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DIRECCIÓN DE NUEVAS CREACIONES

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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ASUNTO	Radicación	09 118 682
	Trámite	011
	Evento	379
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	Oficio	
	Folios	10

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Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☐
		Capítulo II	☒
No. Solicitud Internacional	PCT/US2008/057819		
Fecha de Presentación Internacional	21/Marzo/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 137	22/Octubre/2009
Reivindicaciones:	Folios 138 a 142	22/Octubre/2009
	Folios 385 a 388	02/Agosto/2011
Dibujos:	Folios 144 a 246	22/Octubre/2009
Secuencias:	Folios 247 a 291	22/Octubre/2009
Prioridad:	US 60/896,339	22/Marzo/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: "Anticuerpos Anti-IgE Apoptóticos uniendo IgE unida a la membrana".

El problema planteado en esta solicitud consiste en la necesidad de suministrar anticuerpos anti-IgE que se unan a la IgE unida a membrana para el tratamiento de la alergia.

Para resolver este problema técnico la solicitud en estudio proporciona anticuerpos anti-IgE apoptóticos, o fragmentos funcionales de los mismos, composiciones y métodos para reducir de forma específica las células B que producen IgE y el nivel de IgE total en suero.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 16/42; A61P 37/06

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 9 y 13 no se consideran una invención, de acuerdo con el Art citado.

5. Excepción a la Patentabilidad (Art 20 D 486 literal (c))

El objeto de las reivindicaciones 39 no es patentable de acuerdo con el Art citado.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las Reivindicaciones 1 – 10 y 14 - 17 se refieren a un anticuerpo que se une específicamente al segmento M1' de IgE, que induce la apoptosis en células B que expresan IgE, que elimina específicamente las células B productoras de IgE, que reduce IgE sérico total, que reduce el IgE sérico libre, en el que el IgE es específico al alérgeno, que se une al mismo epítipo al que se une un anticuerpo seleccionado del grupo que comprende: 47H4, 7A6, 26A11, 47H4v5, 7A6v1 y 26A11v6, que se une específicamente a un segmento de IgE de M1' con una afinidad de unión Scatchard que es equivalente a la del anticuerpo anti IgE/M1' 47H4 de murino, que tiene afinidad de entre 0,30 y 0,83 nm, que se une específicamente a un segmento de IgE de M1' con una afinidad de unión Scatchard que es equivalente a la del anticuerpo anti IgE/M1'47H4v5 humanizado, que tiene afinidad alrededor de 1,5 nm, todos éstos son resultados a alcanzar, no definen al anticuerpo porque la invención 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 reemplazarlos por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de esos efectos, que en el caso de un anticuerpo corresponden a su estructura completa, definida por las secuencias que lo conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas ligera y pesada.

La reivindicación independiente 1 que se refiere a un anticuerpo debe indicar las características esenciales del mismo, y puesto que un anticuerpo se caracteriza por su estructura completa, se sugiere incluir las secuencias que lo conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas ligera y pesada.

Las reivindicaciones 18 – 20 y 24 nombran fragmentos de un anticuerpo, los cuales no son válidos puesto que no es una estructura activa, probada, ni definida.

Las reivindicaciones 10, 14, 16, 18 – 20, 24 y 25 se refieren a anticuerpos llamados "47H4", "7A6", "26A11", "47H4v5", "7A6v1", "26A11v6", "26A11 v.1 16" y "47H4v1 6", que son designaciones arbitrarias dadas por el solicitante que no permiten definir al anticuerpo de la solicitud. Se sugiere definir el anticuerpo como ya se menciona por

su estructura completa, mencionando las secuencias que lo conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas ligera y pesada.

La reivindicación 23 nombra fármacos que se pueden encontrar en una composición farmacéutica junto con el anticuerpo que se desean proteger, sin embargo estos son nombrados por su actividad farmacológica lo cual no define inequívocamente un ingrediente activo como tal. Además, en la descripción si bien se nombran, no se evidencia un efecto técnico generado por la combinación reclamada en la presente solicitud tal como sinergia, disminución de dosis, disminución de efectos secundarios etc. Por lo tanto, se sugiere al solicitante eliminar esta reivindicación del alcance del pliego reivindicatorio porque no encuentra soporte en la descripción.

La reivindicación 22 se refiere a una composición que comprende un vehículo farmacológicamente aceptable, sin definir el vehículo inequívocamente, se sugiere realizar las aclaraciones necesarias.

Las reivindicaciones 24 y 25 se refieren a un ácido nucleico que se encuentra definido por el anticuerpo que codifica, se aclara al solicitante que un ácido nucleico se define por su secuencia específica de nucleótidos.

La reivindicación 27 se refiere a un vector en el que el ácido nucleico está ligado en forma operacional. Se aclara que no se está definiendo inequívocamente el vector y no es claro a que se refiere el solicitante con que "está ligado en forma operacional", y por lo tanto se sugiere realizar las aclaraciones necesarias.

La célula anfitriona de la reivindicación 28 no se encuentra caracterizada. Se sugiere indicar las células a las que se refiere el solicitante.

La reivindicación 29 no es concisa porque define una célula únicamente porque es de un mamífero, dando lugar a un sinnúmero de posibilidades en cuanto a células que no encuentran soporte en la descripción. Se sugiere restringir la solicitud a una razonable generalización de lo que se encuentra debidamente soportado.

El proceso de la reivindicación 31 no se encuentra adecuadamente caracterizado puesto que no define los pasos y las condiciones de los mismos. Se sugiere definir el procedimiento por sus características esenciales.

El termino "adecuadas" de la reivindicación 31 es relativo por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término concreto.

En cuanto a los artículos de fabricación las reivindicaciones 32 – 36 se debe señalar que la especificación de medios para facilitar la administración del producto, no implica que el objeto de la esta reivindicación cumpla con el requisito de ser de carácter técnico, ya que el artículo de fabricación es simplemente un medio para presentación comercial del producto, y no representa una unidad funcional de varios componentes necesaria para el propósito final. Se sugiere eliminar estas reivindicaciones del alcance del pliego reivindicatorio.

La reivindicación 38 define a un anticuerpo porque es secretado por un hibridoma, se reitera la sugerencia de definir el anticuerpo por su estructura completa.

