | 4.5 | Insoluble |
| Glycerin | 30.0 | 1.6-32.5 | 5 | | 3.7 | Insoluble |
| Glycerin<br>Polysorbate 80 | 10.0<br>0.6 | 1 6-32 5<br>0.8-4 | 5 | 12 | 6.5<br>Adjust. to 4.5 | Insoluble |
| Glycine | 0.4 | 0.2-2 1 | 5 | | 4.6 | Insoluble |
| Hydroxypropyl β-cyclodextrin | 20.0 | Up to 30.0 | 10 | 99 | 4.6 | Clear solution |
| Lysine Acetate Salt | 2.0 | | 5 | | 3 8 | Insoluble |
| Lysine Free base | 2.0 | | 5 | | 9 2 | When the pH was lowered below 8 the peptide precipitated and the solution turned into gel |
| Mannitol in Citrate buffer | 4 0<br>0 035M | 2.0-10 0 | 5 | 38 | 3.4 | For toxicology studies |
| Mannitol<br>in acetate buffer | 4.0<br>0 05M | 2.0-10.0 | 5 | 32 | 4.3 | For toxicology studies |
| Mannitol,<br>Glycine | 20 0<br>0.4 | 2.0 10.0<br>0.2-2.1 | 5 | 14 | 6.4<br>Adjust. to 4.5 | Insoluble |
| Mannitol,<br>Polysorbate 20 | 20.0<br>0.6 | 2.0-10.0 | 5 | 22 | 6.5<br>Adjust. to 4.5 | Insoluble |
| Monothioglycerol | 1 0 | 0.1 10 0 | 5 | | 4.5 | Turbid solution |
| PEG 400 | 30.0 | Up to 30.0 | 5 | 88 | 4.2 | Slightly opalescent |

| Solubility Enhancer * | % Used | % of Standard amount from the literature | Amount of peptide added (mg/ml) | Assay, % | pH of formulation | Remarks |
|---|---|---|---|---|---|---|
| PEG 400 | 30.0 | Up to 30.0 | 10 | 89 | 4.2 | Turbid solution |
| PEG 400<br>with DMA | 30.0<br>6.0 | Up to 30.0<br>0.012-0.6 | 5 | 94 | 4.2 | Clear solution |
| PEG 400 | 10.0 | 6.0-18.0 | 5 | 58 | 4.2 | Insoluble |
| PEG 400<br>DMA | 10.0<br>10.0 | Up to 30.0<br>0.012-0.6 | 5 | | 4.3 | Insoluble |
| PEG 400<br>Propylene glycol PG | 10.0<br>10.0 | Up to 30.0<br>10.0 | 5 | | 4.1 | Insoluble |
| PEG 400<br>Propylene glycol | 13.0<br>50.0 | Up to 30.0<br>10.0 | 5 | 100 | 4.2 | Clear solution |
| Polysorbate 80 | 1.0 | 0.8-4.0 | 5 | 24 | 7.2<br>Adjust. to 4.5 | Insoluble |
| Polysorbate 80 | 6.0 | 0.8-4.0 | 5 | | 3.9 | Insoluble |
| Polysorbate 80<br>Creatinine | 6.0<br>0.6 | 0.8-4.0<br>up to 0.6 | 5 | | 4.0 | Insoluble |
| Propylene glycol PG<br>DMA | 10.0<br>10.0 | 10.0<br>0.012-0.6 | 5 | | 4.2 | Insoluble |
| Propylene glycol PG | 10.0, 30.0 | 10.0 | 5 | | 4.2 | Insoluble |
| Sodium Carbonate | 1.5 | | 5 | | 11.4 | When the pH was lowered below 8.5 the peptide precipitated and the solution turned into gel |
| Sorbitol | 5.0 | 10.0-25.0 | 5 | | 6.9<br>Adjust. to 4.5 | Turbid solution |

**Table 3 Compound 1 formulations at low peptide concentrations**

| Solubility Enhancer * | % Used | % of Standard amount from the literature | Amount of peptide added (mg/ml) | Assay,% | pH of formulation | Remarks |
|---|---|---|---|---|---|---|
| Albumin (HSA) | 5.0 | 0.4-5.0 | 1.0 | 90 | 6.9 | Clear solution |
| | | | 2.5 | | Adjust. to 4.5 | Turbid solution |
| Arginine | 1.5 | 0.8-1.6 | 1.0 | 24 | 10.6 | When the pH was lowered below 8.5 the peptide precipitated and the solution turned into gel |
| | | | | | Adjust. to 8.5 | |
| Captisol® | 12.0 | Up to 30.0 | 1.0 | 106 | 5.3 | Clear solution |
| | | | 2.5 | 100 | 6.5 to 8.5 | |
| Dextran | 20.0 | 3.0-30.0 | 1.0 | 69 | 4.8 | Turbid solution |
| | | | | | Adjust. to 4.0 | |
| Glycerin | 30.0 | 1.6-32.5 | 1.0 | | 4.8 | Turbid solution |
| | | | | | Adjust. to 4.0 | |
| Mannitol | 4.0 | 0.8-4.0 | 1.0 | 64 | 4.7 | Turbid solution |
| | | | | | Adjust. to 4.0 | |
| Polysorbate 20 | 10.0 | | 1.0 | 95 | 5.8 | Clear solution |
| | | | 2.5 | 88 | Adjust. to 4.8 | Clear with small amount of precipitation |
| Polysorbate 20 | 10.0 | | 2.5 | 115 | 5.1 | Clear solution |
| Mannitol | 2.0 | 2.0-10.0 | | | Adjust. to 4.3 | |
| Polysorbate 80 | 4.0 | 0.8-4.0 | 1.0 | 91 | 5.5 | Clear solution |
| | 6.0-10.0 | | 2.5 | 89 | Adjust. to 5.0 | Slightly turbid solution |
| Polysorbate 80 | 4.0 | 0.8-4.0 | 2.5 | 88 | 5.1 | Slightly turbid solution |
| Mannitol | 2.0 | 2.0-10.0 | | | Adjust. to 4.4 | |
| Propylene glycol PG | 10.0 | 10.0 | 1.0 | 78 | 5.0 | Turbid solution |
| | | | | | Adjust. to 4.4 | |
| Sorbitol | 20.0 | 10.0-25 | 1.0 | 52 | 4.5 | Turbid solution |

**Example 2: Preparation protocol for solution of Compound 1 in Captisol®**

Standard dissolution methods, such as mixing dry Compound 1 and dry Captisol® into water or adding Compound 1 to a prepared solution of Captisol® and water did not result in complete dissolution at the desired concentrations. Several different concentrations of both Compound 1 and Captisol® were tested at various pH levels. However, the following method for producing a solution of Compound 1 in Captisol® resulted in complete dissolution at the desired concentrations.

Materials: Captisol®, Compound 1 and water

Method:

1. Weigh the appropriate amount of Captisol® to give a final concentration of 120 mg/ml.
2. Add 80% of the final amount of water and mix for 10 minutes with a magnetic stirrer.
3. Weigh Compound 1 to give a final concentration of 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml or 0.1 mg/ml.
4. Add the peptide to the Captisol® solution. Mix for 1 hour.
5. Raise the pH to obtain clear solution (in the 2.0 mg/ml formulation there might be a need to raise the pH slightly above 9). pH should be adjusted using HCl 1.0 N and NaOH 1.0 N. Mix for 10 minutes.
6. Correct the pH to the range of 7.5 to 8.5 if needed (using either HCl or NaOH 1.0 N).
7. Add water to final volume.

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8. Filter the solution through a 0.2µ cellulose acetate filter.

9. Record final pH.

10. Dispense into aliquots and store at the proper temperature.

**Example 3: Lyophilization of Compound 1 and Captisol® solution**

The current lyophilization process differs from other lyophilization processes in that the percentage of solids in the formulation is high (12%) whereas lyophilized products normally contain between 5 and 10% solids.

**Equipment**

The freeze drier used was an Edwards lyophilizer Lyoflex 0.6. The equipment IQ/OQ was performed and checked for compliance by quality assurance prior to the process development.

Solutions of Compound 1 and Captisol® at concentrations of 0.5 mg/ml, 1.0mg/ml and 2.5mg/ml of Compound 1 were prepared. The fill-volume was adjusted 1 ml (1.05 gr).

**Main process steps:**

1. Freezing
2. Holding (at low temperature)
3. Drying under vacuum in two stages:
   - 3.1. Primary drying - shelf warming to an upper hold temperature, controlling shelf temperature at the upper hold level.
   - 3.2. Secondary drying - Pressure reduction to a minimal value at the upper hold shelf temperature.

**Batches 1-3**

Freezing - Freezing was from room temperature to -40°C within 2 hours. Shelves were held at -40°C for 3 hours.

Drying - Drying was performed at 110 µbar pressure. Shelf temperature was increased to 20°C over 13 hours and held at that temperature for additional 13 hours.

Total process time was 31 hours.

Results:

Water content results were:

Batch no. 1: 3.8%

Batch no. 2: 4.0% and

Batch no. 3: 4.9%

**Batches 4 and 5**

Since the water content results of the processes leading to batches 1, 2 and 3 were higher then the desired value, it was decided to add a secondary drying step at the same temperature and at low pressure.

Drying - Drying was performed at 110µbar pressure. Shelf temperature was increased to 20°C over 13 hours and held at that temperature for additional 13 hours (Batch 4) or 8 hours (Batch 5). Pressure was decreased to 10µbar for additional 5 hours.

Total process time was 36 hours.

Results:

Water content results were

Batch 4: Placebo: 3.0%,

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1 mg/ml: 3.9%.

Batch 5: Placebo: 4.1%

**Conclusions**

As shown, a satisfactory lyophilization process for Compound 1 with Captisol® was developed. Due to the high percentage of solids and hence the condensed cake, the developed process is longer then the currently available lyophilization cycles for peptides and it exhibits an additional secondary drying stage. Table 4 summarizes the developed process.

Table 4

| Step | Compound 1 (Peptide) with Captisol® |
|---|---|
| Loading | 5°C |
| Freezing | 2 hours to -40°C |
| Hold at low temp. | 3 hours to -40°C |
| Primary Drying: | |
| Warm to 20°C | 13 hours pressure 110µbar |
| Hold at 20°C | 13 hours pressure 110µbar |
| Secondary drying: | |
| Hold at 20°C | 5 hours pressure 10µbar |
| Storage at | -20°C |
| Process time | 36 hours |

**Example 4.**

**Examination of the in-vivo biological activity of the lyophilized compound solution (DP, 1 mg/vial, 12% captisol®)**

The biological activity was monitored by inhibition of IL-2 secretion from Compound 1 reference standard (RS) specific T-cells following subcutaneous (s.c.) treatment with the lyophilized compound solution, i.e. the drug product (DP), at two concentrations. The results of the treatment are compared to those of treating mice with Compound 1 (RS) in phosphate buffered saline (PBS). The results are shown in the tables below and in Figure 2.

**Experimental design:**

1. Immunization — Day 0
   (Compound 1 RS emulsified with CFA, at all four footpads)

2. Treatment
   (s.c. at the back of the neck, in 200 µl solution) — Day 0

3. In-vitro activation with: — Day 10
   a. Compound 1 RS at concentrations of 0; 0.5; 1; 2.5; 5; 10; 25; 50 and 100 µg/ml
   b. a peptide with the reverse order of amino acids of Compound 1 (negative control).
   c. Con A (positive control).

4. Incubation of culture for 20 hrs at 37°C in a humidified 5% $CO_2$ incubator.

5. IL-2 measurement by ELISA.

**Table of experimental Groups:**

| Group | Immunization with | Treatment |
|---|---|---|
| A | 50 μg Compound 1 RS | 50μg Compd. 1 RS in PBS |
| B | | 200μg Compd. 1 RS in PBS |
| C | | 50μg DP(batch 2) |
| D | | 200μg DP (batch 2) |
| F | | Placebo (12 % captisol®) |

## IL-2 Secretion from Compound 1 (DP) Treated Mice Following In-vitro Activation with Compound 1 RS (pg/ml)

| | | | Treated with: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Group | F | A | | B | | C | | D | |
| Activator | Concentration of activator (μg/ml) | 12% captisol® Ampulized | Compd. 1 RS 50 μg/mouse | % Inhib. | Compd. 1 RS 200 μg/mouse | % Inhib. | DP 50 μg/mouse | % Inhib. | DP 200 μg/mouse | % Inhib. |
| Con A | 2.5 | 5,825 | 6,215 | | 5,403 | | 3,537 | | 4,069 | |
| Compd. 1 RS | 0 | BQL | BQL | | BQL | | BQL | | BQL | |
| Compd. 1 RS | 0.5 | 11 | 9 | | 10 | | 8 | | BQL | |
| Compd. 1 RS | 1 | 10 | BQL | | BQL | | BQL | | BQL | |
| Compd. 1 RS | 2.5 | 15 | 8 | 51 | BQL | NA | 6 | 61 | 6 | 62 |
| Compd. 1 RS | 5 | 20 | 9 | 55 | 8 | 60 | 10 | 48 | 7 | 63 |
| Compd. 1 RS | 10 | 25 | 16 | 36 | 11 | 58 | 13 | 48 | 8 | 67 |
| Compd. 1 RS | 25 | 29 | 15 | 48 | 11 | 63 | 16 | 45 | 13 | 58 |
| Compd. 1 RS | 50 | 40 | 21 | 47 | 15 | 62 | 20 | 50 | 12 | 69 |
| Compd. 1 RS | 100 | 42 | 25 | 41 | 18 | 58 | 24 | 43 | 15 | 64 |
| | Average Inhibition (%) (at the range of 5-100 μg/ml) | | | 45.6 | | 60.4 | | 46.8 | | 63.7 |

BQL = Below Quantitation Limit

NA = Not Applicable

Rows 1-4 were not included in the curve

**Example 5: Evaluation of Optimal Dose for Treatment**

The following abbreviations are used in the following description:

| | |
|---|---|
| CFA | Complete Freund's adjuvant |
| Con A | Concanavalin A |
| DP | Drug Product |
| DS | Drug Substance |
| EM-1 | Enriched DCCM-1 Medium |
| EM-3 | Enriched RPMI-1640 + fetal calf serum medium |
| FCS | Fetal Calf Serum |
| IFN-γ | Interferon-gamma |
| LN | Lymph Node |
| PBS | Phosphate Buffered Saline |
| RS | Reference Standard |
| s.c. | Subcutaneous |
| TB | Trypan Blue |
| TGF-β | Transforming Growth Factor-beta |
| WFI | Water for Injection |

**Introduction**

A group of 20 mice were immunized with 50 µg/mouse of Compound 1 RS. The immunized mice were allocated to five treatment groups as follows: placebo, 25, 50, 100 and 200µg/mouse of Compound 1 DP (subcutaneous administration). Ten days post immunization and treatment, LN was extracted and single cell suspension was prepared. The *in-vitro* secretion of IFN-γ and TGF-β by the cultured cells in response to activation with several concentrations of Compound 1 RS was then measured.

**Experimental design**

1. Immunization -Day 0
2. Treatment with Compound 1 DP -Day 0

3. In-vitro activation of LN cells from treated mice -Day 10
4. Collection of culture media (for IFN-γ determination) -Day 12
5. Collection of culture media (for TGF-β determination) -Day 13
6. ELISA for IFN-γ
7. ELISA for TGF-β

**Table 7: Experimental Groups**

| Exp. Group | Treatment | | In-vitro activation | |
|---|---|---|---|---|
| | Article | Mice/group | Cells/well | Compound 1 RS concentration |
| A1 | Control 12% Captisol® | 4 | $2.5 \times 10^6$ | Compound 1 RS 0-100 µg/ml |
| | | | $5 \times 10^6$ | |
| A2 | 25 µg/mouse | 4 | $2.5 \times 10^6$ | |
| | | | $5 \times 10^6$ | |
| A3 | 50 µg/mouse | 4 | $2.5 \times 10^6$ | |
| | | | $5 \times 10^6$ | |
| A4 | 100 µg/mouse | 4 | $2.5 \times 10^6$ | |
| | | | $5 \times 10^6$ | |
| A5 | 200 µg/mouse | 4 | $2.5 \times 10^6$ | |
| | | | $5 \times 10^6$ | |

**Materials and Reagents**

***Animals***

Mice: 20 female BALB/c mice, supplied by Harlan animals breeding center, Rehovot.

Age at immunization (week+days): 10

Average weight of mice included in the experiment: 19.01 gr.

***Materials***

**General reagents**

70% ethanol was prepared from 96% ethanol by diluting with purified $H_2O$.

**Preparation of Compound 1 solutions for immunization**

CFA-Compound 1 RS emulsion (500 µg/ml, 50 µg/mouse) was prepared as follows:

1. 1.874 mg of Compound 1 was dissolved in 1.87 ml of WFI to yield a solution of 1 mg/ml.
2. The solution was tested with a pH indicator strip and found to have a pH of 5.
3. 1.5 ml of the solution were emulsified with 1.5 ml CFA resulting in a final concentration of 500 µg/ml.

**Preparation of solutions for Treatment**

Treatment was by a s.c. injection of 200 µl solution.

**Preparation of 12% captisol® solution**

1.2 gr of captisol® were dissolved in 10 ml of WFI to yield a solution of 12% captisol®.

**Experimental procedure**

**Mice weighing**

Mice were weighed before immunization. Average mice weight: 19.01 ± 0.97 gr

**Immunization**

The immunization was performed by injecting 100 microliters of the emulsion (50 microliters into each hind footpad).

**Treatment**

Following the immunization step the mice were treated by s.c. injection of 200 µl from the designated Compound 1 DP or 12% captisol® treatment solutions, at the back of their neck.

**In-vitro culture**

Mice were sacrificed by cervical dislocation. LN were extracted from the hind legs and were transferred to a sterile petri dish containing about 5 mL RPMI. The cells were extracted by gentle squeezing of the tissue against a 200 micrometer mesh stainless steel net. The cells were collected and centrifuged at 300 G for 10 minutes at RT.

Single cell suspensions were prepared from pooled LN of each experimental group.

2.5 and 5.0 million cells/ml/well suspensions were cultured with Compound 1 RS (0-100 µg/ml) in EM-1.

Secretion of IFN-γ and TGF-β, as indication of cellular response, were determined by ELISA of culture media (48hrs for IFN-γ and 72hrs for TGF-β).

**Table 8: The *in-vitro* experimental groups**

| Experimental Group | Treatment | In-vitro activation | |
|---|---|---|---|
| | Article | Cells/well | Activation substance concentration |
| A1-2.5 | Control 12% Captisol® | $2.5 \times 10^6$ | Compound 1 RS 0; 3.125; 6.25; 12.5; 50 and 100 µg/ml Con A 2.5 µg/ml |
| A1-5 | | $5 \times 10^6$ | |
| A2-2.5 | DP 25 µg/mouse | $2.5 \times 10^6$ | |
| A2-5 | | $5 \times 10^6$ | |
| A3-2.5 | DP 50 µg/mouse | $2.5 \times 10^6$ | |
| A3-5 | | $5 \times 10^6$ | |
| A4-2.5 | DP 100 µg/mouse | $2.5 \times 10^6$ | |
| A4-5 | | $5 \times 10^6$ | |
| A5-2.5 | DP 200 µg/mouse | $2.5 \times 10^6$ | |
| A5-5 | | $5 \times 10^6$ | |

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**Preparation of cell suspensions**

**Table 9: Results of cell counting and preparation of cell suspensions (10 x $10^6$/ml)**

| Grp | Vol. (ml) | Dilutn. factor | Viable cells | Dead cells | %Viable cells | %Dead cells | Average viable cells | Total viable cells (x $10^5$) | EM-1 to add (ml) for suspension of 10 x $10^6$ cells/ml |
|---|---|---|---|---|---|---|---|---|---|
| A1 | 10 | 16 | 115 | -- | 100 | -- | 112 | 179.2 | 17.9 |
| | | | 109 | -- | 100 | -- | | | |
| A2 | 10 | 16 | 50 | 4 | 92.6 | 7.4 | 47.5 | 76 | 7.6 |
| | | | 45 | 2 | 95.7 | 4.3 | | | |
| A3 | 10 | 16 | 80 | 4 | 95.2 | 4.8 | 80.5 | 128.8 | 12.8 |
| | | | 81 | 5 | 94.2 | 5.8 | | | |
| A4 | 10 | 16 | 87 | -- | 100 | -- | 89 | 142 | 14.2 |
| | | | 91 | -- | 100 | -- | | | |
| A5 | 10 | 16 | 120 | 2 | 98.4 | 1.6 | 112.5 | 180 | 18 |
| | | | 105 | 2 | 98.1 | 1.9 | | | |

**Preparation of cell suspensions (5 x $10^6$/ml)**

The 10 x $10^6$ cells/ml suspensions were diluted 1:2 by adding 5 ml EM-1 to 5 ml cells suspension.

**Incubation of LN cells cultures in 48 wells plates**

3 tissue culture plates were prepared. The following was added to each plate.

**Background control (cells incubated with culture media)**

0.5 ml of cells suspension
0.5 ml of culture media (EM-1)

**System positive control (cells stimulated with Con A)**

0.5 ml of cells suspension

0.5 ml of Con A 5 μg/ml in EM-1 (final conc. 2.5 μg/well)

**Cells incubated with Compound 1 activation solutions (samples)**

0.5 ml of cells suspension
0.5 ml of Compound 1 RS 6.25 - 200 μg/ml (final conc. 3.125 - 100 μg/ml/well)

**Incubation of LN cells cultures in 96 wells plates**

After the 48-wells plates were prepared, 96-wells plates were prepared by applying 100 μl from the cell suspension and 100 μl from the activation solutions.

The culture plates were incubated at 37° C in a humidified 5% $CO_2$ incubator, for either 48 or 72 hrs.

**Supernatants Collection**

The cultured plates were centrifuged at 300 g for 10 minutes at RT. Supernatants (850 μl from each well) were transferred either to mirror plates or to tubes. The supernatant was then divided into working aliquots (two aliquots of 200 and one aliquot of 450 μl), in order to avoid repeated freeze/thawing of the samples. Each tube was labeled with the following details:

1. Experimental code and time post incubation.
2. Group and sample number
3. Activator and concentration.
4. Date of sup collection

The supernatants were stored at -20°C until used for ELISA.

**Results**

**Table 10: Summary of Groups**

| Experimental Groups: | | | | |
|---|---|---|---|---|
| Immunization | | Treatment | | In-vitro |
| Exp.Groups | Immunization dose | Sub group | Article | activation |
| A | 50µg/mouse | A1 | 12% Captisol® Placebo control | Compound 1 RS 3.125-100µg/ml |
| | | A2 | Compound 1 25µg/M | |
| | | A3 | Compound 1 50µg/M | |
| | | A4 | Compound 1 100µg/M | |
| | | A5 | Compound 1 200µg/M | |

**Table 11-A: Final cytokine concentrations**

Final cytokine (pg/ml) (2.5 million cells/well)

| Compound 1 concentration | Placebo | 50µg/M | 100µg/M | 200µg/M |
|---|---|---|---|---|
| 3.125µg/ml | 321.3 | 54.1 | 64.5 | 103.9 |
| 6.25µg/ml | 238.8 | 81.8 | 116.1 | 126.1 |
| 12.5µg/ml | 397.1 | 123.1 | 180.9 | 129.0 |
| 25µ/ml | 655.5 | 215.1 | 262.8 | 240.3 |
| 50µg/ml | 573.9 | 292.5 | 518.3 | 378.1 |
| 100µg/ml | 928.0 | 531.8 | 582.7 | 524.1 |
| Con A | 322.6 | 356.2 | 337.4 | BQL |

**Table 11-B: Final cytokine concentrations**

Final cytokine (pg/ml) (5 million cells/well)

| Compound 1 concentration | Placebo | 25µg/M | 50µg/M | 100µg/M | 200µg/M |
|---|---|---|---|---|---|
| 3.125µg/ml | 522.3 | BQL | 76.2 | 90.8 | 204.4 |
| 6.25µg/ml | 634.8 | BQL | 109.2 | 157.8 | 244.1 |
| 12.5µg/ml | 962.8 | 41.9 | 179.5 | 257.1 | 488.1 |
| 25µ/ml | 987.4 | 70.0 | 277.9 | 421.7 | 660.5 |
| 50µg/ml | 1336.8 | 104.2 | 373.4 | 739.7 | 922.5 |
| 100µg/ml | 2010.2 | 185.2 | 547.0 | 995.5 | 1006.2 |
| Con A | 6839.8 | 2995.3 | 4837.0 | 10126.8 | 7722.8 |

The results are also presented in Figures 3-4.

**Observations**

IFN-γ secretion

1. In the placebo group, a linear dose response upon Compound 1 activation in-vitro was shown. This graph resembles the graph obtained for the Ex-vivo model with the same immunization dose (50µg/mouse) and culturing medium (EM-1).
2. There was a dose response upon Compound 1 activation in vitro within all the tested groups.
3. Significant inhibition of IFN-γ secretion was seen with all the doses used for treatment (an average of 95% inhibition with treated dose of 25µg/mouse). A reverse correlation between the dose served for treatment and % inhibition can be found, mainly when $5x10^6$ cells/well were used. When $2.5x10^6$ cells/well were used, treatment of animals with 50µg/mouse gave better inhibition than 100 or 200µg. The point of 25µg is missing (lack of cells).
4. A better inhibition was seen when $5x10^6$ cells/well were used instead of $2.5x10^6$ cells/well.
5. In the linear range of the graphs, SD of % inhibition was low.
6. A technical problem with Con A is apparent when $2.5x10^6$ cells/well were used.

TGF-β secretion

1. In the placebo group, no dose response upon in vitro activation with compound 1 was seen. TGF-β secreted level was below the detection limit of the ELISA in all other treatment groups.

**Example 6: Optimizations of freeze drying cycle with Compound 1 and Captisol® for injection (0, 0.5, 1.0 and 2.5 mg/vial)**

**Purpose**

The purpose of this study was to optimize the freeze drying cycle for Compound 1 with captisol® for injection to improve the shape of the lyophilization cake and avoid collapse and cracking. Thus it was decided to improve and optimize the lyophilization cycle. This cycle is transferred to the production lyophilizers for the manufacturing of the phase I batches.

**Process optimization**

Batches of peptide at concentrations of 0.5 mg/ml 1.0mg/ml, 2.5mg/ml and Placebo were prepared and several freeze drying cycles were performed. The freeze drier used was an Edwards lyophilizer Lyoflex 0.6.

Solubility, water content and cake appearance were tested.
According to the obtained results a new lyophilization cycle for Compound 1 was selected. Due to the high percentage of solids (12%) and hence the condensed cake, the new process is longer than the lyophilization cycle in Example 3 and exhibits an additional primary drying stage. Table 12 summarizes the differences between the processes.

Table 12

| Step | Lyoph. cycle for Compound 1 and Captisol® of Example 3 | New Lyoph. cycle for Compound 1 and Captisol® |
|---|---|---|
| Loading | 5°C | 5°C |
| Freezing | 2 hours to -40°C | 6 hours to -45°C |
| Hold at low temp. | 3 hours to -40°C | 3 hours to -45°C |
| Primary Drying:<br>Stage I<br>Stage II<br><br>Hold at 20°C (25°C) | to 20°C<br>13 hours pressure 110μbar<br>-<br><br>13 hours pressure 110μbar | to -20°C<br>19 hours pressure 150μbar<br>to 25°C<br>13 hours pressure 150μbar<br>8 hours pressure 150μbar |
| Secondary drying:<br>Hold at 20°C | <br>5 hours pressure 10μbar | <br>- |
| Storage at | 5°C | 5°C |
| Process time | 36 hours | 49 hours |

**Example 7: Effect of Compound 1 (administered in Captisol®) on lupus symptoms in the SLE-prone (NZBxNZW)F1 female mouse**

Patients participating in clinical trials are to be treated with Compound 1 using Captisol® (sulfobutyl ether beta-cyclodextrin sodium) as the excipient. For this reason, it was important to determine whether treatment of (NZBxNZW)F1 mice with the formulation of Compound 1 given in Captisol® would have the same beneficial effects on lupus symptoms as observed when this strain of mice was treated with Compound 1 in PBS.

To this end, (NZBxNZW)F1 female mice (about 8 months old) were divided into 3 groups that were treated subcutaneously once a week for 10 weeks either with Captisol® alone (n=8) or with 25 or 50 μg/mouse Compound 1 in Captisol® (n=9 and 10, respectively). These doses were selected since prior studies indicated that doses in this range were more effective in ameliorating SLE symptoms than the higher doses tested (100 and 200 μg/mouse). The

same batch of drug substance was used in this study and in the first Phase I clinical trial with Compound 1.

The mice were followed for anti-dsDNA antibodies and for proteinuria. When the mice were sacrificed, the intensity of ICD was determined in kidneys.

As can be seen in Figure 5, no significant differences between groups could be observed in the levels of dsDNA-specific antibodies after 10 treatment injections.

Table 13 also shows that the beneficial effect of treatment with Compound 1 could be observed starting from the 5$^{th}$ injection and it was sustained up to the 10$^{th}$ injection. The mean levels of proteinuria in the Captisol® control group were consistently higher than in the Compound 1 -treated groups. Table 13 also shows that a reduction in the intensity of ICD was observed in kidneys of both Compound 1 dose groups. There was an overall trend showing that the lower dose (25 µg/mouse) was more effective than the higher dose (50 µg/mouse) in reducing the clinical symptoms of SLE in these mice.

**Table 13. Clinical Symptoms of SLE in (NZBxNZW)F1 Mice Treated with 25 or 50 µg/mouse Compound 1 (in Captisol®)**

| Study Group | Mean Proteinuria ± SEM (g/L) Number of Weeks Following Treatment Initiation | | | | ICD[a] (Mean ± SEM) |
|---|---|---|---|---|---|
| | 5 | 7 | 8 | 10 | |
| Captisol® | 1.81 ± 1.22 (n=8) | 5.74 ± 3.13 (n=8) | 4.5 ± 2.92 (n=7)[b] | 4.46 ± 2.93 (n=7)[b] | 2.29 ± 0.28 (n=7) |
| Compound 1 (50 µg/mouse) | 0.75 ± 0.3 (n=10) | 0.81 ± 0.3 (n=10) | 1.09 ± 0.4 (n=10) | 1.29 ± 0.3 (n=10) | 1.90 ± 0.23 (n=10) |
| Compound 1 (25 µg/mouse) | 0.16 ± 0.05 (n=9) | 1.26 ± 1.09 (n=9) | 0.5 ± 0.31 (n=9) | 0.56 ± 0.3 (n=9) | 1.22[c] ± 0.32 (n=9) |

[a] ICD=Immune Complex Deposits. ICD intensity scale: 0=none; 1=moderate; 2=severe; 3=severe/extremely intense.
[b] The death of one animal with a high level of proteinuria resulted in a lower group mean.
[c] $p<0.05$ (compared to Captisol®-treated control mice; Mann-Whitney).

Figure 6 shows representative sections of one kidney from each treatment group. The top row sections are from a Captisol®-treated mouse, the mid-row sections are from a mouse treated with 50 µg/mouse Compound 1 and the bottom row sections are from a mouse treated with 25 µg/mouse Compound 1. It can be seen that the intensity of immune complex deposits observed in kidney sections of mice treated with Compound 1 (dissolved in Captisol®) at either dose level was much lower than that observed in the control group.

**Example 8: Phase I Clinical Study**

**A Phase I, Multicenter, Randomised, Double-Blind, Placebo Controlled, Single Dose, Four-Arm Study to Assess the Tolerability and Safety of Compound 1 in Captisol® Subcutaneous Injection in SLE Subjects.**

This was the first clinical study with Compound 1 in captisol® in humans, conducted in France. Its main objective was to evaluate tolerability and safety of Compound 1 in captisol®, administered as a single sc injection to SLE subjects. Its

secondary objective was to evaluate immunological responses following a single sc dose of Compound 1 in captisol® in these subjects.

Thirty-six (36) subjects participated in the study. To be eligible for inclusion in the study, SLE patients must have fulfilled at least four criteria used for the diagnosis of lupus by the American College of Rheumatology. Patients must also have had stable, mild/moderate disease and score less than or equal to 10 on the SLE Disease Activity Index, SLEDAI.

Each patient received a single sc injection of reconstituted Compound 1 for injection or its matching placebo (Captisol®) according to the following group assignment:

- **Group A**: Placebo (Captisol®)
- **Group B**: 0.5 mg Compound 1 in Captisol®
- **Group C**: 1 mg Compound 1 in Captisol®
- **Group D**: 2.5 mg Compound 1 in Captisol®

A standard battery of safety tests, including blood and urine collection for laboratory tests, was performed at screening, during the day of dosing, at 24 hours post-dose and at 2, 4 and 8 weeks following dosing. Prior to dosing, and on scheduled follow-up visits, blood samples were withdrawn for SLE-related immunological tests, anti-Compound 1 antibodies and PBL proliferation assay. The following immunology tests were performed:

- Coomb's (direct and indirect)
- C3, C4 and CH50
- Total IgG, IgM and IgA
- ANA, anti-dsDNA (Farr assay), anti-ssDNA
- Anti-ENA (including anti-La, anti-Ro, anti-RNP, anti-Sm)
- Anti-cardiolipin antibodies
- VDRL

- FTA antibodies
- Rheumatoid factor

The safety and tolerability of Compound 1 in captisol® in the subject population was evaluated on the basis of the following criteria:

- Occurrence of AEs, including SLE flare
- Vital signs
- 12-lead ECG
- Changes in physical examination
- Routine clinical laboratory tests
- SLEDAI score
- Immunological test results

**Phase Ia clinical study details**

**Study Principal Investigators and Respective Study Sites:** Six (6) study centers in France:Prof. Jean Charles Piette (Hopital La Pitie Salpetriere, Paris), Prof Oliver Meyer (Hopital Bichat, Paris), Prof. Jean Revuz (Hopital Henri Mondor, Creteil), Prof. Loic Guillevin (Hopital Avicenne, Bobigny), Prof. Eric Hachulla (Hopital Claude Huriez, Lille Cedex), Prof. Xavier Mariette (Hopital Bicetre, Kremlin Bicetre).

**Compound 1 (in captisol®), Placebo, Water for Injection-Ampoules, Dose and Mode of Administration:**
Vials of Compound 1 in Captisol® (120mg/vial) were injected subcutaneously as a single dose per subject in the following dosages:
0.5 mg Compound 1/vial in Captisol®, 1mg Compound 1/vial in Captisol® and 2.5 mg Compound 1/vial in Captisol®.
Placebo for Compound 1: 120 mg Captisol®/vial (identical in appearance to vials of Compound 1 in Captisol®).

**Methodology**

This was a multi-center, randomized; double blind, placebo-controlled, four-arm study, using a single subcutaneous injection of Compound 1 or placebo. SLE patients were screened up to 21 days prior to baseline procedures. Each eligible subject was randomized to one of the 4 treatment groups: subcutaneous injection of either 0.5, 1 or 2.5 mg Compound 1 or its matching placebo. All subjects were admitted to the clinic on pre-dosing day. Each subject received a single dose of one of the above listed treatments. Subjects were discharged from the clinic 24 hours after dosing. Subjects were further monitored at weeks 2, 4 and 8 following dosing. Blood samples (serum and whole blood) for safety laboratory tests were withdrawn at Screening, Dosing Day (pre-dose), Day 2 (post dose), at Weeks 2, 4 and 8 (Termination visit). Blood samples for immunological tests were withdrawn at: Screening, Dosing Day (pre-dose) and at Weeks 4 and 8. Peripheral blood lymphocytes (PBL) proliferation was evaluated at Dosing Day (pre-dose) and at Weeks 2, 4 and 8.

**Number of Subjects (total and for each treatment):**
Thirty six (36) subjects were randomized into this study as follows; 9 subjects into the 0.5 mg treatment group, 9 subjects into 1 mg treatment group, 10 subjects into the 2.5 mg treatment group, and 8 subjects into the placebo treatment group.

**Diagnosis and Main Criteria for Inclusion:**
Eligible subjects for this study were SLE patients who fulfilled at least four diagnostic criteria of the American College of Rheumatology (ACR). Their disease condition had to be stable, mild to moderate with a score equal to or less than 10 on the SLE disease activity index, year 2000 updated (SLEDAI 2K).

Excluded from participation were SLE patients who reported unstable or severe asthma, stroke, acute myocardial infarction, unstable angina, cerebral hemorrhage and pulmonary embolism during the six months prior to study screening. SLE patients

who had any clinically significant or unstable medical or surgical conditions, diabetes mellitus, liver disease (cirrhosis, active hepatitis, portal hypertension, and/or ascites), clinically significant hypertension, a medical history of any malignancy, dialysis, or chronic obstructive pulmonary disease (COPD) were also excluded from study participation.

Also excluded from study participation were SLE patients who underwent plasmapheresis or were treated during the three months prior to screening with one of the drugs listed below: prednisone 30mg/day or greater (or an equivalent dose of another corticosteroid), intravenous corticosteroids, intravenous immunoglobulin G (IgG), oral anticoagulants and any cytotoxic agents (e.g. azathioprine, chlorambucil, cyclophosphamide, mycophenolate mofetil, methothrexate, tacrolimus.

In addition, SLE patients initiating treatment with corticosteroids (more than ± 10 mg/day prednisone, or an equivalent dose of another corticosteroid) and/or anti-malarials, during the last 3 months prior to screening were excluded from the study.

While an effort was made to retain baseline SLE medical treatments throughout the course of the study, investigators could nevertheless change participant medical treatment at any time during the study to maintain and optimize patient welfare.

**Criteria for Evaluation**

**Safety:**

The following safety parameters were assessed at Screening, during the hospitalization and at follow-up visits including Termination visit: vital signs (systolic blood pressure, diastolic blood pressure, pulse, oxygen saturation, temperature and weight), 12-lead ECG, change in physical examination and

clinical routine laboratory safety tests. Adverse events were recorded at the Dosing Day and at each visit thereafter.

**Immunology:**

SLE-related immunological tests were performed at Screening, during the hospitalization and at follow-up visits including Termination visit.

Drug-related immunological responses were followed by using the PBL proliferation assay and anti-Compound 1 antibodies assay at the Dosing Day and at follow-up visits including Termination visit.

**Disease Activity:**

Disease activity assessment using the SLE disease activity index score, year 2000 updated (SLEDAI 2K) was assessed at Screening, during the hospitalization and at follow-up visits including Termination visit.

**Statistical Methods:**

SAS® version 9.0 software was used to analyze and present data collected during this study. No power calculation was performed and no formal hypothesis testing was conducted for this Phase Ia study.

**Adverse Experiences**

The incidence and the frequency of adverse experiences was presented by System Organ Class and preferred terminology according to MedDRA dictionary version 5.0. The data is tabulated by treatment group.

**Clinical Laboratory Data**

Descriptive statistics of laboratory values including number of observations, mean, standard deviation, minimum and maximum were determined for Screening, Day 1 (pre dose), Day 2, Week 2, 4 and

8 are presented by treatment group. Changes from baseline to each time point/visit are also presented for each visit by treatment assignment. Percent of abnormal results (low and high, where applicable) are presented on a parameter basis, by treatment group and visit/time point. Shift analyses from baseline to 24-hours post dose and from baseline to termination visit were performed.

**Vital Signs**

Descriptive statistics for vital signs including number of observations, mean, standard deviation, median, minimum and maximum values were determined for Screening, Day 1 (pre and post dose, and at each time point) Day 2, Weeks 2, 4 and 8 are tabulated by the assigned treatment. Changes from baseline to each time point/visit is presented in by visit and treatment assignment.

**Weight**

Descriptive Statistics of Weight (kg) at baseline, termination and change from baseline is presented by treatment group.

**ECG**

Descriptive statistics of ECG parameters at baseline, termination and changes from baseline are presented. Shift analysis is presented as tables of shift from baseline to termination between normal/abnormal or present/absent ECG parameters. Potentially clinically significant (PCS) QTc (Bazett) measurements were identified according to the predefined criteria. Tables of shift analysis between PCS and non-PCS Absolute QTc (Bazett) and incidence table of PCS change in QTc (Bazett) from baseline to any visit are presented.

**Physical Examination**

Physical examination results are analyzed by incidence of subjects with abnormal or normal findings for each body system

at Baseline and Termination visit. Shift analysis between normal to abnormal and vice versa was also applied. When no change from baseline occurred, it was defined as "other".

**Compound 1 related immunological tests**

For immunological parameters, descriptive statistics, including number of observations, mean, standard deviation, median, minimum and maximum values were calculated and are presented by treatment group and visit. Change from baseline to each follow-up visit is also presented by treatment group. Where applicable, number and percent of subjects with negative/positive results is presented by treatment group and visit.

**SLEDAI 2K**

Descriptive statistics, including mean, standard deviation, median, minimum and maximum values of SLEDAI 2K are presented.

**Results of Phase Ia clinical study:**

Subject Disposition and SLE Characteristics

Thirty six (36) study subjects entered and completed this study per protocol. The majority of subjects (34) in all treatment groups were female (94.4%) and Caucasian (30, 83.3%). The mean age for all treatment groups was 35.6 years (range of means from 32 to 39 years). Most of the subjects (91.7%) had between 4 to 6 American College of Rheumatology (ACR) diagnostic criteria and a mean group SLEDAI 2K score ranged from 2.1 to 4.1.

Safety Results

There was no prominent difference between study drug treatment groups and the placebo group in the incidence of AEs. The most common AEs in all groups were headache, classified as mild or moderate in nature and injection site reaction classified as mild in nature. Dose response was not seen. No serious adverse event (SAE) or AE classified as severe occurred during the study.

No clinically significant effect attributable to study drug was seen for hematology, biochemistry or urinalysis values.

No clinically significant effect attributable to the study drug was seen for vital signs parameters (systolic blood pressure, diastolic blood pressure, pulse, oxygen saturation).

No clinically significant effect attributable to the study drug was seen for temperature and weight.

No differences of clinical significance were seen between Compound 1 -treated groups and placebo for categorical ECG measurements and digitized ECG parameters. No PCS QTc absolute value and no QTc change from baseline > 60 msec was recorded. A similar number of subjects in Compound 1 -treated and placebo groups had QTcB change from baseline between 30 and 60 msec.

No clinically significant effects of Compound 1 on physical exam were noted.

Immunology Results

Evaluation of serum samples from all subjects indicated that a single subcutaneous administration of Compound 1 at the dose levels of 0.5, 1 and 2.5 mg/patient did not induce the development of anti-Compound 1 specific antibodies. Seven subjects had a response to Compound 1 above the cut-off. These elevated levels of antibodies were already present prior to dosing. No increase in the levels of antibodies was observed in the follow up period (two months) of the study. The sera of these subjects were analyzed for the isotype of the reactive antibodies. The response in two of the subjects was associated with the IgM isotype and with the IgG isotype in two others. None of the seven had specific IgE antibodies.

The peripheral blood lymphocytes (PBL) assay showed that 50 % of the subjects (18) were classified as responders (SI>2) with similar distribution in all treatment groups. The T cell response was relatively low and no association between Compound 1 treatment dose or concentration used in the assay and responder/ non-responder status could be detected, taking into consideration that only a single SC dose of the study drug was administered. Also, no indication of increased incidence of responder status over time was observed. The tetanus toxoid (TTX) assay that serves as a safety control shows that the response to TTX was preserved throughout the study period in all treatment groups indicating that Compound 1 in captisol® did not change the immunological response to TTX recall antigen.

The immunological findings are the result of the administration of only a single dose of the study drug Compound 1.

Disease Activity Results

No clinically significant effects of Compound 1 on the SLEDAI score (change of ≥ 3, ≤ 12 points) were noted during the study except for one subject in the 0.5 mg treatment group for whom a change in the SLEDAI score of 2 to 10 points was recorded between baseline and week 4 on the basis of an urinalysis showing pyuria. This urinalysis finding was not confirmed by the investigator as a lupus flare per protocol definition and was resolved with no treatment change.

**Conclusions**

This Phase Ia study showed that a single subcutaneous injected dose of Compound 1 of 0.5, 1 or 2.5 mg in 120 mg Captisol © was safe and well tolerated and allows continuation to a phase Ib multiple dose study.

**Example 9: Phase Ib Clinical Study**

**A Phase I, Multicenter, Bi-National, Randomized, Double-Blind, Four-Arm, Placebo Controlled, Multiple Dose Study to Assess the Tolerability and Safety of Compound 1 in Captisol® Subcutaneous Injections in SLE Subjects**

This study is being performed in order to evaluate the safety and tolerability of repeated Compound 1 sc administration to SLE subjects. The study's secondary objective is to evaluate immunological responses following repeated sc administration of Compound 1 in Captisol® in SLE subjects.

Compound 1 is given in doses of 0.5, 1.0 or 2.5 mg in Captisol®. The investigational product is administered every other day (excluding weekends) for a total of 12 sc injections, i.e. 3 doses a week for 4 weeks. Subjects are monitored on planned visits scheduled at 2, 4, 8 and 12 weeks after start of dosing. Safety and tolerability are evaluated using tests similar to those described in the Phase Ia Clinical Study above.

**Results**

This Phase Ib study shows that multiple subcutaneous injected doses of Compound 1 of 0.5, 1 or 2.5 mg in 120 mg Captisol ® are safe and well tolerated.

What is claimed is:

1. A pharmaceutical composition comprising

an aqueous carrier;

from 0.1 mg/ml to 20 mg/ml of the composition of a pharmaceutically acceptable salt of a peptide having the structural formula

$NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1); and

a substituted β-cyclodextrin in an amount effective to dissolve the peptide in the aqueous carrier,

wherein the composition has a pH between 4 and 9.

2. The pharmaceutical composition of claim 1, wherein the concentration of the salt of the peptide is at least 0.5 mg/ml.

3. The pharmaceutical composition of claim 2, wherein the concentration of the salt of the peptide is from 0.5 mg/ml to 10 mg/ml.

4. The pharmaceutical composition of claim 3, wherein the concentration of the salt of the peptide is from 0.5 mg/ml to 2.5 mg/ml.

5. The pharmaceutical composition of any one of claims 1-4, wherein the composition has a pH between 6.5 and 8.5.

6. The pharmaceutical composition of claim 5, wherein the composition has a pH between 7.5 and 8.5.

7. The pharmaceutical composition of any one of claims 1-6, wherein the pharmaceutically acceptable salt is an acetate salt.

8. The pharmaceutical composition of any one of claims 1-7, wherein the substituted β-cyclodextrin is a hydroxypropyl, a sulfobutyl ether, or a sulfopropyl ether substituted β-cyclodextrin.

9. The pharmaceutical composition of claim 8, wherein the substituted β-cyclodextrin is a sulfobutyl ether substituted β-cyclodextrin.

10. The pharmaceutical composition of claim 7, wherein the substituted β-cyclodextrin is hepta-(sulfobutyl ether)-β-cyclodextrin.

11. The pharmaceutical composition of any one of claims 1-10, further comprising a pharmaceutically acceptable buffer in an amount and of a type suitable to make the pH of the pharmaceutical composition in the range of 4-9.

12. A pharmaceutical composition comprising

an aqueous carrier;

from 0.1 mg/ml to 20 mg/ml of the composition of an acetate salt of a peptide having the structural formula $NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1); and

from 70 mg/ml to 170 mg/ml of the composition of hepta-(sulfobutyl ether)-β-cyclodextrin,

wherein the peptide and the hepta-(sulfobutyl ether)-β-cyclodextrin are dissolved in the aqueous carrier; and

wherein the composition has a pH between 6.5 and 8.5.

13. The pharmaceutical composition of claim 12, wherein the concentration of the acetate salt of the peptide is at least 0.5 mg/ml.

14. The pharmaceutical composition of claim 13, wherein the

concentration of the acetate salt of the peptide is from 0.5 mg/ml to 10 mg/ml.

15. The pharmaceutical composition of claim 13, wherein the concentration of the acetate salt of the peptide is from 0.5 to 2.5 mg/ml.

16. The pharmaceutical composition of claim 13, wherein the concentration of hepta-(sulfobutyl ether)-β-cyclodextrin is 120 mg/ml, and wherein the pH of the composition is between 7.5 and 8.5.

17. The pharmaceutical composition of claim 16, wherein the concentration of the acetate salt of the peptide is 1.0 mg/ml.

18. The pharmaceutical composition of claim 16, wherein the concentration of the acetate salt of the peptide is 2.5 mg/ml.

19. A method of alleviating symptoms of systemic lupus erythematosus (SLE) in a human subject comprising administering to the human subject the pharmaceutical composition of any one of claims 1-18 in an amount effective to alleviate the symptoms of SLE in the human subject.

20. The pharmaceutical composition of any one of claims 1-18 for use in treating SLE in a human subject.

21. A process for manufacturing the pharmaceutical composition of any one of claims 1-18 comprising the steps of:

a) preparing a solution of a substituted β-cyclodextrin in an aqueous carrier at a predetermined concentration;

b) adding a predetermined amount of a pharmaceutically acceptable salt of the peptide $NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1) to the solution of step a);

c) adjusting the pH of the solution of step b) until the peptide dissolves in the solution; and

d) if necessary, adjusting the pH of the solution of step c) to a pH of 4-9, thereby manufacturing the pharmaceutical composition.

22. The process of claim 21, wherein the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of from 70 mg/ml to 170 mg/ml.

23. The process of claim 22, wherein the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of 120 mg/ml.

24. The process of claim 21, wherein the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition of at least 0.1 mg/ml.

25. The process of claim 21, wherein the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition of at least 0.5 mg/ml.

26. The process of claim 21, wherein the predetermined amount of peptide is such which results in a final concentration

of peptide in the pharmaceutical composition of 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml or 0.1 mg/ml.

27. The process of claim 21, wherein step b) further comprises mixing the solution for 1 hour.

28. The process of claim 21, wherein in step c) the pH is adjusted using HCl or NaOH 1.0N.

29. The process of claim 21, further comprising filtering the solution of step d) through a cellulose acetate filter.

30. The process of claim 21, wherein

the predetermined concentration of the substituted β-cyclodextrin is such which results in a final concentration of substituted β-cyclodextrin in the pharmaceutical composition of 120 mg/ml;

the predetermined amount of peptide is such which results in a final concentration of peptide in the pharmaceutical composition of 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml or 0.1 mg/ml;

step b) further comprises mixing the solution for 1 hour; and

in step c) the pH is adjusted using HCl or NaOH 1.0N, further comprising filtering the solution of step d) through a cellulose acetate filter.

31. A pharmaceutical composition prepared by the process of any one of claims 21-30.

32. A process of lyophilizing the pharmaceutical composition of claim 2, comprising the steps of:

a) lowering the temperature of the pharmaceutical composition to -40°C;

b) holding the temperature at -40°C for a predetermined time;

c) raising the temperature of the solution to 20°C;

d) holding the temperature at 20°C for a predetermined time; and

e) reducing the pressure and holding the temperature at 20°C for a predetermined time, thereby lyophilizing the pharmaceutical composition.

33. The process of claim 32, wherein step a) is performed within 2 hours.

34. The process of claim 32, wherein step b) is performed within 3 hours.

35. The process of claim 32, wherein step c) is performed over 13 hours.

36. The process of claim 32, wherein step c) is performed at a pressure of 110µbar.

37. The process of claim 32, wherein step d) is performed over 13 hours.

38. The process of claim 32, wherein step d) is performed at a pressure of 110µbar.

39. The process of claim 32, wherein in step e) the pressure is reduced to 10µbar.

40. The process of claim 32, wherein step e) is performed over 5 hours.

41. The process of claim 32, wherein

step a) is performed within 2 hours;

step b) is performed within 3 hours;

step c) is performed over 13 hours and at a pressure of 110μbar;

step d) is performed over 13 hours and at a pressure of 110μbar; and

step e) is performed over 5 hours and the pressure is reduced to 10μbar.

42. A lyophilized pharmaceutical composition prepared by the process of any one of claims 32-41.

43. A process of lyophilizing the pharmaceutical composition of claim 2, comprising the steps of:

a) lowering the temperature of the pharmaceutical composition to -45°C;
b) holding the temperature at -45°C for a predetermined time;
c) raising the temperature of the solution to -20°C;
d) raising the temperature of the solution to 25°C; and
e) holding the temperature at 25°C for a predetermined time, thereby lyophilizing the pharmaceutical composition.

44. The process of claim 43, wherein step a) is performed within 6 hours.

45. The process of claim 43, wherein step b) is performed within 3 hours.

46. The process of claim 43, wherein step c) is performed over 19 hours.

47. The process of claim 43, wherein step c) is performed at a pressure of 150μbar.

48. The process of claim 43, wherein step d) is performed over 13 hours.

49. The process of claim 43, wherein step d) is performed at a pressure of 150μbar.

50. The process of claim 43, wherein step e) is performed over 8 hours.

51. The process of claim 43, wherein step e) is performed at a pressure of 150μbar.

52. The process of claim 43, wherein

step a) is performed within 6 hours;

step b) is performed within 3 hours;

step c) is performed over 19 hours and at a pressure of 150μbar;

step d) is performed over 13 hours and at a pressure of 150μbar; and

step e) is performed over 8 hours and at a pressure of 150μbar.

53. A lyophilized pharmaceutical composition prepared by the process of any one of claims 43-52.

54. The lyophilized pharmaceutical composition of claim 53, wherein the water content of the composition is less than 5%.

55. The lyophilized pharmaceutical composition of claim 54, wherein the water content of the composition is less than 4.0%.

56. The lyophilized pharmaceutical composition of claim 55, wherein the water content of the composition is less then 3.5%.

57. A lyophilized pharmaceutical composition comprising

a pharmaceutically acceptable salt of a peptide having the structural formula

$NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-COOH (SEQ ID NO:1); and

a substituted β-cyclodextrin.

58. A packaged pharmaceutical composition comprised of:

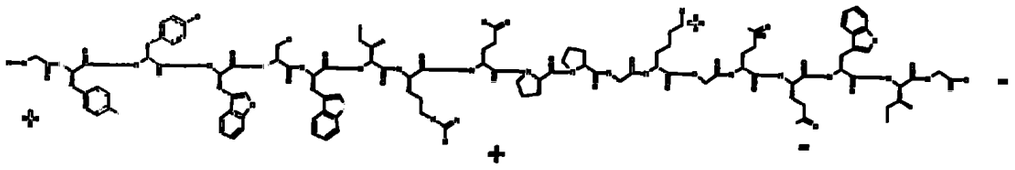
a packaging material; and

a predetermined amount of the lyophilized pharmaceutical composition of claim 57.

WO 2004/064788

**Figure 1**

$^{NH_2}$—G Y Y W S W I R Q P P G K G E E W I G—$^{COOH}$



$^{NH_2}$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly$^{COOH}$

Molecular Weight =2309 59
Exact Mass =2307
Molecular Formula =$C_{111}H_{149}N_{27}O_{28}$
Molecular Composition =C 57 73% H 6 50% N 16 37% O 19 40%

Acetic to be added $CH_3CO_2H$ = $C_2H_4O_2$

$C_{111}H_{149}N_{27}O_{28}$ $C_2H_4O_2$



PCT/US2004/000045

**Figure 2**

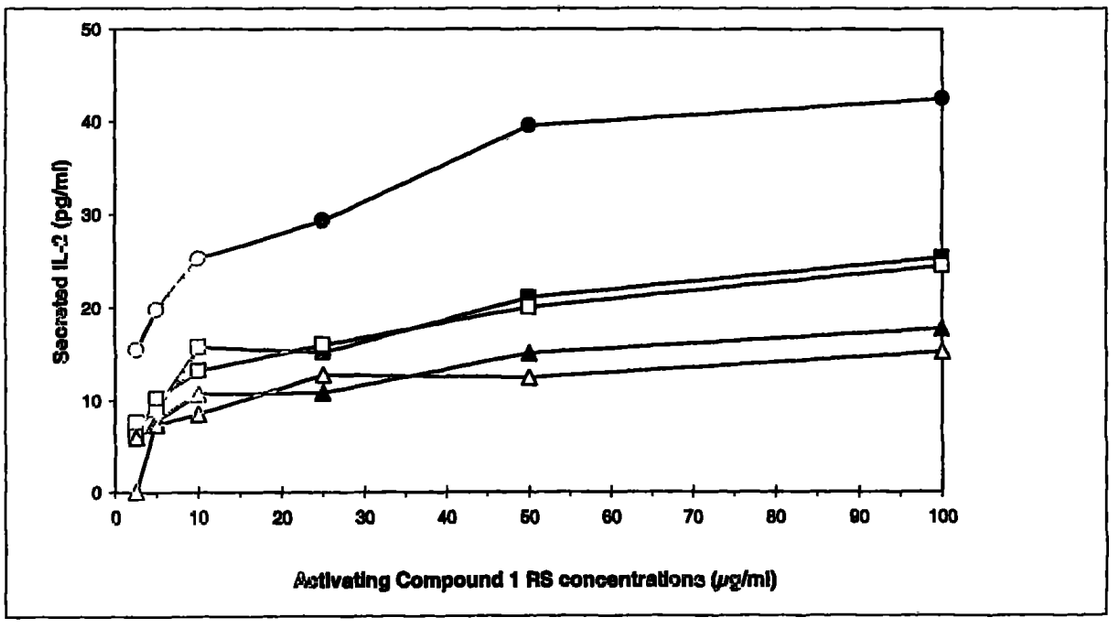

Secreted IL-2 (pg/ml)
0
10
20
30
40
50
0
10
20
30
40
50
60
70
80
90
100
Activating Compound 1 RS concentrations (µg/ml)

Figure 3

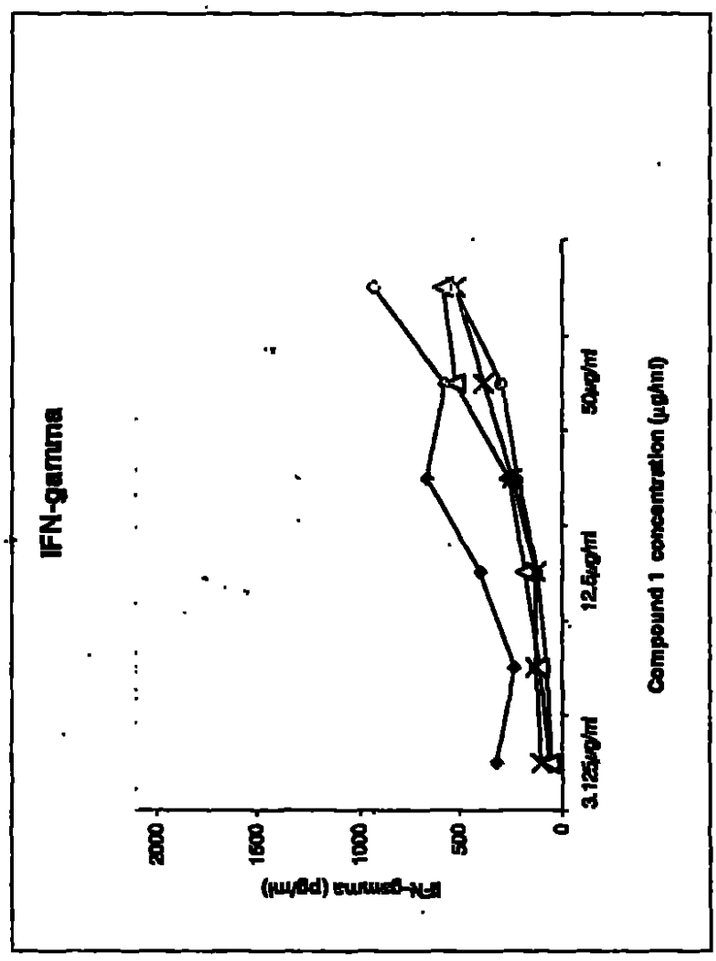

IFN-gamma
IFN-gamma (pg/ml)
2000
1500
1000
500
0
3.125µg/ml
12.5µg/ml
50µg/ml
Compound 1 concentration (µg/ml)

Figure 4

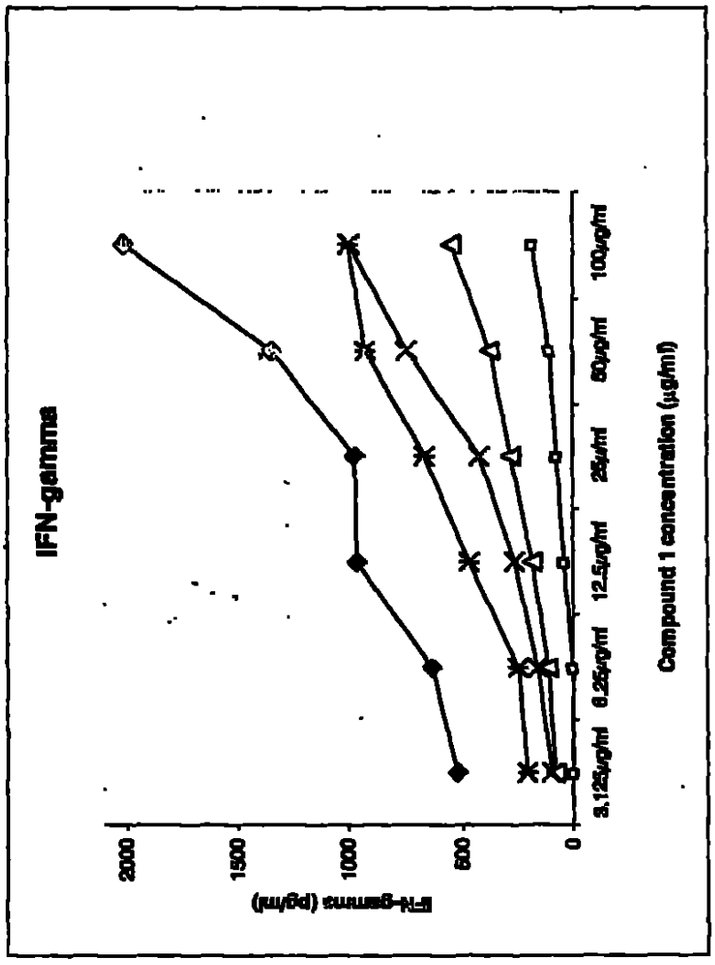
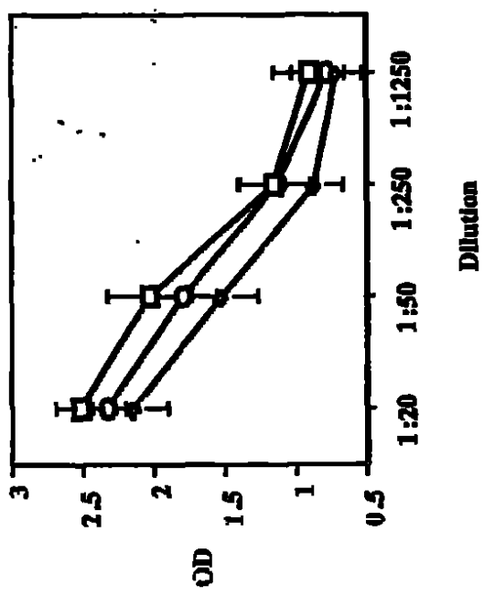

IFN-gamma
IFN-gamma (pg/ml)
2000
1500
1000
500
0
3.125µg/ml
6.25µg/ml
12.5µg/ml
25µg/ml
50µg/ml
100µg/ml
Compound 1 concentration (µg/ml)

Figure 5



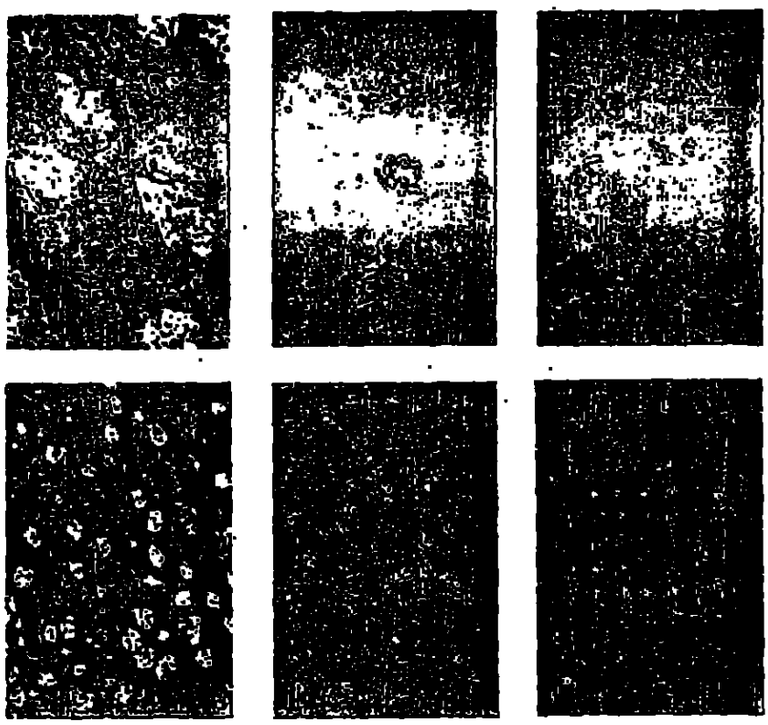


Figure 6

# FORMULACIONES PARENTERALES DE UN PÉPTIDO PARA EL TRATAMIENTO DE LUPUS SISTÉMICO ERITEMATOSO

Esta solicitud reclama el beneficio de la solicitud provisional EUA No. 60/439, 918, presentada el 14 de Enero de 2003, cuyo contenido total se incorpora en la presente invención por referencia.

En toda esta solicitud, se hace referencia a varias publicaciones por medio de citas. Las descripciones de estas publicaciones en tu totalidad se incorporan en la presente invención por referencia dentro de esta solicitud para poder describir más ampliamente el estado de la técnica tal y como se conoce por aquellos expertos en la presente invención, a partir que la fecha de invención se describió y reclamó en la presente.

## ANTECEDENTES DE LA INVENCIÓN

El lupus eritematoso sistémico (SLE), o lupus, es una enfermedad auto-inmune debilitante que se caracteriza por la presencia de un arreglo o disposición de anticuerpos, incluyendo anticuerpos a dsADN, a antígenos nucleicos y a ribonucleoproteínas. El lupus eritematoso sistémico (SLE) afecta aproximadamente de uno a de 2000 individuos en los Estados Unidos (1 de 700 mujeres). La enfermedad principalmente afecta a mujeres jóvenes, con una

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proporción de sexo masculino a sexo femenino de aproximadamente 9:1.

El lupus sistémico puede afectar casi a cualquier órgano o sistema del cuerpo. El lupus sistémico puede incluir períodos en donde pocos síntomas, sino es que ningún síntoma es evidente ("remisión") y otras ocasiones cuando la enfermedad se vuelve más activa ("erupción"). Con mucha frecuencia, cuando la gente menciona "lupus", se refieren a la forma sistémica de la enfermedad.

Los corticoesteroides son el sostén principal en el tratamiento de enfermedades sistémicas auto inmunes. El riesgo de perder la vida, y severamente deshabilitando manifestaciones del SLE, se tratan con altas dosis de glucocorticoides (1-2 mg/kg/día). Los efectos indeseables de los glucocorticoides crónicos incluyen un arreglo de exposición de efectos adversos prominentes tales como la reacción de Cushing, obesidad central, hipertensión, infección, fragilidad capilar, hirsutismo (hipertricosis), osteoporosis acelerada, cataratas, *diabetes mellitus*, miopatía y psicosis. Además de la toxicidad corticoesteroide, el cumplimiento del paciente hacia un régimen de dosificación también plantea un problema muy serio.

Los agentes citotóxicos también se utilizan para controlar enfermedades activas, reduciendo la tasa de

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erupciones de la enfermedad, y reduciendo los requerimientos de esteroides. Los efectos colaterales indeseables de esta última incluyen depresión de médula ósea, infecciones incrementadas con organismos oportunistas, con fallas ováricas irreversibles, alopecia y un riesgo de malignidad incrementado.

El SLE es una enfermedad inflamatoria para la cual hasta la fecha no existe un tratamiento definitivo o cura. La enfermedad resulta en complicaciones crónicas y agudas. Los únicos tratamientos disponibles son paliativos, destinados a aliviar los síntomas agudos y evitar complicaciones crónicas, con frecuencia con efectos secundarios profundos. Por lo tanto, existe una necesidad en este campo que no ha sido cumplido, y que tanto médicos como pacientes darían la bienvenida a nuevos tratamientos que pudieran eliminar potencialmente o reducir las manifestaciones indeseadas de la enfermedad.

Los péptidos que se basan en la región determinante de complementariedad del anticuerpo 16/6Id anti-ADN monoclonal humano que tiene la capacidad de inmunomodular las respuestas asociadas con el SLE se han descrito en la publicación internacional PCT No. WO 02/067848 A2, cuyo contenido total se incorpora en la presente invención. Particularmente, se encontró que la región CDR1 inhibe la respuesta proliferativa de linfocitos

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sanguíneos periféricos (PBL) de los pacientes SLE al mAb 16/6Id anti-ADN humano, y para mejorar las manifestaciones de la enfermedad en ratones afligidos con SLE espontáneo o experimental.

El CDR1 humano, que es el Compuesto 1, que se muestra en la figura 1, es un péptido sintético de 19 aminoácidos que se basa en la región 1 determinante de la complementariedad (CDR1) del mAb anti-ADN humano denotado 16/6 Id (Waisman, A., et al. "Modulation of murine systemic lupues erythematosus with peptides based on complementarity determining regions of pathogenic anti-DNA monoclonal antibodies" "Modulación de lupus sistémico eritematoso de murino con péptidos basados en regiones determinantes de complementariedad de anticuerpos monoclonales anti-ADN patogénicos". (Proc. Natl. Acad. Sci. E.U.A. (1997), 94 (4): 4620-4625).

En modelos SLE experimentales - el tratamiento con ratones Balb/c y ratones propensos a SLE, es decir, ratones (NZBxNZW) $F_1$, ya sea con péptidos basados en mCDR o el Compuesto 1 significativamente redujeron el SLE relacionado, notablemente con depósitos complejos inmunes (ICD) en los riñones, proteinuria y leucopenia. El tratamiento no tuvo efecto en la respuesta de anticuerpos específicos 16/6 Id (Waisman, A., *et al.* "Modulation of murine systemic lupus erythematosus with peptides based on

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complementarity determining regions of pathogenic anti-DNA monoclonal antibodies" ("Modulación del lupus sistémico erimatoso de murino con péptidos basados en regiones que determinan la complementariedad de anticuerpos monoclonales anti-ADN patogénicos"), (Proc. Natl. Acad. Sci. E.U.A. (1997), 94 (4): 620; Eilat, E., *et al.*, "Prevention of systemic lupus erithematosus-like disease in (NZBxNZW) $F_1$ mice by treating with CDR1- and CDR3- based peptides of pathogenic autoantibody" ("Prevención de enfermedad similar del lupus sistémico eritematoso en ratones (NZBxNZW) $F_1$ tratando con péptidos basados en CDR1- y CDR3- de auto-anticuerpos patogénicos") J. Clin Immunol. (200), 20: 268, Eilat, E., *et al.*, "The mechanism by which a peptide Based on complementarity determining region-1 of pathogenic anti-DNA antibody ameliorates experimental SLE" (2001). ("El mecanismo mediante el cual un péptido basado en la región-1 determinante de la complementariedad de anticuerpos anti-ADN patogénicos, disminuye el SLE experimental" (2001), Proc. Natl. Acad. Sci. E.U.A. 98: 1148).

Estos péptidos, como muchos otros péptidos, no son muy solubles. Por lo tanto, en la presente invención se presentan formulaciones que mejoran la solubilidad de los péptidos.

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## SUMARIO DE LA INVENCIÓN

La presente invención provee una composición farmacéutica que comprende:

Un vehículo acuoso; de 0.1 mg/ml a 20 mg/ml de la composición de una sal farmacéuticamente aceptable de un péptido que tiene la fórmula estructural $_{NH2}$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooh (SEC ID NO:1).

Una β ciclodextrina sustituída en una cantidad efectiva para disolver el péptido en el vehículo acuoso, en donde la composición tiene un pH entre 4 y 9.

La invención sujeto también provee una composición farmacéutica que también comprende:

Un vehículo acuoso; de 0.1 mg/ml a 20 mg/ml de la composición de la sal de acetato de un péptido que tiene la fórmula estructural $_{NH2}$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooh (SEC ID NO:1); y de 70 mg/ml a 170 mg/ml de la composición de hepta-sulfobutileter-β-ciclodextrina, en donde el péptido y el hepta-sulfobutileter-β-ciclodextrina se disuelven en el vehículo acuoso; y en donde la solución tiene un pH entre 6.5 y 8.5.

La invención sujeto también provee un método para aliviar los síntomas del lupus sistémico eritematoso (SLE) en un sujeto humano que comprende administrar al sujeto

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humano cualquiera de las composiciones farmacéuticas anteriores en una cantidad efectiva para aliviar los síntomas del SLE en el sujeto humano.

La invención sujeto también provee un procedimiento de fabricación de la composición farmacéutica anterior que comprende los siguientes pasos:

a) Preparación de una solución de un β-ciclodextrina sustituída en un vehículo acuoso a una concentración predeterminante;

b) Agregar una cantidad predeterminante de una sal farmacéuticamente aceptable del péptido $_{NH2}$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooh (SEC ID NO:1)a la solución del paso a);

c) Ajustar el pH de la solución del paso b) hasta que el péptido se disuelva en la solución; y

d) si es necesario, ajustar el pH de la solución del paso c) a un pH de 4-9, de esa manera fabricar la composición farmacéutica.

La invención sujeto también provee un procedimiento de liofilización de la composición farmacéutica anterior, que comprende los pasos de:

a) Reducir la temperatura de una composición farmacéutica a -40°C;

b) Sostener la temperatura a -40°C por un tiempo predeterminado;

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c) Elevar la temperatura de la solución a 20°C;

d) Sostener la temperatura a 20°C por un tiempo predeterminado; y

e) Reducir la presión a 10μbarias, de esa manera liofilizar la composición farmacéutica.

La invención sujeto también provee un procedimiento de liofilización de la composición farmacéutica anterior, que comprende los pasos de:

a) Reducir la temperatura de la composición farmacéutica a -45°C;

b) Sostener la temperatura a -45°C por un tiempo predeterminado;

c) Elevar la temperatura de la solución a -20°C;

d) Elevar la temperatura de la solución a 25°C; y

e) Sostener la temperatura a 25°C por un tiempo predeterminado, de esa manera liofilizar la composición farmacéutica.

## BREVE DESCRIPCIÓN DE LAS FIGURAS

Figura 1.- CDR1 (compuesto 1) humano como sal de acetato que muestra las fórmulas moleculares y estructurales de hCDR1, la secuencia de aminoácidos y los parámetros físicos.

Figura 2.- Secreción de IL-2 de células tomadas de ratones tratados con el Compuesto 1 y con solución de Captisol® después que las células fueron

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posteriormente activadas con una solución del Compuesto 1 en PBS.

-■- Compuesto 1 (RS) 50 µg/ratón

-▲- Compuesto 1 (RS) 200 µg/ratón

-□- DP 50 µg/ratón

-△- DP 200 µg/ratón

-°- 12% Captisol® en ampolleta

Figura 3.- Secreción de IFN-γ de células tomadas de ratones con solución de Compuesto 1 después que la células fueron subsecuentemente activadas con una solución del Compuesto 1 en EM-1 (2.5 x $10^6$ células/pozo).

-◆- Placebo

-●- Compuesto 1 50 µg/ratón (dosis de tratamiento)

-△- Compuesto 1 100 µg/ratón (dosis de tratamiento)

-×- Compuesto 1 200 µg/ratón (dosis de tratamiento)

Figura 4.- Secreción de IFN-γ a partir de células tomadas de ratones tratados con la solución del Compuesto 1, después que las células fueron subsecuentemente activadas con una solución del Compuesto 1 en EM-1 (5 x $10^6$ células/pozo).

-◆- Placebo

-□- Compuesto 1 25 µg/ratón

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-Δ- Compuesto 1 50 µg/ratón

-×- Compuesto 1 100 µg/ratón

-*- Compuesto 1 200 µg/ratón

Figura 5.- Anticuerpos anti-dsADN en ratones (NZBxNZW) F1 después de 10 inyecciones con Compuesto 1 en solución de Captisol® [OD = Densidad óptica; Compuesto 1 (C) = Compuesto 1 disuelto en Captisol®]

-□- Placebo

-◇- Compuesto 1 50 µg/ratón

-o- Compuesto 1 25 µg/ratón

Figura 6.- Las secciones de riñón de ratones (NZBxNZW) F1 que mostraban intensidad de Depósitos complejos inmunes. Las secciones de la fila superior son de ratones tratados con Captisol®, las secciones de la fila media, son de un ratón tratado con el Compuesto 1 con 50 µg/ratón, y las secciones de la fila inferior son de un ratón tratado con 25 µg/ratón Compuesto 1. *Amplificación:* izquierda x100, Derecha: x400. Inmunohistología FITC.

## DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

La presente invención provee una composición farmacéutica que comprende:

un vehículo acuoso;

de 0.1 mg/ml a 20 mg/ml de una sal farmacéuticamente aceptable de de un péptido que tiene la

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fórmula estructural $_{NH2}$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooh(SEC ID NO:1);y

Una β ciclodextrina sustituída en una cantidad efectiva para disolver el péptido en el vehículo acuoso, en donde la composición tiene un pH entre 4 y 9.

En una modalidad, la concentración de sal de acetato del péptido es por lo menos 0.5 mg/ml.

En una modalidad, la concentración de sal del péptido es de 0.5 mg/ml a 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 0.5 mg/ml a 2.5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 2.5 mg/ml a 5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 5 mg/ml a 7 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 7 mg/ml a 8.5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 8.5 mg/ml a 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 9 mg/ml a 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 10 mg/ml a 15 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 15 mg/ml a 20 mg/ml.

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En otra modalidad, la concentración de sal del péptido es de 1.0 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 2.5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 15 mg/ml.

En otra modalidad, la concentración de sal es de 0.1 mg/ml a 0.5 mg/ml.

En otra modalidad, la concentración de sal es de 0.1 mg/ml a 0.2 mg/ml.

En otra modalidad, la concentración de sal es de 0.2 mg/ml a 0.3 mg/ml.

En otra modalidad, la concentración de sal es de 0.3 mg/ml a 0.4 mg/ml.

En otra modalidad, la concentración de sal es de 0.4 mg/ml a 0.5 mg/ml.

En una modalidad adicional, la composición tiene un pH entre 6.5 y 8.5.

En una modalidad adicional, la composición tiene un pH entre 7.5 y 8.5.

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En una modalidad adicional, la composición tiene un pH entre 4 y 5.

En una modalidad adicional, la composición tiene un pH entre 5 y 6.

En una modalidad adicional, la composición tiene un pH entre 6 y 7.

En una modalidad adicional, la composición tiene un pH entre 7 y 8.

En una modalidad adicional, la composición tiene un pH entre 8 y 9.

En otra modalidad, la sal farmacéuticamente aceptable es una sal de acetato.

En otra modalidad, la β-ciclodextrina sustituida es hidroxipropilo, éter sulfobutílico, éter sulfopropílico β-ciclodextrina sustituida.

En una modalidad adicional, la β-ciclodextrina sustituida es una β-ciclodextrina de eter sulfobutílico sustituida.

En una modalidad adicional, la sal farmacéuticamente aceptable es una sal de acetato, y la β-ciclodextrina sustituida es hepta-(sulfobutil éter)- β-ciclodextrina.

En otra modalidad, la composición además comprende un regulador de pH farmacéuticamente aceptable en una cantidad y de un tipo adecuado para lograr que el pH de

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la composición farmacéuticamente sea de la escala de 4-9. El regulador de pH puede ser regulador de pH acetato, regulador de pH de citrato, o carbonato de sodio.

La invención sujeto también provee una composición farmacéutica que también comprende:

Un vehículo acuoso; de 0.1 mg/ml a 20 mg/ml de la composición de la sal de acetato de un péptido que tiene la fórmula estructural $NH_2$-Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooh (SEC ID NO:1); y de 70 mg/ml a 170 mg/ml de la composición de hepta-sulfobutileter-β-ciclodextrina, en donde el péptido y el hepta-sulfobutileter-β-ciclodextrina se disuelven en el vehículo acuoso; y en donde la solución tiene un pH entre 6.5 y 8.5.

En una modalidad, la concentración de sal de acetato del péptido es por lo menos 0.5 mg/ml.

En una modalidad, la concentración de sal de acetato del péptido es de 0.5 mg/ml a 10 mg/ml.

En una modalidad adicional, la concentración de sal de acetato del péptido es de 0.5 mg/ml a 2.5 mg/ml.

En otra modalidad, la concentración de sal es de 0.1 mg/ml a 0.5 mg/ml.

En otra modalidad, la concentración de sal es de 0.1 mg/ml a 0.2 mg/ml.

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En otra modalidad, la concentración de sal es de 0.2 mg/ml a 0.3 mg/ml.

En otra modalidad, la concentración de sal es de 0.3 mg/ml a 0.4 mg/ml.

En otra modalidad, la concentración de sal es de 0.4 mg/ml a 0.5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 5 mg/ml a 7 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 7 mg/ml a 8.5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 8.5 mg/ml a 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 9 mg/ml a 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 10 mg/ml a 15 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 15 mg/ml a 20 mg/ml.

En una modalidad adicional, la concentración de sal de acetato del péptido es de 1.0 mg/ml.

En una modalidad adicional, la concentración de sal de acetato del péptido es de 2.5 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 5 mg/ml.

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En otra modalidad, la concentración de sal del péptido es de 10 mg/ml.

En otra modalidad, la concentración de sal del péptido es de 15 mg/ml.

En otra modalidad, la concentración de hepta-(sulfobutil éter)- β-ciclodextrina es de 120 mg/ml y el pH de la composición es entre 7.5 y 8.5.

La invención sujeto también provee un método para aliviar los síntomas del lupus sistémico eritematoso (SLE) en un sujeto humano que comprende administrar al sujeto humano cualquiera de las composiciones farmacéuticas anteriores en una cantidad efectiva para aliviar los síntomas del SLE en el sujeto humano.

La invención sujeto también provee las composiciones farmacéuticas anteriores para uso en tratamiento de SLE en un sujeto humano.

La invención sujeto también provee un procedimiento de fabricación de la composición farmacéutica anterior que comprende los siguientes pasos:

a) Preparación de una solución de un β-ciclodextrina sustituída en un vehículo acuoso a una concentración predeterminante;

b) Agregar una cantidad predeterminante de una sal farmacéuticamente aceptable del péptido $_{NH2}$-Gly Tyr Tyr

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Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooh (SEC ID NO:1)a la solución del paso a);

c) Ajustar el pH de la solución del paso b) hasta que el péptido se disuelva en la solución; y

d) si es necesario, ajustar el pH de la solución del paso c) a un pH de 4-9, de esa manera fabricar la composición farmacéutica.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida en la composición farmacéutica es de 70 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 80 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 90 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de

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final de β-ciclodextrina sustituida en la composición farmacéutica de 100 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 110 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 120 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 130 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 140 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de

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final de β-ciclodextrina sustituida en la composición farmacéutica de 150 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 160 mg/ml a 170 mg/ml.

En una modalidad del procedimiento, la concentración resultante final de la β-ciclodextrina sustituida y es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica 120 mg/ml.

En otra modalidad, la cantidad predeterminada del péptido es tal que resulta en una concentración final del péptido en la composición farmacéutica que es por lo menos de 0.1 mg/ml.

En otra modalidad, la cantidad predeterminada del péptido es tal que resulta en una concentración final del péptido en la composición farmacéutica que es por lo menos de 0.5 mg/ml.

En otra modalidad, la cantidad predeterminada del péptido es tal que resulta en una concentración final del péptido en la composición farmacéutica que es 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml o 0.1 mg/ml.

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En otra modalidad, la cantidad predeterminada del péptido es tal que resulta en una concentración final del péptido en la composición farmacéutica que es 5 mg/ml, 10 mg/ml o 15 mg/ml.

En otra modalidad del procedimiento, el paso b) que además comprende la mezcla de la solución por 1 hora.

En otra modalidad, en el paso c) el pH se ajusta utilizando HCl o NaOH al 1.0N.

En una modalidad adicional, el procedimiento además comprende filtrar la solución de la etapa d) a través de un filtro de acetato de celulosa.

En otra modalidad del procedimiento anterior, la concentración predeterminada de la β-ciclodextrina sustituida es tal que resulta en una concentración de final de β-ciclodextrina sustituida en la composición farmacéutica de 120 mg/ml;

la cantidad predeterminada de péptido es tal que resulta en una concentración final de péptido en la composición farmacéutica de 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml o 0.1 mg/ml; el paso b) además comprende mezclar la solución por una hora;y en la etapa c) el pH se ajusta utilizando HCl o NaOH 1.0N, que además comprende filtrar la solución de la etapa d) a través de un filtro de acetato de celulosa.

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La presente invención también provee una composición preparada por medio del procedimiento anterior.

La presente invención también provee un procedimiento para liofilizar cualquiera de las composiciones farmacéuticas anteriores que comprende los siguientes pasos:

a) disminuir la temperatura de la composición farmacéutica a -40°C;

b) sostener la temperatura a -40°C durante un tiempo predeterminado;

c) elevar la temperatura de la solución a 20°C;

d) sostener la temperatura a 20°C durante un tiempo predeterminado; y

e) reduciendo la presión y sosteniendo la temperatura a 20°C durante un tiempo predeterminado, liofilizando de esa manera la composición farmacéutica.

En una modalidad, la etapa a) se desarrolla en un periodo de 2 horas.

En otra modalidad, la etapa b) se desarrolla en un periodo de 3 horas.

En una modalidad adicional, la etapa c) se desarrolla en un periodo de más de 13 horas.

En una modalidad adicional, la etapa c) se desarrolla en una presión de 110 µbarias.

En una modalidad adicional, la etapa d) se desarrolla en un periodo de más de 13 horas.

En una modalidad adicional, la etapa d) se desarrolla en una presión de 110 µbarias.

En una modalidad adicional, la etapa e) la presión se reduce a 10 µbarias.

En una modalidad, la etapa e) se desarrolla en un periodo de más de 5 horas.

En otra modalidad del procedimiento,

la etapa a) se desarrolla en un periodo de 2 horas.

la etapa b) se desarrolla en un periodo de 3 horas.

la etapa c) se desarrolla en un periodo de más 13 horas y a una presión de 110 µbarias

la etapa d) se desarrolla en un periodo de más 13 horas y a una presión de 110 µbarias; y

la etapa e) se desarrolla en un periodo de más 5 horas y a una presión de 10 µbarias

La presente invención también provee una composición farmacéutica liofilizada preparada por el procedimiento anterior.

La presente invención también provee un procedimiento para liofilizar la composición farmacéutica anteriore, que comprende las etapas de:

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a) disminuir la temperatura de la composición farmacéutica a -45°C;

b) sostener la temperatura a -45°C durante un tiempo predeterminado;

c) elevar la temperatura de la solución a -20°C;

d) sostener la temperatura a 25°C durante un tiempo predeterminado; y

e) reduciendo la presión y sosteniendo la temperatura a 25°C durante un tiempo predeterminado, liofilizando de esa manera la composición farmacéutica.

En una modalidad, la etapa a) se desarrolla en un periodo de 6 horas.

En una modalidad, la etapa b) se desarrolla en un periodo de 3 horas.

En una modalidad, la etapa c) se desarrolla en un periodo de más de 19 horas.

En una modalidad, la etapa c) se desarrolla en una presión de 150 µbarias.

En una modalidad, la etapa d) se desarrolla en un periodo de más de 13 horas.

En una modalidad, la etapa d) se desarrolla en una presión de 150µbarias.

En una modalidad, la etapa e) se desarrolla en un periodo de más de 8 horas.

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En una modalidad, la etapa e) se desarrolla en una presión de 150µbarias.

En otra modalidad del procedimiento,

la etapa a) se desarrolla en un periodo de 6 horas.

la etapa b) se desarrolla en un periodo de 3 horas.

la etapa c) se desarrolla en un periodo de más 19 horas y a una presión de 150 µbarias

la etapa d) se desarrolla en un periodo de más 13 horas y a una presión de 150 µbarias

la etapa e) se desarrolla en un periodo de más 8 horas y a una presión de 150 µbarias

La presente invención también provee una composición farmacéutica liofilizada preparada por cualquiera de los procedimientos anteriores.

En otra modalidad de la composición farmacéutica liofilizada anterior, el contenido de agua de la composición es menor de 5%.

En una modalidad, el contenido de agua de la composición es menor que 4.0%.

En una modalidad, el contenido de agua de la composición es menor que 3.5%.

La presente invención también provee una composición farmacéutica liofilizada que comprende:

Una sal farmacéuticamente aceptable de un péptido que tiene la fórmula estructural $NH_2$ - Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly - COOH (SEC ID NO: 1); y una β-ciclodextrina sustuida.

La invención sujeto también provee una composición farmacéutica envasada que comprende:

Un material de envase; y una cantidad predeterminada de la composición farmacéutica liofilizada anterior.

Las preparaciones de la presente invención pueden administrarse parenteralmente, tópicamente, o rectalmente. Por supuesto se dan por formas adecuadas para cada ruta de administración. Por ejemplo, se administran mediante inyección, inhalación, ungüento, supositorio, etc. o por administración por medio de inyección, infusión, inhalación; tópica por medio de loción o ungüento; y rectal por medio de supositorios.

Las frases "administración parenteral" y "parenteralmente administrada" como se utiliza en la presente invención son modos de administración diferentes de la administración tópica y enteral, generalmente por inyección e incluye, sin limitación alguna, intravenosa, muscular, intrarterial, intratecal, intracapsular, intraorbital, intracardiaca, intradérmica, intraperitonal, transtraqueal, subcutánea, subcuticular, intraarticular,

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subcapsular, subaracnoidea, intraespinal e inyección e infusión intraesternal.

Las frases "administración sistémica", "sistemáticamente administrada", "administración periférica" y "periféricamente administrada" como se utiliza en la presente invención significa que es la administración de un compuesto, fármaco u otro material diferente del que se inyecta directamente en el sistema nervioso central, de tal forma que entra al sistema del paciente y por lo tanto es sujeto al metabolismo y otros procedimientos similares, por ejemplo, la administración subcutánea.

Los detalles de los procedimientos y la información de la formulación general en excipientes adicionales, se pueden encontrar en Remington: The Science and Practice of Pharmacy, "Remington: La Ciencia y Práctica de la Farmacia", 20a. Edición.

Esta invención se entenderá mejor a partir de los siguientes Detalles Experimentales; sin embargo, aquellos expertos en la técnica apreciarán fácilmente que los métodos y resultados específicos que se describen son simplemente ilustrativos de la invención tal como se analizan más completamente en las reivindicaciones a continuación.

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Detalles experimentales

**EJEMPLO 1**

**Desarrollo de la formulación para el Compuesto 1**

El péptido hCDR1 humano (Compuesto 1) se describe en la Publicación Internacional PCT No. WO 02/067848, publicada el 6 de septiembre de 2002, y puede prepararse por métodos muy conocidos en la técnica, (véase, por ejemplo, "Peptides: Síntesis, Structure and Applications", ("Péptidos: Síntesis, Estructura y Aplicaciones")editado por B. Gutte, Academia Press, 1995: "Peptide Síntesis Protocols" ("Protocolos de Síntesis de Péptido")editado por M. Pennington y B. Duna, Humana Press, 1994; Schnolzer, M. et al., "In situ neutralization in Boc-chemistry solid phase síntesis. Rapid,High yield assembly of difficult sequences" ("Neutralización *In situ* en la síntesis de fase sólida Bio-química. Ensamble de rápido y alto rendimiento de secuencias difíciles") Int. J. Pept. Protein Res. (1992) 40: 180-193).

El compuesto 1 es un polipéptido sintético compuesto de 19 aminoácidos. Es provisto como una sal de acetato. La solubilidad acuosa del péptido ha sido determinada menor que 0.5 mg/ml. La Figura 1 muestra el compuesto 1 como una sal de acetato.

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Para poder desarrollar una formulación con una concentración de péptidos que excedan 2 mg/ml, de preferencia hasta 10 mg/ml, se desarrollaron experimentos con varios incrementadotes de solubilidad. Los experimentos preliminares indicaron que una concentración de 2 mg/ml no se puede lograr tan fácilmente. Para poder desarrollar una formulación para inyección subcutánea, también es deseable que el pH esté en la escala de 4 a 9, y que la solución sea iso-osmótica.

Basándose en estudios de literatura extensa, se adoptaron algunos enfoques principales para poder producir una formulación con una solubilidad máxima. Los factores considerados fueron:

- Ajuste de pH y reguladores de pH
- Solventes
- Co-solventes
- Agentes solubilizantes

Métodos

El compuesto 1 se disolvió en la solución incrementadora de solubilidad elegida, ya sea separada o en combinación con otros excipientes y las soluciones se agitaron por lo menos por una hora. El pH se ajustó si se necesitaba. Las soluciones se examinaron visualmente para calcular la solubilidad, y fueron enviadas para determinación de prueba analítica. Para algunas

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formulaciones elegidas, también se sometió a prueba la actividad biológica.

Resultados

El Cuadro 1 presenta el tipo de incrementadores de solubilidad que se utilizan para el desarrollo de la formulación. Los Cuadros 2 y 3 resumen los experimentos que fueron realizados con los diversos incrementadores de solubilidad. El Cuadro 2 resume el cribado inicial desarrollado con concentraciones de péptidos en la escala de 5 a 10 mg/ml. El trabajo experimental se desarrolló con una concentración más elevada de péptidos y después se repitió con dosis menores (véase cuadro 3).

Las pruebas iniciales indicaron que el compuesto 1 era más soluble a los límites de los niveles de pH deseados, tanto ácidos como básicos, pero eran menos estables en la escala de pH básica. Por lo tanto, se sometieron a prueba varios reguladores de pH y agentes de ajustes de pH, incluyendo el regulado de pH de acetato, el regulador de pH de citrato y carbonato de sodio. Ninguno de los reguladores de pH inicialmente comprobados logró el nivel deseado de solubilidad de los péptidos.

Solamente arriba de pH 9.2 y debajo de pH 3.0, los niveles de solubilidad que se observaron fueron de 2 mg/ml. Sin embargo, en la etapa inicial, las formulaciones con regulador pH de acetato y el regulador de citrato (con

Manitol como agente de tonicidad) fueron seleccionadas para los estudios de toxicología inicial. Estas formulaciones se probaron para la actividad biológica y se comprobaron activos.

Los solventes no acuosos (véase cuadro 1) tal como etanol, glicerina, propilenglicol, cremóforo y sus combinaciones fueron probados, más no incrementaron la solubilidad del Compuesto 1. Una solución de 30% de DMA (dimetil-acetamida) rindió una solubilidad en las escalas deseadas) (5 a 9 mg/ml), pero no fue adecuado para la formulación farmacéutica debido a su perfil de toxicidad. También se observó una solubilidad mejorada utilizando 30% (p/p) de PEG 400 (5 a 9 mg/ml). Esta formulación fue elegida para los estudios de toxicología, pero se comprobó que era tanto inactivo en el ensayo biológico y podría ser también la causa de ciertos efectos adversos en un estudio de toxicidad en ratones. Por lo tanto, se decidió no perseguir más esta formulación. En vista de los experimentos preliminares, no se utilizaron solventes no acuosos en las formulaciones sujeto.

Se probaron varios aminoácidos (ver cuadro 1) incluyendo L-Arginina, ácido Glutámico, L-Glicina, y L-Lisina, para mejorar la solubilidad de la proteína. La solubilidad del péptido en L-Arginina fue al nivel deseado, pero el pH resultante era arriba de 9. Un intento para

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disminuir el pH utilizar una sal de HCl de Arginina resulto en la precipitación del péptido. También se probó Albúmina de suero humano y se mejoró la solubilidad del péptido a concentraciones del péptido bajas (1 mg/ml) (ver cuadro 3). Sin embargo, debido a su inmunogenicidad potencial y la baja solubilidad del péptido, no se utilizó en experimentos adicionales.

Los ingredientes para carga, (véase cuadro 1) incluyendo Mannnitol, Sorbitol y Dextrano, se sometieron a prueba solos y en combinación con otros excipientes, pero no mejoraron la solubilidad del péptido en la solución.

Los co-solventes (véase cuadro 1), incluyendo Polisorbato 20 y Polisorbato 80, se sometieron a pruebas solos y en combinación con otros excipientes. Aunque concentraciones menores de Polisorbatos (hasta el 6%) no mejoraron la solubilidad del péptido, concentraciones más elevadas (hasta el 10% - véase el cuadro 2) mejoraron la solubilidad del péptido hasta 2 mg/ml. Sin embargo, dichas concentraciones elevadas de Polisorbato se consideraron inadecuadas para las formulaciones farmacéuticas.

Dos tipos de ciclodextrinas, tanto aprobadas para su uso en productos parenterales comercializados, se sometieron a prueba también: hidroxipropil-β-ciclodextrina y sulfobutileter-β-ciclodextrina (Captisol). Ambos incrementaron marcadamente la solubilidad del péptido

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(concentraciones en niveles de 10 mg/ml para Hidroxipropil-β-ciclodextrina y 2.5 para Captisol). La actividad biológica de las dos formulaciones de ciclodextrina se sometió a prueba, y se encontró que eran iguales a la actividad del péptido solo.

CAPTISOL® es un derivado de β-ciclodextrina polianinónico comercialmente disponible con una sal de sulfonato de sodio separada de la actividad hidrofóbica por medio de un grupo separador de heterbutílico, o éter sulfobutílico (SBE). CAPTISOL® es el nombre comercial para la preparación CyDex Inc. de sulfobutiléter β-ciclodextrina repta-sustituida de CyDex Inc. (SBE7-β-CD) (www.captisol.com). La estructura de CAPTISOL® permite que las moléculas del fármaco se ajusten en la cavidad hidrofóbica, aislando de esa manera la molécula del fármaco a partir de un solvente acuoso. Debido a que la superficie exterior de CAPTISOL® es hidrofílica, por lo tanto se mejora la solubilidad de la molécula del fármaco formada en complejos. El uso de ciclodextrinas para mejorar la solubilidad de las moléculas del fármaco, se describe en la Patente EUA números 5,134,127 y 5,376,645, cuyos contenidos totales se incorporan en la presente por referencia.

De acuerdo con la literatura del CAPTISOL® de CyDex Inc., es segura cuando se administra parenteralmente y por lo tanto, no muestra la nefrotoxicidad asociada con

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β-ciclodextrina. Con relación a la β-ciclodextrina, el CAPTISOL® provee características de formación de complejos comparables o más elevadas y una superior solubilidad en agua en un exceso de 90 gramos/100 ml - con una mejora de 50 veces.

Conclusiones

Se encontró que varios incrementadores de solubilidad coinciden con la escala de solubilidad deseada: DMA, PEG-400, dimetil-acetamida, polietilenglicol, aceite de castor polioxilado, N-metil-2-pirrolidinona, 1-etenil-2-pirrolidinona, Polisorbato 20, Polisorbato 80, Hidroxipropil-β-ciclodextrina y Sulfobutiléter-β-ciclodextrina (Captisol®). De estos incrementadores de solubilidad, se ha comprobado que ambas ciclodextrinas son superiores con respecto a la solubilidad, actividad biológica y estabilidad. Por lo tanto, se decidió seleccionar Captisol® como el incrementador de solubilidad para su uso en las formulaciones del Ejemplo 5 y para estudiar adicionalmente ambas formulaciones de ciclodextrina. La formulación final para los estudios clínicos del Ejemplo 5, consisten de 120 mg/ml de Captisol en agua con la cantidad deseada de péptido (0.5, 1.0 ó 2.5 mg/ml) y HCl y NaOH para el ajuste de pH.

