

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

2020
Bogotá, D.C.,

Abogado
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ASUNTO	Radicación	09 118 682	
	Trámite	011	
	Evento	379	
	Actuación	519	
	Oficio		E 1 4 6 9 0
	Folios	10	

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 mencionó 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.

Las 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 epítipo 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 epítipo 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.

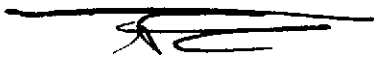
9. Conclusión

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

Item	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 21 AGO. 2012

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

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COOPERMAN, 2003; KATZ & G. PAVEL

Key Words
IgE · mIgE · CemX · 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 IgG (mIgG) on their surface. The ϵ chain of human mIgG

Using synthetic peptides, we found that all five mAbs recognize the same epitope, RADWPGRP, located near the C-terminus of CamX. 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.

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 reprogram B cells to differentially initiate activation of lymphocytes and Ig-secreting plasma cells [2]. Membrane bound Irf (mIrf) is expressed on B cells at certain stages during the process of class switching from naïve (mIrf⁺) to immunocompetent B cells to Irf-expressing B cells and subsequent differentiation to plasma cells in genetically engineered mice lacking the membrane anchoring segment of the α -chain of mIrf (mIrf-anch⁻). The serum IgE level is only 5% of that of wild-type mice [2], suggesting the importance of mIrf, in regulating IgE levels. The functions proposed for mIrf, include class switching [5], affinity maturation [6, 7], memory maintenance [8], and the differentiation of IgE-expressing B cells at the later stages [3].

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 C1q domain of the backbone of an IgE molecule in or near the IgE receptor binding 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 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 mCmX, which extends beyond the CH4 domain, contains an intracellular segment, a transmembrane segment, and an extracellular segment (referred to as migs- α peptide) between the transmembrane segment and CH4 domain of the α -chain [10, 22]. An isoform of the human mCmX contains an extra domain of 52 amino acid (a.a.) residues (named CamX) between the CH4 domain and migs- α peptide [23]. This isoform has been identified in a human IgE-secreting myeloma, SKO-007, and normal blood lymphocytes [23, 24]. A CamX-like domain has not been found in several rodent species studied. At both the mRNA and protein levels, the isoform containing CamX is the dominant one, whereas the conventional form without the CamX domain exists in minor amounts.

isoelectric amounts [3, 25, 26]. The em6 peptide which is expressed in recombinant fusion proteins in the present studies, is composed of the contiguous 57 a.a. CysX domain and the 15 a.a. miga-septide, both of which are unique to human mlgF.

The CcmX-specific mAbs prepared in the present study, which target mIgE and not secreted IgG, can be used to resolve the issue whether the therapeutic anti-IgE antibodies, like Xolair mentioned above, cause the down-regulation of IgE-expressing B cells. If anti CcmX 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 encoding em67 peptide, which can induce antibody response against the CcmX domain and hence mIgE-expressing B cells.

Materials and Methods

Conjugation of Plasmids and Procedures for Transfection

CytA-origes (rm657) was amplified by RT-PCR from rat total RNA of an Ig-secreting cell line, SKO-m07 (ATCC, CRL-9813). A cDNA library was initially derived from 12,266 numbered clones [27], using *wag*-GATC₃Gagcgtgctggctt and gag-TTCGAGccaggcgctgcgt as primers. The amplified product was digested by restriction endonucleases, *Bam*HI and *Xba*I, and ligated between the genomic DNA sequence of CH2-C1B3 domains of human IgG1 and a leucine zipper sequence of CH2-C1B3 domains of human IgM1 FAKELFAKLEPQV (sequence: LEAKIEALEAKIFAI FAKELFAKLEPQV) (FAC) in a plasmid derived from pCDNA3 (Invitrogen, Carlsbad, Calif., USA). Another stable transfectant, used was a CHO cell line transfected by an Fe Expression of mIgE(mIgE-Fc). The cDNA sequence of mFc containing the segment from CH2 domain through the hinge membrane anchor peptide was inserted into pCDNA3 vector. The recombinant plasmid was transfected into CHO cells using a liposome reagent: DMIREC-GINCO BRL, Rockville, Md., USA) according to the manufacturer's instructions. Briefly, 10 µg of plasmid DNA and 10 µg of DMIREC were added to 6 × 10⁶ 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 10% FBS (Calbiochem, San Diego, Calif., USA) and distributed into the individual wells of 96-well plates.

