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ANTIGENIC EPITOPES PRESENT ON MEMBRANE-BOUND BUT NOT SECRETED IgA

Field of the Invention

The invention relates to immune system enhancement through antibody binding of epitopes which are present on B cell-bound but not secreted IgA, leading to increased IgA production by such B cells.

Background

B lymphocytes produce five classes of immunoglobulins, which mediate different functions. IgM is most effective in complement fixation, IgG causes opsonization and cellular cytotoxicity and can cross the placenta. IgA functions on the mucosal surface and IgE mediates degranulation of mast cells and basophils. The function of IgD is still not well understood. These antibodies are present in the blood circulation, with IgG, IgA, and IgM being the major serum components. In addition to secreting immunoglobulins, the B cells also express different isotypes of the immunoglobulins on their cell surfaces at different stages of maturation. IgM and IgD are present on the surface of resting, uncommitted B cells, while often only one of the five classes of immunoglobulins may exist on

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☐ Description	: paged to paged	
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late stage, mature B cells, which secrete the same immunoglobulin isotype as expressed on their cell surface. Teale, J.M., et al., J. Immunol. 1126: 1952-1957 (1981); Gathings, W.E., et al., Immunol. Rev. 57:107-126 (1981); Teale, J.M., Fed. Proc. 41:5-9 (1982).

Numerous pathogenic microorganisms, such as bacteria and viruses, enter the human and animal bodies through the respiratory, gastrointestinal, and genitourinary tracts during air inhalation, food and liquid intake, and sexual contact. The potentially allergenic substances, e.g., tree, grass, and flower pollens, dust mites, fungal particles and animal dander also enter the body through the respiratory tract. Secretory IgA antibodies help to defend against these pathogens and allergens.

IgA is produced by plasma cells located along the mucosal linings of the aforementioned tracts, which are all exposed to the external environment. The α chain and light chain immunoglobulins produced by plasma cells combine with a secretory component produced by the epithelial cells in the mucosal tissues, forming secretory IgA molecules that are secreted to the surface of mucosal layers. See generally J. G. Nedrud et al., "Adjuvants and the Mucosal Immune System", Topics in Vaccine Adjuvant Research, (Spiggs, D. E., Koff, W. C., Eds.) CRC Press, Boca

Raton, Fa. (1990). These secretory IgA molecules bind to the invading pathogens and weaken their ability to penetrate the mucosal layer and to enter the inner tissue and blood stream of the host. See generally J. G. Nedrud et al., "Adjuvants and the Mucosal Immune System", Topics in Vaccine Adjuvant Research, (Spiggs, D. E., Koff, W. C., Eds.) CRC Press, Boca Raton, Fa. (1990). IgA can also bind allergenic substances, thereby preventing the allergens from binding IgE or activating the T cells responsible for delayed-type hypersensitivity.

It has been found that individuals with low IgA production are more prone to various infectious diseases and have a higher tendency to develop allergic diseases than those with normal IgA levels. Thus if the levels of either total IgA or antigen-specific IgA can be increased, the diseases or allergies may be prevented.

It is also well known that various allergenic substances enter through inhalation and food ingestion, causing immediate-type, antibody-mediated hypersensitivities and delayed-type, cell-mediated hypersensitivities. In sensitized individuals, the IgE-mediated reactions against pollens, animal danders, dust mites, and other allergenic antigens cause the common allergic symptoms, such as allergic rhinitis ("hay fever") and extrinsic

asthma. In such allergic responses, the allergens enter the mucosal layers of the respiratory tracts and nasal linings and bind to allergen-specific IgE which is on the surface of basophils and mast cells. The binding of IgE by the allergens on the basophils and mast cell surface causes cross-linking of the underlying IgE Fc receptors, and triggers the release of histamines and other pharmacologic mediators, resulting in various allergic symptoms. In the cell-mediated hypersensitivities, certain T helper cells responsible for delayed-type hypersensitivity are activated. These T cells recruit and activate macrophages, causing inflammatory symptoms.