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**CUADRO 1**

**Incrementadores de solubilidad utilizados para el desarrollo de la formulación del compuesto 1.**

| **Clasificación del incrementador de solubilidad** | **Incrementadores de Solubilidad** |
|---|---|
| Solventes | Cremofor EL, CMC, Etanol, DMA, glicerina, Propilenglicol, PEG 400, Monotioglicerol |
| Co-solventes | Polisorbato 20, Polisorbato 80 |
| Agentes de solubilización | Argenina, HSA, Glicina, Creatinina, Ácido Glutámico, Licina (sal de acetato y base libre), Captisol, Hidroxipropil-β-ciclodextrina, |
| Agentes de formación | Manitol Sorbitol, Dextrosa, Dextranlactosa |
| Agentes de ajuste pH | Regulador de citrato, regulador de acetato, Carbonato de sodio |

**CUADRO 2**

**Lista de co-solventes y estabilizadores evaluados en las formulaciones del péptido del compuesto 1**

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Albúmina (HSA), Dextrosa | 1.5<br>1.5 | 0.4-5.0 | 5 | - | 6.0<br>Ajustar a 4.1 | Insoluble |
| Albúmina (HSA), Polisorbato 80, glicina | 1.0<br>0.6<br>2.0 | 0.4-5.0<br>0.8-4.0<br>0.2-2.1 | 5 | - | 5.0<br>Ajustar a 4.1 | Insoluble |
| Arginina | 1.5 | 0.8-1.6 | 15 | 93 | 9.8 | Solución transparente |
| Arginina HC1 | 2.0 | 0.8-1.6 | 5 | - | 3.5 | Insoluble |
| Arginina<br>Lactosa | 1.5<br>1.5 | 0.8-1.6 | 15 | 93 | 9.8 | Cuando el pH fue reducido bajo 8.5, el péptido se precipitó y la solución se tornó en un gel |
| Captisol | 10.0<br>20.0 | Hasta 30.0 | 10 | 86<br>89 | 4.9 Ajustar a 4.4 | Solución turbia |
| CMC (carboxi-metil celulosa) en acetato | 0.2<br>0.05M | | 5 | 90 | 5.0 | Para estudios toxicológicos |

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Creatinina | 0.8 | Hasta 0.6 | 5 | - | 6.1 Ajustar a 4.1 | Insoluble |
| Cremofor EL<br>Etanol<br>Dimetilacetamida | 15.0<br>10.0<br>6.0 | -10.0<br>0.6-32.9<br>0.012-6.0 | 5 | - | 4.0 | Muy turbia |
| Dextrano | 4.0 a 15.0 | 3.0-30.0 | 5 | - | 3.9 | Insoluble |
| Dimetilacetamida (DMA) | 6.0-20.0 | 0.012-6.0 | 5 | - | 4.6 | Insoluble |
| Dimetilacetamida (DMA) | 25.0 | 0.012-6.0 | 5 | 87 | 5.1 | Solución transparente |
| Dimetilacetamida (DMA) | 30.0 | 0.012-6.0 | 10 | 93 | 5.1 | Solución transparente |
| Etanol | 10.0 | 0.6-32.9 | 5 | - | - | Insoluble |
| Ácido glutámico | 2.0 | | 5 | - | 3.7 | Cuando el pH se incrementó sobre 4, el péptido se precipitó y la solución se tornó en un gel |
| Glicerina | 1.5 | 1.6-32.5 | 5 | - | 4.5 | Insoluble |

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Glicerina | 30.0 | 1.6-32.5 | 5 | - | 3.7 | Insoluble |
| Glicerina, Polisorbato 80 | 10.0<br>0.6 | 1.6-32.5<br>0.8-4 | 5 | 12 | 6.5<br>Ajustar a 4.5 | Insoluble |
| Glicina | 0.4 | 0.2-2.1 | 5 | - | 4.6 | Insoluble |
| Hidroxipropil-β-ciclodextrina | 20.0 | Hasta 30.0 | 10 | 99 | 4.6 | Solución transparente |
| Sal de acetato de lisina | 2.0 | | 5 | - | 3.8 | Insoluble |
| Base libre de Lisina | 2.0 | | 5 | - | 9.2 | Cuando el pH se incrementó sobre 8, el péptido se precipitó y la solución se tornó en un gel |
| Manitol en regulador de pH de citrato | 4.0<br>0.035M | 2.0-10.0 | 5 | 38 | 3.4 | Para estudios de toxicología |
| Manitol en regulador de pH de acetato | 4.0<br>0.05M | 2.0-10.0 | 5 | 32 | 4.3 | Para estudios de toxicología |
| Manitol, Glicina | 20.0<br>0.4 | 2.0-10.0<br>0.2-2.1 | 5 | 14 | 6.4<br>Ajustar a 4.5 | Insoluble |

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Manitol, Polisorbato 20 | 20.0 0.6 | 2.0-10.0 | 5 | 22 | 6.5 Ajustar a 4.5 | Insoluble |
| Monotioglicerol | 1.0 | 0.1-10.0 | 5 | - | 4.5 | Solución turbia |
| PEG 400 | 30.0 | Hasta 30.0 | 5 | 88 | 4.2 | Ligeramente opaca |
| PEG 400 | 30.0 | Hasta 30.00 | 10 | 89 | 4.2 | Solución turbia |
| PEG 400 con DMA | 30.0 6.0 | Hasta 30.0 0.012-0.6 | 5 | 94 | 4.2 | Solución transparente |
| PEG 400 | 10.0 | 6.0-18.0 | 6 | 58 | 4.2 | Insoluble |
| PEG 400 DMA | 10.0 10.0 | Hasta 30.0 0.012-0.6 | 5 | - | 4.3 | Insoluble |
| PEG 400 Propilenglicol PG | 10.0 10.0 | Hasta 30.0 10.0 | 5 | - | 4.1 | Insoluble |
| PEG 400 Propilenglicol | 18 50.0 | Hasta 30.0 10.0 | 5 | 100 | 4.2 | Solución transparente |
| Polisorbato 80 | 1.6 | 0.8-4.0 | 5 | 24 | 7.2 Ajustar a 4.5 | Insoluble |
| Polisorbato 80 | 6.0 | 0.8-4.0 | 5 | - | 3.9 | Insoluble |
| Polisorbato 80 Creatinina | 6.0 0.6 | 0.8-4.0 hasta 0.6 | 5 | - | 4.0 | Insoluble |

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Propilenglicol<br>PG<br>DMA | 10.0<br>10.0 | 10.0<br>0.012-0.6 | 5 | - | 4.2 | Insoluble |
| Propilenglicol<br>PG | 10.0-30.0 | 10.0 | 5 | - | 4.2 | Insoluble |
| Carbonato de sodio | 1.5 | | 5 | - | 11.4 | Cuando el pH se incrementó sobre 8.5, el péptido se precipitó y la solución se tornó en un gel |
| Sorbitol | 5.0 | 10.0-25.0 | 5 | - | 6.9<br>Ajustar a 4.5 | Solución turbia |

**CUADRO 3**

**Formulaciones del compuesto 1 a concentraciones bajas del péptido**

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Albúmina (HSA) | 5.0 | 0.4-5.0 | 1.0<br>2.5 | 90<br>- | 6.9<br>Ajustar a 4.5 | Solución transparente<br>Solución turbia |
| Arginina | 1.5 | 0.8-1.6 | 1.0 | 24 | 10.6<br>Ajustar a 8.5 | Cuando el pH se incrementó sobre 8.5, el péptido se precipitó y la solución se tornó en un gel |
| Captisol® | 12.0 | Hasta 30.0 | 1.0<br>2.5 | 106<br>100 | 5.3<br>6.5 a 8.5 | Solución transparente |
| Dextrano | 20.0 | 3.0-30.0 | 1.0 | 69 | 4.8<br>Ajustar a 4.0 | Solución turbia |
| Glicerina | 30.0 | 1.6-32.5 | 1.0 | - | 4.8<br>Ajustar a 4.0 | Solución turbia |
| Manitol | 4.0 | 0.8-4.0 | 1.0 | 64 | 4.7 Ajustar a 4.0 | Solución turbia |

| Incrementador de solubilidad * | % Utilizado | % de la cantidad estándar a partir de la literatura | Cantidad de péptido agregado (mg/ml) | % Análisis | pH de formulación | Observaciones |
|---|---|---|---|---|---|---|
| Polisorbato 20 | 10.0 | - | 1.0<br>2.5 | 95<br>88 | 5.8<br>Ajustar a 4.8 | Solución transparente<br>Transparente con una pequeña cantidad de precipitación |
| Polisorbato 20<br>Manitol | 10.0<br>2.0 | 2.0-10.0 | 2.5 | 115 | 5.1<br>Ajustar a 4.3 | Solución transparente |
| Polisorbato 80 | 4.0<br>6.0-10.0 | 0.8-4.0 | 1.0<br>2.5 | 91<br>89 | 5.5<br>Ajustar a 5.0 | Solución transparente<br>Solución ligeramente turbia |
| Polisorbato 80<br>Manitol | 4.0<br>2.0 | 0.8-4.0<br>2.0-10.0 | 2.5 | 88 | 5.1<br>Ajustar a 4.4 | Solución ligeramente turbia |
| Propilenglicol PG | 10.0 | 10.0 | 1.0 | 78 | 5.0<br>Ajustar a 4.4 | Solución turbia |
| Sorbitol | 20.0 | 10.0-25 | 1.0 | 52 | 4.5 | Solución turbia |

**EJEMPLO 2**

**Protocolo de preparación para solución del compuesto 1 en Captisol®.**

Los métodos de solución estándar, tales como el mezclado del Compuesto 1 en seco y Captisol® en seco en agua o agregando el Compuesto 1 a una solución preparada de Captisol® y agua no dieron como resultado la completa disolución en las concentraciones deseadas. Diversas concentraciones diferentes tanto el Compuesto 1 como de Captisol® se sometieron a prueba a varios niveles de pH. Sin embargo, el siguiente método para producir una solución del Compuesto 1 en Captisol® resultó en la completa disolución en las concentraciones deseadas.

Materiales: Captisol®, Compuesto 1 y agua.

Método:

1.- Pesar la cantidad apropiada de Captisol® para dar una concentración final de 120 mg/ml.

2.- Agregar 80% de la cantidad final de agua mezclado por 10 minutos con un agitador magnético.

3.- Pesar el Compuesto 1 para dar una concentración final de 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml ó 0.1 mg/ml.

4.- Agregar el péptido a la solución de Captisol®. Mezclar por 1 hora.

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5.- Elevar el pH para obtener una solución transparente (en la formulación de 2.0 mg/ml podría existir la necesidad de elevar el pH ligeramente arriba de 9). El pH se debe ajustar utilizando HCl 1.0 N y NaOH 1.0 N. Mezclar durante 10 minutos.

6.- Corregir el pH a la escala de 7.5 a 8.5 si se necesita (utilizando ya sea HCl o NaOH 1.0 N).

7.- Agregar agua al volumen final.

8.- Filtrar la solución a través de un filtro de acetato de celulosa 0.2µ.

9.- Registrar el pH final.

10.-Dispensar en cantidades alícuotas y almacenar la temperatura adecuada.

**EJEMPLO 3**

**Liofilización del Compuesto 1 y solución de Captisol®.**

El procedimiento de actual de liofilización difiere de otros procedimientos de liofilización ya que el porcentaje de sólidos en la formulación es alto (12%) mientras que los productos liofilizados normalmente contienen entre 5 y 10% de sólidos.

Equipo

La secadora que se utilizó fue una secadora liofilizadora Edwards Lyoflex 0.6. Se desarrolló el IQ/OQ del equipo y se verificó para que tuviera su cumplimiento

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en aseguramiento de calidad antes del desarrollo del procedimiento.

Se prepararon las soluciones del Compuesto 1 y Captisol® en concentraciones de 0.5 mg/ml, 1.0 mg/ml y 2.5 mg/ml del Compuesto 1. El volumen de relleno se ajustó a 1 ml (1.05 gr).

Principales etapas del procedimiento:

1.- Congelar

2.- Sostener (a temperatura baja)

3.- Secar bajo vacío en dos etapas:

3.1 Secado primario - entibiado en el instante para sostener la temperatura, controlar la temperatura de estante al nivel más elevado.

3.2 Secado secundario - Reducción de presión a un valor mínimo a la temperatura superior del estante.

Lotes 1 - 3

Congelación - La congelación fue de temperatura ambiente a -40° C en un periodo de 2 horas. Los estantes se mantuvieron a -40° C durante 3 horas.

Secado - Se desarrolló el secado a una presión de 110 µbarias. La temperatura de estante se incrementó a 20° C sobre 13 horas y se sostuvo a esa temperatura por otras 13 horas adicionales.

El tiempo total del procedimiento fue de 31 horas.

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Resultados:

Los resultados de contenido de agua fueron:

Lote no. 1: 3.8%

Lote no. 2: 4.0% y

Lote no. 3: 4.9%

Lotes 4 y 5

Como los resultados del contenido de agua de los procedimientos que condujeron a los lotes 1, 2 y 3 fueron más elevados que el valor deseado, se decidió agregar una etapa secundaria de secado a la misma temperatura y a una presión baja.

Secado - Se desarrolló el secado a una presión 110 µbarias. Se incrementó la temperatura de estante a 20° C por más de 13 horas y se sostuvo a esa temperatura por otras 13 horas más. (Lote 4) u 8 horas (Lote 5). Se redujo la presión a 10 µbarias durante otras 5 horas adicionales.

Tiempo total del procedimiento de 36 horas.

Resultados:

Los resultados del contenido de agua fueron

Lote 4: Placebo: 3.0%.

1 mg/ml: 3.9%.

Lote 5: Placebo: 4.1%

Conclusiones

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Como se muestra, se desarrolló un procedimiento de liofilización satisfactoria para el Compuesto 1 con Captisol®. Debido al alto porcentaje de sólidos y por lo tanto a la torta condensada, el procedimiento desarrollado es más largo que los ciclos actualmente disponibles de liofilización para péptidos y muestra una etapa secundaria de secado adicional. El Cuadro 4 resume el procedimiento desarrollado.

**CUADRO 4**

| **Etapa** | **Compuesto 1 (Péptido) con Captisol®** |
|---|---|
| Cargado | 5° C |
| Congelado | 2 horas a -40° C |
| Sostener a baja temperatura | 3 horas a -40° C |
| Secado primario:<br>Entibiar a 20° C<br>Sostener a 20° C | <br>13 horas, presión 110 µbar<br>13 horas, presión 110 µbar |
| Secado secundario:<br>Sostener a 20° C | 5 horas, presión 110 µbar |
| Almacenaje a | -20° C |
| Tiempo de procesamiento | 36 horas |

**EJEMPLO 4**

**Reexaminación de la actividad biológica *in vivo* de la solución del compuesto liofilizado (DP, 1 mg/frasco, Captisol® al 12%)**

Se controló la actividad biológica por medio de inhibición de la secreción de IL-2 de las células T

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específicas de estándar de referencia (RS) del Compuesto 1 seguido de tratamiento subcutáneo (s.c.) con la solución del compuesto liofilizado, es decir, el producto del fármaco (DP), a dos concentraciones. Los resultados del tratamiento se comparan con aquellos para tratar ratones con Compuesto 1 (RS) en solución salina regulada con pH de fosfato (PBS). Los resultados se muestran en los cuadros a continuación y en la Figura 2.

Diseño experimental:

1.- Inmunización Día 0

(Compuesto 1 RS emulsificado con CFS, todos ellos en cuatro soportes de pedestal)

2.- Tratamiento Día 0

(s.c. (subcutánea) en la parte trasera del cuello en solución al 200 µl)

3.- Activación *in vitro* con: Día 10

a) Compuesto 1 RS concentraciones de 0; 0.5; 1; 2.5; 5; 10; 25; 50 y 100 µg/ml

b) un péptido con el orden inverso de aminoácidos del Compuesto 1 (control negativo).

c) Con A (control positivo).

4.- Incubación de cultivo durante 20 horas a 37°C en una incubadora de 5% $CO_2$ modificada.

5.- Medición de IL-2 por medio de ELISA.

**CUADRO DE GRUPOS EXPERIMENTALES**

| GRUPO | INMONIZACIÓN CON | TRATAMIENTO |
|---|---|---|
| A | 50 µg Compuesto 1 RS | Compuesto 50µg 1 RS en PBS |
| B | | Compuesto 200µg 1 RS en PBS |
| C | | 50µg DP (lote 2) |
| D | | 200µg DP (lote 2) |
| F | | Placebo (Captisol® al 12 %) |

**Secreción de iL-2 de ratones tratados con el compuesto 1 (dp) después de la activación in-*vitro* con el compuesto 1 RS (pg/ml)**

| | | TRATADO CON: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | GRUPO | F | A | | B | | C | | D | |
| Activador | Concentración de Activador (µg/ml) | Captisol® al 12% en ampolletas | COMP.1 RS 50 µg/ratón | % inhib. | COMP.1 RS 200 µg/ratón | % inhib. | DP 50 µg/ratón | % inhib. | DP 200 µg/ ratón | % inhib. |
| Con A | 2.5 | 5,825 | 6,215 | | 5,403 | | 3,537 | | 4,069 | |
| Comp.1 RS | 0 | BQL | BQL | | BQL | | BQL | | BQL | |
| Comp.1 RS | 0.5 | 11 | 9 | | 19 | | 8 | | BQL | |
| Comp.1 RS | 1 | 10 | BQL | | BQL | | BQL | | BQL | |
| Comp.1 RS | 2.5 | 15 | 8 | 51 | BQL | NA | 6 | 61 | 6 | 62 |
| Comp.1 RS | 5 | 20 | 9 | 55 | 8 | 60 | 10 | 48 | 7 | 63 |
| Comp.1 RS | 10 | 25 | 16 | 38 | 11 | 58 | 13 | 48 | 8 | 67 |
| Comp.1 RS | 25 | 29 | 15 | 48 | 11 | 63 | 16 | 45 | 13 | 56 |
| Comp.1 RS | 50 | 40 | 21 | 47 | 15 | 62 | 20 | 50 | 12 | 69 |
| Comp.1 RS | 100 | 42 | 25 | 41 | 18 | 58 | 24 | 43 | 15 | 64 |
| | Inhibición promedio (%) (en la escala de 5-100µg/ml) | | | 45.6 | | 60.4 | | 46.8 | | 63.7 |

BQL= Debajo del límite de cuantificación
Filas 1-4 no se incluyeron en la curva

NA = No aplica

## EJEMPLO 5

## Evaluación de la dosis óptima para el tratamiento

Se utilizan las siguientes abreviaturas en la siguiente descripción:

| | |
|---|---|
| CFA | Adyuvante completo de Freund |
| Con A | Concanavalina A |
| DP | Producto del fármaco |
| DS | Sustancia del fármaco |
| EM-1 | Medio DCCM-1 enriquecido |
| EM-3 | Medio de suero de becerro fetal RPMI-1640 + |
| FCS | Suero de becerro fetal |
| IFN-γ | Gama interferón |
| LN | Nódulo linfático |
| PBS | Solución salina regulada con fosfato |
| RS | Solución estándar de referencia |
| s.c. | Subcutánea |
| TB | Azul tripano |
| TGF-β | Factor de crecimiento beta de transformación |
| WFI | Agua para inyección |

### Introducción

Se inmunizó un grupo de 20 ratones con 50 µg/ratón de Compuesto 1 RS. Los ratones inmunizados se

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asignaron a cinco grupos de tratamiento conforme a lo que sigue: placebo, 25, 50, 100 y 200 µg/ratón de Compuesto 1 DP (administración subcutánea). Diez días después de la inmunización y el tratamiento, se extrajo LN y se preparó una suspensión unicelular. Se midió la secreción *in vitro* de IFN-γ y TGF-β por la células cultivadas en respuesta a la activación con diferentes concentraciones del Compuesto 1 RS.

Diseño experimental

1.- Inmunización -Día 0

2.- Tratamiento con el Compuesto 1 DP -Día 0

3.- Activación *in vitro* de células LN de los ratones tratados -Día 10

4.- Recopilación de cultivo (para la determinación de IFN-γ) -Día 12

5.- Recopilación de cultivo (para la determinación de TGF-β) -Día 13

6.- ELISA para IFN-γ

7.- ELISA para TGF-β

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**CUADRO 7**
**Grupos experimentales**

| Grupo experimental | Tratamiento | | Activación in-vitro | |
|---|---|---|---|---|
| | Artículo | Ratón/ grupo | Células/ pocillo | Compuesto 1 rs concentración |
| A1 | Control 12% Captisol® | 4 | $\frac{2.5 \times 10^6}{5 \times 10^6}$ | Compuesto 1 RS 0-100 µg/ml |
| A2 | 25 µg/ratón | 4 | $\frac{2.5 \times 10^6}{5 \times 10^6}$ | |
| A3 | 50 µg/ratón | 4 | $\frac{2.5 \times 10^6}{5 \times 10^6}$ | |
| A4 | 100 µg/ratón | 4 | $\frac{2.5 \times 10^6}{5 \times 10^6}$ | |
| A5 | 200 µg/ratón | 4 | $\frac{2.5 \times 10^6}{5 \times 10^6}$ | |

Materiales y reactivos

Animales

Ratones: 20 ratones hembra BALB/c, proveedos por Harlan animals breeding center, Rehoboth.

Edad a la inmunización (semanas + días): 10

Peso promedio de los ratones incluidos en el experimento: 19.01 gr.

Materiales

Reactivos generales

Se preparó etanol al 70% a partir de 96% de etanol diluyendo con $H_2O$ purificada.

Preparación de las soluciones del Compuesto 1 para la inmunización

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Se preparó una emulsión RS del Compuesto CFA-1 (500 µg/ml, 50 µg/ratón) conforme a lo siguiente:

1.- Se disolvieron 1.874 mg del Compuesto 1 en 1.87 ml de WFI para rendir una solución de 1 mg/ml.

2.- Se sometió a prueba la solución con una banda indicadora de pH y se encontró que tenía un pH de 5.

3.- Se emulsificaron 1.5 ml de la solución con CFA de 1.5 ml resultando en una concentración final de 500 µg/ml.

Preparación de las soluciones para el tratamiento

El tratamiento fue por medio de una inyección subcutánea con una solución de 200 µl.

Preparación de la solución de Captisol® al 12%

Se disolvieron 1.2 gr de captisol® en 10 ml de WFI para dar una solución de captisol® al 12%.

Procedimiento experimental

Pesado de los ratones

Se pesaron los ratones antes de la inmunización. El peso promedio de los mismos fue de 19.01 + 0.97 gr.

Inmunización

Se desarrolló la inmunización inyectando 100 microlitros de la emulsión de inmunización (50 microlitros dentro de cada soporte trasero).

Tratamiento

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Seguido de la etapa de inmunización, se trataron los ratones por medio de inyección subcutánea de 200 µl del Compuesto 1 DP o con soluciones del tratamiento con captisol® al 12%, en la parte posterior del cuello de los ratones.

Cultivo *in vitro*

Se sacrificaron los ratones por medio de dislocación cervical. Se extrajo LN de las patas traseras y se transfirieron a una caja de petri estéril conteniendo aproximadamente 5 ml de RPMI. Se extrajeron las células por medio de un apretado delicado del tejido contra una red de acero inoxidable de medida de 200 micrometros red. Se cultivaron las células se centrifugaron a 300 G durante 10 minutos a RT.

Se prepararon suspensiones unicelulares del LN de los pozos de cada grupo experimental.

Se cultivaron suspensiones de 2.5 y 5.0 millones de células/mililitro/pozo con el Compuesto 1 RS (0-100 µg/ml) en EM-1.

Se determinó por medio de ELISA del medio de cultivo la secreción de IFN-γ y TGF-β, como indicación de la respuesta celular (48 horas para IFN-γ y 72 horas para TGF-β).

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**CUADRO 8**

**Grupos Experimentales In-Vitro**

| Grupo experimental | Tratamiento | Activación in-vitro | |
|---|---|---|---|
| | Artículo | Células/pocillo | Concentración de la sustancia de activación |
| A1-2.5 | Control 12% Captisol® | $2.5 \times 10^6$ | Compuesto 1 RS 0; 3.125; 6.25; 12.5; 50 y 0-100 µg/ml Con A 2.5 µg/ml |
| A1-5 | | $5 \times 10^6$ | |
| A2-2.5 | DP 25 µg/ratón | $2.5 \times 10^6$ | |
| A2-5 | | $5 \times 10^6$ | |
| A3-2.5 | DP 50 µg/ratón | $2.5 \times 10^6$ | |
| A3-5 | | $5 \times 10^6$ | |
| A4-2.5 | DP 100 µg/ratón | $2.5 \times 10^6$ | |
| A4-5 | | $5 \times 10^6$ | |
| A5-2.5 | DP 200 | $2.5 \times 10^6$ | |

Preparación de suspensiones celulares

**CUADRO 9**

**Resultados del conteo de células y preparación de las suspensiones de células**

**(10 X $10^6$/ml)**

| Gpo. | Vol. (ml) | Factor Dilut. | Células viable | Células muertas | Células viables% | Células muertas% | Prom. Células viables | Total células viables (x $10^6$) | EM-1 para agregar (ml) para suspensión de 10 x $10^6$ células/ml |
|---|---|---|---|---|---|---|---|---|---|
| A1 | 10 | 16 | 115 / 109 | -- / -- | 100 / 100 | -- / -- | 112 | 179.2 | 17.9 |
| A2 | 10 | 16 | 50 / 45 | 4 / 2 | 92.6 / 95.7 | 7.4 / 4.3 | 47.5 | 76 | 7.6 |
| A3 | 10 | 16 | 80 / 81 | 4 / 5 | 95.2 / 94.2 | 4.8 / 5.8 | 80.5 | 128.8 | 12.8 |
| A4 | 10 | 16 | 87 / 91 | -- / -- | 100 / 100 | -- / -- | 89 | 142 | 14.2 |
| A5 | 10 | 16 | 120 / 105 | 2 / 2 | 98.4 / 98.1 | 1.6 / 1.9 | 112.5 | 180 | 18 |

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Preparación de suspensiones celulares (5 x $10^6$/ml)

Se diluyeron las suspensiones 10 x $10^6$ células/ml al 1:2 agregando 5ml EM-1 a una suspensión de 5 ml de células.

Incubación de cultivos de células LN en placas de 48 pozos

Se prepararon 3 placas de cultivo de tejido. Se agregó lo siguiente a cada placa.

Control antecedente (células incubadas con medio de cultivo)

0.5 ml de suspensión de células

0.5 ml de cultivo medio (EM-1)

Control positivo de sistema (células estimuladas con Con A)

0.5 ml de suspensión de células

0.5 ml de cultivo medio (EM-1)

0.5 ml de Con A 5 µg/ml en EM-1 (concentración final: 2.5 µg/pozo)

Células incubadas con soluciones de activación del Compuesto 1 (muestras)

0.5 ml de suspensión de células

0.5 ml de Compuesto 1 RS 6.25 - 200 µg/ml (concentración final 3.125 - 100 µg/ml/pozo)

Incubación de cultivos de células LN en placas de 96 pozos

Después de que se prepararon las placas con 48 pozos, se prepararon placas con 96 pozos aplicando 100 µl a partir de la suspensión celular y 100 µl de las soluciones de activación.

Las placas de cultivo se incubaron a 37°C en un incubador de $CO^2$, al 5% humidificado, tanto como para 48 como para 72 horas.

Recolección de sobrenadantes

Se centrifugaron las placas cultivadas a 300 g por 10 minutos a RT. Se transfirieron los sobrenadantes (850 µl de cada pozo) a placas espejo o a tubos. El sobrenadante se dividió en cuotas alicotas de funcionamiento (dos alicotas de 200 y una alicota de 450 µl). Para poder evitar un congelamiento/descongelamiento repetido de las muestras. Cada tubo se etiquetó con los siguientes detalles:

1.- Código experimental y tiempo post-incubación.

2.- Número de muestra y grupo.

3.- Activador y concentración.

4.- Fecha de recopilación sobrenadante.

Los sobrenadantes se almacenaron a -20°C hasta que se utilizaron para la prueba ELISA.

**Resultados**

**CUADRO 10**

**Resumen de grupos**

| **Grupos experimentales:** | | | | |
|---|---|---|---|---|
| **Inmunización** | | **Tratamiento** | | **Activación in-vitro** |
| **Grupos exp.** | **Dosis de inmunización** | **Sub-grupo** | **Artículo** | |
| A | 50 µg/ratón | A1 | 12% Captisol® Control Placebo | |
| | | A2 | Compuesto 1 25 µg/M | Compuesto 1 rs |
| | | A3 | Compuesto 1 50µg/M | 3.125-10025 µg/ml |
| | | A4 | Compuesto 1 100µg/M | |
| | | A5 | Compuesto 1 200µg/ml | |

**CUADRO 11-A**

**Concentraciones de Citosina Final**

**CITOSINA FINAL (pg/ml) (2.5 millones de células/pocillo)**

| Concentración de Compuesto 1 | Placebo | 50 µg/M | 100 µg/M | 200 µg/M |
|---|---|---|---|---|
| 3.125 µg/ml | 321.3 | 54.1 | 64.5 | 103.9 |
| 6.25 µg/ml | 238.6 | 81.8 | 116.1 | 126.1 |
| 12.5 µg/ml | 397.1 | 123.1 | 180.9 | 129.0 |
| 25 µg/ml | 655.5 | 215.1 | 262.8 | 240.3 |
| 50 µg/ml | 573.9 | 292.5 | 518.3 | 378.1 |
| 100 µg/ml | 926.0 | 531.8 | 582.7 | 524.1 |
| Con A | 322.6 | 356.2 | 337.4 | BQL |

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**CUADRO 11-B**

**Concentraciones finales de citosina**

**CITOSINA FINAL (pg/ml) (5 millones de células/pozo)**

| Concentración del Compuesto 1 | Placebo | 25 µg/M | 50 µg/M | 100 µg/M | 200 µg/M |
|---|---|---|---|---|---|
| 3.125 µg/ml | 522.3 | BQL | 76.2 | 90.8 | 204.4 |
| 6.25 µg/ml | 634.8 | BQL | 109.2 | 157.8 | 244.1 |
| 12.5 µg/ml | 962.8 | 41.9 | 179.5 | 257.1 | 466.1 |
| 25 µg/ml | 967.4 | 70.0 | 277.9 | 421.7 | 660.5 |
| 50 µg/ml | 1338.8 | 104.2 | 373.4 | 739.7 | 922.5 |
| 100 µg/ml | 2010.2 | 185.2 | 547.0 | 995.5 | 1006.2 |
| Con A | 6839.8 | 2995.3 | 4837.0 | 10126.8 | 7722.8 |

Los resultados también se presentan en las Figuras 3-4.

Observaciones

Secreción de IFN-γ

1.- En el grupo de placebo, se mostró una respuesta lineal a la dosis con la activación de Compuesto 1 *in vitro*. Esta gráfica se asemeje a la gráfica obtenida para el modelo *Ex-vivo* con la misma dosis de inmunización (50 µg/ratón) y medio de cultivo (EM-1).

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2.- Hubo una respuesta a la dosis con la activación del Compuesto 1 *in vitro* con todos los grupos probados.

3.- Se apreció una inhibición significativa de la secreción IFN-γ con todas las dosis utilizadas para el tratamiento (un promedio de inhibición de 95% con la dosis tratada de 25 µg/ratón). Se puede encontrar una correlación inversa entre la dosis servida para el tratamiento y el porcentaje de inhibición, principalmente cuando se utilizaron $5x10^5$ células/pozo. Cuando se utilizaron $2.5x10^6$ células/pozo, el tratamiento con animales con 50 µg/ratón, dieron mejor inhibición que 100 ó 200 µg. El punto de 25 µg es faltante (carencia de células).

4.- Se apreció una mejor inhibición cuando se utilizaron $5x10^6$ células/pozo en lugar de $2.5x10^6$ células/pozo.

5.- En la escala lineal de las gráficas, la SD del % de inhibición fue baja.

6.- El problema técnico con A es aparente cuando se utilizaron $2.5x10^6$ células/pozo.

Secreción de TGF-β

1.- En el grupo de placebo, no se apreció ninguna respuesta a la dosis sobre activación *in vitro* con el

Compuesto 1. El nivel secretado de TGF-β fue debajo del límite de detección de la prueba ELISA en los demás grupos de tratamiento.

**EJEMPLO 6**

**Optimizaciones del ciclo de secado por congelación con el compuesto 1 y Captisol® para la inyección (0, 0.5, 1.0 y 2.5 mg/frasco)**

Objetivo

El objetivo de este estudio fue optimizar el ciclo de secado por congelación para el Compuesto 1 con Captisol® para la inyección para mejorar la forma de la torta de liofilización y evita el colapso de fractura. Por lo tanto se decidió mejorar y optimizar el ciclo de liofilización. Este ciclo se transfiere a los liofilizadores de producción para la manufactura de los lotes de la fase I.

Optimización del procedimiento

Se prepararon lotes del péptido a concentraciones de 0.5 mg/ml 1.0 mg/ml, 2.5 mg/ml y Placebo y se desarrollaron varios ciclos de secado por congelación. La secadora por congelación que se utilizó fue un liofilizador Edwards Lyoflex 0.6.

Se probaron la solubilidad, el contenido de agua y la apariencia de la torta. De acuerdo con los resultados

obtenidos, se seleccionó un nuevo ciclo para el Compuesto 1. Debido al alto porcentaje de sólidos (12%) y por lo tanto de la torta condensada, el nuevo procedimiento es más prolongado que el ciclo de liofilización en el Ejemplo 3 y muestra la etapa adicional primaria de secado. El Cuadro 12 resume las diferencias entre los procedimientos.

**CUADRO 12**

| Etapa | Liofilización, ciclo para el Compuesto 1 y Captisol® del Ejemplo 3 | Nuevo ciclo de liofilización para el Compuesto 1 y Captisol® |
|---|---|---|
| Carga | 5° C | 5° C |
| Congelamiento | 2 horas a -40° C | 6 horas a -45° C |
| Sustentación a baja temperatura | 3 horas a -40° C | 3 horas a -45° C |
| Secado primario:<br>Etapa I<br>Etapa II<br><br>Sostener a 20° C (25° C) | A 20° C presión de 13 horas 110 µbarias<br>-<br><br>presión de 13 horas 110 µbarias | A -20° C 19 horas presión 150 µbarias a 25° C 13 horas presión 150 µbarias 8 horas presión 150 µbarias |
| Secado secundario: | presión de 5 horas 10 µbarias | - |
| Almacenaje a | 5° C | 5° C |
| Tiempo de procedimiento | 36 horas | 49 horas |

**EJEMPLO 7**

**Efecto del Compuesto 1 (administrador en Captisol®) en síntomas de lupus en el ratón hembra SLE propenso a (NZBxNZW) F1.**

Los pacientes que participaron en las pruebas clínicas deben ser tratados por el Compuesto 1 utilizando Captisol® (éter sulfobutílico de beta-ciclodextrina sódica) como excipiente. Por esta razón, era importante determinar si el tratamiento de los ratones (NZBxNZW) F1 con la formulación del Compuesto 1 dado en Captisol®, tendrían los mismos efectos benéficos en síntomas de Lupus como se observó cuando esta sepa de ratones fue tratada con el Compuesto 1 en PBS.

Hasta este extremo, los ratones hembra (NZBxNZW) F1 (aproximadamente de 8 meses de edad), se dividieron en 3 grupos que fueron tratados subcutáneamente una vez a la semana durante 10 semanas ya sea con Captisol® únicamente (n=8) o con 25 o 50 µg/ratón del Compuesto 1 en Captisol (n=9 y 10, respectivamente). Estas dosis fueron seleccionadas ya que los estudios previos indicaron que las dosis en esta escala eran más efectivas para mejorar los síntomas de SLE que las dosis más altas comprobadas (100 y 200 µg/ratón). Se utilizó el mismo lote de la sustancia del fármaco en este estudio y en la primera prueba clínica de la Fase 1 con el Compuesto 1.

Se les dio seguimiento a los ratones para verificar los anticuerpos anti-dsDNA y para proteinura. Cuando se sacrificaron los ratones, se determinó la intensidad de ICD en riñones.

Como se puede apreciar en la Figura 5, no se pudo observar ninguna diferencia significativa entre los grupos en los niveles de anticuerpos especificos a dsDNA después de 10 inyecciones del tratamiento.

El Cuadro 13 también muestra que se pudo observar el efecto benéfico del tratamiento con el Compuesto 1, iniciando desde la 5ª inyección y se sostuvo hasta la 10ª inyección. Los niveles medios de proteinura en el grupo de control con Captisol® fueron consistentemente más elevados que en los grupos tratados con el Compuesto 1. El Cuadro 13 también muestra que se observó una reducción en la intensidad de ICD en riñones de ambos grupos de dosis con el Compuesto 1. Hubo una tendencia general que mostraba que una dosis más baja (25 µg/ratón) era más efectiva que la dosis más elevada (50 µg/ratón) para reducir los síntomas clínicos de SLE en ratones.

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**CUADRO 13**

**Síntomas clínicos de SLE en (NZBXNZW)F1 ratones tratados con 25 ó 50 µg/ratón del Compuesto 1 (en Captisol®)**

| Grupo de estudio | Proteinuria media ± SEM (g/L) Número de semanas seguido del inicio del tratamiento | | | | ICD[a] (Media SEM) |
|---|---|---|---|---|---|
| | 5 | 7 | 8 | 10 | |
| Captisol® | 1.81 ± 1.22 (n=8) | 5.74 ± 3.13 (n=8) | 4.5 ± 2.92 (n=7)[b] | 4.46 ± 2.93 (n=7)[b] | 2.29 ± 0.28 (n=7) |
| Compuesto 1 (50 µg/ratón) | 0.75 ± 0.3 (n=10) | 0.81 ± 0.3 (n=10) | 1.09 ± 0.4 (n=10) | 1.29 ± 0.3 (n=10) | 1.90 ± 0.23 (n=10) |
| Compuesto 1 (25 µg/ratón) | 0.16 ± 0.05 (n=9) | 1.26 ± 1.09 (n=9) | 0.5 ± 0.31 (n=9) | 0.56 ± 0.3 (n=9) | 1.22[c] ± 0.32 (n=9) |

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[a] Depósitos del complejo inmune = ICD. Escala de intensidad ICD: 0 = ninguna; 1 = moderada; 2 = severa; 3 = severa/extremadamente intensa.

[b] Resultó en la muerte de un animal con un alto nivel de proteinura en una media más inferior del grupo.

[c] $p<0.05$ (comparado con los ratones de control tratados con Captisol®; Mann-Whitney).

La Figura 6 muestra secciones representativas de un riñón de cada grupo de tratamiento. Las secciones de la fila superior son de un ratón tratado con Captisol®, las secciones de la fila media son de un ratón tratado con el Compuesto 1 de 50 µg/ratón y las secciones de la fila inferior son de un ratón tratado con el Compuesto 1 25 µg/ratón. Se puede apreciar que la intensidad de los depósitos del complejo inmune observados en secciones de riñón de los ratones tratados con el Compuesto 1 (disuelto en Captisol®) en cualquier nivel de dosis fue mucho menor que el observado en el grupo de control.

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**EJEMPLO 8**

**Fase 1 Estudio clínico**

**Fase 1 A, multicentro, aleatorizado, doble ciego, controlado por Placebo, una sola dosis, estudio de cuatro etapas para evaluar la capacidad de tolerancia y seguridad del Compuesto 1 en inyección subcutánea con Captisol® en sujetos con SLE.**

Este fue el primer estudio clínico con el Compuesto 1 con Captisol® en humanos, que se condujo en Francia. Su objetivo principal fue evaluar la capacidad de tolerancia y seguridad del Compuesto 1 en Captisol , administrado como una sola inyección subcutánea SC a sujetos con SLE. Su objetivo secundario fue evaluar las respuestas inmunológicas siguiendo una sola dosis subcutánea SC del Compuesto 1 en Captisol® en estos sujetos.

Participaron en el estudio 36 (treinta y seis) sujetos. Para ser elegible para la inclusión en el estudio, los pacientes de SLE debieron haber cumplido por lo menos con cuatro criterios utilizados para el diagnóstico de lupus por el Colegio Americano de Reumatología. Los pacientes también debieron haber sido estables, con una enfermedad estable ligera/moderada, y haber apuntado una calificación menor o igual a 10 en el Índice de actividad de la enfermedad SLE, SLEDAT.

Cada paciente recibió una sola inyección subcutánea SC del Compuesto 1 reconstituido para inyección o su placebo coincidente (Captisol®) de acuerdo con la asignación del grupo siguiente.

- **Grupo A**: Placebo (Captisol®)
- **Grupo B**: 0.5 mg del Compuesto en Captisol®
- **Grupo C**: 1 mg del Compuesto en Captisol®
- **Grupo D**: 2.5 mg del Compuesto en Captisol®

Se desarrolló una batería estándar de pruebas de seguridad, incluyendo la recopilación de orina y sangre para las pruebas de laboratorio, esto se elaboró en la etapa de control y monitoreo durante el día de la dosificación, a las 24 horas post-dosis a las 2, 4 y 8 semanas después de la dosificación. Previo a la dosificación, y en las visitas de seguimiento programadas, se retiraron las muestras para las pruebas inmunológicas relacionadas con SLE, para los anticuerpos de anti-Compuesto 1 y para el ensayo de proliferación PBL. Se realizaron las siguientes pruebas de inmunología:

- Prueba de Coomb (directa e indirecta)
- C3, C4 y CH50
- IgG, IgM e IgA total
- ANA, anti-dsDNA (ensayo Farr), anti-ssDNA

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- Anti-ENA (incluyendo anti-La, anti-Ro, anti-RNP, anti-Sm)
- Anticuerpos anti-cardiolipina
- VDRL
- Anticuerpos FTA
- Factor reumatoide

Se evaluó la seguridad y capacidad de tolerancia al Compuesto 1 en Captisol® en la población sujeto, basándose en el siguiente criterio.

- Ocurrencia de AEs, incluyendo erupción de SLE
- Signos vitales
- Electrocardiograma de 12-guías
- Cambios en el examen físico
- Pruebas rutinarias del laboratorio clínico
- Calificación SLEDAI
- Resultados de la prueba inmunológica

Detalles del estudio clínico de la Fase Ia

Investigadores principales del estudio y sitios respectivos del mismo:

6 (seis) centros de estudio en Francia: Profesor Jean Charles Piette (Hospital La Pitie Salpetriere, París), Profesor Oliver Meyer (Hospital Bichat, París), Profesor Jean Revuz (Hospital Henri Mondor, Credeil), Profesor Loic

Guillevin (Hospital Avicente, Bobigny), Profesor Eric Hachulla (Hospital Claude Huriez, Lille Cedex), Profesor Xavier Mariette (Hospital Bicetre, Kremlin Bicetre).

Compuesto 1 (en Captisol®), Placebo, agua para ámpulas de inyección, dosis y modo de administración.

Se inyectaron frascos del Compuesto 1 en Captisol® (120 mg/frasco) subcutáneamente como una sola dosis por sujeto en las siguientes dosificaciones:

0.5 mg del Compuesto 1/frasco en Captisol®, 1 mg del Compuesto/frasco en Captisol® y 2.5 mg del Compuesto 1/frasco en Captisol®.

Placebo para el Compuesto 1: 120 mg del Captisol®/frasco (idéntico en apariencia a los frascos del Compuesto 1 en Captisol®).

Metodología

Esto fue un estudio de cuatro etapas de centros múltiples, aleatorizado; de doble ciego, controlado con placebo, utilizando una sola inyección subcutánea del Compuesto 1 o un placebo. Se monitorearon los pacientes SLE hasta 21 días previos a los procedimientos de línea basal. Cada sujeto elegible fue aleatorizado a uno de los 4 grupos de tratamiento: inyección subcutánea ya sea de 0.5, 1 ó 2.5 mg del Compuesto 1 o su placebo coincidente. Todos los sujetos fueron admitidos en la clínica en el día de la pre-dosificación. Cada sujeto recibió una sola dosis

de uno de los tratamientos que se enlistaron anteriormente. Se dieron de alta los sujetos de la clínica 24 horas después de la dosificación. Después se controlaron los sujetos a la semana 2, 4 y 8 seguido de la dosificación. Las muestras de sangre (suero y sangre total), para las pruebas de laboratorio de seguridad se retiraron en la etapa de Control, Día de dosificación (previa a la dosis), al Día 2 (post-dosis) a la semanas 2, 4 y 8 (visita de Conclusión). Las muestras sanguíneas para las pruebas inmunológicas se retiraron en las etapas de Control, Día de dosificación (pre-dosis) en las semanas 4 y 8; la proliferación de linfocitos sanguíneos periféricos (PBL) se evaluó en los días de dosificación (pre-dosis), y a las semanas 2, 4 y 8.

Número de sujetos (total y para cada tratamiento):

Se clasificaron aleatoriamente 36 (treinta y seis) sujetos en este estudio como sigue: 9 sujetos en el grupo de tratamiento de 0.5 mg, 9 sujetos en el tratamiento de 1 mg y 10 sujetos en el tratamiento de 2.5 mg, y 8 sujetos en el grupo de tratamiento con placebo.

Diagnóstico y criterio principal para la inclusión

Los sujetos elegibles para este estudio fueron pacientes con SLE que complementaron y requisitaron por lo

menos cuatro criterios de diagnóstico del Colegio Americano de Reumatología (ACR). Su condición de enfermedad tenía que ser estable, de ligera a moderada con una calificación igual o menor a 10 en el índice de actividad de la enfermedad de SLE, en el año 2000 actualizado (SLEDAI 2K).

Los pacientes con SLE excluidos de la participación, fueron aquellos que reportaron un asma inestable o severo, infarto, infarto agudo al miocardio, angina inestable, hemorragia cerebral, embolismo pulmonar durante los seis meses previo al monitoreo del estudio. Los pacientes con SLE que tuvieron alguna condición quirúrgica o médica inestable o clínicamente significativa, o diabetes melitus, enfermedad en el hígado (como cirrosis, hepatitis activa, hipertensión portal y/o ascitos; hipertensión clínicamente significativa, alguna historia médica o cualquier malignidad, diálisis o enfermedad pulmonar obstructiva crónica (COPD) también fue excluido de la participación en el estudio.

También se excluyeron en la participación del estudio los pacientes con SLE que sufrieron plasmaféresis o que fueron tratados durante los tres meses previo a la selección con uno de los fármacos enlistados a continuación con prednisona 30 mg/al día o mayor (una dosis equivalente de otro corticoesteroide), con corticoesteroide intravenosos, con inmunoglobina intravenosa G (IgG), con

anticoagulantes orales y cualesquiera agentes citotóxicos (por ejemplo, con azatioprina, clorambucil, ciclofosfamida, mofetil micofenolato, con metotrexato, o con tacrolimus (fármaco inmunosupresor).

Además, los pacientes SLE que iniciaron el tratamiento con corticoesteriodes (más de ± 10 mg/día con prednisona, o una dosis equivalente de otro corticoesteroide) y/o anti-malaria, durante los últimos 3 meses previo a la selección, fueron excluidos del estudio.

Aunque hizo un gran esfuerzo para retener los tratamientos médicos SLE de línea basal a lo largo del curso del estudio, los investigadores sin embargo, no pudieron cambiar el tratamiento médico participante en ningún momento, durante el estudio para poder mantener y optimizar el bienestar del paciente.

Criterios para la evaluación.

Seguridad:

Se evaluaron los siguientes parámetros de seguridad en las etapas de control, selección, o entre la hospitalización y en las visitas de seguimiento incluyendo en la visita de conclusión: signos vitales (presión sanguínea sistólica, presión sanguínea diastólica, pulso, saturación con oxígeno, temperatura y peso), electrocardiograma de 12 guías, cambio en el examen físico y pruebas rutinarias de laboratorio clínicas. Se

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registraron los eventos adversos al día de la dosificación en cada visita posterior a la anterior.

Inmunología:

Se desarrollaron pruebas inmunológicas relacionadas con SLE en la etapa de selección, durante la hospitalización y en las visitas de seguimiento, incluyendo la visita de conclusión.

Las respuestas inmunológicas relacionadas con el fármaco fueron seguidas utilizando el ensayo de proliferación PBL y el ensayo con anticuerpos, anti-Compuesto 1 en el dia de la dosificación y, en las visitas de seguimiento incluyendo la visita de Conclusión.

Actividad de la enfermedad:

La valoración de la actividad de la enfermedad utilizando la puntuación del índice de actividad de enfermedad de SLE, en el año 2000 actualizado (SLEDAT 2K) se evaluó en las etapas de Selección, durante la hospitalización y en las visitas de seguimiento incluyendo la vista de Conclusión.

Métodos estadísticos:

Se utilizó un software SAS® versión 9.0 para analizar y presentar datos recopilados durante este estudio. No se desarrolló ningún cálculo de potencia y no se condujo ninguna prueba de hipótesis formal para este estudio de Fase Ia.

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Experiencias adversas:

Se presentó la incidencia y la frecuencia de experiencias adversas por medio de Clase de órganos del sistema y por la terminología preferida de acuerdo con el diccionario MedDRA versión 5.0. Los datos se tabularon por grupo de tratamiento.

Datos de laboratorio clínico:

Estadísticas descriptivas de valores de laboratorio incluyendo el número de observaciones, desviación estándar, desviación media, mínima y máxima y se determinaron para la etapa de Selección, Día 1, (pre-dosis), Día 2, Semana 2, 4 y 8, y se presentaron por grupo de tratamiento. También se presentaron los cambios de línea basal a cada visita/punto, de tiempo, para cada asignación por tratamiento. El porcentaje de los resultados anormales (bajo y alto, donde aplicaba) se presenta sobre una base de parámetro, por grupo de tratamiento y por punto de visita/periodo. Se desarrollaron análisis de turno desde la línea basal a 24 horas post- dosis y de la línea basal hasta la visita de conclusión.

Signos vitales:

Estadísticas descriptivas para los signos vitales incluyen el número de observaciones, la media, desviación estándar, valores medianos, mínimos y máximos, y se determinaron para la etapa de Selección, Día 1 (pre y post-

dosis, y en cada punto de periodo). Día 2, Semanas 2, 4 y 8, se tabulan por el tratamiento asignado. Los cambios de línea basal a cada punto de periodo/visita, se presentan por visita y por asignación de tratamiento.

Peso:

Estadísticas descriptivas de peso (kilogramos) en la línea basal, terminación y cambio de la línea basal, que se presente por grupo de tratamiento.

Electrocardiograma (ECG)

Estadísticas descriptivas de los parámetros de electrocardiograma en línea basal, terminación y cambios desde la línea basal, son los que se presentan en este párrafo. Se presenta el análisis de turno conforme a los cuadros de turno desde línea basal hasta la conclusión entre parámetros normal/anormal o presente/ausente del electrocardiograma (ECG). Se identificaron las medidas potencial y clínicamente significativas (PCS) QTc (Bazett), de acuerdo con los criterios predefinidos. Se presentan los cuadros de análisis de cambio entre el PCS y el cuadro de incidencia de PCS y QTc no-PCS absoluta (Bazett) de cambio de PCS en QTc (Bazett) desde la línea basal hasta cualquier visita.

Examen físico:

Se analizaron los resultados del examen físico por incidencia de sujetos con hallazgos anormales o

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normales para cada sistema corporal en la visita de Línea basal y en la visita de terminación. Los análisis de cambio entre anormal y normal y viceversa también se aplicaron. Cuando no hubo cambio que ocurriera en la línea basal, se definió como "otros".

Pruebas inmunológicas relacionadas con el Compuesto 1

Para los parámetros inmunológicos, estadísticas descriptivas, incluyendo el número de observaciones, media, desviación estándar, mediana y valores mínimo y máximo, se calcularon en este caso y se presentaron por un grupo de tratamiento y por visita. El cambio desde la línea basal a cada visita de seguimiento también se presenta por un grupo de tratamiento. Cuando aplicaba, se presentó el número y porcentaje de sujetos con resultados negativos/positivos por grupo de tratamiento y visita.

SLEDAI 2K

Estadísticas descriptivas incluyendo desviación estándar, media, valores mínimos y máximos, mediana, de SLEDAI 2K.

Resultados del estudio clínico de fase Ia:

Disposición sujeta y características SLE

Se ingresaron treinta y seis (36) sujetos del estudio y completaron este estudio por protocolo. La mayoría de sujetos (34) en todos los grupos de tratamiento

fueron del sexo femenino (94.4%) y caucásicas (30, 83.3%). La edad media para todos los grupos de tratamiento fue de 35.6 años (rango de media de 32 a 39 años). La mayoría de los sujetos (91.7%) tenían entre 4 a 6 criterios de diagnóstico según el Colegio americano de reumatología (ACR) y una puntuación de grupo de media SLEDAI 2K que variaba de 2.1 a 4.1.

Resultados de seguridad

No hubo diferencia prominente entre los grupos de tratamiento con el fármaco de estudio y el grupo de placebo en la incidencia de AEs. Los AEs más comunes en todos los grupos fueron dolor de cabeza, clasificado de ligero a moderado en naturaleza y una reacción en el sitio de inyección clasificada como ligera naturaleza. No se apreció respuesta a la dosis. No hubo evento adverso grave (SAE) o AE clasificado como grave que haya ocurrido durante el estudio.

No hubo ningún evento clínicamente significativo atribuible al fármaco bajo estudio que se pudiera apreciar para los valores de hematología, bioquímica o análisis de orina.

No hubo efecto clínicamente significativo atribuible al fármaco bajo estudio que se apreciará para los parámetros de signos vitales (presión sanguínea

sistólica, presión sanguínea diastólica), pulso, saturación de oxígeno).

No hubo efecto clínicamente significativo atribuible al fármaco bajo estudio que se pudiera observar para temperatura y peso.

No hubo diferencias clínicamente significativas que se apreciará entre los grupos tratados con el Compuesto 1 y el placebo para las medidas categóricas de electrocardiograma ECG y los parámetros de ECG digitalizados. No hubo valor absoluto QTc de la PCS y no hubo cambio QTc desde línea basal > 60 mseg que se hubiera registrado. Un número similar en sujetos en los grupos tratados con Compuesto 1 y con placebo tuvo cambio QTcB desde línea basal entre 30 a 60 mseg.

No se anotaron efectos clínicamente significativos de Compuesto 1 en el examen físico.

Resultados inmunológicos

La evaluación de muestras de suero de todos los sujetos indicaron que una sola administración subcutánea del Compuesto 1 a los niveles de dosis de 0.5, 1 y 2.5 mg/paciente no indujo el desarrollo de anticuerpos específicos al anti-Compuesto 1. Siete sujetos tuvieron una respuesta al Compuesto 1 sobre el valor límite. Estos niveles elevados de anticuerpos no estaban presentes previo a la dosificación. No hubo incrementos en los niveles de

anticuerpos que se observaran en el periodo de seguimiento (dos meses) del estudio. Los sueros de estos sujetos fueron analizados para el isotipo de los anticuerpos reactivos. La respuesta en dos de los sujetos estuvo asociada con el isotipo IgM y con el isotipo IgG en los otros dos. Ninguno de los siete tenía anticuerpos IgE específicos.

El ensayo de linfocitos sanguíneos periféricos (PBL) mostró que el 50% de los sujetos (18) fueron clasificados como activos a la respuesta (SI>2) con distribución similar en todos los grupos de tratamiento. La respuesta de células T fue relativamente baja y no hubo asociación entre la dosis de tratamiento al Compuesto 1 o la concentración utilizada en el ensayo y se pudo detectar un estatus de activo a respuesta/sin respuesta, tomando en consideración que solamente una dosis subcutánea SC del fármaco bajo estudio fue administrada. Además no se observó indicio de incidencia incrementada del estatus de respuesta durante el transcurso del tiempo. El ensayo de toxoide tetánico (TTX) que sirve como un control de seguridad muestra que la respuesta a TTX es conservó a lo largo de todo el periodo de estudio en todos los grupos de tratamiento indicando que el Compuesto 1 en Captisol® no cambió la respuesta inmunológica al antígeno de respuesta libre TTX.

Los hallazgos inmunológicos son el resultado de la administración de solamente una sola dosis de Compuesto 1 del fármaco bajo estudio.

Resultados de la actividad de la enfermedad

No hubo efecto clínicamente significativo del Compuesto 1 en la puntuación SLEDAI, (cambio de ≥ 3, ≤ 12 puntos) que se pudieren notado durante el estudio excepto para un sujeto en el grupo de tratamiento con 0.5 mg para quien, se les registró un cambio en la puntuación SLEDAI o 2 a 10 puntos entre la línea basal y la semana 4 basándose en un análisis de orina que mostraba pyuri. Este hallazgo en el análisis de orina no fue confirmado por el investigador como una erupción de lupus por definición de protocolo y fue resuelto sin cambio de tratamiento.

Conclusiones

Este estudio de Fase Ia mostró que una sola dosis inyectada subcutáneamente de Compuesto 1 con 0.5, 1 o 2.5 mg en 120 mg de Captisol® era segura y bien tolerada y permitió la continuación hacia el estudio de dosis múltiple de fase Ib.

## EJEMPLO 9

## ESTUDIO CLÍNICO DE FASE IB

La fase I, múltiples centros, binacional, clasificado aleatoriamente, doble ciego, Cuatro etapas controlado con placebo, estudio de dosis múltiple para

evaluar la capacidad de tolerancia y seguridad del Compuesto 1, en inyecciones subcutáneas con Captisol® en sujetos SLE.

Este estudio se desarrolla para evaluar la seguridad y capacidad de tolerancia de la administración repetida subcutánea de Compuesto 1 a sujetos SLE. El objetivo secundario del estudio es evaluar las respuestas inmunológicas seguida la administración subcutánea repetida sc del Compuesto 1 en Captisol® en sujetos con SLE.

El Compuesto 1 se administra en dosis de 0.5, 1.0 o 2.5 mg en Captisol®. El producto bajo investigación es administrado en cualquier otro día (excluyendo fines de semana) para un total de 12 inyecciones subcutáneas sc, es decir, 3 dosis a la semana por 4 semanas. Los sujetos son controlados en visitas planeadas que fueron planeadas a las 2, 4, 8 y 12 semanas después del inicio de la dosificación. Se evaluó la seguridad y la capacidad de tolerancia utilizando pruebas similares a aquellas descritas en el estudio clínica de fase Ia anterior.

Resultados

Este estudio de fase Ib muestra que múltiples dosis inyectadas subcutáneamente del Compuesto 1 de 0.5, 1 o 2.5 mg en 120 mg de Captisol®, son seguros y bien tolerados.

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## NOVEDAD DE LA INVENCIÓN

Habiendo descrito el presente invento, se considera como una novedad y, por lo tanto, se reclama como prioridad lo contenido en las siguientes:

## REIVINDICACIONES

1.- Una composición farmacéutica, que comprende un vehículo acuoso; desde 0.1 mg/ml a 20 mg/ml de la composición de una sal farmaceuticamente aceptable de un péptido que tiene la fórmula estructural $NH_2$ - Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Glu Glu Trp Ile Gly - COOH (SEQ ID NO: 1); y un β-ciclodextrina sustituida en una cantidad efectiva para disolver el péptido en el vehículo acuoso, caracterizado porque la composición tiene un pH entre 4 y 9.

2.- La composición farmacéutica de conformidad con la reivindicación 1, caracterizada porque la concentración de la sal del péptido es de por lo menos 0.5 mg/ml.

3.- La composición farmacéutica de conformidad con la reivindicación 2, caracterizada

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porque la concentración de la sal del péptido es de 0.5 mg/ml a 10 mg/ml.

4.- La composición farmacéutica de conformidad con la reivindicación 3, caracterizada porque la concentración de la sal del péptido es de 0.5 mg/ml a 2.5 mg/ml.

5.- La composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 4, caracterizada porque la composición tiene un pH entre 6.5 y 8.5.

6.- La composición farmacéutica de conformidad con la reivindicación 5, caracterizada porque la composición tiene un pH entre 7.5 y 8.5.

7.- La composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 6, caracterizada porque la sal farmaceuticamente aceptable es una sal de acetato.

8.- La composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 7, caracterizada porque el β-ciclodextrina sustituida es un hidropropil, un éter sulfobutílico, o un éter sulfobutílico β-ciclodextrina sustituida.

9.- La composición farmacéutica de conformidad con la reivindicación 8, caracterizada

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porque el β-ciclodextrina sustituida es un éter sulfobutílico β-ciclodextrina sustituida.

10.- La composición farmacéutica de conformidad con la reivindicación 7, caracterizada porque el β-ciclodextrina sustituida es hepta-(éter sulfobutílico)- β-ciclodextrina.

11.- La composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 10, que además comprende un regulador farmaceuticamente aceptable en una cantidad y de un tipo adecuado para hacer que el pH de la composición farmacéutica se encuentre dentro del rango de 4 a 9.

12.- Una composición farmacéutica, que comprende un vehículo acuoso; desde 0.1 mg/ml a 20 mg/ml de la composición de una sal de acetato de un péptido que tiene la fórmula estructural $NH_2$ - Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Glu Glu Trp Ile Gly - COOH (SEQ ID NO: 1); y de 70 mg/ml a 170 mg/ml de la composición de hepta-(éter sulfobutílico)- β-ciclodextrina, caracterizada porque el péptido y el hepta-(éter sulfobutílico)- β-ciclodextrina se disuelven en el vehículo acuoso; y caracterizada porque la composición tiene un pH entre 6.5 y 8.5.

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13.- La composición farmacéutica de conformidad con la reivindicación 12, caracterizada porque la concentración de la sal de acetato del péptido se de por lo menos 0.5 mg/ml.

14.- La composición farmacéutica de conformidad con la reivindicación 13, caracterizada porque la concentración de la sal de acetato del péptido es de 0.5 mg/ml a 10 mg/ml.

15.- La composición farmacéutica de conformidad con la reivindicación 13, caracterizada porque la concentración de la sal de acetato del péptido es de 0.5 mg/ml a 2.5 mg/ml.

16.- La composición farmacéutica de conformidad con la reivindicación 13, caracterizada porque la concentración de hepta-(éter sulfobutílico)- β-ciclodextrina es de 120 mg/ml, y caracterizada porque el pH de la composición se encuentra entre 7.5 y 8.5.

17.- La composición farmacéutica de conformidad con la reivindicación 16, caracterizada porque la concentración de la sal de acetato del péptido es de 1.0 mg/ml.

18.- La composición farmacéutica de conformidad con la reivindicación 16, caracterizada

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porque la concentración de la sal de acetato del péptido es de 2.5 mg/ml.

19.- Un método para aliviar síntomas de un lupus sistemático eritematoso (SLE) en un sujeto humano que comprende administrar el sujeto humano la composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 18 en una cantidad efectiva para aliviar los síntomas de SLE en el sujeto humano.

20.- La composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 18, para utilizarla en el tratamiento de SLE en un sujeto humano.

21.- Un procedimiento para fabricar la composición farmacéutica de conformidad con cualquiera de las reivindicaciones 1 a 18, que comprende los pasos de: a) preparar una solución de un β-ciclodextrina sustituida en un vehículo acuoso en una concentración predeterminada; b) agregar una cantidad predeterminada de una sal farmaceuticamente aceptable del péptido $NH_2$ - Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Glu Glu Trp Ile Gly - COOH (SEQ ID NO: 1) a la solución del paso a); c) ajustar el pH de la solución del paso b) hasta que el péptido se disuelve en la

solución; y d) si es necesario, ajustar el pH de la solución del paso c) a un pH de 4 a 9, de se modo fabricando la composición farmacéutica.

22.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque la concentración predeterminada del β-ciclodextrina sustituida es tal, que resulta en una concentración final del β-ciclodextrina sustituida en la composición farmacéutica desde 70 mg/ml a 170 mg/ml.

23.- El procedimiento de conformidad con la reivindicación 22, caracterizado porque la concentración predeterminada del β-ciclodextrina sustituida es tal, que resulta en una concentración final del β-ciclodextrina sustituida en la composición farmacéutica de 120 mg/ml.

24.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque la cantidad predeterminada de péptido es tal, que resulta en una concentración final del péptido en la composición farmacéutica de por lo menos 0.1 mg/ml.

25.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque la cantidad predeterminada de péptido es tal, que resulta en

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una concentración final de péptido en la composición farmacéutica de por lo menos 0.5 mg/ml.

26.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque la cantidad predeterminada de péptido es tal, que resulta en una concentración final de péptido en la composición farmacéutica de 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml o 0.1 mg/ml.

27.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque el paso b) además comprende mezclar la solución durante un lapso de una hora.

28.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque en el paso c) el pH se ajusta utilizando HCl o NaOH 1.0N.

29.- El procedimiento de conformidad con la reivindicación 21, que además comprende filtrar la solución del paso d) a través de un filtro de acetato de celulosa.

30.- El procedimiento de conformidad con la reivindicación 21, caracterizado porque la concentración predeterminada del β-ciclodextrina sustituida es tal, que resulta en una concentración final del β-ciclodextrina sustituida en la composición farmacéutica de 120 mg/ml; la cantidad

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predeterminada de péptido es tal, que resulta en una concentración final de péptido en la composición farmacéutica de 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml o 0.1 mg/ml; el paso b) además comprende mezclar la solución durante un lapso de una hora; y en el paso c) el pH se ajusta utilizando HCl p NaOH 1.0N, que además comprende filtrar la solución del paso d) a través de un filtro de acetato de celulosa.

31.- Una composición farmacéutica preparada por el procedimiento de cualquiera de las reivindicaciones 21 a 30.

32.- Un procedimiento para liofilizar la composición farmacéutica de conformidad con la reivindicación 2, que comprende los pasos de: a) disminuir la temperatura de la composición farmacéutica a -40°C; b) mantener la temperatura de la composición farmacéutica a -40°C por un tiempo predeterminado; c) elevar la temperatura de la solución a 20°C; d) mantener la temperatura en 20°C por un tiempo predeterminado; y e) reducir la presión y mantener la temperatura a 20°C por un tiempo predeterminado, de se modo liofilizando la composición farmacéutica.

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33.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso a) se lleva a cabo dentro de un lapso de 2 horas.

34.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso b) se lleva a cabo dentro de un lapso de 3 horas.

35.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso c) se lleva a cabo dentro de un lapso de 13 horas.

36.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso c) se lleva a cabo a una presión de 110µbar.

37.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso d) se lleva a cabo dentro de un lapso de 13 horas.

38.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso d) se lleva a cabo a una presión de 110µbar.

39.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque en el paso e) la presión se reduce a 10µbar.

40.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso e) se lleva a cabo dentro de un lapso de 5 horas.

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41.- El procedimiento de conformidad con la reivindicación 32, caracterizado porque el paso a) se lleva a cabo dentro de un lapso de 2 horas; el paso b) se lleva a cabo dentro de un lapso de 3 horas; el paso c) se lleva a cabo dentro de un lapso de 13 horas y a una presión de 110µbar; el paso d) se lleva a cabo dentro de un lapso de 13 horas y a una presión de 110µbar; y el paso e) se lleva a cabo dentro de un lapso de 5 horas y a una presión de 10µbar.

42.- Una composición farmacéutica liofilizada preparada por el procedimiento de conformidad con cualquiera de las reivindicaciones 32 a 41.

43.- Un procedimiento para liofilizar la composición farmacéutica de conformidad con la reivindicación 2, que comprende los pasos de: a) disminuir la temperatura de la composición farmacéutica a -45°C; b) mantener la temperatura a -45°C por un tiempo predeterminado; c) elevar la temperatura de la solución a -20°C; d) elevar la temperatura de la solución a 25°C; y mantener la temperatura a 25°C por un tiempo predeterminado, de ese modo liofilizando la composición farmacéutica.

44.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso a) se lleva a cabo dentro de un lapso de 6 horas.

45.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso b) se lleva a cabo dentro de un lapso de 3 horas.

46.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso c) se lleva a cabo dentro de un lapso de 19 horas.

47.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso c) se lleva a cabo a una presión de 150µbar.

48.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso d) se lleva a cabo dentro de un lapso de 13 horas.

49.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso d) se lleva a cabo a una presión de 150µbar.

50.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso e) se lleva a cabo dentro de un lapso de 8 horas.

51.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso e) se lleva a cabo a una presión de 150µbar.

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52.- El procedimiento de conformidad con la reivindicación 43, caracterizado porque el paso a) se lleva a cabo dentro de un lapso de 6 horas; el paso b) se lleva a cabo dentro de un lapso de 3 horas; el paso c) se lleva a cabo dentro de un lapso de 19 horas y a una presión de 150µbar; el paso d) se lleva a cabo dentro de un lapso de 13 horas y a una presión de 150µbar; y el paso e) se lleva a cabo dentro de un lapso de 8 horas y a una presión de 150µbar.

53.- Una composición farmacéutica liofilizada preparada por el procedimiento de conformidad con cualquiera de las reivindicaciones 43 a 52.

54.- La composición farmacéutica liofilizada de conformidad con la reivindicación 53, caracterizada porque el contenido de agua de la composición es menor al 5%.

55.- La composición farmacéutica liofilizada de conformidad con la reivindicación 54, caracterizada porque el contenido de agua de la composición es menor al 4.0%.

56.- La composición farmacéutica liofilizada de conformidad con la reivindicación

53, caracterizada porque el contenido de agua de la composición es menor al 3.5%.

57.- Una composición farmacéutica liofilizada, que comprende una sal farmaceuticamente aceptable de un péptido que tiene la fórmula estructural $NH_2$ - Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Glu Glu Trp Ile Gly - COOH (SEQ ID NO: 1); y un β-ciclodextrina sustituida.

58.- Una composición farmacéutica empacada, que comprende: un material de empaque; y una cantidad predeterminada de composición farmacéutica liofilizada de conformidad con la reivindicación 57.

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## RESUMEN DE LA INVENCION

La presente invención provee una composición farmacéutica que comprende un vehículo acuoso de 0.1 mg/ml a 20 mg/ml de la composición de una sal farmacéuticamente aceptable de un péptido que tiene la fórmula estructural $NH_2$-Gly Tyr Tyr Tip Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly-cooH; y una 13-ciclodextrina sustituida en una cantidad efectiva para disolver el péptido en el vehículo acuso en donde la composición tiene un pH entre 4 y 9, un procedimiento para su preparación, y un método para aliviar los síntomas del lupus sistémico eritematoso (SLE) en un ser humano que comprende administrar a dicho ser humano la composición farmacéutica.

$NH_2$ – G Y Y W S W I R Q P P G K G E E W I G – COOH

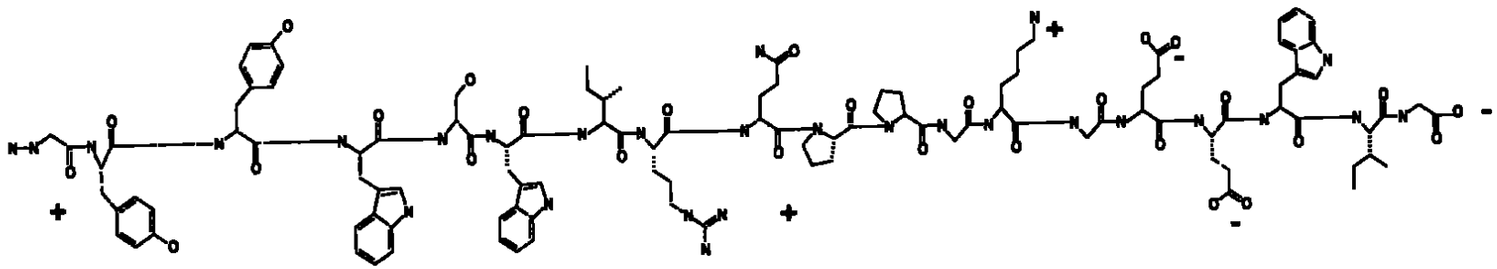


FIGURA 1

$NH_2$ – Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu Glu Trp Ile Gly – COOH

Peso Molecular = 2309.59
Masa Exacta = 2307
Fórmula Molecular = $C_{111}H_{149}N_{27}O_{28}$
Composición Molecular = C 57.73% H 6.50% N 16.37% O 19.40%

**Acético a agregarse:** $CH_3CO_2H = C_2H_4O_2$

**$C_{111}H_{149}N_{27}O_{28}. C_2H_4O_2$**

1/6

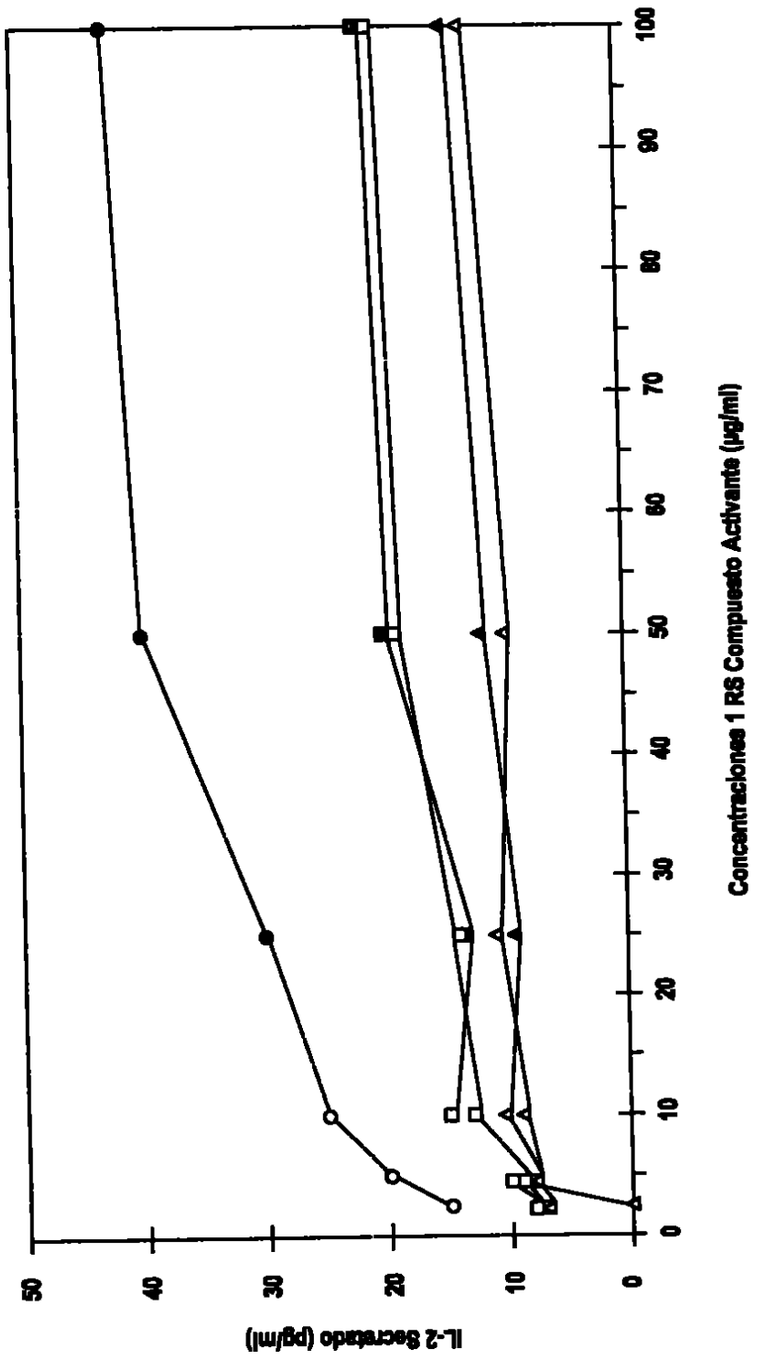

IL-2 Secretado (pg/ml)
0
10
20
30
40
50
Concentraciones 1 RS Compuesto Activante (µg/ml)
0
10
20
30
40
50
60
70
80
90
100

FIGURA 2

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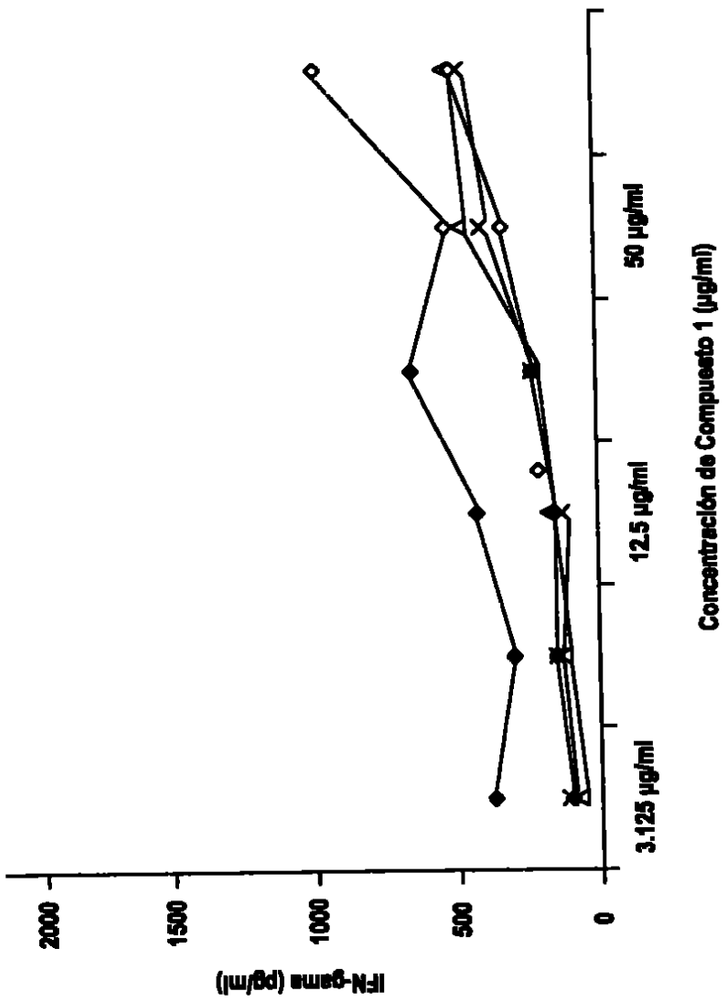

IFN-gama (pg/ml)
2000
1500
1000
500
0
3.125 µg/ml
12.5 µg/ml
50 µg/ml
Concentración de Compuesto 1 (µg/ml)

FIGURA 3

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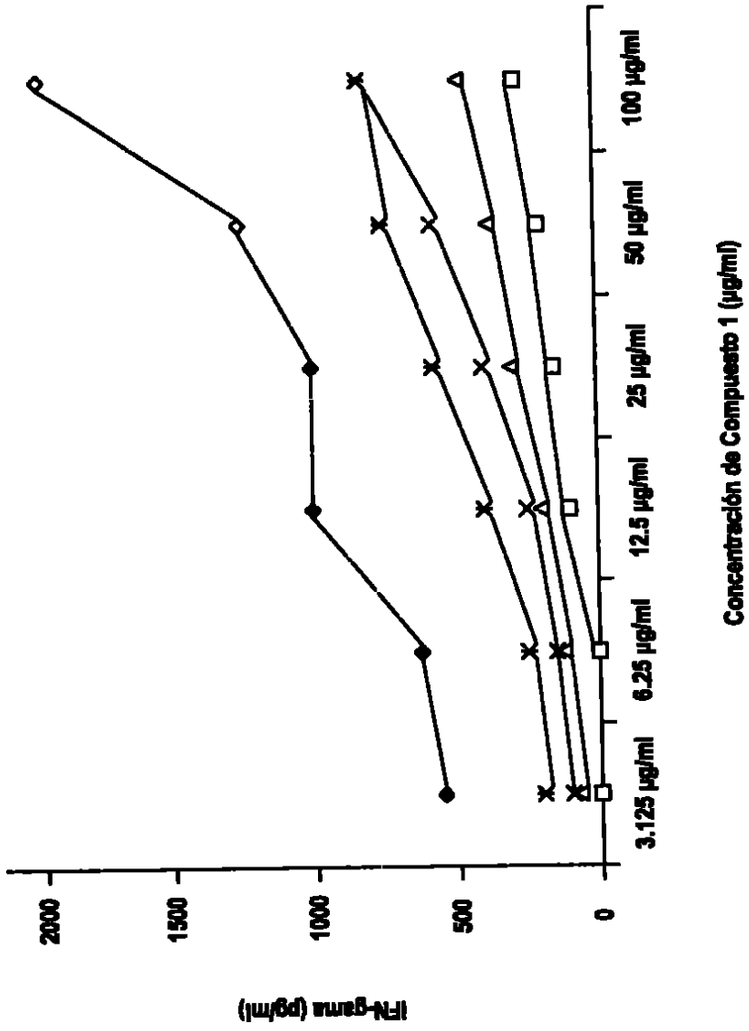

IFN-gama (pg/ml)
2000
1500
1000
500
0
3.125 µg/ml
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
Concentración de Compuesto 1 (µg/ml)

FIGURA 4

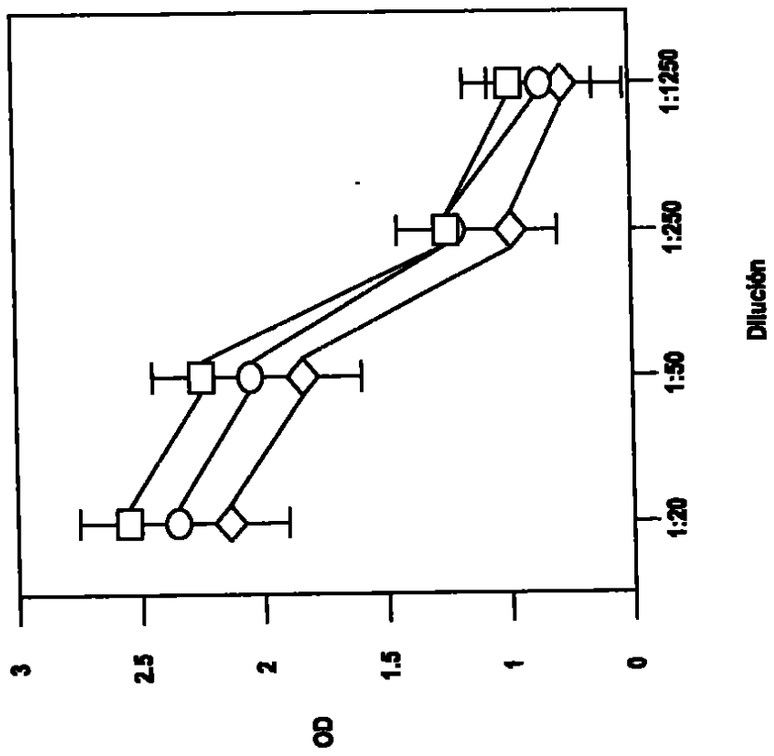



FIGURA 5

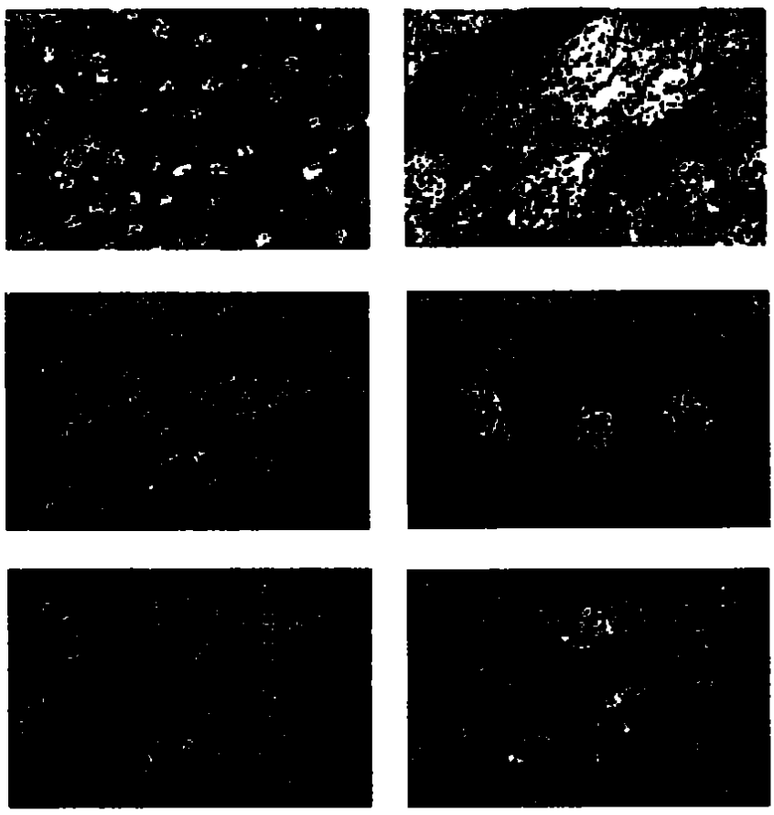


**FIGURA 6**

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference 68518-A-PCT/JPW/JCC | FOR FURTHER ACTION | see Form PCT/ISA/220 as well as, where applicable, item 5 below. |
|---|---|---|
| International application No. PCT/US04/00955 | International filing date (*day/month/year*) 14 January 2004 (14.01.2004) | (Earliest) Priority Date (*day/month/year*) 14 January 2003 (14.01.2003) |
| Applicant TEVA PHARMACEUTICAL INDUSTRIES, LTD. | | |

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the Report**
   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      ☐ The international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.
2. ☒ Certain claims were found unsearchable (See Box No. II)
3. ☐ Unity of invention is lacking (See Box No. III)
4. With regard to the title,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,
   a. the figure of the drawings to be published with the abstract is Figure No. ____
      ☐ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☒ none of the figures is to be published with the abstract.

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/00955

## Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing

   ☒ contained in the international application as filed

   ☐ filed together with the international application in computer readable form

   ☒ furnished subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/00955

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-11, 19-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/00955

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/00, 47/40, 51/10
US CL : 514/2; 530/300; 424/130.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/2; 530/300; 424/130.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 96/30057 MOZES, E. et al. 03 October 1996 (03.10.1996), entire document, particularly claims 1-15. | 1-6, 12-18, and 32-58. |
| Y | US 5,646,131 (BADWAN et al) 08 July 1997 (08.07.1997), entire document, particularly abstract. | 1-6, 12-18, and 32-58. |
| Y | US 4,656,158 (MATSUO et al.) 07 April 1987 (07.04.1987), entire document, particularly claim 5. | 1-6, 12-18, and 32-58. |
| Y, E | US 2004/0127408 A1 (MOZES, E.) 01 July 2004 (01.07.2004), entire document, particularly claims 24, and 29. | 1-6, 12-18, and 32-58. |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 22 November 2004 (22.11.2004) | 30 DEC 2004 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US<br>Commissioner for Patents<br>P.O. Box 1450<br>Alexandria, Virginia 22313-1450<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Anand Desai<br>Telephone No. (571) 272-0947 |

Form PCT/ISA/210 (second sheet) (January 2004)

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# PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
JOHN P. WHITE
COOPER & DUNHAM LLP
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

# PCT

## WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

| | |
|---|---|
| | Date of mailing (*day/month/year*) 30 DEC 2004 |
| Applicant's or agent's file reference<br>68518-A-PCT/JPW/JCC | FOR FURTHER ACTION<br>See paragraph 2 below |

| International application No. | International filing date (*day/month/year*) | Priority date (*day/month/year*) |
|---|---|---|
| PCT/US04/00955 | 14 January 2004 (14.01.2004) | 14 January 2003 (14.01.2003) |

International Patent Classification (IPC) or both national classification and IPC

IPC(7): A61K 38/00, 47/40, 51/10 and US Cl.: 514/2; 530/300; 424/130.1

Applicant

TEVA PHARMACEUTICAL INDUSTRIES, LTD.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I — Basis of the opinion
- ☐ Box No. II — Priority
- ☒ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV — Lack of unity of invention
- ☒ Box No. V — Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI — Certain documents cited
- ☐ Box No. VII — Certain defects in the international application
- ☐ Box No. VIII — Certain observations on the international application

## 2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(*b*) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

| | |
|---|---|
| Name and mailing address of the ISA/ US<br>Mail Stop PCT, Attn: ISA/US<br>Commissioner for Patents<br>P.O. Box 1450<br>Alexandria, Virginia 22313-1450<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Anand Desai<br>Telephone No. (571) 272-0947 |

Form PCT/ISA/237 (cover sheet) (January 2004)

**WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US04/00955

**Box No. I Basis of this opinion**

1. With regard to the language, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This opinion has been established on the basis of a translation from the original language into the following language _____, which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☒ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☒ in written format

☒ in computer readable form

c. time of filing/furnishing

☐ contained in international application as filed.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority for the purposes of search.

3. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

**WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US04/00955

**Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 7-11 and 19-31

because:

☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require an international preliminary examination *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 7-11 and 19-31 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims are improper multiple dependent claims.

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _____

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form
- ☐ has not been furnished
- ☐ does not comply with the standard

the computer readable form
- ☐ has not been furnished
- ☐ does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See Supplemental Box for further details.

**WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US04/00955

**Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Claims | 1-6, 12-18, and 32-58 | YES |
| | Claims | NONE | NO |
| Inventive step (IS) | Claims | NONE | YES |
| | Claims | 1-6, 12-18, and 32-58 | NO |
| Industrial applicability (IA) | Claims | 1-6, 12-18, and 32-58 | YES |
| | Claims | NONE | NO |

2. Citations and explanations:

Claims 1-6, 12-18, and 32-58 lack an inventive step under PCT Article 33(3) as being obvious over Mozes, E. et al. (WO 96/30057) in view of Badwan et al. (US 5,646,131) and further in view of Matsuo et al. (US 4,656,158). WO 96/30057 teaches synthetic peptides and pharmaceutical compositions for the treatment of systemic lupus erythematosus. The peptides are based on the complementarity-determining region of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a systemic lupus erythematosus disease (see page 4, lines 9-19). The peptide inhibits the proliferative response and cytokine secretion of T lymphocytes of that are high responders to systemic lupus erythematosus inducing autoantibodies (see claim 2). The pharmaceutical composition for the treatment of systemic lupus erythematosus comprises an effective amount of a peptide and a pharmaceutically acceptable carrier (see claim 12). WO 96/30057 does not specifically disclose a pharmaceutical composition comprising a peptide and a substituted beta-cyclodextrin in a lyophilized form.
Badwan et al. teaches a method for enhancing the solubilization of a drug with cyclodextrin (see abstract). Matsuo et al. teaches the lyophilized form of a pharmaceutical composition comprising a peptide active agent (see claim 5). Therefore, it would have obvious to prepare a pharmaceutical composition in lyophilized form comprising the peptide disclosed by Mozes, E. et al. to treat systemic lupus erythematosus (current application, claims 1-6, 12-18, and 32-58).

Claims 1-6, 12-18, and 32-58 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

PATENT COOPERATION TREATY

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From the
INTERNATIONAL SEARCHING AUTHORITY

To:
JOHN P. WHITE
COOPER & DUNHAM LLP
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

# PCT

## WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

| | |
|---|---|
| | Date of mailing (*day/month/year*) 30 DEC 2004 |
| Applicant's or agent's file reference<br>68518-A-PCT/JPW/JCC | FOR FURTHER ACTION<br>See paragraph 2 below |

| International application No. | International filing date (*day/month/year*) | Priority date (*day/month/year*) |
|---|---|---|
| PCT/US04/00955 | 14 January 2004 (14.01.2004) | 14 January 2003 (14.01.2003) |

International Patent Classification (IPC) or both national classification and IPC

IPC(7): A61K 38/00, 47/40, 51/10 and US Cl.: 514/2; 530/300; 424/130.1

Applicant

TEVA PHARMACEUTICAL INDUSTRIES, LTD.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I — Basis of the opinion
- ☐ Box No. II — Priority
- ☒ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV — Lack of unity of invention
- ☒ Box No. V — Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI — Certain documents cited
- ☐ Box No. VII — Certain defects in the international application
- ☐ Box No. VIII — Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(*b*) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

| | |
|---|---|
| Name and mailing address of the ISA/ US<br>Mail Stop PCT, Attn: ISA/US<br>Commissioner for Patents<br>P.O. Box 1450<br>Alexandria, Virginia 22313-1450<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Anand Desai<br>Telephone No. (571) 272-0947 |

Form PCT/ISA/237 (cover sheet) (January 2004)

**WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US04/00955

**Box No. I Basis of this opinion**

1. With regard to the language, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language _____, which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material

      ☒ a sequence listing

      ☐ table(s) related to the sequence listing

   b. format of material

      ☒ in written format

      ☒ in computer readable form

   c. time of filing/furnishing

      ☐ contained in international application as filed.

      ☐ filed together with the international application in computer readable form.

      ☒ furnished subsequently to this Authority for the purposes of search.

3. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

**WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US04/00955

**Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 7-11 and 19-31

because:

☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require an international preliminary examination *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 7-11 and 19-31 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims are improper multiple dependent claims.

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _____

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form
- ☐ has not been furnished
- ☐ does not comply with the standard

the computer readable form
- ☐ has not been furnished
- ☐ does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See Supplemental Box for further details.

**WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US04/00955

**Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | |
|---|---|---|
| Novelty (N) | Claims 1-6, 12-18, and 32-58 | YES |
| | Claims NONE | NO |
| Inventive step (IS) | Claims NONE | YES |
| | Claims 1-6, 12-18, and 32-58 | NO |
| Industrial applicability (IA) | Claims 1-6, 12-18, and 32-58 | YES |
| | Claims NONE | NO |

2. Citations and explanations:

Claims 1-6, 12-18, and 32-58 lack an inventive step under PCT Article 33(3) as being obvious over Mozes, E. et al. (WO 96/30057) in view of Badwan et al. (US 5,646,131) and further in view of Matsuo et al. (US 4,656,158). WO 96/30057 teaches synthetic peptides and pharmaceutical compositions for the treatment of systemic lupus erythematosus. The peptides are based on the complementarity-determining region of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a systemic lupus erythematosus disease (see page 4, lines 9-19). The peptide inhibits the proliferative response and cytokine secretion of T lymphocytes of that are high responders to systemic lupus erythematosus inducing autoantibodies (see claim 2). The pharmaceutical composition for the treatment of systemic lupus erythematosus comprises an effective amount of a peptide and a pharmaceutically acceptable carrier (see claim 12). WO 96/30057 does not specifically disclose a pharmaceutical composition comprising a peptide and a substituted beta-cyclodextrin in a lyophilized form.
Badwan et al. teaches a method for enhancing the solubilization of a drug with cyclodextrin (see abstract). Matsuo et al. teaches the lyophilized form of a pharmaceutical composition comprising a peptide active agent (see claim 5). Therefore, it would have obvious to prepare a pharmaceutical composition in lyophilized form comprising the peptide disclosed by Mozes, E. et al. to treat systemic lupus erythematosus (current application, claims 1-6, 12-18, and 32-58).

Claims 1-6, 12-18, and 32-58 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

# PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JOHN P. WHITE
COOPER & DUNHAM LLP
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

JAN - 6

# PCT

## NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

| | |
|---|---|
| | Date of mailing (*day/month/year*) 30 DEC 2004 |
| Applicant's or agent's file reference 68518-A-PCT/JPW/JCC | FOR FURTHER ACTION See paragraphs 1 and 4 below |
| International application No. PCT/US04/00955 | International filing date (*day/month/year*) 14 January 2004 (14.01.2004) |
| Applicant TEVA PHARMACEUTICAL INDUSTRIES, LTD. | |

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19: → 2/28/05

   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes, 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 740 14 35

   IDS: [68518-A]

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

   Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

   The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

   Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

| | |
|---|---|
| Name and mailing address of the ISA/ US<br>Mail Stop PCT, Attn: ISA/US<br>Commissioner for Patents<br>P.O. Box 1450<br>Alexandria, Virginia 22313-1450<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Anand Desai<br>Telephone No. (571) 272-0947 |

Form PCT/ISA/220 (January 2004) *(See notes on accompanying sheet)*

## NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article," "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

## *INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19*

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

**What parts of the international application may be amended ?**

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Preliminary Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

**When ?** Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

**Where not to file the amendments ?**

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

**How ?** Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

**The amendments must be made in the language in which the international application is to be published.**

**What documents must/may accompany the amendments ?**

**Letter (Section 205(b)):**

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

**The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.**

# PATENT COOPERATION TREATY

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From the INTERNATIONAL SEARCHING AUTHORITY

To:
JOHN P. WHITE
COOPER & DUNHAM LLP
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

# PCT

## NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

| | |
|---|---|
| | Date of mailing (*day/month/year*) |
| Applicant's or agent's file reference 68518-A-PCT/JPW/JCC | FOR FURTHER ACTION See paragraphs 1 and 4 below |
| International application No. PCT/US04/00955 | International filing date (*day/month/year*) 14 January 2004 (14.01.2004) |
| Applicant TEVA PHARMACEUTICAL INDUSTRIES, LTD. | |

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   **Filing of amendments and statement under Article 19:**
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   **When?** The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   **Where?** Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 740 14 35

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

   The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

   Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

| | |
|---|---|
| Name and mailing address of the ISA/ US<br>Mail Stop PCT, Attn: ISA/US<br>Commissioner for Patents<br>P.O. Box 1450<br>Alexandria, Virginia 22313-1450<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Anand Desai<br>Telephone No. (571) 272-0947 |

Form PCT/ISA/220 (January 2004) *(See notes on accompanying sheet)*

PCT

# REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference (if desired) (12 characters maximum) 68518-A-PCT/JPW/JCC

**Box No. I TITLE OF INVENTION** PARENTERAL FORMULATIONS OF A PEPTIDE FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS.

**Box No. II APPLICANT** ☐ This person is also inventor

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

TEVA PHARMACEUTICAL INDUSTRIES, LTD.
5 Basel Street
P.O. Box 3190
Petach-Tikva 49131
ISRAEL

Telephone No.: None
Facsimile No.: None
Teleprinter No.: None
Applicant's registration No. with the Office: None

State (that is, country) of nationality: Israel
State (that is, country) of residence: Israel

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☒ the States indicated in the Supplemental Box

**Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

TEVA PHARMACEUTICALS USA, INC.
1090 Horsham Road
North Wales, PA 19454
United States of America

This person is:
☒ applicant only
☐ applicant and inventor
☐ inventor only (If this check-box is marked, do not fill in below.)

Applicant's registration No. with the Office:

State (that is, country) of nationality: United States of America
State (that is, country) of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☒ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

**Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

WHITE, John P.
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
United States of America

Telephone No.: (212) 278-0400
Facsimile No.: (212) 391-0526
Teleprinter No.: None
Agent's registration No. with the Office: 28,678

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

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**Continuation of Box No. III . URTHER APPLICANT(S) AND/OR (FURTHER) IN . .TOR(S)**

*If none of the following sub-boxes is used, this sheet should not be included in the request.*

Name and address: *(Family name followed by given names; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

COHEN-VERED, Sharon
Azar 167
Kfar Saba
ISRAEL

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality: Israel

State *(that is, country)* of residence: Israel

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given names; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

NAFTALI, Esmira
44/6 He-beiyar St.
Rosh HaAyin 48056
ISRAEL

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality: Israel

State *(that is, country)* of residence: Israel

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given names; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

WEINSTEIN, Vera
Rambam St., POB 846
Mevaseret Zion 90805
ISRAEL

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality: Israel

State *(that is, country)* of residence: Israel

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given names; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GILBERT, Adrian
60B Apt. 5, Hankin Street
Ra'anana 43465
ISRAEL

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality: Israel

State *(that is, country)* of residence: Israel

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.

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**Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

*If none of the following sub-boxes is used, this sheet should not be included in the request.*

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KLINGER, Ety
Agadati Street, 16
Tel Aviv 39930
ISRAEL

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: Israel

State *(that is, country)* of residence: Israel

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

---

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

---

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

---

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

---

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

**Supplemental Box** *If the Supplemental Box is not used, this sheet should not be included in the request.*

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*1. If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) If more than two persons are to be indicated as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) If, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes Nos. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) If, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes Nos. II and No. III" (as the case may be); indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) If, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) If, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*

*2. If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such a case, write the name or two-letter code of each designated State concerned and the indication "patent of addition," "certificate of addition," "inventor's certificate of addition" or "utility certificate of addition," the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other patent grant or the date of filing of the parent application (Rules 4.11(a)(iii) and 49bis.1(a) or (b)).*

*3. If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such a case, write "United States of America" or "US" and the indication "continuation" or "continuation-in-part" and the number and the filing date of the parent application (Rules 4.11(a)(iv) and 49bis.1(d)).*

Continuation on Box No. II:

TEVA PHARMACEUTICAL INDUSTRIES, LTD: AP, EA, EP, OA, AE, AG, AL, AM, AT, AU, AZ, BA, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EG, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LI, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, SZ, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW (i.e. All States Except US and BB)

Continuation of Box No. III:

TEVA PHARMACEUTICALS USA, INC.: BB

US United States of America
(continuation-in-part)
US: 60/439,950
filed: 14 January 2003
(14.01.03)

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**Box No. V DESIGNATIONS**

The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

However,

☐ DE Germany is not designated for any kind of national protection

☐ KR Republic of Korea is not designated for any kind of national protection

☐ RU Russian Federation is not designated for any kind of national protection

*(The check-boxes above may be used to exclude (irrevocably) the designations concerned in order to avoid the causing of the effect, under the national law, of an earlier national application from which priority is claimed. See the Notes to Box No. V as to the consequences of such national law provisions in these and certain other States.)*

**Box No. VI PRIORITY CLAIM**

The priority of the following earlier application(s) is hereby claimed:

| Filing date of earlier application (day/month/year) | Number of earlier application | Where earlier application is: | | |
|---|---|---|---|---|
| | | national application: country or Member of WTO | regional application:* regional Office | international application: receiving Office |
| item (1) (14.01.03) 14 January 2003 | 60/439,950 | US | | |
| item (2) | | | | |
| item (3) | | | | |

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

* *Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* ....

**Box No. VII INTERNATIONAL SEARCHING AUTHORITY**

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / US

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date (day/month/year) Number Country (or regional Office)

**Box No. VIII DECLARATIONS**

| The following declarations are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*: | | Number of declarations |
|---|---|---|
| ☐ Box No. VIII (i) | Declaration as to the identity of the inventor | : |
| ☐ Box No. VIII (ii) | Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent | : |
| ☐ Box No. VIII (iii) | Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application | : |
| ☐ Box No. VIII (iv) | Declaration of inventorship (only for the purposes of the designation of the United States of America) | : |
| ☐ Box No. VIII (v) | Declaration as to non-prejudicial disclosures or exceptions to lack of novelty | : |

169

**Box No. IX CHECK LIST; LANGUAGE OF FILING**

This international application contains:

(a) In paper form, the following number of sheets:

| | | |
|---|---|---|
| request (including declaration sheets) | : | 9 |
| description (excluding sequence listing and/or tables related thereto) | : | 60 |
| claims | : | 9 |
| abstract | : | 1 |
| drawings | : | 6 |
| Sub-total number of sheets | : | 85 |
| sequence listing | : | 0 |
| tables related thereto | : | 0 |
| *(for both, actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (c) below)* | | |
| Total number of sheets | : | 85 |

(b) ☐ only in computer readable form (Section 801(a)(i))
- (i) ☐ sequence listing
- (ii) ☐ tables related thereto

(c) ☐ also in computer readable form (Section 801(a)(ii))
- (i) ☐ sequence listing
- (ii) ☐ tables related thereto

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the
- ☐ sequence listing: .....
- ☐ tables related thereto: .....

*(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)*

This international application is accompanied by the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:

| | | Number of items |
|---|---|---|
| 1. ☒ fee calculation sheet | : | 1 |
| 2. ☐ original separate power of attorney | : | |
| 3. ☐ original general power of attorney | : | |
| 4. ☐ copy of general power of attorney; reference number, if any: ..... | : | |
| 5. ☐ statement explaining lack of signature | : | |
| 6. ☐ priority document(s) identified in Box No. VI as item(s): ..... | : | |
| 7. ☐ translation of international application into *(language)*: ..... | : | |
| 8. ☐ separate indications concerning deposited microorganism or other biological material | : | |
| 9. ☐ sequence listing in computer readable form *(indicate type and number of carriers)* | | |
| (i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application) | : | |
| (ii) ☐ *(only where check-box (b)(i) or (c)(i) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter* | : | |
| (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing mentioned in left column | : | |
| 10. ☐ tables in computer readable form related to sequence listing *(indicate type and number of carriers)* | | |
| (i) ☐ copy submitted for the purposes of international search under Section 802(b-*quater*) only (and not as part of the international application) | : | |
| (ii) ☐ *(only where check-box (b)(ii) or (c)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Section 802(b-*quater*) | : | |
| (iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column | : | |
| 11. ☒ other *(specify)*: Express Mail Certificate of Mailing dated: Jan. 142004, bearing Express Mail Label No. EV305641724US | | |

| | |
|---|---|
| Figure of the drawings which should accompany the abstract: | Language of filing of the international application: English |

**Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).*

TEVA PHARMACEUTICAL INDUSTRIES, LTD.

| | | | |
|---|---|---|---|
| NAME: Yehudah Livneh | 22/1/04 DATE | NAME: Irit Pinchasi | DATE |
| TITLE: Manager, Patents Department | | TITLE: Vice President, R&D | |

**For receiving Office use only**

| | |
|---|---|
| 1. Date of actual receipt of the purported international application: | 2. Drawings: ☐ received: |
| 3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application: | |
| 4. Date of timely receipt of the required corrections under PCT Article 11(2): | ☐ not received: |
| 5. International Searching Authority (if two or more are competent): ISA / | 6. ☐ Transmittal of search copy delayed until search fee is paid |

**For International Bureau use only**

Date of receipt of the record copy by the International Bureau:

120

**Box No. IX CHECK LIST; LANGUAGE OF FILING**

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|---|---|---|---|
| (a) In paper form, the following number of sheets: | | | |
| request (including declaration sheets) | 9 | 1. ☒ fee calculation sheet | 1 |
| description (excluding sequence listing and/or tables related thereto) | 60 | 2. ☐ original separate power of attorney | |
| claims | 9 | 3. ☐ original general power of attorney | |
| abstract | 1 | 4. ☐ copy of general power of attorney; reference number, if any: | |
| drawings | 6 | 5. ☐ statement explaining lack of signature | |
| Sub-total number of sheets | 85 | 6. ☐ priority document(s) identified in Box No. VI as item(s): | |
| sequence listing | 0 | 7. ☐ translation of international application into *(language)*: | |
| tables related thereto | 0 | 8. ☐ separate indications concerning deposited microorganism or other biological material | |
| *(for both, actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (c) below)* | | 9. ☐ sequence listing in computer readable form *(indicate type and number of carriers)* | |
| Total number of sheets | 85 | (i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application) | |
| (b) ☐ only in computer readable form (Section 801(a)(i)) | | (ii) ☐ *(only where check-box (b)(i) or (c)(i) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter* | |
| (i) ☐ sequence listing | | (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing mentioned in left column | |
| (ii) ☐ tables related thereto | | 10. ☐ tables in computer readable form related to sequence listing *(indicate type and number of carriers)* | |
| (c) ☐ also in computer readable form (Section 801(a)(ii)) | | (i) ☐ copy submitted for the purposes of international search under Section 802(b-*quater*) only (and not as part of the international application) | |
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| (ii) ☐ tables related thereto | | (iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column | |
| Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the | | 11. ☒ other *(specify)*: Express Mail Certificate of Mailing dated: Jan. 142004, bearing Express Mail Label No. EV305641724US | |
| ☐ sequence listing: | | | |
| ☐ tables related thereto: | | | |
| *(additional copies to be indicated under items 9(i) and/or 10(i), in right column)* | | | |

| Figure of the drawings which should accompany the abstract: | Language of filing of the international application: English |
|---|---|

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TEVA PHARMACEUTICAL INDUSTRIES, LTD.

| | | | |
|---|---|---|---|
| NAME: Yehudah Livneh | DATE | NAME: Irit Pinchasi | DATE 22/2/04 |
| TITLE: Manager, Patents Department | | TITLE: Vice President, R&D | |

**For receiving Office use only**

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|---|---|---|
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| 4. Date of timely receipt of the required corrections under PCT Article 11(2): | | |
| 5. International Searching Authority (if two or more are competent): ISA / | 6. ☐ Transmittal of search copy delayed until search fee is paid | |

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Date of receipt of the record copy by the International Bureau:

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| abstract | 1 | 5. ☐ statement explaining lack of signature | |
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| (i) ☐ sequence listing | | (iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column | |
| (ii) ☐ tables related thereto | | 11. ☒ other *(specify)*: Express Mail Certificate of Mailing dated: Jan. 142004, bearing Express Mail Label No. EV305617240US | |
| Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the | | | |
| ☐ sequence listing: | | | |
| ☐ tables related thereto: | | | |
| *(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)* | | | |

| Figure of the drawings which should accompany the abstract: | Language of filing of the international application: English |
|---|---|

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TEVA PHARMACEUTICALS USA, INC.