Summary of Transcripts

To identify clones expressing mDf-Fe fusion protein, the activity of the culture medium was measured by ELISA. Briefly, the wells of 96-well microtiter plates were coated with 1 µg/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 100 nmol/l mAb in PBS. The plates were then washed with 0.05% Tween-20 in PBS (pH 7.3) 5 times. The culture medium was applied to the wells and the plates were placed at 37°C for 1 h. After five

the α and β components of SMO (Fig. 7) were also used to form SMO-PCs. The products in the α and β were electrotransferred to poly(vinylidene fluoride) in acetonitrile (Mitsubishi, Rockland, Mass., USA) and were then washed with distilled water and dried under vacuum.

$$(e^{2\pi i} - 1)^{-1} \sum_{k=0}^{\infty} \frac{1}{k!} \left(\frac{d}{dx} \right)^k f(x) \Big|_{x=0} = f(0) + f'(0) + \frac{f''(0)}{2} + \dots$$
[illegible]

Ecological Mapping of Invertebrates in the Long-Spanned Forests

Fourteen peptides (designated p1-p14) of 15-aa residues, which contained 11-aa residues in a stepwise fashion, along the entire length of the em67 segment were synthesized at a local facility for the University of California, San Diego (UCSD), (San Diego, CA) and the University of Maryland (College Park, MD). 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 the mAb in PBS. An anti-Ab at 1 µg/ml was then added and the bound mAb was detected as described above. In the second method, plates were first coated with anti-IgT and SSA-0107. All plates were followed to allow mAb 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 anti-mouse IgG.

1. Introduction

1 × 10⁶ SK-0037 (mIgE) cells or transfected CHO cells were washed with PBS containing 2% FCS and 0.1% sodium azide. Cells were resuspended in 100 µl containing 0.05 µg/ml anti-Cu²⁺ mAb or anti-DNP IgG2b in the presence or absence of 10 µg/ml pentadecylamine for which the anti-Cu²⁺ mAb is specific. Cells were washed once again with 37 °C PBS containing 2% FCS and 0.1% sodium azide and then incubated with 100 µl of 45 min at 37 °C. Cells were washed once again with 37 °C PBS containing 2% FCS and 0.1% sodium azide and then incubated with 100 µl of 10 µg/ml FITC-conjugated F(ab')₂ goat anti-mouse IgG1:Fc (Jackson Laboratory, West Grove, Penn., USA) at 4 °C for 40 min. Cells were washed with PBS and analyzed by FACScan (Becton Dickinson, Franklin Lakes, N.J., USA).

Phonogram Vignettes

SKO-007 cells were incubated with 10 µg/ml anti-CamX at 37°C for 2 h. FITC-conjugated goat anti-rabbit 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 in PBS. The fluorescently 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 μ g anti-Cu²⁺ mAb and 500 μ l of protein G-Sepharose CL-4B. After washing with lysis buffer, the beads were boiled in sample buffer, and the eluted material was subjected to 10% SDS-PAGE. The separated proteins were stained by Coomassie blue and the band corresponding to insulin (confirmed by immunoblotting) was cut off and analyzed by mass spectrometry at The Scripps Research Institute in La Jolla, Calif., USA. The procedure consisted of digestion of the protein by trypsin, extraction of the digested peptides, analysis of the

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cells were grown in the presence of IL-2 (Pierce and Warriner, 1989) or IL-2 plus interferon γ (Pierce and Warriner, 1989). The cells were washed with phosphate buffered saline, adherent medium was removed, and the cells were grown in the presence of IL-2 (Pierce and Warriner, 1989) or IL-2 plus interferon γ (Pierce and Warriner, 1989). The medium was replaced with fresh medium containing IL-2 (Pierce and Warriner, 1989) or IL-2 plus interferon γ (Pierce and Warriner, 1989). The cells were harvested at the indicated times and the medium was assayed for IL-2 (Pierce and Warriner, 1989) or IL-2 plus interferon γ (Pierce and Warriner, 1989).

