

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

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ASUNTO Radicación 09-145155
 Trámite 011
 Evento 378
 Actuación 519
 Oficio **E 1 6 7 2 0**
 Folios 008

Patente de Invención ☐ Patente de Modelo de Utilidad ☐
Vía Nacional ☐
Vía PCT (Fase Nacional) ☐ Cap. I ☐ Cap. II ☐

No. Solicitud Internacional PCT/CU2008/000005
Fecha de Presentación Internacional 26 Junio 2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 10 a 26	18/Diciembre/2009
Reivindicaciones:	Folios 27 a 28	18/Diciembre/2009
Dibujos:	Folios 29 a 32	18/Diciembre/2009
Prioridad:	CU 154-2007	29/Junio/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Composición vacunal que comprende un conjugado químico entre el factor de crecimiento epidérmico humano recombinante (hfEGF) y la proteína recombinante P64k" (modificado en el folio 80).

El problema planteado en esta solicitud consiste en la necesidad de suministrar vacunas basadas en el factor de crecimiento epidermal (EGF) para el tratamiento de cáncer, las cuales sean químicamente homogénea, con pureza definida, inmunogenicidad potenciada, actividad clínica incrementada y que permitan disminuir el número de inmunizaciones para su acción terapéutica.

Para resolver este problema técnico la solicitud en estudio proporciona una composición vacunal cuyo principio activo comprende un conjugado proteico hrEGF-

rP64k, caracterizada porque cada molécula de la proteína rP64k tiene enlazada dos moléculas de hrEGF.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 61 K 39/385

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 20 a 23 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque reclama una composición pero sólo define el principio activo de la misma. Se recuerda al solicitante que una composición está definida por sus componentes (Ej. principio activo y excipientes) y proporciones. Se sugiere incluir las reivindicaciones 2 a 5 dentro de la reivindicación 1 de tal forma que se reclamen los demás componentes de la composición en la reivindicación principal o modificar la reivindicación 1 de tal forma que el objeto de la invención se refiera a un compuesto y no a una composición.

Las reivindicaciones 5 y 6 no son claras porque hacen referencia a "Montanide", que es una marca registrada y por tanto no definen al producto porque su composición puede cambiar con el paso del tiempo aunque conserve el mismo nombre. Se sugiere incluir las características estructurales y/o el nombre genérico del producto en cuestión.

Las reivindicaciones 8 a 10 no son claras porque se definen por medio de un efecto o resultado a alcanzar como es el título de anticuerpos anti-EGF. La composición no queda definida por el resultado a alcanzar puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales de la composición que contribuyen a la obtención de ese efecto.

La reivindicación 11 no es clara porque la expresión "un procedimiento adecuado" no es limitativo. Adicionalmente, no se incluyen las condiciones necesarias para llevar a cabo el procedimiento. Se sugiere al solicitante definir el procedimiento por medio de cada una de sus etapas y condiciones y eliminar la expresión mencionada.

Las reivindicaciones 17 y 18 no son claras porque reclaman una composición pero esta se encuentra definida por su proceso de fabricación. Se sugiere al solicitante definir la composición por sus características técnicas esenciales como sus componentes y proporciones.

Descripción (Art 28 D 486)

Dado que esta solicitud se refiere a secuencias de aminoácidos, deben presentarse las listas en forma separada de la descripción, bajo el título: "Listado de secuencias".

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (Junio 29 de 2007) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Gonzales G. et al. Annals of Oncology. 1998;9(4):431-5.	A novel cancer vaccine composed of human-recombinant epidermal growth factor linked to a carrier protein: report of a pilot clinical trial	Abril/1998
D2	WO0245747	Immunotherapeutic combinations for the treatment of tumors	13/Junio/2002

D1<http://www.ncbi.nlm.nih.gov/pubmed?term=Annals%20of%20Oncology%209.%20431-435.%201998> divulga (Pág. 431, summary; Pág. 432, columna 1, párrafos 8 a 10; folio 95 y 95 vuelto) una vacuna para el tratamiento del cáncer que tiene por principio activo un conjugado proteico hrEGF-rP64k y por adyuvante hidróxido de aluminio y el procedimiento para obtenerla.