NAME: Richard S. Egosi DATE: 2/2/04

TITLE: Vice President and General Counsel

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|---|---|
| 1. Date of actual receipt of the purported international application: | 2. Drawings: ☐ received: ☐ not received: |
| 3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application: | |
| 4. Date of timely receipt of the required corrections under PCT Article 11(2): | |
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| abstract | : | 1 |
| drawings | : | 6 |
| Sub-total number of sheets | : | 85 |
| sequence listing | : | 0 |
| tables related thereto | : | 0 |
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(b) ☐ only in computer readable form (Section 801(a)(i))
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- (ii) ☐ tables related thereto

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Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the
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*(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)*

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1. ☒ fee calculation sheet : 1
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3. ☐ original general power of attorney :
4. ☐ copy of general power of attorney; reference number, if any: ..... :
5. ☐ statement explaining lack of signature :
6. ☐ priority document(s) identified in Box No. VI as item(s): ..... :
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8. ☐ separate indications concerning deposited microorganism or other biological material :
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   - (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing mentioned in left column :
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    - (iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column :
11. ☒ other *(specify)*: Express Mail Certificate of Mailing dated: Jan. 142004, bearing Express Mail Label No. EV305641724US

Figure of the drawings which should accompany the abstract: .....

Language of filing of the international application: English

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| | | | |
|---|---|---|---|
| Sharon Cohen-Vered | 08 Feb 04 DATE | Esmira Naftali | 08 Feb 04 DATE |
| Vera Weinstein | DATE | | |

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**Box No. IX CHECK LIST; LANGUAGE OF FILING**

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Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

**Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).*

| Sharon Cohen-Vered | DATE | Esmira Naftali | DATE |
|---|---|---|---|
| Vera Weinstein | | Feb 5, 2004 | |
| Vera Weinstein | DATE | | |

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| | |
|---|---|
| 1. Date of actual receipt of the purported international application: | 2. Drawings: ☐ received: ☐ not received: |
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**Box No. IX CHECK LIST; LANGUAGE OF FILING**

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Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

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| | | | |
|---|---|---|---|
| [signature] | February 9, 2004 | | |
| Adrian Gilbert | DATE | Ety Klinger | DATE |

**For receiving Office use only**

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| 1. Date of actual receipt of the purported international application: | 2. Drawings: ☐ received: ☐ not received: |
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**Box No. IX CHECK LIST; GUAGE OF FILING**

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| ☐ tables related thereto: | | | |
| *(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)* | | | |

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(57) Abstract

Synthetic peptides based on a complementarity-determining region (CDR) of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a systemic lupus erythematosus (SLE)-like disease in mice, and analogs, and salts and chemical derivatives thereof; dual peptides comprising two such peptides or analogs covalently linked to one another either directly or through a short linking chain; peptide polymers comprising a plurality of sequences of said peptide or analog thereof; and peptide polymers attached to a macromolecular carrier, are disclosed, and pharmaceutical compositions comprising them for the treatment of SLE in humans.

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# SYNTHETIC PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEM FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

## Field of the Invention

The present invention relates to synthetic peptides and to pharmaceutical compositions comprising them useful for the treatment of systemic lupus erythematosus (SLE) in humans.

## Background of the Invention

Autoimmune diseases are characterized by immune responses that are directed against self antigens. These responses are maintained by the persistent activation of self-reactive T lymphocytes. T lymphocytes are specifically activated upon recognition of foreign and/or self antigens as a complex with self major histocompatibility complex (MHC) gene products on the surface of antigen-presenting cells (APC).

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown origin and cure. Despite the extensive research on the mechanisms underlying the induction and development of SLE, the information available on the etiology of the disease is very limited due to the heterogeneity of SLE patients on one hand, and the lack of an experimental model in which the induction of the disease could be controlled, on the other hand.

The cause of SLE is unknown and it has heterogeneous clinical manifestations. Furthermore, no specific treatment aimed towards the prevention or cure of SLE is available. Despite the extensive research on the mechanisms underlying the induction of SLE, the information on the etiology of the disease is very limited. Studies on SLE have been performed until recently using peripheral blood lymphocytes (PBL) of patients at different clinical stages and under various treatment protocols. Alternatively, murine strains that develop spontaneous SLE-like disease were investigated as a model for SLE. This kind of analysis led to incomplete and confusing interpretations of the role of various immunological

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and non-immunological factors in either inducing or sustaining the disease, mainly due to the heterogeneity of patients on one hand and the inability to control the induction phase of the disease in murine SLE strains on the other hand.

Several years ago, an animal model of SLE has been established in the laboratory of one of the present inventors. This model, based on the concept of the idiotypic network, developed a wide spectrum of lupus-related autoantibodies and clinical manifestations (Mendlovic et al., 1988). The induction was carried out by the immunization of mouse strains that do not develop any spontaneous autoimmune disorders, with a human anti-DNA monoclonal antibody (mAb) which bears a common idiotype termed 16/6 Id (Shoenfeld et al., 1983). Following immunization, the mice produced antibodies specific to the 16/6 Id, antibodies that bear the 16/6 Id and antibodies directed against different nuclear antigens (dsDNA, ssDNA, Sm, ribonucleoprotein (RNP), Ro, La and others). The serological findings were associated with leukopenia, elevated erythrocyte sedimentation rate, proteinuria, abundance of immune complexes in the kidneys and sclerosis of the glomeruli (Mendlovic et al., 1988), which are typical manifestations of SLE. The present inventors have further shown that the experimental disease could be induced by a murine anti-16/6 Id mAb (Mendlovic et al., 1989) and by the mouse anti-anti 16/6 Id (16/6Id+) mAb (Waisman et al., 1993). The induction of the disease is genetically controlled, and thus is strain dependent (Mendlovic et al., 1990). This unique model for the induction of experimental SLE provides the appropriate tools to clearly dissect the different steps and the linked immune parameters involved in the induction and development of SLE.

SLE is a systemic autoimmune disease characterized by the formation of autoantibodies against self-antigens, such as DNA, Sm, Ro, La, RNP, cardiolipin and histones. The etiology of SLE is unknown, and understanding the mechanism by which these self-antibodies arise might provide insight to this problem. For this purpose, the present inventors have produced a variety of monoclonal autoantibodies derived from C3H.SW mice in which experimental SLE was induced. As a rule, the monoclonal autoantibodies that were capable of eliciting antibodies that bear the 16/6 Id or react with it were found to be pathogenic and thus capable of inducing experimental SLE (Fricke et al., 1990; Sthoeger et al., 1993). Later on, the variable (V) regions of nine autoantibodies that bind either DNA or HeLa nuclear extract (NE), isolated from the C3H.SW mice with experimental SLE, were sequenced (Waisman and Mozes, 1993). Monoclonal antibodies with different specificity

were analyzed in an attempt to determine the connections between the different autoantibodies. Three mAb were found to bind DNA, and were shown to exhibit sequence characteristics of pathogenic anti-DNA antibodies. One of these mAb, designated 2C4C2, was shown to use a heavy (H) chain V region gene ($V_H$) identical to the $V_H$ of anti-DNA mAb isolated from other lupus-prone mice, namely (NZB x NZW)$F_1$. The light (L) chain V region gene ($V_L$) of mAb 2C4C2 is 98% homologous to the $V_L$ of another anti-DNA mAb, also isolated from (NZB x NZW)$F_1$ mice. The other two anti-DNA mAb, designated 5G12-4 and 5G12-6, share 93% of their $V_H$ sequences with that of mAb 2C4C2. Six mAb bound proteins of HeLa NE. The nine mAb use a total of five $V_H$ and four $V_L$ germ-line genes, demonstrating that the autoantibodies induced in mice with experimental SLE do not originate from one B cell clone. Three of the nine $V_H$ and $V_L$ were identical in sequence to germ-line genes, while at least three others had somatic mutations. The latter suggests that these autoantibodies arise in mice by both usage of existing (pre-immune) B cells, and through an antigen-driven process. Furthermore, it appears that autoantibodies found in mice with experimental SLE use genetic elements similar to those used by mAb that were isolated from mouse strains which develop *lupus* spontaneously.

T cells play an important role in the induction and development of experimental SLE. Thus, T cell lines and clones specific to the 16/6 Id were shown to induce experimental SLE in syngeneic recipients similarly to the 16/6 antibody. Therefore, following the inoculation of the activated cells of the lines, the mice developed both the serology and the renal damage which is typical to SLE (Fricke et al., 1991). Furthermore, a 16/6 Id specific T cell line of C3H.SW origin induced SLE in C57BL/6 mice that were shown to be resistant to the induction of the disease following injections with either the 16/6 Id or the anti-16/6 Id mAb (Mendlovic et al., 1990).

In an attempt to identify the pathogenic region of the 16/6 Id, $(Fab')_2$ fragments were prepared of the 16/6 Id mAb and were found to retain the specificity and pathogenic capacity of the whole 16/6 Id molecule (Ruiz et al., 1994).

The mAb 5G12 that was isolated from mice with experimental SLE and was shown to bind DNA and bear the 16/6 Id, is capable of inducing experimental SLE in mice (Waisman et al., 1993). T cells that react specifically to mAb by proliferation, are probably reacting to peptides representing sequences from their complementarity-determining regions (CDR). It is very likely that the T cells recognize the V regions of the above antibodies since they do not

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react with other antibodies that carry the same constant region but have different specificities. Within the variable region, the regions with the highest probability to be recognized are the CDR, since those are the regions that differ the most between the various antibodies. The CDR regions of the $V_H$ sequences of the nine pathogenic murine mAb mentioned above that induce SLE in mice, are boxed in Fig. 1 of Waisman and Mozes, 1993, in which the complete nucleotide and deduced amino acid sequences for the $V_H$ of the nine mAb are presented.

## Summary of the Invention

It is an object of the present invention to provide means for specific treatment of patients with SLE.

For this purpose, the invention provides peptides and analogs thereof based on the CDR regions of pathogenic monoclonal autoantibodies isolated from mice with experimental SLE.

Thus, in one aspect, the invention relates to a synthetic peptide selected from the group consisting of:

(i) a peptide of at least 12 and at most 30 amino acid residues based on a complementarity-determining region (CDR) of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a systemic lupus erythematosus (SLE)-like disease in mice (hereinafter CDR-based peptide), a salt or a chemical derivative thereof;

(ii) an analog of a CDR-based peptide defined in (i), a salt or a chemical derivative thereof;

(iii) a dual synthetic peptide comprising two such peptides of (i) or analogs of (ii) covalently linked to one another either directly or through a short linking chain;

(iv) a peptide polymer comprising a plurality of sequences of said peptide (i) or analog thereof (ii); and

(v) a peptide polymer (iv) attached to a macromolecular carrier.

In one embodiment of this aspect, the synthetic peptide is capable of:

(i) inhibiting specifically the proliferative response and cytokine secretion of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies; or

(ii) inhibiting development of SLE in mice that are susceptible to SLE-induction by pathogenic autoantibodies.

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The synthetic peptides and analogs thereof according to the invention may be selected from the group consisting of peptides having the sequences I to V herein, wherein:

(i) the peptide of sequence I has the formula:

T G Y Y $X_1$ $X_2$ $X_3$ $X_4$ $X_5$ Q S P E K S L E W I G [I]

wherein $X_1$ is Met, Ala or Val; $X_2$ is Gln, Asp, Glu or Arg; $X_3$ is Trp or Ala; $X_4$ is Val or Ser; and $X_5$ is Lys, Glu or Ala;

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(ii) the peptide of sequence II has the formula:

E I N P S T G G $X_6$ $X_7$ $X_8$ $X_9$ $X_{10}$ $X_{11}$ $X_{12}$ K A K A T [II]

wherein $X_6$ and $X_7$ are each Thr, Val or Ala; $X_8$ is Tyr or Phe; $X_9$ is Asn or Asp; $X_{10}$ is Gln or Glu; $X_{11}$ is Lys or Glu, and $X_{12}$ is Phe or Tyr;

(iii) the peptide of sequence III has the formula:

Y Y C A R $X_{13}$ $X_{14}$ $X_{15}$ $X_{16}$ P Y A $X_{17}$ $X_{18}$ Y W G Q G S [III]

wherein $X_{13}$ is Phe, Thr or Gly; $X_{14}$ is Leu, Ala or Ser; $X_{15}$ is Trp or Ala; $X_{16}$ is Glu or Lys; $X_{17}$ is Met or Ala, and $X_{18}$ is Asp, Lys or Ser;

(iv) the peptide of sequence IV has the formula:

G Y N $X_{19}$ $X_{20}$ $X_{21}$ $X_{22}$ $X_{23}$ $X_{24}$ S H G $X_{25}$ $X_{26}$ L E W I G [IV]

wherein $X_{19}$ is Met or Ala; $X_{20}$ is Asn, Asp or Arg; $X_{21}$ is Trp or Ala; $X_{22}$ is Val or Ser; $X_{23}$ is Lys or Glu; $X_{24}$ is Gln or Ala; $X_{25}$ is Lys or Glu, and $X_{26}$ is Ser or Ala; and

(v) the peptide of sequence V has the formula:

Y Y C A R $X_{27}$ $X_{28}$ $X_{29}$ Y G $X_{30}$ $X_{31}$ $X_{32}$ G Q G T L [V]

wherein $X_{27}$ is Ser or Phe; $X_{28}$ is Gly or Ala; $X_{29}$ is Arg, Ala or Glu; $X_{30}$ is Asn or Asp; $X_{31}$ is Tyr or Phe, and $X_{32}$ is Trp, His or Ala.

In preferred embodiments, peptides I to V have the sequences Ia-Va herein:

T G Y Y M Q W V K Q S P E K S L E W I G (Ia)

E I N P S T G G T T Y N Q K F K A K A T (IIa)

Y Y C A R F L W E P Y A M D Y W G Q G S (IIIa)

G Y N M N W V K Q S H G K S L E W I G (IVa)

Y Y C A R S G R Y G N Y W G Q T L (Va)

Peptides Ia to IIIa are based on the CDR1, CDR2 and CDR3 regions, respectively, of the $V_H$ chain of mAb 5G12, and peptides IVa and Va are based on the CDR1 and CDR3 regions, respectively, of the $V_H$ chain of mAb 2C4C2 (Waisman and Mozes, 1993).

In another aspect, the invention relates to pharmaceutical compositions for the treatment of SLE comprising a synthetic peptide or peptide polymer of the invention and a pharmaceutically acceptable carrier.

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In still another aspect, the invention relates to a method of treatment of a SLE patient comprising administering to a SLE patient an effective amount of a synthetic peptide or peptide polymer of the invention.

**Brief Description of the Drawings**

Figs. 1A-B show the presence of anti-DNA antibodies in sera of SJL (1A) and BALB/c (1B) mice immunized with mAb 5G12, peptides Ia and IIIa and a control peptide 278, or non-immunized. Sera of individual SJL or BALB/c mice immunized with either one of the indicated antigens, taken three months after the booster injection, and sera of age-matched naive mice, were tested for anti-ssDNA antibody titers. Following incubation with the diluted sera, goat anti-mouse IgG (γ-chain specific) conjugated to peroxidase was added. Results were expressed as mean OD of each mouse group.

Figs. 2A-B show the presence of HeLa anti-nuclear extract (NE) antibodies in the sera of SJL (2A) and BALB/c (2B) mice immunized with same antigens as in Fig. 1.

Figs. 3A-B show the presence of anti-RNP, Sm, Ro and La antibodies in the sera of SJL (3A) and BALB/c (3B) mice immunized with peptides Ia and IIIa or with control peptide 278, and normal mice.

Figs. 4A-B show the presence of anti-DNA (4A) and anti-HeLa NE (4B) antibodies in the sera of BALB/c mice tolerized with peptide Ia or with control peptide p307, and immunized with either peptide Ia or mAb 5G12.

Figs. 5a-b show *in vivo* inhibition of lymph node cell (LNC) proliferation responses in BALB/c (5a) and SJL (5b) mice to the CDR-based peptides Ia and IIIa, respectively, following treatment with the latter.

Figs. 6a-b show *in vivo* inhibition of LNC to mAb 5G12 in BALB/c (6a) or SJL (6b) mice treated with peptide Ia and IIIa, respectively.

Figs. 7a-b show *in vivo* inhibition of LNC proliferation to the human monoclonal anti-DNA 16/6 Id antibody in BALB/c (7a) and SJL (7b) mice treated with peptide Ia and IIIa, respectively.

Fig. 8 shows binding of peptides Ia and IIIa to the surface of splenic antigen-presenting cells of different mouse strains.

Fig. 9 shows antibody titers in sera of SLE patients and healthy human controls by testing their sera for the ability to bind the peptides Ia, IIa and IIIa, or mAb 5G12 or a control peptide.

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**Detailed Description of the Invention**

The present invention relates to synthetic peptides that are based on the CDR of monoclonal pathogenic autoantibodies isolated from mice with experimental SLE. Such monoclonal antibodies are obtained from supernatants of hybridomas produced by fusion, for example, of spleen cells of C3H.SW mice immunized with an anti-16/6 Id mAb, with X63.653 plasmacytoma cells (Waisman and Mozes, 1993).

Examples of such peptides are those of formulas Ia to Va herein, based on, respectively, the CDR1, CDR2 and CDR3 regions of the heavy chain of mAb 5G12 and the CDR1 and CDR3 regions of the heavy chain of mAb 2C4C2 (Waisman and Mozes, 1993), and analogs thereof .

Analogs of parent peptides Ia-Va contemplated by the invention include substitution, deletion and addition analogs as described herein. Substitution analogs have amino acid substitutions at different positions, these substitutions being made based on the volume, hydrophobic-hydrophilic pattern and charge of the amino acids.

Amino acids may be divided along the lines of volume, hydrophobic-hydrophilic pattern and charge. With respect to volume, those of ordinary skill in the art understand that the amino acids with the largest volume are Trp, Tyr, Phe, Arg, Lys, Ile, Leu , Met and His, while those with the smallest volumes are Gly, Ala, Ser, Asp, Thr and Pro, with others being in between.

With respect to hydrophobic-hydrophilic pattern, it is well known that the amino acids Gly, Ala, Phe, Val, Leu, Ile, Pro, Met and Trp are hydrophobic, whereas all of the remaining amino acids are hydrophilic. Among the hydrophilic amino acids, Ser, Thr, Gln, and Tyr have no charge, while Arg, Lys, His and Asn have a positive charge and Asp and Glu have negative charges.

In selecting peptides to be tested for their potential in inhibiting the proliferative response of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies, it is important that the substitutions be selected from those which cumulatively do not substantially change the volume, hydrophobic-hydrophilic pattern and charge of the corresponding portion of the unsubstituted parent peptide. Thus, a hydrophobic residue may be substituted with a hydrophilic residue, or vice-versa, as long as the total effect does not

substantially change the volume, hydrophobic-hydrophilic pattern and charge of the corresponding unsubstituted parent peptide.

It should be understood that other modifications of the peptides and analogs thereof are also contemplated by the present invention. Thus, the peptide or analog of the present invention is intended to include a "chemical derivative" thereof which retains at least a portion of the function of the peptide which permits its utility in preventing or inhibiting T cell proliferative responses and autoimmune disease.

A "chemical derivative" of a peptide or analog of the present invention contains additional chemical moieties not normally a part of the peptide. Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Many such chemical derivatives and methods for making them are well known in the art.

Also included in the scope of the invention are salts of the peptides and analogs of the invention. As used herein, the term "salts" refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptide molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as those formed for example, with amines, such as triethanolamine, arginine, or lysine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Such chemical derivatives and salts are preferably used to modify the pharmaceutical properties of the peptide insofar as stability, solubility, etc., are concerned.

Examples of peptides and analogs thereof are as follows:

(i) Peptide Ia of the formula :

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T | G | Y | Y | M | Q | W | V | K | Q | S | P | E | K | S | L | E | W | I | G | (Ia) |

and substitution analogs thereof in which Met at position 5 is substituted by either Ala or Val; Gln at position 6 is substituted by either Asp, Glu or Arg; Trp at position 7 is substituted by Ala; Val at position 8 by Ser; and Lys at position 9 is substituted by either

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Glu or Ala; and deletion analogs thereof in which up to 5 amino acid residues are deleted from the C-terminal of peptide Ia.

(ii) Peptide IIa of the formula :

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

E I N P S T G G T T Y N Q K F K A K A T (IIa)

and substitution analogs thereof in which Thr in positions 9 and 10 are each substituted by either Val or Ala; Tyr at position 11 is substituted by Phe; Asn at position 12 is substituted by Asp; Gln at position 13 by Glu; Lys at position 14 by Glu; and Phe at position 15 by Tyr, and deletion analogs thereof in which up to 5 amino acid residues are deleted from the C-terminal of peptide IIa.

(iii) Peptide IIIa of the formula :

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Y Y C A R F L W E P Y A M D Y W G Q G S (IIIa)

and substitution analogs thereof in which Phe at position 6 is substituted by either Thr or Gly; Leu at position 7 is substituted by either Ala or Ser; Trp at position 8 is substituted by Ala; Glu at position 9 is substituted by Lys; Met at position 13 by Ala; and Asp at position 14 by either Lys or Ser; and deletion analogs thereof in which up to 5 amino acid residues are deleted from the C-terminal of peptide IIIa.

(iv) Peptide IVa of the formula :

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

G Y N M N W V K Q S H G K S L E W I G (IVa)

and substitution analogs thereof in which Met at position 4 is substituted by Ala; Asn at position 5 is substituted by either Asp or Arg; Trp at position 6 is substituted by Ala; Val at position 7 by Ser; Lys at position 8 by Glu; Gln at position 9 by Ala; Lys at position 13 by Glu; and Ser at position 14 by Ala; and deletion analogs thereof in which up to 5 amino acid residues are deleted from the C-terminal of peptide IVa.

(v) Peptide Va of the formula :

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Y Y C A R S G R Y G N Y W G Q G T L (V)

and substitution analogs thereof in which Ser at position 6 is substituted by Phe; Gly at position 7 is substituted by Ala; Arg at position 8 is substituted by either Ala or Glu; Asn at position 11is substituted by Asp; Tyr at position 12 by Phe; and Trp at position 13 by

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either His or Ala; and deletion analogs thereof in which up to 5 amino acid residues are deleted from the C-terminal of peptide Va.

Once an analog in accordance with the present invention is produced, its ability to inhibit the proliferative response of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies may be readily determined by those of ordinary skill in the art without undue experimentation using tests such as those described herein. One test which may be readily conducted is for the ability of substituted peptides to inhibit *in vitro* the proliferative responses of certain T cell lines and clones specific to SLE-inducing autoantibodies. The T cell lines and clones may, for example, be the T cell lines and clones specific to the 16/6 Id mAb (Fricks et al., 1991) established from immunized lymph node cells of mice by previously described methodology (Axelrod and Mozes, 1986). Cells are exposed to the stimulating antibody presented on irradiated syngeneic spleen cells in the presence of enriched medium every two weeks. The T cell lines are cloned by the standard limiting dilution technique. The proliferative responses of these T cell lines and clones are tested, for example, by the method described in Materials and Methods, section (g), herein.

Another test which can be conducted in order to select analogs having the desired activity is to test for the ability of the substituted peptides to inhibit the ability of the T cell lines and clones to provide help to peptide-specific B cells in the presence of the parent peptide. The substituted peptides may also be tested for their ability to bind directly, following biotinylation, to MHC Class II products on antigen-presenting cells of the relevant strains. For this purpose, N-terminal biotinylation of the relevant peptides is performed at 0°C with an excess of biotin-N-hydroxysuccinimide in aqueous solution (Mozes et al., 1989). Mouse splenic adherent cells or human peripheral blood lymphocyte (PBL)-adherent cells ($1x10^6$/sample) are incubated with biotinylated peptides in PBS containing 0.1% bovine serum albumin (PBS/BSA) at 37°C for 20 hr, followed by incubation with phycoerythrin-streptavidin for 30 min at 4°C. After each incubation, the cells are washed twice with the above solution. Thereafter, the cells are analyzed by flow cytometry using FACScan. In each analysis, a minimum of 5000 cells are examined (for above procedures, see, for example, Mozes et al., 1989; Zisman et al., 1991).

A further test which can be conducted is to test for the ability of the analogs to inhibit cytokine secretion by the T cell line or by T lymphocytes oh lymph nodes of mice that are high responders to SLE-inducing autoantibodies. The cytokines are detected as

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follows: IL-1 activity is assessed either by ELISA using a pair of capture and detecting antibodies (as described below for IL-4, IL-6, IL-10) or using the LBRM-33(1A5) assay (Conlon, 1983) in which 1A5 cells are stimulated in the presence of phytohemagglutinin (PHA), with either supernatants or recombinant IL-1 at various concentrations to secrete IL-2. Following an overnight incubation, supernatants of 1A5 cells are transferred to the IL-2 dependent cytotoxic T lymphocyte (CTLL) line. Stimulation of the CTLL line by IL-2 is measured after 24 hr by incorporation of $^{3}$[H]-thymidine. IL-2 is directly detected using the IL-2 dependent CTLL line or by ELISA. Levels of IL-4, IL-6, IL-10, INFγ and TNFα in the supernatants are determined by ELISA using antibodies to the various cytokines (Pharmingen, San Diego, Ca., USA) according to the manufacturer's instructions.

Peptides which test positive in one or more of these *in vitro* tests will provide a reasonable expectation of *in vivo* activity. However, *in vivo* tests can also be conducted without undue experimentation. Thus, for example, adult mice may be injected with the candidate peptide at either day -3 or day 0. The mice are then immunized with the disease-inducing autoantibody or with the peptide. Ten days later, lymph node cells of the mice are tested for their ability to proliferate to the immunogen in order to find out the inhibitory capacity of the candidate peptide.

Another such *in vivo* animal test consists in measuring the therapeutic activity directly in the murine model *in vivo* for the production of SLE as described above. The peptides can be injected into the mice in which experimental SLE is induced by different routes at different dosages and at different time schedules. In order to determine the pharmacokinetic parameters of the analogs, including volume of distribution, uptake into antigen-presenting cells and clearance, one can use biotinylated derivatives of the analogs. The concentration of the soluble fraction of the analogs in the various body fluids can be determined by ELISA, using avidin-coated plates and specific anti-peptide antibodies. Cell bound analogs can be analyzed by FACS, using fluorochromo-conjugated avidin or streptavidin. Furthermore, the treated mice can be tested periodically in order to determine the effect of the peptides on the autoantibody responses and on disease manifestations elicited in the mice by the SLE-inducing autoantibody.

Another *in vivo* procedure consists in tolerizing newborn mice with the candidate peptide followed by immunization of the mice with the pathogenic autoantibody, such as 16/6 Id+, or with the same peptide, and following the disease manifestations, such as

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serological findings associated with leukopenia, elevated erythrocyte sedimentation rate, proteinuria, abundance of immune complexes in the kidneys and sclerosis of the glomeruli.

It can thus be seen that, besides the preferred embodiments which have been shown to be operable in the examples herein, those of ordinary skill in the art will be able to determine additional analogs which will also be operable following the guidelines presented herein without undue experimentation.

A relatively simple *in vitro* test can also be conducted in order to assay for the expected therapeutic efficacy of any given substituted peptide on any given SLE patient. In order to assess the ultimate goal of producing peptides that will bind with high affinity to the appropriate MHC Class II molecules but will not lead to further activation of T cells and will therefore have a therapeutic effect on SLE patients, the peptides may be assayed, following biotinylation, for their ability to bind directly to HLA Class II products on antigen-presenting cells in the peripheral blood lymphocytes of the SLE patients. Healthy control donors and control peptides may be used in such assays to verify their specificity.

A *preferred form of the therapeutic agent of the invention is a peptide selected from* the group of peptides of formulas I to V herein, including peptides Ia to Va and substitution and/or deletion analogs thereof.

Another preferred form of the therapeutic agent in accordance with the present invention is the form of a multi-epitope single peptide. Thus, in a preferred embodiment, dual petides consisting of two different peptides selected from the group of peptides of formula I-V herein, are covalently linked to one another, such as by a short stretch of alanine residues or by a putative site for proteolysis by cathepsin. See, for example, U.S. Patent 5,126,249 and European Patent 495,049 with respect to such sites. This will induce site-specific proteolysis of the preferred form into the two desired analogs. Alternatively, a number of the same or different peptides of the present invention may be formed into a peptide polymer, such as, for example, polymerization of the peptides with a suitable polymerization agent, such as 0.1% glutaraldehyde (Audibert et al. (1981), Nature 289:593). The polymer will preferably contain from 5 to 20 peptide residues. Such peptide polymers may also be formed by crosslinking the peptides or attaching multiple peptides to macromolecular carriers. Suitable macromolecular carriers are, for example, proteins, such as tetanus toxoid, and linear or branched copolymers of amino acids, such as a linear copolymer of L-alanine, L-glutamic acid and L-lysine and a branched copolymer of L-

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tyrosine, L-glutamic acid, L-alanine and L-lysine (T,G)-A-L-, or multichain poly-DL-alanine (M. Sela et al. 1955, J. Am. Chem. Soc. 77:6175). The conjugates are obtained, for example, by first coupling the peptide with a water-soluble carbodiimide, such as 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride, and then performing the conjugation with the macromolecular carrier as described by Muller, G.M. et al. (1982) Proc. Natl. Acad. Sci. USA 79:569. The contents of the coupled peptide in each conjugate are determined by amino acid analysis, in comparison to the composition of the carrier alone.

According to one embodiment of the present invention, one or more active peptides may be attached to a suitable macromolecular carrier or may be polymerized in the presence of glutaraldehyde.

The peptides, polymers thereof or their conjugates with suitable macromolecular carriers, will be given to patients in a form that insures their bioavailability, making them suitable for treatment. If more than one peptide analog is found to have significant inhibitory activity, these analogs will be given to patients in a formulation containing a mixture of the peptides.

The invention further includes pharmaceutical compositions comprising at least one synthetic peptide according to the invention, a conjugate thereof with a suitable macromolecular carrier or a polymer thereof optionally with a pharmaceutically acceptable carrier.

Any suitable route of administration is encompassed by the invention, including oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intraperitoneal, intradermal, transdermal or other known routes, including the enteral route.

The dose ranges for the administration of the compositions of the present invention should be large enough to produce the desired effect, whereby, for example, an immune response to the SLE-inducing autoantibody, as measured by T cell proliferation *in vitro*, is substantially prevented or inhibited, and further, where the disease is significantly treated. The doses should not be so large as to cause adverse side effects, such as unwanted cross reactions, generalized immunosuppression, anaphylactic reactions and the like.

Effective doses of the peptides of this invention for use in treating SLE are in the range of about 1μg to 100 mg/kg body weight. The dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

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The synthetic peptides and analogs of the invention, particularly those of sequences I to V herein, are aimed at inhibiting or suppressing specific antigen responses of SLE patients, without affecting all other immune responses. This approach is of the utmost importance since most diagnosed patients are young women that have to be treated for many years and the currently accepted treatment for SLE involves administration of immuno-suppressive agents, such as corticosteroids and/or cytotoxic drugs, that are both non-specific and have multiple adverse side effects.

The present invention will now be described in more detail in the following non-limiting Examples and the accompanying figures:

## EXAMPLES

### Materials and Methods

a) Mice : Mice (BALB/c and SJL/J) were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA and from Olac, Show's farm, Bicesper Oxon, England. Mice were used at the age of 6-12 weeks. In some studies neonatal mice were also used.

b) Human mAb 16/6 Id : The human mAb 16/6 is an anti-DNA antibody originally of the IgM isotype and switched in culture to IgG1. The mAb was derived from a patient and expresses a common idiotype, the 16/6 Id (Shoenfeld et al., 1983; Mendlovic et al., 1988). The hybridoma cells secreting this mAb are routinely grown in culture, and the antibody is isolated from culture supernatants using an affinity column of Protein G coupled to Sepharose™.

c) Production of mouse mAb 5G12 and 2C4C2: Experimental SLE was induced in C3H.SW female mice by immunization with the previously described murine anti-16/6 Id mAb (Mendlovic et al., 1989). Four months later, two mice were sacrificed and their spleen cells were fused with X63.653 plasmacytoma cells. Hybridoma cells that secreted autoantibodies were cloned by limiting dilution in 96-well microtiter plates. The sequence characteristics of nine monoclonal autoantibodies secreted by nine of the hybridoma clones were characterized (Waisman and Mozes, 1993). The mAb designated 5G12 and 2C4C2 were isolated and affinity purified from the hybridoma supernatants using a goat anti-mouse Ig-Sepharose™ 4B column. The 5G12 mAb was found to be an anti-DNA mAb that bear the 16/6 Id and have the IgG2a isotype. The 2C4C2 mAb was found to be an anti-DNA and anti-cardiolipin mAb and to be of the IgM isotype. The nucleotide and deduced amino acid

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sequences for the $V_H$ of both 5G12 and2C4C2 mAb are presented in Fig. 1 of Waisman and Mozes, 1993, in which figure the CDR regions are boxed..

d) Induction of experimental SLE in mice : Mice were injected with the human monoclonal 16/6 Id (1μg/mouse) or the murine 16/6 Id mAb, e.g. mAb 5G12 (20μg/mouse), in complete Freund's adjuvant in the hind footpads. Three weeks following injection, the mice were boosted with the same amount of the immunizing antibody in phosphate-buffered saline (PBS).The mice were then tested for autoantibody production and clinical manifestations characteristic of experimental SLE.

e) Detection of SLE-associated clinical manifestations : The erythrocyte sedimentation rate was determined by diluting the heparinized blood in PBS at a ratio of 1:1. The diluted blood was then passed to a microsampling pipette and the sedimentation was measured 6 hours later. White blood cell counts were determined after the hemolysis of heparinized blood. Proteinuria was measured in a semi-quantitative manner, using a Combistix kit (Ames, Stoke Poges, Slough, U.K.). Immunohistology was performed by incubation of fixed frozen cryostat sections with FITC-labeled antibodies to mouse Ig. Staining was visualized via use of a fluorescent microscope.

f) Enzyme-linked immunosorbent assay (ELISA) : ELISA was utilized for the detection and quantitation of antibodies in experimental mice, and in humans. Polystyrene microtiter plates were coated with the relevant antigen or antibody, and sera dilutions or supernatants derived from the human or mouse cell cultures were added to the blocked plates. Specific binding was determined following the addition of peroxidase-conjugated antibodies against the appropriate immunoglobulin (Ig) (e.g. goat anti-human or goat anti-mouse peroxidase-conjugated antibodies) and the peroxidase substrate. Optical densities were read at 414 nm using an ELISA reader.

g) Proliferative responses of splenic and lymph node cells : Cells ($0.5x10^6$/well) derived from the spleen and lymph nodes of treated and untreated mice were cultured in microtiter plates in the presence of different concentrations of the various immunizing pathogenic autoantibodies. At the end of 96 hours incubation, 0.5 μCi of $^3$H-thymidine was added for an additional 18 hours, after which cells were harvested and radioactivity was counted.

h) Treatment of experimental mice : In order to either prevent induction of experimental SLE or to cure mice afflicted with the disease, the following procedures were

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used: (i) Newborn mice were tolerized with a peptide of the invention (100 μg of the peptide in PBS, intraperitoneally at 24 and 72 hours after birth). Six weeks later, the mice were immunized with the pathogenic autoantibody, e.g. 5G12 (16/6Id+) and examined for disease manifestations; (ii) A first group of adult mice was injected with various concentrations of the peptides before disease induction with the pathogenic autoantibody or pathogenic T cell line; another group was injected with the peptides to be tested for their therapeutic effect six weeks following immunization at the peak of the serological response; and a further group was treated at 4-6 months post-immunization after the establishment of the overt SLE disease. The number of injections with the peptides was determined based on their effect on the disease induction and progression. The effect of the peptide treatment on T cell proliferation, on the autoantibody production and on the disease manifestations was then evaluated.

i) Proliferative responses of T cell lines and clones: T cell lines and clones specific to the 16/6 Id were established from immunized lymph node cells as previously described (Axelrod and Mozes, 1986). Cells were exposed to the stimulating antibody presented on irradiated syngeneic spleen cells in the presence of enriched medium every two weeks. The T cell lines were cloned by the limiting dilution technique. Cells ($10^4$/well) were cultured with $0.5x10^6$ irradiated (3000 rad) syngeneic spleen cells in the presence of different concentrations of either the specific stimulator of the line or control reagents. At the end of 48 hours incubation, 0.5μCi of $^3$H-thymidine were added for an additional 18 hours, after which cells were harvested and radioactivity was counted.

j) Proliferation and cytokine production by peripheral blood lymphocytes (PBL): PBL from human SLE patients and of the appropriate control donors ($2x10^5$/well) were cultured in microtiter plates in enriched medium containing 10% pooled AB sera in the presence of the human or mouse monoclonal 16/6Id antibody, in the presence of peptides of the invention or in the presence of phytohemagglutinin (PHA). The rate of proliferation was evaluated by the incorporation of $^3$[H]-thymidine in the cell culture. Non-relevant peptides were used as specificity controls. Antigen and mitogen-stimulated cytokine production was quantitated in the supernatants of the above cultures using either the cytokine-dependent lines or the appropriate pairs of antibodies in ELISA assays. Inhibition of the proliferative responses was performed *in vitro* by adding increasing doses of the tested peptide analogs into the proliferative culture mixtures.

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k) **Human T cell lines and clones :** Human T cell lines specific to the 16/6Id may be established from PBL of either SLE patients or controls following stimulation *in vitro* with either the human or mouse mAb 16/6 Id or the peptides. The maintenance and cloning of the lines was performed similarly to that described above for the murine T cell lines, with the exception that the stimulation was performed using either autologous irradiated cells or EBV-transformed lines of autologous PBL (used as antigen-presenting cells).

l) **Biotinylation of peptides :** N-terminal biotinylation of the peptides was performed in 0.1N sodium bicarbonate solution at room temperature, with excess of biotinamidocaproate N-hydroxysuccinimide ester (Sigma, St. Louis, MO) dissolved in 1-methyl-2-pyrrolidone (Sigma).

m) **Direct binding of biotinylated peptides to APC :** Spleen cells suspended in RPMI 1640 medium containing 10% FCS were incubated in Petri dishes for 60 min at 37°C. Thereafter, non-adherent cells were removed, the plates were washed, and the adherent cells were collected from the plates using a rubber policeman (Costar, MA, USA). These cells ($1x10^6$/100µl/tube) were incubated with the biotinylated peptides in PBS containing 0.1% BSA (high purity grade, Amresco, OH, USA) for 16 hr at 37°C, followed by incubation with phycoerythrin (PE)-streptavidin (Jackson ImmunoResearch) for 30 min at 4°C. Thereafter the samples were incubated with biotinylated anti-streptavidin (1:60, Vector Laboratories, Burlingame, CA) and for an additional period with PE-streptavidin, all for 30 min at 4°C. The cells were washed twice with cold PBS/BSA solution after each incubation. Thereafter, cells were analyzed by flow cytometry using the FACSort cytometer and CELLQuest software (Beckton-Dickinson, Mountain View, CA). Three antibodies were used for inhibition of binding in these experiments: 34-5-3 (anti-I-$A^b$, Pharmingen, San Diego, CA); MKD6 (anti-I-$A^d$, Beckton-Dickinson) and 10.3.6.2 (anti-I-$A^s$ (Zamvil et al., 1988)).

**Example 1. Synthesis of the peptides**

The synthetic peptides of the invention of the formulas Ia, IIa and IIIa herein as well as control peptides were prepared with an automated synthesizer (Applied Biosystem model 430A, Germany) using the manufacturer's protocols for t-butyloxycarbonyl (BOC) procedure (see Kent et al., 1984; Schnolzer et al., 1992). Briefly, in this procedure, commercially available side-chain protected amino acids were used, the amino acids being

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added at each step with at least 99% efficiency. The protecting groups were removed from the peptides and were cleared from the resin with anhydrous HF. Subsequently, the peptides were purified by extraction with ethyl acetate or isopropyl acetate and by HPLC. The purity of the peptides Ia, IIa and IIIa so obtained was then verified by HPLC and amino acid analysis.

For the preparation of peptides IVa and Va herein and analogs of the peptides Ia to Va of the invention, the same procedure as noted above may be used.

The peptides Ia, IIa and IIIa were then analyzed for their biological activity and other characteristics as set forth in Examples 2-14 below. It is to be understood that the other peptides not so-tested may be subjected to the same analysis.

**Example 2. Detection of anti-DNA antibodies in the sera of mice immunized with peptides Ia and IIIa**

SJL/J and BALB/c female mice (6-8 week old) were immunized with 20 μg of peptide Ia or IIIa of the invention, or with a control peptide designated p278 (the peptide designated Pep 278h described in published PCT International Application WO 94/03208) or with mAb 5G12 emulsified in complete Freund's adjuvant (CFA) in the foot pads. Three weeks later the mice received a booster injection with the same amount of peptide or mAb, in PBS. Thereafter, blood was drawn every two weeks. A fifth group included non-immunized mice.

Fig. 1 depicts the anti-DNA antibodies in the sera of mice three months after the booster injection, and is very similar to the amount of the autoantibodies produced in later periods.

As shown in Fig. 1A, SJL/J mice that were immunized with the peptide IIIa (open circles) show a high level of anti-DNA antibodies, that is higher than that of mice immunized with the whole antibody 5G12 (open boxes). Low levels of anti-DNA antibodies were observed in the sera of SJL/J mice immunized with either the peptide Ia (open diamonds), control peptide p278 (open triangles) or normal non-immunized mice (crossed square).

As shown in Fig. 1B, BALB/c mice that were immunized either with the whole antibody 5G12 (open boxes) or the peptide Ia (open diamonds) show presence of anti-DNA antibodies in the sera. However, sera of BALB/c mice immunized with either the peptide

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IIIa (open circles), p278 (open triangles) or normal non-immunized mice (crossed square) did not show presence of anti-DNA antibodies.

ELISA was utilized to test the presence of the anti-DNA antibodies in the sera of the mice, as follows : Plates (Nunc) were coated for 90 min with 10 μg/ml of methylated BSA. Thereafter the plates were washed (all the washes were 3 times with PBS/0.05% Tween 20 (Sigma)) and incubated for an additional 90 min with 10 μg/ml of single-stranded DNA (calf thymus DNA (Sigma) that was heated for 15 min at 90°C and fast-cooled). The plates were washed and blocked overnight with 1% ovalbumin in PBS (Sigma). Thereafter, the plates were washed and incubated with the sera of the mice diluted in the blocking reagent, followed by wash and incubation with 1:500 dilution of goat anti-mouse IgG (Fc receptor specific) polyclonal antibody conjugated to peroxidase. The plates were then washed and developed using ABTS substrate (Sigma), and the color was read using an ELISA reader at 414 nm. Results are expressed as mean OD of each mouse group (5 mice per group).

**Example 3. Detection of anti-nuclear extract (NE) antibodies in the sera of mice immunized with the peptides Ia and IIIa**

Five groups of mice were immunized according to Example 2, and their sera were tested for the presence of anti-NE antibodies.

As shown in Fig. 2A, SJL/J mice immunized with the mAb 5G12 (open squares) or with the peptide IIIa (open circles) produced a high level of anti-NE antibodies, whereas mice immunized with the peptide Ia (open diamonds) or p278 control peptide (open triangles), or normal non-immunized mice (crossed squares), produced lower levels of anti-NE antibodies.

As shown in Fig. 2B, BALB/c mice immunized with the mAb 5G12 (open squares) or with the peptide Ia (open diamonds) produced high levels of anti-NE antibodies, whereas very low level of anti-NE antibodies was detected in the sera of BALB/c mice immunized with the peptide IIIa (open circles). No anti-NE antibodies were detected in the group of mice immunized with p278 control peptide (open triangles) or in normal non-immunized mice (crossed squares).

ELISA was utilized to test the presence of the anti-NE antibodies in the sera of the mice, as follows: Plates (Nunc) were coated with 5 μg/ml of of HeLa cells NE for 90 min.

Thereafter plates were washed and blocked, and ELISA was continued the next day, as described in Example 2 for anti-DNA antibodies..

**Example 4. Detection of anti-RNP, Sm, Ro and La antibodies in the sera of mice immunized with the peptides Ia and IIIa**

The same sera of the mice as described in Examples 2 and 3 were used for detection of anti-RNP, Sm, Ro and La antibodies .

As shown in Fig. 3A, SJL/J mice immunized with the peptide IIIa (lined box) produced extremely high levels of anti-Ro autoantibodies, antibodies that are typical for SLE in humans. High levels of anti-RNP, anti-Sm and anti-La antibodies were detected not only in SJL/J mice immunized with the peptide IIIa (lined box), but also with the peptide Ia (closed box), as compared to normal mice (open box) or to mice immunized with the control peptide p278 (dotted box).

As shown in Fig. 3B, BALB/c mice immunized with the peptide Ia (closed box) or the peptide IIIa (lined box) produced very high levels of anti-RNP antibodies. However, BALB/c mice immunized with the peptide IIIa (lined box) showed very low levels of anti-Sm, anti-La and anti-Ro antibodies, as compared to BALB/c mice immunized with the peptide Ia (closed box) which produced detectable antibodies in the sera.

Plates were purchased as pre-coated plates and were blocked with 1% ovalbumin in PBS for 2 hr. Thereafter the plates were washed as in Example 2 above, incubated in duplicates with 1:10 diluted sera, washed again and ELISA was carried out as described in Example 2 above.

**Example 5. Clinical manifestations of SLE in mice immunized with the peptides Ia and IIIa**

BALB/c and SJL mice were immunized with mAb 5G12 or with peptides Ia and IIIa, and five months later were checked by two criteria for manifestation of SLE: white blood cell count (WBC) and proteinuria.

(i) White blood cell count (WBC): The mice were bled, their blood was diluted 1:10 with 1% (vol/vol) acetic acid in order to eliminate the red blood cells, and white blood cells were counted under a normal light microscope.

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Proteinuria : The urine of the mice was tested using combisticks (Combistix Kit, Ames) for the presence of protein. High levels of protein in the urine are indicative of kidney damage, a typical manifestation of SLE.

Table 1. Clinical manifestations of mice immunized with the peptides

| Immunization | BALB/c W.B.C.[a] | BALB/c proteinuria[b] | SJL W.B.C.[a] | SJL proteinuria[b] |
|---|---|---|---|---|
| mAb 5G12 | 3800±400 | 0.975±0.08 | Nd[c] | 0.8±0.07 |
| pep Ia | 3375±350 | 0.88±0.076 | Nd[c] | 0.375±0.04 |
| pep IIIa | 3325±400 | 0.30±0.01 | 3300±1343 | 0.9±0.075 |
| p278 | 6470±920 | 0.33±0.02 | 7150±320 | 0.2±0.25 |
| non-immunized | 6800±1200 | 0.1±0 | 8100±475 | 0.5±0 |

a) W.B.C., white blood cell counts per $mm^3$

b) proteinuria (g/l)

c) ND, not done

The results for both WBC and proteinuria are shown in Table 1: Mice immunized with either the mAb 5G12 or the peptides Ia or IIIa had a lower number of white blood cells in comparison to non-immunized mice or those immunized with p278 control peptide. High levels of protein were measured in the urine of both BALB/c and SJL mice immunized with mAb 5G12, of SJL mice immunized with the IIIa peptide and of BALB/c mice immunized with the Ia peptide, while a smaller increase in protein level was detected in the urine of both mice immunized with control peptide p278, of IIIa-immunized BALB/c mice anf of Ia-immunized SJL mice.

Example 6. Specificity of mice response to the peptides

As shown in previous examples, the peptides Ia and IIIa were used for the immunization of different mouse strains, in parallel to their immunization with the whole monoclonal antibody. The draining lymph nodes of the mice proliferated to the immunizing

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peptides to different extents, depending on the mouse strains. Thus, BALB/c mice were found to be high responders to peptide Ia, whereas SJL mice were found to be high responders to peptide IIIa. Both peptides were used in attempts to induce experimental SLE using the protocol utilized for the pathogenic autoantibodies. It was found that SJL mice that were immunized with peptide IIIa and BALB/c mice that were immunized with peptide Ia produced elevated levels of autoantibodies including anti-DNA (see Fig. 1) and anti-NE antibodies (see Fig. 2). Moreover, the immunized mice developed leukopenia and proteinuria (see Table I) similarly to mice in which experimental SLE has been induced using the murine anti-DNA, 16/6Id+ pathogenic 5G12 mAb. Kidney analysis of the peptide-injected mice revealed mild immune complex deposits in part of the mice. These results indicate that peptides Ia and IIIa are important T cell epitopes of the whole molecule of the pathogenic autoantibody.

In order to assess the correlation between the peptides of the invention and T cells, a T cell line specific to peptide IIIa of SJL origin (high responders to the peptide IIIa) was established. The T cells of the line proliferated specifically to the peptide IIIa but not to non-relevant control peptide p278, and upon stimulation with peptide IIIa, secreted the Th1-type cytokines, namely, IL-2, IFNγ and TNFα. Injection of the T cell line into syngeneic healthy mice led to the production of autoantibodies and development of clinical manifestations that are characteristic to mice with experimental SLE. These results confirm the role of the CDR-based peptides of the invention in experimental SLE and demonstrate the role of the peptide-specific T cells in the autoimmune disease.

<u>Example 7. Detection of anti-DNA and anti-NE antibodies in the sera of BALB/c mice tolerized with the peptide Ia and immunized with either peptide Ia or mAb 5G12</u>

In order to further elucidate the role of the peptides in SLE, peptide Ia was utilized for the induction of tolerance in BALB/c mice. Newborn mice were injected twice (at day 1 and 3) with either peptide Ia or a control peptide. Thus, neonatal BALB/c mice, 24 hr old, were injected intraperitoneally (i.p.) with 100 μg of the peptide Ia or the control peptide p307 (a peptide related to myasthenia gravis described in published PCT Application No. W 94/00148) in PBS, and received a second injection 48 hr later with the same amount of peptide. Six to seven weeks after injection, the mice were immunized as described in Example 2 above with either the mAb 5G12 or the peptide Ia. The mice were bled two

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weeks after boost (and then periodically every two weeks) and the sera of the mice were tested for the presence of anti-DNA or anti-NE antibodies, as described in Examples 1 and 2 above. The assays performed to measure these autoantibody titers in the sera of the experimental mice indicated that the mice that were tolerized with peptide Ia did not produce significant titers of antibodies to either DNA or nuclear extract antigens, whereas mice tolerized to the control peptide p307 prior to their immunization with peptide Ia or the mAb 5G12 produced high autoantibody titers.

As shown in Figs. 4A-B, BALB/c mice that were either tolerized with the peptide Ia and then immunized with the mAb 5G12 (half-filled squares), or tolerized with the peptide Ia and then immunized with the same peptide Ia (filled squares) produced lower levels of anti-DNA and anti-NE antibodies in comparison with mice that were tolerized with the non-relevant peptide p307 and then immunized with the mAb 5G12 (filled triangles), or tolerized with peptide 307 and then immunized with peptide Ia (filled circles).

This indicates that neonatal tolerization with the peptide Ia could lower the levels of autoantibodies in the sera of mice later immunized with the peptide Ia or the mAb 5G12.

**Example 8. *In vivo* inhibition of lymph node cell (LNC) proliferation responses to the CDR-based peptides Ia and IIIa**

BALB/c (Fig. 5a) and SJL (Fig. 5b) mice were immunized with peptides Ia and IIIa (20 μg/mouse in CFA i.d. in the hind footpads), respectively. The mice were also injected i.v. with 200 μg of the above peptides in PBS either 3 days prior to immunization (open squares), at the immunization day (open circles) or at both dates (open triangles). Ten days later the mice were sacrificed and their lymph nodes were removed and tested for proliferation in the presence of different concentrations of the immunizing peptide. Control groups were of LNC taken from mice that were immunized but not treated (filled squares), or treated with control peptide, p307 (half filled squares). The culture mixtures were incubated for 96 hours in enriched RPMI medium containing 1% normal mouse serum prior to addition of $^{3}$H-thymidine. Sixteen hours later cells were harvested and radioactivity was counted. Results are expressed as mean CPM of triplicates. SD values did not exceed 10%.

As shown in Figs. 5a-b, both peptides Ia (5a) and IIIa (5b) inhibited proliferative responses of LNC of BALB/c and SJL mice, respectively, when injected to the mice either 3 days prior to, or at the immunization day: Up to 95% of the proliferative capacity of the

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cells was inhibited by the peptides.The inhibition was specific since the proliferative responses of the LNC to Con A were not inhibited by peptides Ia and IIIa (not shown).

**Example 9. *In vivo* inhibition of LNC proliferation of mice immunized with mAb 5G12 and treated with peptides Ia and IIIa.**

BALB/c (Fig. 6a) and SJL (Fig. 6b) mice were immunized with mAb 5G12 (20 μg/mouse in CFA i.d. in the hind footpads) and were injected (200 μg/mouse i.v. in PBS) with either peptide Ia or IIIa, respectively. Proliferation responses to mAb 5G12 were measured in LNC taken from mice that were immunized and not treated (filled squares), treated concomitantly with immunization with the control peptide p307 (half filled squares) or treated with the appropriate CDR-based peptide Ia (6a) or IIIa (6b) (open squares). Proliferation responses to the immunodominant CDR-based peptide Ia and IIIa was also monitored in LNC taken from non-treated mice (filled circles) or from mice treated with the appropriate CDR-based peptide Ia or IIIa (open circles). Results are expressed as mean CPM of triplicates. SD values did not exceed 10%.

As shown in Figs. 6a-b, proliferative responses to mAb 5G12 of LNC taken from mice treated with the appropriate CDR-based peptide were inhibited comparing to the responses of non-treated mice.

**Example 10. *In vivo* inhibition of LNC proliferation to the human monoclonal anti-DNA 16/6Id antibody**

BALB/c (Fig. 7a) and SJL (Fig. 7b) mice were immunized with human mAb 16/6Id (1 μg/mouse in CFA i.d. in the hind footpads) and were injected (200 μg/mouse i.v. in PBS) with either peptide Ia or IIIa, respectively. Proliferation responses to mAb 16/6Id were measured in LNC taken from immunized but not-treated mice (filled squares), from mice treated concomitantly with immunization with the control peptide p307 (half filled squares) or from mice treated with the appropriate CDR-based peptide Ia or IIIa (open squares). Proliferation responses were also shown to the immunodominant CDR-based peptide Ia or IIIa of LNC taken from 16/6Id immunized non-treated mice (filled circles) or from mice treated with the appropriate CDR-based peptide Ia or IIIa (open circles). Results are expressed as mean CPM of triplicates. SD values did not exceed 10%.

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As shown in Figs. 7a-b, proliferative responses to mAb 16/6Id of LNC taken from mice treated with the appropriate CDR-based peptide Ia or IIIa were inhibited comparing to the responses of immunized but not treated mice, or mice treated with the control peptide p307.

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**Example 11. Binding of CDR-based peptides Ia and IIIa to the surface of splenic antigen-presenting cells (APC)**

Splenic adherent cells ($10^6$/100µl/tube) isolated from BALB/c, SJL, C3H.SW or C57BL/6 mice were incubated for 16 hours with biotinylated CDR-based peptide Ia or IIIa followed by incubation with PE-streptavidin for 30 min at 4°C. Thereafter the samples were incubated with biotinylated anti-streptavidin and for an additional period with PE-streptavidin, all at 4°C for 30 min. After washing, the cells were analysed by flow cytometry using the FACSort cytometer and CELLQuest software.

The results are shown in Fig. 8: staining of cells that were incubated with the biotinylated CDR-based peptides is marked by solid lines, and background staining with non-biotinylated peptide is marked by broken lines. Splenic antigen-presenting cells derived from all tested mouse strains (except for C57BL/6 mice that are resistant to induction of SLE) showed significant binding of both CDR-based peptides Ia and IIIa to MHC class II products, indicating that their binding capacity agrees with the susceptibility of the mouse strains to SLE induction.

Binding of the CDR-based peptides Ia and IIIa to APC was determined as described in Materials and Methods herein and the results are shown in Table 2. Binding percentage was about 38-53% for all strains, except for APC from C57BL/6 strain which showed only 19.3% and 8.5% binding with peptides Ia and IIIa, respectively The binding was inhibited by the relevant anti-Ia antibodies showing the specificity of the binding to MHC Class II products. The results are shown in Table 3: Inhibition of binding was specific and ranged from 60% to 100%.

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Table 2. Binding of peptides Ia and IIIa to APC of mice

| Mouse strain | H-2[a] | peptide | % binding |
|---|---|---|---|
| BALB/c | d | Ia | 45.7 |
| BALB/c | d | IIIa | 41.3 |
| SJL | s | Ia | 42.3 |
| SJL | s | IIIa | 38.0 |
| C3H.SW | b | Ia | 42.3 |
| C3H.SW | b | IIIa | 52.9 |
| C57BL/6 | b | Ia | 19.3 |
| C57BL/6 | b | IIIa | 8.5 |

H-2 is the MHC Class II of the mouse

Table 3. Inhibition of binding of peptides Ia and IIIa to APC by anti-Ia mAb

| Mouse strain | H-2 | mAb | %inhibition | |
|---|---|---|---|---|
| | | | pep Ia | pep IIIa |
| BALB/c | d | anti I-$A^d$ (MKD6) | 76.7 | 100 |
| BALB/c | d | anti I-$A^b$ (34-5-3) | 0 | 0 |
| SJL | s | anti I-$A^s$ (10.3.6.2) | 100 | 92.8 |
| SJL | s | anti I-$A^d$ (MKD6) | 0 | 0 |
| C3H.SW | b | anti I-$A^b$ (34-5-3) | 60 | 84.4 |
| C3H.SW | b | anti I-$A^d$(MKD6) | 0 | 25 |
| C57BL/6 | b | anti I-$A^b$ (34-5-3) | 82 | 59.3 |
| C57BL/6 | b | anti I-$A^d$ (MKD6) | 0 | 0 |

2.23

**Example 12. Detection of antibodies against peptides Ia, IIa and IIIa, and anti-16/6 Id antibodies in the sera of SLE patients and healthy controls**

Human SLE patients (32 patients) were bled and their sera were tested by ELISA for their ability to bind the peptides Ia, IIa and IIIa, a control peptide p195-212 (a myasthogenic peptide described in PCT publication No. WO 94/00148) or mAb 5G12.

Detection of the antibodies was conducted on plates that were coated with 10 μg/ml of peptides Ia, IIa, IIIa or p195-212 or mAb 5G12, in PBS for 2 hr, washed and blocked with 1% ovalbumin in PBS for an additional 2 hr. ELISA was continued as described after blockage in Example 2 above, using goat anti-human IgG polyclonal antibody conjugated to peroxidase.

As shown in Fig. 9, SLE patients exhibited significantly higher levels of antibodies that bind either peptide Ia (open squares), IIa (open diamonds) or IIIa (open circles) , or mAb 5G12 (open triangles), in comparison to healthy controls (peptide Ia-healthy - closed diamonds; peptide IIa-healthy - crossed circles; peptide IIIa-healthy - inverted open triangles; 5G12-healthy - half filled squares). No binding could be observed when either sera of patients or controls were tested on plates coated with the non-relevant peptide p195-212 (p195-212-SLE - crossed squares; p195-212-healthy - half filled diamonds). The results indicate a correlation between the whole antibody molecule and the CDR-based peptides on the level of antibody titers.

**Example 13. Proliferation of PBL from SLE patients and healthy controls in the presence of human 16/6 Id mAb and peptides**

Peripheral blood lymphocytes (PBL) were isolated from the blood of SLE patients or healthy controls using ficol gradient. Thereafter, the PBL were incubated in the presence of different concentrations of the peptides Ia, IIa or IIIa, or the human 16/6 Id mAb for 24 hr, when a sample was taken for IL-2 measurement. The assay was continued for a total of 7 days, and $^{3}$H-thymidine was added for the last 16 hr. Proliferation was detected by reading the amount of radioactivity incorporated into the DNA of the cells.

As is seen in Table 4, a lower proportion of the PBL taken from SLE patients reacted to the peptides or to the 16/6 Id mAb, when compared to the healthy controls.The results are expressed in percentage of responder (34% in the first line) and the actual number of patients (11 out of 32: 11/32)

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Similar results were obtained when the levels of the IL-2 produced by the PBL in the presence of the peptides or the 16/6 Id mAb were tested, as shown in the next example.

**Table 4. Proliferation of PBL from SLE Patients and Healthy Controls in Presence of mAb 16/6 Id and Peptides Ia-IIIa**

| | SLE Patients | | Healthy Controls | |
|---|---|---|---|---|
| 16/6 Id | 34% | 11/32 | 72% | 18/25 |
| pep Ia | 21% | 7/32 | 44% | 11/25 |
| pep IIa | 9% | 3/32 | 28% | 7/25 |
| pep IIIa | 31% | 10/32 | 60% | 15/25 |

**Example 14. Production of IL-2 by PBL of SLE patients and healthy controls in the presence of human mAb 16/6 Id and peptides**

PBL were isolated from blood of SLE patients or healthy controls using ficol gradient, and were incubated as in Example 13. A sample of 50 µl was removed 24 hr after the assay was started, and incubated in the presence of IL-2 sensitive cells (CTLD) for 24 hr, after which $^{3}$H-thymidine was added for 16 hr, and the plates were harvested and counted on a beta counter.

As in Table 4, it can also be seen from Table 5 that a lower proportion of the PBL taken from SLE patients reacted to the peptides or to the 16/6 Id mAb, when compared to the healthy controls, thus indicating that the response to the peptide corresponds to that of T cells of the patient to the pathogenic human autoantibody.

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**Table 5. IL-2 Production by PBL of SLE Patients and Healthy Controls in Presence of mAb 16/6 Id and Peptides Ia-IIIa**

| | SLE Patients | | Healthy Controls | |
|---|---|---|---|---|
| 16/6 Id | 31% | 10/32 | 68% | 17/25 |
| pep Ia | 16% | 5/32 | 56% | 14/25 |
| pep IIa | 9% | 3/32 | 32% | 8/25 |
| pep IIIa | 16% | 5/32 | 64% | 16/25 |

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(ii) TITLE OF INVENTION: SYNTHETIC PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEM FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IL 113,159
(B) FILING DATE: 28-MAR-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(D) OTHER INFORMATION:/note= Xaa in position 5 is Met, Ala or Val; Xaa in position 6 is Gln, Asp, Glu, or Arg; Xaa in position 7 is Trp or Ala; Xaa in position 8 is Val or Ser; and Xaa in position 9 is Lys, Glu or Ala.

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Thr Gly Tyr Tyr Xaa Xaa Xaa Xaa Xaa Gln Ser Pro Glu Lys Ser Leu
1 5 10 15

Glu Trp Ile Gly
20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(D) OTHER INFORMATION:/note= Xaa in position 9 is Thr, Val or Ala; Xaa in position 10 is Thr, Val or Ala; Xaa in position 11 is Tyr or Phe; Xaa in position 12 is Asn or Asp; Xaa in position 13 is Gln or Glu; Xaa in position 14 is Lys or Glu; and Xaa in position 15 is Phe or Tyr.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Glu Ile Asn Pro Ser Thr Gly Gly Xaa Xaa Xaa Xaa Xaa Xaa Xaa Lys
1 5 10 15

Ala Lys Ala Thr
20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(D) OTHER INFORMATION:/note= Xaa in position 6 is Phe, Thr or Gly; Xaa in position 7 is Leu, Ala or Ser; Xaa in position 8 is Trp or Ala; Xaa in position 9 is Glu or Lys; Xaa in position 13 is Met or Ala; and Xaa in position 14 is Asp, Lys or Ser.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Tyr Tyr Cys Ala Arg Xaa Xaa Xaa Xaa Pro Tyr Ala Xaa Xaa Tyr Trp
1 5 10 15

Gly Gln Gly Ser
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(D) OTHER INFORMATION:/note= Xaa in position 4 is Met or Ala; Xaa in position 5 is Asn, Asp or Arg; Xaa in position 6 is Trp or Ala; Xaa in position 7 is Val or Ser; Xaa in position 8 is Lys or Glu; Xaa in position 9 is Gln or Ala; Xaa in position 13 is Lys or Glu; and Xaa in position 14 is Ser or Ala.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gly Tyr Asn Xaa Xaa Xaa Xaa Xaa Xaa Ser His Gly Xaa Xaa Leu Glu
1 5 10 15

Trp Ile Gly

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(D) OTHER INFORMATION:/note= Xaa in position 6 is Ser or Phe; Xaa in position 7 is Gly or Ala; Xaa in position 8 is Arg, Ala or Glu; Xaa in position 11 is Asn or Asp; Xaa in position 12 is Tyr or Phe; and Xaa in position 14 is Trp, His or Ala.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Tyr Tyr Cys Ala Arg Xaa Xaa Xaa Tyr Gly Xaa Xaa Xaa Gly Gln Gly
1 5 10 15

Thr Leu

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Thr Gly Tyr Tyr Met Gln Trp Val Lys Gln Ser Pro Glu Lys Ser Leu
1 5 10 15

Glu Trp Ile Gly
20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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1 5 10 15

Ala Lys Ala Thr
20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Tyr Tyr Cys Ala Arg Phe Leu Trp Glu Pro Tyr Ala Met Asp Tyr Trp
1 5 10 15

Gly Gln Gly Ser
20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Gly Tyr Asn Met Asn Trp Val Lys Gln Ser His Gly Lys Ser Leu Glu
1 5 10 15

Trp Ile Gly

(2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Tyr Tyr Cys Ala Arg Ser Gly Arg Tyr Gly Asn Tyr Trp Gly Gln Thr
1 5 10 15

Leu

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CLAIMS:

1. A synthetic peptide selected from the group consisting of:

(i) a peptide of at least 12 and at most 30 amino acid residues based on a complementarity-determining region (CDR) of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a systemic lupus erythematosus (SLE)-like disease in mice (hereinafter CDR-based peptide), a salt or a chemical derivative thereof;

(ii) an analog of a CDR-based peptide defined in (i), a salt or a chemical derivative thereof;

(iii) a dual synthetic peptide comprising two such peptides of (i) or analogs of (ii) covalently linked to one another either directly or through a short linking chain;

(iv) a peptide polymer comprising a plurality of sequences of said peptide (i) or analog thereof (ii); and

(v) a peptide polymer (iv) attached to a macromolecular carrier.

2. A synthetic peptide according to claim 1, capable of:

(i) inhibiting specifically the proliferative response and cytokine secretion of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies; or

(ii) inhibiting development of SLE in mice that are susceptible to SLE-induction by pathogenic autoantibodies.

3. A synthetic peptide according to claim 1 or 2, being selected from the group consisting of peptides having the sequences I to V herein, wherein:

(i) the peptide of sequence I has the formula:

T G Y Y $X_1$ $X_2$ $X_3$ $X_4$ $X_5$ Q S P E K S L E W I G [I]

wherein $X_1$ is Met, Ala or Val; $X_2$ is Gln, Asp, Glu or Arg; $X_3$ is Trp or Ala; $X_4$ is Val or Ser; and $X_5$ is Lys, Glu or Ala;

(ii) the peptide of sequence II has the formula:

E I N P S T G G $X_6$ $X_7$ $X_8$ $X_9$ $X_{10}$ $X_{11}$ $X_{12}$ K A K A T [II]

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wherein $X_6$ and $X_7$ are each Thr, Val or Ala; $X_8$ is Tyr or Phe; $X_9$ is Asn or Asp; $X_{10}$ is Gln or Glu; and $X_{11}$ is Lys or Glu, and $X_{12}$ is Phe or Tyr;

(iii) the peptide of sequence III has the formula:

$$Y\,Y\,C\,A\,R\,X_{13}\,X_{14}\,X_{15}\,X_{16}\,P\,Y\,A\,X_{17}\,X_{18}\,Y\,W\,G\,Q\,G\,S \qquad [III]$$

wherein $X_{13}$ is Phe, Thr or Gly; $X_{14}$ is Leu, Ala or Ser; $X_{15}$ is Trp or Ala; $X_{16}$ is Glu or Lys; $X_{17}$ is Met or Ala, and $X_{18}$ is Asp, Lys or Ser;

(iv) the peptide of sequence IV has the formula:

$$G\,Y\,N\,X_{19}\,X_{20}\,X_{21}\,X_{22}\,X_{23}\,X_{24}\,S\,H\,G\,X_{25}\,X_{26}\,L\,E\,W\,I\,G \qquad [IV]$$

wherein $X_{19}$ is Met or Ala; $X_{20}$ is Asn, Asp or Arg; $X_{21}$ is Trp or Ala; $X_{22}$ is Val or Ser; $X_{23}$ is Lys or Glu; $X_{24}$ is Gln or Ala; $X_{25}$ is Lys or Glu, and $X_{26}$ is Ser or Ala; and

(v) the peptide of sequence V has the formula:

$$Y\,Y\,C\,A\,R\,X_{27}\,X_{28}\,X_{29}\,Y\,G\,X_{30}\,X_{31}\,X_{32}\,G\,Q\,G\,T\,L \qquad [V]$$

wherein $X_{27}$ is Ser or Phe; $X_{28}$ is Gly or Ala; $X_{29}$ is Arg, Ala or Glu; $X_{30}$ is Asn or Asp; $X_{31}$ is Tyr or Phe, and $X_{32}$ is Trp, His or Ala.

4. A peptide according to claim 3, having a sequence Ia of the formula:

T G Y Y M Q W V K Q S P E K S L E W I G (Ia)

5. A peptide according to claim 3, having a sequence IIa of the formula:

E I N P S T G G T T Y N Q K F K A K A T (IIa)

6. A peptide according to claim 3, having a sequence IIIa of the formula:

Y Y C A R F L W E P Y A M D Y W G Q G S (IIIa)

7. A peptide according to claim 3, having a sequence IVa of the formula:

G Y N M N W V K Q S H G K S L E W I G (IVa)

8. A peptide according to claim 3, having a sequence Va of the formula:

Y Y C A R S G R Y G N Y W G Q T L (Va)

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9. A dual synthetic peptide according to claim 1 or 2, in which two different sequences I to V in claim 3 are covalently linked to one another either directly or through a short linking chain.

10. A dual synthetic peptide according to claim 9, in which two different sequences of the peptides Ia to Va are linked covalently.

11. A peptide polymer according to claim 1, containing a plurality of identical sequences selected from the sequences I to V in claim 3.

12. A pharmaceutical composition for the treatment of systemic lupus erythematosus comprising an effective amount of a synthetic peptide or peptide polymer according to any one of claims 1 to 11, and a pharmaceutically acceptable carrier.

13. A pharmaceutical composition for the treatment of systemic lupus erythematosus comprising an effective amount of a mixture of at least two different peptides in accordance with any one of the claims 3 to 10.

14. A method for the treatment of systemic lupus erythematosus comprising administering to a systemic lupus erythematosus patient an effective amount of a peptide or peptide polymer according to any one of claims 1 to 11.

15. A method of selecting peptides capable of inhibiting the proliferative response of T lymphocytes from a SLE patient, comprising:

(i) synthesizing a peptide of at least 12 and at most 30 amino acid residues, having a sequence based on the CDR region of the heavy or light chain of a pathogenic anti-DNA monoclonal antibody that induces a SLE-like disease in mice, or an analog thereof;

(ii) testing said peptide or analog for its ability to inhibit the proliferative response of T cells from a SLE patient, or a T cell line or clone which is specific to the 16/6 Id anti-DNA monoclonal antibody to which the T cells are specific; and

(iii) selecting and producing said peptide only if it is capable of inhibiting said proliferative response.

FIG. 1A

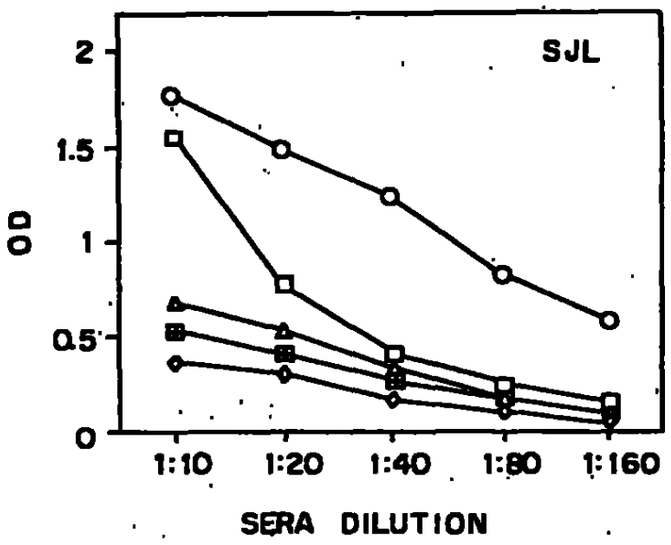
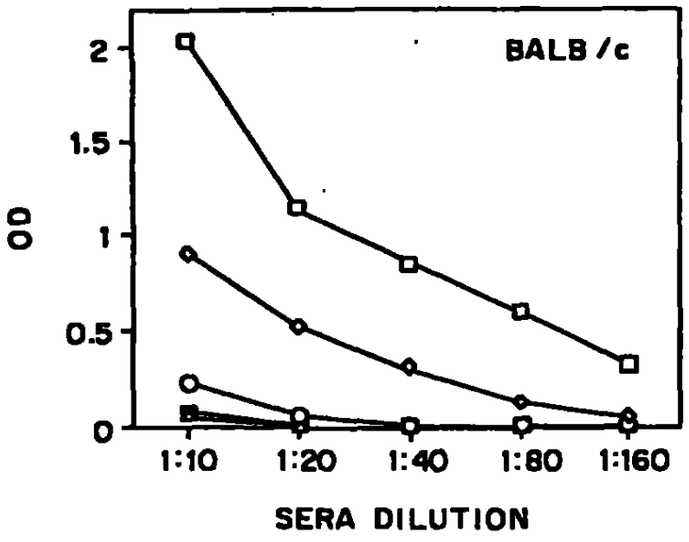
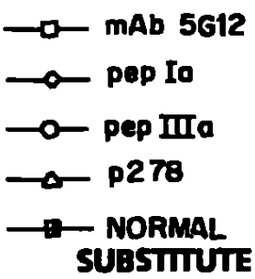

SJL
OD
2
1.5
1
0.5
0
1:10
1:20
1:40
1:80
1:160
SERA DILUTION

FIG. 1B


BALB/c
OD
2
1.5
1
0.5
0
1:10
1:20
1:40
1:80
1:160
SERA DILUTION


mAb 5G12
pep Ia
pep IIIa
p278
NORMAL

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FIG. 2A

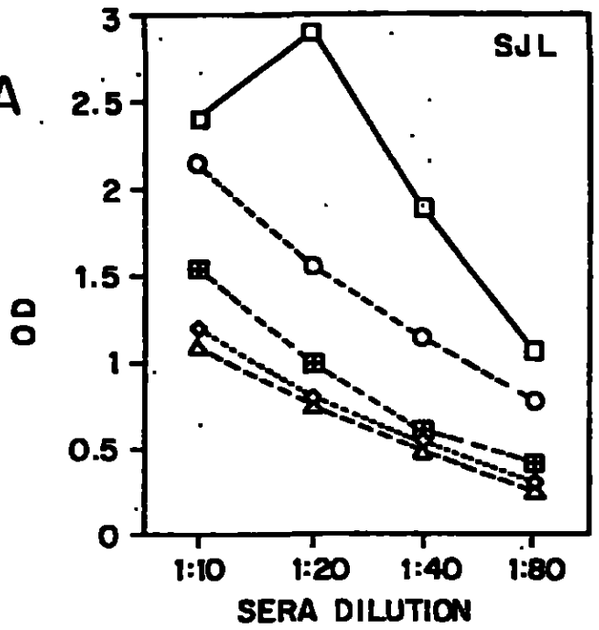



FIG. 2B

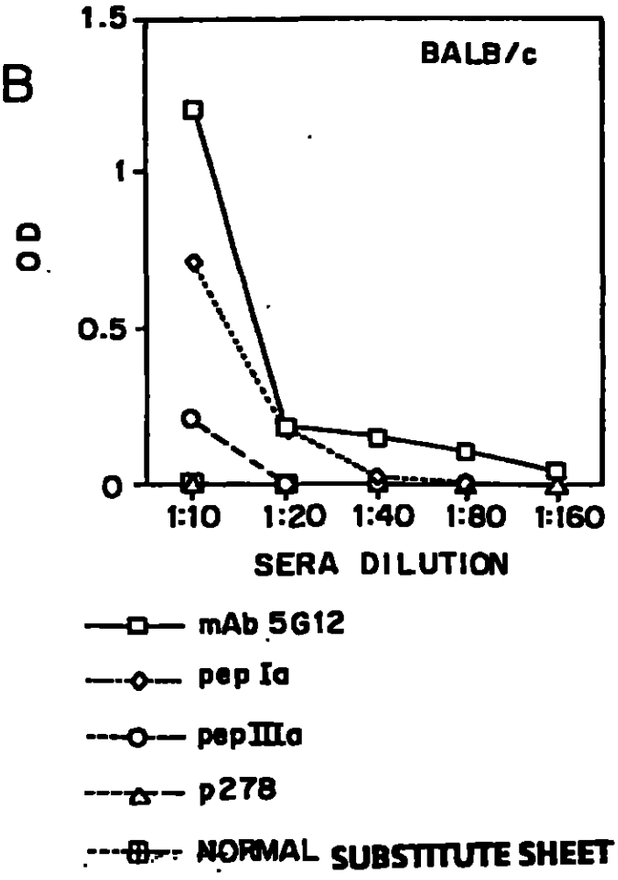


FIG. 3A

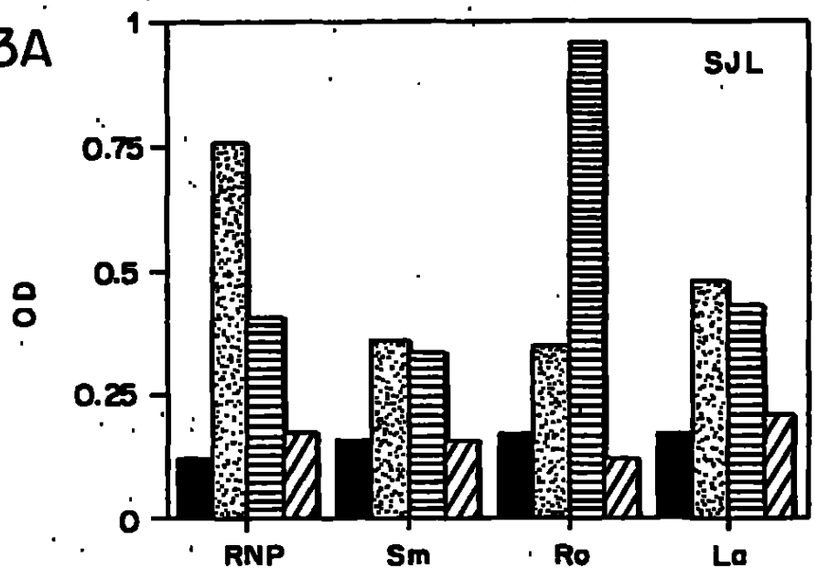
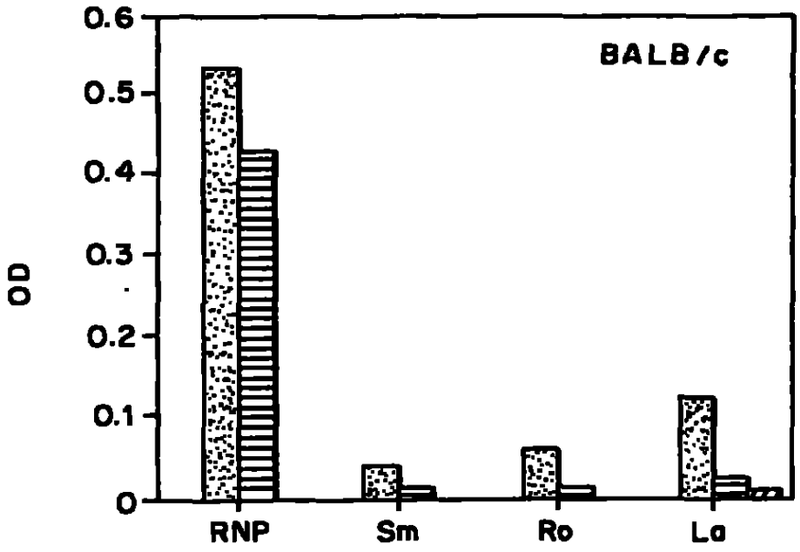



FIG. 3B




NON-IMMUNIZED
pep Ia
pep IIIa
p278

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FIG. 4A

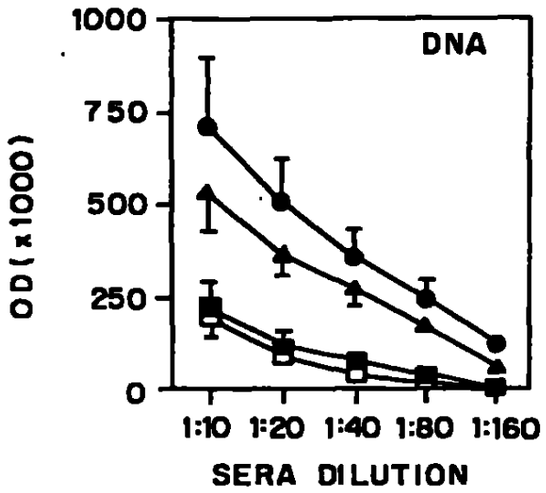
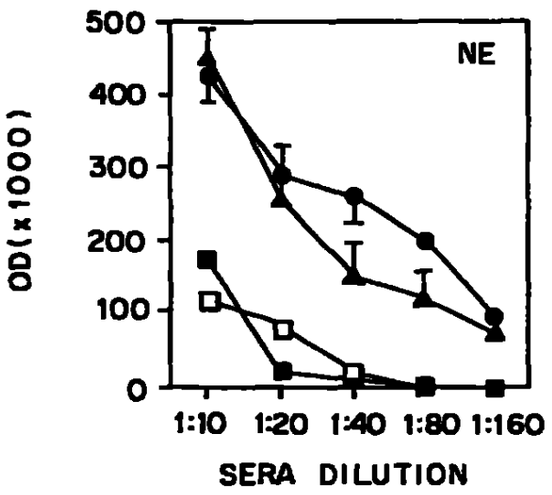
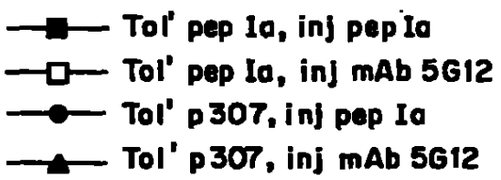



FIG. 4B



220



FIG. 5A

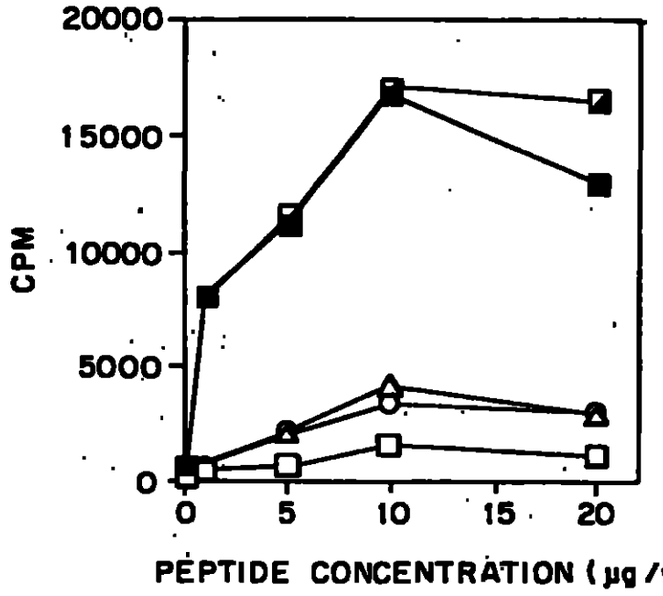
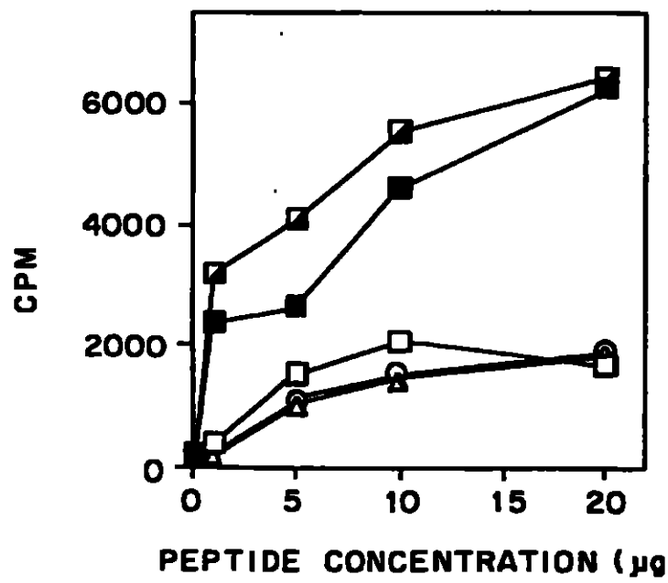



FIG. 5B



221



FIG. 6A

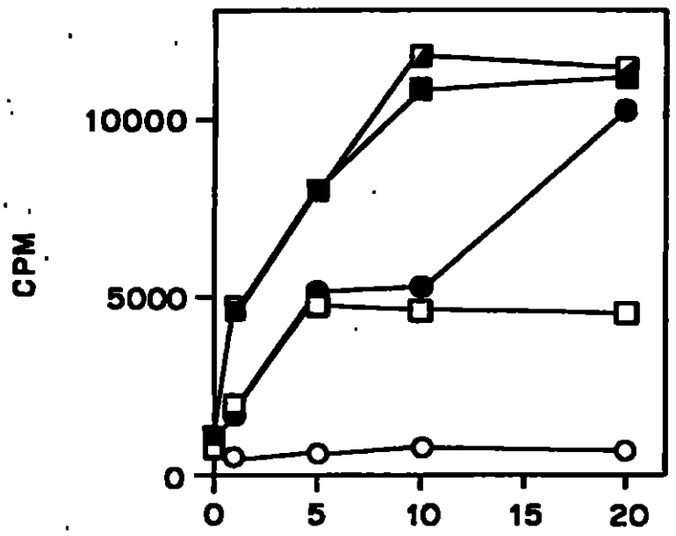
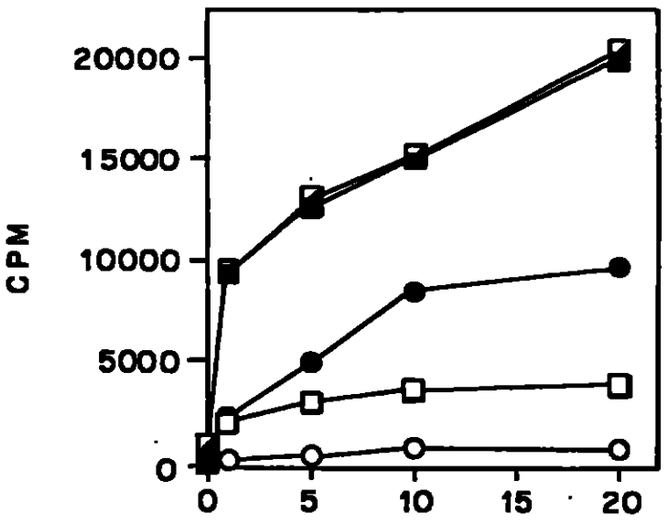



FIG. 6B



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FIG. 7A

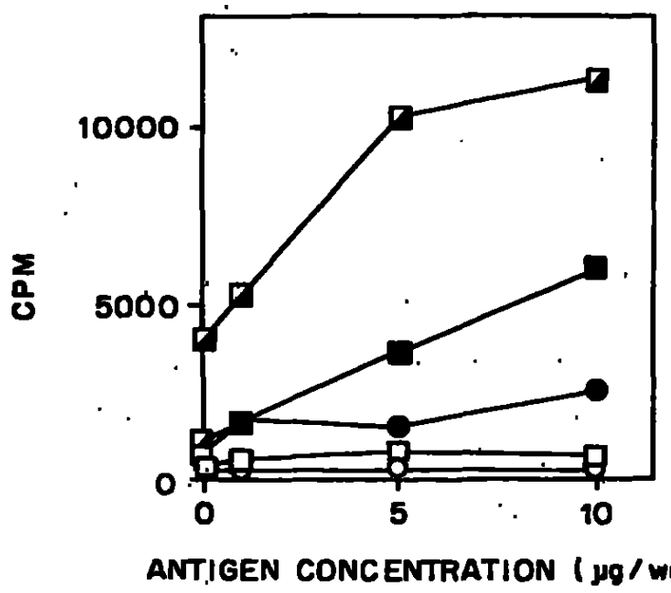
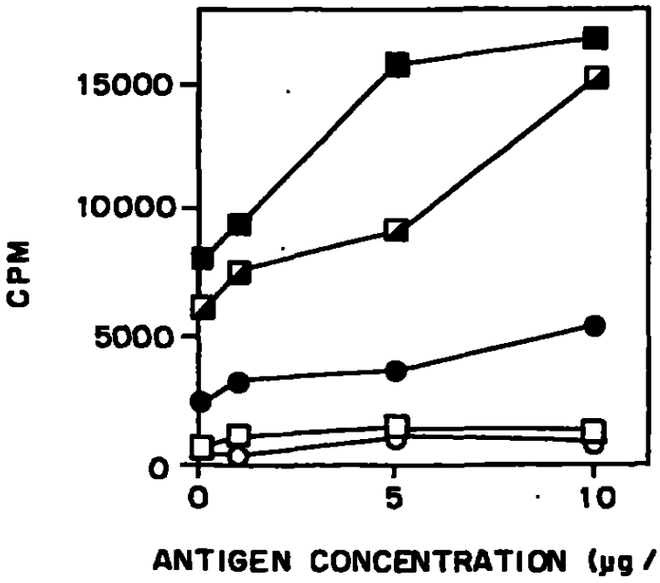



FIG. 7B



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FIG. 8B




FIG. 8D




FIG. 8A




FIG. 8C

FIG.8F

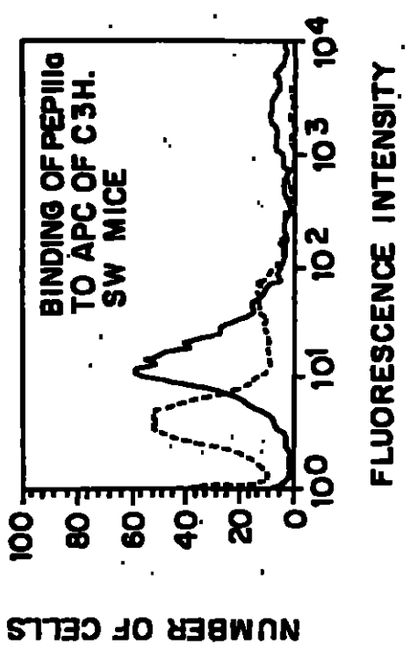



FIG.8H

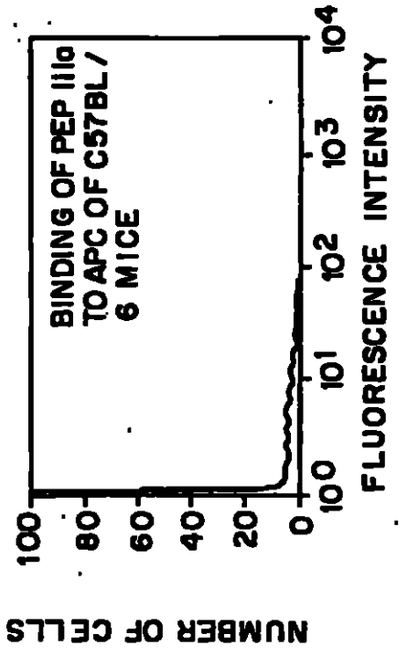



FIG.8E

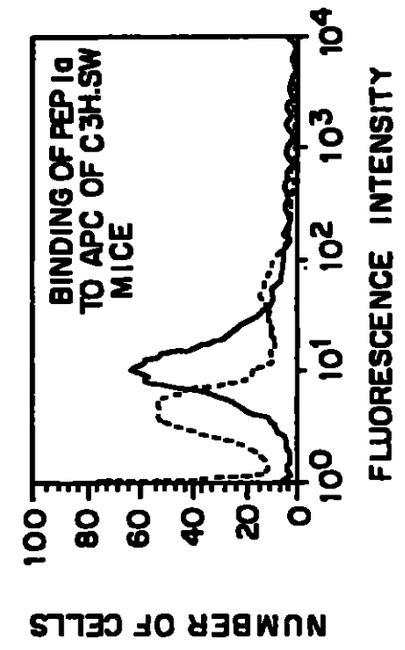
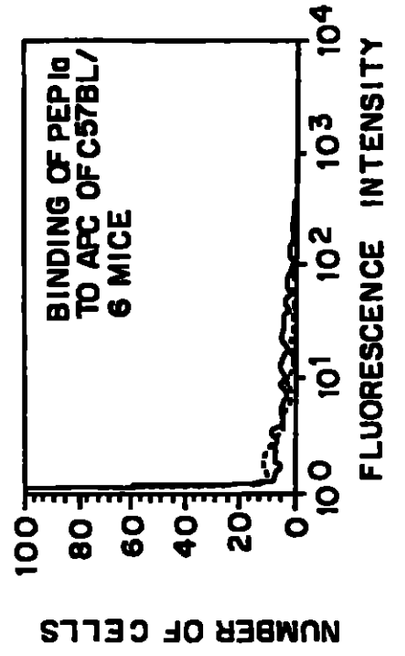


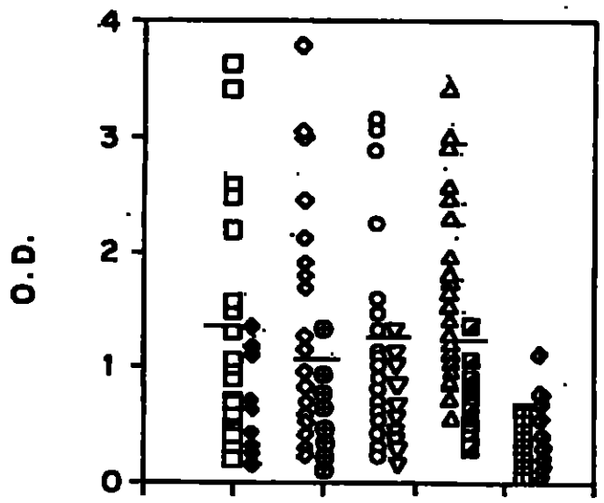

FIG.8G



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FIG. 9




- pep Ia-SLE
- pepIIa-SLE
- pepIIIa-SLE
- 5G12-SLE
- p195-212-SLE
- pep Ia-HEALTHY
- pepIIa-HEALTHY
- pepIIIa-HEALTHY
- 5G12-HEALTHY
- p195-212-HEALTHY

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04206

226

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 51/10; G01N 33/53
US CL :424/130.1; 435/7.1; 514/13; 530/326, 387.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1; 435/7.1; 514/13; 530/326, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WAISMAN, A. et al. Variable region sequences of autoantibodies from mice with experimental systemic lupus erythematosus. Eur. J. Immunol. 1993, Vol. 23, No. 7, pages 1566-1573, see entire document. | 1-15 |
| Y | MENDLOVIC, S. et al. The genetic regulation of the induction of experimental SLE. Immunology. 1990, Vol. 69, pages 228-236, see entire document. | 1-15 |
| Y | FRICKE, H. et al. Idiotype specific T-cell lines inducing experimental systemic lupus erythematosus in mice. Immunology. 1991, Vol. 73, pages 421-427, see entire document. | 1-15 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 09 JULY 1996 | 17 JUL 1996 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>DONALD E. ADAMS, PH.D.<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | STHOEGER, Z.M. et al. Monoclonal anticardiolipin antibodies derived from mice with experimental lupus erythematosus: Characterization and the induction of a secondary antiphospholipid syndrome. J. Clin. Immunol. 1993, Vol. 13, No. 2, pages 127-138, see entire document. | 1-15 |
| Y | WAISMAN, A. et al. The role of the 16/6 idiotype network in the induction and manifestations of systemic lupus erythematosus. Int. Immunol. 1993, Vol. 5, No. 10, pages 1293-1300, see entire document. | 1-15 |

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# United States Patent [19]

**Matsuo et al.**

[11] **Patent Number: 4,656,158**

[45] **Date of Patent: Apr. 7, 1987**


[54] **PEPTIDE, AND PRODUCTION AND USE THEREOF**


[75] Inventors: Hisayuki Matsuo, 6653, Ocuza-Kikura, Kiyotake-cho, Miyazaki-gun, Miyazaki-ken; Kenji Kangawa, Miyazaki, both of Japan

[73] Assignees: Suntory Limited; Hisayuki Matsuo, both of Japan



[21] Appl. No.: 706,382

[22] Filed: Feb. 27, 1985

[30] Foreign Application Priority Data

Mar. 2, 1984 [JP] Japan ..... 59-38817

[51] Int. Cl.[4] ..... A61K 37/02; C07K 7/10; C07K 3/24

[52] U.S. Cl. ..... 514/12; 530/324; 530/344

[58] Field of Search ..... 260/112.5 R; 514/12; 530/324, 344



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de Bold, A. J. et al, "A Rapid and Potent Natriuretic Response to Intravenous Injection of Atrial Myocardial Extract in Rats", Life Sciences, vol. 28, (1981), pp. 89–94.

Keeler, R., "Atrial Natriuretic Factor has a Direct, Prostaglan-Independent Action on Kidneys", Can. J. Physiol. Pharmacol, vol. 60, (1982), pp. 1078–1082.

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Kangawa, K. and Matsuo, H., "Purification and Complete Amino Acid Sequence of α-Human Atrial Natriuretic Polypeptide, (α-haNP)" Biochemical and Biophysical....



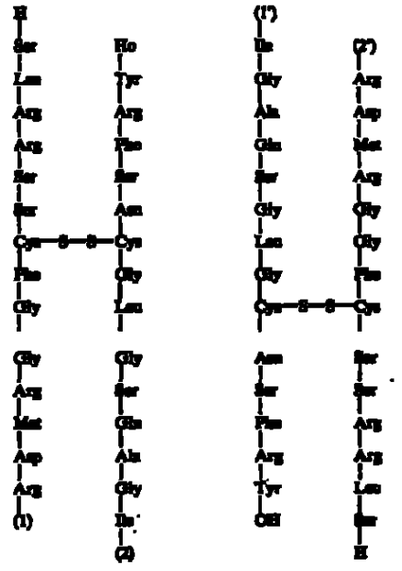
*Primary Examiner*—Delbert R. Phillips

*Attorney, Agent, or Firm*—Burns, Doane, Swecker & Mathis



[57] **ABSTRACT**

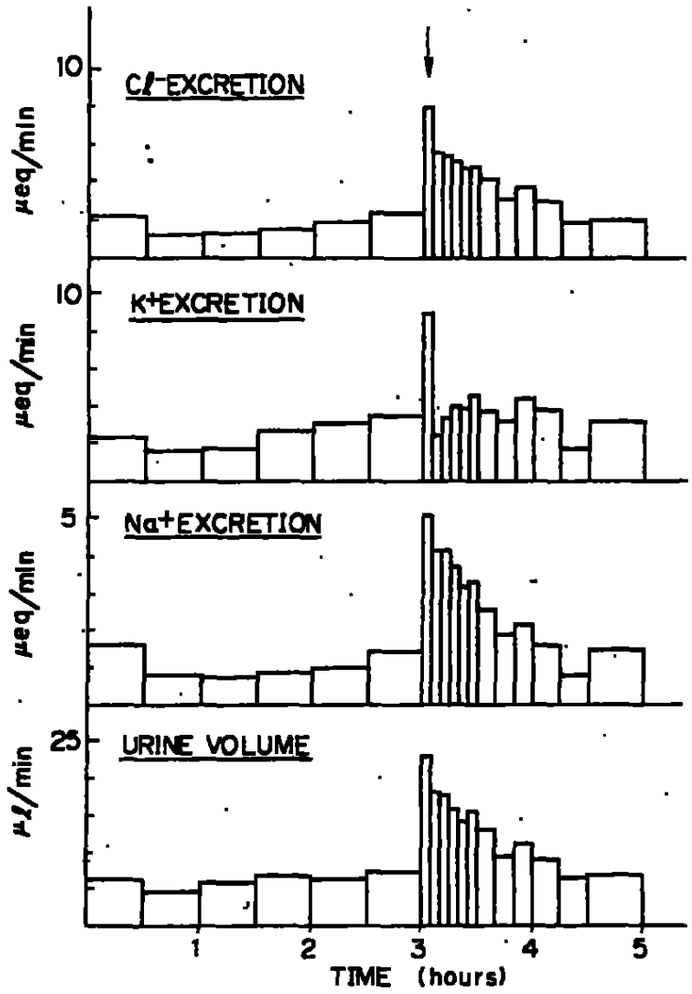
There are disclosed a new peptide β-hANP of the following structure:




and acid addition salt thereof; a diuretic composition and a hypotensor composition containing a β-hANP or an acid addition salt thereof; and processes for the production thereof.


17 Claims, 2 Drawing Figures

Fig. 1


10
Cl⁻EXCRETION
μeq/min
10
K⁺EXCRETION
μeq/min
5
Na⁺EXCRETION
μeq/min
25
URINE VOLUME
μl/min
1
2
3
4
5
TIME (hours)

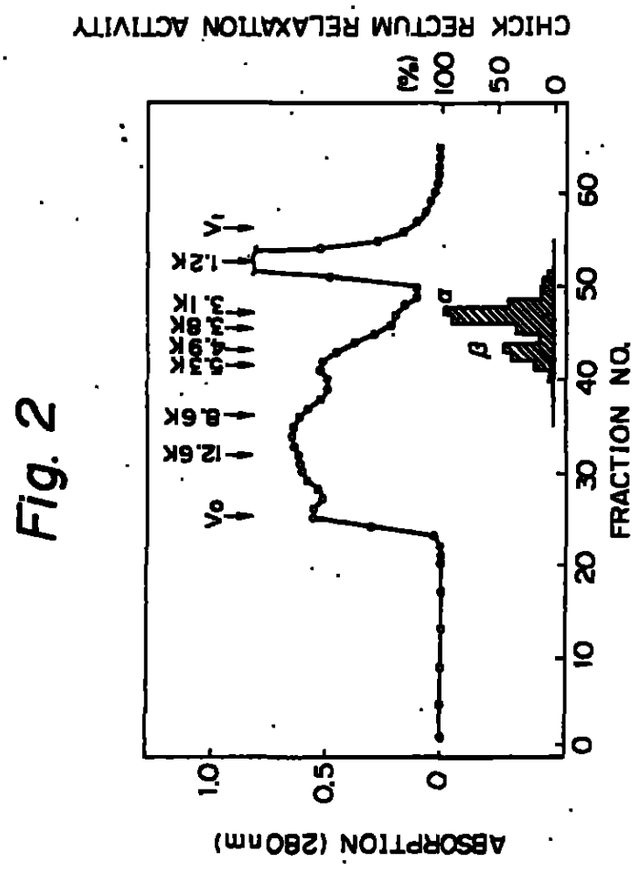

CHICK RECTUM RELAXATION ACTIVITY
(%)
100
50
0
60
50
40
30
20
10
0
FRACTION NO.
1.2K
3.1K
3.8K
4.9K
5.3K
8.6K
12.6K
α
β
1.0
0.5
0
ABSORPTION (280nm)

Fig. 2

# PEPTIDE, AND PRODUCTION AND USE THEREOF

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a novel peptide, a process for the production thereof, and a pharmaceutical composition containing the novel peptide as a diuretic or hypotensor.

2. Description of the Related Art

A normal regulation of the blood pressure in a human body is important for the maintenance of personal health, and various physical and humoral factors contribute to this regulation of the blood pressure. The physical factors include, for example, output, and the elasticity of the walls of blood vessels, etc. The humoral factors include, for example, the renin-angiotensin-aldosterone system, catecholamines, prostaglandins, kinin-kallikrein system, and natriuretic hormones including ouabain-like substances. Herein the term "natriuretic" will denote selective excretion of sodium cation relating to potassium cation.