Descripción (Art 28 D 486)

En las figuras 4A-2, 4A-8, 4A-9, 4A-10, 4A-12, 4A-14, 4A-15, 4A-17 y 4A-20 se encuentran cuadros que no tienen ninguna figura, se sugiere realizar las aclaraciones necesarias.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el certificado de depósito para los hibridomas PTA-8268, PTA-8267, PTA-8266, PTA-8270, PTA-8260, PTA-8265, PTA-8262, PTA-8264, PTA-8269, PTA-8261 y PTA-8263,

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. 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: 22/Marzo/2007 y se encontró el siguiente documento que anticipa el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	CHEN. H. Y., ET AL, International Archives of Allergy and Immunology, Vol. 128, Págs. 315 - 324	Monoclonal Antibodies Against the CεmX Domain of Human Membrane-Bound IgE and Their Potential Use for Targeting IgE-Expressing B Cells	2002

D1 divulga (Pág. 315, resumen) 5 anticuerpos monoclonales que se unen a la IgE unida a membrana, estos anticuerpos reconocen el mismo epítoto localizado cerca del extremo C-terminal CεmX.

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un anticuerpo anti IgE/M1' que se une específicamente al segmento M1' de IgE y que induce la apoptosis en células B que expresan IgE.

La reivindicación 14 consiste en un anticuerpo anti IgE/M1' que se une específicamente a un segmento de IgE de M1' con una afinidad de unión Scatchard que es equivalente a la del anticuerpo anti IgE/M1' 47H4.

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

Características esenciales	D1
Anticuerpo anti IgE/M1'	Anticuerpo anti IgE/M1'

Las características **esenciales** del anticuerpo de la reivindicación 1 habían sido divulgadas previamente en D1, el cual enseña 5 anticuerpos monoclonales que unen a la IgE unida a membrana, específicamente se unen al epítoto RADWPGPP localizado cerca del extremo terminal de CεmX (Pág. 315, Resumen).

La unión específica al segmento M1' de IgE y la inducción de la apoptosis en células B que expresan IgE, no se consideran características esenciales puesto que son resultados a alcanzar.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 – 6, 14 – 17 y 22 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

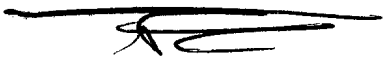
9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	CHEN. H. Y., ET AL, International Archives of Allergy and Immunology, Vol. 128, Págs. 315 - 324	1 – 6, 14 – 17, 22	Novedad

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

29 AGO. 2012

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Monoclonal Antibodies against the C α mX Domain of Human Membrane-Bound IgE and Their Potential Use for Targeting IgE-Expressing B Cells

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Key Words
IgE · mIgE · C α mX · IgE-expressing B cells · Monoclonal antibody · Fc fusion protein

Abstract
Background: IgE mediates immediate-type hypersensitivity reactions responsible for various allergic symptoms. It is secreted by IgE-producing plasma cells, which differentiate from B cells expressing membrane-bound IgE (mIgE) on their surface. The ϵ -chain of human mIgE contains a membrane-anchoring peptide and an extra 52-amino-acid (a.a.)-long domain (referred to as C α mX) between the membrane anchor and the CH4 domain. **Objective:** The study was designed to evaluate the effects of C α mX-specific monoclonal antibodies (mAbs) to target IgE-expressing B cells and decrease IgE production. **Methods:** A C α mX containing IgG1Fc fusion protein was produced in CHO cells and used to immunize mice; five hybridoma clones secreting C α mX-specific mAbs were obtained. **Results:** Characterization of the mAbs using ELISA, immunoprecipitation, and immunoblotting methods showed that they could bind to both native and denatured forms of C α mX. The mAbs exhibited mutual inhibition of binding to mIgE. Epitope map-

ping using synthetic peptides revealed that all five mAbs recognize the same epitope, RADWPGPP, located near the C-terminus of C α mX. Binding of one of the mAbs to mIgE on SKO-007 cells induced the cross-linking of mIgE molecules on the cell surface, resulting in their patching and capping. In vitro functional analysis revealed that mAbs are able to cause complement-mediated cytotoxicity on transfectants expressing the Fc portion of mIgE. **Conclusion:** We have prepared several human mIgE-specific mAbs. The potential of the mAbs on targeting mIgE⁺ B cells was demonstrated by CDC analysis.

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Introduction

The symptoms of most allergic reactions result from the cross-linking of IgE-armed IgE/Fc receptors on mast cells and basophils by multivalent allergens and the subsequent release of mediators by those allergen-sensitized cells. Since IgE plays a central role in those allergic responses, devising treatments to control IgE levels in the body and regulating IgE synthesis has been of great interest to immunologists. The membrane immunoglobulin on mature B cells is essential for B cell functions [1] and can

transduce signals for resting B cells to differentiate into activated lymphoblasts and Ig-secreting plasma cells [2–4]. Membrane-bound IgE (mIgE) is expressed on B cells at certain stages during the process of class switching from mIgM⁺ mIgD⁺ immunocompetent B cells to IgE-expressing B cells and subsequent differentiation to plasma cells. In genetically engineered mice lacking the membrane-anchoring segment of the ϵ -chain of mIgE (m ϵ -chain), the serum IgE level is only 5% of that of wild-type mice [2], suggesting the importance of mIgE in regulating IgE levels. The functions proposed for mIgE include class switching [5], affinity maturation [6, 7], memory maintenance [8], and the differentiation of IgE-expressing B cells at the later stages [2].

Several strategies have been proposed to treat IgE-mediated allergic diseases by downregulating IgE levels [9–14], one of them being the use of unique anti-IgE antibodies, which bind to epitopes in the CH3 domain of the ϵ -chain of an IgE molecule or near the IgE/Fc receptor site [15]. These antibodies are designed to neutralize free IgE and target IgE-expressing B cells [9, 11, 12]. In clinical trials, allergic patients treated with anti-IgE antibodies showed nearly complete depletion of free IgE in the blood and improved allergic symptoms [16–19]. One therapeutic anti-IgE antibody, Xolair, is now pending for marketing approval from the FDA of the USA and regulatory agencies in other countries. The proposed effects of anti-IgE antibodies include the neutralization of free IgE, the downregulation of IgE/Fc receptors on basophils and mast cells [20, 21], and the sweeping of incoming allergens by rapidly accumulating, elevated IgE-anti-IgE complexes [12]. It has also been proposed that the anti-IgE antibodies may downregulate or lyse IgE-expressing B cells although the results from the clinical trials do not confirm this hypothesis.