D2http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=25&ND=4&adjacent=true&locale=en_EP&FT=D&date=20020613&CC=WO&NR=0245747A1&KC=A1 divulga (Pág. 4, líneas 10 – 12; Pág. 6, líneas 25 –29) una vacuna donde el principio activo es el conjugado de EGF con la proteína p64k de *Neisseria meningitidis* y el adyuvante es alumina o Montanide ISA 51.

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 11 consiste en un procedimiento para la obtención de forma sanitaria de la composición vacunal de la reivindicación 1 que comprende un procedimiento adecuado de conjugación covalente del EGF, a la proteína transportadora rP64k y la purificación del mismo, empleando una membrana de ultrafiltración para remover las especies conjugadas biológicamente inactivas y otras impurezas químicas.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Un procedimiento para la obtención de la composición vacunal de la reivindicación 1 que comprende:	Un procedimiento para la obtención de una composición vacunal que comprende:
un procedimiento adecuado de conjugación covalente del EGF, a la proteína transportadora rP64k	un procedimiento adecuado de conjugación covalente del EGF, a la proteína transportadora rP64k (Pág. 432, columna 1, párrafo 8)
y la purificación del mismo, empleando una membrana de ultrafiltración para remover las especies conjugadas biológicamente inactivas y otras impurezas químicas	Purificación del conjugado por medio de diálisis contra solución buffer de fosfato salino (PBS) y posterior filtración a través de una membrana bacteriológica de 0,2µm (Pág. 432, columna 1, párrafos 8 y 9)

Las características **esenciales** del procedimiento de la reivindicación 1 habían sido divulgadas previamente en D1, el cual enseña que el conjugado entre EGF y una proteína transportadora como P64k se obtuvo mezclando estas dos moléculas en una proporción de 10:1 (moles de EGF/moles proteína transportadora) y luego adicionando glutaraldehído suficiente para que la concentración final fuera de 0.05%.

la mezcla se incubó por una hora y luego se dializó contra una solución buffer de fosfato salino (PBS) durante una noche a 4°C. D1 divulga que el conjugado se filtró bajo flujo laminar a través de un filtro bacteriológico de 0,2µm para esterilizarlo y se recuperó en un recipiente apirógeno estéril (Pág. 432, columna 1, párrafos 8 y 9; ver folio 95 vuelto).

En consecuencia, la reivindicación 11 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 12 a 19 no mencionan alguna característica que haga nueva a la composición de la reivindicación 11, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en una composición vacunal cuyo principio activo comprende un conjugado proteico hrEGF-rP64k, caracterizada porque cada molécula de la proteína rP64k tiene enlazada dos moléculas de hrEGF.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Una composición vacunal cuyo principio activo comprende:	Una composición vacunal cuyo principio activo comprende:	Una composición vacunal cuyo principio activo comprende:
un conjugado proteico hrEGF-rP64k,	un conjugado proteico hrEGF-rP64k	un conjugado proteico hrEGF-rP64k
caracterizada porque cada molécula de la proteína rP64k tiene enlazada dos moléculas de hrEGF	No se menciona	No se menciona

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga una vacuna para el tratamiento del cáncer que tiene por principio activo un conjugado proteico hrEGF-rP64k y por adyuvante hidróxido de aluminio (Pág. 431, summary; Pág. 432, columna 1, párrafos 8 a 10; ver folio 95 y 95 vuelto).

D2 divulga una vacuna donde el principio activo es el conjugado de EGF con la proteína p64k de *Neisseria meningitidis* (Pág. 4, líneas 10 – 12). D2 divulga que la vacuna se administra con un adyuvante como alumina o Montanide ISA 51(Pág. 6, líneas 25 –29). Adicionalmente, D2 da a conocer que luego de la vacunación con dichas vacunas en pacientes con cáncer se consiguieron respuestas de anticuerpos anti-EGF con títulos entre 1:8000 (Pág. 9, líneas 2 – 3, 22 – 23) y 1:400000 (Pág. 12, líneas 10 – 12) dependiendo del esquema de vacunación.

La diferencia entre la invención, definida en la reivindicación 1 y la composición de D1 y D2 consiste en que el conjugado está caracterizado porque cada molécula de la proteína rP64k tiene enlazada dos moléculas de hrEGF.