It has been shown that antibodies which bind to epitopes of B cell membrane-bound immunoglobulins can be used to eliminate B cells producing the immunoglobulins. In particular, antibodies specific for the antigenic epitope located on the transmembrane anchoring peptide of B cell membrane-bound (but not secreted) IgE can be used for removing IgE secreting B cells in patients suffering from IgE-mediated allergies, as described in the published international PCT/US88/04706 patent application, filed Dec. 29, 1988.

Antibodies that belong to certain immunoglobulin classes and subclasses, such as murine IgG₂ and human IgG₁, and that bind with an

appropriately high affinity to surface antigens of target cells can lyse those target cells. However, as noted above, not all antibodies specific for target cells will cause cytolysis, and some in fact cause isotype switching, proliferation, and increased or decreased antibody production. See Vitetta, E.D., et al., Immunol. Rev. 52: 211-231 (198); Cooper, M.D., et al., Immunol. Rev. 52:29-53 (1980). In numerous studies, polyclonal antibodies have also been shown to induce B cell proliferation. See Sell, S. and Gell, P.G.H., J. Exp. Med. 122:423-44 (1965); Kishimoto, T., et al., J. Immunol. 115:1179-1184 (1975); Parker, D.C., Nature 258:361-363 (1975); Sieckmann, D.G., et al., J. Exp. Med. 147:814-829; Pure, E. and Vitetta, E.S., J. Immunol. 125:1240-1242 (1980). Unlike antibody-dependent cellular cytotoxicity and complement-mediated cytolysis, this proliferative response does not seem to involve the Fc of the antibodies. It has been shown that F(ab')₂ is more effective than whole antibody in inducing the proliferative effect, Vitetta, E.S. et al., Immunol. Rev. 52:211-231 (1980), indicating the absence of Fc involvement.

Numerous investigators have studied effects of anti-Ig antibodies on the activity of B cells. A review article, "Effects of anti-immunoglobulin sera on B lymphocyte function", Immunol.Rev. Vol. 52, ed. by Moller, G.

(1980), states that anti-Ig antibodies have a variety of effects on B cells, including the ability to cause proliferation of B cells. In addition, anti-IgM and anti-IgD antibodies appear to be able to switch the resting uncommitted B cells to producers of IgG and IgA. These studies show that divalent antibodies which cross-link the surface Ig are required to stimulate B cell proliferation. These effects do not require Fc portions of the antibodies and, in fact, the F(ab')₂ fragments appear to be more effective than whole IgG in stimulating B cell proliferation.

Proliferating B cell with anti-Ig antibodies seem highly desirable for enhancing antibody production in vivo. However, proliferation is difficult to achieve. Because of the large concentrations of IgG, IgM, and IgA antibodies in the circulation, anti-Ig antibodies which are administered will bind to the circulating Ig before they can bind to significant amounts of the surface Ig on B cells. What is needed is a B cell proliferative agent which reacts with membrane-bound immunoglobulins and causes proliferation or B cell modulation without reacting with the secreted, circulating immunoglobulins.

Summary of the Invention

The invention includes the membrane anchoring peptide of human

α chain, antibodies and related products to epitopes on the extracellular segment of this peptide (designated herein as the extracellular *migs*- α epitopes), and prophylactic and therapeutic treatments involving such antibodies and related products. These extracellular peptide segments form, entirely or in part, antigenic epitopes unique to membrane-bound but not secreted IgA.

The membrane anchoring extracellular peptide segment for human IgA has the amino acid sequence:

GSCS(or C)V·ADWQM·PPPYV·VLDL·PQETL·EEEEPG·AN

Peptides including this sequence or an immunologically equivalent sequence (or a epitope thereof) can be conjugated to protein carriers and used to induce formation of endogenous antibodies to the extracellular *migs*- α epitopes. These endogenously produced antibodies modulate the synthesis of IgA and, in particular, increase its synthesis. When the antibodies specific for the extracellular *migs* epitopes are of certain immunoglobulin classes and subclasses, such as mouse IgG₁ or human IgG₂, or in the form of divalent antigen binding fragments such as F(ab')₂, they can be administered to directly enhance IgA synthesis. Further, peptides

representing IgM or IgD, or antibody specific for these peptides, can be used to stimulate IgA production. The peptides and antibodies can be used alone, or combined with vaccines or allergenic antigens, to increase IgA synthesis. The patient population includes those susceptible to infectious diseases and those affected by allergic diseases.