The ‘membrane-anchoring peptide’ of mouse or human m ϵ -chain, which extends beyond the CH4 domain, contains an intracellular segment, a transmembrane segment, and an extracellular segment (referred to as migs- ϵ peptide) between the transmembrane segment and CH4 domain of the ϵ -chain [10, 22]. An isoform of the human m ϵ -chain contains an extra domain of 52 amino acid (a.a.) residues (named C α mX) between the CH4 domain and the migs- ϵ peptide [23]. This isoform has been identified in a human IgE-secreting myeloma, SKO-007, and normal blood lymphocytes [23, 24]. A C α mX-like domain has not been found in several rodent species studied. At both the mRNA and protein levels, the isoform containing C α mX is the dominant one, whereas the conventional form without the C α mX domain exists in minute or unde-

tectable amounts [23, 25, 26]. The cm67 peptide, which is expressed in recombinant fusion proteins in the present studies, is composed of the contiguous 52 a.a. C α mX domain and the 15 a.a. migs- ϵ peptide, both of which are unique to human mIgE.

The C α mX-specific mAbs prepared in the present studies, which target mIgE and not secreted IgE, can be used to resolve the issue whether the therapeutic anti-IgE antibodies, like Xolair mentioned above, cause the downregulation of IgE-expressing B cells. If anti-C α mX mAbs can downregulate the levels of IgE in the body, they should have the potential for treating patients with very high IgE levels, for whom anti-IgE antibodies would not be practical. Positive results may also encourage a therapeutic approach based on developing an immunogen using cm67 peptide, which can induce antibody response against the C α mX domain and hence mIgE-expressing B cells.

Materials and Methods

Construction of Plasmids and Procedure for Transfection

C α mX-migs- ϵ (cm67) was amplified by RT-PCR from the total RNA of an IgE-secreting cell line, SKO-007 (ATCC CRL-8033-1), which was originally derived from U266 myeloma cell line [27], using anti-GATC Cggatggagcgagct and ggcTCCG Gccagcgagct-cggcct as primers. The amplified product was digested by restriction enzymes, *Bam*HI and *Xho*I, and ligated between the genomic DNA sequence of CH2-CH3 domains of human IgG1 and a leucine zipper fragment (sequence: LEAKIFALFAKIFALFAKIFALFAKIFALFAK) in a plasmid derived from pcDNA3 (Invitrogen, Carlsbad, Calif., USA). Another stable transfectant used was a CHO cell line transfected by an Fc portion of mIgE (mIgE-Fc). The cDNA sequence of m ϵ -chain containing the segment from CH2 domain through the membrane anchor peptide was inserted into pcDNA3 vector. The recombinant plasmid was transfected into CHO cells using a liposome reagent DMIRE-C (GIBCO BRL, Rockville, Md., USA) according to the manufacturer’s instructions. Briefly, 10 μ g of plasmid DNA and 10 μ g of DMIRE-C were added to 6×10^5 cells in a 10-cm petri dish and incubated at 37°C for 5 h in fresh IMDM (GIBCO). Afterwards, 10 ml of medium containing 10% fetal bovine serum (FBS) was added and the cells were incubated at 37°C for an additional 48 h. The cells were then resuspended in a medium containing G418 (Calbiochem, San Diego, Calif., USA) and distributed into the individual wells of 96-well plates.

Screening of Transfectants

To identify clones expressing cm67-Fc fusion protein, the expressed protein in the culture medium was measured by ELISA. Briefly, the wells of 96-well microtiter plates were coated with 1 μ g/ml goat anti-human IgG (KPL, Gaithersburg, Md., USA) in coating buffer (100 mM sodium carbonate, pH 9.6) at 37°C for 1 h, followed by 3% nonfat milk in PBS. The plates were then washed with 0.05% Tween-20 in PBS (PBST) 5 times. The culture medium was applied to the wells and the plates were placed at 37°C for 1 h. After five

was then allowed to develop using diaminobenzidine tetrahydrochloride (DAB) as a substrate. For indirect immunofluorescence, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with 1% bovine serum albumin (BSA). The cells were then incubated with the primary antibody (1:100) and the secondary antibody (1:1000) for 1 h each. The cells were then incubated with the DAPI (4',6-diamidino-2-phenylindole) for 10 min. The cells were then mounted on slides and covered with mounting medium. The slides were then viewed under a fluorescence microscope.

Purification of Recombinant Proteins
Selected recombinant cell lines were cultured in DMEM containing 10% of protein-G-fractionated FBS in roller bottles. Recombinant proteins in conditioned medium were purified with protein-A-sepharose CL-4B (Pharmacia, Piscataway, N.J., USA). The purified recombinant protein was analyzed by 12.5% SDS-PAGE.

Preparation of Anti-em67 Hybridomas and mAbs
Six to 8-week-old BALB/c mice were injected intraperitoneally (i.p.) with 50 µg of purified recombinant protein in complete Freund's adjuvant (Sigma, St. Louis, Mo., USA). After 3 weeks, the mice were injected similarly with the same amount of antigen in incomplete Freund's adjuvant (Sigma). Hereafter, an additional 3–4 booster immunizations were performed similarly every 2 weeks. The anti-em67 titers of the sera from the immunized mice were determined by ELISA. Briefly, 96-well plates were coated with 1 µg/ml goat anti-human IgG1 at 4°C overnight and then blocked with 3% normal milk in PBS. 50 µl of SKO-007 cell lysate, prepared by lysing 4×10^6 SKO-007 cells in 1 ml lysis buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.02% Na₂S₂O₈, 1.0% Triton X-100, and 5.0 mM EDTA, pH 8.0), was added to each well. The procedure allowed the mAb and secreted IgE from the SKO-007 cell lysate to be bound to the plates. Serial dilutions of the sera from the immunized mice were then applied to the wells for titrating anti-em67 activities. For preparing hybridomas secreting anti-em67 mAbs, the spleen cells from the immunized mice were fused with mouse NS0 myeloma cells to form hybrids using the routine procedure with polyethylene glycol [28]. The hybrids were selected by culturing in IMDM containing hypoxanthine, aminopterin, and thymidine (HAT, Gibco) and then screened by the same ELISA as described above. The hybridoma secreting anti-em67 mAbs were subcloned. Chosen clones were grown as ascitic tumors in BALB/c mice. The antibodies in collected ascitic fluids were purified using protein G-Sepharose CL-4B (Pharmacia).