El hecho de incluir dos moléculas de hrEGF enlazadas a una proteína rP64k no ha mostrado un efecto técnico sorprendente con respecto a lo divulgado en los documentos D1 y D2 con respecto a los títulos de anticuerpos anti-EGF.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar la composición vacunal conocida en D1 y D2 para conseguir vacunas alternativas con actividad similar frente a la generación de anticuerpos anti-EGF”.

Teniendo en cuenta que los documentos D1 y D2 ya divulgaban composiciones vacunales que tienen por principio activo un conjugado proteico hrEGF-rP64k para el tratamiento de cáncer, la persona del oficio normalmente versada en la materia, habría inferido la generación de combinaciones alternativas de las proteínas hrEGF y rP64k para obtener conjugados con la misma actividad. Por lo tanto, la reivindicación 1 se considera obvia.

Puesto que las reivindicaciones dependientes 2 a 10 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
Las reivindicaciones 1 a 10 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

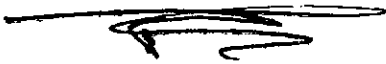
8. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Gónzales G. et al. Annals of Oncology. 1998;9(4):431-5.	11 – 19	Novedad
D1	Gónzales G. et al. Annals of Oncology. 1998;9(4):431-5.	1 – 10	Nivel Inventivo
D2	WO0245747	1 – 10	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
25 SET. 2012

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Original article

A novel cancer vaccine composed of human-recombinant epidermal growth factor linked to a carrier protein: Report of a pilot clinical trial

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Summary

Background: There is evidence of a relationship between epidermal growth factor (EGF) and tumor cell proliferation, such as the overexpression of EGF receptor (EGF-R) in different human tumors, which makes this system an interesting target for cancer treatment. Up to now, passive immunotherapy with monoclonal antibodies against the EGF-R has been assayed in clinics. Our approach consists of active immunotherapy with human EGF (hu-EGF). We conducted a pilot clinical trial to define the safety, toxicity and immunogenicity of vaccination with hu-EGF coupled to a carrier protein.

Patients and methods: Ten patients with histologically proven malignant carcinomas (colon, lung, stomach and prostate) in advanced clinical stages were enrolled. Patients were immu-

nized twice (on days 0 and 15) with hu-EGF linked to either tetanic toxoid (TT, five patients) or P64k *Neisseria Meningitidis* recombinant protein (P64k, five patients), intradermally, using aluminium hydroxide as adjuvant.

Results: In both groups 60% of patients developed anti-EGF antibody titers without evidence of toxicity. Secondary reactions were very mild, limited to erythema and itching at the site of injection, which disappeared without medication.

Conclusions: We conclude that the proposed vaccination with hu-EGF was well tolerated and that antibody titers against self EGF were developed. The results of this trial may be useful in the design of new clinical trials with higher dose immunization protocols and using more effective adjuvants.

Key words: cancer vaccine, epidermal growth factor

Introduction

Epidermal growth factor is a 53-amino acid (molecular weight 6045d) polypeptide which is mitogenic for epithelial cells [1, 2].

Since its discovery in 1962 [3] the relationship between EGF and tumor cell proliferation has held the attention of several scientific teams. Our own staff has been active in this field since 1982, reporting consecutively that high-affinity EGF-R are present in human breast cancer cells [4], that the presence of EGF-R is inversely related to survival [5] and to the presence of estrogen receptors [6], that expression of EGF-R is higher in metastasis than in primary tumors [7], and that expression of EGF-R is frequently associated with expression of Transforming Growth Factor alpha [8] in what could be an autocrine loop.

Simultaneously, other groups have been reporting the overexpression of EGF-R in human tumors in diverse localizations such as lung [9], prostate [10], head and neck [11], ovarian [12] and digestive tract [13, 14].

Several attempts have been made using monoclonal antibodies (MoAb) against EGF-R for the treatment of tumors [15-17].

Radioactive anti-EGF-R MoAb do localize in human tumors [15, 16], but the therapeutic effect of naked anti-EGF-R MoAbs has been marginal up to now.

An alternative approach is EGF deprivation through active immunization, if tolerance against self-EGF can be broken. We have recently shown that this is possible [18], and that antibodies against autologous EGF can be produced in mice and monkeys when the latter are immunized with self-EGF linked to a carrier protein or with EGF of another species.

Moreover, we have also shown that mice with antibody titers against self EGF have an altered biodistribution of EGF and also have better survivals when transplanted with Ehrlich Ascites Tumor cells [18].