Brief Description of the Figures

Figure 1 shows, for genomic DNA, the deduced amino acid sequence and the nucleotide sequence of the membrane exon of two isoforms of human $\alpha 1$ membrane anchoring peptide (one isoform including the amino acids shown in lower case letters) as well as about 1,700 bp of the 5' flanking sequence and about 500 bp in the 3' untranslated region.

Figure 2A shows the nucleotide sequence of the membrane exon in Fig. 1 for human $\alpha 1$ and $\alpha 2$, as well as the corresponding sequence from murine α chain.

Figure 2B shows the amino acid sequences encoded by the membrane exons of Fig 2A, the proposed extracellular segments being underlined.

Detailed Description of the Invention and Its Manner and Process of Making and Using

1. Membrane Anchoring Peptides of B Cell Membrane-bound Immunoglobulins

The membrane-bound immunoglobulins expressed in the surface of B cells differ from the secretory immunoglobulins synthesized by the same cells in that the former have extra peptidic segments extending from the C-termini of the immunoglobulin heavy chains which anchor the immunoglobulins to the cell surface. These extra segments are unique for different isotypes and are referred to herein as the membrane anchoring peptides.

The amino acid sequence data of the eleven membrane-bound immunoglobulins from several species have been determined. See Word, C.J. *et al.*, EMBO J. 2:887-898 (1983); Ishida, N. *et al.*, EMBO J. 1:1117-1123 (1982); Steen, M. L. *et al.*, J. Mol. Biol. 177:19-32 (1984); Rogers, J. *et al.*, Cell, 26:19-27 (1981); Yamawaki-Kataoka, Y. *et al.*, Proc. Natl. Acad. Sci. USA, 79:2008-2012 (1982); Kamaromy, M. *et al.*, Nucleic Acids Res., 11:6775-6785 (1983); Rogers, J. *et al.*, Cell, 20:303-312 (1980); Bernstein, K.E., J. Immunol. 132: 490-495 (1984); Cheng, H. *et al.*, Nature.

296:410-415 (1982); Robbitts, T. H. et al, Nucleic Acids Res. 9:4509-4524.

These sequences indicate certain common features of the membrane anchoring peptides. As shown in Table 1, the membrane anchoring peptide has three segments which are distinguishable based upon their locations in relation to the plasma membrane. Even though these peptides are short, ranging from 41 to 130 amino acid residues, and have often been referred to as the "membrane-bound domain", the peptides are not entirely in the membrane lipid bilayer. In fact, only 25 amino acid residues, largely hydrophobic residues and threonine and serine residues which are located in the middle part of the peptides, are in the lipid bilayer. The C-terminal, hydrophilic segments of 3 to 28 amino acid residues are located on the cytoplasmic side of the membrane. The segments toward the N-terminus, which are connected to the third or fourth constant domains of the immunoglobulin heavy chains (CH_3 or CH_4) are very hydrophilic and are on the extracellular side of the plasma membrane.

Table 1. Key features and properties of peptidic segments unique to membrane-bound immunoglobulins on B cells.

	First segment	Middle segment	Last segment	Total
Immunoglobulin	<u>Length</u>			
<u>Class/Subclass</u>	# Amino acid residues			
Mouse IgA	26	25	14	65
Mouse IgE	19	25	28	72
Rat IgE	19	25	28	72
Mouse IgG ₁	18	25	28	71
Mouse IgG _{2a}	18	25	28	71
Mouse IgG _{2b}	18	25	28	71
Mouse IgG ₃	18	25	28	71
Mouse IgM	13	25	3	41
Human IgM	13	25	3	41
Human IgD	27	25	3	55
Mouse IgD	26	25	3	54
Properties	Hydrophilic Highly acidic	Hydrophobic No charged residues	Hydrophilic	
Physical Location	On exterior surface	In membrane lipid bilayer	On cytoplasmic surface	
Abbreviated Symbols	<u>mb/ec</u> segment	<u>mb/tm</u> segment	<u>mb/ic</u> segment	

* mb stands for membrane-bound; ec for extracellular; tm for transmembrane; and ic for intracellular.