Immunoprecipitation and Immunoblotting Analyses
 1×10^6 SKO-007 cells were lysed with 1 ml of lysis buffer and the resultant lysate was mixed with 5 µg of goat anti-human IgE, anti-em67 mAb, or control anti-DNP IgG2b [29] at 4°C for 1 h. 80 µl protein G-Sepharose CL-4B resin was added to absorb the formed complexes. After the beads were washed with lysis buffer, the bound complexes were eluted by boiling in sample buffer, subjected to SDS-PAGE, and analyzed by incubation with HRP-conjugated goat anti-human IgE (Fig. 2, lanes 1–3). For the analysis of binding of anti-

em67 to mAb, the lysate of SKO-007 cells was subjected to SDS-PAGE. The proteins in the gel were electrotransferred to polyvinylidene fluoride membrane (Millipore, Bedford, Mass., USA) and analyzed with anti-IgE (a) (lane 4) or anti-em67 (lane 5).

Competitive Binding of mAbs to mIgE
Microtiter plates were coated with various anti-em67 mAbs (1 µg/ml) at 4°C overnight and then blocked with 3% normal milk in PBS. SKO-007 cell lysate from 4×10^6 cells was mixed with serial dilutions of an mAb, whose inhibitory activity was to be examined, and the mixture was added to individual wells. The mIgE bound to the mAb coated on the wells was measured by HRP-conjugated goat anti-human IgE in an ELISA as described above.

Epitope Mapping of Anti-em67 mAbs Using Synthetic Peptides
Fourteen peptides (designated p1–p14) of 15-a.a. residues, which overlapped 11-a.a. residues in a stepwise fashion, along the entire 63-a.a. length of the em67 segment were synthesized at La Jolla Institute for Allergy and Immunology (San Diego, Calif., USA). Two methods were employed to map the epitopes of the mAbs. In the first method, plates were coated with one of the peptides at 10 µg/ml and then blocked by 3% normal milk in PBS. An mAb at 1 µg/ml was applied and the bound mAb was detected as described above. In the second method, plates were first coated with anti-IgE and SKO-007 cell lysate was followed to allow mIgE to be captured. Anti-em67 at 1 µg/ml mixed with one of the peptides at 5 µg/ml was added to the individual wells and the bound mAb was detected by HRP-conjugated goat anti-mouse IgG.

Fluorescence Flow Cytometric Analysis
 1×10^6 SKO-007 (mIgE+) cells or transfected CHO cells (mIgE-Fe+) were washed with PBS containing 2% FCS and 0.1% sodium azide. Cells were resuspended in 100 µl buffer containing 10 µg/ml anti-CanX mAb or anti-DNP IgG2b in the presence or absence of p10 peptide (for which the anti-CanX mAbs are specific) at 100 µg/ml for 45 min at 37°C. Cells were washed once with 3–3 ml PBS containing 2% FCS and 0.1% sodium azide and then incubated with 100 µl 10 µg/ml FITC-conjugated F(ab')₂ goat anti-mouse IgG-Fc (Jackson Laboratory, West Grove, Penn., USA) at 4°C for 30 min. Cells were washed with PBS and analyzed by FACScan (Becton Dickinson, Franklin Lakes, N.J., USA).

Fluorescence Microscopy
SKO-007 cells were incubated with 10 µg/ml anti-CanX at 37°C for 2 h. FITC-conjugated goat F(ab')₂ anti-mouse IgG was then added and the cells were incubated at 4°C for 30 min. The cells were washed once and fixed with 1% paraformaldehyde/PBS. The fluorescence-labeled cells were mounted on slides and observed with a confocal microscope (Bio-Rad, Hercules, Calif., USA).

Mass Spectrometric Analysis
The lysate from 1×10^6 SKO-007 cells was mixed with 100 µg anti-CanX mAb and 500 µl of protein G-Sepharose CL-4B. After washing with lysis buffer, the beads were boiled in sample buffer, and the dissolved material was subjected to 10% SDS-PAGE. The separated proteins were stained by Coomassie blue and the band corresponding to mAb chain (confirmed by immunoblotting) was cut out and analyzed by mass spectrometry at The Scripps Research Institute (La Jolla, Calif., USA). The procedure consisted of digestion of the protein by trypsin, extraction of the digested peptides, analysis of the

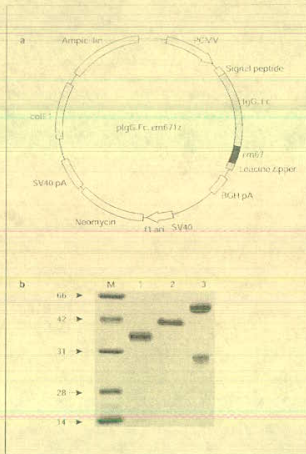


Fig. 1. Construction of the plasmid and analysis of IgG-Fc fusion protein. **a** Recombinant plasmid was constructed by inserting em67 into the 3' end of the IgG-Fc genomic sequence. **b** The purified recombinant protein was analyzed by SDS-PAGE. M = Molecular weight markers; lane 1 = IgG-Fc; lane 2 = em67-Fc; lane 3 = bovine IgG.

masses of the peptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and analysis of the sequences of peptides by liquid chromatography-mass-mass (tandem mass) spectrometry.

Complement-Mediated Cytotoxicity
 4×10^4 CHO cell transfectants expressing mIgE-Fc or SKO-007 cells were placed in individual wells of 96-well plates and the cells were cultured overnight. Twofold serial dilutions (final concentration 0.25–4 µg/ml) of an anti-CanX mAb or a control mAb (anti-DNP IgG2b) were added in the presence of 1:5-fold-diluted baby rabbit complement (Serotec, Raleigh, N.C., USA). After incubation for 2 h at 37°C in 5% CO₂ incubator, live cells were determined using WST-1 assay (Dojindo, Kumamoto, Japan).