Granules morphologically similar to granules present in peptide hormone-producing cells are found in human atrium (J. D. Jamieson and G. E. Palade, *J. Cell Biol.*, 23, 151, 1964). A homogenate of rat atrium and granules contained therein are known to show natriuretic action in rats (A. J. DeBold et. al., *Life Science*, 28, 89, 1981; R. Kesler, *Can. J. Physiol. Pharmacol.*, 60, 1078, 1982). Recently Mark G. Currie et. al. suggested peptide-like substances with a molecular weight of 20,000 to 30,000 or not more than 10,000, present in atrium of humans, rabbits, swine, and rats, and having natriuretic action (*Science*, 221, 71–73, 1983).

Moreover, a peptide consisting of 28 amino acids derived from rat atrium cordis was identified (*Biochem. Biophys. Res. Commun.*, Vol. 117, No. 3, p859–865, 1983). The present inventors found a new peptide consisting of 28 amino acids from human atrium cordis; referred to as "α-human atrial natriuretic polypeptide" and abbreviated as "α-hANP" (*Biochem. Biophys. Res. Commun.*) Vol. 118, No. 1, p131–139, 1984).

## SUMMARY OF THE INVENTION

The present invention provides a new peptide having natriuretic action and hypotensive or antihypertensive action. The peptide according to the present invention is hereinafter referred to as "β-human atrial natriuretic polypeptide" and abbreviated as "β-hANP".

There is also provided a process for production of the peptide.

Another object of the present invention is to provide a pharmaceutical composition containing the peptide as a diuretic or hypotensor.

Other objects and advantages of the present invention will be apparent from the description set forth hereinbelow.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 contains graphs showing the diuretic and natriuretic action of the β-hANP.

FIG. 2 is a chromatogram showing an elution profile wherein β-component is separated from α-component during isolation of β-hANP from human atrium cordis.

## DESCRIPTION OF THE PREFERRED EMBODIMENT

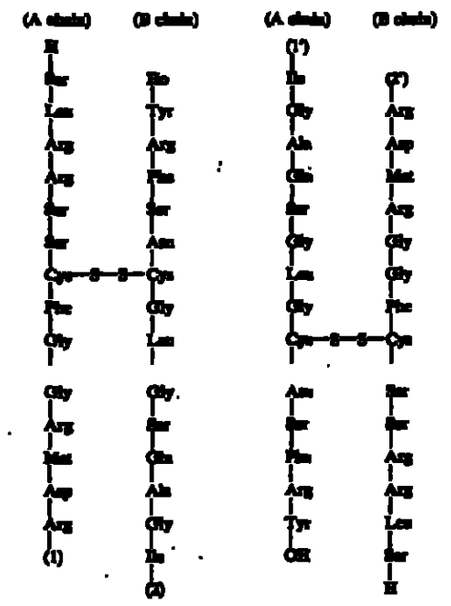
At present, furosemide as a natriuretic agent is used for the treatment of essential hypertension. However, the structure of the furosemide is different to that of the new peptide according to the present invention.

The present inventors isolated a peptide, in substantially pure form, consisting of 56 amino acid residues and having a molecular weight of about 6,000, determined the structure of the peptide and found that the peptide showed notable natriuretic and hypotensive action.

### Structure and Physico-chemical Properties of the β-hANP

(1) Structure

The β-hANP has the structure:




wherein (1) and (1'), and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— a L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end.

(2) Molecular weight: about 6,000 as determined by gel-filtration (6160.78 as calculated).

(3) UV spectrum: Max=275 mμ.

(4) Color reactions: Ehrlich's reaction; negative; Sakaguchi's reaction, positive; Pauly's reaction, positive.

(5) Distinction of basic, acidic, or neutral property: basic.

(6) Solubility in solvents: soluble in water, partially in methanol, and acetic acid; insoluble in ethyl acetate, butyl acetate, ethyl ether, hexane, petroleum ether, benzene, and chloroform.

(7) Amino acid composition by amino acid analysis:

| Amino acid | β-hANP | | RCM β-hANP* | |
|---|---|---|---|---|
| | Found | Calculated | Found | Calculated |
| Asp + Asn | 4.14 | 4 | 2.08 | 2 |
| Ala | 2.06 | 2 | 1.05 | 1 |
| Arg | 9.90 | 10 | 5.02 | 5 |
| Ile | 1.94 | 2 | 0.97 | 1 |
| Gly | 10.00 | 10 | 5.03 | 5 |
| Glu(Gln) | 2.06 | 2 | 1.01 | 1 |
| (Cys)s | | 2 | 0.99 | 1 |
| Ser | 10.18 | 10 | 5.17 | 5 |
| Tyr | 1.92 | 2 | 1.03 | 1 |
| Phe | 4.16 | 4 | 2.09 | 2 |
| Met | 2.18 | 2 | 0.95 | 1 |
| Leu | 3.98 | 4 | 2.06 | 2 |

(8) Determination of the structure

A product obtained after reduction of β-hANP with dithiothreitol to cleave a disulfide bond of the β-hANP and subsequent carboxymethylation of the cleft product, and that obtained from α-hANP by the same procedure provided an identical elution pattern showing a single peak in a liquid chromotography. These carboxymethylated products were digested with trypsin, and each digestion product was subjected to a liquid chromatography. In the chromatography, the digestion products from α-hANP and β-hANP provided an identical elution pattern, i.e., an identical combination of the digestion fragments. Moreover, a molecular weight of β-hANP was about two times that of α-hANP. These facts show that the β-hANP is a dimer of the α-hANP wherein the two α-hANP molecules are bonded through two disulfide bonds.

β-hANP and α-hANP were digested with trypsin, and the digestion products were analyzed by liquid chromatography for the fragment composition. A comparison of the fragment compositions from β-hANP and α-hANP showed that, in the β-hANP, two α-hANP molecules which are directed in reverse to each other are bonded through two disulfide bonds.

(9) Formation of salts: the β-hANP is a basic compound as described in item (5), and can form acid addition salts with an inorganic acid such as hydrochloric acid, sulfuric acid, phosphoric acid, or an organic acid such as formic acid, acetic acid, propionic acid, fumaric acid, and citric acid.

## Physiological Properties of β-hANP

The β-hANP according to the present invention has notable diuretic, natriuretic, and hypotensive or antihypertensive actions.

Test method:

Male rats weighing 300 to 400 grams were anesthetized by intraperitoneal administration of pentobarbital at a dosage of 60 mg/kg, and used for tests of the β-hANP according to the method described in *Life Sciences*, Vol. 28, pp89–94.

To keep the respiratory tract open, a tracheal cannula (PE240 Clay-Adams) was inserted into the trachea. An arterial cannula (PE-50) was inserted into a femoral artery for measurement of the blood pressure, and a venous cannula was inserted into a femoral vein for the administration of Ringer's solution. 1.2 ml of Ringer's solution was infused at a flow rate of 1.2 ml/hour.

A bladder cannula made of silastic tube with a inner diameter of 0.02 inches and an outer diameter of 0.037 inches was inserted into the bladder, and via the cannula, a urine sample was collected into a test tube. The collection of urine was carried out for 30 minutes before administration of the test compound, and every five minutes after the administration.

0.8 n mole of the test compound β-hANP was dissolved in 50 μl of sterilized physiological saline with 5 μg of bacitracin, and the solution was injected into the jugular vein.

The results of the test are set forth in FIG. 1. As shown in the figure, β-hANP shows notable diuretic and natriuretic actions. The actions of β-hANP are comparable to those of α-hANP, but are maintained for a period longer than that of α-hANP. 0.8 n mole of β-hANP provides diuretic and natriuretic actions comparable to those provided by 1.21 μ mole of furosemide, which is a known diuretic agent. However, β-hANP brings about the actions more rapidly than furosemide.

## Use of β-hANP as a pharmaceutical product

Repeated administration of β-hANP does not stimulate production of antibodies, and does not cause anaphylaxis shock. β-hANP consisting of L-amino acids is gradually hydrolized in a body providing the L-amino acids, and therefore shows little toxicity.

Due to the higher diuretic, natriuretic, and blood pressure-lowering or antihypertensive actions, and the lower toxicity, β-hANP is useful as an active ingredient for pharmaceutical compositions such as a diuretic and a hypotensor. β-hANP is administered at 0.1 μg/kg to 1 mg/kg, preferably 1 μg/kg to 100 μg/kg.

β-hANP can be administered in the same manner as conventional peptide type pharmaceuticals. Namely, β-hANP is preferably administered parenterally, for example, intravenously, intramuscularly, intraperitoneally, or subcutaneously. β-hANP, when administered orally, may be proteolytically hydrolyzed. Therefore, oral application is not usually effective. However, β-hANP can be administered orally as a formulation wherein β-hANP is not easily hydrolyzed in a digestive tract, such as liposome-microcapsules. β-hANP may be also administered in suppositories, sublingual tablets, or intranasal spray.

The parenterally administered pharmaceutical composition is an aqueous solution containing about 0.000005 to 5%, preferably 0.00005 to 0.5% of β-hANP, which may contain, in addition to β-hANP as an active ingredient, for example, buffers such as phosphate, acetate, etc., osmotic pressure-adjusting agents such as sodium chloride, sucrose, and sorbitol, etc., antioxidative or anticoxygenic agents, such as ascorbic acid or tocopherol and preservatives, such as antibiotics. The parenterally administered composition also may be a solution readily usable or in a lyophilized form which is dissolved in sterile water before administration.

## Production of β-hANP

β-hANP can be produced by either the extraction of the β-hANP from human atrium or by chemical synthesis.

In the former process, human atrium is homogenized in an acidic aqueous solution such as a phosphate buffer solution, or an acetic acid solution containing hydrochloric acid. Subsequently, β-hANP is purified accord-

ing to a conventional method suitable for the purification of peptide, such as centrifugation, isoelectric point precipitation, solvent extraction, ultrafiltration, gel filtration, adsorption chromatography or high performance liquid chromatography (HPCL), or a combination of such methods. In the above-mentioned methods, chick rectum relaxation activity is conveniently used to select fractions containing β-hANP, because β-hANP has this activity. In the chromatography methods, the β-hANP containing fractions can be also selected by molecular weight (about 5,000 to 6,000). Chemical synthesis is preferable for the industrial production of β-hANP, in which chemical synthesis, a liquid phase method or solid phase method, or a combination thereof can be used. The solid phase method such as Merrifield's method [R. B. Merrifield, *J. Am. Chem. Soc.* 85, 2184 (1963)] is most convenient.

In Merrifield's method, each amino acid is protected preferably with tert-butyloxycarbonyl (Boc) at the α-amino group; a hydroxyl group in tyrosine is protected preferably with 2,6-dichlorobenzyl group ($Cl_2Bzl$); a guanidino group in arginine is protected preferably with a tosyl group (Tos); a hydroxyl group in serine is protected preferably with a benzyl group (Bzl); a β-carboxyl group in aspartic acid is protected preferably with an O-benzyl group (O-Bzl); and a thiol group in cysteine is protected with an acetamidomethyl group (Acm). In the Merrifield method, first a protected derivative of C-terminal amino acid L-tyrosin, i.e., Boc-Tyr($Cl_2Bzl$), is introduced onto a solid phase resin carrier, such as chloromethyl-resin, and subsequently, each suitably protected amino acid is coupled to a terminal amino acid of an intermediate amino acid chain bonded to the resin, in the order of the amino acid sequence of α-hANP. After all the amino acids are coupled in the predetermined order, the protected α-hANP thus obtained is removed from the resin by treatment with hydrogen fluoride, and simultaneously protecting groups other than Acm are also removed. The product is then reduced to obtain $Cys^{7,23}$(Acm)-α-hANP), which is then oxidized with iodine to remove the thiol-protecting group Acm, and simultaneously, to form a disulfide bond. The crude-β-hANP thus obtained is then purified by conventional methods such as gel filtration, reverse phase HPLC, etc., to obtain purified β-hANP.

## EXAMPLES

The present invention will now be further illustrated by, but is by no means limited to, the following examples.

### EXAMPLE 1

Preparation of β-hANP from human atrium cordis

Ten hours after death, 40 g of human atrium cordis was removed and boiled in seven volumes of 1M acetic acid aqueous solution containing 20 mM hydrochloric acid for five minutes, to inactivate protease present in the atrium cordis. The boiled atrium cordis in the acetic acid solution was then cooled to 4° C., and homogenized with a Polytron homogenizer to extract the β-hANP. The homogenate thus obtained was centrifuged at 12000 XG for 30 minutes to obtain 200 ml of a supernatant. To the supernatant, 12 ml of glacial acetic acid was added to the concentration of 1M acetic acid, and acetone was dropwise added in an amount of 66% of the final concentration to precipitate impurities. The mixture thus obtained was centrifuged to obtain 434 ml of supernatant containing β-hANP, which was then evaporated to dryness. The residue thus obtained was dissolved in 100 ml of 1N acetic acid, and the solution was extracted two times with 50 ml of ethyl ether to defat the solution. The aqueous phase thus obtained was lyophilized, and the lyophilizate was redissolved in 100 ml of 1N acetic acid. The solution was then ultrafiltrated with a UM-2 filter (Amicon) to desalt the solution. The desalted solution was concentrated to 50 ml.

The concentrated solution was applied on SP-Sephadex C-25 column (Pharmacia, 8.0×22 cm). The elution was carried out with 1N acetic acid, 2N pyridine solution, aND 2N pyridine-1N acetic acid solution (pH 5.0), in that order, to obtain fractions SP-I, SP-II, and SP-III. The fraction SP-III was lyophilized to obtain 26.6 mg of lyophilizate, which was then dissolved in 1N acetic acid.

The solution thus obtained was gel-filtrated with Sephadex G-25 column (1.2×103 cm) at a flow rate of 5.4 ml/hour, collecting 2 ml of fractions. Thereby, β fractions (fractions No. 42 to 45) which have chick rectum relaxation activity were obtained. The elution profile is shown in FIG. 2. The β fractions were combined for further purification.

The combined fraction was then subjected to cation exchange HPLC in a TSK-CM2SW column (Toyo Soda, 7.5×300 mm). Elution was carried out by linear gradient with (A) 10 mM ammonium formate (pH 6.6)/acetonitrile (90:10) and (B) 1.0M ammonium formate (pH 6.6)/acetonitrile (90:10), after (A) for 12 minutes, changing the concentration of formate from 10 mM to 0.75M for 140 minutes. A set of fractions (No. 66 and 67, retention time 140 to 142 minutes) with chick rectum relaxation activity was obtained. The active fractions were combined and subjected to reverse phase HPLC in a TSK LS-410-ODS-SIL column (φ4.0×250 mm, Toyo Soda). Elution was carried out with (A) water/acetonitrile/10% trifluoroacetic acid (90:10:1) and (B) water/acetonitrile/10% trifluoroacetic acid (40:60:1) as eluents wherein the eluent (A) was used for 15 minutes and then linear gradient from (A) to (B) was used for 80 minutes, at a flow rate of 1.0 ml/min., and pressure of 110 to 130 kg/cm². A main peak was collected and 11 n mole of substantially pure β-hANP was obtained.

### EXAMPLE 3

Preparation of parenteral composition

(A) Injection solution

| Composition | |
|---|---|
| β-hANP | 2 g |
| sodium chloride | 8 g |
| acetic acid | 2 g |
| sterile water | 1 l |

Method

β-hANP and sodium chloride were dissolved in sterile water, an ampule was filled with 5 ml of the solution, and the ampule was then sealed.

(B) Lyophilizate

| Composition | |
|---|---|
| β-hANP | 2 g |

-continued

| Composition | |
|---|---|
| sorbitol | 20 g |

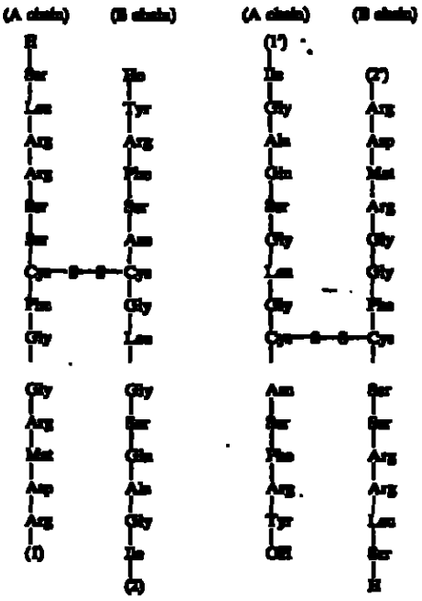
Method

β-hANP and sorbitol were dissolved in 200 ml of sterile water, a vial was filled with 1 ml of the solution, and lyophilized, and the vial was then sealed.

The composition is dissolved in 5 ml of sterile water before parenteral administration.

We claim:

1. A peptide β-hANP having the following formula:




wherein (1) and (1'), are directly bonded and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— ½ L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end, or an acid addition salt thereof.

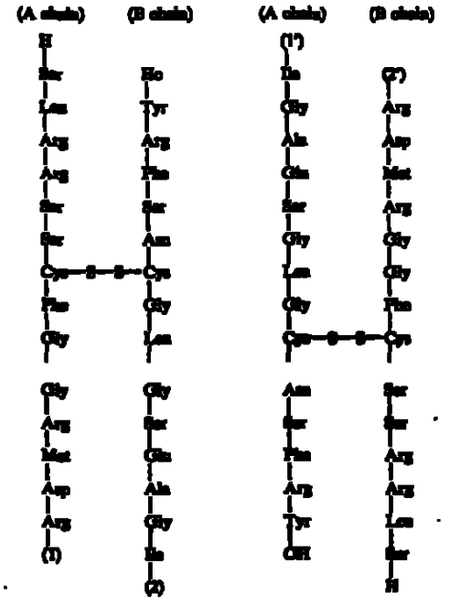
2. A pharmaceutical composition for use as a diuretic or antihypertensive agent comprising a diuretically effective amount or a blood pressure lowering effective amount of a peptide β-hANP according to claim 1 or an acid addition salt thereof with a conventional pharmaceutical additive.

3. The pharmaceutical composition according to claim 2, wherein the composition is a solution for parenteral administration and the conventional pharmaceutical additive is a buffer, an osmotic pressure adjusting agent or a preservative, or a combination thereof.

4. The pharmaceutical composition according to claim 2, wherein the composition is a solution for parenteral administration and contains about 0.000005 to 5% of the β-hANP.

5. The pharmaceutical composition according to claim 2, wherein the composition is in a lyophilized form.

6. A method for promoting diuresis comprising administering a composition comprising a diuretically effective amount of a peptide β-hANP having the following formula:




wherein (1) and (1') are directly bonded, and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— ½ L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end, or an addition salt thereof, with a conventional pharmaceutical additive.

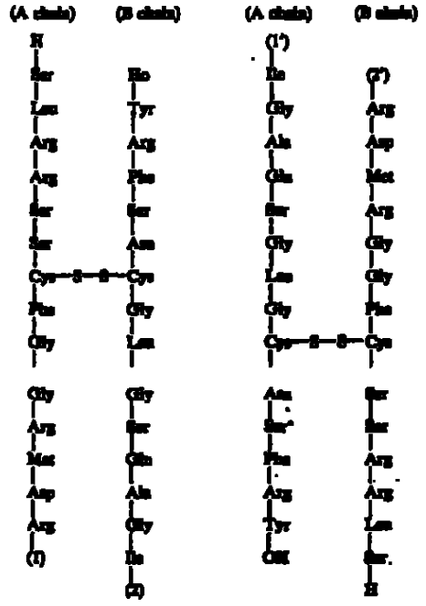
7. The method according to claim 6, wherein the composition comprises about 0.000005 to 5% of the β-hANP.

8. The method according to claim 7, wherein the composition is administered parenterally.

9. The method according to claim 6, wherein the conventional pharmaceutical additive is a buffer, an osmotic pressure adjusting agent, a preservative or a combination thereof.

10. The method according to claim 6, wherein the composition is in lyophilized form.

11. A method for lowering blood pressure comprising administering an antihypertensively effective amount of a peptide β-hANP having the following formula:




wherein (1) and (1') are directly bonded, and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— a L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end, or an addition salt thereof, with a conventional pharmaceutical additive.

12. The method according to claim 11, wherein the composition comprises about 0.000005 to 5% of the β-hANP.

13. The method according to claim 12, wherein the composition is administered parenterally.

14. The method according to claim 11, wherein the conventional pharmaceutical additive is a buffer, an osmotic pressure adjusting agent, a preservative or a combination thereof.

15. The method according to claim 11, wherein the composition is in lyophilized form.

16. A process for the production of a peptide β-hANP or an addition salt thereof according to claim 1 which comprises the following steps:

(a) boiling rat atrium in an acidic solution;

(b) homogenizing said rat atrium to obtain a homogenate;

(c) centrifuging the homogenate to obtain a supernatant containing the β-hANP; and

(d) separating said β-hANP from impurities using a purification process comprising organic solvent-precipitation, adsorption chromatography, ultrafiltration, gel filtration or high performance liquid chromatography to obtain said β-hANP in substantially purified form.

17. The process according to claim 16, further comprising the step (e) converting said β-hANP into an acid addition salt thereof or acid addition salt thereof or acid addition salt into free β-hANP.

* * * * *

US005646131A

# United States Patent [19]

Badwan et al.

[11] Patent Number: 5,646,131

[45] Date of Patent: Jul. 8, 1997

[54] **METHOD FOR SOLUBILIZING DRUGS USING CYCLODEXTRINS AND CARBOXYLIC ACIDS**

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[22] Filed: Jun. 2, 1995

Related U.S. Application Data

[63] Continuation of Ser. No. 199,523, Feb. 22, 1994, abandoned.

[51] Int. Cl.[6] ..... A61K 31/715; A61K 31/54; A61K 31/445; A61K 31/19

[52] U.S. Cl. ..... 514/58; 514/224.3; 514/317; 514/327; 514/568; 536/103

[58] Field of Search ..... 514/58, 224.2, 514/317, 327, 568; 536/103

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*Primary Examiner*—Gary L. Kunz
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*Attorney, Agent, or Firm*—Burns, Doane, Swecker & Mathis, L.L.P.

[57] ABSTRACT

A method for enhancing the solubilization and/or complexation of a drug which is insoluble or sparingly soluble in water with cyclodextrin, comprising combining the drug with cyclodextrin together with a selected hydroxycarboxylic or polycarboxylic acid.

43 Claims, 1 Drawing Sheet

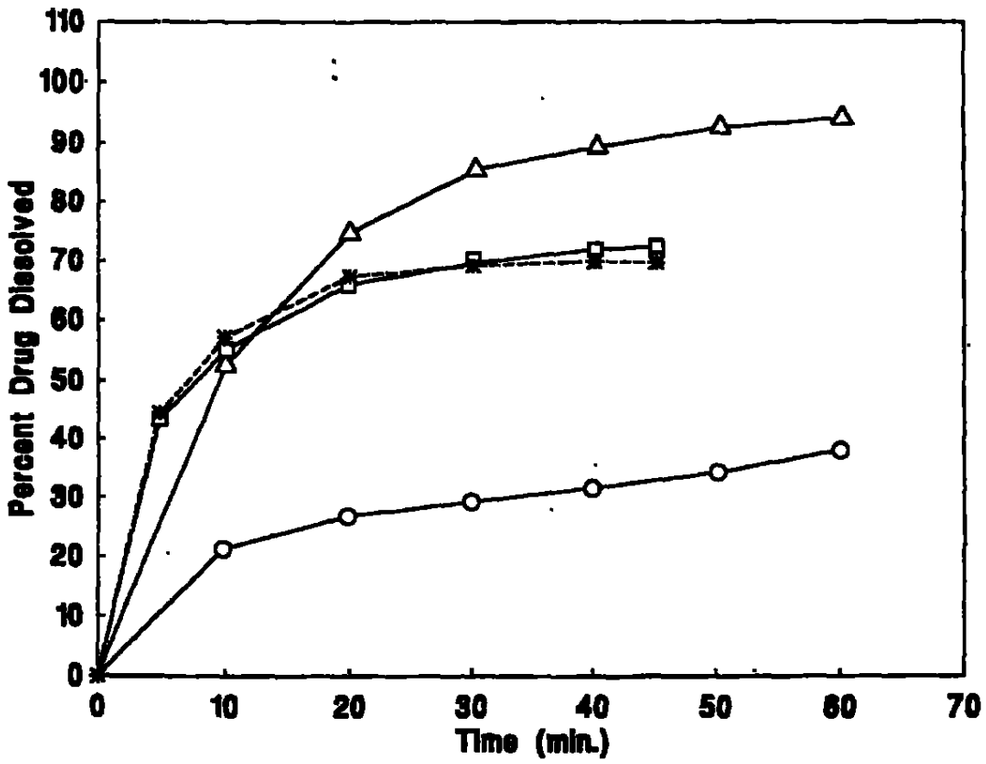

Percent Drug Dissolved
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time (min.)

Figure 1

# METHOD FOR SOLUBILIZING DRUGS USING CYCLODEXTRINS AND CARBOXYLIC ACIDS

This application is a continuation of application Ser. No. 08/199,523, filed Feb. 22, 1994, now abandoned.

## FIELD OF THE INVENTION

This invention relates to a method for increasing the aqueous solubility and/or complexation of drugs which are insoluble or sparingly soluble in water, using cyclodextrins together with selected hydroxy-carboxylic or polycarboxylic acids.

## BACKGROUND OF THE INVENTION

Cyclodextrins are cyclic multicyclopyranose units connected by α-(1,4) linkages. The most widely known cyclodextrins are α, β and γ-cyclodextrins. Derivatives of these cyclodextrins with improved properties are also known and used in the pharmaceutical field. The cyclic nature of the cyclodextrins, the hydrophobic properties of their cavities as well as the hydrophilic character of their outer surfaces, enables them to interact with other chemicals and to produce inclusion compounds which are characterized by improved solubilities and stabilities. Fields of potential applications of cyclodextrins include pharmaceuticals, fine chemicals, industrial chemicals and biological active substances.

Numerous reviews and patents related to the use of cyclodextrins and their derivatives to prepare inclusion complexes of drugs are found in the literature, for example, D. Duchêne, *Cyclodextrins and their Industrial uses*, Editions de Santé, Paris, 1987, Chapter 6 (211–257), Chapter 8 (297–350), Chapter 10 (393–439); D. Duchêne et al, *Acta Pharma Technol.* 36(1)6, 1–6, 1990; D. Duchêne et al, *Drug Dev. Ind. Pharm.*, 16(17), 2487–2499, 1990; C. Hunter et al, European Patent Publication No. EP 0346006, December 1988.

Inclusion complexes prepared to specifically improve water solubility and hence bioavailability of poorly soluble drugs have been reported by workers such as D. D. Chow et al, *Int. J. Pharm.*, 28, 95–101, 1986; R.A. Ménard et al, *Drug Dev. Ind. Pharm.*, 14(11), 1529–1547, 1988; F. J. Otero-Espinar et al, *Int. J. Pharm.*, 75, 37–44, 1991; and Bernard M. Mackiewicz et al, European Patent Publication No. EP 0274444, July 1988. Chemical modifications of cyclodextrins to prepare derivatives that further improve solubility of water insoluble drugs have been described, for example, by J. Pitha, U.S. Pat. No. 4,727,064, February 1988; N. S. Bodor, U.S. Pat. No. 5,024,998, July 1991.

In connection with the use of acids or buffers with cyclodextrins, the effects of the latter on dissociation constants of acidic organic compounds, including non-carboxylic acids, have been reported by K. Connors et al, *J. Pharm. Sci.*, 65 (3), 379–383, 1976, but these workers have not addressed the issue of solubility. As an extension to this work, equilibrium constants of some prostaglandins in the presence of α- and β-cyclodextrins have been determined in phosphate buffer solutions, as reported by K. Uekama et al, *Chem. Pharm. Bull.*, 26 (4), 1195–1200, 1978. Furthermore, β-cyclodextrin inclusion complexes of several non-steroidal anti-inflammatory drugs have been correlated for their dissolution behavior at different pH values by V. Zecchi et al, *Proc. Eur. Congr. Biopharm. Pharmacokinet.* 3rd, (1), 526–531, 1987; V. Zecchi et al, *Pharma Acta Helv.*, 63 (11), 299–302, 1988; Zecchi et al concluded that the dissolution rate of such complexes is scarcely altered by changing the pH. Also, the diffusion of free and β-cyclodextrin-complexed non-steroidal anti-inflammatory drugs have been determined at different pH values by I. Crisanti et al, *Arch. Pharm.*(Weinheim, Germany) 322(4), 207–211, 1989. A naproxen/β-cyclodextrin complex has been examined for solubility in buffered aqueous media by F. J. Otero-Espinar et al, Int. J. *Pharm.*, 79, 149–157, 1992; N. (Calchi et al, *Int. J. Pharm.*, 78, 183–187, 1992; N. Erden, *Int. J. Pharm.*, 48, 83–89, 1988. Dissolution of famotidine, and the β-cyclodextrin inclusion compound of the drug have been reported at pH 7.4 by M. Hassan et al, *Int. J. Pharm.*, 58, 19–24, 1990. The effects of pH on the complexation of hydrochlorothiazide, ibuprofen and diazepam with β-cyclodextrin cyclodextrin have been discussed by F. Ménard et al, *Drug Dev. Ind. Pharm.*, 16(1), 91–113, 1990; these workers have found in their experiments that the relationship between solubility and concentration is the same at different pH levels. The dissolution of tolbutamide/β-cyclodextrin complex has been compared to that of the drug itself and solid dispersions at pH 2 by F. Kedzierowicz et al, *Int. J. Pharm.*, 58, 221–227, 1990.

Pharmaceutical formulations containing cyclodextrins typically contain other ingredients commonly used in pharmaceuticals, for example, pH adjusters (acids, bases, buffers), effervescing agents and the like, to create forms suitable for administration. For example, International Publication No. WO85/02767, July 1985, has disclosed preparing pharmaceutical compositions by dissolving a selected cyclodextrin derivative in water and adding the desired drug to form an inclusion compound, wherein the water "may further comprise physiologically compatible compounds such as sodium chloride, potassium nitrate, glycose, mannitole, sorbitol, xylitol or buffers such as phosphate, acetate or citrate buffer." Hirai et al, U.S. Pat. No. 4,659,696, April 1987, have described various non-oral, non-injectable pharmaceutical compositions containing a drug which is poorly absorbable through the gastrointestinal tract and cyclodextrin, which may contain a variety of excipients and pH adjusters, such as an acid, a base or a buffer solution. As examples of the acid, there are mentioned "inorganic acids (e.g., hydrochloric acid, boric acid, phosphoric acid, carbonic acid, bicarbonic acid, etc.), amino acids and organic acids (e.g., monocarboxylic acids, oxycarboxylic acids, polycarboxylic acids)." Also, Chiral et al, in U.S. Pat. No. 4,603,123, July 1986, in describing pharmaceutical compositions containing piroxicam/cyclodextrin complexes, show an effervescent tablet formulation comprising citric acid and glycine sodium carbonate in equal amounts, but these are indeed typical effervescing agents and, moreover, are added after the drug/cyclodextrin complex has already been formed. Similarly, in C. Hunter, European Patent Publication No. 0346006 relating to pharmaceutical compositions comprising ibuprofen-cyclodextrin complexes, there are examples of non-effervescent, effervescent and slightly effervescent formulations comprising trisodium citrate, citric acid and sodium bicarbonate, but these ingredients are added to the already formed ibuprofen complexes. None of these earlier workers has attached any significance to particular acids, or has recognized that selected acids could be used to improve the solubilization and/or complexation with cyclodextrins of drugs which are insoluble or sparingly soluble in water. And, of course, no such improvement would be present, for example, in the case of Chiral et al's effervescent tablets, since the citric acid component is only present in sufficient amount to effervesce together with the glycine sodium carbonate; there is no additional citric acid present to serve as a solubilizer, as provided by the present invention as described in detail hereinbelow.

Despite the advances in the pharmaceutical arts made possible by the use of cyclodextrins with drugs which are insoluble or only sparingly soluble in water, there remains a need for further improvements in drug/cyclodextrin formulations. β-Cyclodextrin, although considerably less expensive than its derivatives, increases drug solubility much more modestly than the derivatives and, in some instances, does not confer sufficient solubility at a low enough concentration to provide a feasible product, for example, a small enough tablet to be readily swallowed. Thus, there is a real need in this art for a means of significantly increasing the solubility of drugs in the less efficient cyclodextrins. On the other hand, the derivatized cyclodextrins tend to be very expensive; thus, there is also a real need for enhancing the solubility of drugs with such expensive cyclodextrins so that considerably smaller amounts of the cyclodextrins are required to achieve an effect comparable to that which could previously be obtained.

## SUMMARY AND OBJECTS OF THE INVENTION

One object of the present invention is to provide a method for enhancing the solubilizing effects of cyclodextrins on drugs which are insoluble or sparingly soluble in water.

Another object of the present invention is to provide a method for enhancing the complexation of cyclodextrins with drugs which are insoluble or sparingly soluble in water.

Yet another object of the present invention is to provide a means for alleviating drug absorption problems related to the poor solubility of active agents.

Another object of the invention is to provide solid pharmaceutical formulations which are characterized by rapid dissolution and better absorption.

Another object of the invention is to provide non-alcoholic syrups and fast-dissolving tablets, capsules, effervescent tablets and/or sachets.

Still a further object of the invention is to provide effervescent tablets and/or sachets which readily dissolve in situ to solutions, thus providing a means for alleviating swallowing problems, especially in geriatric and pediatric patients.

In accord with these and other objects, the present invention provides a method for enhancing the solubilization and/or complexation of a drug which is insoluble or sparingly soluble in water with cyclodextrin, said method comprising combining said drug with cyclodextrin and an effective solubility-enhancing amount of a saturated or unsaturated $C_2$–$C_6$ carboxylic acid having from one to three —COOH groups and optionally bearing up to four —OH substituents (i.e., bearing from zero to four —OH substituents), provided that when the acid has only one —COOH group, then said acid must bear at least one —OH substituent, or a pharmaceutically acceptable salt of said acid, the weight ratio of cyclodextrin to carboxylic acid being from about 1:100 to about 100: 1.

## BRIEF DESCRIPTION OF THE DRAWING

Other objects and advantages of the present invention will be apparent from the following detailed description and accompanying drawing, in which the FIGURE is a series of regular cartesian plots depicting the dissolution profile of terfenadine from capsules containing the following: Δ60 mg terfenadine, 11 mg β-cyclodextrin and 30 mg citric acid; ○60 mg terfenadine, 0% β-cyclodextrin, 0% citric acid; * 60 mg terfenadine, 11 mg β-cyclodextrin, 0% citric acid; □60 mg terfenadine, 30 mg β-cyclodextrin plus 0% citric acid.

## DETAILED DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS

Here and throughout this description, the following definitions are applicable:

Cyclodextrins for use in the present invention include the natural cyclodextrins and their pharmaceutically acceptable derivatives. Cyclodextrins and their derivatives which have been previously described as useful for complexation with/solubilization of drugs are of particular interest herein. In addition to α-, β- and γ-cyclodextrin, derivatives of these cyclodextrins such as hydroxyalkyl (e.g., hydroxypropyl), carboxamide, diethylaminoacetyl, carboxymethyl and dihydroxyalkyl (e.g., dihydroxypropyl) are of special interest, and these can be prepared by well-known procedures. For a discussion of such suitable cyclodextrin derivatives, see, for example, D. Duchêne et al, *Drug Dev. Ind. Pharm.*, 16(17), 2487–2499, 1990. Presently preferred cyclodextrins for use herein are the natural cyclodextrins, α-, β- and γ-, especially/β-cyclodextrin.

Suitable $C_2$–$C_6$ carboxylic acids for use herein can be straight- or branched-chain alkyl or alkenyl, or can even have a cyclic lactone structure. These acids are polyfunctional, i.e., multifunctional, that is, they have at least two carboxyl functions or at least one carboxyl function and at least one hydroxyl function. Such acids are thus also referred to herein as hydroxy-carboxylic acids or polycarboxylic acids, or simply as polyfunctional carboxylic acids. Appropriate acids include citric acid, tartaric acid, gluteric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid and succinic acid. Presently preferred carboxylic acids for use herein include citric acid, gluteric acid, lactic acid and tartaric acid. An especially preferred acid is citric acid. The acids can be optionally used in whole or in part in the form of their pharmaceutically acceptable salts, particularly their alkali metal salts, such as, for example, trisodium citrate.

Among the drugs which are insoluble or sparingly soluble in water and which are contemplated for use in the present invention, any of those which have been previously complexed with/solubilized by cyclodextrin are of particular interest. Of special interest herein are: non-steroidal anti-inflammatory agents, such as ibuprofen, ketoprofen, piroxicam, tenoxicam, ketorolac and naproxen; antihistamines or antiallergics, for example, terfenadine and cinnarizine; vasodilators, oxytocic agents and abortifacients, such as prostaglandin $E_1$, prostaglandin $E_2$ and prostaglandin $F_{2\alpha}$ and sedatives, tranquilizers, hypnotics, anticonvulsants, anxiolytics, muscle relaxants and antispasmodics, for example, phenobarbital, pentobarbital, secobarbital, butabarbital, butalbital, alpidride, phenytoin, diazepam, oxazepam, cloxazolam, lorazepam, clobazam, nitrazepam, fludiazepam, oxazepam, medazepam, dimethyl diazepam, flunitrazepam, nimetazepam, pentazocine, methaqualone, salbutamol, carbamazepine, baclofen, chlorpromazine and chlordiazepoxide. Especially advantageous for enhancement of solubilization and/or complexation in accord with the present invention are basic drugs which are insoluble or sparingly soluble in water.

It is now well-known that drugs which are insoluble or sparingly soluble in water and which have the required shape and size to fit at least partially into the cavity of the hydrated cyclodextrin molecule will complex with cyclodextrin and that the drug/cyclodextrin inclusion complex which results has improved water solubility as compared to

the drug itself; see, for example, International Publication No. WO85/02767, July 1985. Drugs whose water solubility can be improved by complexation with cyclodextrins exhibit significantly increased complexation and water solubility when treated in accord with the present invention. The polyfunctional carboxylic acids utilized herein make it possible to reduce the amount of cyclodextrin present in the pharmaceutical formulation, which can in turn result in substantial cost savings. Reducing the amount of cyclodextrin can also provide solid dosage forms of more reasonable size for swallowing than heretofore possible, and can result in lower toxicity. Alternatively, rather than reducing the amount of cyclodextrin present, one can instead prepare a formulation which has substantially enhanced solubility and absorption. Obviously, formulations can be made which would share these benefits, i.e., that contain somewhat less cyclodextrin while still exhibiting improved drug solubility and absorption. Preferred pharmaceutical formulations provided by the present invention include non-alcoholic syrups and rapidly dissolving tablets, capsules, effervescent tablets and/or sachets. The latter two solid preparations can be converted in situ to solutions, thus providing a means for alleviating swallowing problems in geriatric and pediatric patients.

In accord with the present invention, it has now been discovered that use of a hydroxy-carboxylic or polycarboxylic acid and/or a salt thereof as defined hereinabove together with α, β or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof results in a dramatic increase in the solubility of drugs which are insoluble or sparingly soluble in water, enhancing the solubilizing and/or complexing ability of the cyclodextrins for the drug through the presence of the selected polyfunctional acid component. Advantageously, the acid, cyclodextrin and drug can be combined with each other in the process of preparing the pharmaceutical composition/formulation, so that the complexing/solubilizing takes place in the course of preparing the final dosage form and need not be carried out separately beforehand. Indeed, in the case of effervescent tablets, sachets and the like, the actual complexation/solubilization will typically occur when the tablet or similar solid dosage form is dissolved in water shortly before administration to the patient.

As noted hereinabove, in preparing the formulations of the invention, the weight ratio of cyclodextrin to selected carboxylic acid and/or salt of said acid is from about 1:100 to about 100: 1. More preferably, the weight ratio of cyclodextrin to acid ingredient is from about 1:50 to 50: 1,most preferably from about 1:5 to about 5:1.

Typical compositions prepared in accord with the present invention comprise the selected cyclodextrin at a weight percent of from about 0.05 to about 8% of the total weight and the selected hydroxy-carboxylic or polycarboxylic acid and/or salt thereof at a concentration ranging from about 0.1 to about 5% total weight. However, in selected solid dosage forms, considerably greater amounts of cyclodextrin and polyfunctional acid are typically used, for example from about 15 to about 30% by weight of cyclodextrin and from about 5 to 10% by weight of the acid as solubilization enhancer. Greater amounts of the acid/salt can be present in such formulations if that ingredient is serving another purpose in the composition, for example, as an effervescing agent.

In preferred embodiments, the present invention provides non-alcoholic aqueous syrups of water-insoluble or sparingly insoluble drugs, which syrups are advantageous over suspensions and/or hydroalcoholic solutions of such drugs; and rapidly dissolving solid dosage forms which can be quickly and conveniently converted in situ to aqueous solutions which can be readily administered to patients who have difficulty in swallowing, such as pediatric and geriatric patients. The pharmaceutical preparations prepared in accord with this invention are stable and non-irritating, have very low systemic or local toxicity and are generally well-suited for oral and parenteral administration, depending of course upon the properties of the selected ingredients, particularly the cyclodextrin and the drug. Formulations for oral use can be enhanced by the addition of a taste-masking agent, flavoring agent and/or sweetener. Other ingredients commonly used in oral dosage forms can also be present. In the case of parenteral formulations, the ingredients will be in an aqueous solution suitable for injection, i.e., sterile and pyrogen free and prepared in accord with accepted pharmaceutical procedure. Again, other ingredients typically used in such formulations can be present.

While not wishing to be bound by any particular theory of operation, applicants believe that the acids which are selected for use herein form aggregates by virtue of the fact that they possess both polar and non-polar groups, that these aggregates combine with the selected cyclodextrin to enhance solubilization/complexation of the drug, and that the selected acid needs to be present in sufficient quantity to form aggregates in order for enhancement of solubility to occur. Thus it appears that the acid participates directly in the drug complex formation, acting as a co-complexing agent with the cyclodextrin and resulting in a new three-way co-complex of drug, cyclodextrin and acid.

Pharmaceutical compositions prepared in accord with the present invention can be used to treat a variety of conditions, depending upon the pharmacological nature of the drug selected for administration. The compositions comprise a pharmacologically/therapeutically effective amount of the selected drug and the amount/ratio of selected cyclodextrin and selected polyfunctional acid or salt thereof described hereinabove. For example, if the selected drug is an antihistaminic or antiallergic, for example, terfenadine, a pharmacologically effective amount thereof will be an amount sufficient to provide an antihistaminic or antiallergic response (e.g., in the treatment of symptoms associated with seasonal allergic rhinitis, such as sneezing, lacrimation, pruritus and rhinorrhea). As a further example, when the selected drug is an anti-inflammatory agent, for example ketoprofen, a pharmacologically effective amount thereof will be an amount sufficient to elicit an anti-inflammatory response (such as in the treatment of rheumatoid arthritis or osteoarthritis), or an amount sufficient to elicit an analgesic effect (since ketoprofen and other non-steroidal anti-inflammatory agents are frequently used for their analgesic as well as their anti-inflammatory properties). In short, the instant compositions are typically used for those purposes for which the drug component of the composition is itself known to be useful.

Generally speaking, the therapeutic dosage ranges for administration of drugs in the pharmaceutical compositions/formulations described herein will be the same as or less than those characteristically used for administration of the drug per se. Naturally, such therapeutic dosage ranges will vary with the size and species of the patient, the condition for which the formulation is administered, the route of administration employed and the like. The quantity of given dosage form needed to deliver the desired dose of active ingredients will of course depend upon the concentration of the drug in the pharmaceutical formulation.

In order to further illustrate the present invention and the advantages thereof, the following specific examples are

given, it being understood that some are intended only as illustrative and in no way limitative of the invention.

The terfenadine stability testing referred to in Examples 2 and 4 hereinbelow was based on the assay method described in a terfenadine monograph by A. Badwan et al appearing in *Analytical Profiles of Drug Substances*, volume 19, ed. K. Florey, Academic Press, Inc., San Diego, Calif., 1990, pp. 627–662. The actual assay method was as follows:

A quantity of the mixed contents of 20 capsules containing the equivalent of 60 mg terfenadine was transferred to a 100 ml volumetric flask. 70 ml of methanol were added and the mixture was shaken for 20 minutes, then methanol was added to volume, and the contents of the flask were mixed and centrifuged. The first derivative ultraviolet absorption spectra of the solutions (1) and (2) using methanol as the blank and a path length of 1 cm in the range of 300–200 nm at a speed of 120 nm/min and $\lambda$=2.0 were recorded. Solution (1) was the sample solution prepared. Solution (2) was a standard solution of terfenadine in the same medium having a known concentration of about 600 μg/ml. The amplitude obtained at about 270 nm was measured for sample and standard solutions. The quantity, in mg of ($C_{32}H_{41}NO_2$), in the portion of capsules content was calculated according to the formula:

$$\frac{D_s}{D_w} \times 100\,C$$

wherein C is the concentration, in μg per ml, of terfenadine in the standard solution, and $D_s$ and $D_w$ are the first derivative amplitude of the solution of sample and standard, respectively.

## EXAMPLE 1

Working models showing the effectiveness of the invention in increasing the solubility of representative water-insoluble or sparingly soluble drugs are shown in TABLE I below. The solubilities (in mg/ml) of the drugs were investigated at 30° C., in water; in water containing 2% β-cyclodextrin; and in aqueous solution of 2% selected cyclodextrin and 1% selected polyfunctional acid or corresponding salt.

## EXAMPLE 2

### Preparation of Syrups

Syrups prepared in accord with the present invention typically contain water-insoluble or sparingly soluble drugs in an amount of from about 6 to about 600 mg/ml of syrup. The syrups generally contain the solubilizing agent, i.e., α,β, γ cyclodextrin or derivative thereof, in an amount of from about 0.1 to about 5% of the syrup and the solubilization augmenting agent, i.e., the hydroxy-carboxylic acid or polycarboxylic acid, in an amount of from about 0.1 to about 5% of the syrup. The syrups are preferably buffered to control the pH value to about pH 3 to 7, more preferably pH 3.5 to 5. The preferred buffering system is a combination of the selected acid and its corresponding salt in an amount of from about 0.1 to about 5% for the acid and a similar range for the corresponding salt. The syrups typically contain sufficient viscosity imparting agents (such as polyvinylpyrrolidone) in an amount of from about 1 to about 3% of the syrup and medium-building agents such as glycerine, propylene glycol or sorbitol in an amount of from about 10 to about 35% of the syrup. These additives are included to render the medium more viscous, thus preventing the solution from spilling over when administered by a spoon or the like. To mask the undesirable taste of the drug, the syrups generally contain sufficient sweetening agents such as sucrose in an amount of from about 20 to about 30% of the syrup. Other sweetening agents such as saccharin sodium could be used in an amount of from about 0.1 to about 0.2% of the syrup.

Additional ingredients such as coloring or flavoring agents can be incorporated to enhance the quality of the syrup, if desired. Water is present as the main vehicular agent in the syrups and is added to the desired volume.

In any event the weight ratio of cyclodextrin to acid in syrup formulations is as broadly described hereinabove, but preferably is from about 1:5 to 5:1, and more preferably from about 3:1 to about 1:1.

TABLE I

Solubilities (mg/ml) of Drugs in Water, in Water Containing 2% β-Cyclodextrin (CD), and in Aqueous Solution of 2% Cyclodextrin, 1% Hydroxy-Carboxylic or Polycarboxylic Acid or Corresponding Salt, at 30° C.

| | | 2% aq. BETA CD soln. | BETA-CYCLODEXTRIN + Citric acid | + Gluconic acid | + Lactic acid | + Tartaric acid | ALPHA-CYCLODEXTRIN + Citric acid | + Gluconic acid | + Lactic acid | + Tartaric acid | GAMMA-CYCLODEXTRIN + Citric acid | + Gluconic acid | + Lactic acid | + Tartaric acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DRUG | Water | | | | | | | | | | | | | |
| Terfenadine | <0.01 | 0.01 | 5.68 | 5.49 | 8.47 | 5.21 | 2.15 | 7.17 | 6.27 | 1.91 | 2.20 | 6.08 | 7.34 | 1.22 |
| Ibuprofen | <0.01 | 0.69 | 2.95* | 3.07* | 1.65* | 1.36* | | | | | | | | |
| Cisapride | 0.005 | 0.015 | 0.55 | 2.07 | 11.4 | 4.38 | | | | | | | | |
| Ketoprofen | 0.005 | 1.64 | 7.06* | | | | | | | | | | | |
| Piroxicam | Insoluble | 0.072 | 0.78* | | | | | | | | | | | |

(*) Solubility in aqueous solution containing 2% β-cyclodextrin and 1% disodium salt.

| Part A: Terfenadine Syrup (6 mg/ml) | |
|---|---|
| Ingredient | Concentration (mg/ml) |
| Terfenadine | 6 |
| Propylene glycol | 250 |
| Glycerin | 100 |
| Sucrose | 500 |
| Citric acid, 1 $H_2O$ (monohydrate) | 10 |
| Trisodium citrate, 2 $H_2O$ (dihydrate) | 10 |
| β-Cyclodextrin | 50 |
| Sodium saccharin | 1 |
| Polyvinylpyrrolidone (K30) | 20 |
| Water | q.s. |

Method of Preparation for 100 ml of Syrup

1. Dissolve citric acid and trisodium citrate in 10 ml of deionized water.
2. Dissolve sucrose and β-cyclodextrin in 10 ml of deionized water.
3. Dissolve polyvinylpyrrolidone and sodium saccharin in 10 ml of deionized water.
4. Mix terfenadine, propylene glycol and glycerin, then, while stirring and heating, add the solutions prepared in steps 1–3. Continue mixing until the drug is dissolved.
5. Add water to the desired volume, then mix for 20 minutes.

A syrup made as described above was tested for stability at room temperature (RT) and at the accelerated temperatures of 55° and 65° C., with the following results.

| | 65° C. | | 55° C. | | RT | |
|---|---|---|---|---|---|---|
| Syrup Time (days) | Assay (%) | pH | Assay (%) | pH | Assay (%) | pH |
| 0 | 99.1 | 4.5 | 99.1 | 4.5 | 99.1 | 4.6 |
| 11 | 99.4 | 4.6 | 99.4 | 4.5 | — | — |
| 27 | 100.0 | 4.5 | 100.9 | 4.5 | — | — |
| 59 | 101.0 | — | — | — | — | — |
| 88 | — | — | — | — | 101.0 | 4.6 |
| 360 | — | — | — | — | 100.0 | 4.6 |

Other examples of syrups made in accord with the present invention are demonstrated by the ketoprofen syrup and cisapride syrup, as described below.

| Ingredient | Concentration (mg/ml) |
|---|---|
| Part B: Ketoprofen Syrup (10 mg/ml) | |
| Ketoprofen | 10 |
| Propylene glycol | 250 |
| Glycerin | 100 |
| Sucrose | 500 |
| Trisodium citrate | 15 |
| β-Cyclodextrin | 30 |
| Polyvinylpyrrolidone (K30) | 20 |
| Water | q.s. |
| Part C: Cisapride Syrup (1 mg/ml) | |
| Cisapride | 1 |
| Propylene glycol | 300 |
| Tartaric acid | 10 |
| β-Cyclodextrin | 20 |
| Sorbitol 70% | 300 |
| Sodium saccharin | 1 |
| Polyvinylpyrrolidone (K30) | 2 |
| Water | q.s. |

Syrups made in accord with the present invention have an advantage over other possible liquid formulations in being devoid of alcohol, an essential ingredient used to increase solubility of water-insoluble drugs. They offer a wider range of concentrations of the water-insoluble drugs compared to other liquid formulations. Consequently, they provide a means for better control of dose administration and greater absorption of said drugs.

## EXAMPLE 3

### Preparation of Effervescent Tablets

Effervescent tablets prepared in accord with the present invention typically contain water-insoluble or sparingly soluble drugs in an amount of from about 10 to about 800 mg per tablet. The tablets generally contain the solubilizing agent α, β, γ-cyclodextrin or derivative thereof in an amount of from about 15 to about 30% of the tablet and the solubilization augmenting agent, preferably citric or tartaric acid, in an amount of from about 5 to about 10% of the tablet. An additional amount of citric acid, from about 15 to about 35% of the tablet, is typically added to the formula to produce the desired effervescent action. The tablets generally contain sodium bicarbonate in an amount of from about 5 to about 30% of the tablet to react with part of the acid and produce the effervescent action. Other additives can be added to the formula to give body to the tablet and help bind the ingredients; e.g., soluble starch or Aerosil™ in an amount of from about 25 to about 125 mg of the tablet. Other additives such as ethyl alcohol or 5% Tween™80 solutions can be added, if desired, in sufficient quantities to granulate the powders. The formula can also contain other ingredients such as a sweetener or a taste-masking agent to improve taste and a soluble lubricating agent such as sodium benzoate to facilitate tablet compression. In any case, the weight ratio of cyclodextrin to acid in effervescent tablets is as broadly described hereinabove, but preferably is from about 1:5 to 5:1, and more preferably from about 1:4 to about 1:1. An illustrative formulation is shown below.

| Part A: Terfenadine Effervescent Tablets (60 ea) | |
|---|---|
| Ingredient | Amount (g) |
| Terfenadine | 0.06 |
| Sodium bicarbonate | 0.75 |
| β-Cyclodextrin | 1.25 |
| Saccharin sodium | 0.05 |
| Sucrose | 1.00 |
| Citric acid, anhydrous | 2.00 |
| Sodium benzoate | 0.15 |
| Flavoring agent | q.s. |

Method of Preparation of Effervescent Tablets

1. Mix β-cyclodextrin with sodium bicarbonate.
2. Mix powdered sugar, saccharin sodium and mixture from Step 1 with terfenadine in a suitable mixer.
3. Granulate resultant mix with ethyl alcohol.
4. Sieve and dry resulting granules.
5. Mix granules with citric acid, sodium benzoate and flavoring agent.
6. Compress into tablets.

Effervescent tablets made as described above, when tested in 100 ml of water, achieved complete dissolution, with effervescence, in 2–4 minutes. Terfenadine assayed at 95–97% of the nominal amount.

Another example of effervescent tablets prepared in accord with the present invention is demonstrated in the following example.

Part B: Cisapride Effervescent Tablets (10 mg)

| Ingredient | Amount (mg) |
|---|---|
| Cisapride (anhydrous) | 10 |
| Citric acid, anhydrous | 65 |
| Tartaric acid | 250 |
| Sodium bicarbonate | 200 |
| Aerosil™ (silica aerogel) | 25 |
| Sodium benzoate | 20 |
| Soluble starch | 25 |
| β-Cyclodextrin | 50 |
| Flavoring agent | q.s. |

Method of Preparation of Cisapride Effervescent Tablets

1. Mix β-cyclodextrin with sodium bicarbonate, soluble starch and cisapride.
2. Granulate the mixture with ethyl alcohol.
3. Sieve and dry the resulting granules.
4. Mix granules with citric acid, tartaric acid, sodium benzoate, the flavoring agent and Aerosil™.
5. Compress into tablets.

The effervescent tablets, when tested in 100 ml of water, dissolved with effervescence in 2–3 minutes, forming a solution containing 10 mg of the drug and of pH=4.

Effervescent tablets made in accord with the present invention provide a means for preparing solutions of water-insoluble drugs in situ. Such solutions are readily absorbed and are well-suited for geriatric and pediatric patients.

## EXAMPLE 4

### Preparation of Fast-Dissolving Capsules

Rapidly dissolving capsules prepared in accordance with the present invention typically contain water-insoluble or sparingly soluble drugs in an amount of from about 6 to about 600 mg of the capsule. The capsules typically contain the solubilizing agent α, β or γ-cyclodextrin or derivative thereof in an amount of from about 0.1 to about 5% of the capsule and the solubilization augmenting hydroxy-carboxylic or polycarboxylic acid in an amount of from about 0.1 to about 5% of the capsule. The formula generally contains additives to give body to the granules, e.g., calcium hydrogen phosphate, and Avicel™ in amounts of from about 25 to about 30% and about 5 to about 10% of the capsule, respectively. Binding agents and/or wetting agents such as PEG 4000, Tween™80, sodium lauryl sulfate and PVP to granulate the powder mix are typically added to the formula in an amount of from about 0.1 to about 5% of the capsule. The formula typically contains disintegrants and swelling agents such as Primojel™, maize starch, and Aerosil™ in an amount of from about 1 to about 10%. The formula can contain other ingredients, such as a sweetener. In any event, the weight ratio of cyclodextrin to acid in rapidly dissolving capsule formulations will be as broadly described hereinabove, but preferably will be from about 1:5 to 5:1, and more preferably from about 3:1 to about 1:1. The following illustrates such a formulation.

Terfenadine Capsule (60 mg)

| Ingredient | Amount (mg) |
|---|---|
| Terfenadine | 60 |
| Polyethylene glycol 4000 | 24 |
| Tween™ 80 | 3 |

-continued

Terfenadine Capsule (60 mg)

| Ingredient | Amount (mg) |
|---|---|
| Calcium hydrogen phosphate | 28 |
| Maize starch | 4 |
| Sodium lauryl sulfate | 1 |
| Avicel™ pH 101 (microcrystalline cellulose) | 72 |
| β-cyclodextrin | 11 |
| Citric acid, anhydrous | 30 |
| Polyvinylpyrrolidone (K30) | 12 |
| Primojel™ | 10 |
| Aerosil™ (silica aerogel) | 8 |

Method of Preparation of Fast-Dissolving Terfenadine Capsules

1. Mix calcium hydrogen phosphate, maize starch, sodium lauryl sulfate and Avicel™ with hot alcoholic solution of terfenadine, PEG and Tween™80. Maintain mixing until powder is dry. Pass through 0.8 mm sieve.
2. Granulate with 20 ml aqueous solution containing PVP and citric acid. Pass granules through 0.8 mm sieves, then dry at 40° C.
3. Add Primojel™, Aerosil™ and cyclodextrin to granules and mix for 10 minutes.
4. Fill capsules with granules.

Capsules made as described above were tested for dissolution and chemical stability with the following results.

| Storage Time (weeks) | 65° C. Assay (%) | 44° C. Assay (%) | RT · Assay (%) |
|---|---|---|---|
| 0 | 99.6 | 99.6 | 99.6 |
| 1 | 98.8 | — | — |
| 2 | 98.5 | — | — |
| 3 | 98.6 | — | — |
| 4 | 97.9 | 100.2 | — |
| 6 | 98.2 | — | — |
| 12 | — | 99.3 | 99.2 |
| 24 | — | 99.0 | 99.4 |

A comparison of the dissolution profile of terfenadine from capsules prepared as in the forgoing example (Δ) and from identical controls devoid of cyclodextrin and citric acid (○), or devoid of citric acid and containing the same (•) or greater (□) amounts of cyclodextrin, is shown in the FIGURE. As is readily apparent from the FIGURE, the control containing 11 mg of β-cyclodextrin (•) had greatly improved dissolution over the control containing no cyclodextrin (○). However, increasing the amount of cyclodextrin in the capsule from 11 mg to 30 mg of β-cyclodextrin (□) did not further improve dissolution to a significant extent. In contrast, the capsule prepared in accord with the present invention containing 11 mg of β-cyclodextrin and 30 mg of citric acid (Δ) substantially improved dissolution of terfenadine over all of the control formulations.

Capsules made in accordance with the invention ensure complete dissolution of the required dose of the water-insoluble drug within an acceptable time span. Therefore, such capsules provide a means to ensure better absorption of the drugs and hence, better bioavailability than comparable dosage forms.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions and changes may be made without departing from the spirit thereof. Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims, including equivalents thereof.

What is claimed is:

1. A method for enhancing the solubilization of a drug which is insoluble or sparingly soluble in water with cyclodextrin, said method comprising combining in water said drug with cyclodextrin and an effective solubility-enhancing amount of saturated or unsaturated $C_2$–$C_6$ carboxylic acid having from one to three —COOH groups and bearing from zero to four —OH substituents, provided that when the acid has only one —COOH group, then said acid must bear at least one —OH substituent, or a pharmaceutically acceptable salt of said acid, the weight ratio of cyclodextrin to carboxylic acid being from about 1:50 to about 50:1.

2. The method according to claim 1, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

3. The method according to claim 2, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ,-cyclodextrin.

4. The method according to claim 2, wherein the cyclodextrin comprises β-cyclodextrin.

5. The method according to claim 1, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, gluteric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

6. The method according to claim 5, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

7. The method according to claim 1, wherein the drug is a non-steroidal anti-inflammatory agent.

8. The method according to claim 7, wherein the drug is ibuprofen, ketoprofen, piroxicam, tenoxicam, ketorolac or naproxen.

9. The method according to claim 1, wherein the drug is an antihistaminic or antiallergic.

10. The method according to claim 9, wherein the drug is terfenadine or cianocirizina.

11. The method according to claim 1, wherein the drug is a vasodilator, oxytocic agent or abortifacient.

12. The method according to claim 11, wherein the drug is prostaglandin $E_1$, prostaglandin $E_2$ or prostaglandin $F_2$.

13. The method according to claim 1, wherein the drug is a sedative, tranquilizer, hypnotic, anticonvulsant, anxiolytic, muscle relaxant or antispasmodic.

14. The method according to claim 13, wherein the drug is phenobarbital, pentobarbital, secobarbital, butobarbital, butalbital, sulpiride, phenytoin, diazepam, oxazepam, etomidate, bromazepam, clobazam, nitrazepam, flodiazepam, lorazepam, medazepam, dimethyl diazepam, flunitrazepam, nimetazepam, pentazocine, methaqualone, salbutamol, carbamazepine, baclofen, chlorpromazine or chlordiazepoxide.

15. The method according to claim 1, wherein the drug is terfenadine, ketoprofen, cisapride or ibuprofen, the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

16. The method of claim 1 wherein the drug is terfenadine.

17. The method according to claim 16, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

18. The method according to claim 17, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

19. The method according to claim 17, wherein the cyclodextrin comprises β-cyclodextrin.

20. The method according to claim 16, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, gluteric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

21. The method according to claim 20, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

22. The method according to claim 16, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

23. The method of claim 1 wherein the drug is ibuprofen.

24. The method according to claim 23, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

25. The method according to claim 24, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

26. The method according to claim 24, wherein the cyclodextrin comprises β-cyclodextrin.

27. The method according to claim 23, wherein the $C_2$–$C_6$ carboxylic acid, comprises citric acid, tartaric acid, gluteric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

28. The method according to claim 27, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

29. The method according to claim 23, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

30. The method of claim 1 wherein the drug is cisapride.

31. The method according to claim 30, wherein the cyclodextrin comprises α- β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

32. The method according to claim 31, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-or β- or γ-cyclodextrin.

33. The method according to claim 31, wherein the cyclodextrin comprises β-cyclodextrin.

34. The method according to claim 30, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, gluteric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

35. The method according to claim 34, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

36. The method according to claim 30, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

37. The method of claim 1 wherein the drug is piroxicam.

38. The method according to claim 37, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

39. The method according to claim 38, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoacetoxyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

40. The method according to claim 38, wherein the cyclodextrin comprises β-cyclodextrin.

41. The method according to claim 37, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

42. The method according to claim 41, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

43. The method according to claim 37, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is trisodium citrate.

* * * * *

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2 4/6

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Mozes (43) Pub. Date: Jul. 1, 2004

(54) SYNTHETIC HUMAN PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEM FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

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(57) ABSTRACT

Synthetic peptides of at least 12 and at most 30 amino acid residues comprising a sequence consisting of, or found within, a complementarity-determining region (CDR) found in the heavy or light chain of the human anti-DNA 16/6Id monoclonal antibody, or a sequence obtained by replacement and/or deletion and/or addition of one or more amino residues to said sequence, and salts, chemical derivatives and polymers of said peptides can be used for immunomodulation of systemic lupus erythematosus-associated responses.

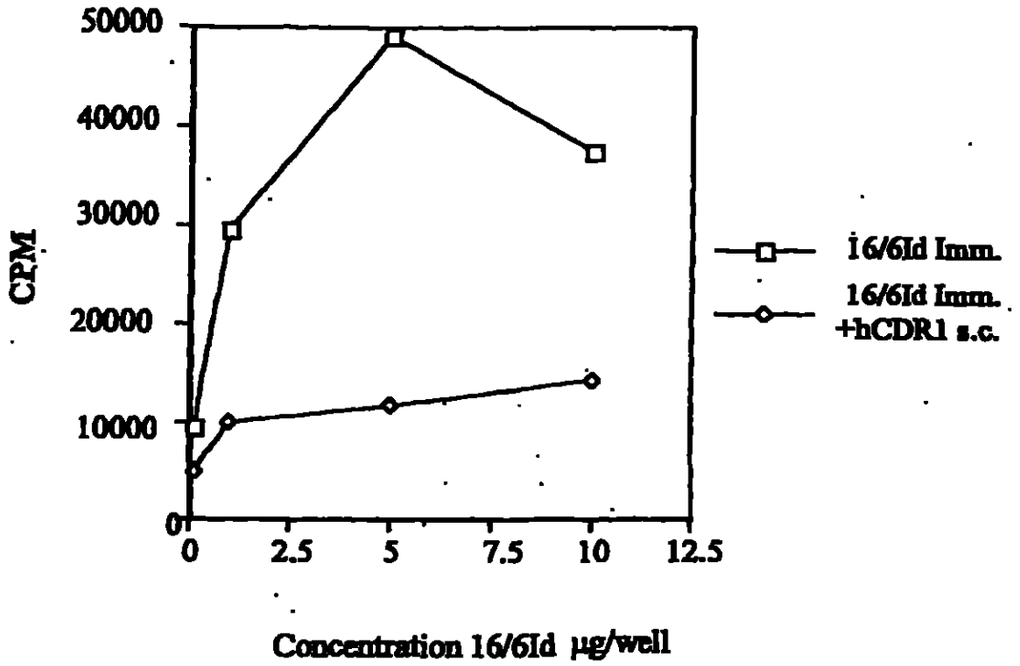

50000
40000
30000
20000
10000
0
CPM
0
2.5
5
7.5
10
12.5
Concentration 16/6Id µg/well
16/6Id Imm.
16/6Id Imm. +hCDR1 s.c.

Fig. 1

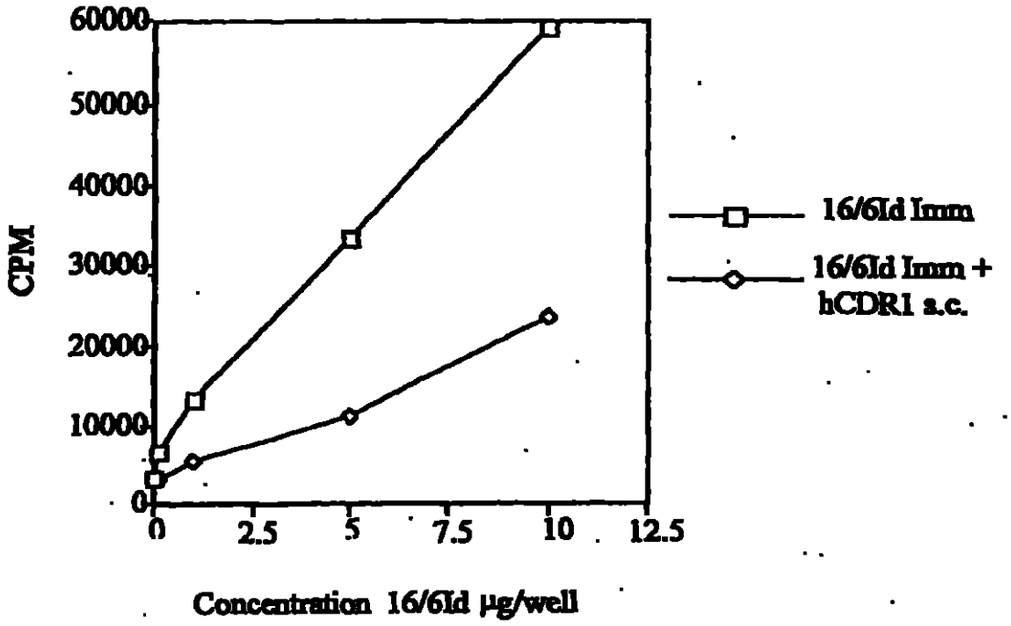

60000
50000
40000
30000
20000
10000
0
CPM
0
2.5
5
7.5
10
12.5
Concentration 16/6Id µg/well
16/6Id Imm
16/6Id Imm + hCDR1 s.c.

Fig. 2

IFN-γ Pg/ml
600
500
400
300
200
100
0
1646d imm.
1646d imm.+hCDR1 +
p259-271 ac

Fig. 3 A


TGF-β pg/ml
500
400
300
200
100
0

Fig. 3 B

250


16/6Id Imm.
16/6Id Imm.+hCDR1sc
16/6Id Imm. + p259-271 sc
400
300
200
100
0
IL-10 pg/ml

Fig. 3 C

251

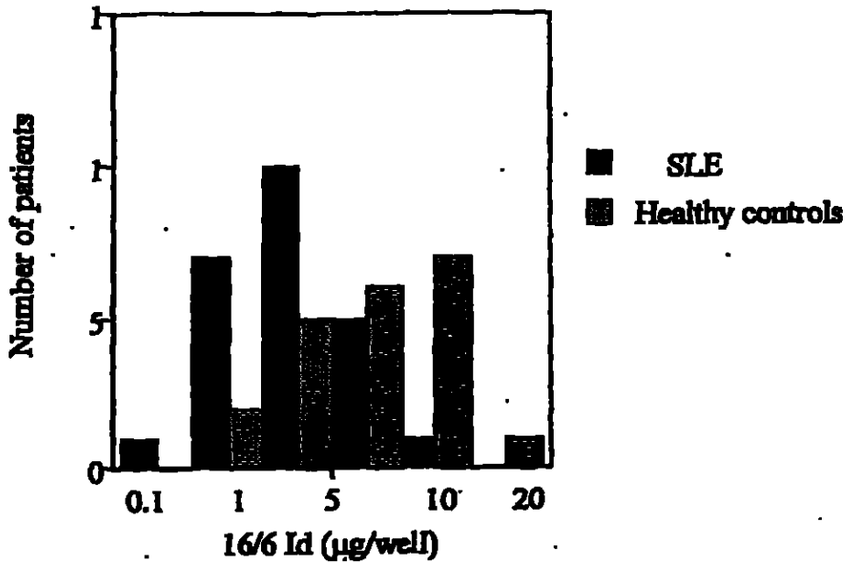



Fig. 4

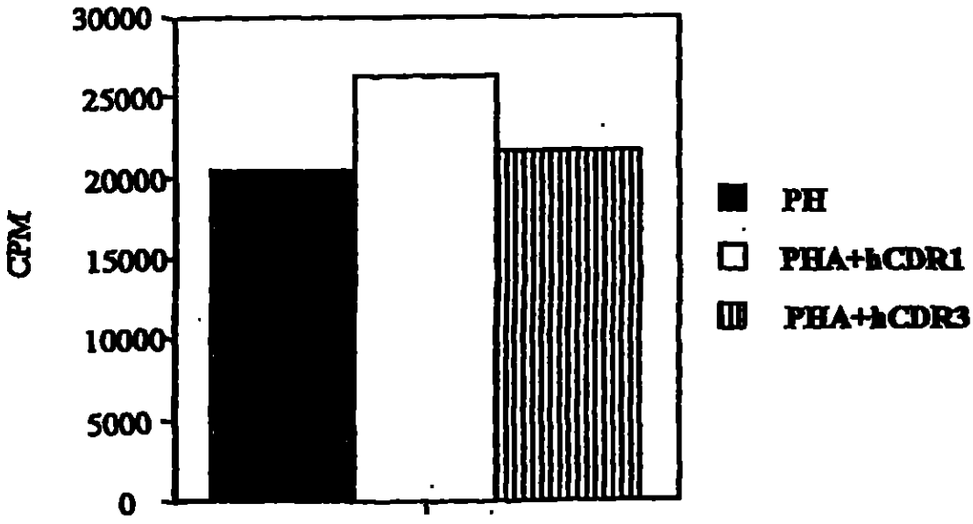



Fig. 5

252

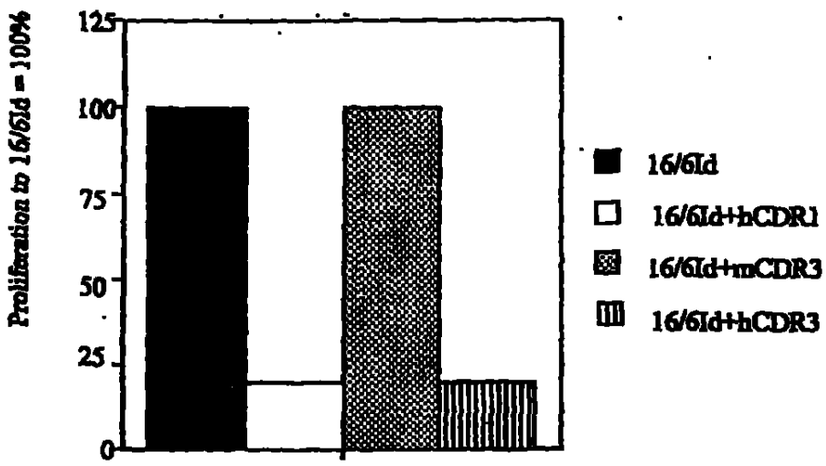



Fig. 6

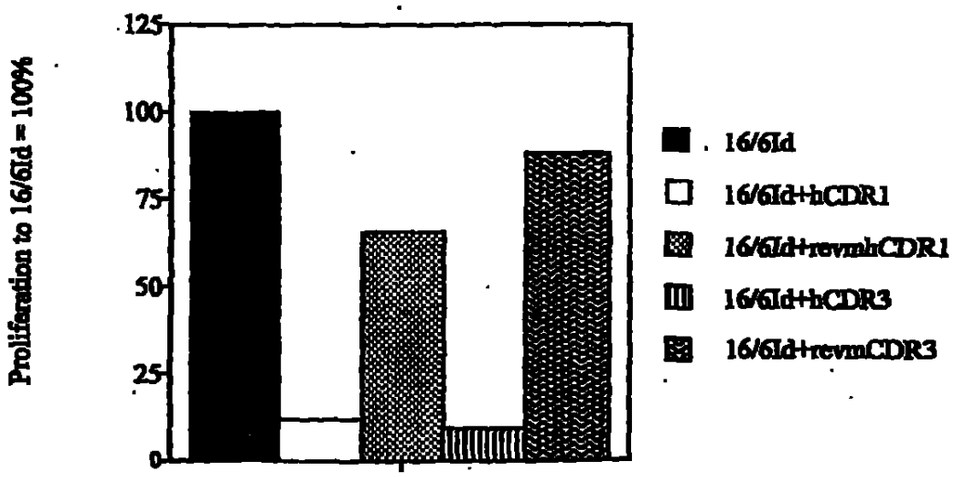



Fig. 7

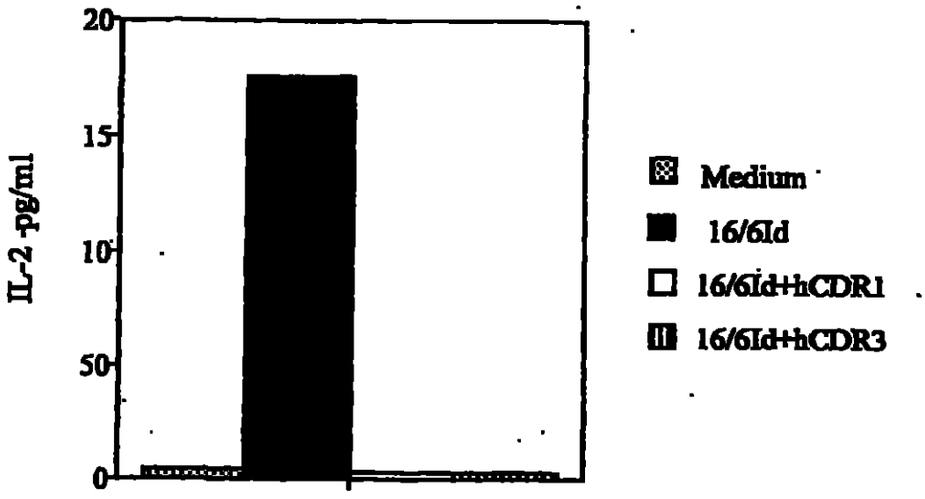

20
15
10
50
0
IL-2 pg/ml
Medium
16/6Id
16/6Id+hCDR1
16/6Id+hCDR3

Fig. 8

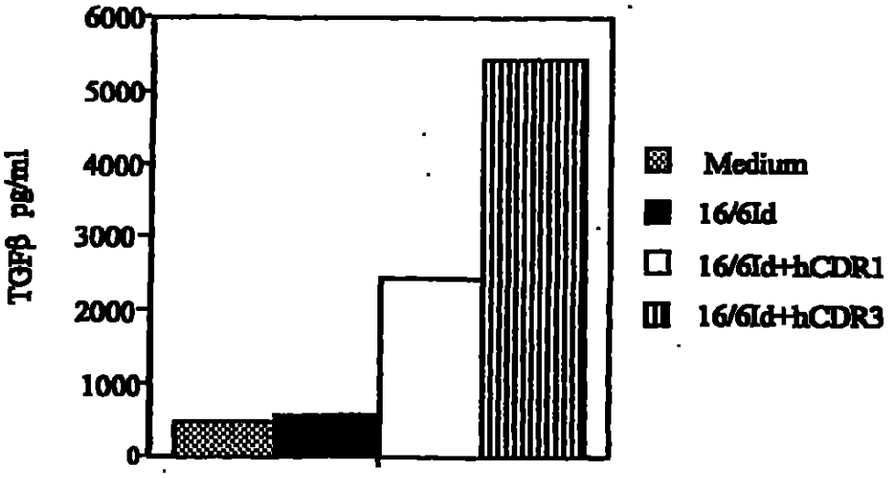

6000
5000
4000
3000
2000
1000
0
TGFβ pg/ml
Medium
16/6Id
16/6Id+hCDR1
16/6Id+hCDR3

Fig. 9

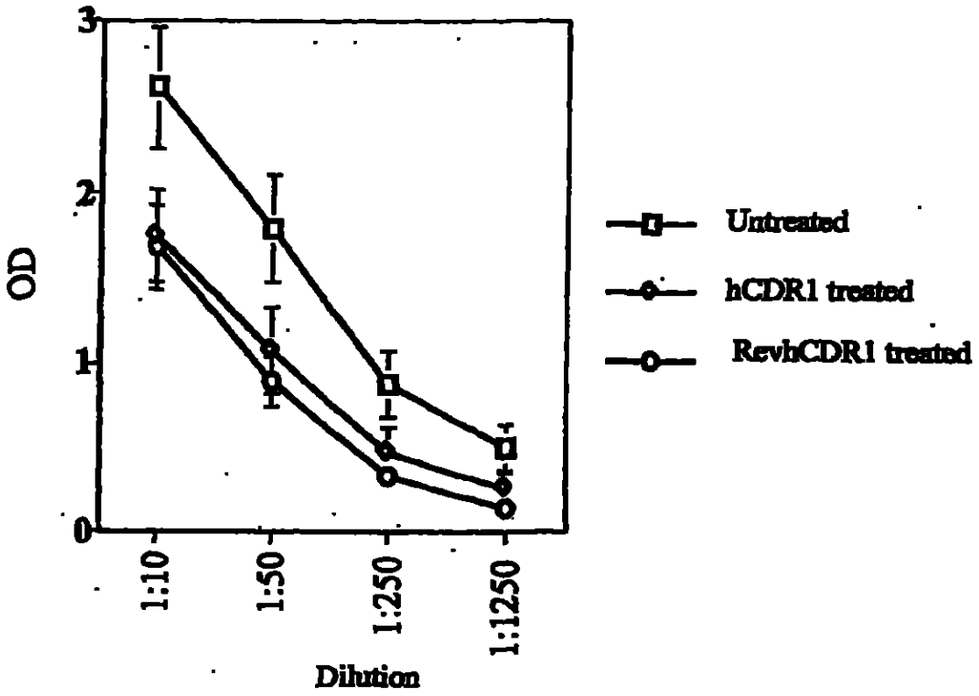

3
2
1
0
OD
1:10
1:50
1:250
1:1250
Dilution
Untreated
hCDR1 treated
RevhCDR1 treated

Fig. 10

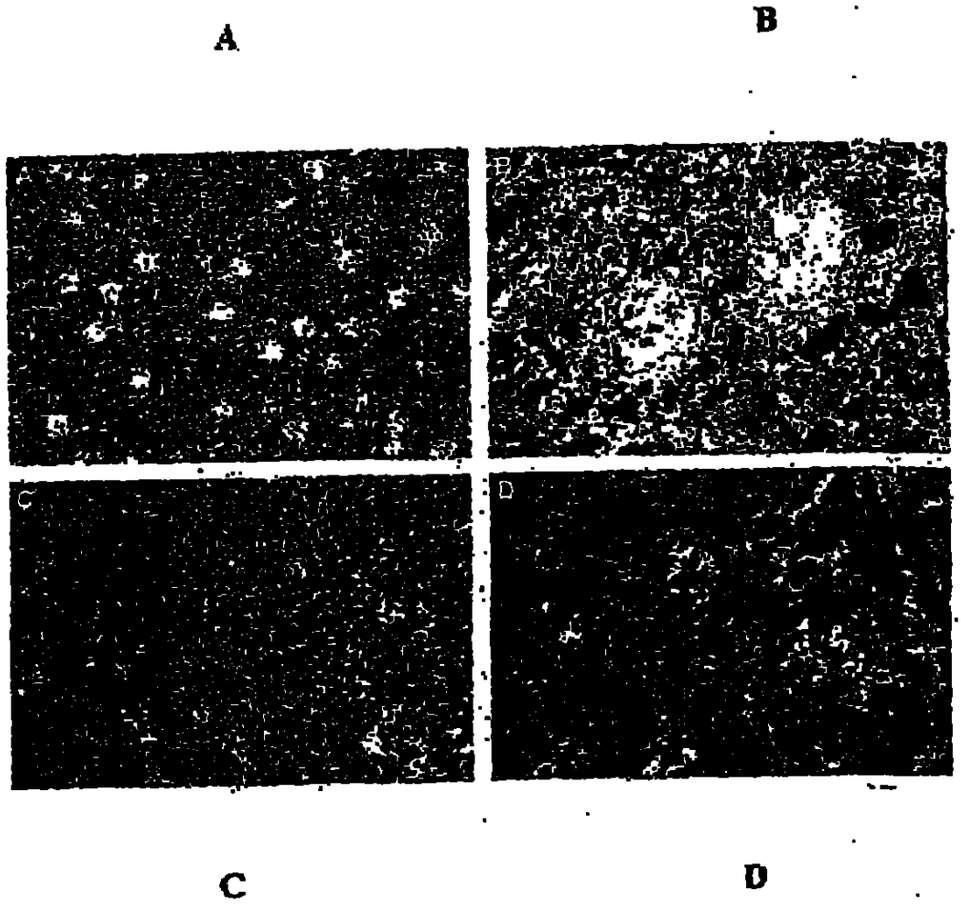



Fig. 11

256

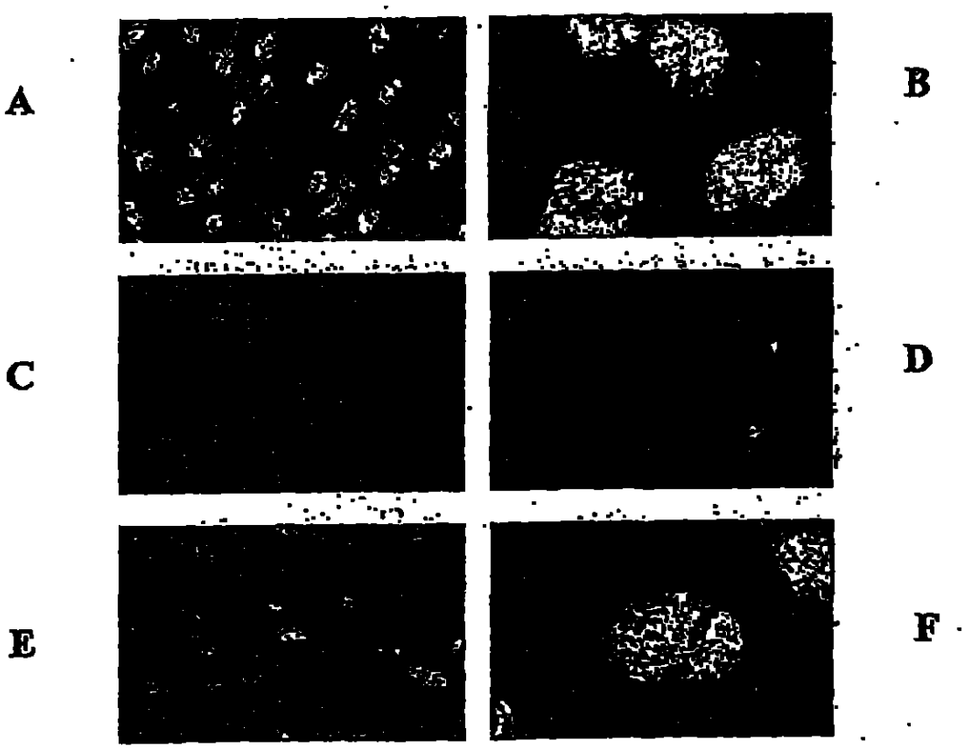



Fig. 12

257




Fig. 13 A




Fig. 13 B

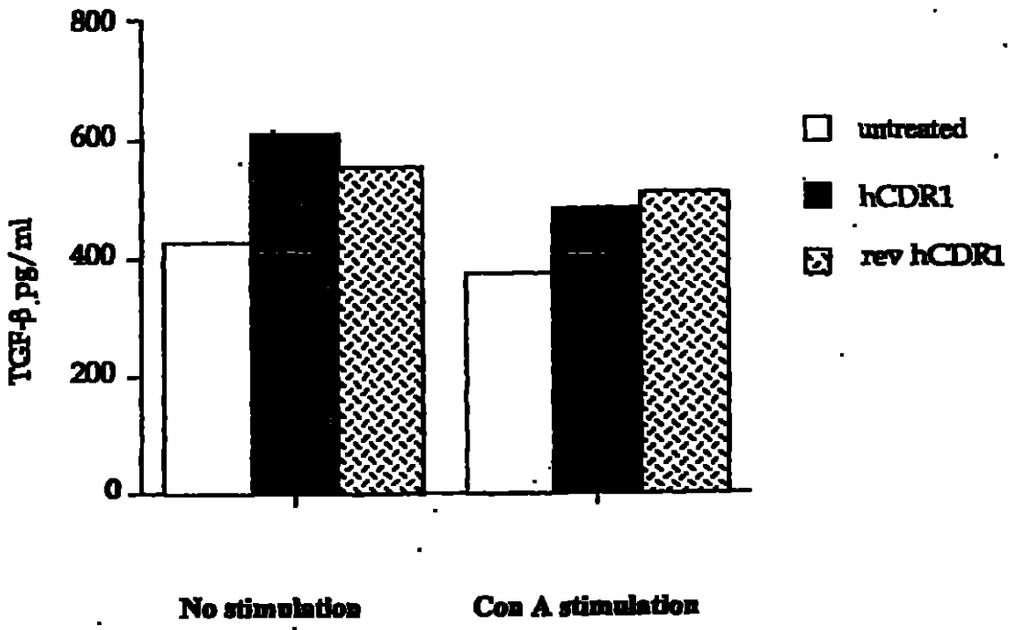

800
600
400
200
0
TGF-β pg/ml
untreated
hCDR1
rev hCDR1
No stimulation
Con A stimulation

Fig. 13 C

259

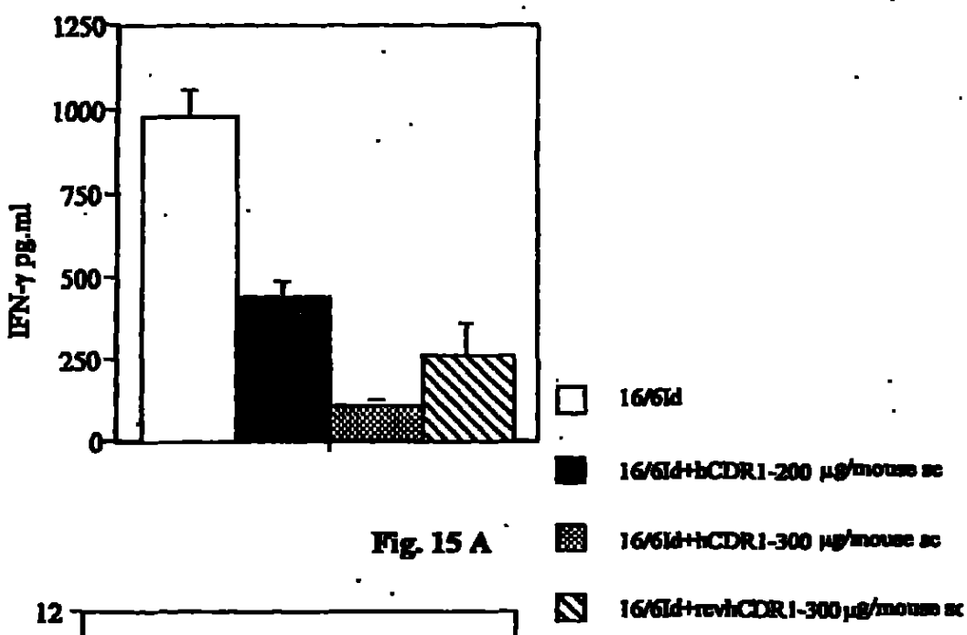



Fig. 15 A

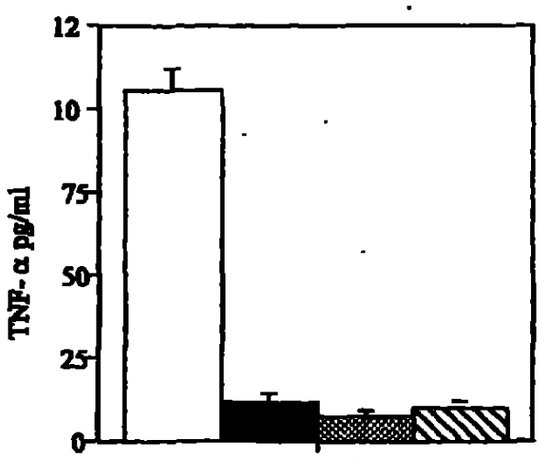



Fig. 15 B

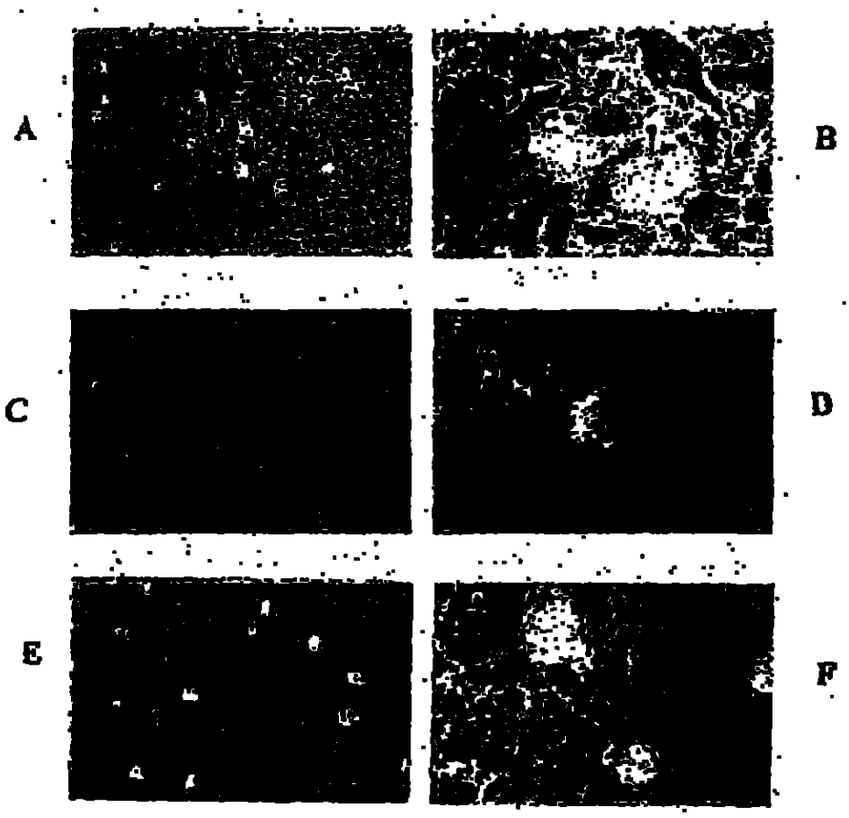



Fig. 14

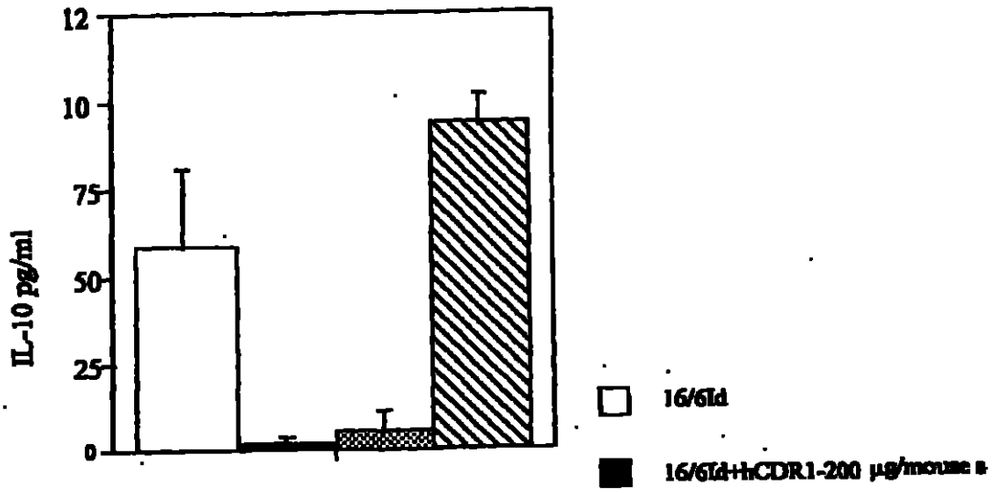



Fig. 15 C

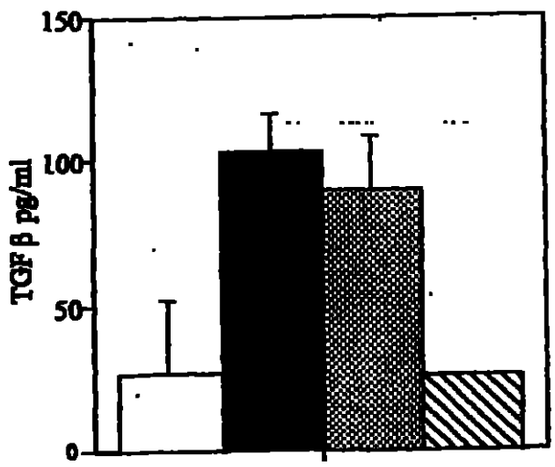



Fig. 15 D

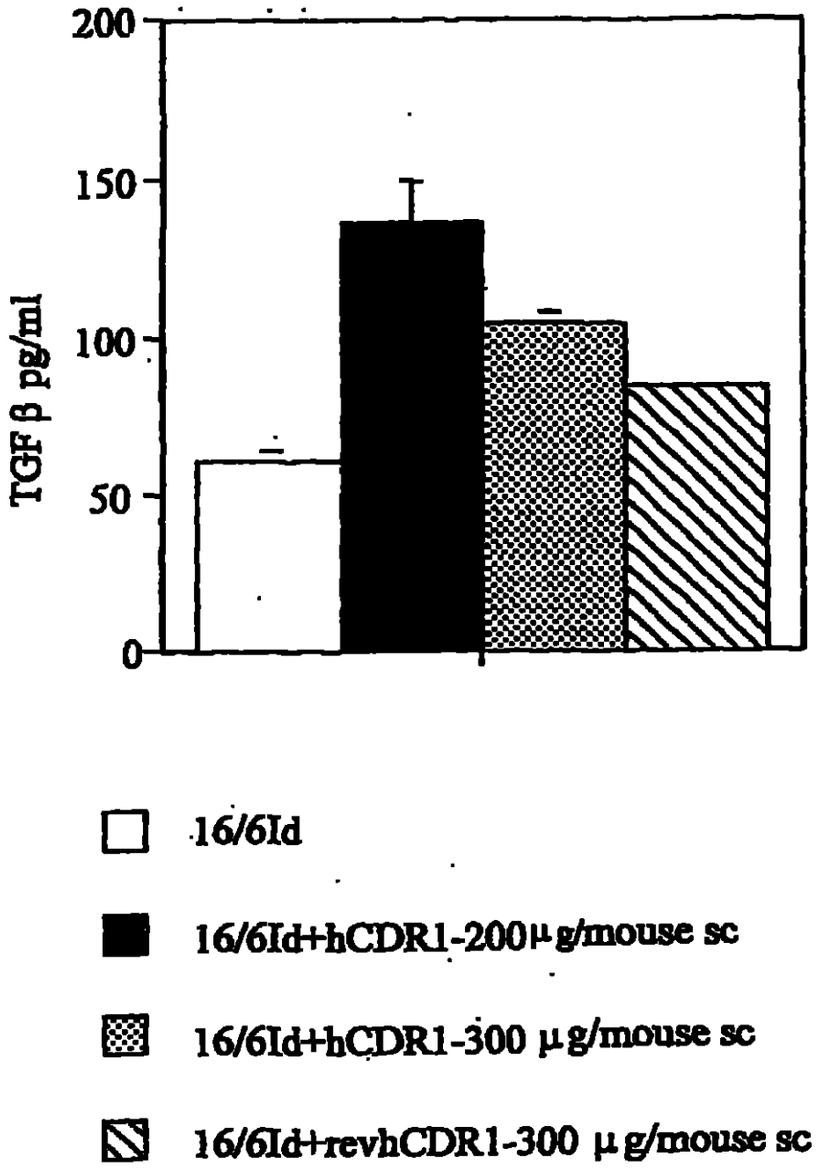

200
150
100
50
0
TGF β pg/ml
16/6Id
16/6Id+hCDR1-200μg/mouse sc
16/6Id+hCDR1-300 μg/mouse sc
16/6Id+revhCDR1-300 μg/mouse sc

Fig. 15 E

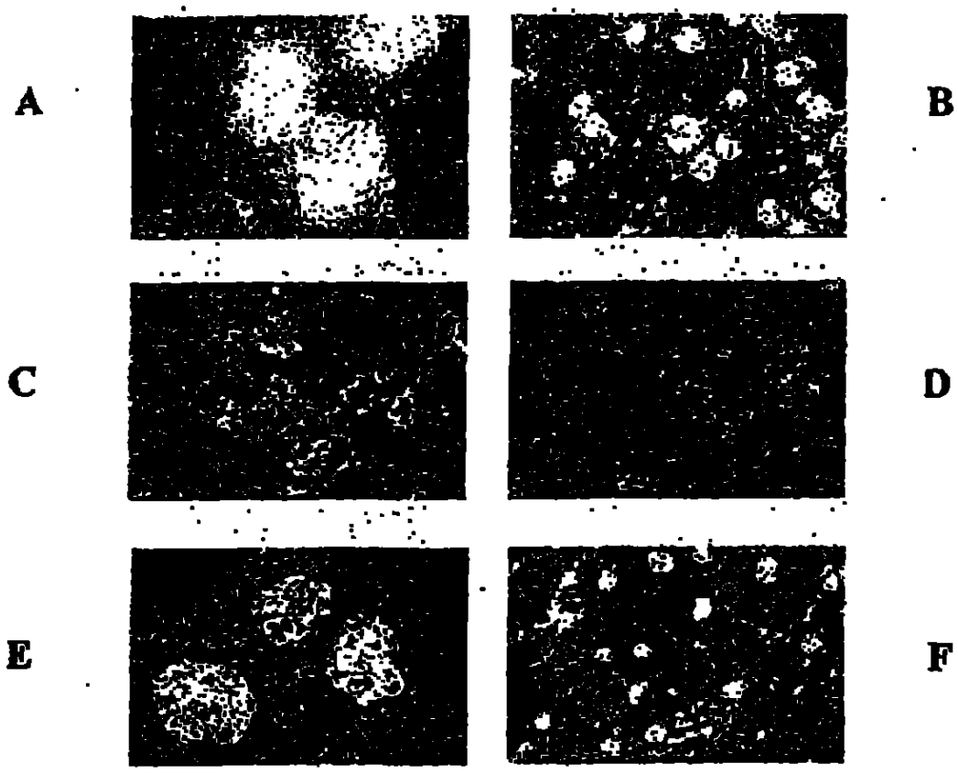



Fig. 16




Fig. 17 A

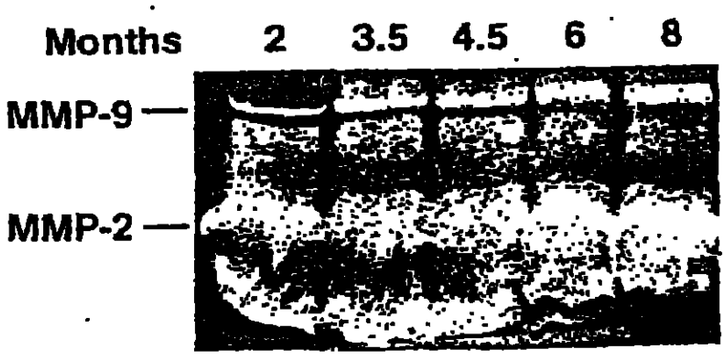



Fig. 17 B

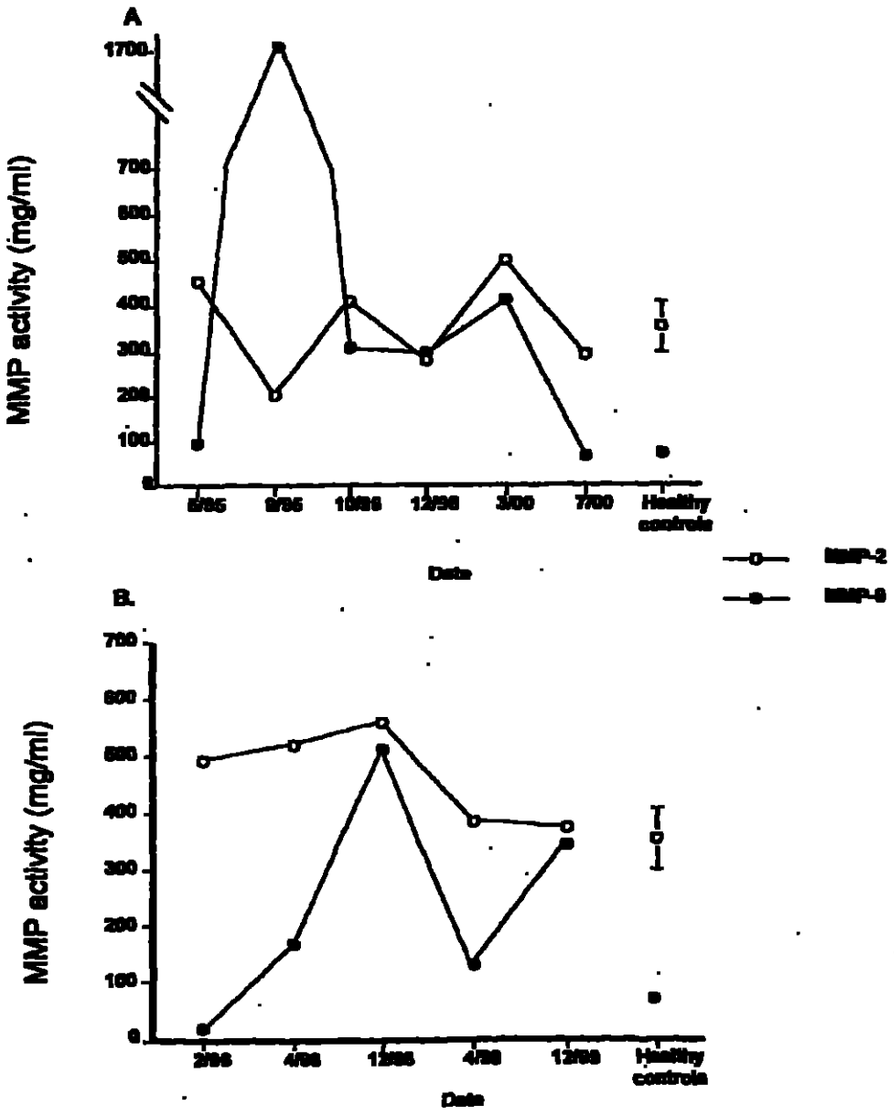
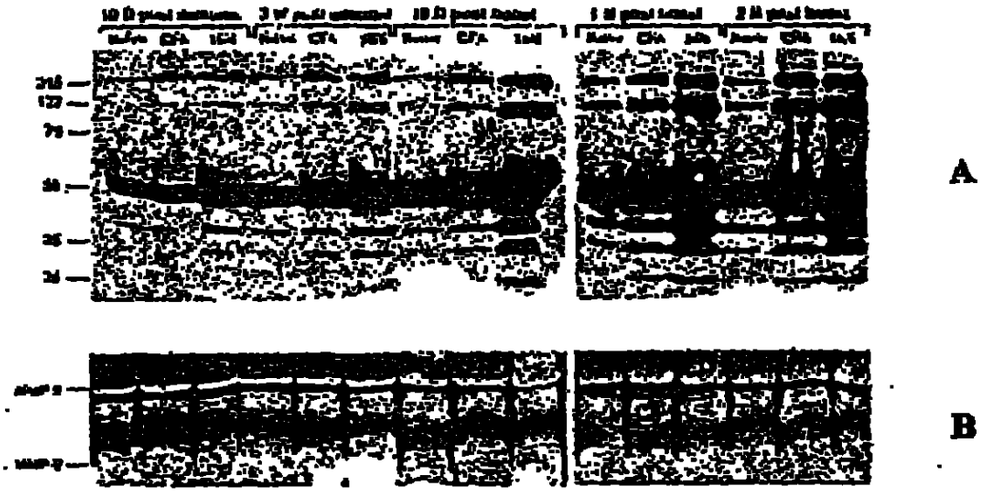