In the present paper we report the results of a pilot clinical trial in which patients with advanced-stage tumors were immunized with human recombinant EGF chemically linked to either TT or to P64k.

Patients and methods

Eligibility criteria

Patients with histologically-proven malignant carcinoma at advanced clinical stages were eligible. All patients had active progressing disease after failure of conventional treatment, and were not eligible for any other modality of onco-specific therapy. Their last onco-specific treatment had occurred at least four weeks prior to their inclusion in this protocol.

Other eligibility criteria were age between 18 and 75 years, WHO

performance status 0-3, normal liver, kidney and bone marrow functions, no pregnancy or lactation, no severe uncontrolled co-morbidities, no second malignancies and no previous history of hypersensitivity to foreign proteins.

All patients gave their written consent after receiving complete information about the product and the protocol.

Study design and patient evaluation

This was an open label non-randomized pilot clinical trial intended to obtain initial information about safety and toxicity. A secondary objective was to measure antibody titers against both EGF and the carrier proteins.

The trial protocol was approved by the Ethics Committee of the Center of Medical Surgical Research (CIMEQ) where the trial was carried out, and it also received clearance from the State Center of Drug Quality Control (CECMED, National Regulatory Authority).

Patients underwent a confirmatory histological or cytological tumor exam, a complete blood count, including differential leukocyte count, platelet count, and hemoglobin, and also glutamic oxalacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase and serum creatinine measurements prior to inclusion in the trial. These parameters were tested monthly during the follow-up period.

Tumor response was evaluated in the third month of inclusion by chest X-ray, abdominal ultrasound, or thoracic and abdominal computerized tomography (CT) scan.

Immunogens

Human Recombinant EGF and P64k [19, 20] were purchased from the Center of Genetic Engineering and Biotechnology of Havana, Cuba. Purified TT was purchased from the Finlay Institute, Havana, Cuba.

The conjugates between hu-EGF and carrier proteins (TT or P64k) were made by mixing them in a proportion of 10:1 (mol EGF/mol carrier protein) and then adding glutaraldehyde to a final concentration of 0.05%. The mixture was incubated for one hour and then dialyzed against phosphate-buffered saline (PBS) overnight at 4 °C.

Before being mixed with aluminum hydroxide, the conjugate solution was sterilized by filtration through 0.2 µm bacteriological filters under laminar flow, and recovered on sterile, depyrogenized recipients.

Aluminum hydroxide was mixed with conjugates, 2 mg/dose, and the adsorption was achieved by constant stirring at room temperature for one hour under sterile conditions. One dose of vaccine is equivalent to 50 µg of hu-EGF.

Vaccine batches were released for use after independent testing for sterility, and assessment for potency through its ability to elicit anti-EGF antibodies in NMRI mice [18].

Immunization protocol

Patients were immunized twice (days 1 and 15) with 50 µg of hu-EGF linked either to tetanic toxoid (five patients) or to P64k (five patients), through intradermal injection at two sites in both arms.

Blood samples were taken on days 0, 21, 28 and monthly thereafter, for antibody titer measurement. A parallel immunization protocol was performed in Green monkeys (*Cercopithecus aethiops*).

Measurement of antibody titers

Serum titers against hu-EGF, TT or P64k were measured through enzyme-linked immunosorbent assay. Microtiter plates were coated overnight at 4 °C either with hu-EGF, TT or P64k (10 µg/ml) in coating buffer (0.1 M sodium carbonate-bicarbonate, pH 9.6), 50 µl/well. The plates were blocked with 5% calf serum in PBS/Tween (0.5%) for one hour at 37 °C. After washing, 50 µl of different patient sera dilutions (in PBS-Tween calf serum) were added to each well and incubated for one hour at 37 °C. After incubation, the plates were

washed with PBS/Tween and filled with 50 µg/well of 1:1000 diluted alkaline phosphatase-labeled antihuman polyvalent immunoglobulin serum, then incubated for one hour at 37 °C. The plates were then washed and color developed with orthophenyldiamine (1 mg/ml in diethanolamine). Absorbance at 405 nm was measured in an ELISA plate reader.

Specificity of antibodies against EGF was determined by adding to immune sera different concentrations of EGF and then testing them in the ELISA assay previously described.