Results

Preparation of em67-Fc Fusion Protein and Anti-em67 mAbs

The expression level of the recombinant Fc fusion protein containing em67 peptide (CanX-migis-ε segment) by the transfectants described in Materials and Methods was approximately $10 \mu\text{g}/1 \times 10^6$ cells/day. Several milligrams of the recombinant protein were obtained from 1 liter of culture medium by using affinity purification with a protein A-Sepharose CL-4B column. Analysis of the purified fusion protein showed that it was not contaminated by bovine IgG (Fig. 1). Probably because em67 represents a small portion of the fusion protein, it was necessary to screen large numbers of hybrids for those secreting em67-specific mAbs. From several thousands of hybridomas screened, five secreting anti-em67 antibodies were obtained. They were designated as a10, a23, a25, and a31. The heavy-chain isotypes of the mAbs secreted by those hybridomas were $\gamma 1$ for a10, a20, a23, a25, and a31 clones and $\gamma 2b$ for a20 clone, whereas the light-chain isotypes for all five clones are κ .

Immunoprecipitation and Immunoblotting of mIgE in mIgE+ Cell Lysates by Anti-em67 mAbs

To characterize the binding specificity of the anti-em67 mAbs, their reactivity with the Triton (detergent) lysate of SKO-007 cells, which should contain mIgE and secreted IgE, was analyzed. In one set of experiments, the SKO-007 cell lysate was mixed with either goat anti-IgE antibodies or anti-em67 mAbs and the immune complexes formed were isolated by protein G-Sepharose CL-4B and subjected to SDS-PAGE. Subsequently, the gels were stained with goat anti-IgE antibodies coupled to colorimetric second antibodies. The results of lanes 1–3 in figure 2 show that three ϵ -related molecular species were pulled down by the goat anti-IgE antibodies [26], while among them, only the largest species was pulled down by anti-em67 mAb, a20. In the other experiments, the SKO-007 cell lysate was subjected to SDS-PAGE and the resultant gels reacted with either goat anti-IgE antibodies or anti-em67 mAbs coupled to colorimetric second antibodies. The results of lanes 4 and 5 in figure 2 also show that among the three ϵ -related molecular species, anti-em67 mAb, a20, could only react with the largest one. The results indicate that the largest ϵ -related chain contains CanX domain and can be recognized by anti-em67 mAbs.

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Mutual Inhibition of Binding of Anti-*cm67* mAbs to mlgE

To analyze the relationship among the epitopes recognized by these mAbs, the effects of one mAb on the binding of another mAb to mlgE were examined. A constant amount of mlgE-containing SKO-007 cell lysate was mixed with increasing concentrations of an mAb and the mixtures were added to microtiter wells coated with another mAb. If the epitope of the mAb in the solution overlaps or is in the close vicinity of that of the mAb on the solid phase, lower amounts of mlgE binding to the plate result. The data show that the binding of any of the anti-*cm67* mAbs to mlgE was inhibited by all other anti-*cm67* mAbs. Figure 3 illustrates the results of experiments using a20 as the solid-phase mAb. Thus, all five mAbs bind either to the same epitope on *cm67* or to epitopes that are in close enough proximity to cause steric hindrance of one another. Among the mAbs tested, a10, a20 and a31 inhibited the binding of other mAbs more efficiently than a25. A control mAb, a16, which is specific for human IgG-Fc, did not inhibit the binding of mlgE to any of the five mAbs coated on the solid phase.

Epitope Mapping of Anti-*cm67* mAbs

Since the anti-*cm67* mAbs could bind to the denatured mlgE in SDS gel (Fig. 2), they might bind to linear epitopes that correspond to peptide segments of contained *cm67*. We synthesized fourteen peptides of 15-a.a. residues that sequentially overlap 11-a.a. residues in a stepwise fashion and employed them to determine the epitopes of the anti-*cm67* mAbs. In one set of ELISA using plates coated with different peptides, it was found that all these anti-*cm67* mAbs bound to peptides p10, p11 and p12, which represent overlapping sequences containing RADWPGPP located near the C-terminal of the *CcmX* domain (Fig. 4). In another set of ELISA analyzing the inhibitory effects of the peptides on the binding of anti-*cm67* mAbs to solid-phase mlgE, it was found that p10, p11 and p12 could inhibit all five mAbs binding to mlgE, with the exception that p10 inhibited a31 poorly (Fig. 5). In summary, the five anti-*cm67* mAbs are all specific to a linear epitope containing RADWPGPP on the *CcmX* domain and not to the 15-a.a. mlgE peptide.

Binding of Anti-*CcmX* mAbs to the Surface of mlgE+ Cells

To examine whether the anti-*CcmX* mAbs could bind to mlgE on the cell surface of B cells, flow cytometric analysis was performed. The results show that a20 could bind to SKO-007 cells (Fig. 6a) and to mlgE-Fc-transfected

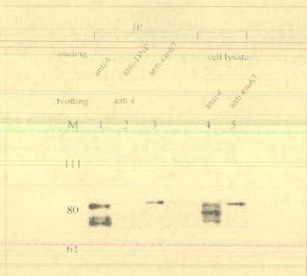


Fig. 2. Immunoprecipitation and immunoblotting analysis of binding of anti-*cm67* to mlgE. SKO-007 cell lysate was treated with goat anti-human IgE (lane 1), anti-DNP IgG2b (lane 2) or anti-*cm67* a20 (lane 3) followed by incubation with protein G-Sepharose CL-4B beads. The absorbed proteins were eluted and loaded onto individual lanes. Alternatively, the lysate was loaded directly, subjected to electrophoresis, and electro-transferred to polyvinylidene membrane (lanes 4 and 5). IgE-related proteins were detected by using HRP-conjugated goat anti-IgE (lanes 1–3), or anti-*cm67* a20 (lanes 4 and 5) followed by HRP-conjugated anti-mouse IgG.

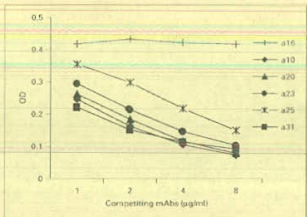


Fig. 3. Mutual inhibition of binding of anti-*cm67* mAbs to mlgE. ELISA plates were coated with a20 mAb. Equal aliquots of SKO-007 cell lysate were mixed with various concentrations of anti-*cm67* mAb a10, a20, a25, or a31, or a negative control mAb, a16, before addition to a20-coated wells. Bound mlgE was detected by HRP-conjugated goat anti-human IgG.