A
MMP activity (mg/ml)
1700
700
600
500
400
300
200
100
0
Healthy controls
Date
MMP-2
MMP-9
B.
MMP activity (mg/ml)
700
600
500
400
300
200
100
0
Healthy controls
Date

Fig. 27



A



B

Fig. 18

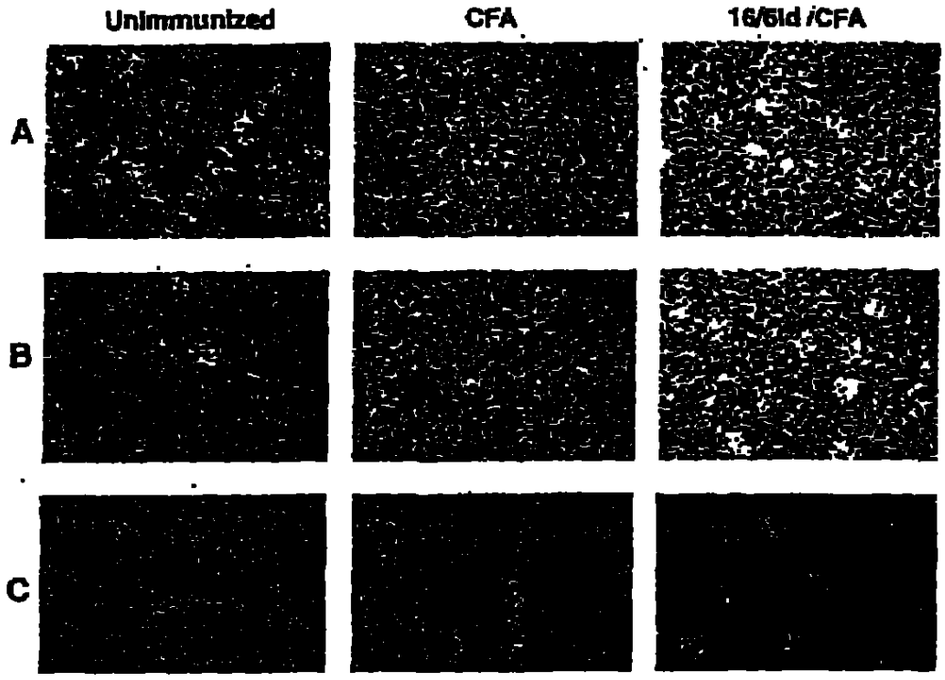



Fig. 19

268

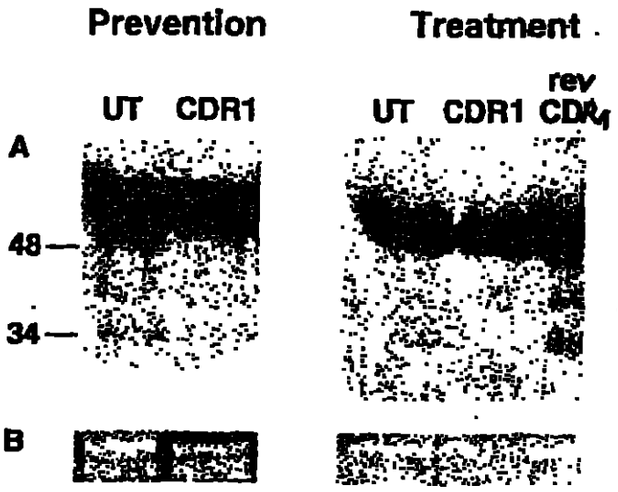



Fig. 20

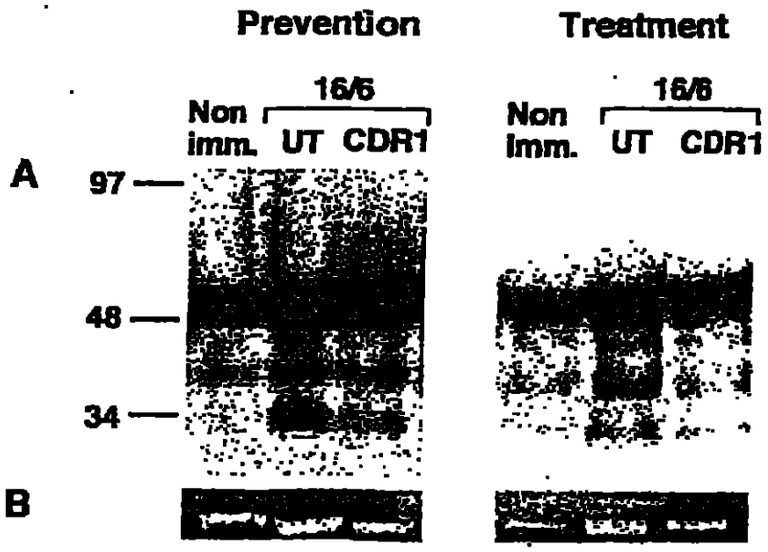



Fig. 21

269

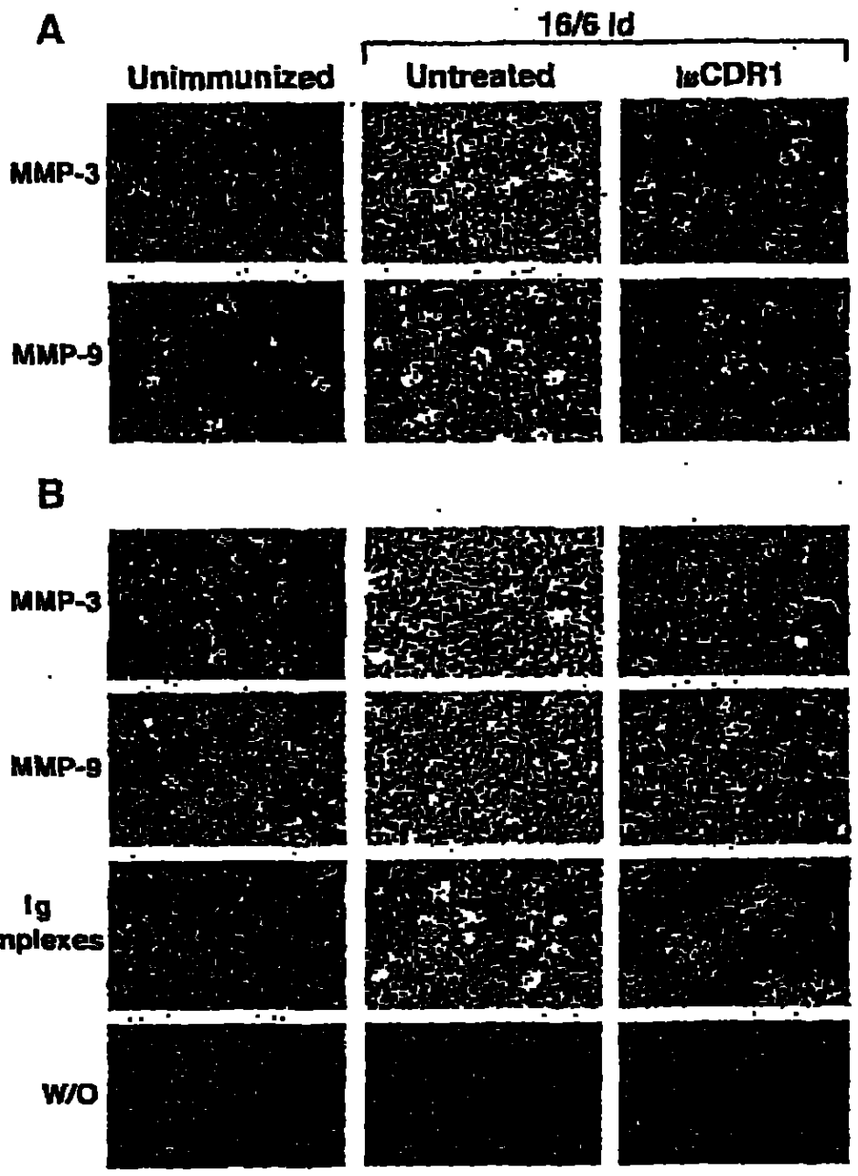

A
16/6 Id
Unimmunized
Untreated
IdCDR1
MMP-3
MMP-9
B
MMP-3
MMP-9
Ig complexes
W/O

Fig. 22

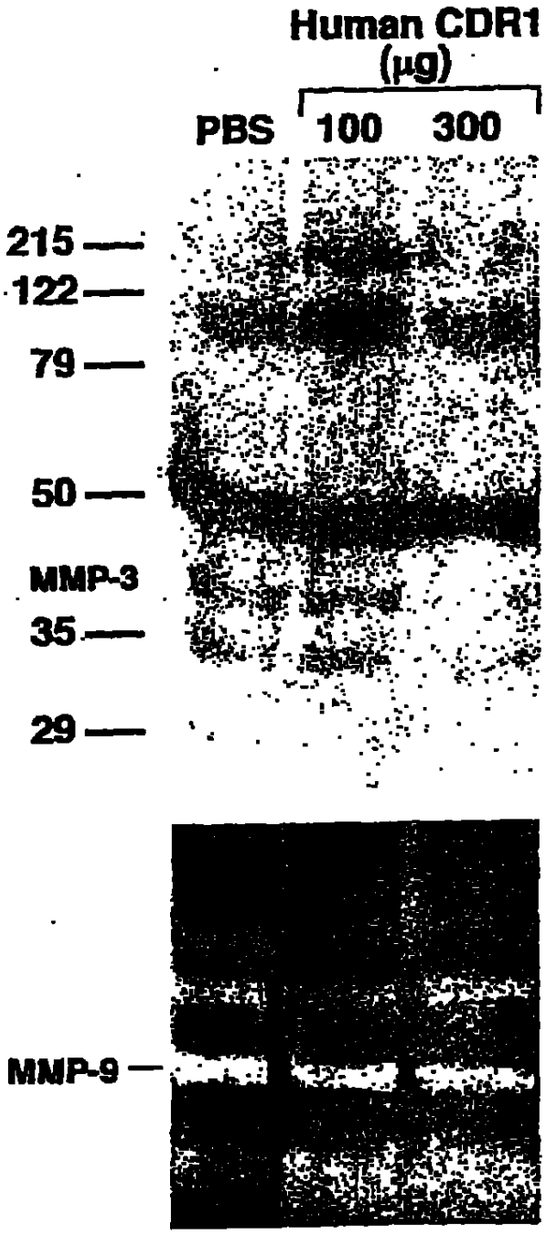



Fig. 23

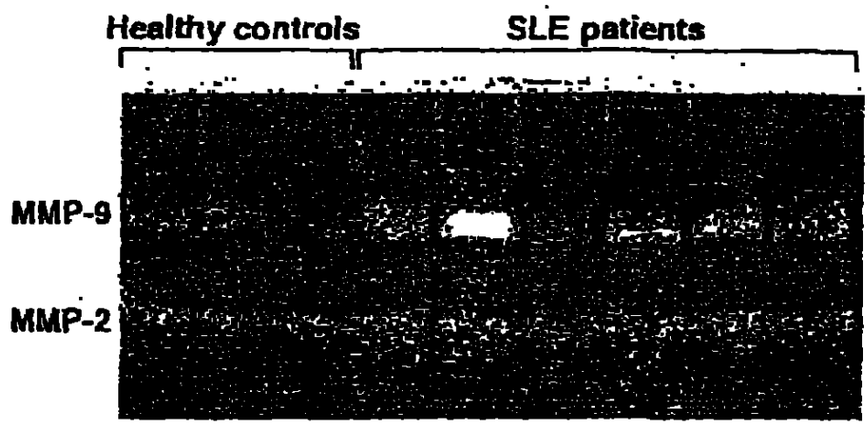

Healthy controls
SLE patients
MMP-9
MMP-2

Fig. 24

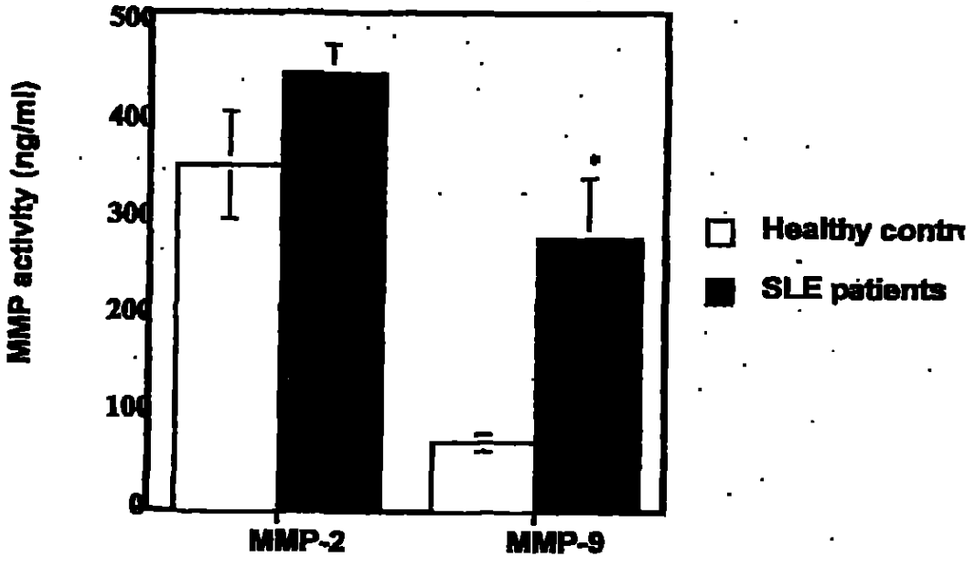

MMP activity (ng/ml)
500
400
300
200
100
0
MMP-2
MMP-9
Healthy contr
SLE patients

Fig. 25

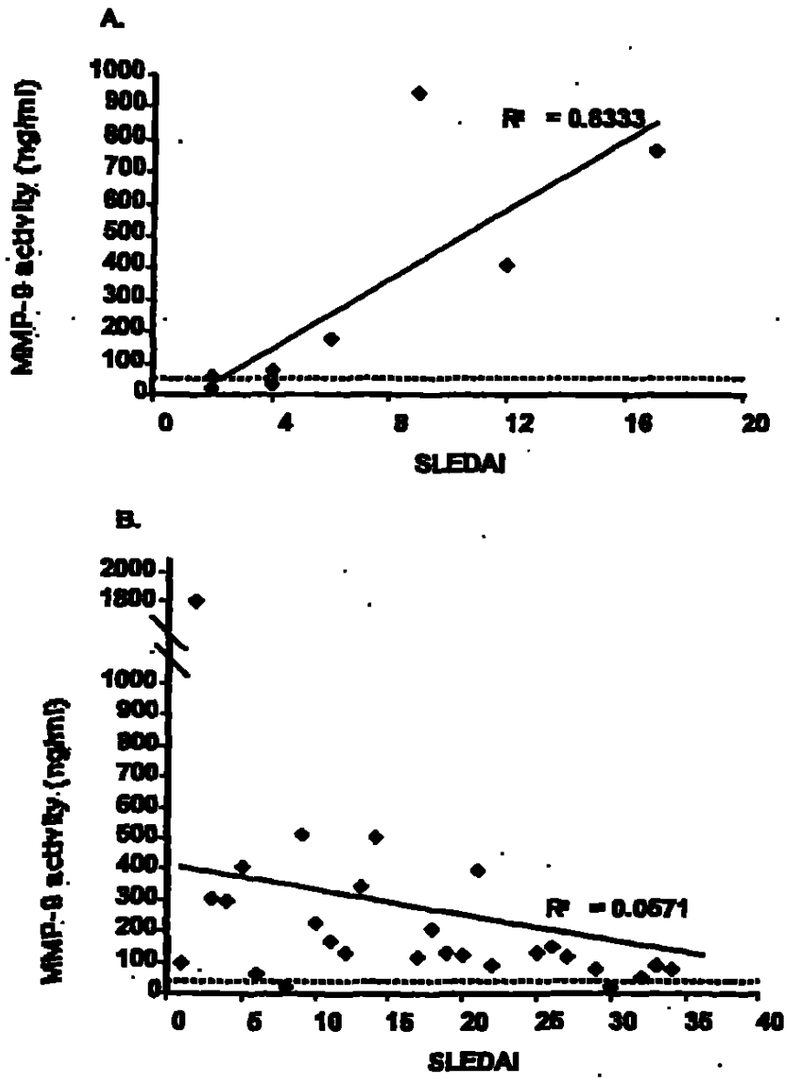

A.
MMP-9 activity (ng/ml)
1000
900
800
700
600
500
400
300
200
100
0
R² = 0.8333
0
4
8
12
16
20
SLEDAI
B.
MMP-9 activity (ng/ml)
2000
1800
1000
900
800
700
600
500
400
300
200
100
0
R² = 0.0571
0
5
10
15
20
25
30
35
40
SLEDAI

Fig. 26

# SYNTHETIC HUMAN PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEM FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

## FIELD OF THE INVENTION

[0001] The present invention relates to synthetic peptides and, more particularly, to synthetic peptides based on the complementarity-determining region (CDR) of a human monoclonal anti-DNA antibody, to pharmaceutical compositions comprising them, and to their use in the immunomodulation of systemic lupus erythematosus (SLE)-associated responses.

[0002] Abbreviations: 16/6Id: human 16/6Id mAb; CDR: complementarity-determining region; CFA: complete Freund's adjuvant; hCDR peptide: a peptide based on a CDR region of the human 16/6Id mAb; hCDR1: the human peptide of the SEQ ID NO:6; hCDR3: the human peptide of the SEQ ID NO:7; human 16/6Id mAb: human pathogenic anti-DNA mAb that bears the 16/6Id; ICD: immune complex deposits; Id: idiotype; LNC: lymph node cells; mAb: monoclonal antibody; MMP: matrix metalloproteinase; mCDR1: the murine peptide of the SEQ ID NO: 1; mCDR3: the murine peptide of the SEQ ID NO3; PBL: peripheral blood lymphocytes; PBS: phosphate-buffered saline; rsv: reversed peptide; SLE: systemic lupus erythematosus; SLEDAI: SLE disease activity index; TGF-β: transforming growth factor-β; UT: untreated.

## BACKGROUND OF THE INVENTION

[0003] Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of an array of autoantibodies, including antibodies to DNA, antibodies to nuclear antigens and antibodies to ribonucleoproteins. The progression of the disease is associated with general clinical manifestations and damage to tissues and organs caused by deposition of immune complexes. Similar to other autoimmune conditions, the etiology of SLE is multifactorial entailing genetic, environmental, hormonal and immunological factors. No specific treatment aimed towards the prevention or cure of SLE is available.

[0004] The human monoclonal anti-DNA antibody termed 16/6Id bears a common idiotype (Shoenfeld et al., 1983). The idiotype was found to have clinical relevance in SLE patients. Thus, the 16/6Id was found to be expressed on anti-DNA antibodies of 54% of SLE patients with active disease (Isenberg et al., 1984) and in affected organs of patients with SLE (Isenberg and Collins, 1985). Mice of inbred strains that do not develop any spontaneous autoimmune diseases were immunized with this human anti-DNA 16/6Id mAb and developed the major hallmarks of SLE in humans and in the spontaneous murine models for this disease (Mendlovic et al., 1988). Thus, following immunization, the mice produced antibodies specific to the 16/6Id, antibodies that bear the 16/6Id and antibodies directed against different nuclear antigens (dsDNA, ssDNA, Sm, ribonucleoprotein, Ro, La and others). The serological findings were associated with leukopenia, elevated erythrocyte sedimentation rate, proteinuria, abundance of immune complexes in the kidneys and sclerosis of the glomeruli (Mendlovic et al., 1988), which are typical manifestations of SLE.

[0005] A murine anti-16/6Id mAb (Ab2) derived from mice with experimental SLE was also capable of inducing the experimental disease in mice (Mendlovic et al., 1989) similar to the 16/6Id (Ab1). Moreover, a murine anti-DNA mAb that expresses the 16/6Id was prepared from mice afflicted with experimental SLE. The antibody Ab3 termed 5G12 reacted with antibodies specific to the 16/6Id. Immunization with the latter antibody resulted in the induction of experimental SLE with similar manifestations as observed following immunization with the human 16/6Id (Ab1) and with the murine anti-16/6Id (Ab2) mAbs (Waisman et al., 1993). These results show the importance of the 16/6Id network in the induction and progression of SLE in mice.

[0006] In order to understand the mechanism by which self-antibodies associated with SLE arise, the present inventor has produced a variety of monoclonal autoantibodies derived from C3HLSW mice in which experimental SLE was induced. As a rule, the monoclonal autoantibodies that were capable of eliciting antibodies that bear the 16/6Id or react with it were found to be pathogenic and thus capable of inducing experimental SLE in mice (Fricke et al., 1990; Shoenfeld et al., 1993).

[0007] Later on, the variable (V) regions of nine autoantibodies that bind either DNA or HeLa nuclear extract (NE), isolated from the C3HLSW mice with experimental SLE, were sequenced (Waisman and Mozes, 1993). Monoclonal antibodies with different specificity were analyzed in an attempt to determine the connections between the different autoantibodies. Three mAb were found to bind DNA, and were shown to exhibit sequence characteristics of pathogenic anti-DNA antibodies. One of these mAb, designated 2C4C2, was shown to use a heavy (H) chain V region gene ($V_H$) identical to the $V_H$ of anti-DNA mAb isolated from other lupus-prone mice, namely (NZB x NZW)$F_1$. The light (L) chain V region gene ($V_L$) of mAb 2C4C2 is 98% homologous to the $V_L$ of another anti-DNA mAb, also isolated from (NZB x NZW)$F_1$ mice. The other two anti-DNA mAb, designated 5G12-4 and 5G12-6 share 93% of their $V_H$ sequences with that of mAb 2C4C2. Based on the analysis of these mAbs, it appeared that autoantibodies found in mice with experimental SLE use genetic elements similar to those used by mAb that were isolated from mouse strains which develop lupus spontaneously.

[0008] T cells play an important role in the induction and development of experimental SLE. Thus, T cell lines and clones specific to the 16/6Id were shown to induce experimental SLE in syngeneic recipients similarly to the 16/6Id antibody. Therefore, following the inoculation of the activated cells of the lines, the mice developed both the serology and the renal damage which is typical to SLE (Fricke et al., 1991).

[0009] As described above, the mAb 5G12 that was isolated from mice with experimental SLE and was shown to bind DNA and bear the 16/6Id, is capable of inducing experimental SLE in mice (Waisman et al., 1995). T cells that react specifically to mAb by proliferation, are probably reacting to peptides representing sequences from their complementarity-determining regions (CDR). It is very likely that the T cells recognize the V regions of the above antibodies since they do not react with other antibodies that carry the same constant region but have different specificities. Within the variable region, the regions with the highest probability to be recognized are the CDR, since those are the regions that differ the most between the various antibodies.

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The CDR regions of the $V_H$ sequences of the nine pathogenic murine mAb mentioned above that induce SLE in mice, are boxed in FIG. 1 of Waisman and Monsa, 1993, in which the complete nucleotide and deduced amino acid sequences for the variable heavy chains ($V_H$) of the nine mAbs are presented.

[0010] International PCT Patent Publication No. WO 96/30057 of the present applicants describes peptides based on the CDR regions of pathogenic mAbs isolated from mice with experimental SLE, in particular peptides Ia to IIIa, based on the CDR1, CDR2 and CDR3 regions, respectively, of the $V_H$ chain of the murine mAb termed 5G12, and peptides IVa and Va, based on the CDR1 and CDR3 regions, respectively, of the $V_H$ chain of the murine mAb termed 2C4C2. These peptides have the sequences substantially as denoted by SEQ ID NO:1 to SEQ ID NO:5 as follows:

T G Y Y M Q W V K Q S P E K S L E W I G (Ia) [SEQ ID NO:1]

E I N P S T G G T T Y N Q K F K A K A T (IIa) [SEQ ID NO:2]

Y Y C A R F L W F P Y A M D Y W G Q G S (IIIa) [SEQ ID NO:3]

G Y E M E W V K Q S H G K S L E W I G (IVa) [SEQ ID NO:4]

Y Y C A R S G N Y G N Y W G Q T L (Va) [SEQ ID NO:5]

[0011] These peptides and, in particular, the peptides Ia and IIIa, herein designated mCDR1 [SEQ ID NO:1] and mCDR3 [SEQ ID NO:3], respectively, were shown by the inventor to be capable, when administered in PBS, of inhibiting T cell priming to either the appropriate mCDR peptide or to the whole anti-DNA 16/6Id mAb of either murine or human origin (Waisman et al., 1997). The peptides mCDR1 and mCDR3 were further shown by the inventor to either prevent or treat an already established SLE that is either induced by the human anti-DNA 16/6Id mAb or that develops spontaneously in the SLE prone mice (NZB x NZW) F1 or MRL/lpr/lpr (Eilat et al., 2000 and 2001).

## SUMMARY OF THE INVENTION

[0012] It has now been found according to the present invention that peptides based on the CDR of the human monoclonal anti-DNA 16/6Id antibody are capable of immunomodulating SLE-associated responses. Thus, peptides based on the CDR1 and CDR3 of the human 16/6Id were tested and shown to inhibit lymph node cell proliferation of mice immunized with the murine peptides mCDR1 (SEQ ID NO:1) and mCDR3 (SEQ ID NO:3) or with the whole human anti-DNA 16/6Id mAb, to inhibit the proliferative response of peripheral blood lymphocytes (PBL) of SLE patients to the human anti-DNA 16/6Id mAb, and to ameliorate disease manifestations of mice afflicted with spontaneous or experimental SLE.

[0013] These findings are completely unexpected because not all CDRs of pathogenic autoantibodies are equally recognized by T cells of patients. As shown previously in the laboratory of the present inventor (Dayan et al., 2000), the CDRs of the anti-DNA autoantibody 2C4C2 are less well recognized by T cells of SLE patients than the peptides based on the CDRs of the anti-DNA antibody 5G12. Furthermore, many of the analogs of the peptides based on the CDRs of the murine autoantibodies described in the above-mentioned WO 96/30057 were shown not to be efficient in their inhibitory effects, and thus the modifications occurring in the sequences of the peptides based on the CDRs of a human monoclonal anti-DNA antibody of the present invention, could not be predicted or suggested to be efficient. In addition, the use of peptides based on a human antibody should be considered as preferable for human use in comparison with peptides based on non-human antibodies.

[0014] The present invention thus relates, in one aspect, to a synthetic peptide selected from the group consisting of:

[0015] (a) a peptide of at least 12 and at most 30 amino acid residues comprising a sequence consisting of, or found within, a complementarity-determining region (CDR) found in the heavy or light chain of the human monoclonal anti-DNA 16/6Id antibody (hereinafter "hCDR sequence"), or a sequence obtained by: (i) replacement of one or more of the amino acid residues of the hCDR sequence by different amino acid residues; (ii) deletion of one or more amino acid residues from the hCDR sequence; and/or (iii) addition of one or more amino acid residues to the hCDR sequence , or a salt or a chemical derivative of said peptide;

[0016] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0017] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0018] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0019] The human monoclonal anti-DNA 16/6Id antibody, herein referred to as "human 16/6Id mAb", is a pathogenic human monoclonal anti-DNA antibody capable of inducing a SLE-like disease in mice.

[0020] A peptide according to the invention comprising a hCDR sequence as defined above is herein referred to as "hCDR peptide".

[0021] In one preferred embodiment, the hCDR peptide includes a sequence of a CDR, more preferably of the CDR1 or CDR3, of the heavy chain of the human 16/6Id mAb, such as, but not being limited to, the peptides herein designated hCDR1 and hCDR3, having the sequences substantially as denoted by SEQ ID NO:6 and SEQ ID NO:7, respectively, as follows:

GYYWSWIRQPPGKGLEWIG [SEQ ID NO:6]

YYCARGLLRGWFDVDYYGMDV [SEQ ID NO:7]

[0022] In another aspect, the invention provides a pharmaceutical composition comprising at least one synthetic peptide or peptide polymer of the invention, and a pharmaceutically acceptable carrier. The pharmaceutical composition is particularly useful for the treatment of SLE and amelioration of the clinical manifestations of the disease, particularly by modulating SLE-associated responses, e.g. down-regulating the levels of P-3 and/or MMP-9, and/or IL-2 and/or IFN-γ activities, or up-regulating the level of TGF-β activity in a patient with SLE.

[0023] In a further aspect, the invention relates to a method for the treatment of SLE comprising administering to an SLE patient an effective amount of a peptide or peptide polymer of the invention. The invention further relates to a method of immunomodulating SLE-associated responses, e.g. down-regulating the levels of MMP-3 and/or MNP-9, and/or IL-2 and/or IFN-γ activities, or up-regulating the level of TGF-β activity in a patient with SLE, comprising administering to an SLE patient an effective amount of a peptide or peptide polymer of the invention.

[0024] In still a further aspect, the invention relates to a method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the levels of MMP-3 and/or MMP-9 in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of MMP-3 and/or MMP-9 correlates with the effectiveness of the drug.

[0025] In still another further aspect, the invention relates to a method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the level of IL-2 and/or IFN-γ in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of IL-2 and/or IFN-γ correlates with the effectiveness of the drug.

[0026] In yet still a further aspect, the invention relates to a method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the level of TGF-β in a blood sample obtained from said patient being treated with said drug, whereby an increased level of TGF-β correlates with the effectiveness of the drug.

[0027] The drug which effectiveness is to be assessed according to any of the above methods may be, for example, without being limited to, a peptide according to the invention or a murine peptide as described in the above-mentioned WO 96/30057.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0028] FIG. 1 shows inhibition of the proliferative responses of lymph node cells of mice immunized with human 16/6Id mAb to various concentrations of the 16/6Id mAb (0.1-10 μg/well), by treatment with 300 μg hCDR1.

[0029] FIG. 2 shows inhibition of the proliferative responses of lymph node cells of mice immunized with human 16/6Id mAb to various concentrations of the 16/6Id mAb (0.1-10 μg/well), by treatment with 50 μg hCDR1.

[0030] FIGS. 3A-C show the cytokine pattern in BALB/c mice immunized with human 16/6Id mab and treated with hCDR1 or with an irrelevant peptide p259-271. FIG. 3A—IFN-γ pattern; FIG. 3B—TGF-β pattern: FIG. 3C—IL-10 pattern.

[0031] FIG. 4 shows the concentrations of the human anti-DNA 16/6Id mAb required for optimal stimulation of PBL of SLE patients and of healthy controls. PBL were stimulated with various concentrations (0.1-40 μg/well) of the 16/6Id mAb. The concentration yielding the highest stimulation index was defined as optimal for triggering a proliferative response.

[0032] FIG. 5 shows proliferation of PBL from one SLE patient stimulated with the mitogen phytohemagglutinin (PHA) in the absence or presence of hCDR1 or hCDR3.

[0033] FIG. 6 shows proliferation of PBL from one SLE patient stimulated with human 16/6Id mAb in the absence or presence of the human peptides hCDR1 or hCDR3 or the murine peptide mCDR3.

[0034] FIG. 7 shows proliferation of PBL from one SLE patient stimulated with human 16/6Id mAb in the absence or presence of the human peptides hCDR1 or hCDR3 or the murine reversed peptides revmCDR1 and revmCDR3.

[0035] FIG. 8 shows inhibition of IL-2 secretion in PBL of SLE patients triggered by the human 16/6Id mAb in the absence or presence of hCDR1 or hCDR3.

[0036] FIG. 9 shows up-regulation of TGF-β secretion in the PBL of one representative SLE patient stimulated with the human 16/6Id mAb in the absence or presence of hCDR1 or hCDR3.

[0037] FIG. 10 shows anti-DNA autoantibody levels in (NZB x NZW)F1 mice untreated or treated with 300 μg hCDR1 or with the reversed peptide revhCDR1 (used as control).

[0038] FIGS. 11A-11D are photos showing representative kidney sections of SLE-prone (NZB x NZW)F1 mice that were treated starting at the age of 5 and half months with PBS (11A, 11B) or with 100 μg hCDR1 (11C, 11D). The sections are of mice sacrificed at the age of 9 months. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (11A, 11C x 100; 11B, 11D x 400).

[0039] FIGS. 12A-12F are photos showing representative kidney sections of SLE-prone (NZB x NZW)F1 mice that were treated with PBS (12A, 12B) or with 300 μg hCDR1 (12C, 12D), or with the reversed peptide revhCDR1 (12E, 12F). The sections are of mice sacrificed at the age of 9 months. For the detection of immune complex Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (12A, 12C, 12E x 100; 12B, 12D, 12F x 400).

[0040] FIGS. 13A-13C show the cytokine pattern as measured by ELISA in supernatants of Con A-stimulated cultures of splenocytes of SLE-prone (NZB x NZW)F1 mice that were untreated or treated with hCDR1 or with the reversed peptide revhCDR1. FIG. 13A—IFN-γ pattern; FIG. 13B—IL-10 pattern; FIG. 13C—TGF-β pattern.

[0041] FIGS. 14A-14F are photos showing representative kidney sections of BALB/c mice with 16/6Id-induced experimental SLE treated with PBS (14A,14B) or with 200 μg hCDR1 (14C, 14D), or with the reversed peptide revhCDR1 (14E, 14F). The sections are of mice sacrificed at the age of 9 months. For the detection of immune complex Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (14A, 14C, 14E x 100; 14B, 14D, 14F x 400).

[0042] FIGS. 15A-15E show the cytokine pattern as measured by ELISA in supernatants of 16/6Id-stimulated lymph node cultures of BALB/c mice with 16/6Id induced experimental SLE that were untreated or treated with hCDR1 (200 or 300 μg ) or with the reversed peptide revhCDR1. FIG. 15A—IFN-γ pattern; FIG. 15B—TNF-α; FIG. 15C—IL-10 pattern; FIG. 15D—TGF-β pattern; FIG. 15E—TGF-β pattern, measured in supernatants of 16/6Id-triggered spleen cells.

[0043] FIGS. 16A-16F are photos showing representative kidney sections of SLE-prone (NZB x NZW)F1 mice untreated (16A, 16B) or mice recipients of splenocytes of mice treated either with 300 μg hCDR1 (16C, 16D) or with the reverse peptide revhCDR1 (16E, 16F). For the detection of immune complex Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (16A, 16C, 16E x 400; 16B, 16D, 16F x 100).

[0044] FIGS. 17A-17B depict kinetics of appearance of MMP-3, MMP-2, and MMP-9 in the sera of (NZB x NZW)F1 mice. (NZB x NZW)F1 mice (10 mice/group) were bled at the indicated time points. Pooled sera (4 μL) of mice from each group were tested for MMP-3 expression levels using Western blot (FIG. 17A) or for MMP-9 and MMP-2 activities using gel zymography (FIG. 17B). The results represent 4 similar experiments.

[0045] FIGS. 18A-18B depict kinetics of appearance of MMP-3, MMP-2, and MMP-9 in the sera of BALB/c mice. Non-immunized BALB/c mice (10 mice/group) or mice that were immunized with PBS/CFA (10 mice/group) or with the 16/6Id (in CFA; 10 mice/group), were bled at the indicated time points. Pooled sera of mice (4 vl) from each group were tested for MMP-3 expression levels using Western blot (FIG. 18A) or for MMP-9 and MMP-2 activities using gel zymography (FIG. 18B). The results are representative of 3 similar experiments.

[0046] FIGS. 19A-19C depict immunostaining of kidney sections of immunized BALB/c mice for MMP-3 and MMP-9. Non-immunized BALB/c mice or mice that were immunized with PBS/CFA or with the 16/6Id (in CFA), (3 mice/group), were sacrificed 5.5 months following their boost with the 16/6Id. Kidneys were removed, and their 5 μm cryostat sections were immunostained for P-3 (19A) and P-9 (19B). A control staining to the efficiency of blocking, was performed (19C). (x200). The results are representative of 3 similar experiments.

[0047] FIGS. 20A-20B depict levels of MMP-3 and MMP-9 in sera of (NZB x NZW)F1 mice treated with mCDR1. In prevention experiments, mice (10/group) were given weekly s.c. injections with mCDR1 starting at the age of 2 months during 10 weeks. The results represent sera taken 4 months after the end of the treatment. In treatment experiments, mice (10/group) were injected s.c. with either PBS or 250 μg/mouse of mCDR1 starting at the age of 5 months. The results represent sera taken 3 weeks after the end of the treatment. Pooled sera of each experimental group were tested for MMP-3 levels by Western blot analysis (20A) and for MMP-9 activity using gel zymography (20B). UT—untreated. The results are representative of 2 similar experiments.

[0048] FIGS. 21A-22B depict levels of MMP-3 and MMP-9 in 16/6Id-immunized BALB/c mice treated with mCDR1. In prevention experiments, mice (8/group) were treated i.v. with mCDR1 (100 μg/mouse). The results shown are of sera taken 4.5 months after the end of the treatment. In treatment experiments, mice (8/group) were treated s.c. with 100 μg/mouse mCDR1. The results are of sera obtained at the end of treatment. Pools of sera of each experimental group were tested for MMP-3 by Western blot analysis (21A) and for MMP-9 activity by gel zymography (21B). UT —untreated. The results are representative of 2 similar experiments.

[0049] FIGS. 22A-22B depict immunostaining for MMP-3 and MMP-9 of kidney sections of 16/6Id immunized BALB/c mice that were treated with mCDR1 for prevention (22A) or treatment (22B) of experimental SLE. Mice were sacrificed 8 months following disease induction, and their kidneys were removed. Cryostat sections (5 μm) were prepared and immunostained for MMP-3, MMP-9 and for the presence of immune complex deposits (x200). (W/O)—control staining to the efficiency of blocking, without the first antibody. The results are representative of 2 similar experiments.

[0050] FIGS. 23A-23B depict levels of MMP-3 and P-9 in sera of (NZB x NZW)F1 mice treated with hCDR1. In treatment experiments, mice (10/group) were injected s.c. with either PBS or 100 μg or 30 μg/mouse of hCDR1 once a week for ten weeks starting at the age of 7 months. The results represent sera collected at the middle of the treatment. Pooled sera of each experimental group were tested for MMP-3 levels by Western blot analysis (23A) and for MMP-9 activity using gel zymography (23B). The results are representative of 2 similar experiments.

[0051] FIG. 24 depicts a representative gel showing activity of MMP-2 and MMP-9 in sera of SLE patients and healthy controls. Sera (5 μl) of 40 individual SLE patients and 25 healthy controls were analysed for their MMP-2 or MMP-9 activities by gel zymography. The figure shows representative results with serum samples of the two groups.

[0052] FIG. 25 depicts a graph showing quantitative analysis of MMP-2 and MMP-9 activities in sera of SLE patients (dark columns) and healthy controls (white columns). Thirty-six serum samples of SLE patients and 15 serum samples of healthy controls were tested for MMP-2 or MMP-9 activity using specific activity assay kits. Results are expressed as the mean ± s.e.m. *P=0.0302.

[0053] FIGS. 26A-26B are graphs showing MMP-9 activity levels and disease activity indices (SLEDAI) in patients with SLE. Thirty-five serum samples from 8 males (FIG.

26A) and 27 females (FIG. 26B) SLE patients were tested for MMP-9 activity by a specific activity assay kit. The distribution of MMP-9 activity according to the SLEDAI of the patients is presented. The dashed line represents the activity of MMP-9 in healthy controls.

[0054] FIGS. 27A-27B are graphs showing pattern of MMP-2 (white circles) and MMP-9 (black circles) activities in sera of two SLE patients sampled during 4-6 years of disease. The sera were tested for MMP-2 or MMP-9 activities by specific activity assay kits. The assays were performed in duplicate.

## DETAILED DESCRIPTION OF THE INVENTION

[0055] The present invention relates, in one aspect, to synthetic peptides comprising a sequence consisting of, or found within, a CDR found in the heavy or light chain of the pathogenic human monoclonal anti-DNA 16/6Id antibody (herein identified as "human anti-DNA 16/6Id mAb" or "human 16/6Id mAb"), which antibody induces a SLE-like disease in mice.

[0056] The synthetic peptides of the invention derived from a CDR of the human 16/6Id mAb, herein identified as hCDR peptides, are preferably based on a CDR found in the heavy chain of the human 16/6Id mAb.

[0057] The CDR regions of the $V_H$ sequences of the human 16/6Id mAb are boxed in FIG. 4A of Waisman et al., 1995. The CDR regions of the heavy chains of the human 16/6Id mAb have the sequences substantially as denoted by SEQ ID NO: 8 to SEQ ID NO: 10, as follows:

CDR1: FGGTYWS [SEQ ID NO: 8]

CDR2: KIHHSGSTYYNPSLKS [SEQ ID NO: 9]

CDR3: GLLRGDEEDYDYTYGMDV [SEQ ID NO: 10]

[0058] The hCDR peptides of the invention contain at least 12 and at most 30 amino acid residues and comprise, preferably, a sequence identical to a sequence selected from the group consisting of SEQ ID NO: 8, 9 and 10, or more preferably are sequence found within said SEQ ID NO: 8, 9 or 10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of the SEQ ID NO: 8, 9 and 10 sequences by different amino acid residues; (ii) deletion of one or more amino acid residues from the SEQ ID NO: 8, 9 and 10 sequences; or (iii) addition of one or more amino acid residues to the SEQ ID NO: 8, 9 and 10 sequences.

[0059] The hCDR peptides of the invention, besides the hCDR sequence, contain further amino acid residues, preferably amino acid residues of the sequences of the human 16/6Id mAb flanking the hCDR sequences or sequences obtained by replacement of one or more of the amino acid residues of the hCDR flanking sequences by different amino acid residues, by deletion of one or more amino acid residues from the hCDR flanking sequences or by addition of one or more amino acid residues to the hCDR flanking sequences.

[0060] Thus, in one embodiment, the present invention provides a synthetic peptide based on the CDR1 of the heavy chain of the human 16/6Id mAb, said CDR1 region being of the sequence substantially as denoted by SEQ ID NO:8, said peptide being selected from the group consisting of:

[0061] (a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO: 8, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:8 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:8; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:8, or a salt or a chemical derivative of said peptide;

[0062] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0063] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0064] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0065] In one preferred embodiment of the invention, a peptide based on the CDR1 of the heavy chain of the human 16/6Id mAb is a peptide of the sequence substantially as denoted by SEQ ID NO: 11:

$X_1$YYWSWI$X_2$Q$X_3$PF$X_4$$X_5$G$X_6$EWIG [SEQ ID NO:11]

[0066] wherein $X_1$ is G or TG; $X_2$ is R or K; $X_3$ is P or S; $X_4$ is G or E; $X_5$ is K or D; and $X_6$ is R, L or S.

[0067] In a more preferred embodiment, the peptide of SEQ ID NO:11 is the 19-mer peptide herein designated "hCDR1 peptide" or simply "hCDR1", of the sequence substantially as denoted by SEQ ID NO:6:

GYYWSWIRQPPGKGEEWIG [SEQ ID NO:6]

[0068] In hCDR1, the sequence GYYWS comprised within SEQ ID NO:8, is followed by the natural sequence of the CDR1 of the heavy chain of the human 16/6Id mAb, with the exception that the natural leucine (L) residue of the mAb sequence was replaced by a glutamic acid residue (E) (bold) at position 15 of the peptide hCDR1.

[0069] In another embodiment, the peptide of SEQ ID NO:11 is an analog of the hCDR1 peptide obtained by replacement and/or addition of amino acid residues to the sequence of the hCDR1 peptide, examples thereof being the peptides of the sequences substantially as denoted by SEQ ID NO:12 to SEQ ID NO:18 (wherein the amino acids replaced or added are represented in bold):

GYYWSWIRQPPGKGLEWIG [SEQ ID NO:12]

GYYWSWIRQPPGKGSEWIG [SEQ ID NO:13]

GYYWSWIRQPPGDGEEWIG [SEQ ID NO:14]

-continued

GYYWSWIRQPPGKGLEWIG [SEQ ID NO:15]

GYYWSWIRQSPGKGLEWIG [SEQ ID NO:16]

GYYWSWIRQPPSKGLEWIG [SEQ ID NO:17]

FGYYWSWIRQPPGKGLEWIG [SEQ ID NO:18]

[0070] In a further embodiment, the invention provides a synthetic peptide based on the CDR3 of the heavy chain of the human 16/6Id mAb, said CDR3 region being of the sequence substantially as denoted by SEQ ID NO:10, said peptide being selected from the group consisting of:

[0071] (a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:10 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO: 10; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:10, or a salt or a chemical derivative of said peptide;

[0072] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0073] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0074] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0075] In one preferred embodiment of the invention, the peptide based on the CDR3 of the heavy chain of the human 16/6Id mAb is a peptide of the sequence substantially as denoted by SEQ ID NO:19:

$$YYCARX_1LLX_2X_3X_4X_5X_6DVDYX_7GX_8DV \quad \text{[SEQ. ID NO:19]}$$

[0076] wherein $X_1$ is G or F; $X_2$ is R or A; $X_3$ is G or A; $X_4$ is G or A; $X_5$ is W or A; $X_6$ is N or A; $X_7$ is Y or W; and $X_8$ is M or Q.

[0077] In a more preferred embodiment, the peptide of SEQ ID NO: 19 is the peptide herein designated "hCDR3 peptide" or simply "hCDR3", of the SEQ ID NO:7:

YYCARGLLRGGWNDVDYYGMDV [SEQ ID NO:7]

[0078] In hCDR3, the sequence GLLRGGWND-VDYYYGMDV [SEQ ID NO:10] of the CDR3 region of the heavy chain of the human 16/6Id mAb is modified by deletion of one of the tyrosine (Y) residues and is preceded by the natural sequence of the mAb.

[0079] In another embodiment, the peptide of SEQ ID NO:19 is an analog of the hCDR3 peptide obtained by replacement and/or addition of amino acid residues to the sequence of the hCDR3 peptide, examples thereof being the peptides of the sequences SEQ ID NO:20 to SEQ ID NO:27 (wherein the amino acids replaced or added are represented in bold):

YYCARGLLRGGWADVDYYGMDV [SEQ ID NO:20]

YYCARGLLRGGADVDYYGMDV [SEQ ID NO:21]

YYCARGLLRGAWDVDYYGMDV [SEQ ID NO:22]

YYCARGLLRAGWDVDYYGMDV [SEQ ID NO:23]

YYCARGLLAGGWDVDYYGMDV [SEQ ID NO:24]

YYCARFLLRGGWDVDYYGMDV [SEQ ID NO:25]

YYCARGLLRGGWDVDYYGQDV [SEQ ID NO:26]

YYCARGLLRGGWDVDYWGMDV [SEQ ID NO:27]

[0080] In still a further embodiment, the invention provides a synthetic peptide based on the CDR2 of the heavy chain of the human 16/6Id mAb, said CDR2 region being of the sequence substantially as denoted by SEQ ID NO:9, said peptide being selected from the group consisting of:

[0081] (a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:9, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:9 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:9; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:9, or a salt or a chemical derivative of said peptide;

[0082] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0083] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0084] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0085] The synthetic peptides of the present invention have 12-30, preferably 17-23, most preferably 19-22, amino acid residues, and may be manufactured by chemical synthesis or by recombinant technology by methods well known in the art.

[0086] When preparing analogs as described above obtained by substitution of amino acid residues, it is important that the substitutions be selected from those which cumulatively do not substantially change the volume, hydrophobic-hydrophilic pattern and charge of the corresponding portion of the unsubstituted parent peptide. Thus, a hydrophobic residue may be substituted with a hydrophilic residue, or vice-versa, as long as the total effect does not substantially change the volume, hydrophobic-hydrophilic pattern and charge of the corresponding unsubstituted parent peptide.

[0087] The present invention also includes chemical derivatives of a peptide of the invention. The "chemical derivative" contains additional chemical moieties not normally a part of the peptide and is encompassed by the invention as long as it retains at least a portion of the function of the peptide which permits its utility in preventing or inhibiting T cell proliferative responses and autoimmune disease. For example, a chemical derivative may result from the reaction of an organic derivatizing agent capable of reacting with selected side chains or terminal residues of said peptide, and will preferably retain at least a portion of the function of the peptide to inhibit specifically the proliferative response and cytokine secretion of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies. Among these chemical derivatives, the amides are of particular interest, both amides of carboxyl groups at the C-terminus and amides of free carboxyl groups of aspartic or glutamic acid residues. Many such chemical derivatives and methods for making them are well known in the art.

[0088] Also included in the scope of the invention are salts of the peptides and analogs of the invention. As used herein, the term "salts" refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptide molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as those formed for example, with amines, such as triethanolamine, arginine, or lysine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Such chemical derivatives and salts are preferably used to modify the pharmaceutical properties of the peptide insofar as stability, solubility, etc., are concerned.

[0089] The hCDR peptides according to the invention may be selected by testing for their potential in inhibiting the proliferative response of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies. Once a peptide in accordance with the present invention is produced, its ability to inhibit the proliferative response of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies may be readily determined by those of ordinary skill in the art without undue experimentation using tests such as those described herein. One test which may be readily conducted is for the ability of the peptides to inhibit in vitro the proliferative responses of certain T cell lines and clones specific to SLE-inducing autoantibodies. The T cell lines and clones may be, for example, the T cell lines and clones specific to the 16/6Id mAb (Fricke et al., 1991) established from immunized lymph node cells of mice by previously described methodology (Axelrod, O. and Mozes, E. *Immunobiology*, 172, 99 (1986)). Cells are exposed to the stimulating antibody presented on irradiated syngeneic spleen cells in the presence of enriched medium every two weeks. The T cell lines are cloned by the standard limiting dilution technique. The proliferative responses of these T cell lines and clones are tested, for example, by the method described in WO 96/30057, in Materials and Methods, section (g).

[0090] Another test which can be conducted in order to select analogs having the desired activity is to test for the ability of the synthetic peptides to inhibit the ability of the T cell lines and clones to provide help to peptide-specific B cells in the presence of the parent peptide. The synthetic peptides may also be tested for their ability to bind directly, following biotinylation, to MHC Class II products on antigen-presenting cells of the relevant strains. For this purpose, N-terminal biotinylation of the relevant peptides is performed at 0° C. with an excess of biotin-N-hydroxysuccinimide in aqueous solution (Mozes et al., 1989). Mouse splenic adherent cells or PBL-adherent cells ($1\times10^6$/sample) are incubated with biotinylated peptides in PBS containing 0.1% bovine serum albumin (PBS/BSA) at 37° C. for 20 hr, followed by incubation with phycoerythrin-streptavidin for 30 min at 4° C. After each incubation, the cells are washed twice with the above solution. Thereafter, the cells are analyzed by flow cytometry using FACScan. In each analysis, a minimum of 5000 cells are examined (for above procedures, see, for example, Mozes et al., 1989).

[0091] A further test which can be conducted is to test for the ability of the peptides to inhibit cytokine secretion by the T cell line or by T lymphocytes or lymph node cells of mice that are high responders to SLE-inducing autoantibodies. The cytokines are detected as follows: IL-1 activity is assessed by ELISA using a pair of capture and detecting antibodies (as described below for IL-4, IL-6, IL-10). IL-2 is directly detected using the IL-2 dependent CTLL line or by ELISA. Levels of IL-4, IL-6, IL-10, INF-γ and TNF-α in the supernatants are determined by ELISA using antibodies to the various cytokines (Pharmingen, San Diego, Calif., USA) according to the manufacturer's instructions. In addition, the ability of the peptides to elevate the level of secretion of the immunosuppressive cytokine TGF-β can be assessed by ELISA as described herein in the Examples.

[0092] Peptides which test positive in one or more of these in vitro tests will provide a reasonable expectation of in vivo activity. However, in vitro tests can also be conducted without undue experimentation. Thus, for example, adult mice may be injected with the candidate peptide at either day −3 or day 0. The mice are then immunized with the disease-inducing autoantibody or with the peptide. Ten days later, lymph node cells of the mice are tested for their ability to

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proliferate to the immunogen in order to find out the inhibitory capacity of the candidate peptide.

[0093] Another such in vivo animal test consists of measuring the therapeutic activity directly in the murine model in vivo for the production of SLE as described above. The peptides can be injected into the mice in which experimental SLE is induced by different routes at different dosages and at different time schedules. Furthermore, the treated mice can be tested periodically in order to determine the effect of the peptides on the autoantibody responses and on disease manifestations elicited in the mice by the SLE-inducing autoantibody.

[0094] Another in vivo procedure consists of assessing the candidate peptide ability to treat mice that spontaneously develop SLE e.g. (NZB x NZW)F1 mice, as described herein in the Examples.

[0095] It can thus be seen that, besides the preferred embodiments which have been shown to be operable in the examples herein, those of ordinary skill in the art will be able to determine additional analogs which will also be operable following the guidelines presented herein without undue experimentation.

[0096] In another preferred embodiment, the present invention provides a multi-epitope single peptide such as a dual peptide. In one embodiment, the dual peptide consists of two different peptides based on the same CDR such as two different peptides including the sequence of the CDR1 (SEQ ID NO:8) or of the CDR3 (SEQ ID NO:10) of the heavy chain of the human 16/6Id mab.

[0097] In another and more preferred embodiment, the dual peptide consists of two different peptides each based on a different CDR such as one peptide including the sequence of CDR1 (SEQ ID NO:8) and another including the sequence of the CDR3 (SEQ ID NO:10) of the heavy chain of the human 16/6Id mAb.

[0098] The dual peptide according to the invention preferably consists of two different peptides, one being a peptide of SEQ ID NO:11 and the other being a peptide of SEQ ID NO:19, more preferably one peptide selected from the group consisting of SEQ ID NO:6 and NO:12-18 and another peptide selected from the group consisting of SEQ ID NO:7 and NO:20-27, most preferably one peptide being the peptide of SEQ ID NO:6 and the other being the peptide of SEQ ID NO:7, the two different peptides being covalently linked to one another either directly or by a short linking chain such as a stretch of alanine residues or by a putative site for proteolysis by cathepsin. See, for example, U.S. Pat. No. 5,126,249 and European Patent 495049 with respect to such sites.

[0099] In yet another preferred embodiment, the present invention provides a multi-epitope single peptide comprising a number of the same or different peptides of the present invention in the form of a peptide polymer, obtained, for example, by polymerization of the peptides with a suitable polymerization agent, such as 0.1% glutaraldehyde (Audibert et al., 1981, Nature 289:593). The polymer will preferably contain from 5 to 20 peptide residues, preferably a peptide of SEQ ID NO: 6, 7 and 11-27. Such peptide polymers may also be formed by crosslinking the peptides or attaching multiple peptides to macromolecular carriers. Suitable macromolecular carriers are, for example, proteins, such as tetanus toxoid, and linear or branched copolymers of amino acids, such as a linear copolymer of L-alanine, L-glutamic acid and L-lysine and a branched copolymer of L-tyrosine, L-glutamic acid, L-alanine and L-lysine (T,G)-A-L-, or multichain poly-DL-alanine (M. Sela et al., 1955, J. Am. Chem. Soc. 77:6175). The conjugates are obtained, for example, by first coupling the peptide with a water-soluble carbodiimide, such as 1-ethyl-3-(3'-dimethylamino-propyl)carbodiimide hydro-chloride, and then performing the conjugation with the macromolecular carrier as described by Muller, G. M. et al. (1982) Proc. Natl. Acad. Sci. USA 79:569. The contents of the coupled peptide in each conjugate are determined by amino acid analysis, in comparison to the composition of the carrier alone.

[0100] According to a further embodiment of the present invention, one or more active peptides may be attached to a suitable macromolecular carrier or may be polymerized in the presence of glutaraldehyde.

[0101] The peptides, polymers thereof or their conjugates with suitable macromolecular carriers, are administered to patients in a form that insures their bioavailability, making them suitable for treatment. If more than one peptide of the invention is found to have significant inhibitory activity, these peptides may be given to patients in a formulation containing a mixture thereof.

[0102] The invention thus further relates to pharmaceutical compositions comprising at least one synthetic peptide or peptide polymer according to the invention, optionally with a pharmaceutically acceptable carrier.

[0103] In one preferred embodiment, the pharmaceutical compositions comprises at least one synthetic peptide of the invention, more preferably a peptide selected from the group consisting of peptides hCDR1 [SEQ ID NO:6] and hCDR3 [SEQ ID NO:7] and peptides obtained by substitution and/or addition of amino acid residues in the hCDR1 and hCDR3 sequences, in particular a peptide selected from the group consisting of peptides of SEQ ID NO:12 to SEQ ID NO:18 and of SEQ ID NO:20 to SEQ ID NO:27.

[0104] Any suitable route of administration is encompassed by the invention, including oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intraperitoneal, intradermal, transdermal or other known routes, including the enteral route. In preferred embodiments, the peptides of the invention are administered by oral, intranasal or subcutaneous routes.

[0105] The dose ranges for the administration of the compositions of the present invention should be large enough to produce the desired effect, whereby, for example, an immune response to the SLE-inducing autoantibody, as measured by T cell proliferation in vitro, is substantially prevented or inhibited, and further, where the disease is significantly treated. The doses should not be so large as to cause adverse side effects, such as unwanted cross reactions, generalized immunosuppression, anaphylactic reactions and the like.

[0106] Effective doses of the peptides of this invention for use in treating SLE are in the range of about 1 μg to 1 mg and up to 100 mg/kg body weight.

[0107] The synthetic human peptides of the invention are aimed at inhibiting or suppressing specific antigen responses

of SLE patients, without affecting all other immune responses. This approach is of the utmost importance since most diagnosed patients are young women that have to be treated for many years and the currently accepted treatment for SLE involves administration of immunosuppressive agents, such as corticosteroids and/or cytotoxic drugs, that are both non-specific and have multiple adverse side effects.

[0108] The invention further relates to a method for the treatment of systemic lupus erythematosus (SLE) comprising administering to a SLE patient an effective amount of a peptide or peptide polymer of the invention. In one preferred embodiment, the method comprises administering the peptide of the SEQ ID NO:6. In another preferred embodiment, the method comprises administering the peptide of the SEQ ID NO:7.

[0109] The invention still further relates to method of immunomodulation of SLE-associated responses in a SLE patient which comprises administering to said SLE patient an effective amount of an effective amount of a peptide or peptide polymer of the invention. In one embodiment, the method comprises down-regulating the levels of matrix metalloproteinase (MMP)-3 and/or MMP-9 activities in a SLE patient. In another embodiment, the method comprises immunomodulating the level of a cytokine activity in a SLE patient, particularly down-regulating the level of IL-2 and/or IFN-γ activity and/or up-regulating the level of TGF-β activity, in a SLE patient. In one preferred embodiment, the method comprises administering the peptide of the SEQ ID NO: 6. In another preferred embodiment, the method comprises administering the peptide of the SEQ ID NO: 7.

[0110] The invention also provides methods for assessing the effectiveness of a drug in the treatment of a SLE-patient which comprises measuring at different intervals of time the levels of MMP-3, MMP-9, IL-2 , IFN-γ and/or TGF-γ in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of MMP-3, MMP-9, IL-2 and/or IFN-γ or an increased level of TGF-β,correlates with the effectiveness of the drug.

[0111] The invention still further relates to the use of a peptide of or peptide polymer of the invention for the preparation of a pharmaceutical composition, in particular for treatment of SLE, more particularly for immunomodulation of SLE-associated responses in a SLE patient such as down-regulation of MMP-3 and/or MMP-9 and/or IL-2 and/or IFN-γ or up-regulation of TGF-β levels in a SLE-patient.

[0112] The present invention will now be described in more detail in the following non-limiting Examples and the accompanying figures.

## EXAMPLES

[0113] Materials and Methods

[0114] Mice. Female (NZB x NZW)F1 mice were obtained from the Jackson Laboratory (Bar Harbor, Me.). Female mice of the BALB/c inbred strain at the age of 6-8 weeks were obtained from the Experimental Animal Unit, The Weizmann Institute of Science, Rehovot, Israel.

[0115] Anti-DNA monoclonal antibody. The human anti-DNA mAb that bears the 16/6Id (IgG1/k) was previously characterized (Shoenfeld et al., 1982; Waisman et al., 1995). The mAb was secreted by hybridoma cells that were grown in culture and were purified by using a protein G-Sepharose column (Pharmacia, Fine Chemicals, Uppsala, Sweden).

[0116] Synthetic peptides. The synthetic murine peptides mCDR1 (SEQ ID NO:1) and mCDR3 (SEQ ID NO:3) as well as the reversed peptides that were synthesized in the reversed order of mCDR1 and mCDR3 and in the reversed order of the human peptide hCDR1, identified herein as revmCDR1 [SEQ ID NO:28], revmCDR3 [SEQ ID NO: 29], and revhCDR1 [SEQ ID NO: 30], respectively, used as control, were prepared as previously described (WO 96/30057 or using an automated synthesizer (Applied Biosystems model 430A, Germany) by using the company's protocols for t-butyloxycarbonyl (t-Boc) technique.

[0117] The reversed peptides have the sequences:

G I W E L S K E P S Q K V W Q R Y Y G T revmCDR1 [SEQ ID NO:28]

S G Q G W Y D R A Y P E W L F R A C Y Y revmCDR3 [SEQ ID NO:29]

G I W E K G K P P Q R I W S W Y Y G revhCDR1 [SEQ ID NO:30]

[0118] Induction and treatment of experimental SLE. In order to induce experimental SLE, BALB/c mice were immunized with 1-2 μg of the human mAb 16/6Id and boosted 3 weeks later. For the prevention of experimental SLE, mice were given hCDR1 or hCDR3 (mCDR1 or revmCDR1 as control peptide in Example 12) intravenously (i.v.) or s.c. concomitant with the immunization, and were injected weekly thereafter for 5 weeks. Treatment of an established disease started three and a half months following disease induction with the 16/6Id, when clinical manifestations were already observed. In Example 12, mice received 10 weekly injections (i.v. or s.c.) of mCDR1 or revmCDR1 at a dose of 100 μg/mouse.

[0119] Prevention and treatment of SLE-like disease in (NZB x NZW)F1 mice with the hCDR1 or mCDR1 peptide. For prevention of SLE, mice at the age of 2 months, before disease manifestations are observed, were injected s.c. with hCDR1 (or mCDR1 in Example 12, 250 μg/mouse) once a week for 10 weeks. To treat an established disease, mice at the age of 5-7 months were injected with hCDR1 (in Example 12, mCDR1 s.c., 250 μg/mouse) once a week for 10 weeks.

[0120] Proliferative Responses. PBL were isolated from heparinized venous blood by Ficoll-Hypaque (Pharmacia) density-gradient centrifugation. All assays were performed, in triplicate, in flat-bottomed microtiter plates (Falcon, Becton Dickinson, Oxnard, Calif., USA) in which $2\times10^5$ PBL were cultured in enriched RPMI-1640 as described (Dayan et al., 2000). The PBL were exposed to various concentrations (0.1-40 μg/well) of the human anti-DNA 16/6 Id mAb with and without the addition of the various CDR-based

peptides at a concentration of at least 10-fold excess over that of the 16/6Id. Phytohemagglutinin (PH-A; 2 μg/well) was used as a control for culture conditions at each experiment. The cultures were incubated in 7.5% $CO_2$ at 37° C. for 6 days. Eighteen hours before the cells were harvested, [$^3$H]-thymidine (0.5 μ Ci of 5 Ci/mmol)(Nuclear Research Center, Negev, Israel) was added to all cultures. Results are expressed as the mean thymidine incorporation in counts per minute (CPM) of triplicate culture ± SD, or as stimulation index (S.I.; the ratio of mean CPM at the optimal concentration of the human 16/6Id to the mean CPM in the presence of medium alone). A S.I. >2 was considered a positive response (Dayan et al., 2000). Inhibition (the ratio of mean CPM in the presence of the 16/6Id and various CDR-based peptides to the mean CPM with the 16/6Id without the CDR-based peptide) above 50% was considered positive.

[0121] Induction of Cytokine Production. Mice that were immunized with the human 16/6Id mAb and either treated or not with the CDR-based peptide, were killed at different periods during or after treatment with the peptide. Splenocytes and lymph node cells (LNC) were harvested and incubated (5×10$^6$/ml) in the presence of the 16/6Id. Supernatants were collected after 48 and 72 h.

[0122] Assessment of Cytokines in the Supernatants. Supernatants were collected 48 hours following the initiation of the cultures and stored at −70° C. Measurements of IL-2, IL-10, IFN-γ, and TNF-α were performed by ELISA by using the relevant standards, capture and detecting Abs (Pharmingen) according to the manufacturer's instructions. TGF-β was determined by an ELISA. Briefly, plates were coated with the recombinant human TGF-β1 sRII/Fc chimera (R & D Systems Inc., Minneapolis, Minn., USA), and the second Ab used was the biotinylated anti-human TGF-β1 antibody (R & D Systems Inc.). The substrate solution used was TMB color Reagent (Helix Diagnostics, West Sacramento, Calif.), and enzyme activity was evaluated by using 570- and 630-nm filters.

[0123] Detection of SLE-Associated Clinical and Pathological Manifestations. Proteinuria was measured semiquantitatively by using Combistix kit (Ames Division, Bayer Diagnostics, Newbury, U.K.). White blood cells (WBC, for leukopenia) were counted after a 10-fold dilution of heparinized blood in distilled water containing 1% acetic acid (vol/vol). For immunohistology analysis, frozen kidney sections (6 μm) were fixed and stained with FITC-conjugated goat Abs to mouse IgG (γ-chain specific; Sigma). ELISA. For measuring anti-DNA Abs, 96-well Maxisorb microtiter plates (Nunc) were coated with either methylated BSA or polyL-lysine (Sigma). The plates were then washed and coated with either 10 μg/ml of denatured calf thymus DNA (Sigma) or λ-phage double-stranded DNA (Boehringer Mannheim, 5 μg/ml). After incubation with different dilutions of sera, goat anti-mouse IgG (γ-chain specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch) was added to the plates, followed by the addition of the substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). Results were read by using an ELISA reader.

[0124] Measurement of activities of MMP-2 and MMP-9. MMP activity was tested by gelatin zymography. Pooled sera of individual mice of the different experimental groups were separated by an 8% SDS-PAGE polymerized with 1 mg/ml gelatin. Following electrophoresis, gels were washed once for 30 min in 2.5% Triton X-100 to remove the SDS, and once for 30 min in the reaction buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl2 and 0.02% (w/v) Brij 35 (pH 7.5). The reaction buffer was changed to a fresh one, and the gels were incubated at 37° C. for 24 h. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie brilliant blue.

[0125] Western blot analysis of MMP-3 in sera. Samples of 5 μl of each serum were loaded on 12% SDS/PAGE, separated under reducing conditions, and transferred to nitrocellulose. The blots were probed (0.5 μg/ml, 1 hr, room temperature) with anti-MMP-3 antibodies (Oncogene Research Products, MA, USA) and developed using chemiluminescence.

[0126] Immunostaining of kidney sections for A MMP-3 or MMP-9. For immunostaining of MMP-3 or MMP-9, kidney sections (5 μm) were fixed with cold acetone (5 min at room temperature), washed twice in PBS, permeabilized (1 min at room temperature) in 0.05% Triton X-100 (diluted in PBS), and washed twice in PBS. In order to get a specific anti-MMP staining and to avoid the staining of immune complex deposits by the FITC-labeled goat anti-mouse, kidney sections were blocked (1 hr at room temperature) with unlabeled goat anti-mouse IgG+IgM (diluted 1:1 in 1% BSA/PBS; (Jackson ImmunoResearch Laboratories) and washed 3 times with PBS containing 0.05% Tween. Monoclonal anti-MMP-9 (1:100; Chemicon International, Inc.) or anti-MMP-3 (1:50; Oncogene Research Products) antibody diluted in 1% BSA/PBS were added for 30 min, at room temperature. For all immunostaining procedures, FITC-labeled goat anti-mouse IgG+IgM (Jackson ImmunoResearch Laboratories), diluted 1:30 in 1% BSA/PBS (30 min at room temperature), was used.

[0127] Statistical analysis. Results are presented as mean ± SD. Chi-square, Wilcoxon, Mann-Whitney and t-tests were employed for statistical analysis. P <0.05 was considered significant.

## EXAMPLE 1.

[0128] Synthesis of the human peptides hCDR1 and hCDR3

[0129] The human hCDR1 (SEQ ID NO:1) and hCDR3 (SEQ ID NO:3) peptides were prepared by methods well-known in the art, for example, by chemical solid phase or solution phase synthesis using an automated synthesizer by using the manufacturer's protocols for t-butyloxycarbonyl (t-Boc), fluorenylmethoxycarbonyl (Fmoc) or other alpha-amino acid protecting group procedure essentially as described (see, for example, Peptides: Synthesis, Structure and Applications, ed. by B. Gutte, Academic Press, 1995; Peptide Synthesis Protocols, ed. by M. Pennington and B. Dunn, Humana Press, 1994; Schnolzer M. et al., In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int. J. Pept. Protein Res. 40: 180-193, 1992).

## EXAMPLE 2.

[0130] In vivo inhibition of lymph node cell (LNC) proliferation of mice immunized with mCDR1 and mCDR3 and treated with hCDR1 and hCDR3

[0131] In order to determine the inhibitory efficacy of the human peptides hCDR1 and hCDR3, we first tested their

ability to inhibit the in vivo priming of mice with the murine peptides mCDR1 and mCDR3.

[0132] To this end, BALB/c and SJL mice were immunized with mCDR1 and mCDR3, respectively. The immunizing murine peptides were injected (10 μg/mouse) in CFA intradermally in the hind footpads. Concomitant with the immunization, groups of BALB/c mice were injected subcutaneously (s.c.) with 200 μg in PBS of hCDR1 and groups of SJL mice were similarly injected with hCDR3. Ten days after the immunization, mice were sacrificed and their lymph nodes were harvested and the cells were tested for their ability to proliferate following triggering with the immunizing peptides. Briefly, LNCs of immunized mice ($0.5\times10^6$/well) were cultured (in triplicates) in the presence of different concentrations (1-20 μg /well) of the murine immunizing peptides in enriched RPMI-1640 medium supplemented with 1% normal mouse serum. Following four days of incubation, $^3$H-thymidine was added for additional 16 hours. Cells were then harvested and radioactivity was counted using a β-counter.

[0133] The results in Tables 1A and 1B represent the maximum % inhibition of proliferation of LNCs of mice immunized with mCDR1 and mCDR3 and treated with hCDR1 and hCDR3, respectively. The inhibition was calculated based on the proliferation of LNCs of mice that were not treated with the inhibitory peptides hCDR1 and hCDR3. It can be seen that hCDR1 and hCDR3 were capable of inhibiting the proliferative responses to the immunizing murine CDR peptides.

TABLE 1A

| Inhibition by hCDR1 of the proliferation of BALB/c-derived LNCs to mCDR1 | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR1 | 55% |

[0134]

TABLE 1B

| Inhibition by hCDR3 of the proliferation of SJL-derived LNCs to mCDR3 | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR3 | 55% |

EXAMPLE 3.

[0135] In vivo inhibition of LNC proliferation of mice immunized with the human anti-DNA 16/6Id mAb and treated with hCDR1 and hCDR3

[0136] Because our aim was to test the inhibitory capacity of the peptides based on the CDR of the human 16/6Id autoantibody, it was of importance to find out whether peptides hCDR1 and hCDR3 are capable of inhibiting the priming to the whole molecule of the human 16/6Id mAb. To this end, BALB/c and SJL mice were primed with the human 16/6Id mAb (2 μg/mouse) in CFA intradermaly in the hind footpads. The priming was done concomitant with the s.c. administration in PBS of 200 μg/mouse of hCDR1 to groups of BALB/c mice and of hCDR3 to SJL mice. Ten days following immunization, the mice were sacrificed and their LNCs were tested in vitro for their ability to proliferate to different concentrations (0.1-10 pg/well) of the human anti-DNA 16/6Id mAb.

[0137] Representative results of these experiments are shown in Tables 2A and 2B. The results are presented as maximum % inhibition of the proliferation to the immunizing human 16/6Id mAb of lymph node cells of mice immunized and treated with the peptides hCDR1 and hCDR3 as compared with mice that were immunized with the 16/6Id mAb but were not treated with the peptides. As can be seen, both peptides hCDR1 and hCDR3 were capable of inhibiting efficiently the priming to the human 16/6Id mAb.

TABLE 2A

| Inhibition by hCDR1 of the proliferation of BALB/c-derived LNC to the human 16/6Id mAb. | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR1 | 68% |

[0138]

TABLE 2B

| Inhibition by hCDR3 of the proliferation of SJL-derived LNC to the human 16/6Id mAb. | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR3 | 69% |

[0139] Further experiments demonstrated that a nasal administration of as low as 10 or even 2 μg/mouse of the peptide hCDR1 or hCDR3, concomitant with the immunization with the human 16/6Id mAb, inhibited by up to 100% the proliferative responses of lymph node cells to the immunizing antibody.

[0140] In another experiment, BALB/c mice were immunized with 1 μg human 16/6Id in CFA, intradermally in the hind footpads, and either were injected with hCDR1 s.c., 300 μg/mouse in PBS, or were not further treated. Ten days later the mice were sacrificed and their LNC were tested for their ability to proliferate in vitro to the human 16/6Id. Thus, popliteal LNCs ($0.5\times10^6$) were incubated in the presence of various concentrations (0.1-10 μg/well) of the human anti-DNA 16/6Id mAb. At the end of 4 days incubation, $^3$H-thymidine was added to the cultures for the last 18 hours of incubation. Cells were then harvested and radioactivity counted.

[0141] FIG. 1 shows the results of such experiments and demonstrates that hCDR1 inhibited efficiently the proliferative responses of lymph node cells of the treated mice. The % inhibition of proliferation at the various concentrations of 16/6Id mAb was as follows: 0.1 μg/well—47%; 1 μg/well—66%; 5 μg/well—76%; and 10 μg/well—62%.

[0142] The same experiment above was repeated with 50 μg hCDR1/mouse in PBS. FIG. 2 shows that hCDR1 is very effective in inhibiting the in vivo priming of mice with the whole anti-DNA 16/6Id macromolecule and even a s.c. injection with as little as 50 μg of hCDR1 inhibited significantly the ability of lymph node cells to proliferate to the 16/6Id autoantibody. The % inhibition of proliferation at the various concentrations of 16/6Id mAb was as follows: 0.1 μg/well—98%; 1 μg/well—76%; 5 μg/well—73%; and 10 μg/well—64%.

EXAMPLE 4.

[0143] Peptide hCDR1 immunomodulates cytokine production

[0144] The lymph node cells of the BALB/c mice treated with 50 μg of hCDR1 of Example 3 were also stimulated with the human 16/6Id mAb for cytokine production and the supernatants were tested for cytokine (INF-γ TGF-β and IL-10) secretion, by ELISA.

[0145] FIG. 3 shows results of a representative experiment. It can be seen that hCDR1 down-regulated the production of INF-γ (FIG. 3A) and up-regulated the secretion of TGF-β (FIG. 3B) and IL-10 (FIG. 3C). It should be noted that a peptide used as control (p259-271, that was shown to be a myasthenogenic peptide) did not affect significantly INF-γ and IL-10 production and was less effective in up-regulating TGF-β.

EXAMPLE 5.

[0146] Peptides hCDR1 and hCDR3 inhibit the proliferative response of PBL of SLE patients to the human 16/6Id mAb.

[0147] Sixty-two patients, 9 males (14.5%) and 53 females (85.5%) with SLE participated in our study. The mean age at diagnosis was 32.95±12.92 (range 12-61) years and the mean follow-up period was 10.98±10.76 (range 1-32) years. All patients fulfilled at least 4 of the American College of Rheumatology (ACR) revised diagnostic criteria for SLE (Tan et al., 1982). Patients were recruited from three Israeli Medical Centers (Kaplan, Rehovot; Ichilov, Tel Aviv; Assaf-Harofeh, Rishon Lezion). Disease activity was determined according to the SLEDAI lupus activity index (Bombardier et al., 1992). A control group of 36 sex- and age-matched healthy control volunteers was studied concomitantly with the SLE patients. The study was approved by the Ethical Committee of the Medical Center.

[0148] It was of interest to investigate whether the peptides hCDR1 and hCDR3, which are based on the CDR1 and CDR3 of the human 16/6Id mAb, are capable of inhibiting the specific proliferative responses of PBL of SLE patients to the human 16/6Id mAb. To this end, we first had to identify the patients whose PBL could be stimulated to proliferate by the human 16/6Id mAb (responders).

[0149] Therefore, PBL of 62 consecutive SLE patients were cultured in the presence of the human 16/6Id and their proliferative responses and ability to secrete IL-2 were determined. PBL of 24 out of the total of 62 (39%) and of 23 out of 55 (42%) SLE patients tested responded (SI >2, range 2-5.6) by proliferation and by IL-2 secretion (SI >2, range 2-60), respectively. The frequency of responders in the group of SLE patients was lower than that observed in the group of healthy donors that was tested as control. Thus, PBL of 21 out of a total of 36 (58%) healthy donors responded by proliferation to the 16/6Id. The extent of proliferation (SI levels) was similar for the SLE patients and for the healthy controls who responded to the 16/6Id. However, as shown in FIG. 4, the optimal response to the 16/6Id of PBL of the control donors was observed at higher concentrations of 16/6Id as compared to the SLE patients.

[0150] No differences could be demonstrated between gender and age of SLE patients that responded to the 16/6Id and of the non-responder group of patients. However, the patients whose PBL proliferated in response to the 16/6Id were sick for a shorter period of time (a mean of 9.78±8.36 vs. 11.73±12.06 years for responders and non-responders, respectively; P:≤0.036). Table 3 summarizes the clinical characterization of the 16/6 Id responder and non-responder groups of SLE patients. As can be seen in the Table, both groups were similar in most SLE related clinical manifestations. The SLE disease activity score (SLEDAM) and the number of SLE diagnostic criteria were also similar in the two groups. Nevertheless, a higher frequency of neurological (both seizures and psychosis) and hematological involvement and a lower rate of renal involvement were noted in the responder group of patients in comparison to the group of non-responders. However, probably because of low number of patients in the relevant subgroups, the above differences did not reach statistical significance. Moreover, relatively less responder patients were determined between those treated with either steroids or cytotoxic agents at the time of the study. It is noteworthy that significantly more patients who never received steroids responded to the 16/6Id in comparison to the non-responder group (54% vs 21%; P=0.023).

[0151] It is noteworthy that the efficacy of the CDR-based peptides to inhibit the proliferative responses of PBL of healthy donors to the 16/6Id was much lower than that observed for PBL of SLE patients (not shown).

TABLE 3

Clinical and laboratory characterization of SLE patients.

| | All Patients | Responders | Non-responders |
|---|---|---|---|
| **A: Diagnostic Criteria*** | | | |
| Number of Patients (%) | 62 (100) | 24 (39) | 38 (61) |
| Malar rash | 19/62 (30.1) | 8/24 (33.3) | 11/38 (29) |
| Discoid rash | 9/62 (15) | 3/24 (12.5) | 6/38 (16) |
| Photosensitivity | 21/62 (34) | 9/24 (37.5) | 12/38 (32) |
| Mucosal ulcers | 17/62 (27.4) | 8/24 (33.3) | 9/38 (23.7) |
| Arthritis | 46/62 (74.2) | 18/24 (75.2) | 27/38 (71) |
| Serositis | 14/62 (22.6) | 5/24 (20.8) | 9/38 (23.7) |
| Neurologic disorder* | 5/62 (8.1) | 4/24 (16.7) | 1/38 (2.7) |
| Renal disorder* | 24/62 (38.8) | 7/24 (29.2) | 17/38 (44.8) |
| Hematological Disorder* | 44/62 (71) | 19/24 (79.2) | 25/38 (65.8) |
| ANA | 61/62 (98.4) | 24/24 (100) | 37/38 (92.1) |
| α-dsDNA | 54/62 (87.1) | 19/24 (79.2) | 35/38 (92.1) |
| APLA | 35/62 (56.5) | 12/24 (50.0) | 23/38 (60.53) |
| **B: Disease Activity** | | | |
| SLEDAI Score | 6.65 ± 5.12 | 7.29 ± 1.06 | 6.34 ± 0.84 |
| Number of ACR diagnostic criteria | 5.44 ± 1.39 | 5.54 ± 0.33 | 5.34 ± 0.2 |
| **C: Current treatment†** | | | |
| NSAIDS | 17/62 (27.4) | 6/24 (25) | 11/38 (29) |
| Anti-Malarial | 37/62 (59.7) | 15/24 (62.5) | 22/38 (57.9) |
| Steroids* | 33/62 (53.2) | 11/24 (45.8) | 22/38 (57.9) |
| Cytotoxic* | 10/62 (16.1) | 2/24 (8.3) | 8/38 (21) |

*Clinical involvement was defined according to the ACR revised criteria. Anti-nuclear antibodies (ANA) and anti-dsDNA antibodies were determined by Hep2 cells and Crithidia lucilea, respectively. Anti-phospholipid antibodies (APLA) were defined as reactivity in one or more of the following assays: false positive VDRL, lupus anti-coagulant (LAC) or ELISA for anticardiolipin antibodies.

†The anti-malarial agent, hydroxychloroquine, was used at a dose of 200–400 mg/day; Steroid treatment was defined as a daily dose ≥ 5 mg of prednisone; cytotoxic agents used were cyclophosphamide (0.75–1.0 g/m²; monthly) or azathioprine (100–150 mg/day).

*Parameters for which tendency was observed towards differences between the two groups of responder and non-responder SLE patients.

[0152] To test the ability of the peptides hCDR1 and hCDR3 to inhibit the proliferative response of PBL of SLE patients to the human 16/6Id mAb, PBL ($2\times10^5$/well) of SLE patients were stimulated in vitro in triplicates with different concentrations (0.1-20 μg/well) of the human 16/6Id mAb in the absence or presence of the peptides hCDR1 and hCDR3 (either 50 or 100 μg/well). Following 6 days of incubation, $^3$H-thymidine (0.5 μCi of 5 Ci/mmol) was added to each well for additional 18 hours of incubation. Cells were then harvested and radioactivity was counted using a β-counter. Results were expressed as mean counts per minute (cpm) of triplicate cultures. Stimulation indices (the ratio of mean cpm at the optimal concentration of 16/6Id to mean cpm without 16/6Id) were then calculated. A stimulation index (SI) >2 was considered positive.

[0153] PBL of 24 out of the total of 62 (39%) SLE patients were found to proliferate to the 16/6Id mAb. The ability of the peptides hCDR1 and hCDR3 to inhibit the proliferative responses to the whole molecule of the 16/6Id autoantibody was tested on PBL of 19 responders SLE patients.

[0154] Table 4 shows the results of these experiments. Inhibition of above 50% of the proliferative capacity was considered positive. The Table represents the highest positive inhibition capacity for each peptide. It can be seen that the human hCDR1 and hCDR3 inhibited the proliferation of PBL of 16/19 (84.2%) and 15/19 (78.9%), respectively, of the 19 responders tested. Both peptides inhibited the proliferation of PBL of 18/19 (95%) of responders tested. It can also be seen in the Table that the magnitudes of inhibitions were similar for both peptides. Thus, it can be concluded that peptides based on CDR1 and CDR3 of the human 16/6Id mAb are efficient inhibitors of the proliferation of PBL of SLE patients to the human 16/61d mAb.

TABLE 4

Inhibition of proliferation of PBL of SLE patients by peptides hCDR1 and hCDR3.

| | | Percent Inhibition | |
|---|---|---|---|
| Number | Initials | hCDR1 | hCDR3 |
| 1. | B. L. | 62 | <50 |
| 2. | M. D. | 70 | 75 |
| 3. | T. L. | 69 | <50 |
| 4. | Z. D. | <50 | <50 |
| 5. | N. N. | 88.5 | 87.5 |
| 6. | S. S. | 80 | 80 |
| 7. | S. H. | 76 | 70.4 |
| 8. | S. D. | 58 | 56 |
| 9. | A. N. | 69.5 | 65 |
| 10. | I. I. | 68.2 | 71.8 |
| 11. | L. J. | <50 | 72 |
| 12. | V. L. | 82 | 86 |
| 13. | M. S. | 63 | 64 |
| 14. | D. S. | 56 | 74 |
| 15. | Z. A. | 63 | 69 |
| 16. | B. M. | <50 | 68 |
| 17. | S. N. | 70.5 | 77.8 |
| 18. | G. M. | 51.5 | <50 |
| 19. | N. I. | 63 | 60.8 |
| Mean ± SD | | 68.12 ± 9.57 | 71.82 ± 8.44 |

EXAMPLE 6.

[0155] Specificity of the inhibitory capacity of hCDR1 and hCDR3

[0156] It is important to demonstrate that the inhibitory effects of the hCDR-based peptides are specific to SLE-associated responses. To this end the peptides hCDR1 or hCDR3 were added to cultures of PBL of SLE patients that were stimulated with the mitogen phytohemagglutinin (PHA, 2 μg/ml). The results of such an experiment performed with PBL of one SLE patient is shown in FIG. 5. The peptides hCDR1 and hCDR3 could not inhibit the proliferative responses (expressed in cpm) of the PBL to the mitogen PHA and the proliferative responses were similarly high in the absence (black column) or presence of either hCDR1 or hCDR3.

[0157] In another experiment, cultures of PBL of SLE patients were stimulated with the human 16/6Id mAb and then incubated with the human peptides hCDR1 or hCDR3 or with the murine peptide mCDR3 as a control. The results of such an experiment performed with PBL of one SLE patient are shown in FIG. 6. As shown in FIG. 6, whereas both peptides hCDR1 and hCDR3 based on the human autoantibody inhibited efficiently the proliferative responses of PBL to the human 16/6Id mAb, the peptide mCDR3 based on the CDR3 of the murine antibody did not inhibit the proliferation.

[0158] Two additional control peptides were used in these experiments, namely peptides synthesized at the reversed order of the murine MCDR1 and mCDR3 pieptides (revmCDR1 and revmCDR3), and the results are shown in FIG. 7. It can be seen that the two reversed peptides failed to inhibit significantly the proliferative responses of the PBL of the SLE patient to human 16/6Id mAb while peptides hCDR1 and hCDR3 did inhibit efficiently the proliferation, demonstrating that the inhibition of proliferation by the human hCDR-based peptides is specific to the peptides and to the SLE-associated T cell responses.

EXAMPLE 7.

[0159] Down-regulation of the secretion of IL-2 by PBL of SLE patients in the presence of the peiptides hCDR1 and hCDR3

[0160] It was of interest to find out whether the hCDR peptides are capable of inhibiting IL-2 secretion by PBL of SLE patients following stimulation with the human 16/6Id mAb. Such inhibition might also suggest that the human CDR-based peptides inhibit the proliferative responses to the 16/6Id mAb at least partially by down-regulating IL-2 secretion. To this end, PBL of SLE patients were incubated with the human 16/6Id mAb in the absence or presence of the peptides hCDR1 or hCDR3. Supernatants of the cultures were collected following 48 hours of incubation. Assays to determine levels of IL-2 in the supernatants were performed using the CTLL IL-2 dependent line. Briefly, cells of the CTLL line ($2\times10^4$/well) were incubated in the presence of the different supernatants for 24 hours, followed by the addition of $^3$H-thymidine for an additional 18-hour incubation period. Cells were then harvested and radioactivity counted using a β-counter. Results were calculated based on recombinant human IL-2 used as a standard. The ability of the peptides to inhibit the IL-2 secretion of PBL of 23 responders stimulated by the human 16/6Id was tested. The results, summarized in Table 5, show that hCDR1 and hCDR3 inhibited the secretion of IL-2 by PBL of 21/23 and 19/23 patients, respectively. Inhibition of proliferative responses of PBL directly correlated with IL-2 inhibition by the CDR-based peptides. Thus, inhibition of IL-2 secretion was observed in all cases where inhibition of proliferation were determined.

[0161] The results obtained with PBL of one SLE patient represented in FIG. 8 (secretion of IL-2 is expressed in

pg/ml) show that both hCDR1 and hCDR3 inhibited 100% of the IL-2 secretion by PBL of a SLE patient triggered by the human 16/6Id mAb.

TABLE 5

Inhibition of IL-2 secretion by hCDR1 and hCDR3

| Peptide | Inhibitory Activity* % | Maximum Inhibition % |
|---|---|---|
| hCDR1 | 91 (21/23) | 84 ± 31 |
| hCDR3 | 83 (19/23) | 78 ± 34 |

*IL-2 secretion in the presence of 16/6 Id alone was considered as 100%. Inhibition of 50% or more was considered significant.

EXAMPLE 8.

[0162] Up-regulation of the secretion of the immunosuppressive cytokine TGF-β by CDR-based peptides

[0163] In attempts to shed light on the mechanisms by which the human CDR-based peptides inhibit the proliferative responses to the human monoclonal anti-DNA 16/6Id antibody, the levels of the immunosuppressive cytokine TGF-β in the supernatants of the cell cultures were determined. The rationale behind these experiments is based on our previous findings of elevated levels of TGF-β in cultures of splenocytes of mice with SLE either induced with the human anti-DNA 16/6Id mAb or spontaneous {(NZB x NZW) F1 mice} following treatment with the peptides based on mouse CDR (Elias et al., 2001). The elevation in the levels of TGF-β correlated with amelioration of disease manifestations in the treated mice.

[0164] For this purpose, supernatants were removed from cultures of PBL of various SLE patients following 48 hours incubation with the human 16/6Id mAb in the absence or presence of the peptides hCDR1 or hCDR3. TGF-β was determined by ELISA according to the manufacturer's instructions. Briefly, Maxisorb plates (Nunc) were coated with recombinant human TGFβaRII/Fc chimera (R&D Systems) diluted in PBS (100 ng/ml). After blocking, cell supernatants were added. After 18 hours incubation the detecting biotinylated anti-human TGF-β antibody (R&D Systems) was added. The substrate solution used was the TMB Colour Reagent (Helix Diagnostics) and enzyme activity was evaluated by the MRX ELISA reader using the 570 nm and 630 nm filters. The results are summarized in Table 6.

[0165] The results in FIG. 9 demonstrate that peptides hCDR1 and hCDR3 triggered a significant up-regulation in the secretion of TGF-β (expressed in pg/ml) by the PBL of one representative SLE patient that were stimulated with the pathogenic human 16/6Id mAb.

TABLE 6

Up-regulation of TGF-β secretion of 16/6 Id-induced stimulation of PBL of SLE patients with hCDR1 and hCDR3 peptides.

| Peptide | Up-regulation of TGF-β % | Maximum Up-regulation % |
|---|---|---|
| hCDR1 | 100 (19/19) | 305 ± 221 |
| hCDR3 | 100 (19/19) | 338 ± 242 |

[0166] Secretion of TGF-β in the presence of 16/6Id alone (mean 636±25 βg/ml) was considered as 100%. Results are expressed as percent secretion above that in the presence of 16/6Id alone.

EXAMPLE 9.

[0167] Immunomodulation of SLE manifestations in mice by hCDR1: Amelioration of disease manifestations following treatment of (NZB x NZW)F1 mice with hCDR1.

[0168] As shown above, the peptides based on the CDR of the human anti-DNA 16/6Id mAb were capable of inhibiting the priming of lymph node cells to the 16/6Id mAb and the proliferative responses of PBL of SLE patients to the 16/6Id mAb with similar efficiency. It was thus of interest to find out if these peptides can immunomodulate an SLE-like disease in animal models.

[0169] The experiments aimed at addressing the ability of the human peptides to treat an established SLE disease were performed first with the hCDR1 peptide. To this end, a few experiments were designed in which SLE-prone (NZB x NZW)F1 mice were treated with the hCDR1 peptide at the age of 5 and a half months, when manifestations of the SLE-like disease (anti-dsDNA, proteinuria, etc) are already observed. The hCDR1 peptide was administered in PBS s.c. weekly, for 10 weeks. The efficacy of different doses (50, 100 and 200 μg/mouse) of the hCDR1 peptide was tested. Control groups were injected with the vehicle PBS. The treatment led to a moderate reduction in the anti-dsDNA autoantibody titers. Thus, at a 1:1250 sera dilutions, O.D. values of 0.586±0.1, 0.2710.1, 0.37±0.1 and 0.29±0.1 were measured at the end of the treatment for sera of PBS-treated mice, mice treated with 50 μg/mouse, 100 μg/mouse and 200 μg/mouse hCDR1, respectively.

[0170] We performed another experiment in which 7-month old (NZB x NZW)F1 mice were treated weekly for 10 weeks with 300 μg of hCDR1 injected s.c. in PBS. A mild reduction in the anti-DNA antibody titers could be observed in sera of hCDR1-treated mice. Nevertheless, Table 7 shows that treatment with hCDR1 resulted in a reduction in proteinuria and in a significant decrease in the immune complex deposits (ICDs) in the kidneys of the treated mice. The results in the Table express the intensity of ICDs where 0–no ICD; 1 –moderate ICD; 2–severe ICD and 3–severe and extremely intense ICD.

TABLE 7

Clinical manifestations of (NZB x NZW)F1 mice treated with hCDR1 at the age of 7 months

| Treatment | Proteinuria g/L ± SEM | Immune Complex Deposits ± SEM |
|---|---|---|
| Untreated | 4.80 ± 2.56 | 2.57 ± 0.29 |
| hCDR1 300 μg/mouse | 1.08 ± 0.38 | 1.44 ± 0.41 p = 0.085 |

p was calculated in comparison to untreated group of mice.

[0171] We then performed an additional experiment using 400 μg/mouse of hCDR1 to find out if more beneficial effects can be achieved by increasing the dose of the peptide. Treatment with 400 μg/mouse of hCDR1 had no greater effect on the anti-DNA antibody titers and, as can be seen in Table 8, the effect on the kidney disease was similar to that observed following treatment with the 300 μg dose.

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TABLE 8

Clinical manifestations of (NZB × NZW)F1 mice treated with hCDR1 at the age of 6 months

| Treatment | Proteinuria g/L ± SEM | Immune Complex Deposits ± SEM |
|---|---|---|
| Untreated | 4.59 ± 2.53 | 2 ± 0.29 |
| hCDR1 400 μg/mouse | 0.77 ± 0.32 | 1.42 ± 0.19 p = 0.05 |

p was calculated in comparison to untreated group of mice.

[0172] We have therefore performed an additional experiment in which mice were treated with 300 μg of hCDR1 at the age of 7 months and used a control peptide, namely, the reversed hCDR1. The aim of the experiment was to find out if, in addition to the amelioration in the clinical manifestations, treatment with hCDR1 immunomodulates cytokine production. FIG. 10 demonstrates the mild reduction in anti-DNA autoantibody levels. Table 9 shows the proteinuria measured at different time points.

TABLE 9

Clinical manifestations of (NZB × NZW)F1 mice treated with hCDR1 at the age of 7 months

| | Proteinuria g/L ± SEM Following treatment No: | | |
|---|---|---|---|
| Treatment | 5 | 7 | 9 |
| Untreated | 2.65 ± 1.76 | 3.89 ± 2.73 | 7.55 ± 3.95 |
| hCDR1 300 μg/mouse | 0.61 ± 0.23 | 0.65 ± 0.32 | 1.02 ± 0.39 p = 0.05 |
| revhCDR1 300 μg/mouse | 6.94 ± 2.53 | 6.76 ± 2.91 | 5.99 ± 3.08 |

[0173] The effect of the treatment with hCDR1 can be seen at all measurements. Kidney damage is one of the main manifestations of the SLE-like disease in the (NZB x NZW)F1 mice. Ten-week treatment with the hCDR1 peptide reduced significantly the kidney disease. The 50 μg dose was less efficient than the 100 and 200 μg doses in treating the kidney disease. The latter two doses were similarly efficient.

[0174] FIGS. 11A-11D are photos showing representative kidney sections of mice treated with the 100 μg dose of hCDR1. Thus, mice at the age of 9 months were sacrificed and their kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections of 5 μm were air dried and fixed in acetone. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ-chain specific). FIGS. 11A and 11B show kidney sections of a mouse of the PBS-treated group (control) whereas FIGS. 11C and 11D show kidney sections of a mouse treated with 100 μg of hCDR1. It can be seen in the figures that the treatment reduced the number of immune complexes as well as their intensity (11A,11C x 100; 11B, 11D x 400).

[0175] Similar results were obtained when groups of (NZB x NZW)F1 mice were treated with hCDR1 at the age of 7 months when their full blown disease is already observed. The mice were treated for 10 weeks with either 100 μg/mouse or 300 μg/mouse. Treatment with both doses led to a moderate reduction in the anti-DNA autoantibody titers, similar to the above described results. A reduction in proteinuria in comparison to the PBS-treated group was measured as well. The kidney disease was ameliorated following treatment with both doses; nevertheless, a more significant effect was determined in the group of mice treated with the 300 μg dose. FIGS. 12A-12F are representative mouse kidney sections of each group where A, B represent an untreated mouse; C, D represent a kidney of a hCDR1-treated mouse and E, F represent a kidney section of a mouse treated with the reversed hCDR1. A, C, E x100 and B, D, F x 400.

[0176] FIGS. 13A-13C show the cytokine (INF-γ, IL-10 and TGF-β) pattern as measured by ELISA in supernatants of Con A-stimulated cultures of splenocytes of mice of the 3 groups taken at the end of treatment (10 weekly s.c. injections). It can be seen that INF-γ (FIG. 13A) and IL-10 (FIG. 13B, to a lesser extent) were down-regulated in the treated mice. An increased secretion of TGF-β could be seen in the supernatants of non-stimulated cells (FIG. 13C, left). Con A did not trigger the cells to secrete more TGF-β (FIG. 13C, right).

[0177] The results summarized above indicate that long term treatment with hCDR1 ameliorates disease manifestations and immunomodulates cytokine secretion.

Example 10.

[0178] Treatment of BALB/c mice with the human hCDR1 peptide following induction of experimental SLE

[0179] It was of interest to find out whether the hCDR1 peptide is capable of treating experimental SLE induced in mice with the human anti-DNA 16/6Id mAb. Therefore, BALB/c mice were immunized and boosted with the 16/6Id pathogenic autoantibody. Three and a half months following the booster injection, when the mice developed already disease manifestations, they were divided into three groups. One group was not treated, the second group was treated with 100 μg/mouse of the hCDR1 and the third group was treated for 10 weeks with 300 μg/mouse of the hCDR1 peptide. The mice were followed for disease manifestations.

[0180] Table 10 presents the results obtained in testing individual mice at the end of treatment. An amelioration was observed for all measured clinical manifestations with both doses used for the treatment [anti-dsDNA autoantibodies, white blood cells counts (WBC) and urine protein]. Analysis of kidneys of mice sacrificed at the end of the experiment (7 months after booster injections) demonstrated immune complex deposits in kidneys of 9/10 mice that were immunized with the 16/6Id mAb and were not treated. In contrast, immune complex deposits were observed in kidneys of only 3/10 and 2/9 mice that were immunized with the 16/61d mAb and treated with 100 μg/mouse and 300 μg/mouse hCDR1, respectively. Thus, it is shown that the human peptide hCDR1 is capable of treating an established experimental SLE.

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TABLE 10

The effect of treatment of BALB/c mice afflicted with experimental SLE with 100 μg or 300 μg hCDR1 peptide.

| | Control, healthy mice Mean ± SD | 16/6Id injected Mean ± SD | 16/6Id injected + hCDR1 100 μg Mean ± SD | 16/6Id injected + hCDR1 300 μg Mean ± SD |
|---|---|---|---|---|
| Proteinuria g/l | 0.12 ± 0.16 | 0.65 ± 0.36 | 0.3 ± 0.02 | 0.34 ± 0.25 |
| WBC/mm$^3$ | 7440 ± 960 | 3260 ± 920 | 6090 ± 2160* | 5890 ± 2460* |
| Anti-dsDNA O.D. (1:50) | 0.1 ± 0.05 | 1.1 ± 0.6 | 0.3 ± 0.2* | 0.55 ± 0.3* |

*significantly different ($p < 0.05$) from 16/6Id injected group.

[0181] In another experiment, BALB/c mice were immunized and boosted with the human 16/6Id for induction of experimental SLE. Two months after the boost, mice were divided into groups (8 mice per group). The mice were treated with 200, 300 or 400 μg/mouse for 10 weeks. The mice were followed for antibody production, leukopenia, proteinuria and, when sacrificed, two months after the end of treatment, their kidneys were analyzed for immune complex deposits. The results, summarized in Table 11, show no effect of treatment on the 16/6Id specific antibody response.

TABLE 11

Effect of treatment of BALB/c mice afflicted with experimental SLE with 200 μg, 300 μg or 400 μg hCDR1 peptide.

| | 16/6Id Mean ± SD | 16/6Id + hCDR1 200 μg/ mouse Mean ± SD | 16/6Id + hCDR1 300 μg/ mouse Mean ± SD | 16/6Id + hCDR1 400 μg/ mouse Mean ± SD | Control Mean ± SD |
|---|---|---|---|---|---|
| Proteinuria g/l | 0.5 ± 0.34 | 0.13 ± 0.16 p = 0.0131 | 0.16 ± 0.16 p = 0.0269 | 0.11 ± 0.15 p = 0.0070 | 0.03 ± 0.09 p = 0.0002 |
| WBC/mm$^3$ | 2871 ± 1205 | 5625 ± 1659 p = 0.0047 | 4677 ± 1508 p = 0.0269 | 4012 ± 1421 p = 0.0760 | 7280 ± 352 p = 0.0001 |
| Intensity of ICD in kidneys | 1.86 ± 0.38 | 0.57 ± 0.97 p = 0.0189 | 0.75 ± 0.71 p = 0.0047 | 0.71 ± 0.76 p = 0.0055 | 0 |
| Anti-dsDNA antibodies at a dilution of: 1:250 | 1.16 ± 0.37 | 0.69 ± 0.39 p = 0.0189 | 0.45 ± 0.24 p = 0.0005 | 0.8 ± 0.39 p = 0.0603 | 0.039 ± 0.025 p = 0.0001 |
| Anti-16/6 Id antibodies at a dilution of 1:1000 | 2.55 ± 0.09 | 2.7 ± 0.12 | 2.8 ± 0.11 | 2.75 ± 0.1 | 0 |

[0182] However, treatment affected anti-DNA antibody titers, leukopenia, levels of proteinuria and, more importantly, immune complex deposits in the kidneys. The control group of the experiment is of BALB/c mice of the same age that were not immunized or treated at all. This experiment suggested that hCDR1 is effective in treating experimental SLE and no advantage of the 400 μg dose could be seen.