Pacificization of Reconciliation Processes

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

$$y_{\alpha}^{\beta} = \frac{1}{\Gamma(\beta)} \int_0^{\alpha} (\alpha - \tau)^{\beta-1} y(\tau) d\tau, \quad \beta \in \mathbb{N}, \quad y(0) = y_0.$$

6- to 8-week-old BALB/c mice were injected intraperitoneally (i.p.) and subcutaneously (two sites) with 50 μ g purified recombinant protein in complete Freund's adjuvant (Sigma, Saint Louis, Mo., USA). After 3 weeks, the mice were injected similarly with the same amount of antigen in incomplete Freund's adjuvant (Sigma) thereafter. An additional 1:4 booster immunization was performed every 2 weeks. The anti-m6p titers of the sera from the immunized mice were determined by ELISA. Briefly, 96-well plates were coated with 1 μ g/ml goat anti-human IgG at 4°C overnight and blocked by 3% normal milk in PBS 50 μ l of 1:1000 buffer (serum dilution) for 1 h at 37°C. Then, 50 μ l of 1:1000 buffer (serum dilution) was added to each well. After 1 h at 37°C, the plates were washed with 0.05 M Tris-HCl, 0.15 M NaCl, 0.02% NaN₃, 1.0% Triton X-100, pH 7.4, and 5.0 M EDTA, pH 8.0, was added to each well. The procedure followed the mIpT and secreted IgG from the SK-6-007 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-erythrin.

For preparing hybridomas secreting anti-m67 mAbs, the spleen cells from the tumorized mice were fused with mouse NS0 myeloma cells to form hybridomas 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 hybridomas secreting anti-m67⁺ 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 (Phar-

Immune, Neuroendocrine and Pharmacokinetic Indices

$\times 10^5$ SK-OAG cells were lysed with 1 ml of lysis buffer and the resultant lysate was mixed with 3 μ g of anti-human IgG, anti-mouse IgG mAb, or control anti-DNP IgG [29] at 4°C for 1 h. 80 μ l of protein A-Sepharose 4B (Pharmacia) was added to absorb the formed immune complexes. Cell debris was 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 IgG (Fig. 2, lanes 1–3). For the analysis of binding of anti-

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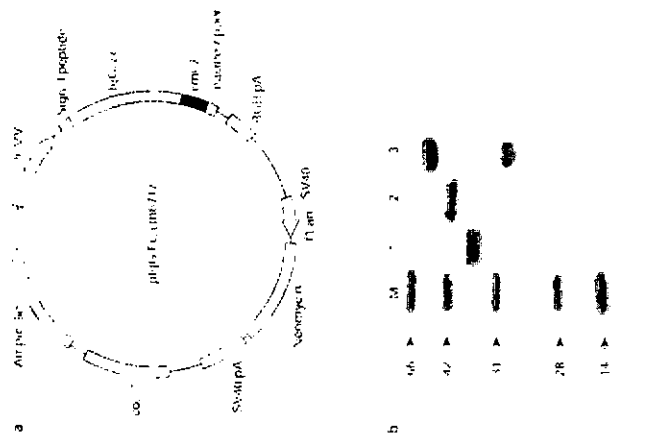


Fig. 1. Construction of the plasmid and analysis of IgG₁Fe fusion protein. **a**, Recombinant plasmid was constructed by inserting *em67* into the 3' end of the IgG₁Fe genomic sequence. **b**, The purified recombinant protein was analyzed by SDS-PAGE. M = Molecular weight markers; lane 1 = IgG₁Fe; lane 2 = *em67*Fe; 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 (LC-MS/MS) spectrometry.

Contribution: We have developed a

cells (4×10^4 CHO cell transfectants expressing $\text{mgE}::\text{Fc}$ or $\text{SKO}::\text{Fc}$) were placed in individual wells of 96-well plates and the cells were cultured overnight. Toward serial dilutions (fluid concentrations of 0.25–4 $\mu\text{g/ml}$) of an anti-CmV mAb or a control mAb (anti- β -galactosidase) were added in the presence of 15-fold-diluted baby rabbit complement (Serotec, Raleigh, N.C., USA). After incubation for 1 h at 37°C in 5% CO_2 incubator, five cells were determined using a ST-4 assay (Dainippon, Kumamoto, Japan).