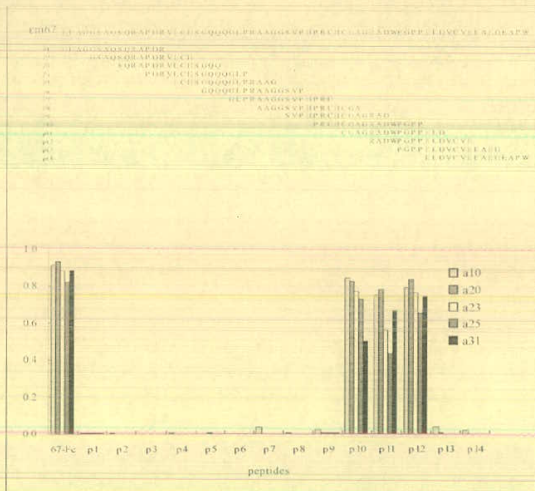


Fig. 4. Epitope mapping of mAbs using synthetic peptides. Plates were coated with 10 µg/ml of peptides or *cm67*-Fc (b7-1c). Aliquots of various anti-*cm67* mAbs at 1 µg/ml were added and the bound mAb was detected by HRP-goat anti-mouse IgG.

CHO cells (Fig. 6c), and such binding could be inhibited by p10 peptide (Fig. 6b, d). Results with other mAbs were similar (data not shown). The binding of a20 to SKO-007 cells was also examined by confocal immunofluorescence microscopy. As shown in Figure 7, patching of the reacted antigen on the cell membrane (Fig. 7a) occurred on most of the cells. It should be noted that the fluorescent second antibody, FITC-conjugated F(ab')₂ goat anti-mouse IgG, was added at 4°C and the cells were swiftly washed with cold medium and fixed with 1% paraformaldehyde/PBS. This procedure prevented patching and capping observed

due to cross-linking induced by the second antibody. The results suggest that a20 could induce cross-linking of mlgE+ on the cell surface.

Analysis of Anti-*CcmX*-Precipitated mlgE by Mass Spectrometry

To verify that the mlgE recognized by the anti-*CcmX* mAbs contains *CcmX*, one of the mAbs, a20, was used to immunoprecipitate mlgE from SKO-007 cell lysate and the isolated protein was analyzed by mass spectrometry. Trypsin-digested fragments were analyzed by MALDI-TOF and sequenced by tandem mass spectrometry. The

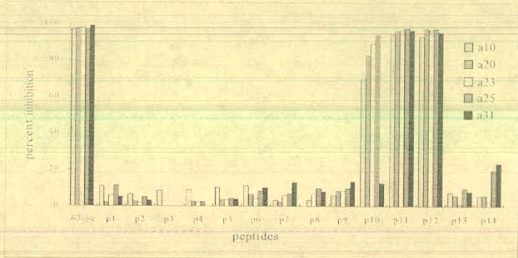


Fig. 5. Inhibition of binding of anti-em67 mAb to mIgE by synthetic peptides. Plates were first coated with goat anti-IgE and SKO-007 cell lysate was applied to allow mIgE to be captured (Materials and Methods). Aliquots of mAbs at 1 µg/ml mixed with 5 µg/ml of the inhibitors, peptides or em67-Fc (67-Fc), were added and the bound mAb was detected by HRP-goat anti-mouse IgG. The inhibitory effect was expressed as the percentage of reduced OD compared to maximum binding.

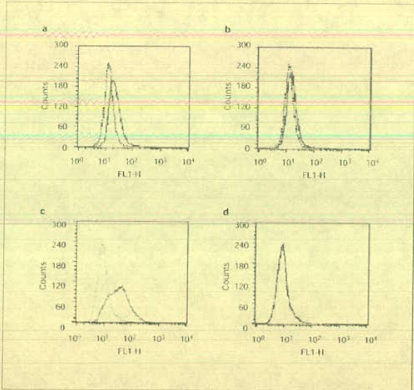


Fig. 6. Binding of anti-CemX mAb on the surface of mIgE+ cells. SKO-007 (**a**) and CHO transfectant (**b**) cells were incubated with either control (anti-DNP IgG2b, dotted line) or anti-CemX a20 (solid line) at 10 µg/ml in the absence (**a**) or presence of an inhibiting peptide (**b**, **d**). After incubation for 30 min at 37 °C, the cells were labeled with FITC-conjugated F(ab')₂ goat anti-mouse IgG and analyzed by fluorescence flow cytometry.

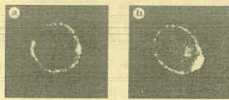


Fig. 7. Induction of patching (**a**) and capping (**b**) on mIgE+ cells by an anti-CemX mAb. SKO-007 cells were incubated with 10 µg/ml of anti-CemX a20 mAb for 2 h at 37 °C. After washing, the cells were labeled with FITC-conjugated F(ab')₂ goat anti-mouse IgG at 4 °C for 30 min and observed with a confocal microscope.

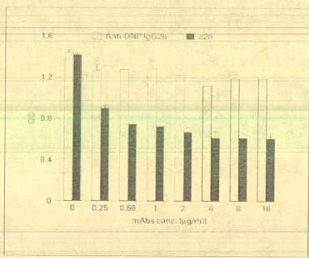


Fig. 8. Induction of complement-mediated cytotoxicity by anti-CemX on mIgE-Fc transfectants. 4×10^4 cells were cultured in a 96-well plate overnight, and 0–16 µg/ml of anti-CemX a20 (**■**) or control, anti-DNP IgG2b (**□**) were added together with 1:5-fold-diluted baby rabbit complement and the mixtures were cultured at 37 °C for 2 h. Live cells were determined by a WST-1 assay.

results of MALDI-TOF reveal that 14 of the 27 detected peptides matched fragments of the m-chain (data not shown). The results of tandem mass spectrometry resolved 13 peptide sequences that match to the m-chain, and two of them, a 17-a.a. residue and a 10-a.a. residue, were found to be identical to peptide segments of the CemX domain that had previously been derived from a nucleotide sequence of cDNA (data not shown).

Induction of Complement-Mediated Cytotoxicity of mIgE-Fc Transfectants by Anti-CemX mAb

To study whether anti-CemX mAbs could be used to target mIgE+ B cell, mIgE-Fc-transfected CHO cells were examined for their susceptibility to complement-mediated cytotoxicity in the presence of anti-CemX a20 mAb and baby rabbit complement. The results show that over 50% of the target cells could be lysed, as compared to the result with an irrelevant control antibody under the same condition (fig. 8). The anti-CemX mAb a20 was effective at a concentration as low as 0.25 µg/ml.