[0183] Therefore, an additional experiment was performed in which mice were treated with 200 and 300 μg /mouse. A control peptide was used in this experiment, the reversed hCDR1. The aim of this experiment has been, in addition to the comparison between the 200 and 300 μg treatment doses, to study the effect of treatment on the cytokine production in the treated mice. Sixty BALB/c mice were immunized and boosted with the 16/6Id. Three months after the boost, the mice were divided into groups (15 mice per group) that either were not further treated or were weekly treated with 200 or 300 μg of hCDR1. An additional group was treated with 300 μg of reversed hCDR1. A fifth group (control) was not immunized and was not further treated. Mice were followed for antibody production and disease manifestations. As seen in Table 12, the anti-16/6Id antibody levels (at a dilution of 1:10000) in sera of the mice did not differ between groups. Treatment with both 200 and 300 μg reduced the anti-DNA antibody levels as can be seen in the Table (the sera were diluted 1:1250). Table 12 also summarizes the clinical manifestations in the different groups of mice. It can be seen that, although both doses were effective in reducing leukopenia and proteinuria, the effect of the 200 μg dose in this experiment did not reach significance (mice were sacrificed a month after the end of treatment).

TABLE 12

Effect of treatment of BALB/c mice afflicted with experimental SLE with 200 μg or 300 μg hCDR1 or 300 μg rev-hCDR1

| | 16/6Id Mean ± SD | 16/6Id + hCDR1 200 μg/ mouse Mean ± SD | 16/6Id + hCDR1 300 μg/ mouse Mean ± SD | 16/6Id + rev-hCDR1 300 μg/ mouse Mean ± SD | Control Mean ± SD |
|---|---|---|---|---|---|
| Proteinuria g/l | 0.58 ± 0.34 | 0.17 ± 0.15<br>p = 0.0946 | 0.085 ± 0.01<br>p = 0.0088 | 0.8 ± 0.3<br>p > 0.5 | 0.031 ± 0.09 |
| WBC/mm³ | 2800 ± 300 | 4900 ± 800<br>p = 0.004 | 5800 ± 300<br>p = 0.004 | 4180 ± 831<br>p = 0.0079 | 7900 ± 400 |
| Intensity of ICD in kidneys | 1.6 ± 0.55 | 1 ± 0.63<br>p = 0.0887 | 0.5 ± 0.5<br>p = 0.0192 | 0.85 ± 0.75<br>p = 0.0628 | 0 |
| Anti-dsDNA antibodies 1:1250 | 0.47 ± 0.3 | 0.23 ± 0.1<br>p = 0.0234 | 0.2 ± 0.1<br>p = 0.0145 | 0.35 ± 0.3<br>p = 0.343 | 0.002 ± 0.002 |
| Anti-16/6 Id antibodies 1:10000 | 0.89 ± 0.1 | 0.96 ± 0.2 | 0.93 ± 0.1 | 0.85 ± 0.13 | 0.03 ± 0.1 |

[0184] FIGS. 14A-F represent one kidney of each group where A,B represent an untreated mouse; C,D represent a kidney of a hCDR1-treated mouse and E,F represent a kidney section of a mouse treated with the reversed hCDR1. Pictures A,C,E x 100 and B,D,F x 400.

[0185] FIGS. 15A-E shows levels of the different cytokines in supernatants of 16/6Id stimulated lymph node cultures of the treated mice. The latter tests were performed at the end of treatment (after 10 treatment injections). It can be seen that treatment with hCDR1 (both doses) down regulated INF-γ (FIG. 15A), IL-10 (FIG. 15C) and TNF-α (FIG. 15B). On the other hand, hCDR1 up-regulated the levels of TGF-β. Because splenocytes usually secrete more TGF-β (FIG. 15D) than lymph node cells, TGF-β was also measured in supernatants of 16/6Id triggered spleen cells of mice of the different groups (FIG. 15E).

[0186] Thus, hCDR1 ameliorates disease manifestations and immunomodulates cytokine production in mice afflicted with an induced experimental SLE.

EXAMPLE 11.

[0187] Transfer of the beneficial effects on lupus manifestations by splenocytes of hCDR1-treated mice.

[0188] It was of importance to investigate whether the beneficial effects of treatment with hCDR1, that are manifested by the down-regulation of disease manifestations, can be transferred by spleen cells of treated mice. To this end, we have performed an experiment in which 8-month old (NZB x NZW)F1 mice, that suffer already from the full-blown lupus-like disease, were divided into 2 groups. Group 1 was not treated and mice of group 2 were transferred with $20\times10^6$ spleen cells of 3-month old (NZB x NZW)F1 mice that were injected 3 times (i.e., every other day) with 300 μg/mouse of hCDR1. All splenocytes were injected intraperitoneally. Mice were tested for anti-dsDNA autoantibody production, disease manifestations, and were sacrificed 4 weeks after cell transfer. Transfer of cells of hCDR1-treated mice caused a mild reduction in the levels of anti-DNA antibodies.

[0189] Table 13 demonstrates that transfer of splenocytes of hCDR1-treated mice resulted in a significantly lower proteinuria and in a reduction in immune complexes in the kidneys.

TABLE 13

Clinical lupus manifestation in mice recipient of spleen cells of mice treated with hCDR1

| Group No. | Treatment | Proteinuria g/L ± SEM | I.C.D ± SEM |
|---|---|---|---|
| 1 | Non-treated | 15.07 ± 4.92 | 2.25 ± 0.47 |
| 2 | $20\times10^6$ spleen cells from hCDR1 treated mice | 0.97 ± 0.67<br>p = 0.05 | 0.75 ± 0.47<br>p = 0.05 |

The p-values are calculated in comparison to gr1 of untreated mice

[0190] The experiment was repeated a few more times using revhCDR1 as control. The results were similar to those in the first experiment, namely, cell transfer of the treated mice affected slightly anti-DNA antibody titres and significantly proteinuria and kidney damage. The clinical manifestations of a representative experiment are shown in Table 14.

TABLE 14

Clinical lupus manifestation in mice recipient of spleen cells of mice treated with hCDR1

| Group No. | Treatment | Proteinuria g/L ± SEM | I.C.D ± SEM |
|---|---|---|---|
| 1 | Non-treated | 10.9 ± 3.05 | 2.3 ± 0.26 |
| 2 | $20\times10^6$ spleen cells from hCDR1 treated mice | 2.91 ± 1.6<br>p = 0.0402 | 1.33 ± 0.35<br>p = 0.034 |
| 3 | $20\times10^6$ spleen cells from revhCDR1 treated mice | 5.38 ± 2.34<br>p = 0.0931 | 1.7 ± 0.23<br>p = 0.065 |

The p-values are calculated in comparison to gr1 of untreated mice

[0191] It can be seen that the down-regulating effect of treatment with hCDR1, could be transferred by spleen cells of the treated mice.

[0192] FIGS. 16A-F represent kidney sections of mice of group 1 (A, B—untreated mice), group 2 (C, D—recipients of splenocytes of 2-month old mice treated with hCDR1)

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and group 3 (E, F—recipients of splenocytes of 2-month old mice treated with revbCDR1). A,C,E x 400; B,D,F x 100.

[0193] The results of the above experiments indicate that the ameliorating effects on disease manifestations of bCDR1 can be transferred by immunocytes of healthy mice that were treated with bCDR1.

EXAMPLE 12.

[0194] Down-regulation of MMP-3 and MMP-9 by the murine mCDR peptides.

[0195] Matrix metalloproteinases (MMPs) (Shingleton et al., 1996; Goetzl et al., 1996; Mannova et al., 1998) constitute a family of zinc containing endoproteinases that play an important role in the remodelling of extracellular matrix in normal tissues, and also contribute to pathological processes. They share structural domains but differ in substrate specificity, cellular sources and inducibility.

[0196] The MMPs are synthesized as zymogen-like latent precursors and are converted subsequently to an active form. P-2 and MMP-9, which are both gelatinases, can degrade type IV collagen, denatured collagens, types V, VII, X and XII collagens, vitronectin, aggrecan, galectin-3 and elastin. MMP-2 is the most widely expressed UP. It is produced by a variety of cells and is frequently elevated in malignant tumor metastasis.

[0197] In terms of protein and domain structure, MMP-9 is the largest and most complex member identified so far. MMP-9 expression is characterized by a complex regulation with tight control at the levels of gene transcription and protein secretion (such as cytokines, chemokines, eicosanoids) inflammatory mediators, and action of tissue inhibitors of metalloproteinases (TIMPs). In addition, MMP-9 activity is modulated by the activation of pro-MMP-9, by components of the plasminogen activation system or other MMPs (Guedez et al., 1996).

[0198] The involvement of MMPs in autoimmune diseases was demonstrated in various autoimmune diseases such as multiple sclerosis (Cuzzoci et al., 1999) and its animal model experimental autoimmune encephalomyelitis (EAE) (Gijbels et al., 1994), rheumatoid arthritis (Keyszer et al., 1999), Guillain-Barre syndrome (Creange et al., 1999), experimental bullous pemphigoid (Liu et al., 1998) and experimental autoimmune neuritis (Hughes et al., 1998). Serum levels of MMP-3 and TIMP-2 in patients with lupus nephritis were reported to be significantly higher than those of healthy controls, but no correlation with disease activity was noted (Zucker, 1999; Keyszer et al., 1999).

[0199] The potential importance of the many activities of MMPs in inflammatory responses has been suggested by the inhibitory effects of MMP inhibitors in several animal models of autoimmune diseases. Specific inhibition of MMPs in vivo suppresses edema, pathologic tissue proliferation, and damage to specialized tissue structures in several inflammatory and autoimmune diseases (Gijbels et al., 1994; Wallace et al., 1999; Conway et al., 1995).

[0200] According to the present invention, we show that levels of P-3 and MMP-9 are elevated both in sera and in kidneys of mice afflicted with either the spontaneous or the induced experimental SLE. We also demonstrate that treatment of SLE-afflicted mice with the CDR-based murine peptides, that ameliorate manifestations of lupus in mice, diminished the levels of MMP-3 and MMP-9 in the serum and kidneys of the treated mice and also caused amelioration of disease manifestations. The same effects are expected to be presented by the human hCDR peptides of the invention.

EXAMPLE 12(i)

[0201] Kinetics of appearance of MMP-3, MMP-2 and MMP-9 in sera of (NZBxNZW)F1 mice.

[0202] We first examined if the development of spontaneous SLE in (NZB x NZW)F1 mice is associated with changes in the levels of MMP-3, P-2 and MMP-9 in their sera. We thus followed the levels of the latter starting at the age of 2 months, before disease manifestations are observed, until the age of 8 months when the mice suffer from a full blown disease. The results are shown in FIG. 17. As can be seen in FIG. 17A, both the 34 kd and the 40 kd forms of MMP-3 in sera of 2-month old mice are very low as detected by Western blot analysis. The levels of all forms are gradually elevated towards the age of 8 months, the last time point tested. Similarly, FIG. 17B shows that MMP-9 activity, assessed by gel zymography, is low at the age of 2 months and is gradually elevated in sera of mice with the progression of disease, until the age of 8 months. It is also seen in FIG. 17B that levels of MMP-2 are not changed significantly with the progression of disease.

EXAMPLE 12(ii)

[0203] Kinetics of appearance of MMP-3, MMP-2 and MMP-9 in sera of BALB/c mice that were immunized with 16/6Id.

[0204] Previous results from our laboratory have shown that SLE can be induced in BALB/c mice following immunization with the 16/6Id (Mendlovic et al., 1988; Weisman et al., 1993). Therefore, it was of interest to test if the induced model of SLE resembles the (NZB x NZW)F1 model with respect to the changes in P-3 and MMP-9. We thus looked at levels of MMP-3 and MMP-9 in this experimental model of SLE. The results demonstrated in FIG. 18 indicate that the levels of MMP-3 (all isoforms) were elevated 10 days after the boost (4.5 weeks post-immunization), and were higher than in control unimmunized or CFA-immunized BALB/c mice of the same age (FIG. 18A). The levels of MMP-3 increased with aging in all groups, however, the latter were always higher in the 16/6Id immunized mice than in the control groups. In contrast, no induced changes in MMP-9 activity could be detected until 2 months after the boost (FIG. 18B). Higher activity of MMP-9 in the 16/6Id immunized mice than in non immunized mice, could be observed at about 4 months after the boost.

EXAMPLE 12(iii)

[0205] Specific up regulation of MMP-3 and MMP-9 in kidney sections of BALB/c mice that were immunized with the 16/6Id.

[0206] Since immunization of BALB/c mice with the 16/6Id leads to clinical manifestations characteristic of SLE including kidney damage (Mendlovic et al., 1988; Weisman et al., 1993), and because kidney damage in (NZB x NZW)F1 mice was reported to be associated with elevation

in the levels of MMP-3 and MMP-9 (Nakamura et al., 1993), we looked at the expression of these enzymes in kidneys of BALB/c mice that were immunized with 16/6Id. As controls, we used mice that were immunized with CFA and aged matched unimmunized mice. Both control groups were injected with PBS at the time point of the boost. Two months and 5 months after the boost with 16/6Id, kidney sections of mice were immunostained for MMP-3 or MMP-9. FIG. 19 represents immunohistology of kidneys taken 5 months after the boost. As can be seen in the figure, immunization of mice with CFA up-regulated the expression of both MMP-3 (19A) and MMP-9 (19B) in the kidneys, in the glomeruli and in the surrounding tissue. Nevertheless, immunization with 16/6Id in CFA further elevated significantly the expression levels of these two MMPs. This elevation was observed already two months after the boost with the 16/6Id (data not shown). The nonspecific background levels of the staining are also demonstrated (19C).

EXAMPLE 12(iv)

[0207] The murine mCDR1 peptide down regulates levels of MMP-3 and activity of MMP-9 in sera of (NZB x NZW)F1 mice.

[0208] Since we could show that levels of MMP-3 and MMP-9 are elevated in both experimental models of SLE, it was of interest to examine if the amelioration in the clinical symptoms of SLE observed following treatment with the mCDR1 peptide (Eilat et al., 2000; Eilat et al., 2001), is accompanied by a reduction in levels of MMP-3 and MMP-9. We thus treated (NZB x NZW)F1 mice that develop SLE-like disease spontaneously, at the age of 2 months (before clinical manifestations are observed) by injecting them weekly with mCDR1 in PBS (250 μg/mouse) s.c. for 10 weeks. This prevention protocol resulted in the amelioration of all clinical symptoms (Eilat et al., 2000). As can be seen in FIG. 20, there is a reduction in the two lower bands of MMP-3 (20A) and in MMP-9 activity (20B) in the serum. This reduction is associated with the reduction in the clinical manifestations (Eilat et al., 2000). As seen in FIG. 20A, the 45 kd form of MMP-3 was not affected.

[0209] It was also of interest to find out whether the mCDR1 peptide is capable of down regulating elevated levels of MMP-3 and activity of MMP-9. We thus looked at the levels of MMP-3 and at MMP-9 activity in the sera of (NZB x NZW)F1 mice that were treated with mCDR1 at the time that disease manifestations were already observed. As a control peptide we used the reversed mCDR1. As can be seen (FIG. 20, at the right), treatment of sick mice with mCDR1, but not with revmCDR1, specifically down-regulated the levels of MMP-3 (20A) and the activity of MMP-9 (20B) in the sera.

EXAMPLE 12(v)

[0210] The mCDR1 peptide down-regulates levels of MMP-3 and activity of MMP-9 in sera of 16/6Id immunized mice.

[0211] As we have demonstrated, activity of MMP-9 is elevated in both experimental models [(NZB x NZW)F1 and 16/6Id-induced] of SLE. Because the mCDR1 peptide was shown to ameliorate the disease in both models, and to down-regulate MMP-3 levels and MMP-9 activity in sera of treated (NZB x NZW)F1 mice, we further looked for the effect of treatment on these enzymes in sera of 16/6Id immunized BALB/c mice. FIG. 21 represents results of a prevention and a treatment experiments. The figure shows that mCDR1 could down regulate MMP-3 and MMP-9 in this experimental model as well. Down regulation of levels of MMP-3 and of MMP-9 activity was observed for both the prevention and the treatment protocols. Thus, in vivo administration of mCDR1, either at the stage of disease induction or at the stage of a full blown disease, could reduce the levels of MMP-3 (21A) and MMP-9 (21B). This reduction was specific, since treatment with the reversed mCDR1 did not affect MMP-3 or MMP-9 activity (not shown). Preliminary results, using the MMP-9 activity assay system (from Amersham-Pharmacia Biotech UK Limited, England) show that the activity of sera from unimmunized BALB/c mice is comparable to 7 ng/ml of pure active MMP-9. Immunization with the 16/6Id elevates the activity to 16 ng/ml and treatment with the mCDR1 peptide down regulates it almost to the normal levels (8.5 ng/ml).

EXAMPLE 12(vi)

[0212] Effect of treatment with the mCDR1 peptide on the levels of MMP-3 and MMP-9 in kidney sections of 16/6Id immunized BALB/c mice.

[0213] Because MMP-3 and MMP-9 were shown to be elevated in kidneys of mice with induced experimental SLE, it was of interest to look for the effect of treatment with the mCDR1 peptide on the expression of the latter MMPs in the kidneys of the treated mice. FIG. 22 demonstrates that in both prevention (FIG. 22A) and treatment (FIG. 22B) experiments, mCDR1 down-regulated the expression levels of both MMP-3 and MMP-9 in the kidneys of the treated mice. The reduction in the levels of MMP was observed in both the glomeruli and interstitium. The observed reduction in MMP expression correlated with the reduction in staining for immune complex deposits using anti-Ig (FIG. 22B).

[0214] Because of the apparent involvement of MMP-3 and MMP-9 in the pathogenesis of SLE, we tested whether the human hCDR1 peptide is capable of down-regulating the levels of the latter in correlation with the amelioration of disease manifestations. To this end, pooled sera of groups of (NZB x NZW)F1 mice that were treated with the hCDR1 by s.c. injections of 100 μg or 300 μg/mouse once a week for ten weeks were tested for MMPs levels at various periods during the treatment. FIG. 23 represents results of an experiment in which mice were treated starting at the age of 7 months when all clinical manifestations were observed. The results are of sera collected at the middle of treatment, after 5 injections of hCDR1. The results of FIG. 23 indicate that treatment of mice with 300 μg hCDR1 down regulated the levels of MMP-3 and the activity of MMP-9. These results are in agreement with the significant amelioration of disease manifestations following treatment with hCDR1.

[0215] Discussion

[0216] The above results show that both MMP-3 and MMP-9 are elevated in sera and in kidneys of murine models of SLE. The murine mCDR1 peptide could down regulate the levels of MMP-9 and MMP-3 in both sera and kidneys of SLE-afflicted mice. It is demonstrated that in the spontaneous model of SLE, up regulation of both MMP-9 and MNP-3 in the serum occurs at the first 3.5 months. In the induced model the elevation in the levels of MMP-3 in sera

is very early (10 days after the boost with the 16/6Id), while the elevation of MMP-9 activity occurs about 3-4 months later. Based on the results with the 16/6Id induced model that allows us to follow processes at the first stages of the disease, it seems that MMP-3 is involved in disease induction while MMP-9 is involved in disease progression.

[0217] Our results showing the elevation of P-3 in the mouse models of SLE, are in agreement with results demonstrating that MMP-3 is significantly increased in sera of patients with SLE (Kotajima et al., 1998) and that MMP-3 transcript is significantly increased with the progression of nephritis in (NZB x NZW)F1 mice (Nakamura et al., 1993). Taken together, these results suggest that MMP-3 may contribute to the development of glomerular injury in lupus nephritis.

[0218] Our results show that activity levels of MP-2 in sera are not elevated significantly with disease progression in both the induced and the spontaneous models of SLE. These results are compatible with those reported previously (Zucker, 1999) that MMP-2 levels were not increased in SLE. Accordingly, this enzyme was not modulated by treatment with the CDR1-based peptide (data not shown). Levels of MMP-2 were secreted constitutively and did not change in other pathological conditions (like optic neuritis and multiple sclerosis) as well, whereas levels of MMP-9 were elevated relatively to the healthy controls (Gijbels et al., 1992; Paemen et al., 1994).

[0219] Interestingly, the mCDR1 peptide, that specifically and directly affects SLE-associated T-cell responses, could down regulate levels of MMP-3 and MMP-9 that are already up regulated (treatment protocol) as well as to prevent their elevation (prevention protocol).

[0220] MMP-9 activity in sera was neither reported in SLE patients nor in animal models of SLE. This is the first time that a correlation between SLE and MP-9 is shown and that MMP-9 is demonstrated to be elevated in sera and kidneys of SLE-afflicted mice. Although the elevation in sera appears relatively late (around 4 months after the boost), the elevation of P-9 in the kidneys is observed at early stages after disease induction (2 months) (data not shown). It is further shown herein for the first time that peptides that ameliorate SLE manifestations in mice (mCDR1 and hCDR1 as shown above), also down regulate MMP-9 both in sera and in kidneys. The latter data were also supported by preliminary results, using the MMP-9 activity assay system that showed that immunization with the 16/6Id elevated MMP-9 activity, whereas treatment with the CDR1-based peptide down regulated it to the activity levels of unimmunized mice.

[0221] It is also demonstrated here for the first time that MMP-3 and MMP-9 differ in the kinetics of their induction in experimental models of autoimmune diseases as shown here specifically for SLE. This may indicate the different roles of the above MMPs in the pathogenesis of the disease. Our results demonstrate that peptides that immunomodulate SLE-associated T-cell responses and down regulate disease manifestations can control the secretion (although not necessarily by the T cells themselves) of those MMPs in sera and kidneys. These results indicate that MMP-3 and MMP-9 play a role in the pathogenesis of SLE and may serve as surrogate markers of disease progression on one hand, and of disease amelioration by a given treatment, on the other hand.

EXAMPLE 13.

[0222] Activity of MMP-9 (but not of MMP-2) is elevated in sera of SLE patients

[0223] In the present example, we determined the levels of MMP-9 and MMP-2 in sera of 40 patients with SLE and we demonstrate that MMP-9 but not MMP-2 activity is significantly elevated in sera of SLE patients compared to healthy controls. High MMP-9 activity correlated with the presence of discoid rash, Raynaud phenomenon, pneumonitis, mucosal ulcers and the presence of anti-phospholipid antibodies (APLA). In addition, elevated levels of MMP-9 correlated with SLE activity in the group of male patients.

[0224] Materials and Methods

[0225] Patients. Forty patients, 32 females and 8 males with SLE participated in this study. All patients revealed at least four of the revised diagnostic criteria of the American College of Rheumatism (ACR) for the diagnosis of SLE (Winchester, 1996). Twenty-five sex- and age-matched healthy volunteers served as a control group in our studies. The mean age of patients at diagnosis was 29±9.7 (range 15-46) years and the mean follow-up period was 11±10 (range 1-32) years. Disease activity was determined according to the SLEDAI lupus activity index (Bombardier et al., 1992) and by the BILAG index (Hay et al., 1993). The study was approved by the ethic committee of the Kaplan Medical Center, Rehovot, Israel.

[0226] Measurement of MMP-2 and MMP-9 by activity assay, kits. Activities of MMP-2 and MMP-9 were measured by specific Biotrak MMP-2 or MMP-9 activity assay kits (Amersham Phalmacia Biotech UK Limited, UK) according to the manufacturer's instructions. Sera were diluted 1:100 and 1:32 for the determination of MMP-2 and MMP-9 activities, respectively. The appropriate standards were added in each assay. In order to measure the total content of the MMPs, activation of the pro form of the MMPs was performed using p-aminophenylmercuric acetate (APMA).

[0227] Measurement of MMP-2 and MMP-9 activities by gel Zymography. MMP-2 and MMP-9 activities were tested by gelatin zymography. A 5 μl sample of serum was separated by an 8% SDS-PAGE gel polymerized with 1 mg/ml gelatin. Gels were washed once for 30 min in 2.5% Triton X-100 to remove the SDS, and once for 30 min in the reaction buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$ and 0.02% (w/v) Brij 35 (pH 7.5). The reaction buffer was changed to a fresh one, and the gels were incubated at 37° C. for 24 h. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie brilliant blue and was quantified by densitometry.

[0228] Statistical analyses. The data were evaluated using chi-square or Fisher exact tests, unpaired t-test and two tailed P-values. Pearson, Spearman and multivariate analyses were also used.

EXAMPLE 13 (i)

[0229] Activity of MMP-9 but not of MMP-2 is elevated in SLE

[0230] As described above, MMP-9 was shown to be involved in several autoimmune diseases as well as in animal models of SLE. Thus, we were interested in studying whether MMP-9 is also elevated in sera of SLE patients. For

this purpose, we examined sera of 40 SLE patients and of 25 healthy controls by gel zymography, in which both MMP-9 and MMP-2 activities can be visualized. A representative gel is shown in FIG. 24. As can be seen in this figure, levels of MMP-9 are elevated in the sera of SLE patients when compared to healthy controls. Densitometric analysis of the zymograms of sera of 40 SLE patients and 25 healthy controls indicated that the mean MMP-9 activity for SLE patients was 109 ±5.6 densitometry units and for the healthy controls, 76.5±4.2 densitometry units (P=0.0001). Activity values of above 85 densitometry units (mean of healthy controls +2 s.e.) were considered high. The results demonstrated high activity levels of MMP-9 in 68% of the SLE patients. Only 3% of healthy controls exhibited high MMP-9 activity (P=0.001). Densitometric analysis of MMP-2 levels in the same serum samples revealed that the differences in MMP-2 activity between sera of SLE patients and of healthy controls were not significant. Thus values of 109±7 and of 123±5 (mean activity densitometry units ± s.e.) were determined for healthy controls and SLE patients, respectively (P=0.0531). To quantify the activity levels of MMP-9 and MMP-2 in the serum further, we used activity assay kits.

[0231] FIG. 25 shows that the activity of MMP-9 is elevated by threefold in sera of SLE patients compared with sera of healthy controls, and this elevation is statistically significant (P=0.0302). In contrast, the differences in the levels of MMP-2 between the two groups are not significant (P=0.1254).

[0232] Since we, as well as others (Hoshara et al., 1998 and 1999) detected high P-9 levels in sera of patients with non-SLE chronic renal failure (e.g. diabetes mellitus, hypertension) probably due to the retention of the enzyme, we analysed the correlation between levels of MMP-9 and kidney function in the group of SLE patients tested. No correlation was observed between creatinine levels and MMP-9 levels ($r^2$=0.01), indicating that the elevated levels of MMP-9 in SLE patients were not the result of retention of the enzyme due to renal impairment.

EXAMPLE 13(ii)

[0233] Correlation of MMP-9 activity with clinical and laboratory parameters

[0234] The elevation in the activity levels of MMP-9 in sera of SLE patients prompted us to look for possible correlation between clinical and laboratory parameters, and serum MMP-9 levels. Statistical analysis (chi-square or Fisher exact tests) was performed by investigating the number of patients with high and normal MMP-9 levels for each clinical manifestation (Table 15) as well as by taking into consideration the actual mean activity levels of MMP-9 for patients with or without a certain clinical symptom. The results were similar by both analyses. It is noteworthy that for all clinical symptoms, the percent of patients with elevated MMP-9 levels is much higher than that in the group of healthy controls. Levels of MMP-9 did not correlate with gender, duration of disease or the age of its onset (Pearson, Spearman).

[0235] Table 15 shows the clinical and laboratory characteristics of the SLE patients according to their MMP-9 activity levels (lower or equal to healthy controls=normal). High levels of MMP-9 correlated significantly with the presence of Raynaud phenomenon (P=0.0138) and APLA (P=0.041). A strong correlation could be observed with pneumonitis, discoid rash, neurological disorders and mucosal ulcers. However, the number of patients with the latter manifestations was too small to perform a statistical analysis. Multivariate analysis revealed that Raynaud phenomenon and low complement (C3, C4) levels are positively correlated with high MMP-9 levels (P=0.0001 and 0.0137, respectively). In contrast, photosensitivity, arthritis and hematological disorders are negatively correlated with MMP-9 activity levels (P=0.0381, 0.0014 and 0.0065, respectively).

TABLE 15

Clinical characteristics of SLE patients with high and normal MMP-9 activities according to their MMP-9 levels.

| | | MMP-9 LEVELS (%) | |
|---|---|---|---|
| | | High | Normal |
| Number of Patients (%) | 40 (100) | 27 (68) | 13 (32) |
| Photosensitivity | 13 | 8 (62) | 5 (38) |
| Mucosal ulcers | 9 | 8 (89) | 1 (11) |
| Malar rash | 9 | 7 (78) | 2 (22) |
| Discoid rash | 5 | 5 (100) | 0 (0) |
| Raynaud phenomenon | 8 | 8 (100) | 0 (0) |
| Vasculitis | 18 | 14 (78) | 4 (22) |
| Arthritis | 31 | 21 (68) | 10 (32) |
| Serositis | 9 | 7 (78) | 2 (22) |
| Pneumonitis | 4 | 4 (100) | 0 (0) |
| Neurological disorders | 4 | 4 (100) | 0 (0) |
| Renal disorder | 16 | 11 (69) | 5 (31) |
| Hematological Disorders | 29 | 18 (62) | 11 (38) |
| ANA | 40 | 27 (68) | 13 (32) |
| anti-DNA | 36 | 24 (67) | 12 (33) |
| APLA | 25 | 20 (80) | 5 (20) |
| Low complement (C3, C4) | 30 | 21 (70) | 9 (30) |

[0236] Clinical involvement was defined according to the ACR revised criteria (Winchester, 1996). Anti-nuclear antibodies (ANA) and anti-ds DNA antibodies were determined by using Hep2 cells and Crithidia luciliae, respectively. Anti-phospholipid antibodies (APLA) were defined as reactivity with one or more of the following assays: false positive VDR, lupus anti-coagulant (LAC) or ELISA for anticardiolipin antibodies.

[0237] We also looked for a possible correlation between SLEDAI and MMP-9 activity in male (FIG. 26A) and female patients (FIG. 26B). Interestingly, the correlation coefficient was significant and positive for men ($r^2$=0.6333) but insignificant and negative for women ($r^2$=0.0571). Similar results were obtained using the BILAG scoring system. Thus, a positive correlation coefficient between MMP-9 activity and BILAG scores was observed for men ($r^2$=0.6442) and an insignificant one for women.

[0238] It was also of interest to determine whether a correlation exists between the use of various treatment modalities by the patients and MMP-9 activity. As can be seen in Table 16(A), there was no significant correlation between the current treatment of the patients and MMP-9 activity. However, when we looked at treatment of patients at any time during their disease course (Table 16(B)), high MMP-9 levels were associated with usage of cytotoxic agents (82%).

TABLE 16

Treatment modalities of SLE patients according to their MMP-9 levels.

| | Total Number of Patients | MMP-9 Levels (%) High | Normal |
|---|---|---|---|
| **A. Current treatment.** | | | |
| Cytotoxic agents | 8 | 6 (75) | 2 (25) |
| Steroids | 23 | 17 (74) | 6 (26) |
| Anti-Malarial | 21 | 14 (67) | 7 (33) |
| NSAID | 7 | 5 (71) | 2 (29) |
| **B. Treatment along the follow up period.** | | | |
| Cytotoxic agents | 17 | 14 (82) | 3 (18) |
| Steroids | 29 | 19 (66) | 10 (34) |
| Anti-Malarial | 26 | 16 (62) | 10 (38) |
| NSAID | 18 | 12 (67) | 6 (33) |

[0239] The anti-malarial agent hydroxychloroquine was used at dose of 200-400 mg/day. Steroid treatment was defined as a daily dose >5 mg of prednisone. Cytotoxic agents used were cyclophosphamide (0.5-1 g/m$^2$ monthly) or azathioprine (100-150 mg/day).

EXAMPLE 13(iii)

[0240] Variations in MMP-9 activity in serum samples taken from individual SLE patients at different time points

[0241] Since disease activity varies over time, we measured the activity levels of MMP-9 and MM-2 in the serum of individual patients that were sampled during 4-6 years of follow-up. Sera of nine patients taken at different time points were analysed. Levels of MMP-2 did not vary significantly between patients and healthy controls. In 5 out of the 9 patients tested, variations in MMP-9 activity in serum samples of individual patients could be observed with time. The results for 2 representative SLE patients are shown in FIG. 27. As can be seen, MMP-9 activity, but not MMP-2 activity, has been changing with time in the same patients. These changes were not associated with disease activity indices as determined by either the SLEDAI or BILAG systems. Changes in MMP-9 activity were not detected in sera of 5 healthy controls that were sampled at different time points (data not shown). In 4 other SLE patients, no substantial changes in MMP-9 or MMP-2 activity were observed with time, and MMP-9 activity levels remained either high or low, depending on the individual patient.

[0242] Discussion

[0243] The present study demonstrates for the first time the involvement of MMP-9 in human SLE. We show that the activity of MMP-9, but not MMP-2, is significantly elevated in sera of 68% of SLE patients compared with healthy controls. High MMP-9 levels correlated with Raynaud phenomenon, pneumonitis, neurological disorders, discoid rash and the presence of APLA. Changes in MMP-9 activity were observed in serum of the same patient at different periods of the disease. MMP-9 activity levels did not correlate with disease activity index (SLEDAI, BILAG) in female patients, but correlated with SLE activity in the group of male patients.

[0244] The present study shows that activity levels of MMP-2 are not elevated significantly in sera of SLE patients. These results are compatible with those reported previously (Zucker, 1999) that MMP-2 levels were not increased in SLE. Levels of MMP-2 were also constitutive and unchanged in other pathological conditions (like optic neuritis and multiple sclerosis) in which levels of MMP-9 were elevated relatively to the healthy controls (Gijbels et al., 1992; Paemen et al., 1994).

[0245] Involvement of an additional MMP, namely, MMP-3 was suggested in the pathogenesis of SLE, since it was significantly increased in sera of patients with SLE (Kotajima et al., 1998). The frequency of SLE patients with elevated MMP-9 activity (68%) shown in the present example, resembles the frequencies reported (Kotajima et al., 1998) for high MMP-3 levels in SLE (76%) and in RA (82%) patients. Furthermore, the MMP-3 transcript was shown to increase significantly with the progression of nephritis in (NZB x NZW)F1 mice (Nakamura et al., 1993).

[0246] The origin of the elevated MMPs in sera of SLE patients is not known. MMP-9 has been shown to be secreted by peripheral blood cells such as T cells, neutrophils, and macrophages (for review, see Goetzl et al., 1996). The fact that no correlation was found between MMP-9 activity levels and the number of peripheral blood cells in the patients may suggest that MMP-9 was not secreted by peripheral blood immune cells but rather, by SLE-affected organs like kidneys or lungs/pleura. The observation that all SLE patients with pneumonitis exhibited high MMP-9 activity levels may suggest the diseased lung as a source of high MMP-9 levels. Moreover, the association between cytotoxic treatment, which represents the severity of SLE-related organ impairment, and high levels of MMP-9 in the sera may also support the notion that the diseased organs are the source of MMP-9 activity in SLE patients. Nevertheless, the possibility still exists that less peripheral blood lymphocytes secreted higher activity levels of MMP-9.

[0247] TNF-α and IL-1 were shown to play an important role in the pathogenesis of SLE both in the human disease (Dean et al., 2000) and in murine models (Segal et al., 1997; Theofilopoulos et al., 1999; Eilat et al., 2001). It has been shown in several systems that these cytokines induce MMP-9 production (Gaedez et al., 1996), and thus, it is possible that the induction of the latter MMPs is part of the pathogenic effect of these cytokines in SLE. It has been reported that levels of MMP-9, that are secreted spontaneously by peripheral blood monocytes of healthy individuals, were upregulated upon exposure to TNF-α and IL-1β (Saren et al., 1996). In addition, MMPs of both T cells and macrophages facilitate secretion of TNF-α by cleavage of the membrane-bound form (Gearing et al., 1994). Thus, these examples demonstrate the mutual regulatory effects of MMP on the proinflammatory cytokines and vice versa. Nevertheless, the fact that in the sera of some of the patients the activity levels of MMP-9 remained within the normal range during the follow-up period, whereas high activity levels of MMP-9 were measured in the sera of most patients, may suggest the involvement of genetic factors in the regulation of the latter.

[0248] The results herein indicate that MMP-9 might play a role in the pathogenesis of SLE, and that measurement of plasma/serum activity levels of this metalloproteinase may

provide important information when monitoring patients treated with drugs that interfere with MMP-9 activity.

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SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 30

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<213> ORGANISM: Murine

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1 5 10 15

Trp Ile Gly

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Leu

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<211> LENGTH: 19
<212> TYPE: PRT
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<400> SEQUENCE: 6

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Trp Ile Gly

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<400> SEQUENCE: 8

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<212> TYPE: PRT
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<400> SEQUENCE: 9

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Asp Val

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<222> LOCATION: (1)..(19)
<223> OTHER INFORMATION: Xaa in position 1 is Gly or Thr Gly; Xaa in position 8 is Arg or Lys; Xaa in position 10 is Pro or Ser; Xaa in position 12 is Gly or Glu; Xaa in position 13 is Lys or Asp; Xaa in position 15 is Glu, Leu or Ser.

<400> SEQUENCE: 11

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1 5 10 15

Trp Ile Gly

<210> SEQ ID NO 12
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<212> TYPE: PRT
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<223> OTHER INFORMATION: A peptide of SEQ ID NO: 11 wherein Xaa(1) is Gly, Xaa(8) is Arg, Xaa(10) is Pro, Xaa(12) is Gly, Xaa(13) is Lys, and Xaa(15) is Leu.

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Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu
1 5 10 15

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Trp Ile Gly

<210> SEQ ID NO 13
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1 5 10 15

Trp Ile Gly

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1 5 10 15

Trp Ile Gly

<210> SEQ ID NO 15
<211> LENGTH: 19
<212> TYPE: PRT
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1 5 10 15

Trp Ile Gly

<210> SEQ ID NO 16
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Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Ser Pro Gly Lys Gly Glu Glu
1 5 10 15

Trp Ile Gly

<210> SEQ ID NO 17

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<400> SEQUENCE: 17

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1 5 10 15

Trp Ile Gly

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<223> OTHER INFORMATION: A peptide of SEQ ID NO: 11 wherein Xaa(1) is Thr Gly, Xaa(8) is Arg, Xaa(10) is Pro, Xaa(12) is Gly, Xaa(13) is Lys, and Xaa(15) is Glu.

<400> SEQUENCE: 18

Thr Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Glu
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Glu Trp Ile Gly
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<210> SEQ ID NO 19
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<212> TYPE: PRT
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<223> OTHER INFORMATION: Peptide based on CDR3 of the human 16/6Id mAb.
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(22)
<223> OTHER INFORMATION: Xaa in position 6 is Gly or Xaa; Xaa in position 9 is Arg or Ala; Xaa in position 10 is Gly or Ala; Xaa in position 11 is Gly or Ala; Xaa in position 12 is Trp or Ala; Xaa in position 13 is Asn or Ala; Xaa in position 18 is Tyr or Trp; Xaa in position 20 is Met or Gln.

<400> SEQUENCE: 19

Tyr Tyr Cys Ala Arg Xaa Leu Leu Xaa Xaa Xaa Xaa Xaa Asp Val Asp
1 5 10 15

Tyr Xaa Gly Xaa Asp Val
20

<210> SEQ ID NO 20
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<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Gly, Xaa(9) is Arg, Xaa(10) is Gly, Xaa(11) is Gly, Xaa(12) is Trp, Xaa(13) is Ala, Xaa(18) is Tyr, and Xaa(20) is Met.

<400> SEQUENCE: 20

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1 5 10 15

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Tyr Tyr Gly Met Asp Val
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<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Phe, Xaa(9) is Arg, Xaa(10) is Gly, Xaa(11) is Gly, Xaa(12) is Trp, Xaa(13) is Asn, Xaa(18) is Tyr, and Xaa(20) is Met.

<400> SEQUENCE: 25

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1 5 10 15

Tyr Tyr Gly Met Asp Val
20

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<400> SEQUENCE: 26

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1 5 10 15

Tyr Tyr Gly Gln Asp Val
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<400> SEQUENCE: 27

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Tyr Trp Gly Met Asp Val
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<210> SEQ ID NO 28
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide prepared in the reversed order of SEQ ID NO: 1.

<400> SEQUENCE: 28

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Tyr Tyr Gly Thr
20

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<210> SEQ ID NO 29
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide prepared in reversed order of SEQ ID NO: 3.

<400> SEQUENCE: 29

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Ala Cys Tyr Tyr
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<210> SEQ ID NO 30
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Gly Ile Trp Gln Gln Gly Lys Gly Pro Pro Gln Arg Ile Trp Ser Trp
1 5 10 15

Tyr Tyr Gly

1. A synthetic peptide selected from the group consisting of:

(a) a peptide of at least 12 and at most 30 amino acid residues comprising a sequence consisting of, or found within, a complementarity-determining region (CDR) found in the heavy or light chain of the human monoclonal anti-DNA 16/6Id antibody (hereinafter "hCDR sequence"), or a sequence obtained by: (i) replacement of one or more of the amino acid residues of the hCDR sequence by different amino acid residues; (ii) deletion of one or more amino acid residues from the hCDR sequence; and/or (iii) addition of one or more amino acid residues to the hCDR sequence , or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

2. A synthetic peptide according to claim 1, wherein said hCDR sequence comprises a sequence consisting of, or found within, a CDR found in the heavy chain of the human monoclonal anti-DNA 16/6Id antibody.

3. A synthetic peptide according to claim 1 or 2, comprising further amino acid residues.

4. A synthetic peptide according to claim 3, wherein said further amino acid residues are amino acid residues of the sequences of the human 16/6Id mAb flanking the hCDR sequences, or sequences obtained by replacement of one or more of the amino acid residues of the hCDR flanking sequences by different amino acid residues, by deletion of one or more amino acid residues from the hCDR flanking sequences and/or by addition of one or more amino acid residues to the hCDR flanking sequences.

5. A synthetic peptide according to any one of claims 2 to 4, selected from the group consisting of:

(a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:8, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:8 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:8;and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:8, or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

6. A synthetic peptide according to claim 5, comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:8, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:8 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:8; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:8.

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7. A synthetic peptide according to claim 6 selected from a peptide consisting of the sequence of the SEQ ID NO: 11.

8. A synthetic peptide according to claim 7 selected from the peptides consisting of the sequences of the SEQ ID NO:12 to NO:18.

9. The synthetic peptide according to claim 7 consisting of the sequence of the SEQ ID NO:6.

10. The peptide consisting of the sequence of SEQ ID NO:6.

11. A synthetic peptide according to any one of claims 2 to 4, selected from the group consisting of:

(a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:10 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:10; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:10, or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

12. A synthetic peptide according to claim 11, comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:10 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:10; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:10.

13. A synthetic peptide according to claim 12 selected from a peptide consisting of the sequence of the SEQ ID NO:19.

14. A synthetic peptide according to claim 13 selected from the peptides consisting of the sequences of the SEQ ID NO:20 to NO:27.

15. The synthetic peptide according to claim 13 consisting of the sequence of the SEQ ID NO:7.

16. The peptide consisting of the sequence of SEQ ID NO:7.

17. A synthetic peptide according to any one of claims 2 to 4, selected from the group consisting of:

(a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:9, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:9 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:9; and/or (iii) addition of one or more amino-acid residues to said SEQ ID NO:9, or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

18. A synthetic peptide according to claim 17, comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:9, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:9 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:9; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:9.

19. A dual synthetic peptide according to claim 2, in which two different peptides each comprising a sequence consisting of, or found within, a different CDR of the heavy chain of the human 16/6Id mAb, are covalently linked to one another either directly or through a short linking chain.

20. A dual synthetic peptide according to claim 19, in which a peptide according to claim 6 is covalently linked to a peptide according to claim 12.

21. The dual synthetic peptide according claim 20, in which a peptide of SEQ ID NO:11 is covalently linked to a peptide of SEQ ID NO:19.

22. The dual synthetic peptide, in which the peptide of SEQ ID NO:6 is covalently linked to the peptide of SEQ ID NO:7.

23. A peptide polymer according to any one of claims 1 to 4, containing a plurality of identical sequences selected from the sequences of SEQ ID NO:6, 7, and 11-27.

24. A pharmaceutical composition comprising at least one synthetic peptide or peptide polymer according to any one of claims 1 to 23, and a pharmaceutically acceptable carrier.

25. A pharmaceutical composition comprising the peptide of the sequence of SEQ ID NO:6, and a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising the peptide of the sequence of SEQ ID NO:7, and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition according to any one of claims 24 to 26, for the treatment of systemic lupus elythematosus.

28. A pharmaceutical composition according to any one of claims 24 to 27, adapted for oral, intravenous, subcutaneous, intrarticular, intramuscular, inhalation, intranasal, intrathecal, intraperitoneal, intradermal, transdermal or enteral administration.

29. A method for the treatment of systemic lupus erythematosus (SLE) comprising administering to a SLE patient an effective amount of a peptide or peptide polymer according to any one of claims 1 to 23.

30. A method according to claim 29, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:6.

31. A method according to claim 29, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:7.

32. A method for immunomodulation of systemic lupus erythematosus (SLE)-associated responses in a SLE patient, which comprises administering to said SLE patient an effective amount of an effective amount of a peptide or peptide polymer according to any one of claims 1 to 23.

33. A method according to claim 32, which comprises down-regulating the levels of matrix metalloproteinase (MMP)-3 and/or MMP-9 activities in a SLE patient.

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34. A method according to claim 32, which comprises immunomodulating the level of a cytokine activity in a SLE patient.

35. A method according to claim 34, which comprises down-regulating the level of IL-2 and/or IFN-γ activity in a SLE patient.

36. A method according to claim 34, which comprises up-regulating the level of TGF-β activity in a SLE patient.

37. A method according to any one of claims 32 to 36, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:6.

38. A method according to any one of claims 32 to 36, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:7.

39. A method for assessing the effectiveness of a drug in the treatment of a SLE-patient which comprises measuring at different intervals of time the levels of MMP-3 and/or MMP-9 in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of MMP-3 and/or MMP-9 correlates with the effectiveness of the drug.

40. A method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the levels of IL-2 and/or IFN-γ in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of IL-2 and/or IFN-γ correlates with the effectiveness of the drug.

41. A method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the level of TGF-β in a blood sample obtained from said patient being treated with said drug, whereby an increased level of TGF-β correlates with the effectiveness of the drug.

* * * * *

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Mozes (43) Pub. Date: Jul. 1, 2004

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(54) SYNTHETIC HUMAN PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEM FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

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(30) Foreign Application Priority Data

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(52) U.S. Cl. ..... 514/12; 514/13; 514/14; 530/324; 530/325; 530/326; 424/130.1; 530/388.1

(57) ABSTRACT

Synthetic peptides of at least 12 and at most 30 amino acid residues comprising a sequence consisting of, or found within, a complementarity-determining region (CDR) found in the heavy or light chain of the human anti-DNA 16/6Id monoclonal antibody, or a sequence obtained by replacement and/or deletion and/or addition of one or more amino residues to said sequence, and salts, chemical derivatives and polymers of said peptides can be used for immunomodulation of systemic lupus erythematosus-associated responses.

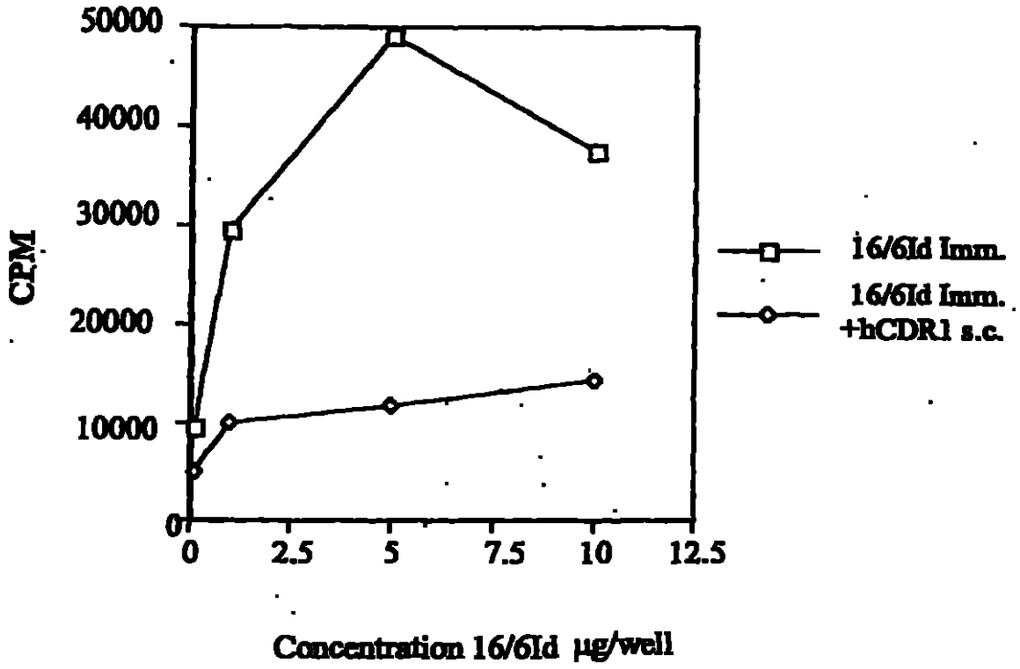

50000
40000
30000
20000
10000
0
CPM
0
2.5
5
7.5
10
12.5
Concentration 16/6Id µg/well
16/6Id Imm.
16/6Id Imm. +hCDR1 s.c.

Fig. 1

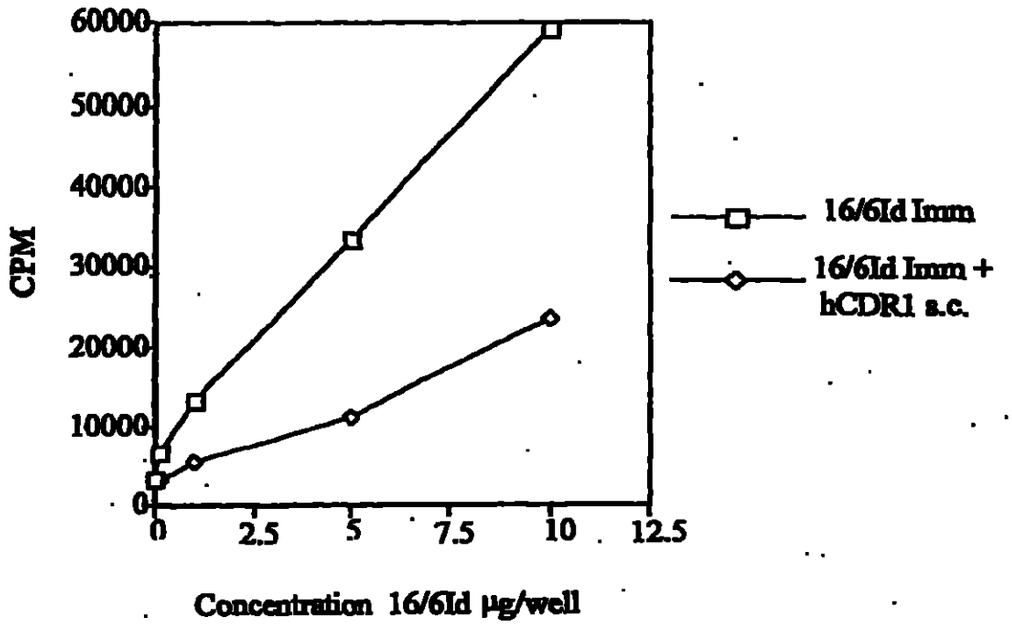

60000
50000
40000
30000
20000
10000
0
CPM
0
2.5
5
7.5
10
12.5
Concentration 16/6Id µg/well
16/6Id Imm
16/6Id Imm + hCDR1 s.c.

Fig. 2

305




Fig. 3 A




Fig. 3 B

309


400
300
200
100
0
IL-10 pg/ml
16/6Id Imm.
16/6Id Imm.+hCDR1sc
16/6Id Imm. +
p259-271 sc

Fig. 3C

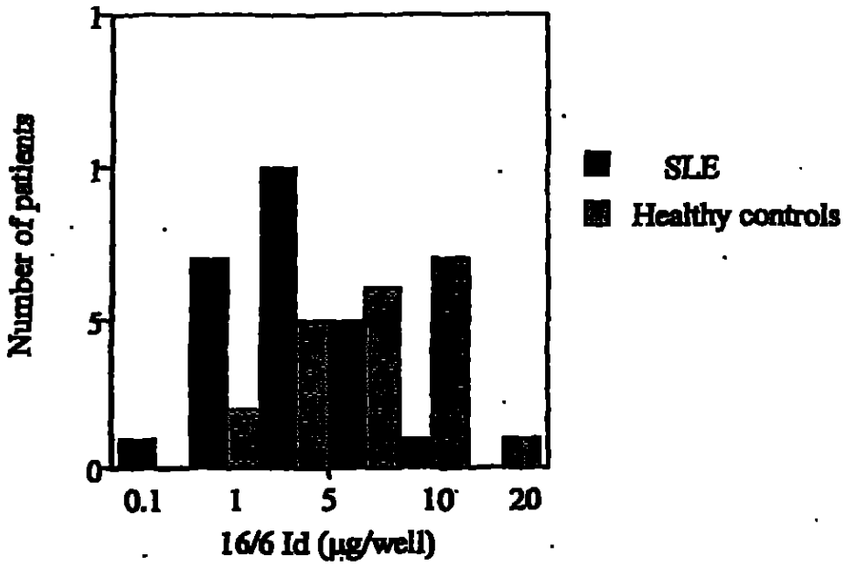

Number of patients
0
5
1
1
0.1
1
5
10
20
16/6 Id (µg/well)
SLE
Healthy controls

Fig. 4

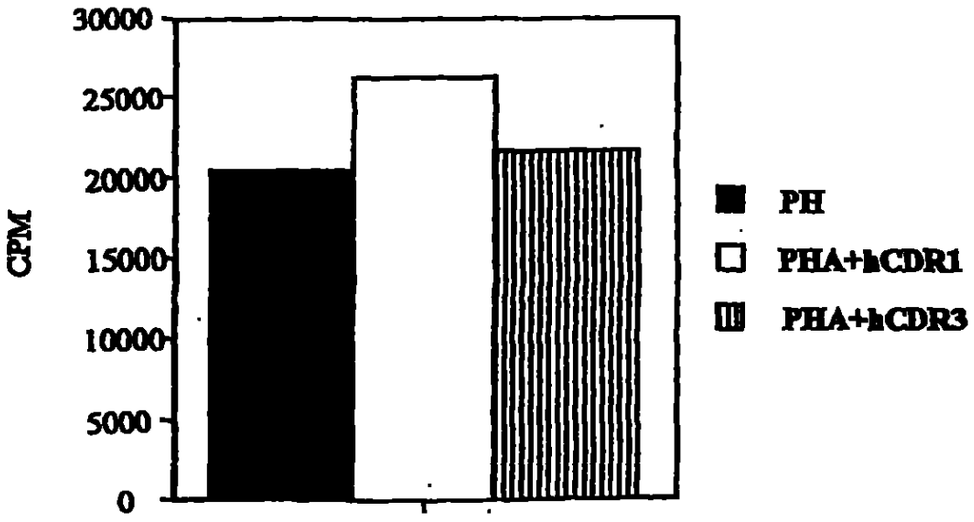

CPM
0
5000
10000
15000
20000
25000
30000
PH
PHA+hCDR1
PHA+hCDR3

Fig. 5

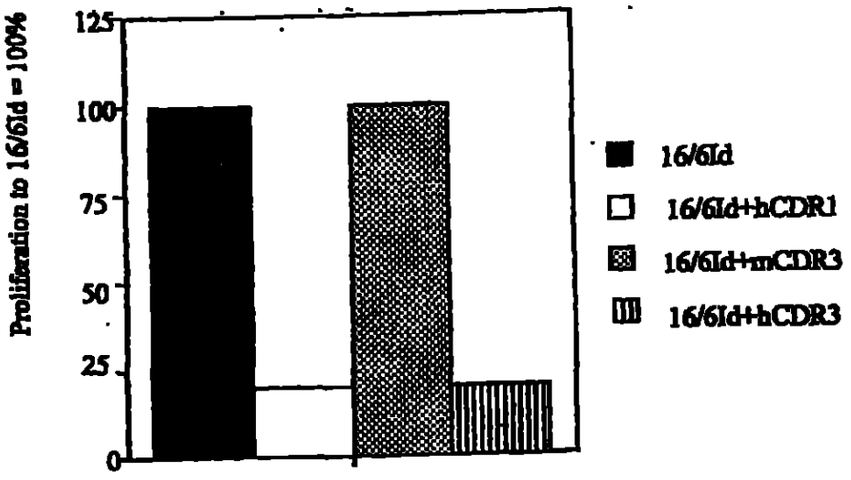

Proliferation to 16/6Id = 100%
125
100
75
50
25
0
16/6Id
16/6Id+hCDR1
16/6Id+mCDR3
16/6Id+hCDR3

Fig. 6

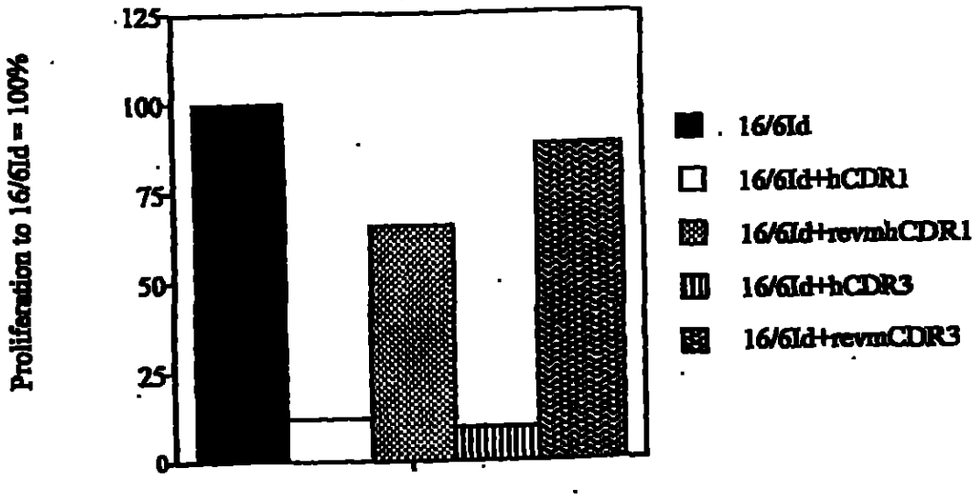

Proliferation to 16/6Id = 100%
125
100
75
50
25
0
16/6Id
16/6Id+hCDR1
16/6Id+revmhCDR1
16/6Id+hCDR3
16/6Id+revmCDR3

Fig. 7

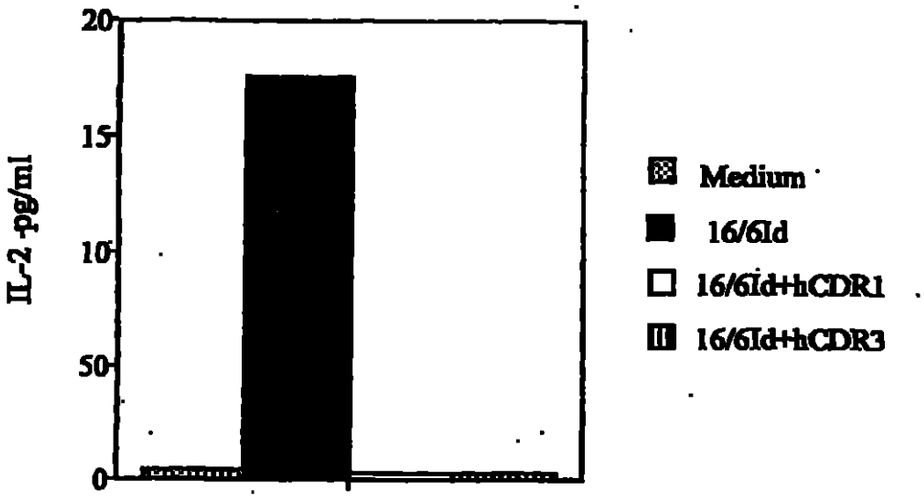

20
15
10
50
0
IL-2 pg/ml
Medium
16/6Id
16/6Id+hCDR1
16/6Id+hCDR3

Fig. 8

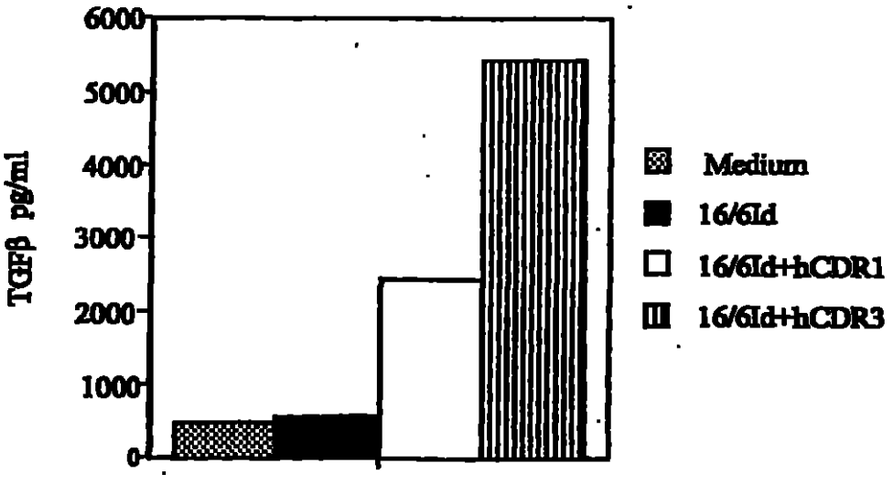

6000
5000
4000
3000
2000
1000
0
TGFβ pg/ml
Medium
16/6Id
16/6Id+hCDR1
16/6Id+hCDR3

Fig. 9

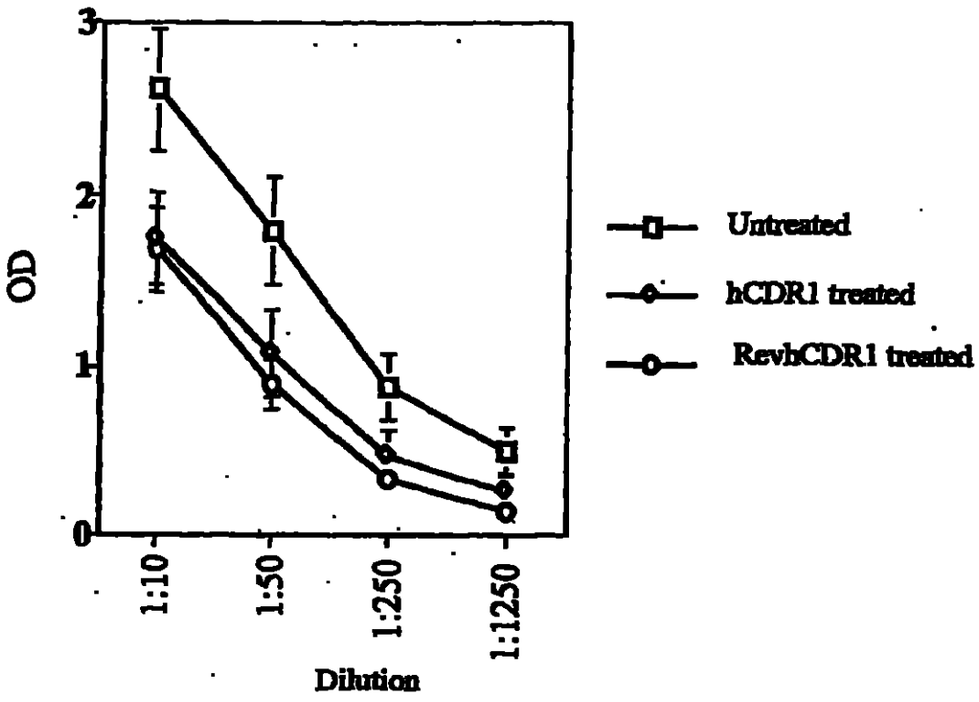
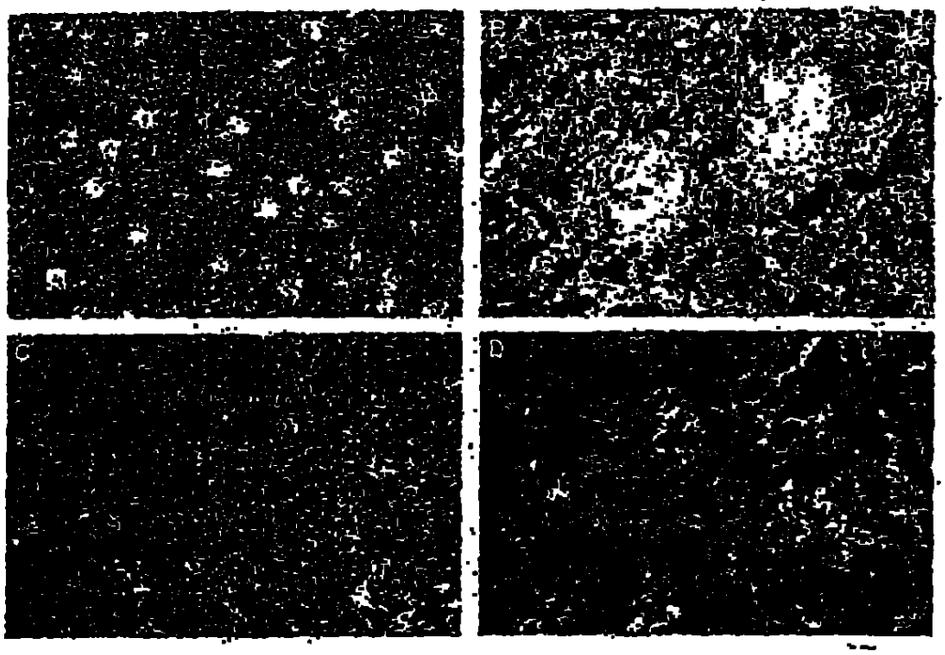

3
2
1
0
OD
1:10
1:50
1:250
1:1250
Dilution
Untreated
hCDR1 treated
RevhCDR1 treated

Fig. 10

314

A

B



C

D

Fig. 11

315

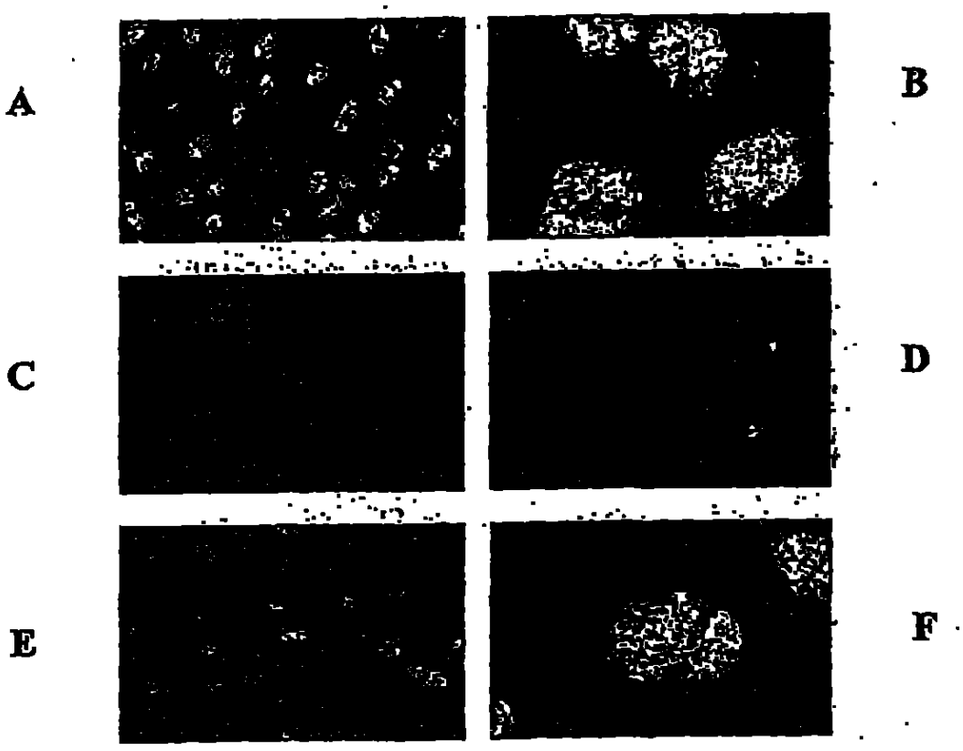



Fig. 12

Fig. 13 A




Fig. 13 B

317

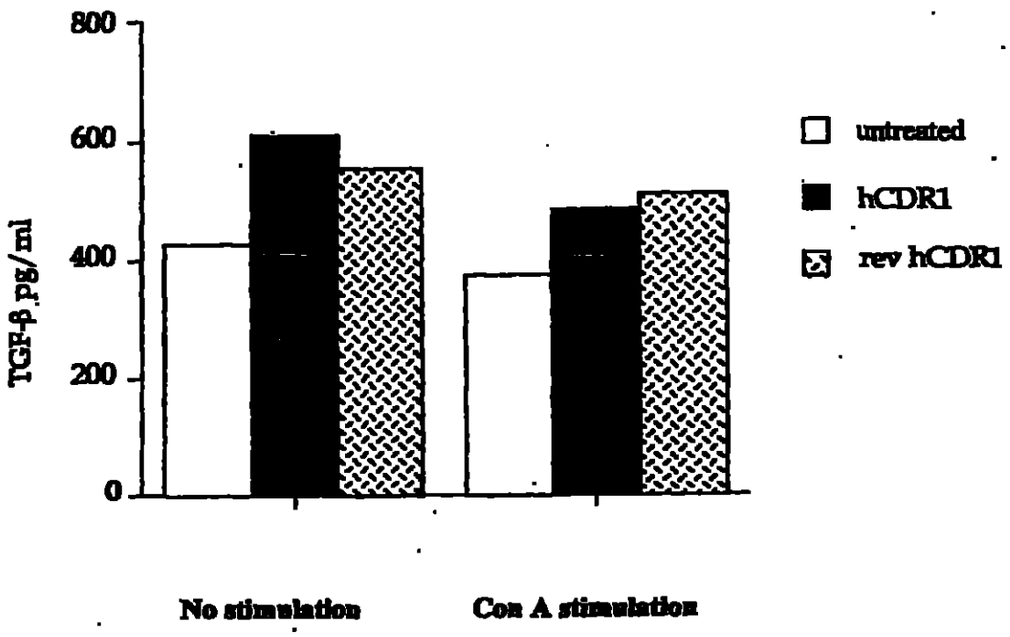

800
600
400
200
0
TGF-β pg/ml
untreated
hCDR1
rev hCDR1
No stimulation
Con A stimulation

Fig. 13 C

318

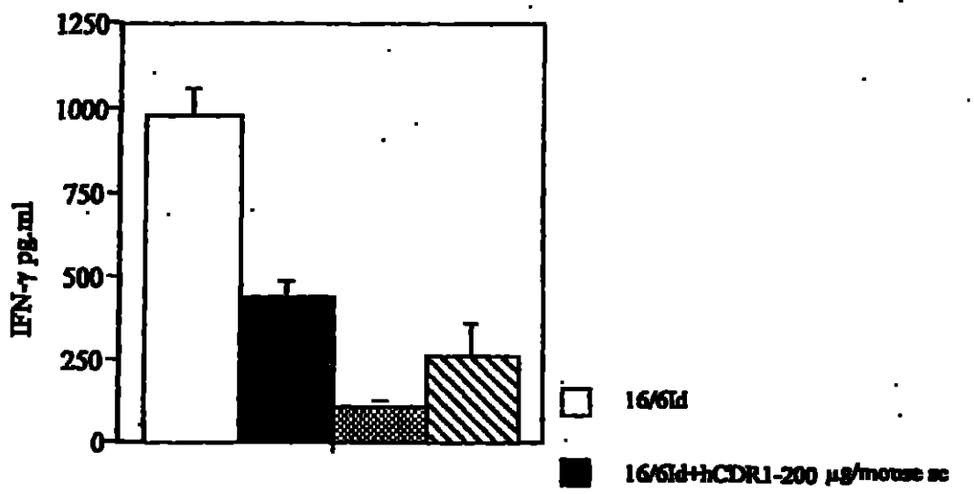



Fig. 15 A

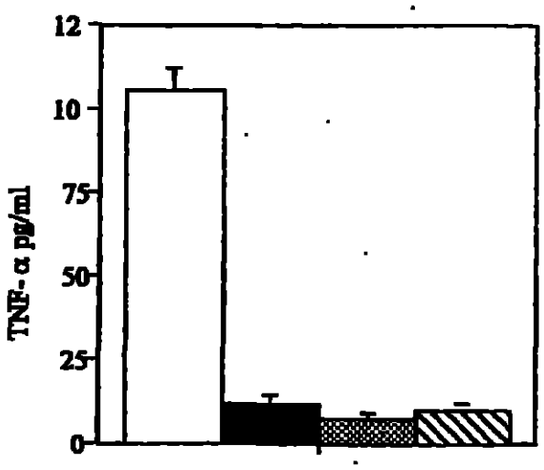



Fig. 15 B

319

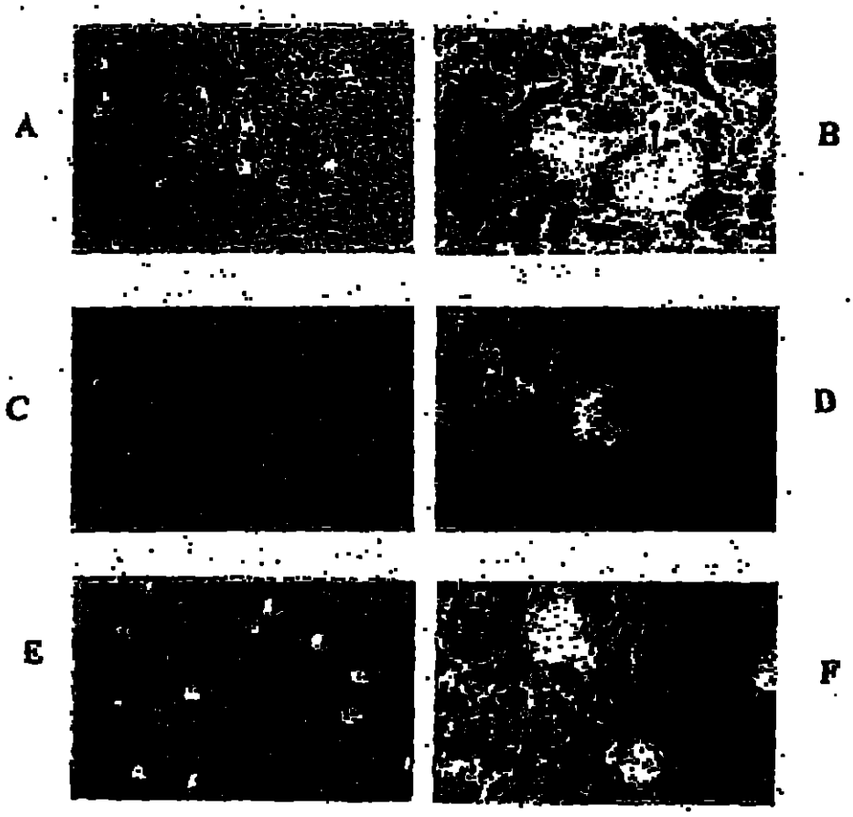



Fig. 14

320

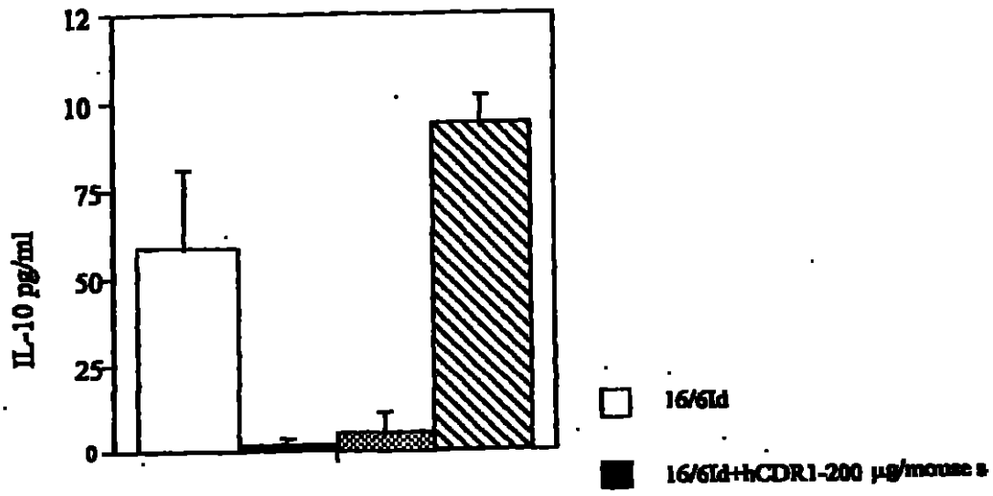



Fig. 15 C

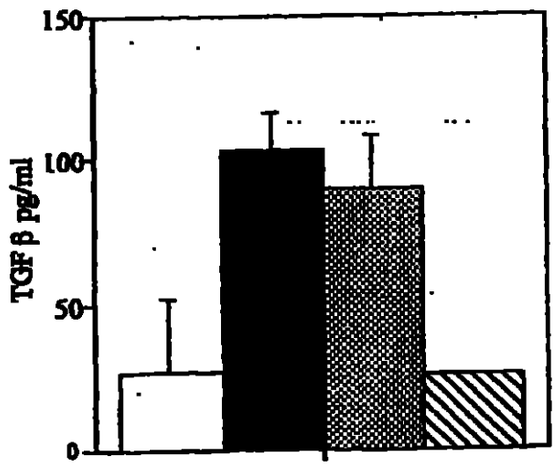



Fig. 15 D

321

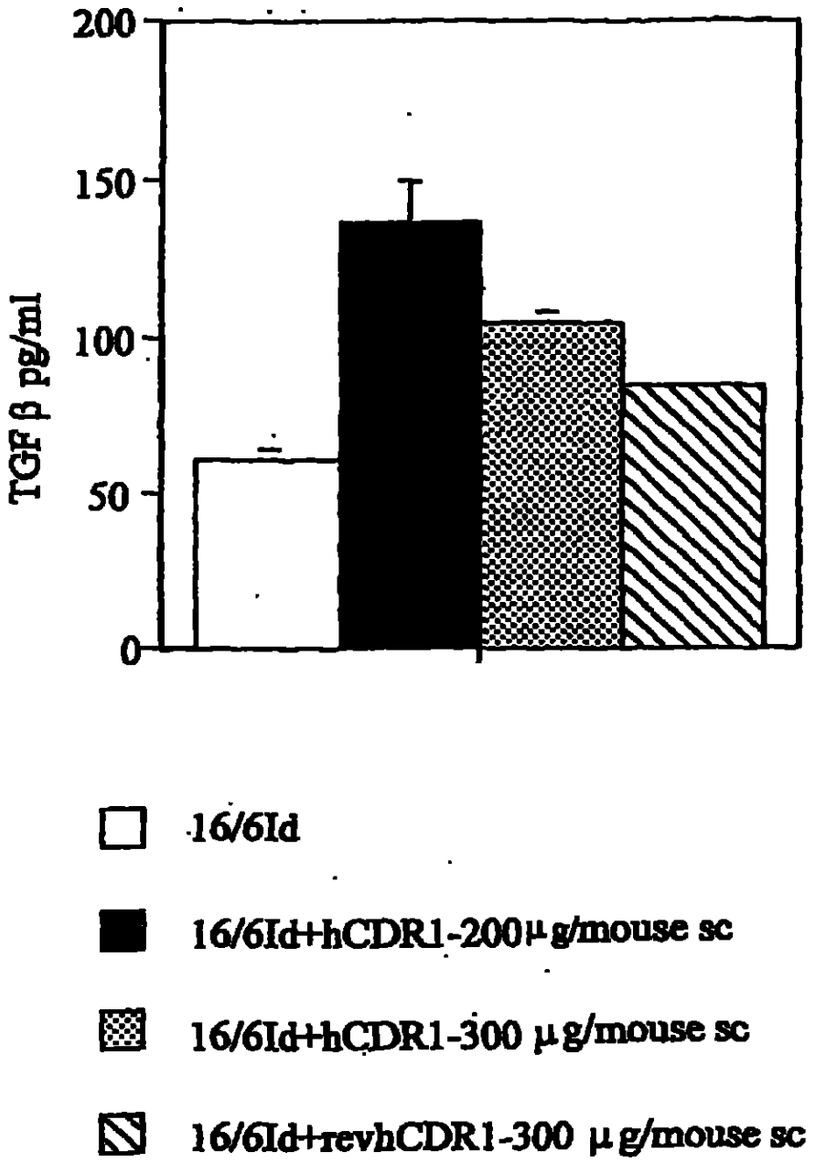

200
150
100
50
0
TGF β pg/ml
16/6Id
16/6Id+hCDR1-200μg/mouse sc
16/6Id+hCDR1-300 μg/mouse sc
16/6Id+revhCDR1-300 μg/mouse sc

**Fig. 15 E**

322

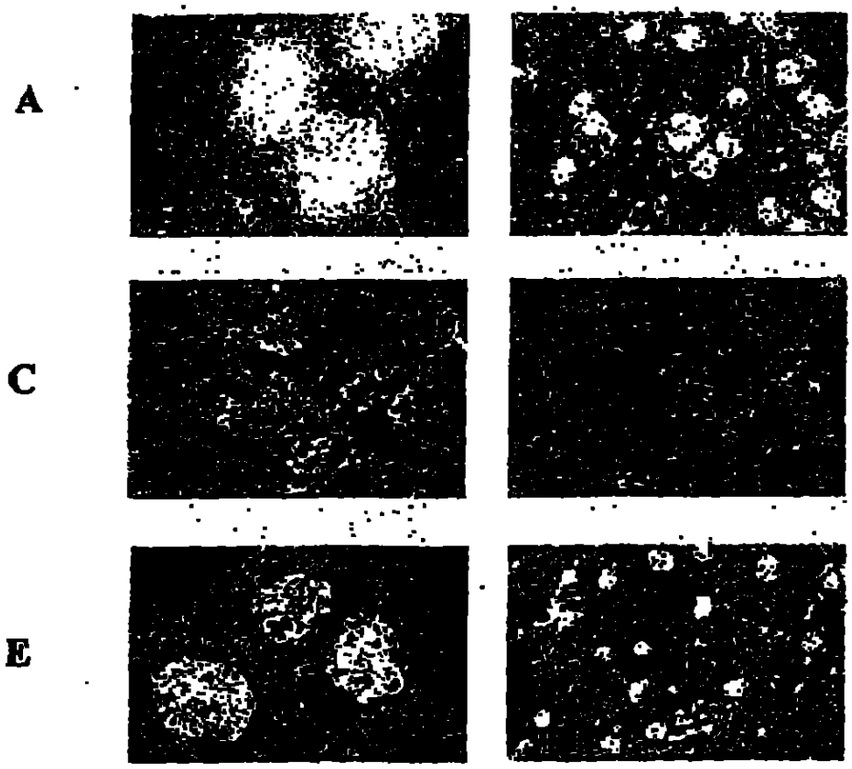



Fig. 16




323

Fig. 17 A

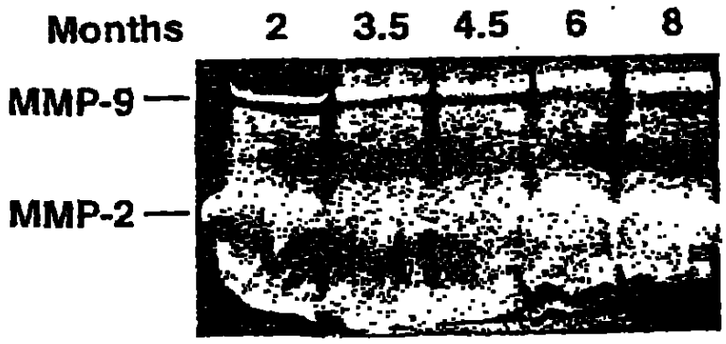
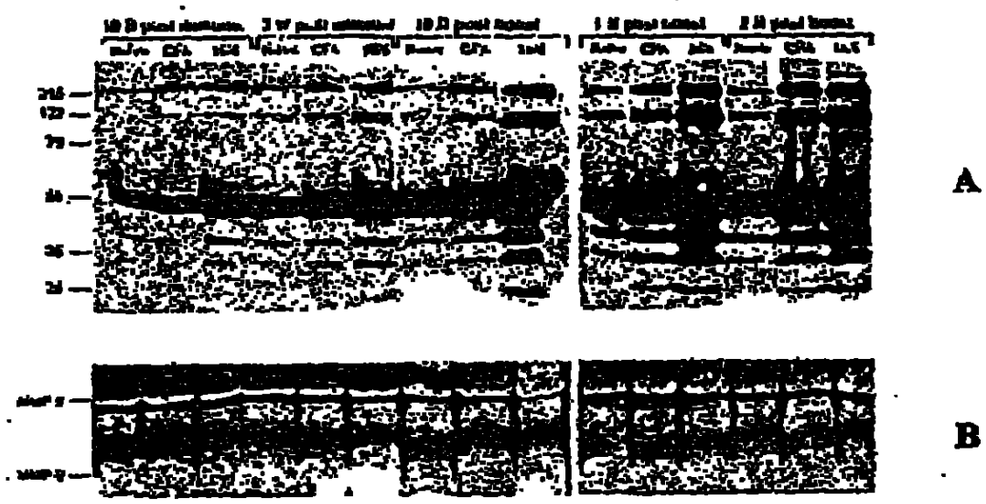



Fig. 17 B



A



B

Fig. 18

325

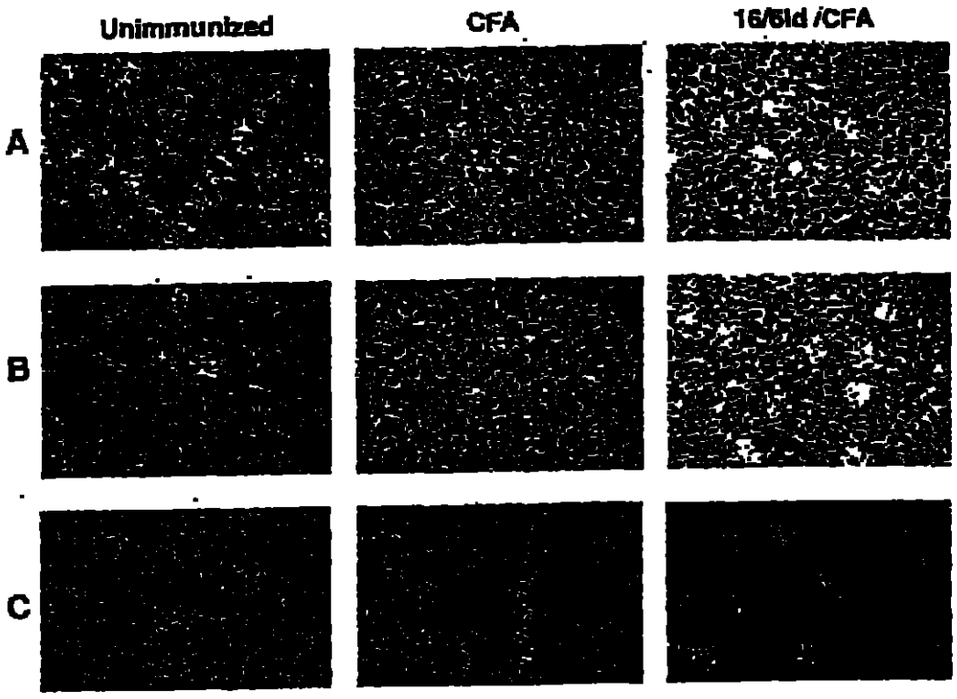



Fig. 19

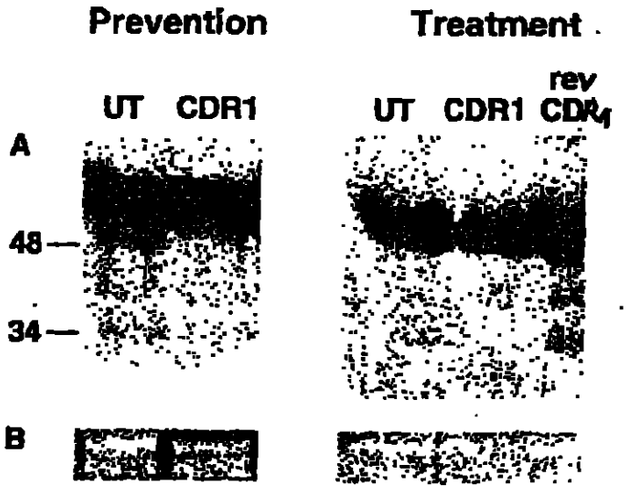

Prevention
Treatment
UT CDR1
UT CDR1 rev CDR1
A
48
34
B

Fig. 20

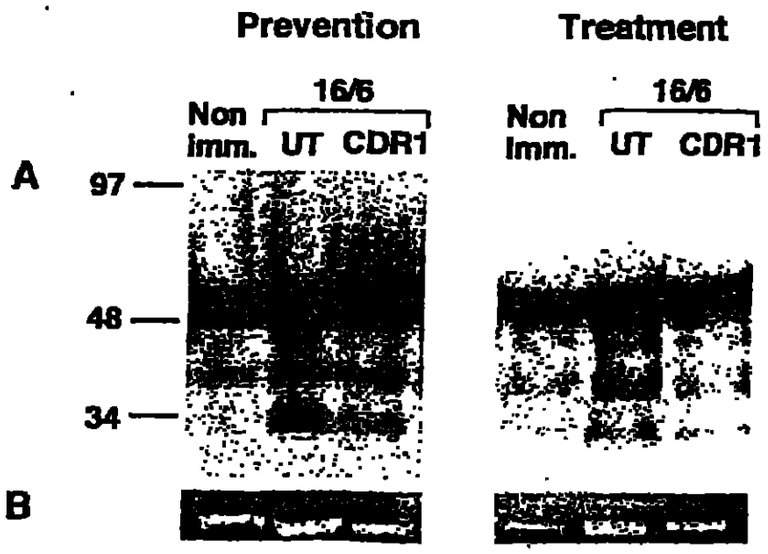

Prevention
Treatment
16/6
Non Imm.
UT CDR1
16/6
Non Imm.
UT CDR1
A
97
48
34
B

Fig. 21

327

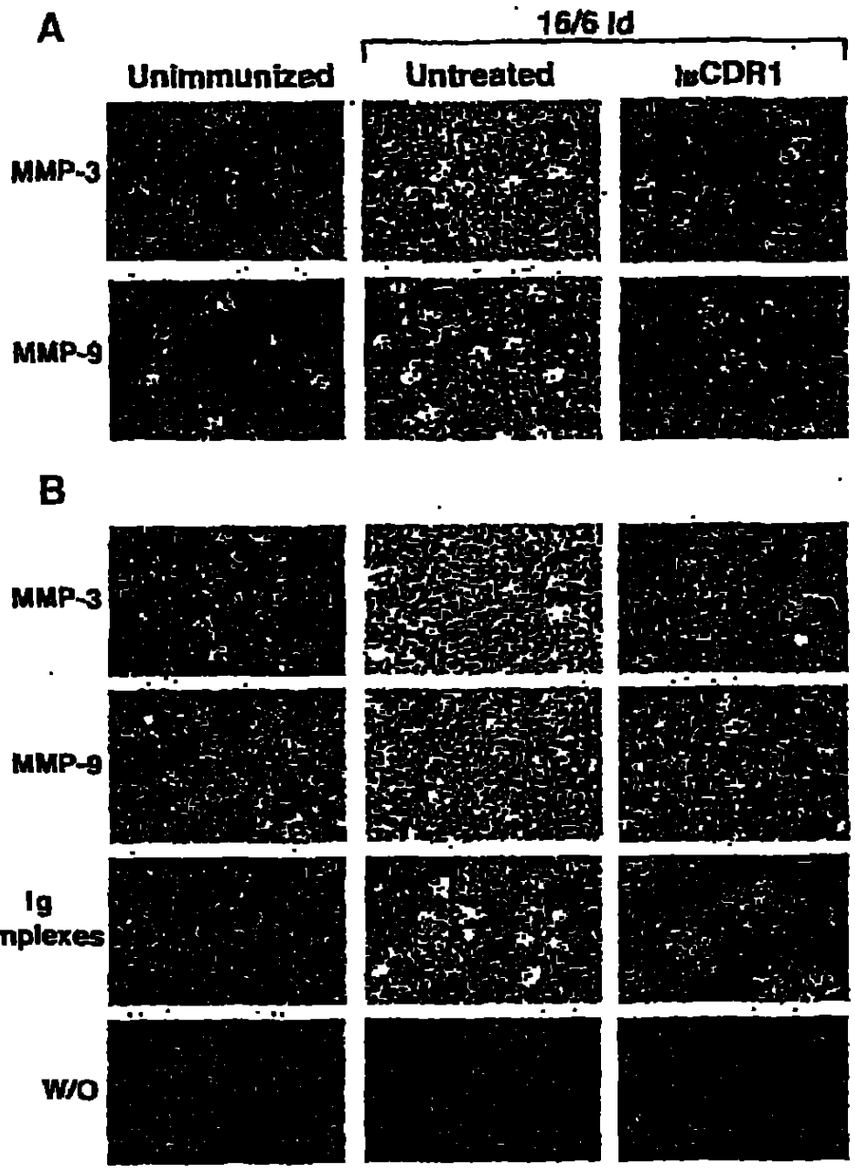

A
16/6 Id
Unimmunized
Untreated
IgCDR1
MMP-3
MMP-9
B
MMP-3
MMP-9
Ig complexes
W/O

Fig. 22

328

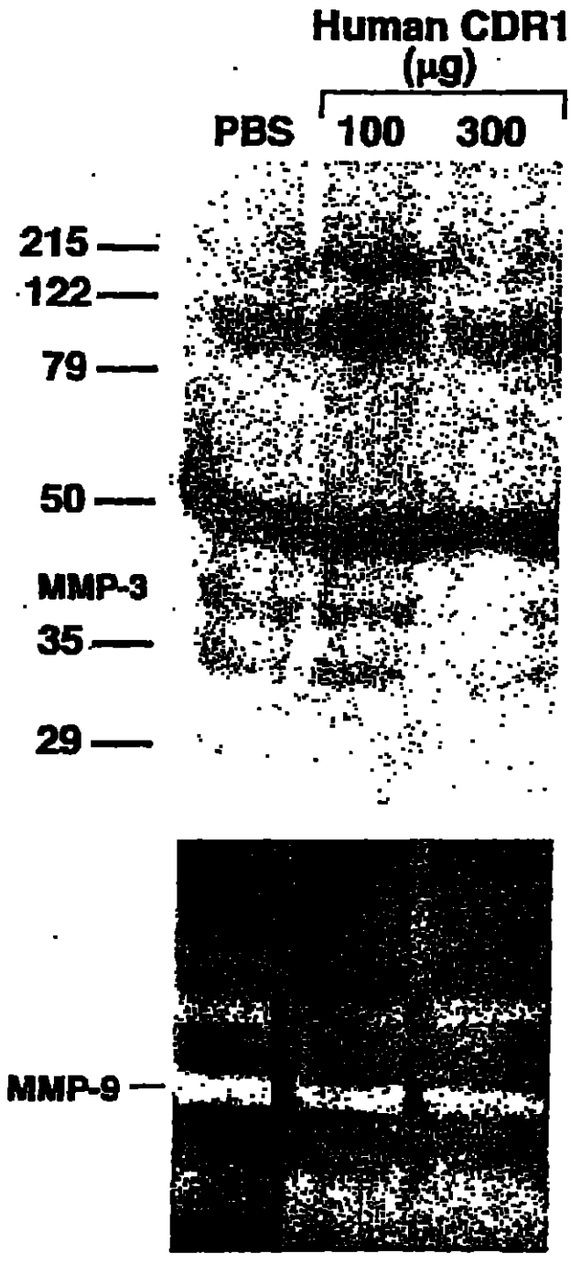
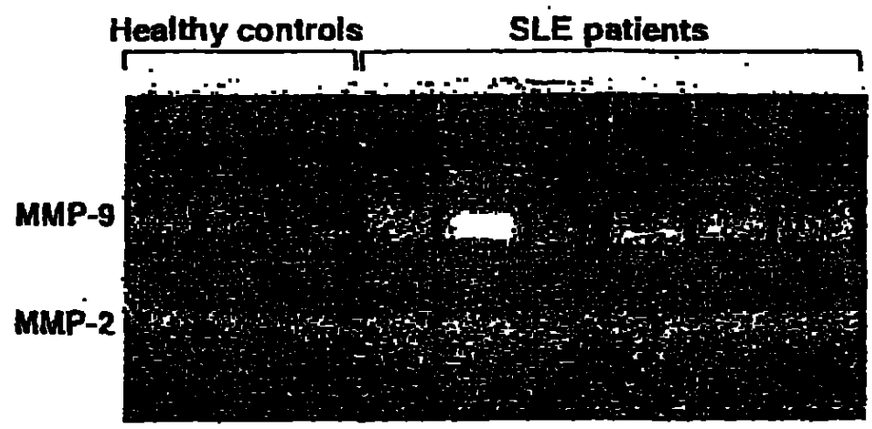



Fig. 23




329

Fig. 24

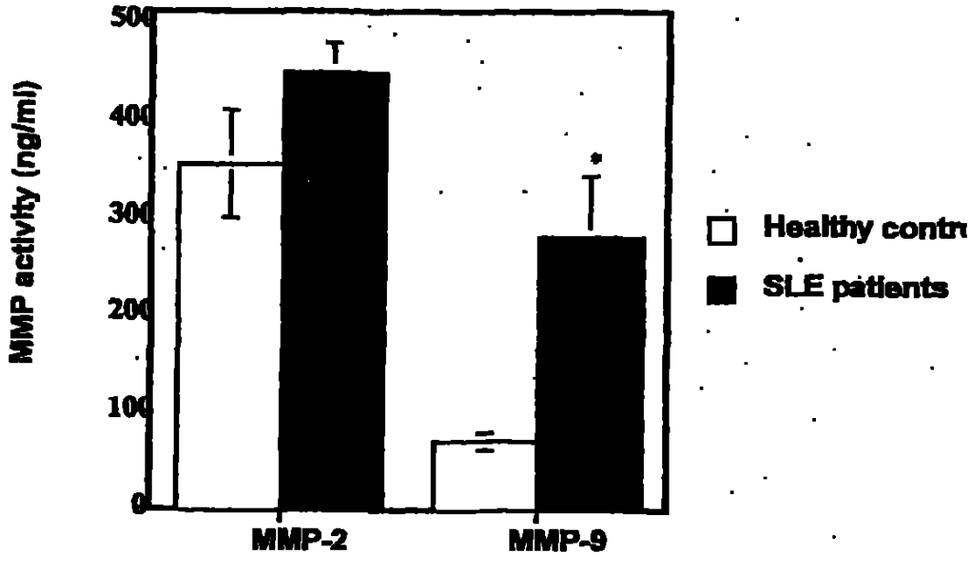



Fig. 25

330

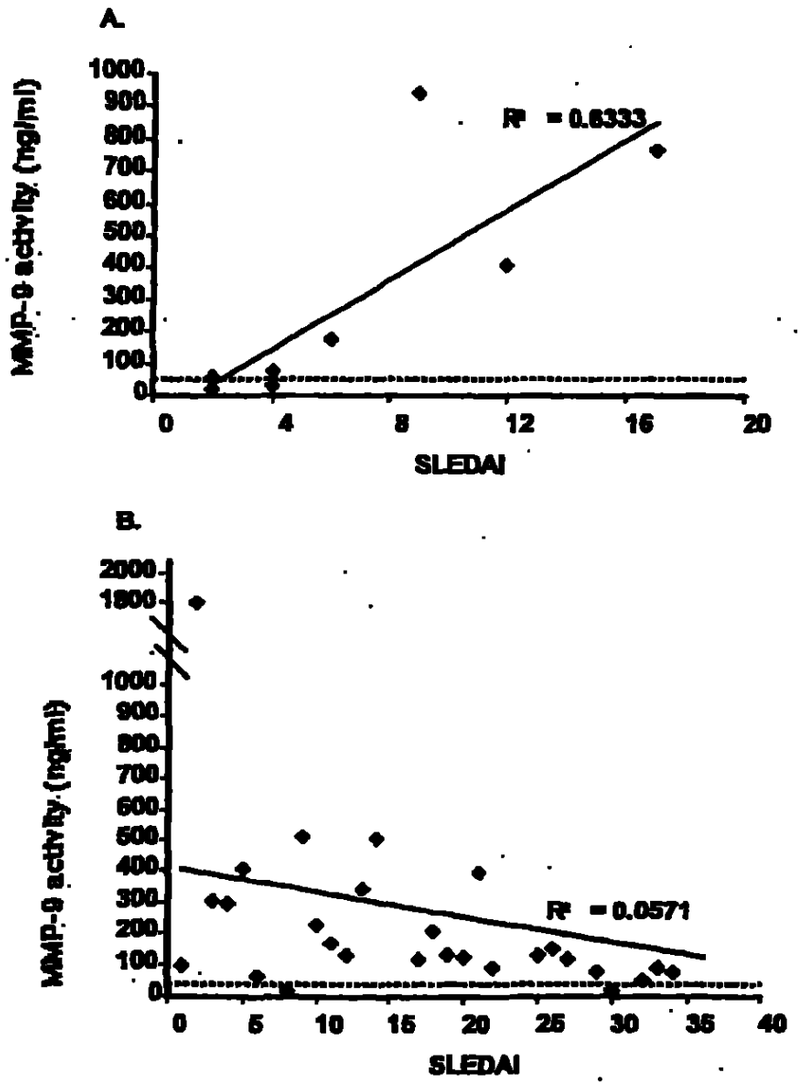

A.
MMP-9 activity (ng/ml)
1000
900
800
700
600
500
400
300
200
100
0
R² = 0.6333
0
4
8
12
16
20
SLEDAI
B.
MMP-9 activity (ng/ml)
2000
1800
1000
900
800
700
600
500
400
300
200
100
0
R² = 0.0571
0
5
10
15
20
25
30
35
40
SLEDAI

Fig. 26

331

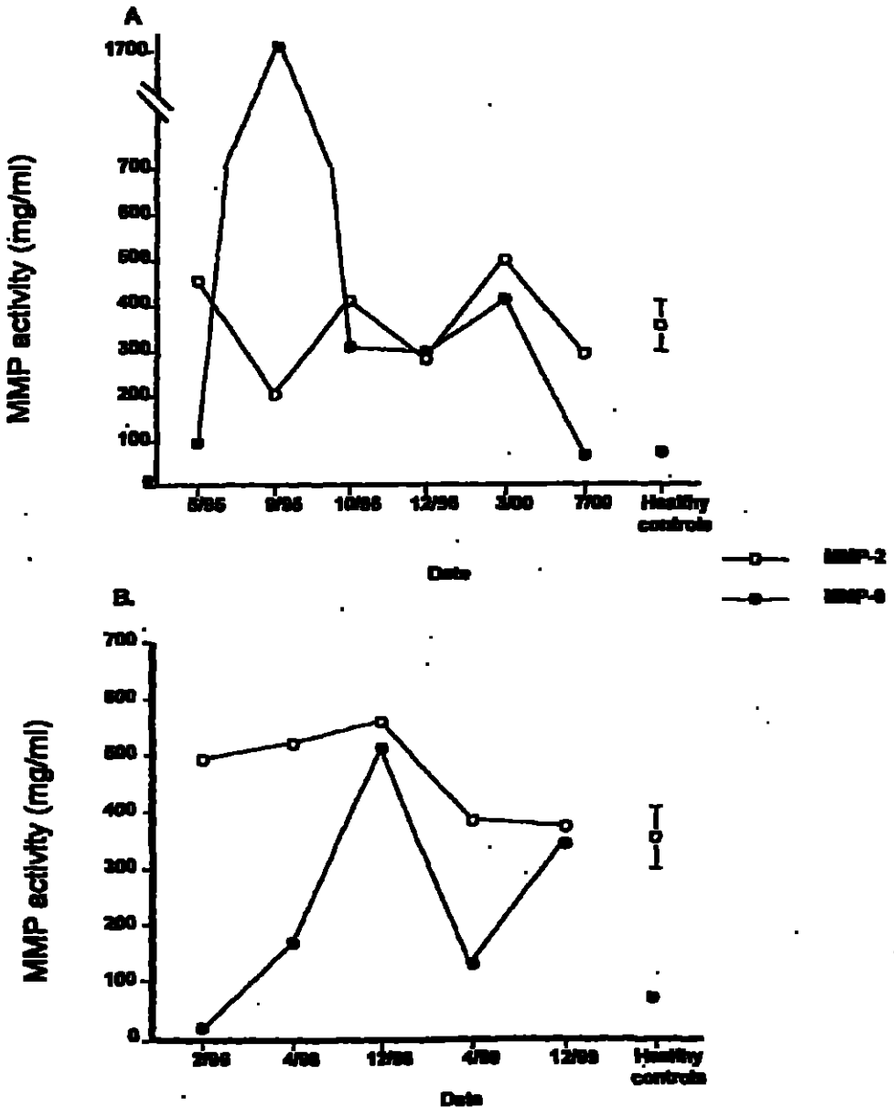

A
MMP activity (mg/ml)
1700
700
600
500
400
300
200
100
0
Healthy controls
Date
MMP-2
MMP-9
B.
MMP activity (mg/ml)
700
600
500
400
300
200
100
0
Healthy controls
Date

Fig. 27

332

# SYNTHETIC HUMAN PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEM FOR THE TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

## FIELD OF THE INVENTION

[0001] The present invention relates to synthetic peptides and, more particularly, to synthetic peptides based on the complementarity-determining region (CDR) of a human monoclonal anti-DNA antibody, to pharmaceutical compositions comprising them, and to their use in the immunomodulation of systemic lupus erythematosus (SLE)-associated responses.