Determination of immunoglobulin class

Immunoglobulin class was determined through enzyme-linked immunosorbent assay. Microtiter plates were coated with hu-EGF (10 µg/ml) and blocked, as previously described. After incubation with patient sera at different dilutions and washings, 1:5000 diluted alkaline phosphatase-labeled goat anti-human IgG or goat anti-human IgM (Jackson), were added to each well and incubated for one hour at 37 °C. Then the plates were washed and color developed with orthophenyldiamine for absorbance measurement.

Assay for EGF/EGF-R binding inhibition by sera from immunized patients

Ehrlich ascites tumor cells (10^6) were incubated for one hour at room temperature with 10^5 cpm ^{125} I-EGF in the presence of different dilutions of sera from immunized patients. Binding inhibition by an excess of nonradioactive EGF was used as a positive control. The incubation was stopped by the addition of 1.5 ml of PBS and centrifugation at 1000 rpm during 10 min. The sedimented cells were washed and dried, and radioactivity was counted in a gamma counter.

Determination of CD3, CD4 and CD8 subpopulations

Purified anti-human CD3, CD4 and CD8 FITC-conjugated antibodies (generated and produced in the Center of Molecular Immunology, Havana, Cuba) were used for the immunofluorescence assay. Total fresh blood samples (100 µl) supplemented with EDTA 5% were mixed with 20 µl of the purified MoAbs and the erythrocytes were then lysed by adding 2 ml of a lysis solution for human blood (0.15 M Ammonium chloride, 0.01 M Potassium carbonate, 0.8×10^{-4} M EDTA). Fluorescence was analyzed on a Fluorescence Activated Cell Scan (Orthoclone) and the data were processed using a program with a scatter gated on lymphocytes excluding monocytes, polymorphonuclear cells and dead cells.

Results

Patient characteristics

Ten patients, four of them male and six female, with a mean of 59.6 years (range 49 to 71), were enrolled in the trial; all of them had histologically-confirmed advanced-stage carcinomas. Primary tumors were located in colon (four cases), lung (three cases), stomach (two cases) and prostate (one case). The tumor histology was adenocarcinoma in eight patients and epidermoid carcinoma in two patients.

The patients were ineligible for any other standard anti-cancer treatment: eight of them previously had surgery, combined in three cases with radiotherapy, one had received radiotherapy alone and one hormone therapy.

Immunogenicity of the EGF vaccine

Antibody titers against self-EGF showed a significant increase after vaccination in 60% of the patients. This figure was the same for patients immunized with hu-EGF linked to tetanic toxoid and with hu-EGF linked to P64k (Table 1). Antibody titers were more than 80% suppressed if EGF (0.1 µg/ml) was added to serum samples, indicating the specificity of the antibody measurement.

Patients immunized with the EGF-P64k preparation showed lower antibody titers against the carrier protein than those immunized with EGF-TT (Table 2a, b).

Peak values of anti-EGF antibodies were obtained between days 21 and 28 after first immunization and decreased thereafter. The two carrier proteins showed similar kinetics (Figure 1a, b).

We also compared antibody responses obtained in humans with those obtained in monkeys immunized with the same vaccine preparation and on the same vaccination schedule. When the EGF-TT preparation was used, humans showed lower anti-EGF antibody titers (geometric mean 1:1,320) than monkeys (geometric mean 1:14,142), but humans showed higher anti-TT antibody titers (1:527,803) than monkeys (1:150,000). When the EGF-P64 preparation was used, antibody response in humans was lower than in monkeys against both the EGF (humans 1:631, monkeys 1:14,142) and the carrier protein (humans 1:1,320, monkeys 1:31,623).

Antibody titers against both EGF and carrier protein were in all patients IgG. No IgM class response was detectable.

Sera from the patient who achieved the highest antibody titers against EGF (patient 6 in Table 1), inhibited by 95% the binding of radioactive EGF to its membrane receptor.

Lymphocyte subpopulations (CD3, CD4, CD8) were counted before and after vaccinations, but no relationship between these data and the antibody response could be demonstrated in this small series.

Toxicity and clinical evaluation

Secondary reactions were very mild and limited to erythema and itching at the site of injection, and it disappeared without medication.