Discussion

In this study, an immunogen in the form of an Fc fusion protein containing em67 unique to human mIgE was expressed in a mammalian expression system. By employing this fusion protein as an immunogen, murine mAbs specific for the CemX domain were generated. The mAbs bind to a linear epitope in the CemX domain and recognize both the native and denatured forms of mIgE. One of the mAbs, which was studied more extensively, was shown to be capable of inducing the cross-linkage of

mIgE on B cells as well as complement-mediated cytotoxicity of transfectants expressing mIgE-Fc on the surface.

The rationale for employing the Fc of human IgG1 as a fusion partner for em67 is as follows [30, 31]: (1) to provide T-cell-reactive peptide segments for eliciting T cell help; (2) to possibly preserve the native conformation of the CemX domain through dimer formation, and (3) to simplify the purification of the expressed product by employing protein A affinity purification. mAbs against the linear peptide epitope may also be prepared with em67 immunogens in other forms of molecular constructs although we have not been successful in attempts at using em67 peptide in a fusion protein with bacterial maltose binding protein.

The mAbs were characterized by various assays. The results indicate that the mAbs can detect the existence of CemX on B cells by immunoblotting and immunofluorescent methods, and hence can be employed to detect, enumerate, and isolate mIgE-expressing B cells. These mAbs do not bind to FcεRII-bound IgE and should not recognize FcεRII-expressing B cells, which do not express mIgE, or other FcεRII-expressing cells such as monocytes.

Epitope mapping of the five anti-CcmX mAbs showed that they bind to a linear peptide, RADWPGPP, located near the C-terminus of the CcmX domain. These results have several implications. First, the conformation of this sequence in the cm67-Fc fusion protein and mIgE is similar to that in the synthetic peptide. Alternatively, synthetic peptides containing the epitope may be induced to fit the binding site of the Fc of the mAbs. Second, this peptide segment in CcmX is exposed on the surface of the protein. The RADWPGPP sequence indicates that, owing to its proline and glycine residues in the last four residues, it possibly bears a turn structure on the surface of a folded protein. Third, this peptide region is possibly a dominant immunogenic epitope site.

Epitope mapping has also proven that the peptide sequences previously deduced from cDNA sequencing exist in the CcmX domain. Tandem mass analysis provided convincing evidence that CcmX is an integral part of the human mu-chain. The results on the peptide masses yielded by MALDI-TOF also indicate that there is no posttranslational modification on these peptides in the mu-chain.

In this study, the ability of anti-CcmX mAbs on targeting and lysing mIgE+ B cells was examined. The mAb a20 could induce patching and capping of mIgE on the surface of SKO-007 cells although such cross-linking of mIgE did not affect cell growth [unpubl. obs.]. This is possibly due to the low expression level of mIgE on SKO-007 cells or to the fact that the cells are of a terminally differentiated myeloma cell line. The low expression level of mIgE on SKO-007 cells was also shown by the low level of fluorescence staining by the anti-CcmX mAbs in flow cytometric analysis. The results are consistent with the finding that the anti-CcmX mAbs did not mediate complement-dependent cytotoxicity on SKO-007 cells (data not shown), probably because a certain expression level of surface molecules is required for effective cytotoxicity. In contrast, anti-CcmX mAb a20 was able to cause complement-dependent cytotoxicity on the mIgE-Fc-expressing transfectants, which expressed relatively higher levels of mIgE-Fc on the surface than SKO-007 cells.

The desired pharmacological targets for anti-CcmX antibodies are mIgE-bearing memory B cells involved in secondary immune responses that generate IgE. The mIgE levels on memory B cells responsible for secondary IgE responses should be higher than those on IgE-secreting plasma cells. The effects of anti-CcmX mAbs on lysis or downregulation of primary mIgE+ B cells and mIgE+ memory B cells remain to be investigated. Studies are now in progress to investigate the potential of anti-CcmX antibodies in causing the reduction of mIgE-bearing B cells and hence serum IgE levels in transgenic mice (SM mouse)™ developed by Kohn-Brevery, Co., Ltd and Medarex, Inc.), which harbor human immunoglobulin genes.

In light of the probably successful development of an anti-IgE antibody (Xolair) for the treatment of asthma and allergic rhinitis [12, 16–19], the pharmacological development of anti-CcmX mAbs may not seem convincing. However, studies on CcmX antibodies will be valuable for the following reasons. The clinical investigations on anti-IgE antibodies have not resolved the question whether the antibodies inhibit or lyse mIgE-expressing B cells and hence downregulate IgE production. The primary reasons for failing to attain a conclusion are that anti-IgE antibodies deplete all free IgE in the blood and that IgE-secreting plasma cells, which do not express mIgE and are not targets of anti-IgE antibodies, are very long-lived in the bone marrow, secreting IgE into the blood, and thus obscuring observation [12]. As anti-CcmX mAbs target only mIgE and not secreted IgE, they should help resolve the question whether mIgE-expressing B cells are inhibited or downregulated.

If anti-CcmX mAbs can indeed inhibit or downregulate IgE synthesis in vivo, these antibodies, after being properly humanized, may be used to treat allergic patients with very high IgE levels because treatment with anti-IgE antibodies is not practical. In patients with high blood IgE levels, the total amount of mIgE on the relatively small numbers of mIgE-expressing B cells should be much less than that of free IgE. Therefore, much less anti-CcmX mAb than anti-IgE mAb will be required for treating those patients. Perhaps more intriguingly, positive results on the effects of anti-CcmX mAbs on inhibiting mIgE-expressing B cells and decreasing IgE synthesis in vivo should support a therapeutic approach that develops an immunogen based on CcmX peptide or cm67 peptide in combination with a foreign peptide for eliciting T cell help. Such an immunogen, if successfully developed, will be able to induce specific autologous antibodies in patients that target mIgE-expressing B cells and hence achieve pharmacological effects similar to those rendered by anti-IgE antibodies or anti-CcmX mAbs.