[0002] Abbreviations: 16/6Id: human 16/6Id mAb; CDR: complementarity-determining region; CFA: complete Freund's adjuvant; hCDR peptide: a peptide based on a CDR region of the human 16/6Id mAb; hCDR1: the human peptide of the SEQ ID NO:6; hCDR3: the human peptide of the SEQ ID NO:7; human 16/6Id mAb: human pathogenic anti-DNA mAb that bears the 16/6Id; ICD: immune complex deposits; Id: idiotype; LNC: lymph node cells; mAb: monoclonal antibody; MMP: matrix metalloproteinase; mCDR1: the murine peptide of the SEQ ID NO: 1; mCDR3: the murine peptide of the SEQ ID NO3; PBL: peripheral blood lymphocytes; PBS: phosphate-buffered saline; rev: reversed peptide; SLE: systemic lupus erythematosus; SLEDAI: SLE disease activity index; TGF-β: transforming growth factor-β; UT: untreated.

## BACKGROUND OF THE INVENTION

[0003] Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of an array of autoantibodies, including antibodies to DNA, antibodies to nuclear antigens and antibodies to ribonucleoproteins. The progression of the disease is associated with general clinical manifestations and damage to tissues and organs caused by deposition of immune complexes. Similar to other autoimmune conditions, the etiology of SLE is multifactorial entailing genetic, environmental, hormonal and immunological factors. No specific treatment aimed towards the prevention or cure of SLE is available.

[0004] The human monoclonal anti-DNA antibody termed 16/6Id bears a common idiotype (Shoenfeld et al., 1983). The idiotype was found to have clinical relevance in SLE patients. Thus, the 16/6Id was found to be expressed on anti-DNA antibodies of 54% of SLE patients with active disease (Isenberg et al., 1984) and in affected organs of patients with SLE (Isenberg and Collins, 1985). Mice of inbred strains that do not develop any spontaneous autoimmune diseases were immunized with this human anti-DNA 16/6Id mAb and developed the major hallmarks of SLE in humans and in the spontaneous murine models for this disease (Mendlovic et al., 1988). Thus, following immunization, the mice produced antibodies specific to the 16/6Id, antibodies that bear the 16/6Id and antibodies directed against different nuclear antigens (dsDNA, ssDNA, Sm, ribonucleoprotein, Ro, La and others). The serological findings were associated with leukopenia, elevated erythrocyte sedimentation rate, proteinuria, abundance of immune complexes in the kidneys and sclerosis of the glomeruli (Mendlovic et al., 1988), which are typical manifestations of SLE.

[0005] A murine anti-16/6Id mAb (Ab2) derived from mice with experimental SLE was also capable of inducing the experimental disease in mice (Mendlovic et al., 1989) similar to the 16/6Id (Ab1). Moreover, a murine anti-DNA mAb that expresses the 16/6Id was prepared from mice afflicted with experimental SLE. The antibody Ab3 termed 5G12 reacted with antibodies specific to the 16/6Id. Immunization with the latter antibody resulted in the induction of experimental SLE with similar manifestations as observed following immunization with the human 16/6Id (Ab1) and with the murine anti-16/6Id (Ab2) mAbs (Waisman et al., 1993). These results show the importance of the 16/6Id network in the induction and progression of SLE in mice.

[0006] In order to understand the mechanism by which self-antibodies associated with SLE arise, the present inventor has produced a variety of monoclonal autoantibodies derived from C3HLSW mice in which experimental SLE was induced. As a rule, the monoclonal autoantibodies that were capable of eliciting antibodies that bear the 16/6Id or react with it were found to be pathogenic and thus capable of inducing experimental SLE in mice (Fricke et al., 1990; Sthoeger et al., 1993).

[0007] Later on, the variable (V) regions of nine autoantibodies that bind either DNA or HeLa nuclear extract (NE), isolated from the C3HLSW mice with experimental SLE, were sequenced (Waisman and Mozes, 1993). Monoclonal antibodies with different specificity were analyzed in an attempt to determine the connections between the different autoantibodies. Three mAb were found to bind DNA, and were shown to exhibit sequence characteristics of pathogenic anti-DNA antibodies. One of these mAb, designated 2C4C2, was shown to use a heavy (H) chain V region gene ($V_H$) identical to the $V_H$ of anti-DNA mAb isolated from other lupus-prone mice, namely (NZB x NZW)$F_1$. The light (L) chain V region gene ($V_L$) of mAb 2C4C2 is 98% homologous to the $V_L$ of another anti-DNA mAb, also isolated from (NZB x NZW)$F_1$ mice. The other two anti-DNA mAb, designated 5G12-4 and 5G12-6 share 93% of their $V_H$ sequences with that of mAb 2C4C2. Based on the analysis of these mAbs, it appeared that autoantibodies found in mice with experimental SLE use genetic elements similar to those used by mAb that were isolated from mouse strains which develop lupus spontaneously.

[0008] T cells play an important role in the induction and development of experimental SLE. Thus, T cell lines and clones specific to the 16/6Id were shown to induce experimental SLE in syngeneic recipients similarly to the 16/6Id antibody. Therefore, following the inoculation of the activated cells of the lines, the mice developed both the serology and the renal damage which is typical to SLE (Fricke et al., 1991).

[0009] As described above, the mAb 5G12 that was isolated from mice with experimental SLE and was shown to bind DNA and bear the 16/6Id, is capable of inducing experimental SLE in mice (Waisman et al., 1993). T cells that react specifically to mAb by proliferation, are probably reacting to peptides representing sequences from their complementarity-determining regions (CDR). It is very likely that the T cells recognize the V regions of the above antibodies since they do not react with other antibodies that carry the same constant region but have different specificities. Within the variable region, the regions with the highest probability to be recognized are the CDR, since these are the regions that differ the most between the various antibodies.

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The CDR regions of the $V_H$ sequences of the nine pathogenic murine mAb mentioned above that induce SLE in mice, are boxed in FIG. 1 of Waisman and Monne, 1993, in which the complete nucleotide and deduced amino acid sequences for the variable heavy chains ($V_H$) of the nine mAbs are presented.

[0010] International PCT Patent Publication No. WO 96/30057 of the present applicants describes peptides based on the CDR regions of pathogenic mAbs isolated from mice with experimental SLE, in particular peptides Ia to IIIa, based on the CDR1, CDR2 and CDR3 regions, respectively, of the $V_H$ chain of the murine mAb termed 5G12, and peptides IVa and Va, based on the CDR1 and CDR3 regions, respectively, of the $V_H$ chain of the murine mAb termed 2C4C2. These peptides have the sequences substantially as denoted by SEQ ID NO:1 to SEQ ID NO:5 as follows:

| | | |
|---|---|---|
| T G Y Y M Q W V K Q S P E K S L E W I G | (Ia) | [SEQ ID NO:1] |
| E I N P S T G G T Y Y N Q K F K A K A T | (IIa) | [SEQ ID NO:2] |
| Y Y C A R F L W E P Y A M D Y W G Q G S | (IIIa) | [SEQ ID NO:3] |
| G Y E F T N Y V K Q S H G K S L E W I G | (IVa) | [SEQ ID NO:4] |
| Y Y C A R S G R Y G N Y W G Q T L | (Va) | [SEQ ID NO:5] |

[0011] These peptides and, in particular, the peptides Ia and IIIa, herein designated mCDR1 [SEQ ID NO:1] and mCDR3 [SEQ ID NO:3], respectively, were shown by the inventor to be capable, when administered in PBS, of inhibiting T cell priming to either the appropriate mCDR peptide or to the whole anti-DNA 16/6Id mAb of either murine or human origin (Waisman et al., 1997). The peptides mCDR1 and mCDR3 were further shown by the inventor to either prevent or treat an already established SLE that is either induced by the human anti-DNA 16/6Id mAb or that develops spontaneously in the SLE prone mice (NZB x NZW) F1 or MRL/lpr/lpr (Eilat et al., 2000 and 2001).

## SUMMARY OF THE INVENTION

[0012] It has now been found according to the present invention that peptides based on the CDR of the human monoclonal anti-DNA 16/6Id antibody are capable of immunomodulating SLE-associated responses. Thus, peptides based on the CDR1 and CDR3 of the human 16/6Id were tested and shown to inhibit lymph node cell proliferation of mice immunized with the murine peptides mCDR1 (SEQ ID NO:1) and mCDR3 (SEQ ID NO:3) or with the whole human anti-DNA 16/6Id mAb, to inhibit the proliferative response of peripheral blood lymphocytes (PBL) of SLE patients to the human anti-DNA 16/6Id mAb, and to ameliorate disease manifestations of mice afflicted with spontaneous or experimental SLE.

[0013] These findings are completely unexpected because not all CDRs of pathogenic autoantibodies are equally recognized by T cells of patients. As shown previously in the laboratory of the present inventor (Dayan et al., 2000), the CDRs of the anti-DNA autoantibody 2C4C2 are less well recognized by T cells of SLE patients than the peptides based on the CDRs of the anti-DNA antibody 5G12. Furthermore, many of the analogs of the peptides based on the CDRs of the murine autoantibodies described in the above-mentioned WO 96/30057 were shown not to be efficient in their inhibitory effects, and thus the modifications occurring in the sequences of the peptides based on the CDRs of a human monoclonal anti-DNA antibody of the present invention, could not be predicted or suggested to be efficient. In addition, the use of peptides based on a human antibody should be considered as preferable for human use in comparison with peptides based on non-human antibodies.

[0014] The present invention thus relates, in one aspect, to a synthetic peptide selected from the group consisting of:

[0015] (a) a peptide of at least 12 and at most 30 amino acid residues comprising a sequence consisting of, or found within, a complementarity-determining region (CDR) found in the heavy or light chain of the human monoclonal anti-DNA 16/6Id antibody (hereinafter "hCDR sequence"), or a sequence obtained by: (i) replacement of one or more of the amino acid residues of the hCDR sequence by different amino acid residues; (ii) deletion of one or more amino acid residues from the hCDR sequence; and/or (iii) addition of one or more amino acid residues to the hCDR sequence , or a salt or a chemical derivative of said peptide;

[0016] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0017] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0018] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0019] The human monoclonal anti-DNA 16/6Id antibody, herein referred to as "human 16/6Id mAb", is a pathogenic human monoclonal anti-DNA antibody capable of inducing a SLE-like disease in mice.

[0020] A peptide according to the invention comprising a hCDR sequence as defined above is herein referred to as "hCDR peptide".

[0021] In one preferred embodiment, the hCDR peptide includes a sequence of a CDR, more preferably of the CDR1 or CDR3, of the heavy chain of the human 16/6Id mAb, such as, but not being limited to, the peptides herein designated hCDR1 and hCDR3, having the sequences substantially as denoted by SEQ ID NO:6 and SEQ ID NO:7, respectively, as follows:

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GYYWSWIRQPPGKGLEWIG [SEQ ID NO:6]

YYCARGLLRGWNDVDYYGRDV [SEQ ID NO:7]

[0022] In another aspect, the invention provides a pharmaceutical composition comprising at least one synthetic peptide or peptide polymer of the invention, and a pharmaceutically acceptable carrier. The pharmaceutical composition is particularly useful for the treatment of SLE and amelioration of the clinical manifestations of the disease, particularly by modulating SLE-associated responses, e.g. down-regulating the levels of P-3 and/or MMP-9, and/or IL-2 and/or IFN-γ activities, or up-regulating the level of TGF-β activity in a patient with SLE.

[0023] In a further aspect, the invention relates to a method for the treatment of SLE comprising administering to an SLE patient an effective amount of a peptide or peptide polymer of the invention. The invention further relates to a method of immunomodulating SLE-associated responses, e.g. down-regulating the levels of MMP-3 and/or MNP-9, and/or IL-2 and/or IFN-γ activities, or up-regulating the level of TGF-β activity in a patient with SLE, comprising administering to an SLE patient an effective amount of a peptide or peptide polymer of the invention.

[0024] In still a further aspect, the invention relates to a method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the levels of MMP-3 and/or MMP-9 in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of MMP-3 and/or MMP-9 correlates with the effectiveness of the drug.

[0025] In still another further aspect, the invention relates to a method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the level of IL-2 and/or IFN-γ in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of IL-2 and/or IFN-γ correlates with the effectiveness of the drug.

[0026] In yet still a further aspect, the invention relates to a method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the level of TGF-β in a blood sample obtained from said patient being treated with said drug, whereby an increased level of TGF-β correlates with the effectiveness of the drug.

[0027] The drug which effectiveness is to be assessed according to any of the above methods may be, for example, without being limited to, a peptide according to the invention or a murine peptide as described in the above-mentioned WO 96/30057.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0028] FIG. 1 shows inhibition of the proliferative responses of lymph node cells of mice immunized with human 16/6Id mAb to various concentrations of the 16/6Id mAb (0.1-10 μg/well), by treatment with 300 μg hCDR1.

[0029] FIG. 2 shows inhibition of the proliferative responses of lymph node cells of mice immunized with human 16/6Id mAb to various concentrations of the 16/6Id mAb (0.1-10 μg/well), by treatment with 50 μg hCDR1.

[0030] FIGS. 3A-C show the cytokine pattern in BALB/c mice immunized with human 16/6Id mab and treated with hCDR1 or with an irrelevant peptide p259-271. FIG. 3A—IFN-γ pattern; FIG. 3B—TGF-β pattern: FIG. 3C—IL-10 pattern.

[0031] FIG. 4 shows the concentrations of the human anti-DNA 16/6Id mAb required for optimal stimulation of PBL of SLE patients and of healthy controls. PBL were stimulated with various concentrations (0.1-40 μg/well) of the 16/6Id mAb. The concentration yielding the highest stimulation index was defined as optimal for triggering a proliferative response.

[0032] FIG. 5 shows proliferation of PBL from one SLE patient stimulated with the mitogen phytohemagglutinin (PHA) in the absence or presence of hCDR1 or hCDR3.

[0033] FIG. 6 shows proliferation of PBL from one SLE patient stimulated with human 16/6Id mAb in the absence or presence of the human peptides hCDR1 or hCDR3 or the murine peptide mCDR3.

[0034] FIG. 7 shows proliferation of PBL from one SLE patient stimulated with human 16/6Id mAb in the absence or presence of the human peptides hCDR1 or hCDR3 or the murine reversed peptides revmCDR1 and revmCDR3.

[0035] FIG. 8 shows inhibition of IL-2 secretion in PBL of SLE patients triggered by the human 16/6Id mAb in the absence or presence of hCDR1 or hCDR3.

[0036] FIG. 9 shows up-regulation of TGF-β secretion in the PBL of one representative SLE patient stimulated with the human 16/6Id mAb in the absence or presence of hCDR1 or hCDR3.

[0037] FIG. 10 shows anti-DNA autoantibody levels in (NZB x NZW)F1 mice untreated or treated with 300 μg hCDR1 or with the reversed peptide revhCDR1 (used as control).

[0038] FIGS. 11A-11D are photos showing representative kidney sections of SLE-prone (NZB x NZW)F1 mice that were treated starting at the age of 5 and half months with PBS (11A, 11B) or with 100 μg hCDR1 (11C, 11D). The sections are of mice sacrificed at the age of 9 months. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (11A, 11C x 100; 11B, 11D x 400).

[0039] FIGS. 12A-12F are photos showing representative kidney sections of SLE-prone (NZB x NZW)F1 mice that were treated with PBS (12A, 12B) or with 300 μg hCDR1 (12C, 12D), or with the reversed peptide revhCDR1 (12E, 12F). The sections are of mice sacrificed at the age of 9 months. For the detection of immune complex Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (12A, 12C, 12E x 100; 12B, 12D, 12F x 400).

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[0040] FIGS. 13A-13C show the cytokine pattern as measured by ELISA in supernatants of Con A-stimulated cultures of splenocytes of SLE-prone (NZB x NZW)F1 mice that were untreated or treated with hCDR1 or with the reversed peptide revhCDR1. FIG. 13A—IFN-γ pattern; FIG. 13B—IL-10 pattern; FIG. 13C—TGF-β pattern.

[0041] FIGS. 14A-14F are photos showing representative kidney sections of BALB/c mice with 16/6Id-induced experimental SLE treated with PBS (14A,14B) or with 200 μg hCDR1 (14C, 14D), or with the reversed peptide revhCDR1 (14E, 14F). The sections are of mice sacrificed at the age of 9 months. For the detection of immune complex Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (14A, 14C, 14E x 100; 14B, 14D, 14F x 400).

[0042] FIGS. 15A-15E show the cytokine pattern as measured by ELISA in supernatants of 16/6Id-stimulated lymph node cultures of BALB/c mice with 16/6Id induced experimental SLE that were untreated or treated with hCDR1 (200 or 300 μg ) or with the reversed peptide revhCDR1. FIG. 15A—IFN-γ pattern; FIG. 15B—TNF-α; FIG. 15C—IL-10 pattern; FIG. 15D—TGF-β pattern; FIG. 15E—TGF-β pattern, measured in supernatants of 16/6Id-triggered spleen cells.

[0043] FIGS. 16A-16F are photos showing representative kidney sections of SLE-prone (NZB x NZW)F1 mice untreated (16A, 16B) or mice recipients of splenocytes of mice treated either with 300 μg hCDR1 (16C, 16D) or with the reverse peptide revhCDR1 (16E, 16F). For the detection of immune complex Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ chain specific) (16A, 16C, 16E x 400; 16B, 16D, 16F x 100).

[0044] FIGS. 17A-17B depict kinetics of appearance of MMP-3, MMP-2, and MMP-9 in the sera of (NZB x NZW)F1 mice. (NZB x NZW)F1 mice (10 mice/group) were bled at the indicated time points. Pooled sera (4 μl.) of mice from each group were tested for MMP-3 expression levels using Western blot (FIG. 17A) or for MMP-9 and MMP-2 activities using gel zymography (FIG. 17B). The results represent 4 similar experiments.

[0045] FIGS. 18A-18B depict kinetics of appearance of MMP-3, MMP-2, and MMP-9 in the sera of BALB/c mice. Non-immunized BALB/c mice (10 mice/group) or mice that were immunized with PBS/CFA (10 mice/group) or with the 16/6Id (in CFA; 10 mice/group), were bled at the indicated time points. Pooled sera of mice (4 vl) from each group were tested for MMP-3 expression levels using Western blot (FIG. 18A) or for MMP-9 and MMP-2 activities using gel zymography (FIG. 18B). The results are representative of 3 similar experiments.

[0046] FIGS. 19A-19C depict immunostaining of kidney sections of immunized BALB/c mice for MMP-3 and MMP-9. Non-immunized BALB/c mice or mice that were immunized with PBS/CFA or with the 16/6Id (in CFA), (3 mice/group), were sacrificed 5.5 months following their boost with the 16/6Id. Kidneys were removed, and their 5 μm cryostat sections were immunostained for P-3 (19A) and P-9 (19B). A control staining to the efficiency of blocking, was performed (19C). (x200). The results are representative of 3 similar experiments.

[0047] FIGS. 20A-20B depict levels of MMP-3 and MMP-9 in sera of (NZB x NZW)F1 mice treated with mCDR1. In prevention experiments, mice (10/group) were given weekly s.c. injections with mCDR1 starting at the age of 2 months during 10 weeks. The results represent sera taken 4 months after the end of the treatment. In treatment experiments, mice (10/group) were injected s.c. with either PBS or 250 μg/mouse of mCDR1 starting at the age of 5 months. The results represent sera taken 3 weeks after the end of the treatment. Pooled sera of each experimental group were tested for MMP-3 levels by Western blot analysis (20A) and for MMP-9 activity using gel zymography (20B). UT—untreated. The results are representative of 2 similar experiments.

[0048] FIGS. 21A-22B depict levels of MMP-3 and MMP-9 in 16/6Id-immunized BALB/c mice treated with mCDR1. In prevention experiments, mice (8/group) were treated i.v. with mCDR1 (100 μg/mouse). The results shown are of sera taken 4.5 months after the end of the treatment. In treatment experiments, mice (8/group) were treated s.c. with 100 μg/mouse mCDR1. The results are of sera obtained at the end of treatment. Pools of sera of each experimental group were tested for MMP-3 by Western blot analysis (21A) and for MMP-9 activity by gel zymography (21B). UT —untreated. The results are representative of 2 similar experiments.

[0049] FIGS. 22A-22B depict immunostaining for MMP-3 and MMP-9 of kidney sections of 16/6Id immunized BALB/c mice that were treated with mCDR1 for prevention (22A) or treatment (22B) of experimental SLE. Mice were sacrificed 8 months following disease induction, and their kidneys were removed. Cryostat sections (5 μm) were prepared and immunostained for MMP-3, MMP-9 and for the presence of immune complex deposits (x200). (W/O)—control staining to the efficiency of blocking, without the first antibody. The results are representative of 2 similar experiments.

[0050] FIGS. 23A-23B depict levels of MMP-3 and P-9 in sera of (NZB x NZW)F1 mice treated with hCDR1. In treatment experiments, mice (10/group) were injected s.c. with either PBS or 100 μg or 30 μg/mouse of hCDR1 once a week for ten weeks starting at the age of 7 months. The results represent sera collected at the middle of the treatment. Pooled sera of each experimental group were tested for MMP-3 levels by Western blot analysis (23A) and for MMP-9 activity using gel zymography (23B). The results are representative of 2 similar experiments.

[0051] FIG. 24 depicts a representative gel showing activity of MMP-2 and MMP-9 in sera of SLE patients and healthy controls. Sera (5 μl) of 40 individual SLE patients and 25 healthy controls were analysed for their MMP-2 or MMP-9 activities by gel zymography. The figure shows representative results with serum samples of the two groups.

[0052] FIG. 25 depicts a graph showing quantitative analysis of MMP-2 and MMP-9 activities in sera of SLE patients (dark columns) and healthy controls (white columns). Thirty-six serum samples of SLE patients and 15 serum samples of healthy controls were tested for MMP-2 or MMP-9 activity using specific activity assay kits. Results are expressed as the mean ± s.e.m. *P=0.0302.

[0053] FIGS. 26A-26B are graphs showing MMP-9 activity levels and disease activity indices (SLEDAI) in patients with SLE. Thirty-five serum samples from 8 males (FIG.

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26A) and 27 females (FIG. 26B) SLE patients were tested for MMP-9 activity by a specific activity assay kit. The distribution of MMP-9 activity according to the SLEDAI of the patients is presented. The dashed line represents the activity of MMP-9 in healthy controls.

[0054] FIGS. 27A-27B are graphs showing pattern of MMP-2 (white circles) and MMP-9 (black circles) activities in sera of two SLE patients sampled during 4-6 years of disease. The sera were tested for MMP-2 or MMP-9 activities by specific activity assay kits. The assays were performed in duplicate.

## DETAILED DESCRIPTION OF THE INVENTION

[0055] The present invention relates, in one aspect, to synthetic peptides comprising a sequence consisting of, or found within, a CDR found in the heavy or light chain of the pathogenic human monoclonal anti-DNA 16/6Id antibody (herein identified as "human anti-DNA 16/6Id mAb" or "human 16/6Id mAb"), which antibody induces a SLE-like disease in mice.

[0056] The synthetic peptides of the invention derived from a CDR of the human 16/6Id mAb, herein identified as hCDR peptides, are preferably based on a CDR found in the heavy chain of the human 16/6Id mAb.

[0057] The CDR regions of the $V_H$ sequences of the human 16/6Id mAb are boxed in FIG. 4A of Waisman et al., 1995. The CDR regions of the heavy chains of the human 16/6Id mAb have the sequences substantially as denoted by SEQ ID NO: 8 to SEQ ID NO: 10, as follows:

CDR1: [redacted] [SEQ ID NO: 8]

CDR2: [redacted] [SEQ ID NO: 9]

CDR3: [redacted] [SEQ ID NO: 10]

[0058] The hCDR peptides of the invention contain at least 12 and at most 30 amino acid residues and comprise, preferably, a sequence identical to a sequence selected from the group consisting of SEQ ID NO: 8, 9 and 10, or more preferably are sequence found within said SEQ ID NO: 8, 9 or 10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of the SEQ ID NO: 8, 9 and 10 sequences by different amino acid residues; (ii) deletion of one or more amino acid residues from the SEQ ID NO: 8, 9 and 10 sequences; or (iii) addition of one or more amino acid residues to the SEQ ID NO: 8, 9 and 10 sequences.

[0059] The hCDR peptides of the invention, besides the hCDR sequence, contain further amino acid residues, preferably amino acid residues of the sequences of the human 16/6Id mAb flanking the hCDR sequences or sequences obtained by replacement of one or more of the amino acid residues of the hCDR flanking sequences by different amino acid residues, by deletion of one or more amino acid residues from the hCDR flanking sequences or by addition of one or more amino acid residues to the hCDR flanking sequences.

[0060] Thus, in one embodiment, the present invention provides a synthetic peptide based on the CDR1 of the heavy chain of the human 16/6Id mAb, said CDR1 region being of the sequence substantially as denoted by SEQ ID NO:8, said peptide being selected from the group consisting of:

[0061] (a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO: 8, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:8 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:8; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:8, or a salt or a chemical derivative of said peptide;

[0062] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0063] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0064] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0065] In one preferred embodiment of the invention, a peptide based on the CDR1 of the heavy chain of the human 16/6Id mAb is a peptide of the sequence substantially as denoted by SEQ ID NO: 11:

$X_1$YYWSWI$X_2$QX$_3$PX$_4$X$_5$GX$_6$EWIG [SEQ ID NO:11]

[0066] wherein $X_1$ is G or TG; $X_2$ is R or K; $X_3$ is P or S; $X_4$ is G or E; $X_5$ is K or D; and $X_6$ is R, L or S.

[0067] In a more preferred embodiment, the peptide of SEQ ID NO:11 is the 19-mer peptide herein designated "hCDR1 peptide" or simply "hCDR1", of the sequence substantially as denoted by SEQ ID NO:6:

GYYWSWIRQPPGKGEEWIG [SEQ ID NO:6]

[0068] In hCDR1, the sequence GYYWS comprised within SEQ ID NO:8, is followed by the natural sequence of the CDR1 of the heavy chain of the human 16/6Id mAb, with the exception that the natural leucine (L) residue of the mAb sequence was replaced by a glutamic acid residue (E) (bold) at position 15 of the peptide hCDR1.

[0069] In another embodiment, the peptide of SEQ ID NO:11 is an analog of the hCDR1 peptide obtained by replacement and/or addition of amino acid residues to the sequence of the hCDR1 peptide, examples thereof being the peptides of the sequences substantially as denoted by SEQ ID NO:12 to SEQ ID NO:18 (wherein the amino acids replaced or added are represented in bold):

GYYWSWIRQPPGKGLEWIG [SEQ ID NO:12]

GYYWSWIRQPPGKGSEWIG [SEQ ID NO:13]

GYYWSWIRQPPGDGEEWIG [SEQ ID NO:14]

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-continued

GYYWSWIRQPPGKGLEWIG [SEQ ID NO:15]

GYYWSWIRQSPGKGLEWIG [SEQ ID NO:16]

GYYWSWIRQPPRKGLEWIG [SEQ ID NO:17]

FGYYWSWIRQPPGKGLEWIG [SEQ ID NO:18]

[0070] In a further embodiment, the invention provides a synthetic peptide based on the CDR3 of the heavy chain of the human 16/6Id mAb, said CDR3 region being of the sequence substantially as denoted by SEQ ID NO:10, said peptide being selected from the group consisting of:

[0071] (a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:10 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO: 10; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:10, or a salt or a chemical derivative of said peptide;

[0072] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

[0073] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0074] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0075] In one preferred embodiment of the invention, the peptide based on the CDR3 of the heavy chain of the human 16/6Id mAb is a peptide of the sequence substantially as denoted by SEQ ID NO:19:

$$YYCARX_1LLX_2X_3X_4X_5X_6DVDYX_7GX_8DV \quad \text{[SEQ. ID NO:19]}$$

[0076] wherein $X_1$ is G or F; $X_2$ is R or A; $X_3$ is G or A; $X_4$ is G or A; $X_5$ is W or A; $X_6$ is N or A; $X_7$ is Y or W; and $X_8$ is M or Q.

[0077] In a more preferred embodiment, the peptide of SEQ ID NO: 19 is the peptide herein designated "hCDR3 peptide" or simply "hCDR3", of the SEQ ID NO:7:

YYCARGLLRGGWNDVDYYGMDV [SEQ ID NO:7]

[0078] In hCDR3, the sequence GLLRGGWNDVDYYYGMDV [SEQ ID NO:10] of the CDR3 region of the heavy chain of the human 16/6Id mAb is modified by deletion of one of the tyrosine (Y) residues and is preceded by the natural sequence of the mAb.

[0079] In another embodiment, the peptide of SEQ ID NO:19 is an analog of the hCDR3 peptide obtained by replacement and/or addition of amino acid residues to the sequence of the hCDR3 peptide, examples thereof being the peptides of the sequences SEQ ID NO:20 to SEQ ID NO:27 (wherein the amino acids replaced or added are represented in bold):

YYCARGLLRGGWADVDYYGMDV [SEQ ID NO:20]

YYCARGLLRGGANDVDYYGMDV [SEQ ID NO:21]

YYCARGLLRGAWNDVDYYGMDV [SEQ ID NO:22]

YYCARGLLRAGWNDVDYYGMDV [SEQ ID NO:23]

YYCARGLLAGGWNDVDYYGMDV [SEQ ID NO:24]

YYCARFLLRGGWNDVDYYGMDV [SEQ ID NO:25]

YYCARGLLRGGWNDVDYYGQDV [SEQ ID NO:26]

YYCARGLLRGGWNDVDYWGMDV [SEQ ID NO:27]

[0080] In still a further embodiment, the invention provides a synthetic peptide based on the CDR2 of the heavy chain of the human 16/6Id mAb, said CDR2 region being of the sequence substantially as denoted by SEQ ID NO:9, said peptide being selected from the group consisting of:

[0081] (a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:9, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:9 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:9; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:9, or a salt or a chemical derivative of said peptide;

[0082] (b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

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[0083] (c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

[0084] (d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

[0085] The synthetic peptides of the present invention have 12-30, preferably 17-23, most preferably 19-22, amino acid residues, and may be manufactured by chemical synthesis or by recombinant technology by methods well known in the art.

[0086] When preparing analogs as described above obtained by substitution of amino acid residues, it is important that the substitutions be selected from those which cumulatively do not substantially change the volume, hydrophobic-hydrophilic pattern and charge of the corresponding portion of the unsubstituted parent peptide. Thus, a hydrophobic residue may be substituted with a hydrophilic residue, or vice-versa, as long as the total effect does not substantially change the volume, hydrophobic-hydrophilic pattern and charge of the corresponding unsubstituted parent peptide.

[0087] The present invention also includes chemical derivatives of a peptide of the invention. The "chemical derivative" contains additional chemical moieties not normally a part of the peptide and is encompassed by the invention as long as it retains at least a portion of the function of the peptide which permits its utility in preventing or inhibiting T cell proliferative responses and autoimmune disease. For example, a chemical derivative may result from the reaction of an organic derivatising agent capable of reacting with selected side chains or terminal residues of said peptide, and will preferably retain at least a portion of the function of the peptide to inhibit specifically the proliferative response and cytokine secretion of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies. Among these chemical derivatives, the *amides* are of particular interest, both amides of carboxyl groups at the C-terminus and amides of free carboxyl groups of aspartic or glutamic acid residues. Many such chemical derivatives and methods for making them are well known in the art.

[0088] Also included in the scope of the invention are salts of the peptides and analogs of the invention. As used herein, the term "salts" refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptide molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as those formed for example, with amines, such as triethanolamine, arginine, or lysine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Such chemical derivatives and salts are preferably used to modify the pharmaceutical properties of the peptide insofar as stability, solubility, etc., are concerned.

[0089] The hCDR peptides according to the invention may be selected by testing for their potential in inhibiting the proliferative response of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies. Once a peptide in accordance with the present invention is produced, its ability to inhibit the proliferative response of T lymphocytes of mice that are high responders to SLE-inducing autoantibodies may be readily determined by those of ordinary skill in the art without undue experimentation using tests such as those described herein. One test which may be readily conducted is for the ability of the peptides to inhibit in vitro the proliferative responses of certain T cell lines and clones specific to SLE-inducing autoantibodies. The T cell lines and clones may be, for example, the T cell lines and clones specific to the 16/6Id mAb (Fricke et al., 1991) established from immunized lymph node cells of mice by previously described methodology (Axelrod, O. and Mozes, E. *Immunobiology*, 172, 99 (1986)). Cells are exposed to the stimulating antibody presented on irradiated syngeneic spleen cells in the presence of enriched medium every two weeks. The T cell lines are cloned by the standard limiting dilution technique. The proliferative responses of these T cell lines and clones are tested, for example, by the method described in WO 96/30057, in Materials and Methods, section (g).

[0090] Another test which can be conducted in order to select analogs having the desired activity is to test for the ability of the synthetic peptides to inhibit the ability of the T. cell lines and clones to provide help to peptide-specific B cells in the presence of the parent peptide. The synthetic peptides may also be tested for their ability to bind directly, following biotinylation, to MHC Class II products on antigen-presenting cells of the relevant strains. For this purpose, N-terminal biotinylation of the relevant peptides is performed at 0° C. with an excess of biotin-N-hydroxysuccinimide in aqueous solution (Mozes et al., 1989). Mouse splenic adherent cells or PBL-adherent cells ($1\times10^6$/sample) are incubated with biotinylated peptides in PBS containing 0.1% bovine serum albumin (PBS/BSA) at 37° C. for 20 hr, followed by incubation with phycoerythrin-streptavidin for 30 min at 4° C. After each incubation, the cells are washed twice with the above solution. Thereafter, the cells are analyzed by flow cytometry using FACScan. In each analysis, a minimum of 5000 cells are examined (for above procedures, see, for example, Mozes et al., 1989).

[0091] A further test which can be conducted is to test for the ability of the peptides to inhibit cytokine secretion by the T cell line or by T lymphocytes or lymph node cells of mice that are high responders to SLE-inducing autoantibodies. The cytokines are detected as follows: IL-1 activity is assessed by ELISA using a pair of capture and detecting antibodies (as described below for IL-4, IL-6, IL-10). IL-2 is directly detected using the IL-2 dependent CTLL line or by ELISA. Levels of IL-4, IL-6, IL-10, INF-γ and TNF-α: in the supernatants are determined by ELISA using antibodies to the various cytokines (Pharmingen, San Diego, Calif., USA) according to the manufacturer's instructions. In addition, the ability of the peptides to elevate the level of secretion of the immunosuppressive cytokine TGF-β can be assessed by ELISA as described herein in the Examples.

[0092] Peptides which test positive in one or more of these in vitro tests will provide a reasonable expectation of in vivo activity. However, in vitro tests can also be conducted without undue experimentation. Thus, for example, adult mice may be injected with the candidate peptide at either day -3 or day 0. The mice are then immunized with the disease-inducing autoantibody or with the peptide. Ten days later, lymph node cells of the mice are tested for their ability to

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proliferate to the immunogen in order to find out the inhibitory capacity of the candidate peptide.

[0093] Another such in vivo animal test consists of measuring the therapeutic activity directly in the murine model in vivo for the production of SLE as described above. The peptides can be injected into the mice in which experimental SLE is induced by different routes at different dosages and at different time schedules. Furthermore, the treated mice can be tested periodically in order to determine the effect of the peptides on the autoantibody responses and on disease manifestations elicited in the mice by the SLE-inducing autoantibody.

[0094] Another in vivo procedure consists of assessing the candidate peptide ability to treat mice that spontaneously develop SLE e.g. (NZB x NZW)F1 mice, as described herein in the Examples.

[0095] It can thus be seen that, besides the preferred embodiments which have been shown to be operable in the examples herein, those of ordinary skill in the art will be able to determine additional analogs which will also be operable following the guidelines presented herein without undue experimentation.

[0096] In another preferred embodiment, the present invention provides a multi-epitope single peptide such as a dual peptide. In one embodiment, the dual peptide consists of two different peptides based on the same CDR such as two different peptides including the sequence of the CDR1 (SEQ ID NO:8) or of the CDR3 (SEQ ID NO:10) of the heavy chain of the human 16/6Id mab.

[0097] In another and more preferred embodiment, the dual peptide consists of two different peptides each based on a different CDR such as one peptide including the sequence of CDR1 (SEQ ID NO:8) and another including the sequence of the CDR3 (SEQ ID NO:10) of the heavy chain of the human 16/6Id mAb.

[0098] The dual peptide according to the invention preferably consists of two different peptides, one being a peptide of SEQ ID NO:11 and the other being a peptide of SEQ ID NO:19, more preferably one peptide selected from the group consisting of SEQ ID NO:6 and NO:12-18 and another peptide selected from the group consisting of SEQ ID NO:7 and NO:20-27, most preferably one peptide being the peptide of SEQ ID NO:6 and the other being the peptide of SEQ ID NO:7, the two different peptides being covalently linked to one another either directly or by a short linking chain such as a stretch of alanine residues or by a putative site for proteolysis by cathepsin. See, for example, U.S. Pat. No. 5,126,249 and European Patent 495049 with respect to such sites.

[0099] In yet another preferred embodiment, the present invention provides a multi-epitope single peptide comprising a number of the same or different peptides of the present invention in the form of a peptide polymer, obtained, for example, by polymerization of the peptides with a suitable polymerization agent, such as 0.1% glutaraldehyde (Audibert et al., 1981, Nature 289:593). The polymer will preferably contain from 5 to 20 peptide residues, preferably a peptide of SEQ ID NO: 6, 7 and 11-27. Such peptide polymers may also be formed by crosslinking the peptides or attaching multiple peptides to macromolecular carriers. Suitable macromolecular carriers are, for example, proteins, such as tetanus toxoid, and linear or branched copolymers of amino acids, such as a linear copolymer of L-alanine, L-glutamic acid and L-lysine and a branched copolymer of L-tyrosine, L-glutamic acid, L-alanine and L-lysine (T,G)-A-L-, or multichain poly-DL-alanine (M. Sela et al., 1955, J. Am. Chem. Soc. 77:6175). The conjugates are obtained, for example, by first coupling the peptide with a water-soluble carbodiimide, such as 1-ethyl-3-(3'-dimethylamino-propyl)carbodiimide hydro-chloride, and then performing the conjugation with the macromolecular carrier as described by Muller, G. M. et al. (1982) Proc. Natl. Acad. Sci. USA 79:569. The contents of the coupled peptide in each conjugate are determined by amino acid analysis, in comparison to the composition of the carrier alone.

[0100] According to a further embodiment of the present invention, one or more active peptides may be attached to a suitable macromolecular carrier or may be polymerized in the presence of glutaraldehyde.

[0101] The peptides, polymers thereof or their conjugates with suitable macromolecular carriers, are administered to patients in a form that insures their bioavailability, making them suitable for treatment. If more than one peptide of the invention is found to have significant inhibitory activity, these peptides may be given to patients in a formulation containing a mixture thereof.

[0102] The invention thus further relates to pharmaceutical compositions comprising at least one synthetic peptide or peptide polymer according to the invention, optionally with a pharmaceutically acceptable carrier.

[0103] In one preferred embodiment, the pharmaceutical compositions comprises at least one synthetic peptide of the invention, more preferably a peptide selected from the group consisting of peptides hCDR1 [SEQ ID NO:6] and hCDR3 [SEQ ID NO:7] and peptides obtained by substitution and/or addition of amino acid residues in the hCDR1 and hCDR3 sequences, in particular a peptide selected from the group consisting of peptides of SEQ ID NO:12 to SEQ ID NO:18 and of SEQ ID NO:20 to SEQ ID NO:27.

[0104] Any suitable route of administration is encompassed by the invention, including oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intraperitoneal, intradermal, transdermal or other known routes, including the enteral route. In preferred embodiments, the peptides of the invention are administered by oral, intranasal or subcutaneous routes.

[0105] The dose ranges for the administration of the compositions of the present invention should be large enough to produce the desired effect, whereby, for example, an immune response to the SLE-inducing autoantibody, as measured by T cell proliferation in vitro, is substantially prevented or inhibited, and further, where the disease is significantly treated. The doses should not be so large as to cause adverse side effects, such as unwanted cross reactions, generalized immunosuppression, anaphylactic reactions and the like.

[0106] Effective doses of the peptides of this invention for use in treating SLE are in the range of about 1 μg to 1 mg and up to 100 mg/kg body weight.

[0107] The synthetic human peptides of the invention are aimed at inhibiting or suppressing specific antigen responses

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of SLE patients, without affecting all other immune responses. This approach is of the utmost importance since most diagnosed patients are young women that have to be treated for many years and the currently accepted treatment for SLE involves administration of immunosuppressive agents, such as corticosteroids and/or cytotoxic drugs, that are both non-specific and have multiple adverse side effects.

[0108] The invention further relates to a method for the treatment of systemic lupus erythematosus (SLE) comprising administering to a SLE patient an effective amount of a peptide or peptide polymer of the invention. In one preferred embodiment, the method comprises administering the peptide of the SEQ ID NO:6. In another preferred embodiment, the method comprises administering the peptide of the SEQ ID NO:7.

[0109] The invention still further relates to method of immunomodulation of SLE-associated responses in a SLE patient which comprises administering to said SLE patient an effective amount of an effective amount of a peptide or peptide polymer of the invention. In one embodiment, the method comprises down-regulating the levels of matrix metalloproteinase (MMP)-3 and/or MMP-9 activities in a SLE patient. In another embodiment, the method comprises immunomodulating the level of a cytokine activity in a SLE patient, particularly down-regulating the level of IL-2 and/or IFN-γ activity and/or up-regulating the level of TGF-β activity, in a SLE patient. In one preferred embodiment, the method comprises administering the peptide of the SEQ ID NO: 6. In another preferred embodiment, the method comprises administering the peptide of the SEQ ID NO: 7.

[0110] The invention also provides methods for assessing the effectiveness of a drug in the treatment of a SLE-patient which comprises measuring at different intervals of time the levels of MMP-3, MMP-9, IL-2 , IFN-γ and/or TGF-γ in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of MMP-3, MMP-9, IL-2 and/or IFN-γ or an increased level of TGF-β,correlates with the effectiveness of the drug.

[0111] The invention still further relates to the use of a peptide of or peptide polymer of the invention for the preparation of a pharmaceutical composition, in particular for treatment of SLE, more particularly for immunomodulation of SLE-associated responses in a SLE patient such as down-regulation of MMP-3 and/or MMP-9 and/or IL-2 and/or IFN-γ or up-regulation of TGF-β levels in a SLE-patient.

[0112] The present invention will now be described in more detail in the following non-limiting Examples and the accompanying figures.

## EXAMPLES

[0113] Materials and Methods

[0114] Mice. Female (NZB x NZW)F1 mice were obtained from the Jackson Laboratory (Bar Harbor, Me.). Female mice of the BALB/c inbred strain at the age of 6-8 weeks were obtained from the Experimental Animal Unit, The Weizmann Institute of Science, Rehovot, Israel.

[0115] Anti-DNA monoclonal antibody. The human anti-DNA mAb that bears the 16/6Id (IgG1/k) was previously characterized (Shoenfeld et al., 1982; Waisman et al., 1995). The mAb was secreted by hybridoma cells that were grown in culture and were purified by using a protein G-Sepharose column (Pharmacia, Fine Chemicals, Uppsala, Sweden).

[0116] Synthetic peptides. The synthetic murine peptides mCDR1 (SEQ ID NO:1) and mCDR3 (SEQ ID NO:3) as well as the reversed peptides that were synthesized in the reversed order of mCDR1 and mCDR3 and in the reversed order of the human peptide hCDR1, identified herein as revmCDR1 [SEQ ID NO:28], revmCDR3 [SEQ ID NO: 29], and revhCDR1 [SEQ ID NO: 30], respectively, used as control, were prepared as previously described (WO 96/30057 or using an automated synthesizer (Applied Biosystems model 430A, Germany) by using the company's protocols for t-butyloxycarbonyl (t-Boc) technique.

[0117] The reversed peptides have the sequences:

G I W E L S K E P S Q K V W Q R Y Y G T revmCDR1 [SEQ ID NO:28]

S G Q G W Y D K A Y P E W L F E A C Y Y revmCDR3 [SEQ ID NO:29]

G I W E G K G P P Q E I W S W Y Y G revhCDR1 [SEQ ID NO:30]

[0118] Induction and treatment of experimental SLE. In order to induce experimental SLE, BALB/c mice were immunized with 1-2 μg of the human mAb 16/6Id and boosted 3 weeks later. For the prevention of experimental SLE, mice were given hCDR1 or hCDR3 (mCDR1 or revmCDR1 as control peptide in Example 12) intravenously (i.v.) or s.c. concomitant with the immunization, and were injected weekly thereafter for 5 weeks. Treatment of an established disease started three and a half months following disease induction with the 16/6Id, when clinical manifestations were already observed. In Example 12, mice received 10 weekly injections (i.v. or s.c.) of mCDR1 or revmCDR1 at a dose of 100 μg/mouse.

[0119] Prevention and treatment of SLE-like disease in (NZB x NZW)F1 mice with the hCDR1 or mCDR1 peptide. For prevention of SLE, mice at the age of 2 months, before disease manifestations are observed, were injected s.c. with hCDR1 (or mCDR1 in Example 12, 250 μg/mouse) once a week for 10 weeks. To treat an established disease, mice at the age of 5-7 months were injected with hCDR1 (in Example 12, mCDR1 s.c., 250 μg/mouse) once a week for 10 weeks.

[0120] Proliferative Responses. PBL were isolated from heparinized venous blood by Ficoll-Hypaque (Pharmacia) density-gradient centrifugation. All assays were performed, in triplicate, in flat-bottomed microtiter plates (Falcon, Becton Dickinson, Oxnard, Calif., USA) in which $2\times10^5$ PBL were cultured in enriched RPMI-1640 as described (Dayan et al., 2000). The PBL were exposed to various concentrations (0.1-40 μg/well) of the human anti-DNA 16/6 Id mAb with and without the addition of the various CDR-based

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peptides at a concentration of at least 10-fold excess over that of the 16/6Id. Phytohemagglutinin (PH-A; 2 μg/well) was used as a control for culture conditions at each experiment. The cultures were incubated in 7.5% $CO_2$ at 37° C. for 6 days. Eighteen hours before the cells were harvested, [$^3$H]-thymidine (0.5 μ Ci of 5 Ci/mmol)(Nuclear Research Center, Negev, Israel) was added to all cultures. Results are expressed as the mean thymidine incorporation in counts per minute (CPM) of triplicate culture ± SD, or as stimulation index (S.I.; the ratio of mean CPM at the optimal concentration of the human 16/6Id to the mean CPM in the presence of medium alone). A S.I. >2 was considered a positive response (Dayan et al., 2000). Inhibition (the ratio of mean CPM in the presence of the 16/6Id and various CDR-based peptides to the mean CPM with the 16/6Id without the CDR-based peptide) above 50% was considered positive.

[0121] Induction of Cytokine Production. Mice that were immunized with the human 16/6Id mAb and either treated or not with the CDR-based peptide, were killed at different periods during or after treatment with the peptide. Splenocytes and lymph node cells (LNC) were harvested and incubated ($5\times10^6$/ml) in the presence of the 16/6Id. Supernatants were collected after 48 and 72 h.

[0122] Assessment of Cytokines in the Supernatants. Supernatants were collected 48 hours following the initiation of the cultures and stored at −70° C. Measurements of *IL-2*, IL-10, IFN-γ, and TNF-α were performed by ELISA by using the relevant standards, capture and detecting Abs (Pharmingen) according to the manufacturer's instructions. TGF-β was determined by an ELISA. Briefly, plates were coated with the recombinant human TGF-β1 sRII/Fc chimera (R & D Systems Inc., Minneapolis, Minn., USA), and the second Ab used was the biotinylated anti-human TGF-β1 antibody (R & D Systems Inc.). The substrate solution used was TMB color Reagent (Helix Diagnostics, West Sacramento, Calif.), and enzyme activity was evaluated by using 570- and 630-nm filters.

[0123] Detection of SLE-Associated Clinical and Pathological Manifestations. Proteinuria was measured semiquantitatively by using Combistix kit (Ames Division, Bayer Diagnostics, Newbury, U.K.). White blood cells (WBC, for leukopenia) were counted after a 10-fold dilution of heparinized blood in distilled water containing 1% acetic acid (vol/vol). For immunohistology analysis, frozen kidney sections (6 μm) were fixed and stained with FITC-conjugated goat Abs to mouse IgG (γ-chain specific; Sigma). ELISA. For measuring anti-DNA Abs, 96-well Maxisorb microtiter plates (*Nunc*) were coated with either methylated BSA or polyL-lysine (Sigma). The plates were then washed and coated with either 10 μg/ml of denatured calf thymus DNA (Sigma) or λ-phage double-stranded DNA (Boehringer Mannheim, 5 μg/ml). After incubation with different dilutions of sera, goat anti-mouse IgG (γ-chain specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch) was added to the plates, followed by the addition of the substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). Results were read by using an ELISA reader.

[0124] Measurement of activities of MMP-2 and MMP-9. MMP activity was tested by gelatin zymography. Pooled sera of individual mice of the different experimental groups were separated by an 8% SDS-PAGE polymerized with 1 mg/ml gelatin. Following electrophoresis, gels were washed once for 30 min in 2.5% Triton X-100 to remove the SDS, and once for 30 min in the reaction buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl2 and 0.02% (w/v) Brij 35 (pH 7.5). The reaction buffer was changed to a fresh one, and the gels were incubated at 37° C. for 24 h. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie brilliant blue.

[0125] Western blot analysis of MMP-3 in sera. Samples of 5 μl of each serum were loaded on 12% SDS/PAGE, separated under reducing conditions, and transferred to nitrocellulose. The blots were probed (0.5 μg/ml, 1 hr, room temperature) with anti-MMP-3 antibodies (Oncogene Research Products, MA, USA) and developed using chemiluminescence.

[0126] Immunostaining of kidney sections for A MMP-3 or MMP-9. For immunostaining of MMP-3 or MMP-9, kidney sections (5 μm) were fixed with cold acetone (5 min at room temperature), washed twice in PBS, permeabilized (1 min at room temperature) in 0.05% Triton X-100 (diluted in PBS), and washed twice in PBS. In order to get a specific anti-MMP staining and to avoid the staining of immune complex deposits by the FITC-labeled goat anti-mouse, kidney sections were blocked (1 hr at room temperature) with unlabeled goat anti-mouse IgG+IgM (diluted 1:1 in 1% BSA/PBS; (Jackson ImmunoResearch Laboratories) and washed 3 times with PBS containing 0.05% Tween. Monoclonal anti-MMP-9 (1:100; Chemicon International, Inc.) or anti-MMP-3 (1:50; Oncogene Research Products) antibody diluted in 1% BSA/PBS were added for 30 min, at room temperature. For all immunostaining procedures, FITC-labeled goat anti-mouse IgG+IgM (Jackson ImmunoResearch Laboratories), diluted 1:30 in 1% BSA/PBS (30 min at room temperature), was used.

[0127] Statistical analysis. *Results* are presented as mean ± SD. Chi-square, Wilcoxon, Mann-Whitney and t-tests were employed for statistical analysis. P <0.05 was considered significant.

## EXAMPLE 1.

[0128] Synthesis of the human peptides hCDR1 and hCDR3

[0129] The human hCDR1 (SEQ ID NO:1) and hCDR3 (SEQ ID NO:3) peptides were prepared by methods well-known in the art, for example, by chemical solid phase or solution phase synthesis using an automated synthesizer by using the manufacturer's protocols for t-butyloxycarbonyl (t-Boc), fluorenylmethoxycarbonyl (Fmoc) or other alpha-amino acid protecting group procedure essentially as described (see, for example, Peptides: Synthesis, Structure and Applications, ed. by B. Gutte, Academic Press, 1995; Peptide Synthesis Protocols, ed. by M. Pennington and B. Dunn, Humana Press, 1994; Schnolzer M. et al., In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int. J. Pept. Protein Res. 40: 180-193, 1992).

## EXAMPLE 2.

[0130] In vivo inhibition of lymph node cell (LNC) proliferation of mice immunized with mCDR1 and mCDR3 and treated with hCDR1 and hCDR3

[0131] In order to determine the inhibitory efficacy of the human peptides hCDR1 and hCDR3, we first tested their

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ability to inhibit the in vivo priming of mice with the murine peptides mCDR1 and mCDR3.

[0132] To this end, BALB/c and SJL mice were immunized with mCDR1 and mCDR3, respectively. The immunizing murine peptides were injected (10 μg/mouse) in CFA intradermally in the hind footpads. Concomitant with the immunization, groups of BALB/c mice were injected subcutaneously (s.c.) with 200 μg in PBS of hCDR1 and groups of SJL mice were similarly injected with hCDR3. Ten days after the immunization, mice were sacrificed and their lymph nodes were harvested and the cells were tested for their ability to proliferate following triggering with the immunizing peptides. Briefly, LNCs of immunized mice ($0.5\times10^6$/well) were cultured (in triplicates) in the presence of different concentrations (1-20 μg /well) of the murine immunizing peptides in enriched RPMI-1640 medium supplemented with 1% normal mouse serum. Following four days of incubation, $^3$H-thymidine was added for additional 16 hours. Cells were then harvested and radioactivity was counted using a β-counter.

[0133] The results in Tables 1A and 1B represent the maximum % inhibition of proliferation of LNCs of mice immunized with mCDR1 and mCDR3 and treated with hCDR1 and hCDR3, respectively. The inhibition was calculated based on the proliferation of LNCs of mice that were not treated with the inhibitory peptides hCDR1 and hCDR3. It can be seen that hCDR1 and hCDR3 were capable of inhibiting the proliferative responses to the immunizing murine CDR peptides.

TABLE 1A

| Inhibition by hCDR1 of the proliferation of BALB/c-derived LNCs to mCDR1 | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR1 | 55% |

[0134]

TABLE 1B

| Inhibition by hCDR3 of the proliferation of SJL-derived LNCs to mCDR3 | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR3 | 55% |

EXAMPLE 3.

[0135] In vivo inhibition of LNC proliferation of mice immunized with the human anti-DNA 16/6Id mAb and treated with hCDR1 and hCDR3

[0136] Because our aim was to test the inhibitory capacity of the peptides based on the CDR of the human 16/6Id autoantibody, it was of importance to find out whether peptides hCDR1 and hCDR3 are capable of inhibiting the priming to the whole molecule of the human 16/6Id mAb. To this end, BALB/c and SJL mice were primed with the human 16/6Id mAb (2 μg/mouse) in CFA intradermaly in the hind footpads. The priming was done concomitant with the s.c. administration in PBS of 200 μg/mouse of hCDR1 to groups of BALB/c mice and of hCDR3 to SJL mice. Ten days following immunization, the mice were sacrificed and their LNCs were tested in vitro for their ability to proliferate to different concentrations (0.1-10 μg/well) of the human anti-DNA 16/6Id mAb.

[0137] Representative results of these experiments are shown in Tables 2A and 2B. The results are presented as maximum % inhibition of the proliferation to the immunizing human 16/6Id mAb of lymph node cells of mice immunized and treated with the peptides hCDR1 and hCDR3 as compared with mice that were immunized with the 16/6Id mAb but were not treated with the peptides. As can be seen, both peptides hCDR1 and hCDR3 were capable of inhibiting efficiently the priming to the human 16/6Id mAb.

TABLE 2A

| Inhibition by hCDR1 of the proliferation of BALB/c-derived LNC to the human 16/6Id mAb. | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR1 | 89% |

[0138]

TABLE 2B

| Inhibition by hCDR3 of the proliferation of SJL-derived LNC to the human 16/6Id mAb. | |
|---|---|
| Inhibitor | % Inhibition |
| hCDR3 | 89% |

[0139] Further experiments demonstrated that a nasal administration of as low as 10 or even 2 μg/mouse of the peptide hCDR1 or hCDR3, concomitant with the immunization with the human 16/6Id mAb, inhibited by up to 100% the proliferative responses of lymph node cells to the immunizing antibody.

[0140] In another experiment, BALB/c mice were immunized with 1 μg human 16/6Id in CFA, intradermally in the hind footpads, and either were injected with hCDR1 s.c., 300 μg/mouse in PBS, or were not further treated. Ten days later the mice were sacrificed and their LNC were tested for their ability to proliferate in vitro to the human 16/6Id. Thus, popliteal LNCs ($0.5\times10^6$) were incubated in the presence of various concentrations (0.1-10 μg/well) of the human anti-DNA 16/6Id mAb. At the end of 4 days incubation, $^3$H-thymidine was added to the cultures for the last 18 hours of incubation. Cells were then harvested and radioactivity counted.

[0141] FIG. 1 shows the results of such experiments and demonstrates that hCDR1 inhibited efficiently the proliferative responses of lymph node cells of the treated mice. The % inhibition of proliferation at the various concentrations of 16/6Id mAb was as follows: 0.1 μg/well—47%; 1 μg/well—66%; 5 μg/well—76%; and 10 μg/well—62%.

[0142] The same experiment above was repeated with 50 μg hCDR1/mouse in PBS. FIG. 2 shows that hCDR1 is very effective in inhibiting the in vivo priming of mice with the whole anti-DNA 16/6Id macromolecule and even a s.c. injection with as little as 50 μg of hCDR1 inhibited significantly the ability of lymph node cells to proliferate to the 16/6Id autoantibody. The % inhibition of proliferation at the various concentrations of 16/6Id mAb was as follows: 0.1 μg/well—98%; 1 μg/well—76%; 5 μg/well—73%; and 10 μg/well—64%.

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EXAMPLE 4.

[0143] Peptide hCDR1 immunomodulates cytokine production

[0144] The lymph node cells of the BALB/c mice treated with 50 μg of hCDR1 of Example 3 were also stimulated with the human 16/6Id mAb for cytokine production and the supernatants were tested for cytokine (INF-γ TGF-β and IL-10) secretion, by ELISA.

[0145] FIG. 3 shows results of a representative experiment. It can be seen that hCDR1 down-regulated the production of INF-γ (FIG. 3A) and up-regulated the secretion of TGF-β (FIG. 3B) and IL-10 (FIG. 3C). It should be noted that a peptide used as control (p259-271, that was shown to be a myasthenogenic peptide) did not affect significantly INF-γ and IL-10 production and was less effective in up-regulating TGF-β.

EXAMPLE 5.

[0146] Peptides hCDR1 and hCDR3 inhibit the proliferative response of PBL of SLE patients to the human 16/6Id mAb.

[0147] Sixty-two patients, 9 males (14.5%) and 53 females (85.5%) with SLE participated in our study. The mean age at diagnosis was 32.95±12.92 (range 12-61) years and the mean follow-up period was 10.98±10.76 (range 1-32) years. All patients fulfilled at least 4 of the American College of Rheumatology (ACR) revised diagnostic criteria for SLE (Tan et al., 1982). Patients were recruited from three Israeli Medical Centers (Kaplan, Rehovot; Ichilov, Tel Aviv; Asaf-Harofeh, Rishon Lezion). Disease activity was determined according to the SLEDAI lupus activity index (Bombardier et al., 1992). A control group of 36 sex- and age-matched healthy control volunteers was studied concomitantly with the SLE patients. The study was approved by the Ethical Committee of the Medical Center.

[0148] It was of interest to investigate whether the peptides hCDR1 and hCDR3, which are based on the CDR1 and CDR3 of the human 16/6Id mAb, are capable of inhibiting the specific proliferative responses of PBL of SLE patients to the human 16/6Id mAb. To this end, we first had to identify the patients whose PBL could be stimulated to proliferate by the human 16/6Id mAb (responders).

[0149] Therefore, PBL of 62 consecutive SLE patients were cultured in the presence of the human 16/6Id and their proliferative responses and ability to secrete IL-2 were determined. PBL of 24 out of the total of 62 (39%) and of 23 out of 55 (42%) SLE patients tested responded (SI >2, range 2-5.6) by proliferation and by IL-2 secretion (SI >2, range 2-60), respectively. The frequency of responders in the group of SLE patients was lower than that observed in the group of healthy donors that was tested as control. Thus, PBL of 21 out of a total of 36 (58%) healthy donors responded by proliferation to the 16/6Id. The extent of proliferation (SI levels).was similar for the SLE patients and for the healthy controls who responded to the 16/6Id. However, as shown in FIG. 4, the optimal response to the 16/6Id of PBL of the control donors was observed at higher concentrations of 16/6Id as compared to the SLE patients.

[0150] No differences could be demonstrated between gender and age of SLE patients that responded to the 16/6Id and of the non-responder group of patients. However, the patients whose PBL proliferated in response to the 16/6Id were sick for a shorter period of time (a mean of 9.78±8.36 vs. 11.73±12.06 years for responders and non-responders, respectively; P:≤0.036). Table 3 summarizes the clinical characterization of the 16/6 Id responder and non-responder groups of SLE patients. As can be seen in the Table, both groups were similar in most SLE related clinical manifestations. The SLE disease activity score (SLEDAI) and the number of SLE diagnostic criteria were also similar in the two groups. Nevertheless, a higher frequency of neurological (both seizures and psychosis) and hematological involvement and a lower rate of renal involvement were noted in the responder group of patients in comparison to the group of non-responders. However, probably because of low number of patients in the relevant subgroups, the above differences did not reach statistical significance. Moreover, relatively less responder patients were determined between those treated with either steroids or cytotoxic agents at the time of the study. It is noteworthy that significantly more patients who never received steroids responded to the 16/6Id in comparison to the non-responder group (54% vs 21%; P=0.023).

[0151] It is noteworthy that the efficacy of the CDR-based peptides to inhibit the proliferative responses of PBL of healthy donors to the 16/6Id was much lower than that observed for PBL of SLE patients (not shown).

TABLE 3

Clinical and laboratory characteristics of SLE patients.

| | All Patients | Responders | Non-responders |
|---|---|---|---|
| **A: Diagnostic Criteria*** | | | |
| Number of Patients (%) | 62 (100) | 24 (39) | 38 (61) |
| Malar rash | 19/62 (30.1) | 8/24 (33.3) | 11/38 (29) |
| Discoid rash | 9/62 (15) | 3/24 (12.5) | 6/38 (16) |
| Photosensitivity | 21/62 (34) | 9/24 (37.5) | 12/38 (32) |
| Mucosal ulcers | 17/62 (27.4) | 8/24 (33.3) | 9/38 (23.7) |
| Arthritis | 46/62 (74.2) | 19/24 (79.2) | 27/38 (71) |
| Serositis | 14/62 (22.6) | 5/24 (20.8) | 9/38 (23.7) |
| Neurologic disorder[a] | 5/62 (8.1) | 4/24 (16.7) | 1/38 (2.7) |
| Renal disorder[b] | 24/62 (38.8) | 7/24 (29.2) | 17/38 (44.8) |
| Hematological Disorder[c] | 44/62 (71) | 19/24 (79.2) | 25/38 (65.8) |
| ANA | 61/62 (98.4) | 24/24 (100) | 37/38 (92.1) |
| α-dsDNA | 54/62 (87.1) | 19/24 (79.2) | 35/38 (92.1) |
| APLA | 35/62 (56.5) | 12/24 (50.0) | 23/38 (60.53) |
| **B: Disease Activity** | | | |
| SLEDAI Score | 6.65 ± 5.12 | 7.29 ± 1.06 | 6.24 ± 0.84 |
| Number of ACR diagnostic criteria | 5.44 ± 1.39 | 5.54 ± 0.39 | 5.34 ± 0.2 |
| **C: Current treatment†** | | | |
| NSAIDS | 17/62 (27.4) | 6/24 (25) | 11/38 (29) |
| Anti-Malarial | 37/62 (59.7) | 15/24 (62.5) | 22/38 (57.9) |
| Steroids[d] | 33/62 (53.2) | 11/24 (45.8) | 22/38 (57.9) |
| Cytotoxic[e] | 10/62 (16.1) | 2/24 (8.3) | 8/38 (21) |

*Clinical involvement was defined according to the ACR revised criteria. Anti-nuclear antibodies (ANA) and anti-dsDNA antibodies were determined by Hep2 cells and Crithidia luciliae, respectively. Anti-phospholipid antibodies (APLA) were defined as reactivity in one or more of the following assays: false positive VDRL, lupus anti-coagulant (LAC) or ELISA for anti-cardiolipin antibodies.

†The anti-malarial agent, hydroxychloroquine, was used at a dose of 200–400 mg/day; Steroid treatment was defined as a daily dose ≥ 5 mg of prednisone; cytotoxic agents used were cyclophosphamide (0.75–1.0 g/m², monthly) or methotrexate (300–150 mg/day).

[a]Parameters for which tendency was observed towards differences between the two groups of responder and non-responder SLE patients.

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[0152] To test the ability of the peptides hCDR1 and hCDR3 to inhibit the proliferative response of PBL of SLE patients to the human 16/6Id mAb, PBL ($2x10^5$/well) of SLE patients were stimulated in vitro in triplicates with different concentrations (0.1-20 μg/well) of the human 16/6Id mAb in the absence or presence of the peptides hCDR1 and hCDR3 (either 50 or 100 μg/well). Following 6 days of incubation, $^3$H-thymidine (0.5 μCi of 5 Ci/mmol) was added to each well for additional 18 hours of incubation. Cells were then harvested and radioactivity was counted using a β-counter. Results were expressed as mean counts per minute (cpm) of triplicate cultures. Stimulation indices (the ratio of mean cpm at the optimal concentration of 16/6Id to mean cpm without 16/6Id) were then calculated. A stimulation index (SI) >2 was considered positive.

[0153] PBL of 24 out of the total of 62 (39%) SLE patients were found to proliferate to the 16/6Id mAb. The ability of the peptides hCDR1 and hCDR3 to inhibit the proliferative responses to the whole molecule of the 16/6Id autoantibody was tested on PBL of 19 responders SLE patients.

[0154] Table 4 shows the results of these experiments. Inhibition of above 50% of the proliferative capacity was considered positive. The Table represents the highest positive inhibition capacity for each peptide. It can be seen that the human hCDR1. and hCDR3 inhibited the proliferation of PBL of 16/19 (84.2%) and 15/19 (78.9%), respectively, of the 1.9 responders tested. Both peptides inhibited the proliferation of PBL of 18/19 (95%) of responders tested. It can also be seen in the Table that the magnitudes of inhibitions were similar for both peptides. Thus, it can be concluded that peptides based on CDR1 and CDR3 of the human 16/6Id mAb are efficient inhibitors of the proliferation of PBL of SLE patients to the human 16/6Id mAb.

TABLE 4

Inhibition of proliferation of PBL of SLE patients by peptides hCDR1 and hCDR3.

| | | Percent Inhibition | |
|---|---|---|---|
| Number | Initials | hCDR1 | hCDR3 |
| 1. | B. L. | 62 | <50 |
| 2. | M. D. | 70 | 75 |
| 3. | T. L. | 69 | <50 |
| 4. | Z. D. | <50 | <50 |
| 5. | N. N. | 88.5 | 87.5 |
| 6. | S. S. | 80 | 80 |
| 7. | S. H. | 76 | 70.4 |
| 8. | S. D. | 58 | 56 |
| 9. | A. N. | 69.5 | 65 |
| 10. | L. I. | 68.2 | 71.8 |
| 11. | L. J. | <50 | 72 |
| 12. | V. L. | 82 | 86 |
| 13. | M. S. | 63 | 64 |
| 14. | D. S. | 56 | 74 |
| 15. | Z. A. | 63 | 69 |
| 16. | B. M. | <50 | 68 |
| 17. | S. N. | 70.5 | 77.8 |
| 18. | G. M. | 51.5 | <50 |
| 19. | N. I. | 63 | 60.8 |
| Mean ± SD | | 68.12 ± 9.57 | 71.83 ± 8.44 |

EXAMPLE 6.

[0155] Specificity of the inhibitory capacity of hCDR1 and hCDR3

[0156] It is important to demonstrate that the inhibitory effects of the hCDR-based peptides are specific to SLE-associated responses. To this end the peptides hCDR1 or hCDR3 were added to cultures of PBL of SLE patients that were stimulated with the mitogen phytohemagglutinin (PHA, 2 μg/ml). The results of such an experiment performed with PBL of one SLE patient is shown in FIG. 5. The peptides hCDR1 and hCDR3 could not inhibit the proliferative responses (expressed in cpm) of the PBL to the mitogen PHA and the proliferative responses were similarly high in the absence (black column) or presence of either hCDR1 or hCDR3.

[0157] In another experiment, cultures of PBL of SLE patients were stimulated with the human 16/6Id mAb and then incubated with the human peptides hCDR1 or hCDR3 or with the murine peptide mCDR3 as a control. The results of such an experiment performed with PBL of one SLE patient are shown in FIG. 6. As shown in FIG. 6, whereas both peptides hCDR1 and hCDR3 based on the human autoantibody inhibited efficiently the proliferative responses of PBL to the human 16/6Id mAb, the peptide mCDR3 based on the CDR3 of the murine antibody did not inhibit the proliferation.

[0158] Two additional control peptides were used in these experiments, namely peptides synthesized at the reversed order of the murine MCDR1 and mCDR3 peptides (revm-CDR1 and revmCDR3), and the results are shown in FIG. 7. It can be seen that the two reversed peptides failed to inhibit significantly the proliferative responses of the PBL of the SLE patient to human 16/6Id mAb while peptides hCDR1 and hCDR3 did inhibit efficiently the proliferation, demonstrating that the inhibition of proliferation by the human hCDR-based peptides is specific to the peptides and to the SLE-associated T cell responses.

EXAMPLE 7.

[0159] Down-regulation of the secretion of IL-2 by PBL of SLE patients in the presence of the peptides hCDR1 and hCDR3

[0160] It was of interest to find out whether the hCDR peptides are capable of inhibiting IL-2 secretion by PBL of SLE patients following stimulation with the human 16/6Id mAb. Such inhibition might also suggest that the human CDR-based peptides inhibit the proliferative responses to the 16/6Id mAb at least partially by down-regulating IL-2 secretion. To this end, PBL of SLE patients were incubated with the human 16/6Id mAb in the absence or presence of the peptides hCDR1 or hCDR3. Supernatants of the cultures were collected following 48 hours of incubation. Assays to determine levels of IL-2 in the supernatants were performed using the CTLL IL-2 dependent line. Briefly, cells of the CTLL line ($2x10^4$/well) were incubated in the presence of the different supernatants for 24 hours, followed by the addition of $^3$H-thymidine for an additional 18-hour incubation period. Cells were then harvested and radioactivity counted using a β-counter. Results were calculated based on recombinant human IL-2 used as a standard. The ability of the peptides to inhibit the IL-2 secretion of PBL of 23 responders stimulated by the human 16/6Id was tested. The results, summarized in Table 5, show that hCDR1 and hCDR3 inhibited the secretion of IL-2 by PBL of 21/23 and 19/23 patients, respectively. Inhibition of proliferative responses of PBL directly correlated with IL-2 inhibition by the CDR-based peptides. Thus, inhibition of IL-2 secretion was observed in all cases where inhibition of proliferation were determined.

[0161] The results obtained with PBL of one SLE patient represented in FIG. 8 (secretion of IL-2 is expressed in

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pg/ml) show that both hCDR1 and hCDR3 inhibited 100% of the IL-2 secretion by PBL of a SLE patient triggered by the human 16/6Id mAb.

TABLE 5

Inhibition of IL-2 secretion by hCDR1 and hCDR3

| Peptide | Inhibitory Activity* % | Maximum inhibition % |
|---|---|---|
| hCDR1 | 91 (21/23) | 84 ± 21 |
| hCDR3 | 83 (19/23) | 78 ± 34 |

*IL-2 secretion in the presence of 16/6 Id alone was considered as 100%. Inhibition of 50% or more was considered significant.

EXAMPLE 8.

[0162] Up-regulation of the secretion of the immunosuppressive cytokine TGF-β by CDR-based peptides

[0163] In attempts to shed light on the mechanisms by which the human CDR-based peptides inhibit the proliferative responses to the human monoclonal anti-DNA 16/6Id antibody, the levels of the immunosuppressive cytokine TGF-β in the supernatants of the cell cultures were determined. The rationale behind these experiments is based on our previous findings of elevated levels of TGF-β in cultures of splenocytes of mice with SLE either induced with the human anti-DNA 16/6Id mAb or spontaneous {(NZB x NZW) F1 mice} following treatment with the peptides based on mouse CDR (Elias et al., 2001). The elevation in the levels of TGF-β correlated with amelioration of disease manifestations in the treated mice.

[0164] For this purpose, supernatants were removed from cultures of PBL of various SLE patients following 48 hours incubation with the human 16/6Id mAb in the absence or presence of the peptides hCDR1 or hCDR3. TGF-β was determined by ELISA according to the manufacturer's instructions. Briefly, Maxisorb plates (Nunc) were coated with recombinant human TGFβsRII/Fc chimera (R&D Systems) diluted in PBS (100 ng/ml). After blocking, cell supernatants were added. After 18 hours incubation the detecting biotinylated anti-human TGF-β antibody (R&D Systems) was added. The substrate solution used was the TMB Colour Reagent (Helix Diagnostics) and enzyme activity was evaluated by the MRX ELISA reader using the 570 nm and 630 nm filters. The results are summarized in Table 6.

[0165] The results in FIG. 9 demonstrate that peptides hCDR1 and hCDR3 triggered a significant up-regulation in the secretion of TGF-β (expressed in pg/ml) by the PBL of one representative SLE patient that were stimulated with the pathogenic human 16/6Id mAb.

TABLE 6

Up-regulation of TGF-β secretion of 16/6 Id-induced stimulation of PBL of SLE patients with hCDR1 and hCDR3 peptides

| Peptide | Up-regulation of TGF-β % | Maximum Up-regulation % |
|---|---|---|
| hCDR1 | 100 (19/19) | 305 ± 221 |
| hCDR3 | 100 (19/19) | 338 ± 242 |

[0166] Secretion of TGF-β in the presence of 16/6Id alone (mean 636±25 βg/ml) was considered as 100%. Results are expressed as percent secretion above that in the presence of 16/6Id alone.

EXAMPLE 9.

[0167] Immunomodulation of SLE manifestations in mice by hCDR1: Amelioration of disease manifestations following treatment of (NZB x NZW)F1 mice with hCDR1.

[0168] As shown above, the peptides based on the CDR of the human anti-DNA 16/6Id mAb were capable of inhibiting the priming of lymph node cells to the 16/6Id mAb and the proliferative responses of PBL of SLE patients to the 16/6Id mAb with similar efficiency. It was thus of interest to find out if these peptides can immunomodulate an SLE-like disease in animal models.

[0169] The experiments aimed at addressing the ability of the human peptides to treat an established SLE disease were performed first with the hCDR1 peptide. To this end, a few experiments were designed in which SLE-prone (NZB x NZW)F1 mice were treated with the hCDR1 peptide at the age of 5 and a half months, when manifestations of the SLE-like disease (anti-dsDNA, proteinuria, etc) are already observed. The hCDR1 peptide was administered in PBS s.c. weekly, for 10 weeks. The efficacy of different doses (50, 100 and 200 μg/mouse) of the hCDR1 peptide was tested. Control groups were injected with the vehicle PBS. The treatment led to a moderate reduction in the anti-dsDNA autoantibody titers. Thus, at a 1:1250 sera dilutions, O.D. values of 0.586±0.1, 0.2710.1, 0.37±0.1 and 0.29±0.1 were measured at the end of the treatment for sera of PBS-treated mice, mice treated with 50 μg/mouse, 100 μg/mouse and 200 μg/mouse hCDR1, respectively.

[0170] We performed another experiment in which 7-month old (NZB x NZW)F1 mice were treated weekly for 10 weeks with 300 μg of hCDR1 injected s.c. in PBS. A mild reduction in the anti-DNA antibody titers could be observed in sera of hCDR1-treated mice. Nevertheless, Table 7 shows that treatment with hCDR1 resulted in a reduction in proteinuria and in a significant decrease in the immune complex deposits (ICDs) in the kidneys of the treated mice. The results in the Table express the intensity of ICDs where 0=no ICD; 1 =moderate ICD; 2=severe ICD and 3=severe and extremely intense ICD.

TABLE 7

Clinical manifestations of (NZB x NZW)F1 mice treated with hCDR1 at the age of 7 months

| Treatment | Proteinuria g/L ± SEM | Immune Complex Deposits ± SEM |
|---|---|---|
| Untreated | 4.80 ± 2.56 | 2.57 ± 0.29 |
| hCDR1 300 μg/mouse | 1.08 ± 0.38 | 1.44 ± 0.41 p = 0.035 |

p was calculated in comparison to untreated group of mice.

[0171] We then performed an additional experiment using 400 μg/mouse of hCDR1 to find out if more beneficial effects can be achieved by increasing the dose of the peptide. Treatment with 400 μg/mouse of hCDR1 had no greater effect on the anti-DNA antibody titers and, as can be seen in Table 8, the effect on the kidney disease was similar to that observed following treatment with the 300 μg dose.

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TABLE 8

Clinical manifestations of (NZB × NZW)F1 mice treated with hCDR1 at the age of 6 months

| Treatment | Proteinuria g/L ± SEM | Immune Complex Deposits ± SEM |
|---|---|---|
| Untreated | 4.89 ± 2.53 | 2 ± 0.29 |
| hCDR1 400 μg/mouse | 0.77 ± 0.32 | 1.42 ± 0.19 p = 0.05 |

p was calculated in comparison to untreated group of mice.

[0172] We have therefore performed an additional experiment in which mice were treated with 300 μg of hCDR1 at the age of 7 months and used a control peptide, namely, the reversed hCDR1. The aim of the experiment was to find out if, in addition to the amelioration in the clinical manifestations, treatment with hCDR1 immunomodulates cytokine production. FIG. 10 demonstrates the mild reduction in anti-DNA autoantibody levels. Table 9 shows the proteinuria measured at different time points.

TABLE 9

Clinical manifestations of (NZB × NZW)F1 mice treated with hCDR1 at the age of 7 months

| | Proteinuria g/L ± SEM Following treatment No. | | |
|---|---|---|---|
| Treatment | 5 | 7 | 9 |
| Untreated | 2.65 ± 1.76 | 3.89 ± 2.73 | 7.55 ± 3.55 |
| hCDR1 300 μg/mouse | 0.61 ± 0.33 | 0.65 ± 0.32 | 1.02 ± 0.39 p = 0.05 |
| revhCDR1 300 μg/mouse | 6.94 ± 2.53 | 6.76 ± 2.91 | 5.99 ± 3.08 |

[0173] The effect of the treatment with hCDR1 can be seen at all measurements. Kidney damage is one of the main manifestations of the SLE-like disease in the (NZB x NZW)F1 mice. Ten-week treatment with the hCDR1 peptide reduced significantly the kidney disease. The 50 μg dose was less efficient than the 100 and 200 μg doses in treating the kidney disease. The latter two doses were similarly efficient.

[0174] FIGS. 11A-11D are photos showing representative kidney sections of mice treated with the 100 μg dose of hCDR1. Thus, mice at the age of 9 months were sacrificed and their kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections of 5 μm were air dried and fixed in acetone. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (γ-chain specific). FIGS. 11A and 11B show kidney sections of a mouse of the PBS-treated group (control) whereas FIGS. 11C and 11D show kidney sections of a mouse treated with 100 μg of hCDR1. It can be seen in the figures that the treatment reduced the number of immune complexes as well as their intensity (11A,11C x 100; 11B, 11D x 400).

[0175] Similar results were obtained when groups of (NZB x NZW)F1 mice were treated with hCDR1 at the age of 7 months when their full blown disease is already observed. The mice were treated for 10 weeks with either 100 μg/mouse or 300 μg/mouse. Treatment with both doses led to a moderate reduction in the anti-DNA autoantibody titers, similar to the above described results. A reduction in proteinuria in comparison to the PBS-treated group was measured as well. The kidney disease was ameliorated following treatment with both doses; nevertheless, a more significant effect was determined in the group of mice treated with the 300 μg dose. FIGS. 12A-12F are representative mouse kidney sections of each group where A, B represent an untreated mouse; C, D represent a kidney of a hCDR1-treated mouse and E, F represent a kidney section of a mouse treated with the reversed hCDR1. A, C, E x100 and B, D, F x 400.

[0176] FIGS. 13A-13C show the cytokine (INF-γ, IL-10 and TGF-β) pattern as measured by ELISA in supernatants of Con A-stimulated cultures of splenocytes of mice of the 3 groups taken at the end of treatment (10 weekly s.c. injections). It can be seen that INF-γ (FIG. 13A) and IL-10 (FIG. 13B, to a lesser extent) were down-regulated in the treated mice. An increased secretion of TGF-β could be seen in the supernatants of non-stimulated cells (FIG. 13C, left). Con A did not trigger the cells to secrete more TGF-β (FIG. 13C, right).

[0177] The results summarized above indicate that long term treatment with hCDR1 ameliorates disease manifestations and immunomodulates cytokine secretion.

Example 10.

[0178] Treatment of BALB/c mice with the human hCDR1 peptide following induction of experimental SLE

[0179] It was of interest to find out whether the hCDR1 peptide is capable of treating experimental SLE induced in mice with the human anti-DNA 16/6Id mAb. Therefore, BALB/c mice were immunized and boosted with the 16/6Id pathogenic autoantibody. Three and a half months following the booster injection, when the mice developed already disease manifestations, they were divided into three groups. One group was not treated, the second group was treated with 100 μg/mouse of the hCDR1 and the third group was treated for 10 weeks with 300 μg/mouse of the hCDR1 peptide. The mice were followed for disease manifestations.

[0180] Table 10 presents the results obtained in testing individual mice at the end of treatment. An amelioration was observed for all measured clinical manifestations with both doses used for the treatment [anti-daDNA autoantibodies, white blood cells counts (WBC) and urine protein]. Analysis of kidneys of mice sacrificed at the end of the experiment (7 months after booster injections) demonstrated immune complex deposits in kidneys of 9/10 mice that were immunized with the 16/6Id mAb and were not treated. In contrast, immune complex deposits were observed in kidneys of only 3/10 and 2/9 mice that were immunized with the 16/6Id mAb and treated with 100 μg/mouse and 300 μg/mouse hCDR1, respectively. Thus, it is shown that the human peptide hCDR1 is capable of treating an established experimental SLE.

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TABLE 10

The effect of treatment of BALB/c mice afflicted with experimental SLE with 100 μg or 300 μg hCDR1 peptide.

| | Control, healthy mice Mean ± SD | 16/6Id injected Mean ± SD | 16/6Id injected + hCDR1 100 μg Mean ± SD | 16/6Id injected + hCDR1 300 μg Mean ± SD |
|---|---|---|---|---|
| Proteinuria g/l | 0.12 ± 0.16 | 0.65 ± 0.36 | 0.3 ± 0.02 | 0.34 ± 0.25 |
| WBC/mm³ | 7440 ± 960 | 3260 ± 920 | 6090 ± 2160* | 5890 ± 2660* |
| Anti-dsDNA O.D. (1:50) | 0.1 ± 0.05 | 1.1 ± 0.6 | 0.3 ± 0.2* | 0.55 ± 0.3* |

*significantly different ($p < 0.05$) from 16/6Id injected group.

[0181] In another experiment, BALB/c mice were immunized and boosted with the human 16/6Id for induction of experimental SLE. Two months after the boost, mice were divided into groups (8 mice per group). The mice were treated with 200, 300 or 400 μg/mouse for 10 weeks. The mice were followed for antibody production, leukopenia, proteinuria and, when sacrificed, two months after the end of treatment, their kidneys were analyzed for immune complex deposits. The results, summarized in Table 11, show no effect of treatment on the 16/6Id specific antibody response. addition to the comparison between the 200 and 300 μg treatment doses, to study the effect of treatment on the cytokine production in the treated mice. Sixty BALB/c mice were immunized and boosted with the 16/6Id. Three months after the boost, the mice were divided into groups (15 mice per group) that either were not further treated or were weekly treated with 200 or 300 μg of hCDR1. An additional group was treated with 300 μg of reversed hCDR1. A fifth group (control) was not immunized and was not further

TABLE 11

Effect of treatment of BALB/c mice afflicted with experimental SLE with 200 μg, 300 μg or 400 μg hCDR1 peptide.

| | 16/6Id Mean ± SD | 16/6Id + hCDR1 200 μg/mouse Mean ± SD | 16/6Id + hCDR1 300 μg/mouse Mean ± SD | 16/6Id + hCDR1 400 μg/mouse Mean ± SD | Control Mean ± SD |
|---|---|---|---|---|---|
| Proteinuria g/l | 0.5 ± 0.34 | 0.13 ± 0.16<br>p = 0.0131 | 0.16 ± 0.16<br>p = 0.0209 | 0.11 ± 0.15<br>p = 0.0070 | 0.03 ± 0.09<br>p = 0.0002 |
| WBC/mm³ | 2871 ± 1203 | 5625 ± 1659<br>p = 0.0047 | 4677 ± 1508<br>p = 0.0289 | 4012 ± 1421<br>p = 0.0760 | 7280 ± 352<br>p = 0.0001 |
| Intensity of ICD in kidneys | 1.86 ± 0.38 | 0.57 ± 0.97<br>p = 0.0189 | 0.75 ± 0.71<br>p = 0.0047 | 0.71 ± 0.76<br>p = 0.0095 | 0 |
| Anti-dsDNA antibodies at a dilution of: 1:250 | 1.16 ± 0.37 | 0.69 ± 0.39<br>p = 0.0189 | 0.45 ± 0.24<br>p = 0.0003 | 0.8 ± 0.39<br>p = 0.0603 | 0.029 ± 0.025<br>p = 0.0001 |
| Anti-16/6 Id antibodies at a dilution of 1:1000 | 2.55 ± 0.09 | 2.7 ± 0.12 | 2.8 ± 0.11 | 2.75 ± 0.1 | 0 |

[0182] However, treatment affected anti-DNA antibody titers, leukopenia, levels of proteinuria and, more importantly, immune complex deposits in the kidneys. The control group of the experiment is of BALB/c mice of the same age that were not immunized or treated at all. This experiment suggested that hCDR1 is effective in treating experimental SLE and no advantage of the 400 μg dose could be seen.

[0183] Therefore, an additional experiment was performed in which mice were treated with 200 and 300 μg /mouse. A control peptide was used in this experiment, the reversed hCDR1. The aim of this experiment has been, in treated. Mice were followed for antibody production and disease manifestations. As seen in Table 12, the anti-16/6Id antibody levels (at a dilution of 1:10000) in sera of the mice did not differ between groups. Treatment with both 200 and 300 μg reduced the anti-DNA antibody levels as can be seen in the Table (the sera were diluted 1:1250). Table 12 also summarizes the clinical manifestations in the different groups of mice. It can be seen that, although both doses were effective in reducing leukopenia and proteinuria, the effect of the 200 μg dose in this experiment did not reach significance (mice were sacrificed a month after the end of treatment).

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TABLE 12

Effect of treatment of BALB/c mice afflicted with experimental SLE with 200 μg or 300 μg hCDR1 or 300 μg revhCDR1

| | 16/6Id Mean ± SD | 16/6Id + hCDR1 200 μg/mouse Mean ± SD | 16/6Id + hCDR1 300 μg/mouse Mean ± SD | 16/6Id + rev-hCDR1 300 μg/mouse Mean ± SD | Control Mean ± SD |
|---|---|---|---|---|---|
| Proteinuria g/l | 0.58 ± 0.34 | 0.17 ± 0.15<br>p = 0.0366 | 0.085 ± 0.01<br>p = 0.0088 | 0.8 ± 0.3<br>p > 0.5 | 0.031 ± 0.09 |
| WBC/mm³ | 2800 ± 300 | 4900 ± 800<br>p = 0.004 | 5800 ± 300<br>p = 0.004 | 4180 ± 881<br>p = 0.0079 | 7500 ± 400 |
| Intensity of ICD in kidneys | 1.6 ± 0.55 | 1 ± 0.63<br>p = 0.0887 | 0.5 ± 0.5<br>p = 0.0152 | 0.85 ± 0.75<br>p = 0.0628 | 0 |
| Anti-dsDNA antibodies 1:1250 | 0.47 ± 0.3 | 0.23 ± 0.1<br>p = 0.0234 | 0.2 ± 0.1<br>p = 0.0145 | 0.35 ± 0.3<br>p = 0.343 | 0.002 ± 0.002 |
| Anti-16/6 Id antibodies 1:10000 | 0.89 ± 0.1 | 0.96 ± 0.2 | 0.93 ± 0.1 | 0.95 ± 0.13 | 0.03 ± 0.1 |

[0184] FIGS. 14A-F represent one kidney of each group where A,B represent an untreated mouse; C,D represent a kidney of a hCDR1-treated mouse and E,F represent a kidney section of a mouse treated with the reversed hCDR1. Pictures A,C,E x 100 and B,D,F x 400.

[0185] FIGS. 15A-E shows levels of the different cytokines in supernatants of 16/6Id stimulated lymph node cultures of the treated mice. The latter tests were performed at the end of treatment (after 10 treatment injections). It can be seen that treatment with hCDR1 (both doses) down regulated INF-γ (FIG. 15A), IL-10 (FIG. 15C) and TNF-α (FIG. 15B). On the other hand, hCDR1 up-regulated the levels of TGF-β. Because splenocytes usually secrete more TGF-β (FIG. 15D) than lymph node cells, TGF-β was also measured in supernatants of 16/6Id triggered spleen cells of mice of the different groups (FIG. 15E).

[0186] Thus, hCDR1 ameliorates disease manifestations and immunomodulates cytokine production in mice afflicted with an induced experimental SLE.

EXAMPLE 11.

[0187] Transfer of the beneficial effects on lupus manifestations by splenocytes of hCDR1-treated mice.

[0188] It was of importance to investigate whether the beneficial effects of treatment with hCDR1, that are manifested by the down-regulation of disease manifestations, can be transferred by spleen cells of treated mice. To this end, we have performed an experiment in which 8-month old (NZB x NZW)F1 mice, that suffer already from the full-blown lupus-like disease, were divided into 2 groups. Group 1 was not treated and mice of group 2 were transferred with $20\times10^6$ spleen cells of 3-month old (NZB x NZW)F1 mice that were injected 3 times ( s.c., every other day) with 300 μg/mouse of hCDR1. All splenocytes were injected intraperitoneally. Mice were tested for anti-dsDNA autoantibody production, disease manifestations, and were sacrificed 4 weeks after cell transfer. Transfer of cells of hCDR1-treated mice caused a mild reduction in the levels of anti-DNA antibodies.

[0189] Table 13 demonstrates that transfer of splenocytes of hCDR1-treated mice resulted in a significantly lower proteinuria and in a reduction in immune complexes in the kidneys.

TABLE 13

Clinical lupus manifestation in mice recipient of spleen cells of mice treated with hCDR1

| Group No. | Treatment | Proteinuria g/L ± SEM | I.C.D ± SEM |
|---|---|---|---|
| 1 | Non-treated | 15.07 ± 4.92 | 2.25 ± 0.47 |
| 2 | $20\times10^6$ spleen cells from hCDR1 treated mice | 0.97 ± 0.67<br>p = 0.05 | 0.75 ± 0.47<br>p = 0.05 |

The p-values are calculated in comparison to g1 of untreated mice

[0190] The experiment was repeated a few more times using revhCDR1 as control. The results were similar to those in the first experiment, namely, cell transfer of the treated mice affected slightly anti-DNA antibody titers and significantly proteinuria and kidney damage. The clinical manifestations of a representative experiment are shown in Table 14.

TABLE 14

Clinical lupus manifestation in mice recipient of spleen cells of mice treated with hCDR1

| Group No. | Treatment | Proteinuria g/L ± SEM | I.C.D ± SEM |
|---|---|---|---|
| 1 | Non-treated | 10.9 ± 3.05 | 2.3 ± 0.26 |
| 2 | $20\times10^6$ spleen cells from hCDR1 treated mice | 2.91 ± 1.6<br>p = 0.0402 | 1.33 ± 0.35<br>p = 0.034 |
| 3 | $20\times10^6$ spleen cells from revhCDR1 treated mice | 5.38 ± 2.34<br>p = 0.0931 | 1.7 ± 0.23<br>p = 0.065 |

The p-values are calculated in comparison to g1 of untreated mice

[0191] It can be seen that the down-regulating effect of treatment with hCDR1, could be transferred by spleen cells of the treated mice.

[0192] FIGS. 16A-F represent kidney sections of mice of group 1 (A, B—untreated mice), group 2 (C, D—recipients of splenocytes of 2-month old mice treated with hCDR1)

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and group 3 (E, F—recipients of splenocytes of 2-month old mice treated with revhCDR1). A,C,E x 400; B,D,F x 100.

[0193] The results of the above experiments indicate that the ameliorating effects on disease manifestations of hCDR1 can be transferred by immunocytes of healthy mice that were treated with hCDR1.

EXAMPLE 12.

[0194] Down-regulation of MMP-3 and MMP-9 by the murine mCDR peptides.

[0195] Matrix metalloproteinases (MMPs) (Shingleton et al., 1996; Gosti et al., 1996; Masson et al., 1998) constitute a family of zinc containing endoproteinases that play an important role in the remodeling of extracellular matrix in normal tissues, and also contribute to pathological processes. They share structural domains but differ in substrate specificity, cellular sources and inducibility.

[0196] The MMPs are synthesized as zymogen-like latent precursors and are converted subsequently to an active form. P-2 and MMP-9, which are both gelatinases, can degrade type IV collagen, denatured collagens, types V, VII, X and XII collagens, vitronectin, aggrecan, galectin-3 and elastin. MMP-2 is the most widely expressed UP. It is produced by a variety of cells and is frequently elevated in malignant tumor metastasis.

[0197] In terms of protein and domain structure, MMP-9 is the largest and most complex member identified so far. MMP-9 expression is characterized by a complex regulation with tight control at the levels of gene transcription and protein secretion (such as cytokines, chemokines, eicosanoids) inflammatory mediators, and action of tissue inhibitors of metalloproteinases (TIMPs). In addition, MMP-9 activity is modulated by the activation of pro-MMP-9, by components of the plasminogen activation system or other MMPs (Gaedez et al., 1996).

[0198] The involvement of MMPs in autoimmune diseases was demonstrated in various autoimmune diseases such as multiple sclerosis (Ozenci et al., 1999) and its animal model experimental autoimmune encephalomyelitis (EAE) (Gijbels et al., 1994), rheumatoid arthritis (Keyszer et al., 1999), Guillain-Barre syndrome (Creange et al., 1999), experimental bullous pemphigoid (Liu et al., 1998) and experimental autoimmune neuritis (Hughes et al., 1998). Serum levels of MMP-3 and TIMP-2 in patients with lupus nephritis were reported to be significantly higher than those of healthy controls, but no correlation with disease activity was noted (Zackar, 1999; Keyszer et al., 1999).

[0199] The potential importance of the many activities of MMPs in inflammatory responses has been suggested by the inhibitory effects of MMP inhibitors in several animal models of autoimmune diseases. Specific inhibition of MMPs in vivo suppresses edema, pathologic tissue proliferation, and damage to specialized tissue structures in several inflammatory and autoimmune diseases (Gijbels et al., 1994; Wallace et al., 1999; Conway et al., 1995).

[0200] According to the present invention, we show that levels of P-3 and MMP-9 are elevated both in sera and in kidneys of mice afflicted with either the spontaneous or the induced experimental SLE. We also demonstrate that treatment of SLE-afflicted mice with the CDR-based murine peptides, that ameliorate manifestations of lupus in mice, diminished the levels of MMP-3 and MMP-9 in the serum and kidneys of the treated mice and also caused amelioration of disease manifestations. The same effects are expected to be presented by the human hCDR peptides of the invention.

EXAMPLE 12(i)

[0201] Kinetics of appearance of MMP-3, MMP-2 and MMP-9 in sera of (NZBxNZW)F1 mice.

[0202] We first examined if the development of spontaneous SLE in (NZB x NZW)F1 mice is associated with changes in the levels of MMP-3, P-2 and MMP-9 in their sera. We thus followed the levels of the latter starting at the age of 2 months, before disease manifestations are observed, until the age of 8 months when the mice suffer from a full blown disease. The results are shown in FIG. 17. As can be seen in FIG. 17A, both the 34 kd and the 40 kd forms of MMP-3 in sera of 2-month old mice are very low as detected by Western blot analysis. The levels of all forms are gradually elevated towards the age of 8 months, the last time point tested. Similarly, FIG. 17B shows that MMP-9 activity, assessed by gel zymography, is low at the age of 2 months and is gradually elevated in sera of mice with the progression of disease, until the age of 8 months. It is also seen in FIG. 17B that levels of MMP-2 are not changed significantly with the progression of disease.

EXAMPLE 12(ii)

[0203] Kinetics of appearance of MMP-3, MMP-2 and MMP-9 in sera of BALB/c mice that were immunized with 16/6Id.

[0204] Previous results from our laboratory have shown that SLE can be induced in BALB/c mice following immunization with the 16/6Id (Mendlovic et al., 1988; Waisman et al., 1993). Therefore, it was of interest to test if the induced model of SLE resembles the (NZB x NZW)F1 model with respect to the changes in P-3 and MMP-9. We thus looked at levels of MMP-3 and MMP-9 in this experimental model of SLE. The results demonstrated in FIG. 18 indicate that the levels of MMP-3 (all isoforms) were elevated 10 days after the boost (4.5 weeks post-immunization), and were higher than in control unimmunized or CFA-immunized BALB/c mice of the same age (FIG. 18A). The levels of MMP-3 increased with aging in all groups, however, the latter were always higher in the 16/6Id immunized mice than in the control groups. In contrast, no induced changes in MMP-9 activity could be detected until 2 months after the boost (FIG. 18B). Higher activity of MMP-9 in the 16/6Id immunized mice than in non immunized mice, could be observed at about 4 months after the boost.

EXAMPLE 12(iii)

[0205] Specific up regulation of MMP-3 and MMP-9 in kidney sections of BALB/c mice that were immunized with the 16/6Id.

[0206] Since immunization of BALB/c mice with the 16/6Id leads to clinical manifestations characteristic of SLE including kidney damage (Mendlovic et al., 1988; Waisman et al., 1993), and because kidney damage in (NZB x NZW)F1 mice was reported to be associated with elevation

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in the levels of MMP-3 and MMP-9 (Nakamura et al., 1993), we looked at the expression of these enzymes in kidneys of BALB/c mice that were immunized with 16/6Id. As controls, we used mice that were immunized with CFA and aged matched unimmunized mice. Both control groups were injected with PBS at the time point of the boost. Two months and 5 months after the boost with 16/6Id, kidney sections of mice were immunostained for MMP-3 or MMP-9. FIG. 19 represents immunohistology of kidneys taken 5 months after the boost. As can be seen in the figure, immunization of mice with CFA up-regulated the expression of both MMP-3 (19A) and MMP-9 (19B) in the kidneys, in the glomeruli and in the surrounding tissue. Nevertheless, immunization with 16/6Id in CFA further elevated significantly the expression levels of these two MMPs. This elevation was observed already two months after the boost with the 16/6Id (data not shown). The nonspecific background levels of the staining are also demonstrated (19C).

EXAMPLE 12(iv)

[0207] The murine mCDR1 peptide down regulates levels of MMP-3 and activity of MMP-9 in sera of (NZB x NZW)F1 mice.

[0208] Since we could show that levels of MMP-3 and MMP-9 are elevated in both experimental models of SLE, it was of interest to examine if the amelioration in the clinical symptoms of SLE observed following treatment with the mCDR1 peptide (Bilat et al., 2000; Bilat et al., 2001), is accompanied by a reduction in levels of MMP-3 and MMP-9. We thus treated (NZB x NZW)F1 mice that develop SLE-like disease spontaneously, at the age of 2 months (before clinical manifestations are observed) by injecting them weekly with mCDR1 in PBS (250 μg/mouse) s.c. for 10 weeks. This prevention protocol resulted in the amelioration of all clinical symptoms (Bilat et al., 2000). As can be seen in FIG. 20, there is a reduction in the two lower bands of MMP-3 (20A) and in MMP-9 activity (20B) in the serum. This reduction is associated with the reduction in the clinical manifestations (Bilat et al., 2000). As seen in FIG. 20A, the 45 kd form of MMP-3 was not affected.

[0209] It was also of interest to find out whether the mCDR1 peptide is capable of down regulating elevated levels of MMP-3 and activity of MMP-9. We thus looked at the levels of MMP-3 and at MMP-9 activity in the sera of (NZB x NZW)F1 mice that were treated with mCDR1 at the time that disease manifestations were already observed. As a control peptide we used the reversed mCDR1. As can be seen (FIG. 20, at the right), treatment of sick mice with mCDR1, but not with revmCDR1, specifically down-regulated the levels of MMP-3 (20A) and the activity of MMP-9 (20B) in the sera.

EXAMPLE 12(v)

[0210] The mCDR1 peptide down-regulates levels of MMP-3 and activity of MMP-9 in sera of 16/6Id immunized mice.

[0211] As we have demonstrated, activity of MMP-9 is elevated in both experimental models [(NZB x NZW)F1 and 16/6Id-induced] of SLE. Because the mCDR1 peptide was shown to ameliorate the disease in both models, and to down-regulate MMP-3 levels and MMP-9 activity in sera of treated (NZB x NZW)F1 mice, we further looked for the effect of treatment on these enzymes in sera of 16/6Id immunized BALB/c mice. FIG. 21 represents results of a prevention and a treatment experiments. The figure shows that mCDR1 could down regulate MMP-3 and MMP-9 in this experimental model as well. Down regulation of levels of MMP-3 and of MMP-9 activity was observed for both the prevention and the treatment protocols. Thus, in vivo administration of mCDR1, either at the stage of disease induction or at the stage of a full blown disease, could reduce the levels of MMP-3 (21A) and MMP-9 (21B). This reduction was specific, since treatment with the reversed mCDR1 did not affect MMP-3 or MMP-9 activity (not shown). Preliminary results, using the MMP-9 activity assay system (from Amersham-Pharmacia Biotech UK Limited, England) show that the activity of sera from unimmunized BALB/c mice is comparable to 7 ng/ml of pure active MMP-9. Immunization with the 16/6Id elevates the activity to 16 ng/ml and treatment with the mCDR1 peptide down regulates it almost to the normal levels (8.5 ng/ml).