The hematological data (hemoglobin concentration, leukocyte and platelet counting) and the blood chemical data (glutamic - oxalacetic and glutamic - pyruvic transaminases, alkaline phosphatase, and creatinin) also remained within normal ranges during the immunization and follow-up period. The clinical outcome of each patient is detailed in Table 3.

Base-line antibody titer in healthy people

The measurement of some level of antibody titer against self-EGF in serum before immunization prompted us to check this in a normal population sample. It was found

Table 1. Antibody titers against EGF.

Pts	Day 0	Day 21	Day 28	Day 60	Day 90	Day 120
(a) Patients immunized with EGF-TT						
1	1000	500	1000	500		
2	500	2000*	2000*			
3	500	1000*	1000*	500		
4	1000	1000	2000*	500	500	500
5	1000	1000	1000	100	100	100
(b) Patients immunized with EGF-P64k						
6	100	1000*	4000*	2000*	1000*	1000*
7	100	500*	500*	100	100	100
8	100	500*	1000*	100	100	100
9	500	500	500	500	500	500
10	100	100	100	100	100	100

Numbers are antibody titers (defined as the maximal sera dilution which, by the ELISA test, produced an optical density higher than that of the negative control (PBS) plus five standard deviations). Each serum was tested in triplicate.

* Those measurements in which antibody titers at least doubled after vaccination.

Table 2. Antibody titers against carrier proteins.

Pts	Day 0	Day 21	Day 28	Day 60	Day 90	Day 120
(a) Patients immunized with EGF-TT						
1	10000	800000*	800000*	200000*		
2	1000	800000*	800000*			
3	200000	800000*	800000*	400000*		
4	1000	800000*	400000*	400000*	400000*	400000*
4	1000	200000*	200000*	100000*	100000*	100000*
(b) Patients immunized with EGF-P64k						
5	1000	2000*	2000*	2000*	500	500
7	1000	2000*	2000*	2000*	2000*	1000
8	500	2000*	4000*	2000*	100	100
9	500	500*	500*	500*	500	500
10	500	100*	100*	100*	100	100

Numbers are antibody titers (as defined in Table 1). Each serum was tested in triplicate.

* Those measurements in which antibody titers at least doubled after vaccination.

that sera from healthy blood donors contained measurable titers of antibodies against self-EGF in 61% of cases.

These antibodies were EGF-specific in the sense that they could be more than 80% adsorbed by the addition of EGF to the serum at a concentration of 0.1 µg/µl.

On the other hand, only 9% of serum samples contained antibodies against insulin and 17% against serum albumin. These natural autoantibodies against self-EGF were also IgG class.

Discussion

To our knowledge, this is the first report of an attempt to induce an antibody response against self-EGF in human beings.

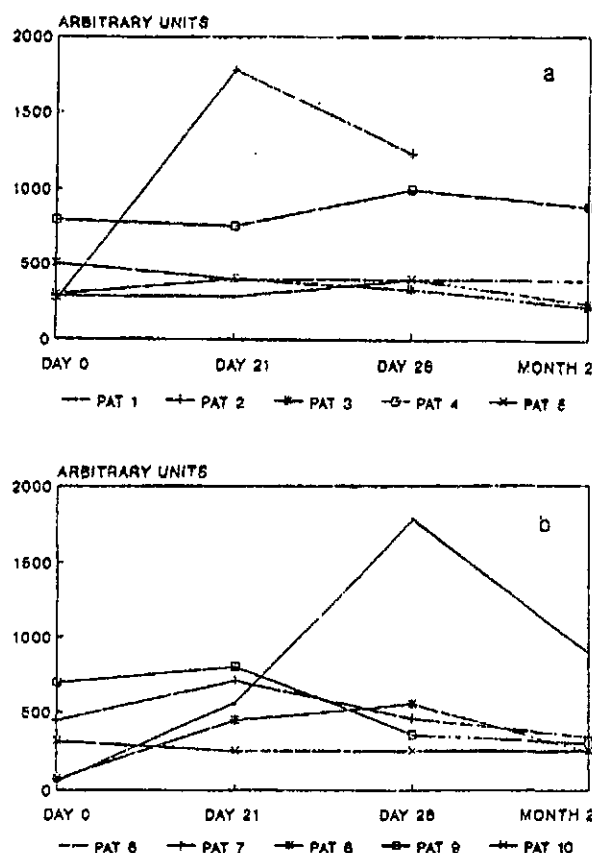


Figure 1 Antibody response against EGF in patients treated with a) EGF-TT, b) EGF-P64k. Abscissa is time after first immunization. Ordinate is serum anti-EGF antibody concentration in arbitrary units. An arbitrary unit was defined as the amount of antibody which, in our ELISA system, produces an optical density equal to that of the internal standard. The internal standard was serum from a previously immunized monkey, diluted 1:40,000. Each line in the figure represents data from a different patient. Each is the mean of triplicate measurements. In the interest of simplicity, standard deviations are not included, but they were consistently below 10% of the mean value.