123 216 300 384 468 552 636 720 804 888 972 1056 1140 1224 1308 1392 1476 1560 1644 1728 1812 1896 1980 2064 2148 2232 2316 2400 2484 2568 2652 2736 2820 2904 2988 3072 3156 3240 3324 3408 3492 3576 3660 3744 3828 3912 3996 4080 4164 4248 4332 4416 4500 4584 4668 4752 4836 4920 5004 5088 5172 5256 5340 5424 5508 5592 5676 5760 5844 5928 6012 6096 6180 6264 6348 6432 6516 6600 6684 6768 6852 6936 7020 7104 7188 7272 7356 7440 7524 7608 7692 7776 7860 7944 8028 8112 8196 8280 8364 8448 8532 8616 8700 8784 8868 8952 9036 9120 9204 9288 9372 9456 9540 9624 9708 9792 9876 9960 10044 10128 10212 10296 10380 10464 10548 10632 10716 10800 10884 10968 11052 11136 11220 11304 11388 11472 11556 11640 11724 11808 11892 11976 12060 12144 12228 12312 12396 12480 12564 12648 12732 12816 12900 12984 13068 13152 13236 13320 13404 13488 13572 13656 13740 13824 13908 13992 14076 14160 14244 14328 14412 14496 14580 14664 14748 14832 14916 15000 15084 15168 15252 15336 15420 15504 15588 15672 15756 15840 15924 16008 16092 16176 16260 16344 16428 16512 16596 16680 16764 16848 16932 17016 17100 17184 17268 17352 17436 17520 17604 17688 17772 17856 17940 18024 18108 18192 18276 18360 18444 18528 18612 18696 18780 18864 18948 19032 19116 19200 19284 19368 19452 19536 19620 19704 19788 19872 19956 20040 20124 20208 20292 20376 20460 20544 20628 20712 20796 20880 20964 21048 21132 21216 21300 21384 21468 21552 21636 21720 21804 21888 21972 22056 22140 22224 22308 22392 22476 22560 22644 22728 22812 22896 22980 23064 23148 23232 23316 23400 23484 23568 23652 23736 23820 23904 23988 24072 24156 24240 24324 24408 24492 24576 24660 24744 24828 24912 25000

Results

Preparation of sub- β_1 Fusion Protein and Anti-sub- β_1 Antibody

The expression level of the recombinant E₂ fusion protein containing em67 peptide (C₁emX-mig-s-segment) by transfectants described in Materials and Methods was approximately 1×10^4 p.p.1 $\times 10^4$ cells/day. Several milligrams of the recombinant protein were obtained from 1 liter of culture medium by using affinity purification with protein A-Sepharose C1-4B column. Analysis of the purified fusion protein showed that it was not contaminated by bovine IgG (fig. 1). Probably because em67 gene encodes 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, a31. The heavy-chain isotypes of the mAbs secreted by these hybridomas are μ for a10, a20, a23, a25, and a31 and δ for a11, a21, a24, a26, a27, a28, a29, a30, a32, and a33. The light-chain isotypes are κ for a10, a20, a23, a25, and a31 and λ for a11, a21, a24, a26, a27, a28, a29, a30, a32, and a33.

immunoprecipitation and immunoblotting of mAb in
 10⁶ + Cell Lysates by: 101-5967 mAb

to characterize the binding specificity of the anti- ϵ 7 mAbs, their reactivity with the Triton (detergent) eluted of SKO-007 cells, which should contain mIgE and anti-IgE, was analyzed. In one set of experiments, the 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 colometric 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-cell lysate was subjected to SDS-PAGE and the resulting gels reacted with either goat anti-IgE antibodies or anti-em67 mAbs coupled to colormetric 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 an X domain and can be recognized by anti-em67

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Characterization of Binding of Anti-em67 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-em67 mAbs to mlgE was inhibited by all other anti-em67 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 em67 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-em67 mAbs

Since the anti-em67 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 em67. 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-em67 mAbs. In one set of ELISA using plates coated with different peptides, it was found that all these anti-em67 mAbs bound to peptides p10, p11 and p12, which represent overlapping sequences containing RADWPQPP located near the C-terminal of the CamX domain (Fig. 4). In another set of ELISA analyzing the inhibitory effects of the peptides on the binding of anti-em67 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-em67 mAbs are all specific to a linear epitope containing RADWPQPP on the CamX domain and not to the 15-a.a. mlgE-6 peptide.