EXAMPLE 12(vi)

[0212] Effect of treatment with the mCDR1 peptide on the levels of MMP-3 and MMP-9 in kidney sections of 16/6Id immunized BALB/c mice.

[0213] Because MMP-3 and MMP-9 were shown to be elevated in kidneys of mice with induced experimental SLE, it was of interest to look for the effect of treatment with the mCDR1 peptide on the expression of the latter MMPs in the kidneys of the treated mice. FIG. 22 demonstrates that in both prevention (FIG. 22A) and treatment (FIG. 22B) experiments, mCDR1 down-regulated the expression levels of both MMP-3 and MMP-9 in the kidneys of the treated mice. The reduction in the levels of MMP was observed in both the glomeruli and interstitium. The observed reduction in MMP expression correlated with the reduction in staining for immune complex deposits using anti-Ig (FIG. 22B).

[0214] Because of the apparent involvement of MMP-3 and MMP-9 in the pathogenesis of SLE, we tested whether the human hCDR1 peptide is capable of down-regulating the levels of the latter in correlation with the amelioration of disease manifestations. To this end, pooled sera of groups of (NZB x NZW)F1 mice that were treated with the hCDR1 by s.c. injections of 100 μg or 300 μg/mouse once a week for ten weeks were tested for MMPs levels at various periods during the treatment. FIG. 23 represents results of an experiment in which mice were treated starting at the age of 7 months when all clinical manifestations were observed. The results are of sera collected at the middle of treatment, after 5 injections of hCDR1. The results of FIG. 23 indicate that treatment of mice with 300 μg hCDR1 down regulated the levels of MMP-3 and the activity of MMP-9. These results are in agreement with the significant amelioration of disease manifestations following treatment with hCDR1.

[0215] Discussion

[0216] The above results show that both MMP-3 and MMP-9 are elevated in sera and in kidneys of murine models of SLE. The murine mCDR1 peptide could down regulate the levels of MMP-9 and MMP-3 in both sera and kidneys of SLE-afflicted mice. It is demonstrated that in the spontaneous model of SLE, up regulation of both MMP-9 and MNP-3 in the serum occurs at the first 3.5 months. In the induced model the elevation in the levels of MMP-3 in sera

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is very early (10 days after the boost with the 16/6Id), while the elevation of MMP-9 activity occurs about 3-4 months later. Based on the results with the 16/6Id induced model that allows us to follow processes at the first stages of the disease, it seems that MMP-3 is involved in disease induction while MMP-9 is involved in disease progression.

[0217] Our results showing the elevation of P-3 in the mouse models of SLE, are in agreement with results demonstrating that MMP-3 is significantly increased in sera of patients with SLE (Kotajima et al., 1998) and that MMP-3 transcript is significantly increased with the progression of nephritis in (NZB x NZW)F1 mice (Nakamura et al., 1993). Taken together, these results suggest that MMP-3 may contribute to the development of glomerular injury in lupus nephritis.

[0218] Our results show that activity levels of MP-2 in sera are not elevated significantly with disease progression in both the induced and the spontaneous models of SLE. These results are compatible with those reported previously (Zucker, 1999) that MMP-2 levels were not increased in SLE. Accordingly, this enzyme was not modulated by treatment with the CDR1-based peptide (data not shown). Levels of MMP-2 were secreted constitutively and did not change in other pathological conditions (like optic neuritis and multiple sclerosis) as well, whereas levels of MMP-9 were elevated relatively to the healthy controls (Gijbels et al., 1992; Pasmen et al., 1994).

[0219] Interestingly, the mCDR1 peptide, that specifically and directly affects SLE-associated T-cell responses, could down regulate levels of MMP-3 and MMP-9 that are already up regulated (treatment protocol) as well as to prevent their elevation (prevention protocol).

[0220] MMP-9 activity in sera was neither reported in SLE patients nor in animal models of SLE. This is the first time that a correlation between SLE and MP-9 is shown and that MMP-9 is demonstrated to be elevated in sera and kidneys of SLE-afflicted mice. Although the elevation in sera appears relatively late (around 4 months after the boost), the elevation of P-9 in the kidneys is observed at early stages after disease induction (2 months) (data not shown). It is further shown herein for the first time that peptides that ameliorate SLE manifestations in mice (mCDR1 and hCDR1 as shown above), also down regulate MMP-9 both in sera and in kidneys. The latter data were also supported by preliminary results, using the MMP-9 activity assay system that showed that immunization with the 16/6Id elevated MMP-9 activity, whereas treatment with the CDR1-based peptide down regulated it to the activity levels of unimmunized mice.

[0221] It is also demonstrated here for the first time that MMP-3 and MMP-9 differ in the kinetics of their induction in experimental models of autoimmune diseases as shown here specifically for SLE. This may indicate the different roles of the above MMPs in the pathogenesis of the disease. Our results demonstrate that peptides that immunomodulate SLE-associated T-cell responses and down regulate disease manifestations can control the secretion (although not necessarily by the T cells themselves) of those MMPs in sera and kidneys. These results indicate that MMP-3 and MMP-9 play a role in the pathogenesis of SLE and may serve as surrogate markers of disease progression on one hand, and of disease amelioration by a given treatment, on the other hand.

## EXAMPLE 13.

[0222] Activity of MMP-9 (but not of MMP-2) is elevated in sera of SLE patients

[0223] In the present example, we determined the levels of MMP-9 and MMP-2 in sera of 40 patients with SLE and we demonstrate that MMP-9 but not MMP-2 activity is significantly elevated in sera of SLE patients compared to healthy controls. High MMP-9 activity correlated with the presence of discoid rash, Raynaud phenomenon, pneumonitis, mucosal ulcers and the presence of anti-phospholipid antibodies (APLA). In addition, elevated levels of MMP-9 correlated with SLE activity in the group of male patients.

[0224] Materials and Methods

[0225] Patients. Forty patients, 32 females and 8 males with SLE participated in this study. All patients revealed at least four of the revised diagnostic criteria of the American College of Rheumatism (ACR) for the diagnosis of SLE (Winchester, 1996). Twenty-five sex- and age-matched healthy volunteers served as a control group in our studies. The mean age of patients at diagnosis was 29±9.7 (range 15-48) years and the mean follow-up period was 11±10 (range 1-32) years. Disease activity was determined according to the SLEDAI lupus activity index (Bombardier et al., 1992) and by the BILAG index (Hay et al., 1993). The study was approved by the ethic committee of the Kaplan Medical Center, Rehovot, Israel.

[0226] Measurement of MMP-2 and MMP-9 by activity assay, kits. Activities of MMP-2 and MMP-9 were measured by specific Biotrak MMP-2 or MMP-9 activity assay kits (Amersham Pharmacia Biotech UK Limited, UK) according to the manufacturer's instructions. Sera were diluted 1:100 and 1:32 for the determination of MMP-2 and MMP-9 activities, respectively. The appropriate standards were added in each assay. In order to measure the total content of the MMPs, activation of the pro form of the MMPs was performed using p-aminophenylmercuric acetate (APMA).

[0227] Measurement of MMP-2 and MMP-9 activities by gel Zymography. MMP-2 and MMP-9 activities were tested by gelatin zymography. A 5 μl sample of serum was separated by an 8% SDS-PAGE gel polymerized with 1 mg/ml gelatin. Gels were washed once for 30 min in 2.5% Triton X-100 to remove the SDS, and once for 30 min in the reaction buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$ and 0.02% (w/v) Brij 35 (pH 7.5). The reaction buffer was changed to a fresh one, and the gels were incubated at 37° C. for 24 h. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie brilliant blue and was quantified by densitometry.

[0228] Statistical analyses. The data were evaluated using chi-square or Fisher exact tests, unpaired t-test and two tailed P-values. Pearson, Spearman and multivariate analyses were also used.

## EXAMPLE 13 (i)

[0229] Activity of MMP-9 but not of MMP-2 is elevated in SLE

[0230] As described above, MMP-9 was shown to be involved in several autoimmune diseases as well as in animal models of SLE. Thus, we were interested in studying whether MMP-9 is also elevated in sera of SLE patients. For

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this purpose, we examined sera of 40 SLE patients and of 25 healthy controls by gel zymography, in which both MMP-9 and MMP-2 activities can be visualized. A representative gel is shown in FIG. 24. As can be seen in this figure, levels of MMP-9 are elevated in the sera of SLE patients when compared to healthy controls. Densitometric analysis of the zymograms of sera of 40 SLE patients and 25 healthy controls indicated that the mean MMP-9 activity for SLE patients was 109±5.6 densitometry units and for the healthy controls, 76.5±4.2 densitometry units (P=0.0001). Activity values of above 85 densitometry units (mean of healthy controls +2 s.e.) were considered high. The results demonstrated high activity levels of MMP-9 in 68% of the SLE patients. Only 3% of healthy controls exhibited high MMP-9 activity (P=0.001). Densitometric analysis of MMP-2 levels in the same serum samples revealed that the differences in MMP-2 activity between sera of SLE patients and of healthy controls were not significant. Thus values of 109±7 and of 123±5 (mean activity densitometry units ± s.e.) were determined for healthy controls and SLE patients, respectively (P=0.0531). To quantify the activity levels of MMP-9 and MMP-2 in the serum further, we used activity assay kits.

[0231] FIG. 25 shows that the activity of MMP-9 is elevated by threefold in sera of SLE patients compared with sera of healthy controls, and this elevation is statistically significant (P=0.0302). In contrast, the differences in the levels of MMP-2 between the two groups are not significant (P=0.1254).

[0232] Since we, as well as others (Ebihara et al., 1998 and 1999) detected high F-9 levels in sera of patients with non-SLE chronic renal failure (e.g. diabetes mellitus, hypertension) probably due to the retention of the enzyme, we analysed the correlation between levels of MMP-9 and kidney function in the group of SLE patients tested. No correlation was observed between creatinine levels and MMP-9 levels ($r^2$=0.01), indicating that the elevated levels of MMP-9 in SLE patients were not the result of retention of the enzyme due to renal impairment.

EXAMPLE 13(ii)

[0233] Correlation of MMP-9 activity with clinical and laboratory parameters

[0234] The elevation in the activity levels of MMP-9 in sera of SLE patients prompted us to look for possible correlation between clinical and laboratory parameters, and serum MMP-9 levels. Statistical analysis (chi-square or Fisher exact tests) was performed by investigating the number of patients with high and normal MMP-9 levels for each clinical manifestation (Table 15) as well as by taking into consideration the actual mean activity levels of MMP-9 for patients with or without a certain clinical symptom. The results were similar by both analyses. It is noteworthy that for all clinical symptoms, the percent of patients with elevated MMP-9 levels is much higher than that in the group of healthy controls. Levels of MMP-9 did not correlate with gender, duration of disease or the age of its onset (Pearson, Spearman).

[0235] Table 15 shows the clinical and laboratory characteristics of the SLE patients according to their MMP-9 activity levels (lower or equal to healthy controls=normal). High levels of MMP-9 correlated significantly with the presence of Raynaud phenomenon (P=0.0138) and APLA (P=0.041). A strong correlation could be observed with pneumonitis, discoid rash, neurological disorders and mucosal ulcers. However, the number of patients with the latter manifestations was too small to perform a statistical analysis. Multivariate analysis revealed that Raynaud phenomenon and low complement (C3, C4) levels are positively correlated with high MMP-9 levels (P=0.0001 and 0.0137, respectively). In contrast, photosensitivity, arthritis and hematological disorders are negatively correlated with MMP-9 activity levels (P=0.0381, 0.0014 and 0.0065, respectively).

TABLE 15

Clinical characteristics of SLE patients with high and normal MMP-9 activities according to their MMP-9 levels.

| | | MMP-9 LEVELS (%) | |
|---|---|---|---|
| | | High | Normal |
| Number of Patients (%) | 40 (100) | 27 (68) | 13 (32) |
| Photosensitivity | 13 | 8 (62) | 5 (38) |
| Mucosal ulcers | 9 | 8 (89) | 1 (11) |
| Malar rash | 9 | 7 (78) | 2 (22) |
| Discoid rash | 5 | 5 (100) | 0 (0) |
| Raynaud phenomenon | 8 | 8 (100) | 0 (0) |
| Vasculitis | 18 | 14 (78) | 4 (22) |
| Arthritis | 31 | 21 (68) | 10 (32) |
| Serositis | 9 | 7 (78) | 2 (22) |
| Pneumonitis | 4 | 4 (100) | 0 (0) |
| Neurological disorders | 4 | 4 (100) | 0 (0) |
| Renal disorder | 16 | 11 (69) | 5 (31) |
| Hematological Disorders | 29 | 18 (62) | 11 (38) |
| ANA | 40 | 27 (68) | 13 (32) |
| ds-DNA | 36 | 24 (67) | 12 (33) |
| APLA | 25 | 20 (80) | 5 (20) |
| Low complement (C3, C4) | 30 | 21 (70) | 9 (30) |

[0236] Clinical involvement was defined according to the ACR revised criteria (Winchester, 1996). Anti-nuclear antibodies (ANA) and anti-ds DNA antibodies were determined by using Hep2 cells and Crithidia lucilias, respectively. Anti-phospholipid antibodies (APLA) were defined as reactivity with one or more of the following assays: false positive VDR, lupus anti-coagulant (LAC) or ELISA for anticardiolipin antibodies.

[0237] We also looked for a possible correlation between SLEDAI and MMP-9 activity in male (FIG. 26A) and female patients (FIG. 26B). Interestingly, the correlation coefficient was significant and positive for men ($r^2$=0.6333) but insignificant and negative for women ($r^2$=0.0571). Similar results were obtained using the BILAG scoring system. Thus, a positive correlation coefficient between MMP-9 activity and BILAG scores was observed for men ($r^2$=0.6442) and an insignificant one for women.

[0238] It was also of interest to determine whether a correlation exists between the use of various treatment modalities by the patients and MMP-9 activity. As can be seen in Table 16(A), there was no significant correlation between the current treatment of the patients and MMP-9 activity. However, when we looked at treatment of patients at any time during their disease course (Table 16(B)), high MMP-9 levels were associated with usage of cytotoxic agents (82%).

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TABLE 16

Treatment modalities of SLE patients according to their MMP-9 levels

| | Total Number of Patients | MMP-9 Levels (%) High | Normal |
|---|---|---|---|
| **A. Current treatment** | | | |
| Cytotoxic agents | 8 | 6 (75) | 2 (25) |
| Steroids | 23 | 17 (74) | 6 (26) |
| Anti-Malarial | 21 | 14 (67) | 7 (33) |
| NSAID | 7 | 5 (71) | 2 (29) |
| **B. Treatment along the follow up period** | | | |
| Cytotoxic agents | 17 | 14 (82) | 3 (18) |
| Steroids | 29 | 19 (66) | 10 (34) |
| Anti-Malarial | 26 | 16 (62) | 10 (38) |
| NSAID | 18 | 12 (67) | 6 (33) |

[0239] The anti-malarial agent hydroxychloroquine was used at dose of 200-400 mg/day. Steroid treatment was defined as a daily dose >5 mg of prednisone. Cytotoxic agents used were cyclophosphamide (0.5-1 g/m$^2$ monthly) or azathioprine (100-150 mg/day).

EXAMPLE 13(iii)

[0240] Variations in MMP-9 activity in serum samples taken from individual SLE patients at different time points

[0241] Since disease activity varies over time, we measured the activity levels of MMP-9 and MM-2 in the serum of individual patients that were sampled during 4-6 years of follow-up. Sera of nine patients taken at different time points were analysed. Levels of MMP-2 did not vary significantly between patients and healthy controls. In 5 out of the 9 patients tested, variations in MMP-9 activity in serum samples of individual patients could be observed with time. The results for 2 representative SLE patients are shown in FIG. 27. As can be seen, MMP-9 activity, but not MMP-2 activity, has been changing with time in the same patients. These changes were not associated with disease activity indices as determined by either the SLEDAI or BILAG systems. Changes in MMP-9 activity were not detected in sera of 5 healthy controls that were sampled at different time points (data not shown). In 4 other SLE patients, no substantial changes in MMP-9 or MMP-2 activity were observed with time, and MMP-9 activity levels remained either high or low, depending on the individual patient.

[0242] Discussion

[0243] The present study demonstrates for the first time the involvement of MMP-9 in human SLE. We show that the activity of MMP-9, but not MMP-2, is significantly elevated in sera of 68% of SLE patients compared with healthy controls. High MMP-9 levels correlated with Raynaud phenomenon, pneumonitis, neurological disorders, discoid rash and the presence of APLA. Changes in MMP-9 activity were observed in serum of the same patient at different periods of the disease. MMP-9 activity levels did not correlate with disease activity index (SLEDAI, BILAG) in female patients, but correlated with SLE activity in the group of male patients.

[0244] The present study shows that activity levels of MMP-2 are not elevated significantly in sera of SLE patients. These results are compatible with those reported previously (Zucker, 1999) that MMP-2 levels were not increased in SLE. Levels of MMP-2 were also constitutive and unchanged in other pathological conditions (like optic neuritis and multiple sclerosis) in which levels of MMP-9 were elevated relatively to the healthy controls (Gijbels et al., 1992; Peeters et al., 1994).

[0245] Involvement of an additional MMP, namely, MMP-3 was suggested in the pathogenesis of SLE, since it was significantly increased in sera of patients with SLE (Kotajima et al., 1998). The frequency of SLE patients with elevated MMP-9 activity (68%) shown in the present example, resembles the frequencies reported (Kotajima et al., 1998) for high MMP-3 levels in SLE (76%) and in RA (82%) patients. Furthermore, the MMP-3 transcript was shown to increase significantly with the progression of nephritis in (NZB x NZW)F1 mice (Nakamura et al., 1993).

[0246] The origin of the elevated MMPs in sera of SLE patients is not known. MMP-9 has been shown to be secreted by peripheral blood cells such as T cells, neutrophils, and macrophages (for review, see Goetzl et al., 1996). The fact that no correlation was found between MMP-9 activity levels and the number of peripheral blood cells in the patients may suggest that MMP-9 was not secreted by peripheral blood immune cells but rather, by SLE-affected organs like kidneys or lungs/pleura. The observation that all SLE patients with pneumonitis exhibited high MMP-9 activity levels may suggest the diseased lung as a source of high MMP-9 levels. Moreover, the association between cytotoxic treatment, which represents the severity of SLE-related organ impairment, and high levels of MMP-9 in the sera may also support the notion that the diseased organs are the source of MMP-9 activity in SLE patients. Nevertheless, the possibility still exists that less peripheral blood lymphocytes secreted higher activity levels of MMP-9.

[0247] TNF-α and IL-1 were shown to play an important role in the pathogenesis of SLE both in the human disease (Dean et al., 2000) and in murine models (Segal et al., 1997; Theofilopoulos et al., 1999; Eilat et al., 2001). It has been shown in several systems that these cytokines induce MMP-9 production (Gaedez et al., 1996), and thus, it is possible that the induction of the latter MMPs is part of the pathogenic effect of these cytokines in SLE. It has been reported that levels of MMP-9, that are secreted spontaneously by peripheral blood monocytes of healthy individuals, were upregulated upon exposure to TNF-α and IL-1β (Saren et al., 1996). In addition, MMPs of both T cells and macrophages facilitate secretion of TNF-α by cleavage of the membrane-bound form (Gearing et al., 1994). Thus, these examples demonstrate the mutual regulatory effects of MMP on the proinflammatory cytokines and vice versa. Nevertheless, the fact that in the sera of some of the patients the activity levels of MMP-9 remained within the normal range during the follow-up period, whereas high activity levels of MMP-9 were measured in the sera of most patients, may suggest the involvement of genetic factors in the regulation of the latter.

[0248] The results herein indicate that MMP-9 might play a role in the pathogenesis of SLE, and that measurement of plasma/serum activity levels of this metalloproteinase may

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provide important information when monitoring patients treated with drugs that interfere with MMP-9 activity.

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Gly, Xaa(9) is Arg, Xaa(10) is Ala, Xaa(11) is Gly, Xaa(12) is Trp, Xaa(13) is Asn, Xaa(18) is Tyr, and Xaa(20) is Met.

<400> SEQUENCE: 23

Tyr Tyr Cys Ala Arg Gly Leu Leu Arg Ala Gly Trp Asn Asp Val Asp
1 5 10 15

Tyr Tyr Gly Met Asp Val
20

<210> SEQ ID NO 24
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Gly, Xaa(9) is Ala, Xaa(10) is Gly, Xaa(11) is Gly, Xaa(12) is Trp, Xaa(13) is Asn, Xaa(18) is Tyr, and Xaa(20) is Met.

<400> SEQUENCE: 24

Tyr Tyr Cys Ala Arg Gly Leu Leu Ala Gly Gly Trp Asn Asp Val Asp
1 5 10 15

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-continued

Tyr Tyr Gly Met Asp Val
20

<210> SEQ ID NO 25
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Nle, Xaa(9) is Arg, Xaa(10) is Gly, Xaa(11) is Gly, Xaa(12) is Trp, Xaa(13) is Asn, Xaa(18) is Tyr, and Xaa(20) is Met.

<400> SEQUENCE: 25

Tyr Tyr Cys Ala Arg Nle Leu Leu Arg Gly Gly Trp Asn Asp Val Asp
1 5 10 15

Tyr Tyr Gly Met Asp Val
20

<210> SEQ ID NO 26
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Gly, Xaa(9) is Arg, Xaa(10) is Gly, Xaa(11) is Gly, Xaa(12) is Trp, Xaa(13) is Asn, Xaa(18) is Tyr, and Xaa(20) is Gln.

<400> SEQUENCE: 26

Tyr Tyr Cys Ala Arg Gly Leu Leu Arg Gly Gly Trp Asn Asp Val Asp
1 5 10 15

Tyr Tyr Gly Gln Asp Val
20

<210> SEQ ID NO 27
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A peptide of SEQ ID NO: 19 wherein Xaa(6) is Gly, Xaa(9) is Arg, Xaa(10) is Gly, Xaa(11) is Gly, Xaa(12) isTrp, Xaa(13) is Asn, Xaa(18) is Trp, and Xaa(20) is Met.

<400> SEQUENCE: 27

Tyr Tyr Cys Ala Arg Gly Leu Leu Arg Gly Gly Trp Asn Asp Val Asp
1 5 10 15

Tyr Trp Gly Met Asp Val
20

<210> SEQ ID NO 28
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide prepared in the reversed order of SEQ ID NO: 1.

<400> SEQUENCE: 28

Gly Ile Trp Gln Leu Ser Lys Glu Pro Ser Gln Lys Val Trp Gln Met
1 5 10 15

Tyr Tyr Gly Thr
20

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-continued

<210> SEQ ID NO 29
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> OTHER INFORMATION: Synthetic peptide prepared in reversed order of SEQ ID NO: 3.

<400> SEQUENCE: 29

Ser Gly Gln Gly Trp Tyr Asp Met Ala Tyr Pro Gln Trp Leu Phe Arg
1 5 10 15

Ala Cys Tyr Tyr
20

<210> SEQ ID NO 30
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> OTHER INFORMATION: Synthetic peptide prepared in the reversed order of SEQ ID NO: 6.

<400> SEQUENCE: 30

Gly Ile Trp Gln Gln Gly Lys Gly Pro Pro Gln Arg Ile Trp Ser Trp
1 5 10 15

Tyr Tyr Gly

1. A synthetic peptide selected from the group consisting of:

(a) a peptide of at least 12 and at most 30 amino acid residues comprising a sequence consisting of, or found within, a complementarity-determining region (CDR) found in the heavy or light chain of the human monoclonal anti-DNA 16/6Id antibody (hereinafter "hCDR sequence"), or a sequence obtained by: (i) replacement of one or more of the amino acid residues of the hCDR sequence by different amino acid residues; (ii) deletion of one or more amino acid residues from the hCDR sequence; and/or (iii) addition of one or more amino acid residues to the hCDR sequence , or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

2. A synthetic peptide according to claim 1, wherein said hCDR sequence comprises a sequence consisting of, or found within, a CDR found in the heavy chain of the human monoclonal anti-DNA 16/6Id antibody.

3. A synthetic peptide according to claim 1 or 2, comprising further amino acid residues.

4. A synthetic peptide according to claim 3, wherein said further amino acid residues are amino acid residues of the sequences of the human 16/6Id mAb flanking the hCDR sequences, or sequences obtained by replacement of one or more of the amino acid residues of the hCDR flanking sequences by different amino acid residues, by deletion of one or more amino acid residues from the hCDR flanking sequences and/or by addition of one or more amino acid residues to the hCDR flanking sequences.

5. A synthetic peptide according to any one of claims 2 to 4, selected from the group consisting of:

(a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:8, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:8 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:8;and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:8, or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

6. A synthetic peptide according to claim 5, comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:8, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:8 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:8; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:8.

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7. A synthetic peptide according to claim 6 selected from a peptide consisting of the sequence of the SEQ ID NO: 11.

8. A synthetic peptide according to claim 7 selected from the peptides consisting of the sequences of the SEQ ID NO:12 to NO:18.

9. The synthetic peptide according to claim 7 consisting of the sequence of the SEQ ID NO:6.

10. The peptide consisting of the sequence of SEQ ID NO:6.

11. A synthetic peptide according to any one of claims 2 to 4, selected from the group consisting of:

(a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:10 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:10; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:10, or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

12. A synthetic peptide according to claim 11, comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:10, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:10 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:10; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:10.

13. A synthetic peptide according to claim 12 selected from a peptide consisting of the sequence of the SEQ ID NO:19.

14. A synthetic peptide according to claim 13 selected from the peptides consisting of the sequences of the SEQ ID NO:20 to NO:27.

15. The synthetic peptide according to claim 13 consisting of the sequence of the SEQ ID NO:7.

16. The peptide consisting of the sequence of SEQ ID NO:7.

17. A synthetic peptide according to any one of claims 2 to 4, selected from the group consisting of:

(a) a peptide comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:9, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:9 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:9; and/or (iii) addition of one or more amino-acid residues to said SEQ ID NO:9, or a salt or a chemical derivative of said peptide;

(b) a dual synthetic peptide comprising two different ones of said peptide of (a) covalently linked to one another either directly or through a short linking chain;

(c) a peptide polymer comprising a plurality of sequences of said peptide of (a); and

(d) a peptide of (a) or a peptide polymer of (c) attached to a macromolecular carrier.

18. A synthetic peptide according to claim 17, comprising a sequence consisting of, or found within, the sequence of SEQ ID NO:9, or a sequence obtained by: (i) replacement of one or more of the amino acid residues of said SEQ ID NO:9 by different amino acid residues; (ii) deletion of one or more amino acid residues from said SEQ ID NO:9; and/or (iii) addition of one or more amino acid residues to said SEQ ID NO:9.

19. A dual synthetic peptide according to claim 2, in which two different peptides each comprising a sequence consisting of, or found within, a different CDR of the heavy chain of the human 16/6Id mAb, are covalently linked to one another either directly or through a short linking chain.

20. A dual synthetic peptide according to claim 19, in which a peptide according to claim 6 is covalently linked to a peptide according to claim 12.

21. The dual synthetic peptide according claim 20, in which a peptide of SEQ ID NO:11 is covalently linked to a peptide of SEQ ID NO:19.

22. The dual synthetic peptide, in which the peptide of SEQ ID NO:6 is covalently linked to the peptide of SEQ ID NO:7.

23. A peptide polymer according to any one of claims 1 to 4, containing a plurality of identical sequences selected from the sequences of SEQ ID NO:6, 7, and 11-27.

24. A pharmaceutical composition comprising at least one synthetic peptide or peptide polymer according to any one of claims 1 to 23, and a pharmaceutically acceptable carrier.

25. A pharmaceutical composition comprising the peptide of the sequence of SEQ ID NO:6, and a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising the peptide of the sequence of SEQ ID NO:7, and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition according to any one of claims 24 to 26, for the treatment of systemic lupus elythematosus.

28. A pharmaceutical composition according to any one of claims 24 to 27, adapted for oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intraperitoneal, intradermal, transdermal or enteral administration.

29. A method for the treatment of systemic lupus erythematosus (SLE) comprising administering to a SLE patient an effective amount of a peptide or peptide polymer according to any one of claims 1 to 23.

30. A method according to claim 29, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:6.

31. A method according to claim 29, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:7.

32. A method for immunomodulation of systemic lupus erythematosus (SLE)-associated responses in a SLE patient, which comprises administering to said SLE patient an effective amount of an effective amount of a peptide or peptide polymer according to any one of claims 1 to 23.

33. A method according to claim 32, which comprises down-regulating the levels of matrix metalloproteinase (MMP)-3 and/or MMP-9 activities in a SLE patient.

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34. A method according to claim 32, which comprises immunomodulating the level of a cytokine activity in a SLE patient.

35. A method according to claim 34, which comprises down-regulating the level of IL-2 and/or IFN-γ activity in a SLE patient.

36. A method according to claim 34, which comprises up-regulating the level of TGF-β activity in a SLE patient.

37. A method according to any one of claims 32 to 36, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:6.

38. A method according to any one of claims 32 to 36, which comprises administering to said SLE patient an effective amount of the peptide of the SEQ ID NO:7.

39. A method for assessing the effectiveness of a drug in the treatment of a SLE-patient which comprises measuring at different intervals of time the levels of MMP-3 and/or MMP-9 in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of MMP-3 and/or MMP-9 correlates with the effectiveness of the drug.

40. A method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the levels of IL-2 and/or IFN-γ in a blood sample obtained from said patient being treated with said drug, whereby a decreased level of IL-2 and/or IFN-γ correlates with the effectiveness of the drug.

41. A method for assessing the effectiveness of a drug in the treatment of a SLE patient which comprises measuring at different intervals of time the level of TGF-β in a blood sample obtained from said patient being treated with said drug, whereby an increased level of TGF-β correlates with the effectiveness of the drug.

* * * * *

US005646131A

# United States Patent [19]

Badwan et al.

[11] Patent Number: 5,646,131

[45] Date of Patent: Jul. 8, 1997

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[54] METHOD FOR SOLUBILIZING DRUGS USING CYCLODEXTRINS AND CARBOXYLIC ACIDS

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[73] Assignee: The Arab Company For Drug Industries And Medical Appliances (ACDIMA), Amman, Jordan

[21] Appl. No.: 469,631

[22] Filed: Jun. 2, 1995

Related U.S. Application Data

[63] Continuation of Ser. No. 199,523, Feb. 22, 1994, abandoned.

[51] Int. Cl.[6] ..... A61K 31/715; A61K 31/54; A61K 31/445; A61K 31/19

[52] U.S. Cl. ..... 514/58; 514/224.3; 514/317; 514/327; 514/568; 536/103

[58] Field of Search ..... 514/58, 224.2, 514/317, 327, 568; 536/103

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*Primary Examiner*—Gary L. Kunz
*Assistant Examiner*—Kathleen Kahler Finda
*Attorney, Agent, or Firm*—Burns, Doane, Swecker & Mathis, L.L.P.

[57] ABSTRACT

A method for enhancing the solubilization and/or complexation of a drug which is insoluble or sparingly soluble in water with cyclodextrin, comprising combining the drug with cyclodextrin together with a selected hydroxy-carboxylic or polycarboxylic acid.

43 Claims, 1 Drawing Sheet

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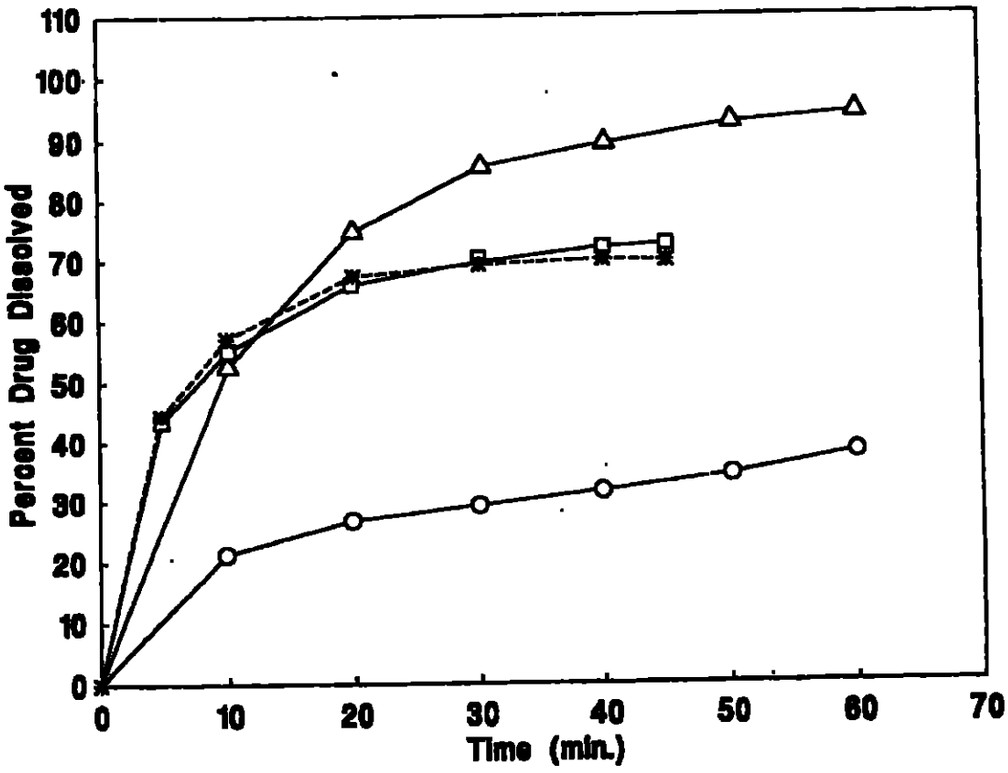

Percent Drug Dissolved
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time (min.)

Figure 1

# METHOD FOR SOLUBILIZING DRUGS USING CYCLODEXTRINS AND CARBOXYLIC ACIDS

This application is a continuation of application Ser. No. 08/199,523, filed Feb. 22, 1994, now abandoned.

## FIELD OF THE INVENTION

This invention relates to a method for increasing the aqueous solubility and/or complexation of drugs which are insoluble or sparingly soluble in water, using cyclodextrins together with selected hydroxy-carboxylic or polycarboxylic acids.

## BACKGROUND OF THE INVENTION

Cyclodextrins are cyclic multicyclopyranose units connected by α-(1,4) linkages. The most widely known cyclodextrins are α, β and γ-cyclodextrins. Derivatives of these cyclodextrins with improved properties are also known and used in the pharmaceutical field. The cyclic nature of the cyclodextrins, the hydrophobic properties of their cavities as well as the hydrophilic character of their outer surfaces, enables them to interact with other chemicals and to produce inclusion compounds which are characterized by improved solubilities and stabilities. Fields of potential applications of cyclodextrins include pharmaceuticals, fine chemicals, industrial chemicals and biological active substances.

Numerous reviews and patents related to the use of cyclodextrins and their derivatives to prepare inclusion complexes of drugs are found in the literature, for example, D. Duchêne, *Cyclodextrins and their Industrial uses*, Editions de Santé, Paris, 1987, Chapter 6 (211–257), Chapter 8 (297–350), Chapter 10 (393–439); D. Duchêne et al, *Acta Pharma Technol.* 36(1)6, 1–6, 1990; D. Duchêne et al, *Drug Dev. Ind. Pharm.*, 16(17), 2487–2499, 1990; C. Hunter et al, European Patent Publication No. EP 0346006, December 1988.

Inclusion complexes prepared to specifically improve water solubility and hence bioavailability of poorly soluble drugs have been reported by workers such as D. D. Chow et al, *Int. J. Pharm.*, 28, 95–101, 1986; F. A. Ménard et al, *Drug Dev. Ind. Pharm.*, 14(11), 1529–1547, 1988; F. J. Otero-Espinar et al, *Int. J. Pharm.*, 75, 37–44, 1991; and Berand M. Markarian et al, European Patent Publication No. EP 0274444, July 1988. Chemical modifications of cyclodextrins to prepare derivatives that further improve solubility of water insoluble drugs have been described, for example, by J. Pitha, U.S. Pat. No. 4,727,064, February 1988; N. S. Bodor, U.S. Pat. No. 5,024,998, July 1991.

In connection with the use of acids or buffers with cyclodextrins, the effects of the latter on dissociation constants of acidic organic compounds, including non-carboxylic acids, have been reported by K. Connors et al, *J. Pharm. Sci.*, 65 (3), 379–383, 1976, but these workers have not addressed the issue of solubility. As an extension to this work, equilibrium constants of some prostaglandins in the presence of α- and β-cyclodextrins have been determined in phosphate buffer solutions, as reported by K. Uekama et al, *Chem. Pharm. Bull.*, 26 (4), 1195–1200, 1978. Furthermore, β-cyclodextrin inclusion complexes of several non-steroidal anti-inflammatory drugs have been correlated for their dissolution behavior at different pH values by V. Zecchi et al, Proc. *Eur. Congr. Biopharm. Pharmacokinet.* 3rd, (1), 526–531, 1987; V. Zecchi et al, *Pharma Acta Helv.*, 63 (11), 299–302, 1988; Zecchi et al concluded that the dissolution rate of such complexes is scarcely altered by changing the pH. Also, the diffusion of free and β-cyclodextrin-complexed non-steroidal anti-inflammatory drugs have been determined at different pH values by I. Orienti et al, *Arch Pharm.*(Weinheim, Germany) 322(4), 207–211, 1989. A naproxen/β-cyclodextrin complex has been examined for solubility in buffered aqueous media by F. J. Otero-Espinar et al, Int. J. *Pharm.*, 79, 149–157, 1992; N. (Celebi et al, *Int. J. Pharm.*, 78, 183–187, 1992; N. Erden, *Int. J. Pharm.*, 48, 83–89, 1988. Dissolution of famotidine, and the β-cyclodextrin inclusion compound of the drug have been reported at pH 7.4 by M. Hassan et al, *Int. J. Pharm.*, 58, 19–24, 1990. The effects of pH on the complexation of hydroclorothiazide, ibuprofen and diazepam with β-cyclodextrin cyclodextrin have been discussed by F. Menard et al, *Drug Dev. Ind. Pharm.*, 16(1), 91–113, 1990; these workers have found in their experiments that the relationship between solubility and concentration is the same at different pH levels. The dissolution of tolbutamide/β-cyclodextrin complex has been compared to that of the drug itself and solid dispersions at pH 2 by F. Kedzierewicz et al, *Int. J. Pharm.*, 58, 221–227, 1990.

Pharmaceutical formulations containing cyclodextrins typically contain other ingredients commonly used in pharmaceuticals, for example, pH adjusters (acids, bases, buffers), effervescing agents and the like, to create forms suitable for administration. For example, International Publication No. WO85/02767, July 1985, has disclosed preparing pharmaceutical compositions by dissolving a selected cyclodextrin derivative in water and adding the desired drug to form an inclusion compound, wherein the water "may further comprise physiologically compatible compounds such as sodium chloride, potassium nitrate, glycose, mannitole, sorbitol, xylitol or buffers such as phosphate, acetate or citrate buffer." Hirai et al, U.S. Pat. No. 4,659,696, April 1987, have described various non-oral, non-injectable pharmaceutical compositions containing a drug which is poorly absorbable through the gastrointestinal tract and cyclodextrin, which may contain a variety of excipients and pH adjusters, such as an acid, a base or a buffer solution. As examples of the acid, there are mentioned "inorganic acids (e.g., hydrochloric acid, boric acid, phosphoric acid, carbonic acid, bicarbonic acid, etc.), amino acids and organic acids (e.g., monocarboxylic acids, oxycarboxylic acids, polycarboxylic acids)." Also, Chiesi et al, in U.S. Pat. No. 4,603,123, July 1986, in describing pharmaceutical compositions containing piroxicam/cyclodextrin complexes, show an effervescent tablet formulation comprising citric acid and glycine sodium carbonate in equal amounts, but these are indeed typical effervescing agents and, moreover, are added after the drug/cyclodextrin complex has already been formed. Similarly, in C. Hunter, European Patent Publication No. 0346006 relating to pharmaceutical compositions comprising ibuprofen-cyclodextrin complexes, there are examples of non-effervescent, effervescent and slightly effervescent formulations comprising trisodium citrate, citric acid and sodium bicarbonate, but these ingredients are added to the already formed ibuprofen complexes. None of these earlier workers has attached any significance to particular acids, or has recognized that selected acids could be used to improve the solubilization and/or complexation with cyclodextrins of drugs which are insoluble or sparingly soluble in water. And, of course, no such improvement would be present, for example, in the case of Chiesi et al's effervescent tablets, since the citric acid component is only present in sufficient amount to effervesce together with the glycine sodium carbonate; there is no additional citric acid present to serve as a solubilizer, as provided by the present invention as described in detail hereinbelow.



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Despite the advances in the pharmaceutical arts made possible by the use of cyclodextrins with drugs which are insoluble or only sparingly soluble in water, there remains a need for further improvements in drug/cyclodextrin formulations. β-Cyclodextrin, although considerably less expensive than its derivatives, increases drug solubility much more modestly than the derivatives and, in some instances, does not confer sufficient solubility at a low enough concentration to provide a feasible product, for example, a small enough tablet to be readily swallowed. Thus, there is a real need in this art for a means of significantly increasing the solubility of drugs in the less efficient cyclodextrins. On the other hand, the derivatized cyclodextrins tend to be very expensive; thus, there is also a real need for enhancing the solubility of drugs with such expensive cyclodextrins so that considerably smaller amounts of the cyclodextrins are required to achieve an effect comparable to that which could previously be obtained.

## SUMMARY AND OBJECTS OF THE INVENTION

One object of the present invention is to provide a method for enhancing the solubilizing effects of cyclodextrins on drugs which are insoluble or sparingly soluble in water.

Another object of the present invention is to provide a method for enhancing the complexation of cyclodextrins with drugs which are insoluble or sparingly soluble in water.

Yet another object of the present invention is to provide a means for alleviating drug absorption problems related to the poor solubility of active agents.

Another object of the invention is to provide solid pharmaceutical formulations which are characterized by rapid dissolution and better absorption.

Another object of the invention is to provide non-alcoholic syrups and fast-dissolving tablets, capsules, effervescent tablets and/or sachets.

Still a further object of the invention is to provide effervescent tablets and/or sachets which readily dissolve in situ to solutions, thus providing a means for alleviating swallowing problems, especially in geriatric and pediatric patients.

In accord with these and other objects, the present invention provides a method for enhancing the solubilization and/or complexation of a drug which is insoluble or sparingly soluble in water with cyclodextrin, said method comprising combining said drug with cyclodextrin and an effective solubility-enhancing amount of a saturated or unsaturated $C_2$–$C_6$ carboxylic acid having from one to three —COOH groups and optionally bearing up to four —OH substituents (i.e., bearing from zero to four —OH substituents), provided that when the acid has only one —COOH group, then said acid must bear at least one —OH substituent, or a pharmaceutically acceptable salt of said acid, the weight ratio of cyclodextrin to carboxylic acid being from about 1:100 to about 100: 1.

## BRIEF DESCRIPTION OF THE DRAWING

Other objects and advantages of the present invention will be apparent from the following detailed description and accompanying drawing, in which the FIGURE is a series of regular cartesian plots depicting the dissolution profile of terfenadine from capsules containing the following: Δ60 mg terfenadine, 11 mg β-cyclodextrin and 30 mg citric acid; ○60 mg terfenadine, 0% β-cyclodextrin, 0% citric acid; * 60 mg terfenadine, 11 mg β-cyclodextrin, 0% citric acid; □60 mg terfenadine, 30 mg β-cyclodextrin plus 0% citric acid.

## DETAILED DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS

Here and throughout this description, the following definitions are applicable:

Cyclodextrins for use in the present invention include the natural cyclodextrins and their pharmaceutically acceptable derivatives. Cyclodextrins and their derivatives which have been previously described as useful for complexation with/solubilization of drugs are of particular interest herein. In addition to α-, β- and γ-cyclodextrin, derivatives of these cyclodextrins such as hydroxyalkyl (e.g., hydroxypropyl), carboxamide, diethylaminoethyl, carboxymethyl and dihydroxyalkyl (e.g., dihydroxypropyl) are of special interest, and these can be prepared by well-known procedures. For a discussion of such suitable cyclodextrin derivatives, see, for example, D. Duchêne et al, *Drug Dev. Ind. Pharm.*, 16(17), 2487–2499, 1990. Presently preferred cyclodextrins for use herein are the natural cyclodextrins, α-, β- and γ-, especially β-cyclodextrin.

Suitable $C_2$–$C_6$ carboxylic acids for use herein can be straight- or branched-chain alkyl or alkenyl, or can even have a cyclic lactone structure. These acids are polyfunctional, i.e., multifunctional, that is, they have at least two carboxyl functions or at least one carboxyl function and at least one hydroxyl function. Such acids are thus also referred to herein as hydroxy-carboxylic acids or polycarboxylic acids, or simply as polyfunctional carboxylic acids. Appropriate acids include citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid and succinic acid. Presently preferred carboxylic acids for use herein include citric acid, glutaric acid, lactic acid and tartaric acid. An especially preferred acid is citric acid. The acids can be optionally used in whole or in part in the form of their pharmaceutically acceptable salts, particularly their alkali metal salts, such as, for example, trisodium citrate.

Among the drugs which are insoluble or sparingly soluble in water and which are contemplated for use in the present invention, any of those which have been previously complexed with/solubilized by cyclodextrin are of particular interest. Of special interest herein are: non-steroidal anti-inflammatory agents, such as ibuprofen, ketoprofen, piroxicam, tenoxicam, ketorolac and naproxen; antihistaminics or antiallergics, for example, terfenadine and cinnarizine; vasodilators, cytocic agents and abortifacients, such as prostaglandin $E_1$, prostaglandin $E_2$ and prostaglandin $F_{2\alpha}$ and sedatives, tranquilizers, hypnotics, anticonvulsants, anxiolytics, muscle relaxants and antispasmodics, for example, phenobarbital, pentobarbital, secobarbital, butabarbital, butalbital, alprazolam, phenytoin, diazepam, oxazepam, etomidate, lorazepam, clobazam, nitrazepam, fludiazepam, oxazepam, medazepam, flunitrazepam, nimetazepam, pentazocine, methaqualone, salbutamol, carbamazepine, baclofen, chlorpromazine and chlordiazepoxide. Especially advantageous for enhancement of solubilization and/or complexation in accord with the present invention are basic drugs which are insoluble or sparingly soluble in water.

It is now well-known that drugs which are insoluble or sparingly soluble in water and which have the required shape and size to fit at least partially into the cavity of the hydrated cyclodextrin molecule will complex with cyclodextrin and that the drug/cyclodextrin inclusion complex which results has improved water solubility as compared to

the drug itself; see, for example, International Publication No. WO85/02767, July 1985. Drugs whose water solubility can be improved by complexation with cyclodextrins exhibit significantly increased complexation and water solubility when treated in accord with the present invention. The polyfunctional carboxylic acids utilized herein make it possible to reduce the amount of cyclodextrin present in the pharmaceutical formulation, which can in turn result in substantial cost savings. Reducing the amount of cyclodextrin can also provide solid dosage forms of more reasonable size for swallowing than heretofore possible, and can result in lower toxicity. Alternatively, rather than reducing the amount of cyclodextrin present, one can instead prepare a formulation which has substantially enhanced solubility and absorption. Obviously, formulations can be made which would share these benefits, i.e., that contain somewhat less cyclodextrin while still exhibiting improved drug solubility and absorption. Preferred pharmaceutical formulations provided by the present invention include non-alcoholic syrups and rapidly dissolving tablets, capsules, effervescent tablets and/or sachets. The latter two solid preparations can be converted in situ to solutions, thus providing a means for alleviating swallowing problems in geriatric and pediatric patients.

In accord with the present invention, it has now been discovered that use of a hydroxy-carboxylic or polycarboxylic acid and/or a salt thereof as defined hereinabove together with α, β or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof results in a dramatic increase in the solubility of drugs which are insoluble or sparingly soluble in water, enhancing the solubilizing and/or complexing ability of the cyclodextrins for the drug through the presence of the selected polyfunctional acid component. Advantageously, the acid, cyclodextrin and drug can be combined with each other in the process of preparing the pharmaceutical composition/formulation, so that the complexing/solubilizing takes place in the course of preparing the final dosage form and need not be carried out separately beforehand. Indeed, in the case of effervescent tablets, sachets and the like, the actual complexation/solubilization will typically occur when the tablet or similar solid dosage form is dissolved in water shortly before administration to the patient.

As noted hereinabove, in preparing the formulations of the invention, the weight ratio of cyclodextrin to selected carboxylic acid and/or salt of said acid is from about 1:100 to about 100: 1. More preferably, the weight ratio of cyclodextrin to acid ingredient is from about 1:50 to 50: 1,most preferably from about 1:5 to about 5:1.

Typical compositions prepared in accord with the present invention comprise the selected cyclodextrin at a weight percent of from about 0.05 to about 8% of the total weight and the selected hydroxy-carboxylic or polycarboxylic acid and/or salt thereof at a concentration ranging from about 0.1 to about 5% total weight. However, in selected solid dosage forms, considerably greater amounts of cyclodextrin and polyfunctional acid are typically used, for example from about 15 to about 30% by weight of cyclodextrin and from about 5 to 10% by weight of the acid as solubilization enhancer. Greater amounts of the acid/salt can be present in such formulations if that ingredient is serving another purpose in the composition, for example, as an effervescing agent.

In preferred embodiments, the present invention provides non-alcoholic aqueous syrups of water-insoluble or sparingly insoluble drugs, which syrups are advantageous over suspensions and/or hydroalcoholic solutions of such drugs; and rapidly dissolving solid dosage forms which can be quickly and conveniently converted in situ to aqueous solutions which can be readily administered to patients who have difficulty in swallowing, such as pediatric and geriatric patients. The pharmaceutical preparations prepared in accord with this invention are stable and non-irritating, have very low systemic or local toxicity and are generally well-suited for oral and parenteral administration, depending of course upon the properties of the selected ingredients, particularly the cyclodextrin and the drug. Formulations for oral use can be enhanced by the addition of a taste-masking agent, flavoring agent and/or sweetener. Other ingredients commonly used in oral dosage forms can also be present. In the case of parenteral formulations, the ingredients will be in an aqueous solution suitable for injection, i.e., sterile and pyrogen free and prepared in accord with accepted pharmaceutical procedure. Again, other ingredients typically used in such formulations can be present.

While not wishing to be bound by any particular theory of operation, applicants believe that the acids which are selected for use herein form aggregates by virtue of the fact that they possess both polar and non-polar groups, that these aggregates combine with the selected cyclodextrin to enhance solubilization/complexation of the drug, and that the selected acid needs to be present in sufficient quantity to form aggregates in order for enhancement of solubility to occur. Thus it appears that the acid participates directly in the drug complex formation, acting as a co-complexing agent with the cyclodextrin and resulting in a new three-way co-complex of drug, cyclodextrin and acid.

Pharmaceutical compositions prepared in accord with the present invention can be used to treat a variety of conditions, depending upon the pharmacological nature of the drug selected for administration. The compositions comprise a pharmacologically/therapeutically effective amount of the selected drug and the amount/ratios of selected cyclodextrin and selected polyfunctional acid or salt thereof described hereinabove. For example, if the selected drug is an antihistaminic or antiallergic, for example, terfenadine, a pharmacologically effective amount thereof will be an amount sufficient to provoke an antihistaminic or antiallergic response (e.g., in the treatment of symptoms associated with seasonal allergic rhinitis, such as sneezing, lacrimation, pruritus and rhinorrhea). As a further example, when the selected drug is an anti-inflammatory agent, for example ketoprofen, a pharmacologically effective amount thereof will be an amount sufficient to elicit an anti-inflammatory response (such as in the treatment of rheumatoid arthritis or osteoarthritis), or an amount sufficient to elicit an analgesic effect (since ketoprofen and other non-steroidal anti-inflammatory agents are frequently used for their analgesic as well as their anti-inflammatory properties). In short, the instant compositions are typically used for those purposes for which the drug component of the composition is itself known to be useful.

Generally speaking, the therapeutic dosage ranges for administration of drugs in the pharmaceutical compositions/formulations described herein will be the same as or less than those characteristically used for administration of the drug per se. Naturally, such therapeutic dosage ranges will vary with the size and species of the patient, the condition for which the formulation is administered, the route of administration employed and the like. The quantity of given dosage form needed to deliver the desired dose of active ingredients will of course depend upon the concentration of the drug in the pharmaceutical formulation.

In order to further illustrate the present invention and the advantages thereof, the following specific examples are

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given, it being understood that some are intended only as illustrative and in no way limitative of the invention.

The terfenadine stability testing referred to in Examples 2 and 4 hereinbelow was based on the assay method described in a terfenadine monograph by A. Badwan et al appearing in *Analytical Profiles of Drug Substances*, volume 19, ed. K. Florey, Academic Press, Inc., San Diego, Calif., 1990, pp. 627–662. The actual assay method was as follows:

A quantity of the mixed contents of 20 capsules containing the equivalent of 60 mg terfenadine was transferred to a 100 ml volumetric flask. 70 ml of methanol were added and the mixture was shaken for 20 minutes, then methanol was added to volume, and the contents of the flask were mixed and centrifuged. The first derivative ultraviolet absorption spectra of the solutions (1) and (2) using methanol as the blank and a path length of 1 cm in the range of 300–200 nm at a speed of 120 nm/min and $\lambda$=2.0 were recorded. Solution (1) was the sample solution prepared. Solution (2) was a standard solution of terfenadine in the same medium having a known concentration of about 600 μg/ml. The amplitude obtained at about 270 nm was measured for sample and standard solutions. The quantity, in mg of ($C_{32}H_{41}NO_2$), in the portion of capsules content was calculated according to the formula:

$$\frac{D_s}{D_{st}} \times 100\,C$$

wherein C is the concentration, in μg per ml, of terfenadine in the standard solution, and $D_s$ and $D_{st}$ are the first derivative amplitude of the solution of sample and standard, respectively.

## EXAMPLE 1

Working models showing the effectiveness of the invention in increasing the solubility of representative water-insoluble or sparingly soluble drugs are shown in TABLE I below. The solubilities (in mg/ml) of the drugs were investigated at 30° C., in water; in water containing 2% β-cyclodextrin; and in aqueous solution of 2% selected cyclodextrin and 1% selected polyfunctional acid or corresponding salt.

## EXAMPLE 2

### Preparation of Syrups

Syrups prepared in accord with the present invention typically contain water-insoluble or sparingly soluble drugs in an amount of from about 6 to about 600 mg/ml of syrup. The syrups generally contain the solubilizing agent, i.e., α,β, γ cyclodextrin or derivative thereof, in an amount of from about 0.1 to about 5% of the syrup and the solubilization augmenting agent, i.e., the hydroxy-carboxylic acid or polycarboxylic acid, in an amount of from about 0.1 to about 5% of the syrup. The syrups are preferably buffered to control the pH value to about pH 3 to 7, more preferably pH 3.5 to 5. The preferred buffering system is a combination of the selected acid and its corresponding salt in an amount of from about 0.1 to about 5% for the acid and a similar range for the corresponding salt. The syrups typically contain sufficient viscosity imparting agents (such as polyvinylpyrrolidone) in an amount of from about 1 to about 3% of the syrup and medium-building agents such as glycerine, propylene glycol or sorbitol in an amount of from about 10 to about 35% of the syrup. These additives are included to render the medium more viscous, thus preventing the solution from spilling over when administered by a spoon or the like. To mask the undesirable taste of the drug, the syrups generally contain sufficient sweetening agents such as sucrose in an amount of from about 20 to about 30% of the syrup. Other sweetening agents such as saccharin sodium could be used in an amount of from about 0.1 to about 0.2% of the syrup.

Additional ingredients such as coloring or flavoring agents can be incorporated to enhance the quality of the syrup, if desired. Water is present as the main vehicular agent in the syrups and is added to the desired volume.

In any event the weight ratio of cyclodextrin to acid in syrup formulations is as broadly described hereinabove, but preferably is from about 1:5 to 5:1, and more preferably from about 3:1 to about 1:1.

TABLE I

Solubilities (mg/ml) of Drugs in Water, in Water Containing 2% β-Cyclodextrin (CD), and in Aqueous Solution of 2% Cyclodextrin, 1% Hydroxy-Carboxylic or Polycarboxylic Acid or Corresponding Salt, at 30° C.

| | | 2% aq. BETA-CYCLODEXTRIN + | | | | ALPHA-CYCLODEXTRIN, + | | | | GAMMA-CYCLODEXTRIN + | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BETA CD soln. | Citric acid | Gluconic acid | Lactic acid | Tartaric acid | Citric acid | Gluconic acid | Lactic acid | Tartaric acid | Citric acid | Gluconic acid | Lactic acid | Tartaric acid |
| DRUG | Water | | | | | | | | | | | | | |
| Terfenadine | <0.01 | 0.01 | 5.68 | 5.49 | 8.87 | 5.21 | 2.15 | 7.17 | 6.27 | 1.91 | 2.20 | 6.08 | 7.24 | 1.22 |
| Ketoprofen | <0.01 | 0.69 | 2.85* | 3.00* | 1.65* | 1.36* | | | | | | | | |
| Cinnarizine | 0.008 | 0.015 | 0.55 | 2.07 | 11.4 | 4.38 | | | | | | | | |
| Ketoprofen | 0.025 | 1.64 | 7.06* | | | | | | | | | | | |
| Fluconazole | Insoluble | 0.072 | 0.78* | | | | | | | | | | | |

(*) Solubility in aqueous solution containing 2% β-cyclodextrin and 1% titanium salt.

| Part A: Terfenadine Syrup (5 ml/ml) | |
|---|---|
| Ingredient | Concentration (mg/ml) |
| Terfenadine | 6 |
| Propylene glycol | 250 |
| Glycerin | 100 |
| Sucrose | 500 |
| Citric acid, 1 $H_2O$ (monohydrated) | 10 |
| Trisodium citrate, 2 $H_2O$ (dihydrated) | 10 |
| β-Cyclodextrin | 50 |
| Sodium saccharin | 1 |
| Polyvinylpyrrolidone (K30) | 20 |
| Water | q.s. |

Method of Preparation for 100 ml of Syrup

1. Dissolve citric acid and trisodium citrate in 10 ml of deionized water.
2. Dissolve sucrose and β-cyclodextrin in 10 ml of deionized water.
3. Dissolve polyvinylpyrrolidone and sodium saccharin in 10 ml of deionized water.
4. Mix terfenadine, propylene glycol and glycerin, then, while stirring and heating, add the solutions prepared in steps 1–3. Continue mixing until the drug is dissolved.
5. Add water to the desired volume, then mix for 20 minutes.

A syrup made as described above was tested for stability at room temperature (RT) and at the accelerated temperatures of 55° and 65° C., with the following results.

| | 65° C. | | 55° C. | | RT | |
|---|---|---|---|---|---|---|
| Storage Time (days) | Assay (%) | pH | Assay (%) | pH | Assay (%) | pH |
| 0 | 99.1 | 4.5 | 99.1 | 4.5 | 99.1 | 4.6 |
| 11 | 99.4 | 4.6 | 99.4 | 4.5 | — | — |
| 27 | 103.0 | 4.5 | 103.5 | 4.5 | — | — |
| 55 | 101.0 | — | — | — | — | — |
| 89 | — | — | — | — | 101.0 | 4.6 |
| 360 | — | — | — | — | 100.0 | 4.6 |

Other examples of syrups made in accord with the present invention are demonstrated by the ketoprofen syrup and cisapride syrup, as described below.

| Ingredient | Concentration (mg/ml) |
|---|---|
| Part B: Ketoprofen Syrup (10 mg/ml) | |
| Ketoprofen | 10 |
| Propylene glycol | 250 |
| Glycerin | 100 |
| Sucrose | 500 |
| Trisodium citrate | 15 |
| β-Cyclodextrin | 20 |
| Polyvinylpyrrolidone (K30) | 20 |
| Water | q.s. |
| Part C: Cisapride Syrup (1 mg/ml) | |
| Cisapride | 1 |
| Propylene glycol | 500 |
| Tartaric acid | 10 |
| β-Cyclodextrin | 20 |
| Sorbitol 70% | 500 |
| Sodium saccharin | 1 |
| Polyvinylpyrrolidone (K30) | 2 |
| Water | q.s. |

Syrups made in accord with the present invention have an advantage over other possible liquid formulations in being devoid of alcohol, an essential ingredient used to increase solubility of water-insoluble drugs. They offer a wider range of concentrations of the water-insoluble drugs compared to other liquid formulations. Consequently, they provide a means for better control of dose administration and greater absorption of said drugs.

## EXAMPLE 3

### Preparation of Effervescent Tablets

Effervescent tablets prepared in accord with the present invention typically contain water-insoluble or sparingly soluble drugs in an amount of from about 10 to about 800 mg per tablet. The tablets generally contain the solubilizing agent α, β, γ-cyclodextrin or derivative thereof in an amount of from about 15 to about 30% of the tablet and the solubilization augmenting agent, preferably citric or tartaric acid, in an amount of from about 5 to about 10% of the tablet. An additional amount of citric acid, from about 15 to about 35% of the tablet, is typically added to the formula to produce the desired effervescent action. The tablets generally contain sodium bicarbonate in an amount of from about 5 to about 30% of the tablet to react with part of the acid and produce the effervescent action. Other additives can be added to the formula to give body to the tablet and help bind the ingredients; e.g., soluble starch or Aerosil™ in an amount of from about 25 to about 125 mg of the tablet. Other additives such as ethyl alcohol or 5% Tween™80 solutions can be added, if desired, in sufficient quantities to granulate the powders. The formula can also contain other ingredients such as a sweetener or a taste-masking agent to improve taste and a soluble lubricating agent such as sodium benzoate to facilitate tablet compression. In any case, the weight ratio of cyclodextrin to acid in effervescent tablets is as broadly described hereinabove, but preferably is from about 1:5 to 5:1, and more preferably from about 1:4 to about 1:1. An illustrative formulation is shown below.

| Part A: Terfenadine Effervescent Tablet (83 mg) | |
|---|---|
| Ingredient | Amount (g) |
| Terfenadine | 0.06 |
| Sodium bicarbonate | 0.75 |
| β-Cyclodextrin | 1.25 |
| Saccharin sodium | 0.05 |
| Sucrose | 1.00 |
| Citric acid, anhydrous | 2.00 |
| Sodium benzoate | 0.15 |
| Flavoring agent | q.s. |

Method of Preparation of Effervescent Tablets

1. Mix β-cyclodextrin with sodium bicarbonate.
2. Mix powdered sugar, saccharin sodium and mixture from Step 1 with terfenadine in a suitable mixer.
3. Granulate resultant mix with ethyl alcohol.
4. Sieve and dry resulting granules.
5. Mix granules with citric acid, sodium benzoate and flavoring agent.
6. Compress into tablets.

Effervescent tablets made as described above, when tested in 100 ml of water, achieved complete dissolution, with effervescence, in 2–4 minutes. Terfenadine assayed at 95–97% of the nominal amount.

Another example of effervescent tablets prepared in accord with the present invention is demonstrated in the following example.

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Part B: Cisapride Effervescent Tablets (10 mg)

| Ingredient | Amount (mg) |
|---|---|
| Cisapride (anhydrous) | 10 |
| Citric acid, anhydrous | 65 |
| Tartaric acid | 250 |
| Sodium bicarbonate | 200 |
| Aerosil™ (silica aerogel) | 25 |
| Sodium benzoate | 20 |
| Soluble starch | 25 |
| β-Cyclodextrin | 30 |
| Flavoring agent | q.s. |

Method of Preparation of Cisapride Effervescent Tablets

1. Mix β-cyclodextrin with sodium bicarbonate, soluble starch and cisapride.
2. Granulate the mixture with ethyl alcohol.
3. Sieve and dry the resulting granules.
4. Mix granules with citric acid, tartaric acid, sodium benzoate, the flavoring agent and Aerosil™.
5. Compress into tablets.

The effervescent tablets, when tested in 100 ml of water, dissolved with effervescence in 2-3 minutes, forming a solution containing 10 mg of the drug and of pH=4.

Effervescent tablets made in accord with the present invention provide a means for preparing solutions of water-insoluble drugs in situ. Such solutions are readily absorbed and are well-suited for geriatric and pediatric patients.

## EXAMPLE 4

### Preparation of Fast-Dissolving Capsules

Rapidly dissolving capsules prepared in accordance with the present invention typically contain water-insoluble or sparingly soluble drugs in an amount of from about 6 to about 600 mg of the capsule. The capsules typically contain the solubilizing agent α, β or γ,-cyclodextrin or derivative thereof in an amount of from about 0.1 to about 5% of the capsule and the solubilization augmenting hydroxy-carboxylic or polycarboxylic acid in an amount of from about 0.1 to about 5% of the capsule. The formula generally contains additives to give body to the granules, e.g., calcium hydrogen phosphate, and Avicel™ in amounts of from about 25 to about 30% and about 5 to about 10% of the capsule, respectively. Binding agents and/or wetting agents such as PEG 4000, Tween™80, sodium lauryl sulfate and PVP to granulate the powder mix are typically added to the formula in an amount of from about 0.1 to about 5% of the capsule. The formula typically contains disintegrants and swelling agents such as Primojel™, maize starch, and Aerosil™ in an amount of from about 1 to about 10%. The formula can contain other ingredients, such as a sweetener. In any event, the weight ratio of cyclodextrin to acid in rapidly dissolving capsule formulations will be as broadly described hereinabove, but preferably will be from about 1:5 to 5:1, and more preferably from about 3:1 to about 1:1. The following illustrates such a formulation.

Terfenadine Capsule (60 mg)

| Ingredient | Amount (mg) |
|---|---|
| Terfenadine | 60 |
| Polyethylene glycol 4000 | 24 |
| Tween™ 80 | 3 |

-continued

Terfenadine Capsule (60 mg)

| Ingredient | Amount (mg) |
|---|---|
| Calcium hydrogen phosphate | 28 |
| Maize starch | 4 |
| Sodium lauryl sulfate | 1 |
| Avicel™ pH 101 (microcrystalline cellulose) | 72 |
| β-cyclodextrin | 11 |
| Citric acid, anhydrous | 30 |
| Polyvinylpyrrolidone (K30) | 12 |
| Primojel™ | 10 |
| Aerosil™ (silica aerogel) | 8 |

Method of Preparation of Fast-Dissolving Terfenadine Capsules

1. Mix calcium hydrogen phosphate, maize starch, sodium lauryl sulfate and Avicel™ with hot alcoholic solution of terfenadine, PEG and Tween™80. Maintain mixing until powder is dry. Pass through 0.8 mm sieve.
2. Granulate with 20 ml aqueous solution containing PVP and citric acid. Pass granules through 0.8 mm sieves, then dry at 40° C.
3. Add Primojel™, Aerosil™ and cyclodextrin to granules and mix for 10 minutes.
4. Fill capsules with granules.

Capsules made as described above were tested for dissolution and chemical stability with the following results.

| Storage Time (weeks) | 65° C. Assay (%) | 44° C. Assay (%) | RT Assay (%) |
|---|---|---|---|
| 0 | 99.6 | 99.6 | 99.6 |
| 1 | 98.8 | — | — |
| 2 | 98.5 | — | — |
| 3 | 98.6 | — | — |
| 4 | 97.8 | 100.2 | — |
| 6 | 98.2 | — | — |
| 12 | — | 99.3 | 99.2 |
| 24 | — | 99.0 | 99.4 |

A comparison of the dissolution profile of terfenadine from capsules prepared as in the forgoing example (Δ) and from identical controls devoid of cyclodextrin and citric acid (○), or devoid of citric acid and containing the same (*) or greater (□) amounts of cyclodextrin, is shown in the FIGURE. As is readily apparent from the FIGURE, the control containing 11 mg of β-cyclodextrin (*) had greatly improved dissolution over the control containing no cyclodextrin (○). However, increasing the amount of cyclodextrin in the capsule from 11 mg to 30 mg of β-cyclodextrin (□) did not further improve dissolution to a significant extent. In contrast, the capsule prepared in accord with the present invention containing 11 mg of β-cyclodextrin and 30 mg of citric acid (Δ) substantially improved dissolution of terfenadine over all of the control formulations.

Capsules made in accordance with the invention ensure complete dissolution of the required dose of the water-insoluble drug within an acceptable time span. Therefore, such capsules provide a means to ensure better absorption of the drugs and hence, better bioavailability than comparable dosage forms.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions and changes may be made without departing from the spirit thereof. Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims, including equivalents thereof.

What is claimed is:

1. A method for enhancing the solubilization of a drug which is insoluble or sparingly soluble in water with cyclodextrin, said method comprising combining in water said drug with cyclodextrin and an effective solubility-enhancing amount of saturated or unsaturated $C_2$–$C_6$ carboxylic acid having from one to three —COOH groups and bearing from zero to four —OH substituents, provided that when the acid has only one —COOH group, then said acid must bear at least one —OH substituent, or a pharmaceutically acceptable salt of said acid, the weight ratio of cyclodextrin to carboxylic acid being from about 1:50 to about 50:1.

2. The method according to claim 1, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

3. The method according to claim 2, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

4. The method according to claim 2, wherein the cyclodextrin comprises β-cyclodextrin.

5. The method according to claim 1, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

6. The method according to claim 5, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

7. The method according to claim 1, wherein the drug is a non-steroidal anti-inflammatory agent.

8. The method according to claim 7, wherein the drug is ibuprofen, ketoprofen, piroxicam, tenoxicam, ketorolac or naproxen.

9. The method according to claim 1, wherein the drug is an antihistaminic or antiallergic.

10. The method according to claim 9, wherein the drug is terfenadine or cinnarizine.

11. The method according to claim 1, wherein the drug is a vasodilator, oxytocic agent or abortifacient.

12. The method according to claim 11, wherein the drug is prostaglandin $E_1$, prostaglandin $E_2$ or prostaglandin $F_2$.

13. The method according to claim 1, wherein the drug is a sedative, tranquilizer, hypnotic, anticonvulsant, anxiolytic, muscle relaxant or antispasmodic.

14. The method according to claim 13, wherein the drug is phenobarbital, pentobarbital, secobarbital, butabarbital, butalbital, sulpiride, phenytoin, diazepam, oxazepam, etomidate, bromazepam, clobazam, nitrazepam, fludiazepam, lorazepam, medazepam, dimethyl diazepam, flunitrazepam, nimetazepam, pentazocine, methaqualone, salbutamol, carbamazepine, baclofen, chlorpromazine or chlordiazepoxide.

15. The method according to claim 1, wherein the drug is terfenadine, ketoprofen, cisapride or ibuprofen, the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

16. The method of claim 1 wherein the drug is terfenadine.

17. The method according to claim 16, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

18. The method according to claim 17, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

19. The method according to claim 17, wherein the cyclodextrin comprises β-cyclodextrin.

20. The method according to claim 16, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

21. The method according to claim 20, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

22. The method according to claim 16, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

23. The method of claim 1 wherein the drug is ibuprofen.

24. The method according to claim 23, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

25. The method according to claim 24, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

26. The method according to claim 24, wherein the cyclodextrin comprises β-cyclodextrin.

27. The method according to claim 23, wherein the $C_2$–$C_6$ carboxylic acid, comprises citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

28. The method according to claim 27, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

29. The method according to claim 23, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

30. The method of claim 1 wherein the drug is cisapride.

31. The method according to claim 30, wherein the cyclodextrin comprises α- β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

32. The method according to claim 31, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-or β- or γ-cyclodextrin.

33. The method according to claim 31, wherein the cyclodextrin comprises β-cyclodextrin.

34. The method according to claim 30, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

35. The method according to claim 34, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

36. The method according to claim 30, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartaric acid or a combination of citric acid and trisodium citrate.

37. The method of claim 1 wherein the drug is piroxicam.

38. The method according to claim 37, wherein the cyclodextrin comprises α-, β- or γ-cyclodextrin or a pharmaceutically acceptable derivative thereof.

39. The method according to claim 38, wherein the cyclodextrin derivative comprises a hydroxyalkyl, carboxamide, diethylaminoethyl, carboxymethyl or dihydroxyalkyl derivative of α-, β- or γ-cyclodextrin.

40. The method according to claim 38, wherein the cyclodextrin comprises β-cyclodextrin.

41. The method according to claim 37, wherein the $C_2$–$C_6$ carboxylic acid comprises citric acid, tartaric acid, glutaric acid, lactic acid, ascorbic acid, glycolic acid, mevalonic acid, malic acid, tartronic acid, maleic acid, fumaric acid, malonic acid or succinic acid, or a pharmaceutically acceptable salt thereof.

42. The method according to claim 41, wherein the $C_2$–$C_6$ carboxylic acid or salt is citric acid, trisodium citrate, tartartic acid or a combination of citric acid and trisodium citrate.

43. The method according to claim 37, wherein the cyclodextrin is β-cyclodextrin and the $C_2$–$C_6$ carboxylic acid or salt is trisodium citrate.

* * * * *

# United States Patent [19]

**Matsuo et al.**

[11] **Patent Number: 4,656,158**

[45] **Date of Patent: Apr. 7, 1987**


[54] **PEPTIDE, AND PRODUCTION AND USE THEREOF**


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[21] Appl. No.: **706,382**

[22] Filed: **Feb. 27, 1985**

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[51] Int. Cl.[4] ..... A61K 37/02; C07K 7/10; C07K 3/24

[52] U.S. Cl. ..... 514/12; 530/324; 530/344

[58] Field of Search ..... 260/112.5 R; 514/12; 530/324, 344



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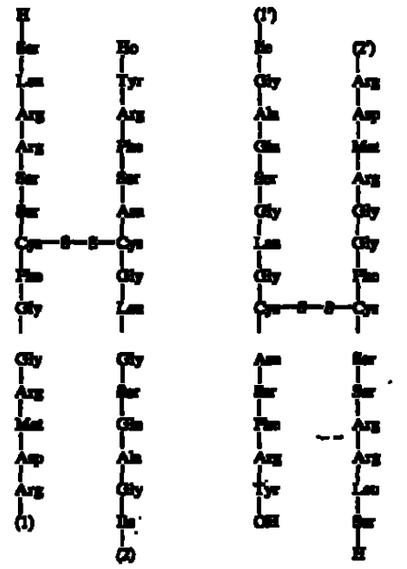
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[57] **ABSTRACT**

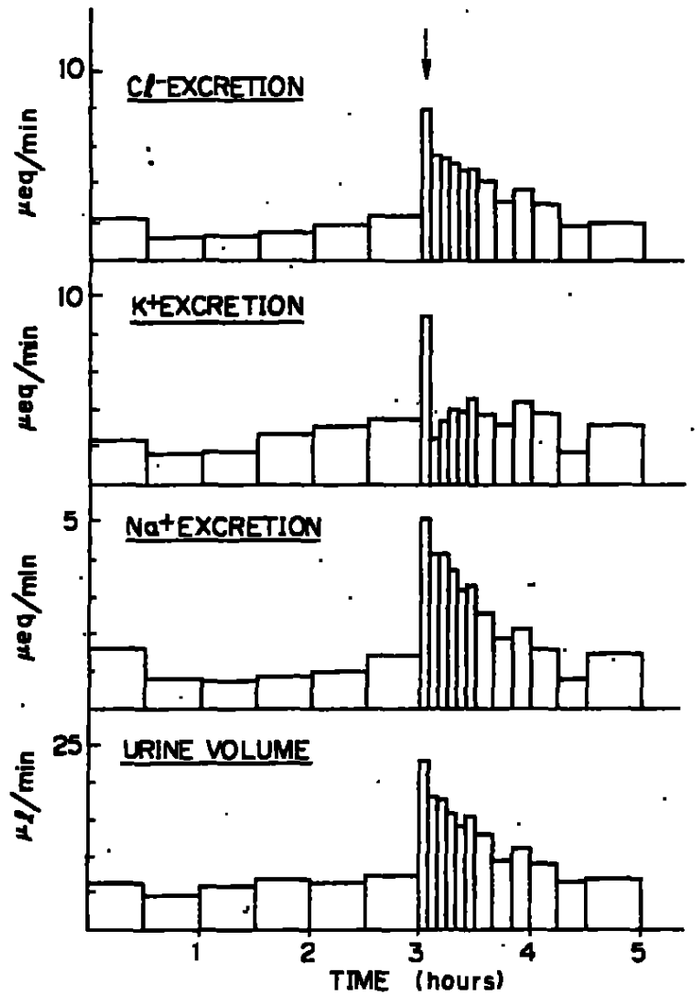
There are disclosed a new peptide β-hANP of the following structure:



and acid addition salt thereof; a diuretic composition and a hypotensor composition containing a β-hANP or an acid addition salt thereof; and processes for the production thereof.


**17 Claims, 2 Drawing Figures**

Fig. 1


10
Cl⁻EXCRETION
μeq/min
10
K⁺EXCRETION
μeq/min
5
Na⁺EXCRETION
μeq/min
25
URINE VOLUME
μl/min
1
2
3
4
5
TIME (hours)

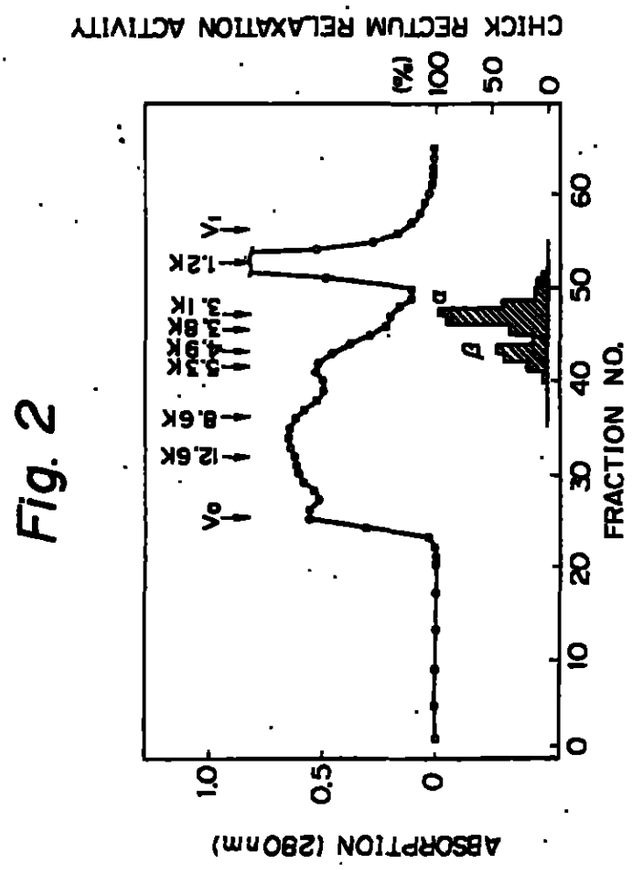

CHICK RECTUM RELAXATION ACTIVITY
(%)
100
50
0
60
50
40
30
20
10
0
FRACTION NO.
$V_t$
1.2K
3.1K
8K
9K
8.6K
12.6K
$V_o$
α
β
1.0
0.5
0
ABSORPTION (280nm)

Fig. 2

# PEPTIDE, AND PRODUCTION AND USE THEREOF

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a novel peptide, a process for the production thereof, and a pharmaceutical composition containing the novel peptide as a diuretic or hypotensor.

2. Description of the Related Art

A normal regulation of the blood pressure in a human body is important for the maintenance of personal health, and various physical and humoral factors contribute to this regulation of the blood pressure. The physical factors include, for example, output, and the elasticity of the walls of blood vessels, etc. The humoral factors include, for example, the renin-angiotensin-aldosterone system, catecholamines, prostaglandins, kinin-kallikrein system, and natriuretic hormones including ouabain-like substances. Herein the term "natriuretic" will denote selective excretion of sodium cation relating to potassium cation.

Granules morphologically similar to granules present in peptide hormone-producing cells are found in human atrium (J. D. Jamieson and G. E. Palade, *J. Cell Biol.*, 23, 151, 1964). A homogenate of rat atrium and granules contained therein are known to show natriuretic action in rats (A. J. DeBold et. al., *Life Science*, 28, 89, 1981; R. Kesler, *Can. J. Physiol. Pharmacol.*, 60, 1078, 1982). Recently Mark G. Currie et. al. suggested peptide-like substances with a molecular weight of 20,000 to 30,000 or not more than 10,000, present in atrium of humans, rabbits, swine, and rats, and having natriuretic action (*Science*, 221, 71–73, 1983).

Moreover, a peptide consisting of 28 amino acids derived from rat atrium cardis was identified (*Biochem. Biophys. Res. Commun.*, Vol. 117, No. 3, p859–865, 1983). The present inventors found a new peptide consisting of 28 amino acids from human atrium cardis; referred to as "α-human atrial natriuretic polypeptide" and abbreviated as "α-hANP" (*Biochem. Biophys. Res. Commun.*) Vol. 118, No. 1, p131–139, 1984).

## SUMMARY OF THE INVENTION

The present invention provides a new peptide having natriuretic action and hypotensive or antihypertensive action. The peptide according to the present invention is hereinafter referred to as "β-human atrial natriuretic polypeptide" and abbreviated as "β-hANP".

There is also provided a process for production of the peptide.

Another object of the present invention is to provide a pharmaceutical composition containing the peptide as a diuretic or hypotensor.

Other objects and advantages of the present invention will be apparent from the description set forth hereinbelow.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 contains graphs showing the diuretic and natriuretic action of the β-hANP.

FIG. 2 is a chromatogram showing an elution profile wherein β-component is separated from α-component during isolation of β-hANP from human atrium cardis.

## DESCRIPTION OF THE PREFERRED EMBODIMENT

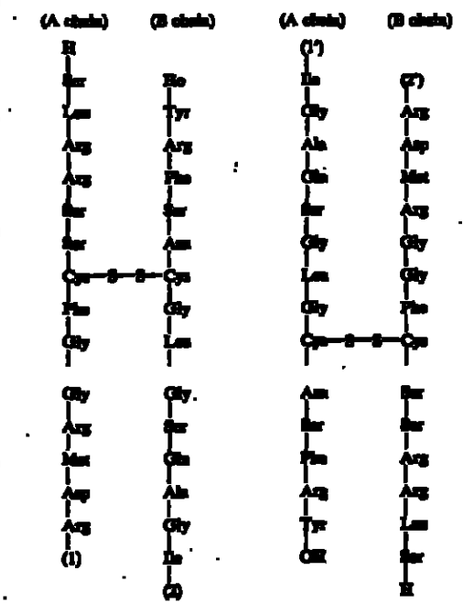
At present, furosemide as a natriuretic agent is used for the treatment of essential hypertension. However, the structure of the furosemide is different to that of the new peptide according to the present invention.

The present inventors isolated a peptide, in substantially pure form, consisting of 36 amino acid residues and having a molecular weight of about 6,000, determined the structure of the peptide and found that the peptide showed notable natriuretic and hypotensive action.

### Structure and Physico-chemical Properties of the β-hANP

(1) Structure

The β-hANP has the structure:




wherein (1) and (1'), and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— ‖ L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end.

(2) Molecular weight: about 6,000 as determined by gel-filtration (6160.78 as calculated).

(3) UV spectrum: Max=275 mμ.

(4) Color reactions: Ehrlich's reaction; negative; Sakaguchi's reaction, positive; Pauly's reaction, positive.

(5) Distinction of basic, acidic, or neutral property: basic.

(6) Solubility in solvents: soluble in water, partially in methanol, and acetic acid; insoluble in ethyl acetate, butyl acetate, ethyl ether, hexane, petroleum ether, benzene, and chloroform.

(7) Amino acid composition by amino acid analysis:

| Amino acid | β-hANP Found | β-hANP Calculated | RCM β-hANP* Found | RCM β-hANP* Calculated |
|---|---|---|---|---|
| Asp + Asn | 4.14 | 4 | 2.08 | 2 |
| Ala | 2.06 | 2 | 1.05 | 1 |
| Arg | 9.90 | 10 | 5.02 | 5 |
| Ile | 1.94 | 2 | 0.97 | 1 |
| Gly | 10.00 | 10 | 5.05 | 5 |
| Glu(Gln) | 2.06 | 2 | 1.01 | 1 |
| (Cys)h | | 2 | 0.99 | 1 |
| Ser | 10.18 | 10 | 5.17 | 5 |
| Tyr | 1.92 | 2 | 1.00 | 1 |
| Phe | 4.16 | 4 | 2.09 | 2 |
| Met | 2.18 | 2 | 0.97 | 1 |
| Leu | 3.98 | 4 | 2.06 | 2 |

(8) Determination of the structure

A product obtained after reduction of β-hANP with dithiothreitol to cleave a disulfide bond of the β-hANP and subsequent carboxymethylation of the cleft product, and that obtained from α-hANP by the same procedure provided an identical elution pattern showing a single peak in a liquid chromatography. These carboxymethylated products were digested with trypsin, and each digestion product was subjected to a liquid chromatography. In the chromatography, the digestion products from α-hANP and β-hANP provided an identical elution pattern, i.e., an identical combination of the digestion fragments. Moreover, a molecular weight of β-hANP was about two times that of α-hANP. These facts show that the β-hANP is a dimer of the α-hANP wherein the two α-hANP molecules are bonded through two disulfide bonds.

β-hANP and α-hANP were digested with trypsin, and the digestion products were analyzed by liquid chromatography for the fragment composition. A comparison of the fragment compositions from β-hANP and α-hANP showed that, in the β-hANP, two α-hANP molecules which are directed in reverse to each other are bonded through two disulfide bonds.

(9) Formation of salts: the β-hANP is a basic compound as described in item (5), and can form acid addition salts with an inorganic acid such as hydrochloric acid, sulfuric acid, phosphoric acid, or an organic acid such as formic acid, acetic acid, propionic acid, succinic acid, and citric acid.

## Physiological Properties of β-hANP

The β-hANP according to the present invention has notable diuretic, natriuretic, and hypotensive or antihypertensive actions.

Test method:

Male rats weighing 300 to 400 grams were anesthetized by intraperitoneal administration of pentobarbital at a dosage of 60 mg/kg, and used for tests of the β-hANP according to the method described in *Life Science*, Vol. 28, pp89–94.

To keep the respiratory tract open, a tracheal cannula (PE240 Clay-Adams) was inserted into the trachea. An arterial cannula (PE-50) was inserted into a femoral artery for measurement of the blood pressure, and a venous cannula was inserted into a femoral vein for the administration of Ringer's solution. 1.2 ml of Ringer's solution was infused at a flow rate of 1.2 ml/hour.

A bladder cannula made of silastic tube with a inner diameter of 0.02 inches and an outer diameter of 0.037 inches was inserted into the bladder, and via the cannula, a urine sample was collected into a test tube. The collection of urine was carried out for 30 minutes before administration of the test compound, and every five minutes after the administration.

0.8 n mole of the test compound β-hANP was dissolved in 50 μl of sterilized physiological saline with 5 μg of bacitracin, and the solution was injected into the jugular vein.

The results of the test are set forth in FIG. 1. As shown in the figure, β-hANP shows notable diuretic and natriuretic actions. The actions of β-hANP are comparable to those of α-hANP, but are maintained for a period longer than that of α-hANP. 0.8 n mole of β-hANP provides diuretic and natriuretic actions comparable to those provided by 1.21 μ mole of furosemide, which is a known diuretic agent. However, β-hANP brings about the actions more rapidly than furosemide.

## Use of β-hANP as a pharmaceutical product

Repeated administration of β-hANP does not stimulate production of antibodies, and does not cause anaphylaxis shock. β-hANP consisting of L-amino acids is gradually hydrolized in a body providing the L-amino acids, and therefore shows little toxicity.

Due to the higher diuretic, natriuretic, and blood pressure-lowering or antihypertensive actions, and the lower toxicity, β-hANP is useful as an active ingredient for pharmaceutical compositions such as a diuretic and a hypotensor. β-hANP is administered at 0.1 μg/kg to 1 mg/kg, preferably 1 μg/kg to 100 μg/kg.

β-hANP can be administered in the same manner as conventional peptide type pharmaceuticals. Namely, β-hANP is preferably administered parenterally, for example, intravenously, intramuscularly, intraperitoneally, or subcutaneously. β-hANP, when administered orally, may be proteolytically hydrolyzed. Therefore, oral application is not usually effective. However, β-hANP can be administered orally as a formulation wherein β-hANP is not easily hydrolyzed in a digestive tract, such as liposome-microcapsules. β-hANP may be also administered in suppositories, sublingual tablets, or intranasal spray.

The parenterally administered pharmaceutical composition is an aqueous solution containing about 0.000005 to 5%, preferably 0.00005 to 0.5% of β-hANP, which may contain, in addition to β-hANP as an active ingredient, for example, buffers such as phosphate, acetate, etc., osmotic pressure-adjusting agents such as sodium chloride, sucrose, and sorbitol, etc., antioxidative or antioxygenic agents, such as ascorbic acid or tocopherol and preservatives, such as antibiotics. The parenterally administered composition also may be a solution readily usable or in a lyophilized form which is dissolved in sterile water before administration.

## Production of β-hANP

β-hANP can be produced by either the extraction of the β-hANP from human atrium or by chemical synthesis.

In the former process, human atrium is homogenized in an acidic aqueous solution such as a phosphate buffer solution, or an acetic acid solution containing hydrochloric acid. Subsequently, β-hANP is purified accord-

ing to a conventional method suitable for the purification of peptide, such as centrifugation, isoelectric point precipitation, solvent extraction, ultrafiltration, gel filtration, adsorption chromatography or high performance liquid chromatography (HPCL), or a combination of such methods. In the above-mentioned methods, chick rectum relaxation activity is conveniently used to select fractions containing β-hANP, because β-hANP has this activity. In the chromatography methods, the β-hANP containing fractions can be also selected by molecular weight (about 5,000 to 6,000). Chemical synthesis is preferable for the industrial production of β-hANP, in which chemical synthesis, a liquid phase method or solid phase method, or a combination thereof can be used. The solid phase method such as Merrifield's method [R. B. Merrifield, *J. Am. Chem. Soc.* 85, 2184 (1963)] is most convenient.

In Merrifield's method, each amino acid is protected preferably with tert-butyloxycarbonyl (Boc) at the α-amino group; a hydroxyl group in tyrosine is protected preferably with 2,6-dichlorobenzyl group ($Cl_2Bzl$); a guanidino group in arginine is protected preferably with a tosyl group (Tos); a hydroxyl group in serine is protected preferably with a benzyl group (Bzl); a β-carboxyl group in aspartic acid is protected preferably with an O-benzyl group (O-Bzl); and a thiol group in cysteine is protected with an acetoamidomethyl group (Acm). In the Merrifield method, first a protected derivative of C-terminal amino acid L-tyrosin, i.e., Boc-Tyr ($Cl_2Bzl$), is introduced onto a solid phase resin carrier, such as chloromethyl-resin, and subsequently, each suitably protected amino acid is coupled to a terminal amino acid of an intermediate amino acid chain bonded to the resin, in the order of the amino acid sequence of α-hANP. After all the amino acids are coupled in the predetermined order, the protected α-hANP thus obtained is removed from the resin by treatment with hydrogen fluoride, and simultaneously protecting groups other than Acm are also removed. The product is then reduced to obtain Cys $^{7,23}$(Acm)-α-hANP), which is then oxidized with iodine to remove the thiol-protecting group Acm, and simultaneously, to form a disulfide bond. The crude-β-hANP thus obtained is then purified by conventional methods such as gel filtration, reverse phase HPLC, etc., to obtain purified β-hANP.

## EXAMPLES

The present invention will now be further illustrated by, but is by no means limited to, the following examples.

### EXAMPLE 1

Preparation of β-hANP from human atrium cordis

Ten hours after death, 40 g of human atrium cordis was removed and boiled in seven volumes of 1M acetic acid aqueous solution containing 20 mM hydrochloric acid for five minutes, to inactivate proteases present in the atrium cordis. The boiled atrium cordis in the acetic acid solution was then cooled to 4° C., and homogenized with a Polytron homogenizer to extract the β-hANP. The homogenate thus obtained was centrifuged at 12000 XG for 30 minutes to obtain 200 ml of a supernatant. To the supernatant, 12 ml of glacial acetic acid was added to the concentration of 1M acetic acid, and acetone was dropwise added in an amount of 66% of the final concentration to precipitate impurities. The mixture thus obtained was centrifuged to obtain 424 ml of supernatant containing β-hANP, which was then evaporated to dryness. The residue thus obtained was dissolved in 100 ml of 1N acetic acid, and the solution was extracted two times with 50 ml of ethyl ether to defat the solution. The aqueous phase thus obtained was lyophilized, and the lyophilizate was redissolved in 100 ml of 1N acetic acid. The solution was then ultrafiltrated with a UM-2 filter (Amicon) to desalt the solution. The desalted solution was concentrated to 30 ml.

The concentrated solution was applied on SP-Sephadex C-25 column (Pharmacia, 8.0×22 cm). The elution was carried out with 1N acetic acid, 2N pyridine solution, aND 2N pyridine-1N acetic acid solution (pH 5.0), in that order, to obtain fractions SP-I, SP-II, and SP-III. The fraction SP-III was lyophilized to obtain 26.6 mg of lyophilizate, which was then dissolved in 1N acetic acid.

The solution thus obtained was gel-filtrated with Sephadex G-25 column (1.2×103 cm) at a flow rate of 5.4 ml/hour, collecting 2 ml of fractions. Thereby, β fractions (fractions No. 42 to 45) which have chick rectum relaxation activity were obtained. The elution profile is shown in FIG. 2. The β fractions were combined for further purification.

The combined fraction was then subjected to cation exchange HPLC in a TSK-CM2SW column (Toyo Soda, 7.5×300 mm). Elution was carried out by linear gradient with (A) 10 mM ammonium formate (pH 6.6)/acetonitrile (90:10) and (B) 1.0M ammonium formate (pH 6.6)/acetonitrile (90:10), after (A) for 12 minutes, changing the concentration of formate from 10 mM to 0.75M for 140 minutes. A set of fractions (No. 66 and 67, retention time 140 to 142 minutes) with chick rectum relaxation activity was obtained. The active fractions were combined and subjected to reverse phase HPLC in a TSK LS-410-OD8-SIL column (ϕ4.0×250 mm, Toyo Soda). Elution was carried out with (A) water/acetonitrile/10% trifluoroacetic acid (90:10:1) and (B) water/acetonitrile/10% trifluoroacetic acid (40:60:1) as eluents wherein the eluent (A) was used for 15 minutes and then linear gradient from (A) to (B) was used for 80 minutes, at a flow rate of 1.0 ml/min., and pressure of 110 to 130 kg/cm$^2$. A main peak was collected and 11 n mole of substantially pure β-hANP was obtained.

### EXAMPLE 3

Preparation of parenteral composition

(A) Injection solution

| Composition | |
|---|---|
| β-hANP | 2 g |
| sodium chloride | 8 g |
| acetic acid | 2 g |
| sterile water | 1 l |

Method

β-hANP and sodium chloride were dissolved in sterile water, an ampule was filled with 5 ml of the solution, and the ampule was then sealed.

(B) Lyophilizate

| Composition | |
|---|---|
| β-hANP | 2 g |

-continued

| Composition | |
|---|---|
| sorbitol | 20 g |

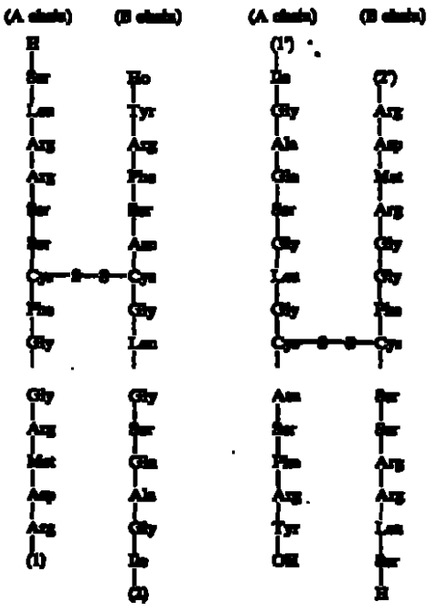
Method

β-hANP and sorbitol were dissolved in 200 ml of sterile water, a vial was filled with 1 ml of the solution, and lyophilized, and the vial was then sealed.

The composition is dissolved in 5 ml of sterile water before parenteral administration.

We claim:

1. A peptide β-hANP having the following formula:




wherein (1) and (1'), are directly bonded and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— ‖ L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end, or an acid addition salt thereof.

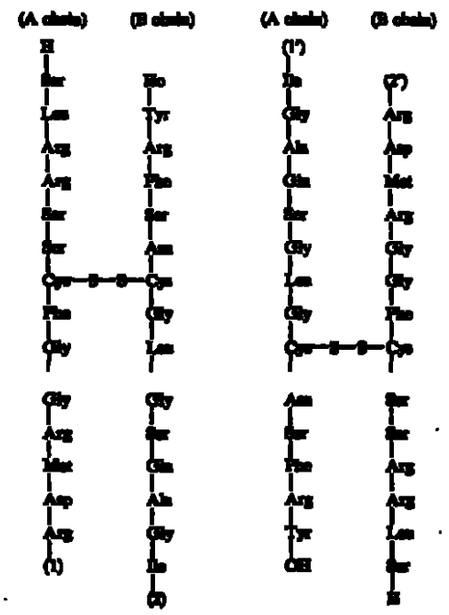
2. A pharmaceutical composition for use as a diuretic or antihypertensive agent comprising a diuretically effective amount or a blood pressure lowering effective amount of a peptide β-hANP according to claim 1 or an acid addition salt thereof with a conventional pharmaceutical additive.

3. The pharmaceutical composition according to claim 2, wherein the composition is a solution for parenteral administration and the conventional pharmaceutical additive is a buffer, an osmotic pressure adjusting agent or a preservative, or a combination thereof.

4. The pharmaceutical composition according to claim 2, wherein the composition is a solution for parenteral administration and contains about 0.000005 to 5% of the β-hANP.

5. The pharmaceutical composition according to claim 2, wherein the composition is in a lyophilized form.

6. A method for promoting diuresis comprising administering a composition comprising a diuretically effective amount of a peptide β-hANP having the following formula:




wherein (1) and (1') are directly bonded, and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— ‖ L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end, or an addition salt thereof, with a conventional pharmaceutical additive.

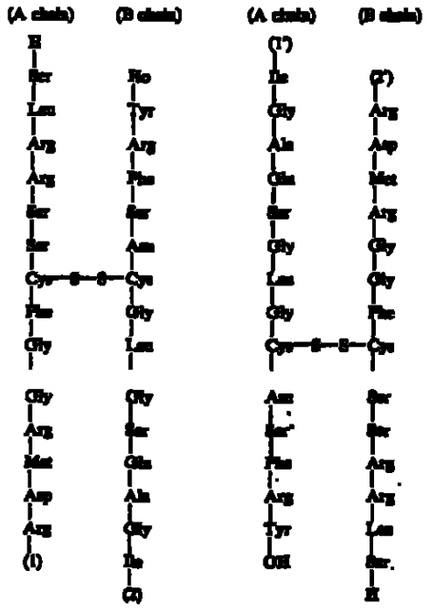
7. The method according to claim 6, wherein the composition comprises about 0.000005 to 5% of the β-hANP.

8. The method according to claim 7, wherein the composition is administered parenterally.

9. The method according to claim 6, wherein the conventional pharmaceutical additive is a buffer, an osmotic pressure adjusting agent, a preservative or a combination thereof.

10. The method according to claim 6, wherein the composition is in lyophilized form.

11. A method for lowering blood pressure comprising administering an antihypertensively effective amount of a peptide β-hANP having the following formula:




wherein (1) and (1') are directly bonded, and (2) and (2') are directly bonded; Asp represents L-aspartic acid, Asn represents L-asparagine, Ala represents L-alanine, Arg represents L-arginine, Ile represents L-isoleucine, Gly represents glycine, Gln represents L-glutamine, Cys represents together with —S— a L-cystine, Ser represents L-serine, Tyr represents L-tyrosine, Phe represents L-phenylalanine, Met represents L-methionine, and Leu represents L-leucine; and the peptide chain A has an amino-terminal at the left end and a carboxy-terminal at the right end, and the peptide chain B has an amino-terminal at the right end and a carboxy-terminal at the left end, or an addition salt thereof, with a conventional pharmaceutical additive.

12. The method according to claim 11, wherein the composition comprises about 0.000005 to 5% of the β-hANP.

13. The method according to claim 12, wherein the composition is administered parenterally.

14. The method according to claim 11, wherein the conventional pharmaceutical additive is a buffer, an osmotic pressure adjusting agent, a preservative or a combination thereof.

15. The method according to claim 11, wherein the composition is in lyophilized form.

16. A process for the production of a peptide β-hANP or an addition salt thereof according to claim 1 which comprises the following steps:

(a) boiling rat atrium in an acidic solution;

(b) homogenizing said rat atrium to obtain a homogenate;

(c) centrifuging the homogenate to obtain a supernatant containing the β-hANP; and

(d) separating said β-hANP from impurities using a purification process comprising organic solvent-precipitation, adsorption chromatography, ultrafiltration, gel filtration or high performance liquid chromatography to obtain said β-hANP in substantially purified form.

17. The process according to claim 16, further comprising the step (e) converting said β-hANP into an acid addition salt thereof or acid addition salt thereof or acid addition salt into free β-hANP.

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Tramite : 002 PATENTES D 1 REGISTRO 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

384

# REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL TRAMITE

SUPERINDUSTRIA Y COMERCIO(COPIA)
Radicacion : 05079319 00000000 Folios: 383
Fecha (AMD): 2005-08-10 17:18:52
Tramite : 011 PCT-PATENT 378 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

PATENTE DE INVENCIÓN ✓ MODELO DE UTILIDAD ( ) ( )

- Indicación de que se solicita la concesión de una patente. ✓ ( )
- Datos de identificación del solicitante o de la persona que se presenta la solicitud. ✓ ( )
- Descripción de la invención. ✓ ( )
- Dibujos de ser estos pertinentes. ✓ ( )
- Comprobante de Pago de las tasas establecidas.

COMPLETA ( ✓ ) INCOMPLETA ( ) ( )

DISEÑO INDUSTRIAL ( )

- Indicación de que se solicita el registro de Diseño Industrial ( ) ( )
- Datos de identificación del solicitante o de la persona que presenta ( ) ( )
- la solicitud.
- Representación gráfica o fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño. ( ) ( )
  Comprobante de pago de las tasas establecidas ( ) ( )

COMPLETA ( ) INCOMPLETA ( ) ( )

ESQUEMA DE TRAZADO ( )

- Indicación de que solicita el registro de un Esquema de trazado. ( ) ( )
- Datos de identificación del solicitante o de la persona que presenta
  La solicitud. ( ) ( )
  Representación gráfica del esquema de trazado ( ) ( )
  Comprobante de pago de las tasas establecidas. ( ) ( )

COMPLETA ( ) INCOMPLETA ( ) ( )

Firma Responsable:

Fecha:

385

**REPUBLICA DE COLOMBIA**

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**Bogotá,**
**2020**

**Doctor(a)**
**RODRÍGUEZ D´ALEMAN DILIA MARÍA**

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|---|---|---|
| **REFERENCIA** | **Radicación No.** | **5 79319** |
| | **Solicitud internacional** | **US2004/000955** |
| | **Fecha inicio fase nacional** | **14/08/2005** |
| | **Trámite** | **11** |
| | **Evento** | **378** |
| | **Actuación** | **430** |
| | **Oficio No.** | **09474** |

8604

**Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:**

**1. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante. (Art. 26-k) Decisión 486.**

**2. La solicitud no contiene el poder debidamente otorgado, con el lleno de los requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente, deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando ésta fuere persona jurídica.**

**En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.**

**Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Unica No.10 de 2001.**

**ALIX CARMENZA CESPEDES DE VERGEL**
**Jefe de la división de Nuevas Creaciones**

**El anterior requerimiento fue notificado por fijación en lista de fecha**
**adpa1**