In this trial, immunization with human-recombinant EGF linked to a carrier protein elicited an increase in antibody titers against self-EGF in 60% of patients, which reached peak values 3–4 weeks after immunization and decreased thereafter.

The use of tetanic toxoid as carrier protein somehow 'deviates' the immune response, eliciting many more antibodies against the carrier than against EGF, as is to be expected, since all patients have at some time in their lives had anti-tetanic vaccinations.

The use of P64k protein of *Neisseria meningitidis* as carrier protein yielded a different antibody profile, with titers against the carrier P64k protein closer to those against EGF. This is probably because patients had not previously been exposed to *Neisseria meningitidis*.

The antibody response against both EGF conjugates in monkeys is in accord with this interpretation. The fact that patients before immunization (and also normal donors) had some measurable titer of IgG antibodies against self-EGF (but not against self insulin), is very interesting. It has been shown that natural autoantibodies do not recognize self proteins randomly, but are directed to a biased subset of self proteins [23], and based on this fact it has been speculated that a kind of 'immunological homunculus' exists, integrated by self molecular components which are overexpressed in the pattern of recognition of anturial autoantibodies [24], although its physiological relevance is unknown. Epidermal growth factor could be connected to this part of the immune system.

The advanced clinical stage of patients included in this pilot trial precluded a long-term follow-up. However, during the observation time (range 2–6 months) there was neither clinical nor laboratory evidence of toxicity. Except for minor local itching and erythema, the vaccination procedure was very well tolerated.

It should be noted that there was no evidence of skin or mucous membrane toxicity. The lack of toxicity of

Table 3 Characteristics and clinical outcome of patients in the two treatment groups.

Patients treated with EGF-TT					Patients treated with EGF-P64k				
Pts	Tumour	Type	Stage	Clinical outcome	Pts	Tumour	Type	Stage	Clinical evaluation
1	Colon	Adenocarcinoma	IV	Died in month 3; multiple hepatic metastases appeared	7	Colon	Adenocarcinoma	IV	Clinical stabilization up to month 3; hepatic metastasis appeared; still alive on month 7
4	Colon	Adenocarcinoma	IV	Clinical stabilization up to month 8; increase in primary lesions; still alive at month 8	8	Colon	Adenocarcinoma	IV	Clinical stabilization up to month 5; hepatic metastases appeared; still alive on month 6
2	Stomach	Adenocarcinoma	IV	Died in month 2; multiple hepatic appeared	9	Stomach	Undifferentiated carcinoma	IV	Pulmonar metastases appeared; still alive on month 6
3	Lung	Carcinoma	IV	Died in month 2; progression of primary lesion	6	Lung	Adenocarcinoma	IV	Died on month 4; progression of primary lesion
5	Prostatic cancer	Adenocarcinoma	IV	Died in month 5; clinical stability up to month 4; hepatic metastases appeared	10	Lung	Adenocarcinoma	IV	Died on month 6; clinical and radiologic stability up to month 5

EGF-immunization in adult animals has been previously reported [21, 22], but this should be re-evaluated in more intensive immunization protocols.

The evaluation of antitumor activity was not the goal of this trial, nor were objective remissions to be expected with any procedure at such advanced stages. No reductions in size of tumor lesions were recorded. However, most patients experienced subjective clinical improvement after vaccination and during the time when anti-EGF antibody titers were present.

In conclusion, vaccination with self EGF linked to a carrier protein is feasible, safe and immunogenic in humans. As this procedure has anti-tumor activity in animals [22], it deserves more extensive clinical evaluation testing higher doses and more effective adjuvants in patients at less advanced clinical stages.

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Received 18 September 1997; accepted 15 January 1998.

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