Binding of Anti-CamX mAbs to the Surface of mlgE+ Cells

To examine whether the anti-CamX 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

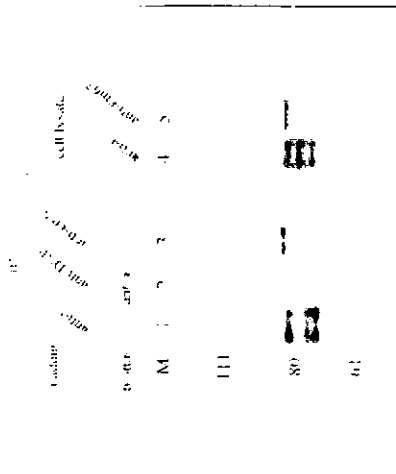


Fig. 2. Immunoprecipitation and immunoblotting analysis of binding of anti-em67 to mlgE. SKO-007 cell lysate was treated with goat anti-human IgG (lane 1), anti-DNP IgG2b (lane 2) or anti-em67 a20 (lanes 3-5) followed by incubation with protein G-Sepharose (CL-4B beads). The absorbed proteins were eluted and loaded onto individual lanes. Alternatively, the lysate was loaded directly, subjected to electrophoresis, and electro-transferred to polyvinylidene difluoride (lanes 4 and 5). IgE-related proteins were detected by using HRP-conjugated goat anti-IgG (lanes 1-3), or anti-em67 a20 (lanes 4 and 5) followed by HRP-conjugated anti-mouse IgG.

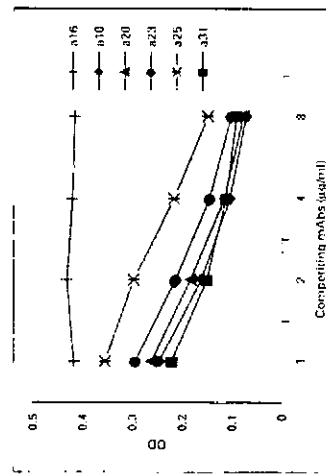


Fig. 3. Mutual inhibition of binding of anti-em67 mAbs to mlgE. ELISA plates were coated with a20 mAb. Equal aliquots of SKO-007 cell lysate were mixed with various concentrations of anti-em67 mAb, a10, a20, a25, or a31, or a negative control mAb, a16 before addition to a20-coated wells. Bound mlgE was detected by HRP-conjugated goat anti-human IgG.

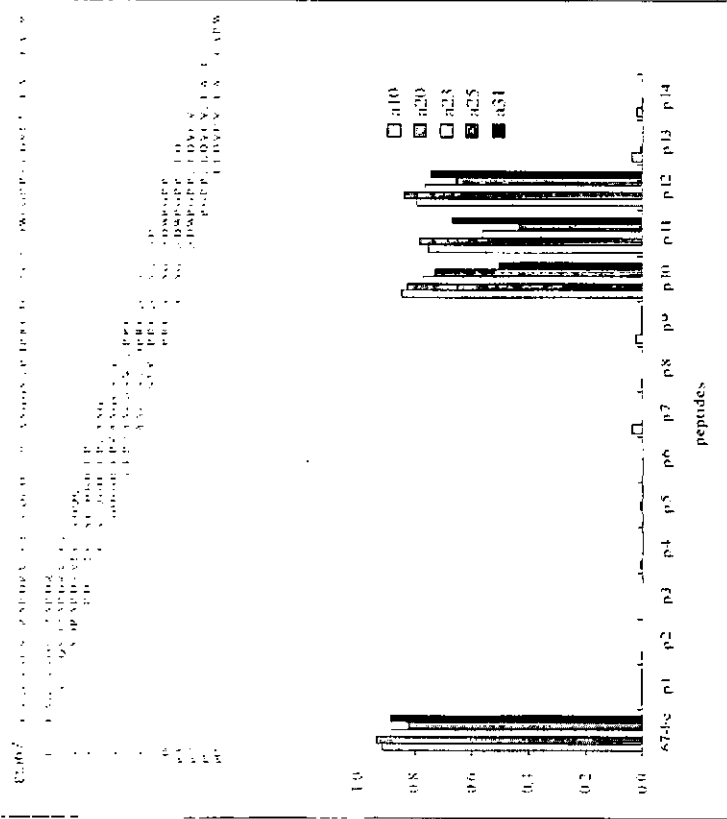


Fig. 4. Epitope mapping of mAbs using synthetic peptides. Plates were coated with 10 μg/ml of peptides or em67-Fc (67-Fc). Aliquots of various anti-em67 mAbs at 1 μg/ml were added and the bound mAb was detected by HRP-goat anti-mouse IgG.