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References

1. Lipo KP, Kohn R, Rajewsky R. In vivo activation of surface immunoglobulin on mature B cells by soluble antigen: peptide stimulation of cell death. *Cell* 1997;90:1073–1083.
2. Adachi G, Nakaiike I, Lantieri MC. Effect of bromelain on the cytotoxicity of mIgE on the IgE-expressing mouse. *Life Sci* 1997;76:409–417.
3. Kandel J, Schreier E, Rapovsky K. The mouse gamma 1 heavy chain membrane expression and cytoplasmic tail is IgG1 responses. *Science* 1987;236:413–415.
4. Tardieu P. Antigen presentation by mature B cells: The story is in the tail. *Science* 1997;276:374–377.
5. Coffman RL, Lubman DA, Robinson P. Mechanism and regulation of immunoglobulin isotype switching. *Annu Rev Immunol* 1993;54:229–278.
6. Marafioti S, Huang G, Pan DR, Martin SM, Goh R, Nishi MH, Chaplin DD. Affinity maturation without germinal centres in hyper-IgM-syndrome-deficient mice. *Nature* 1996;382:462–464.
7. Borch C, Berger A, Anel M. Maturation of the immune response in germinal centres. *Cell* 1991;67:121–128.
8. Virella ES, Bortat MT, Burger C, Kepner M, Lee WT, Yan XM. Memory B and T cells. *Annu Rev Immunol* 1991;9:193–217.
9. Chang TW, Davis FM, Sun NC, Sun CR, MacGladwin DW, Hamilton RG. Monoclonal antibodies specific for human IgE-producing B cells: A potential therapeutic for IgE-mediated allergic diseases. *Biotechnology (NY)* 1990;8:122–126.
10. Davis FM, Gosselt LA, Chang TW. An epitope on membrane-bound but not secreted IgE: Implications for isotype-specific regulation. *Biotechnology (NY)* 1991;9:33–36.
11. Davis FM, Gosselt LA, Pankas KL, Liao RS, Sun LK, Kuo YW, Chang NT, Chang TW, Warner K, Kuo J, Rinkmann V, Tomlin H, Subramanian N, Hensler C. Can anti-IgE be used to treat allergy? *Springer Semin Immunopathol* 1993;15:31–73.
12. Chang TW. The pharmacological basis of anti-IgE therapy. *Int Biotechnol* 2000;18:157–162.
13. Imaioka K. Reduction of the IgE antibody response. *Int Arch Allergy Appl Immunol* 1989;89:8–13.
14. Tanaka H, Nagai H, Maeda Y. Effect of anti-IgE and anti-IgE antibodies on allergic rhinitis hyperresponsiveness in mice. *Life Sci* 1992;52:PL169–174.
15. Wilets JD, Tish C. Prediction of an anti-IgE binding site on IgE. *Protein Eng* 1996;10:421–427.
16. Tish C. Reducing IgE levels as a strategy for the treatment of asthma. *Clin Exp Allergy* 2000;30(suppl 1):16–21.
17. Adachi G, Rah S, Hahnela T, Asaad G, Rosenblatt J, Zetterstrom O, Byrne A, Chapman K, Thirifery J, Coppo GD, Sandstrom T. Recombinant humanized mAb-E15, an anti-IgE mAb, has been polyclonally induced against allergic rhinitis. *J Allergy Clin Immunol* 2000;106:253–259.
18. Midgren H, Eck RB, Su JO, Reinhardt JD, Bush RA, Watrous ML, Metzger WJ. Treatment of allergic asthma with monoclonal anti-IgE antibody. *Am J Med* 1999;24:1166–1173.
19. Dancow P, Boncompagni E. Anti-IgE therapy for asthma. *Am J Respir Crit Care Med* 1997;155:1825–1827.
20. MacGladwin DW, Bochner BS, Adelman DC, Jarman PM, Togias A, McKenzie White J, Sterbinsky SA, Hamilton RG, Lidstenm LM. Down-regulation of FcεR1 expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol* 1997;158:1438–1445.
21. Yamaguchi M, Soyama K, Yano K, Lantz CS, Noben Trauth N, Ra C, Costa D, Galli SJ. IgE on human FcεR1 receptor: Expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells. Synergistic effect of H-4 and IgE on human mast cell FcεR1 expression and mediator release. *J Immunol* 1999;162:5435–5445.
22. Imaioka N, Ueda S, Hayashida H, Morita T, Hongo T. The nucleotide sequence of the mouse immunoglobulin epsilon gene. Comparison with the human epsilon gene sequence. *EMBO J* 1982;1:1117–1123.
23. Peng C, Davis FM, Sun LK, Liao RS, Kuo YW, Chang TW. A new isoform of human membrane-bound IgE. *J Immunol* 1997;158:1284–1286.
24. Saito A, Zhang K, Ma J-E. Human epsilon mRNA as a membrane sequence: evidence for two membrane forms of IgE and a novel second secreted IgE. *Trans Assoc Am Physicians* 1997;109:51–59.
25. Rucita FD, Efremov DG, Burrows DR. Characterization and expression of alternatively spliced IgE heavy chain transcripts produced by peripheral blood lymphocytes. *J Immunol* 1995;154:209–218.
26. Diaz-Sanchez D, Zhang X, Nigam TB, Saito A. Differential regulation of alternative splicing of epsilon messenger RNA variants. *J Immunol* 1995;155:1930–1941.
27. Nilsson S, Bonnichsen H, Johansson SO, Ponnert J. Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgE) cell lines from an IgE myeloma patient. *Exp Immunol* 1979;7:477–489.
28. Gelfer ML, Margules DH, Schaff M. A simple method for polyethylene glycol-mediated hybridization of mouse myeloma cells. *Somatic Cell Genet* 1977;3:231–236.
29. Liu JT, Bohn JW, Ferry EL, Yamanashi H, Molinaro CA, Sherman LA, Krimm SR, Katz DH. Monoclonal (dinitrophenyl)-specific murine IgE antibody: Preparation, isolation, and characterization. *J Immunol* 1980;124:2728–2737.
30. Shou J-C, Huang J, Chang TW. Inducing specific reactivity against B cells to mice by immunizing with an Fc fusion protein containing anti-IgE. *Cancer Immunol Immunother* 2002;51:145–153.
31. Huang J, Shou J-C, Wu S-C, Chang TW. Down-regulation of B cells by immunization with a fusion protein of a self CD20 peptide and a foreign IgG-Fc fragment. *Immunol Lett* 2002;81:49–58.

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