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, and capping (Fig. 7b) was observed on some of the cells. It should be noted that the fluorescent second antibody, FITC-conjugated Fab' 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-CamX-Precipitated mlgE by Mass Spectrometry

To verify that the mlgE recognized by the anti-CamX mAbs contains CamX, 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

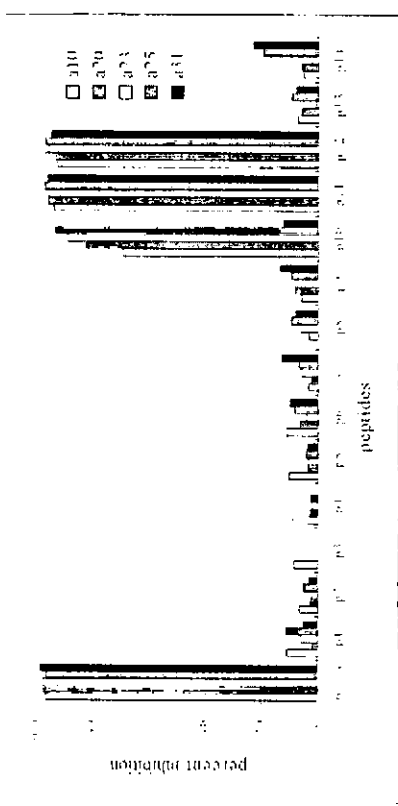


Fig. 5. Inhibition of binding of anti-anti-CemX mAbs to mlgE by synthetic peptides. Plates were first coated with goat anti-mlgE and SKO-107 cell lysate was applied to allow mlgE to be captured (Materials and Methods). Aliquots of mAbs at 1 μ g/ml mixed with 5 μ g/ml of the inhibitors, peptides or anti63-1 (67-10), 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.

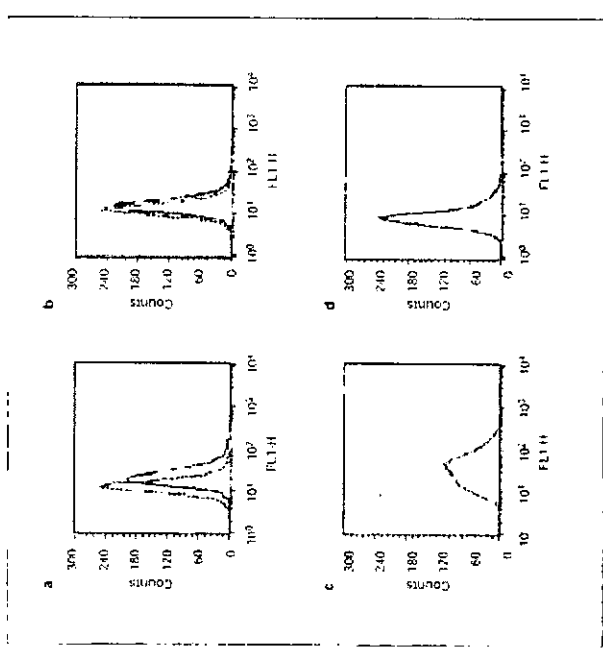


Fig. 6. Binding of anti-anti-CemX mAb on the surface of mlgE+ cells. SKO-107 (a, b) and CHIO transfectant (c, d) cells were incubated with either control anti-DNP IgG2b, detected by FITC-conjugated Fab2, or presence of an inhibiting peptide (a, c) or presence of an anti-anti-CemX a20 (solid line) at 10 μ g/ml in the absence (dashed line) for 30 min at 37°C. The cells were labeled with FITC-conjugated Fab2, goat anti-mouse IgG1 and analyzed by fluorescence flow cytometry.

Anti-CemX mAbs Specific for mlgE

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Fig. 7. Induction of pitting (a) and pitting (b) on mlgE+ cells by anti-CemX mAb SKO-107. Cells were incubated with 10 μ g/ml of anti-CemX a20 mAb or 7 h at 37°C. After washing the cells were observed with FITC-conjugated Fab2; goat anti-mouse IgG1 at 37°C for 30 min and observed with a confocal microscope.

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 mlgE-Fe Transfectants by Anti-CemX mAb

To study whether anti-CemX mAbs could be used to target mlgE+ B cell, mlgE-Fe-transfected CHIO 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 μ g/ml.

Discussion

In this study, an immunogen in the form of an Fe fusion protein containing emf7 unique to human mlgE 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 mlgE. One of the mAbs, which was studied more extensively, was shown to be capable of inducing the cross-linkage of

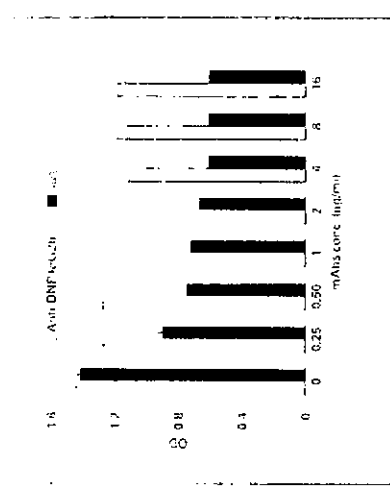


Fig. 8. Induction of complement-mediated cytotoxicity by anti-CemX mlgE-Fe transfectants. 4×10^5 cells were cultured in a 96-well plate overnight, and 0.16 μ g/ml of anti-CemX a20 (■) or control, anti-DNP IgG2b (□) were added together with 15-fold diluted baby rabbit complement and the mixtures were cultured at 37°C for 2 h. Live cells were determined by a WST-1 assay.

mlgE on B cells as well as complement-mediated cytotoxicity of transfectants expressing mlgE-Fe on the surface.

The rationale for employing the Fe of human IgG1 as a fusion partner for emf7 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 emf7 immunogens in other forms of molecular constructs although we have not been successful in attempts at using emf7 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 mlgE-expressing B cells. These mAbs do not bind to Fc γ RII-bound IgE and should not recognize Fc γ RII-expressing B cells, which do not express mlgE, or other Fc γ RII-expressing cells such as monocytes.

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Epitope mapping of the five anti-Cu₂N mAbs showed that they bind to a linear peptide, RA-DWpGPP, located near the C-terminus of the Cu₂N domain. These results have several implications. First, the conformation of this sequence in the waf-24c fusion protein and nlp1 is similar to that in the synthetic peptide. Alternatively, synthetic peptides containing this epitope may be induced to fit the binding site of the Fc of the mAbs. Second, this peptide segment in Cu₂N is exposed on the surface of the protein. The RA-DWpGPP 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 miz-chain. The results on the peptide masses yielded by MALDI-TOF also indicate that there is no posttranslation modification on these peptides in the miz-chain.

In this study, the ability of anti-CemX mAbs on targeting and lysing mlgE- β cells was examined. The mAb $\alpha 20$ could induce patching and capping of mlgE on the surface of SKO-007 cells although such cross-linking of mlgE did not affect cell growth [unpubl. obs.]. This is possibly due to the low expression level of mlgE on SKO-007 cells or to the fact that the cells are of a terminally differentiated myeloma cell line. The low expression level of mlgE on SKO-007 cells was also shown by the low level of fluorescence staining by the anti-CemX mAbs in flow cytometric analysis. The results are consistent with the finding that the anti-CemX 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-CemX mAb $\alpha 20$ was able to cause complement-dependent cytotoxicity on the mlgE-Fe-expressing transfectants, which expressed relatively higher levels of mlgE-Fe on the surface than SKO-007 cells.

The desired pharmacological targets for anti-CemX 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-CemX 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-CemX auto-

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bioassay in causing the reduction of adjuvanted BCG vaccines. Hence serum IgG levels of tuberculin (Mantoux test) developed by Knitt Brewery, Co. Ltd. (McArdle, Inc.), which harbor human immunoglobulin genes

In light of the probably successful development of an anti-IgE antibody (Nofar) for the treatment of asthma and allergic rhinitis [11, 16–19], the pharmacological development of anti-CmX mAbs may not seem convincing. However, studies on CmX 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, 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-CmX 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-CamX 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 IgE-expressing B cells should be much less than that of free IgE. Therefore, much less anti-CamX mAb than anti-IgE mAb will be required for treating those patients. Perhaps more intriguingly, positive results on the effects of anti-CamX mAbs on inhibiting mIgE-expressing B cells and decreasing IgE synthesis *in vivo* should support a therapeutic approach that develops an immunogen based on CamX peptide or em67 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-CamX mAbs.

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- [illegible]

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