The shortest extracellular segments of the membrane-bound pieces of the immunoglobulins (designated mb/ec segments) have 13 amino acid residues (mouse and human μ chains). See Table 2. The mb/ec segments of all immunoglobulins contain high proportions of charged, acidic, amino acid residues. The charged amino acid residues and polar hydrophilic residues account for a very high percentage of the amino acids in the mb/ec segment (Table 3). This indicates that all the mb/ec segments are exposed and of sufficient length to be accessible by antibodies. The immunoglobulin heavy chains evolved before the various mammalian species, including mice, rats, and humans. Thus, it is probably the murine α .mb/ec to which the human α .mb/ec will be most related. The murine α .mb/ec has 26 amino acid residues, including 5 Glu and 2 Asp residues, both of which are acidic. This indicates that the proposed similar human α .mb/ec segment also has acidic residues, and is exposed and accessible to antibodies.

Table 2. The amino acid sequences of the exterior portion of peptidic segments unique to membrane-bound immunoglobulins (mb/ec segments).

		<u>Mb/ec</u> segment					
		26	21	16	11	6	1
Mouse	IgA	E · RQEPL · SYVLL · DQSQD · ILEEE · APGAS					
Mouse	IgE		· ELDI · QDLCI · EEVEG · EELEE				
Rat	IgE		ELDI · QDLCT · EEVEG · EELEE				
Mouse	IgG ₁		GLQ · LDETC · AEAQD · GELDG				
Mouse	IgG _{2a}		GLD · LDDVC · AEAQD · GELDG				
Mouse	IgG _{2b}		GLD · LDDIC · AEAKD · GELDG				
Mouse	IgG ₃		ELE · LNGTC · AEAQD · GELDG				
Mouse	IgM			EGE · VNAEE · EGFEN			
Human	IgM			EGE · VNAEE · EGFEN			
Human	IgD	YL · AMTPL · IPQSK · DENSD · DYTTF · DDVGS					
Mouse	IgD	I · VNTIQ · HSCIM · DEQSD · SYMDL · EEENG					

Table 3. Composition of charged amino acid residues and polar, hydrophilic amino acid residues in the exterior portion of peptidic segments unique to membrane-bound immunoglobulins (mb/ec segments).

	Acidic residues	Basic residues	Polar residues	Total hydro- philic residues	Proportion of hydro- philic residues
# Amino acid residues					
Mouse IgA	7	1	7	15	58
Mouse IgE	10	0	2	12	63
Rat IgE	10	0	2	12	63
Mouse IgG ₁	6	0	4	10	56
Mouse IgG _{2a}	7	0	2	9	50
Mouse IgG _{2b}	7	1	1	9	50
Mouse IgG ₃	6	0	4	10	56
Mouse IgM	6	0	2	8	61
Human IgM	6	0	2	8	61
Human IgD	6	1	8	15	56
Mouse IgD	7	0.5	9	16.5	63
Acidic residues:	E (Glu), D (Asp)				
Basic residues:	K (Lys), R (Arg), H (His); His is partially charged.				
Polar residues:	S (Ser), T (Thr), C (Cys), Q (Gln), N (Asn)				

4. The Amino Acid Sequence of the mb/ec Segment of Human IgA (α .mb/ec segment)

The DNA sequence corresponding to the human α .mb/ec segment was determined as set forth in the exemplification below. The nucleotide sequence of the exon encoding the peptide segment and the amino acid sequence of the segment are shown in Figures 1 and 2.

The α .mb/ec peptides of this invention include the amino acid sequence shown in Figure 2, any immunologically active portion of the sequence or any epitopes contained in this sequence. In addition, the α .mb/ec peptides include any modifications of the amino acid sequence of Figure 2 in which amino acids have been added, inserted, deleted or substituted without detracting significantly from the immunological activity of the peptide. The immunological activity of the peptide includes reactivity with antibody and the ability (alone or conjugated to a carrier) to induce an immune response, including inducing an antibody response which is crossreactive with both the α .mb/ec segment and with native IgA on the cell membrane.

Such immunogenic peptides can be synthesized by conventional

techniques, such as with the Ramp system (DuPont DeNemours & Co.) which applies Fmoc chemistry. Alternatively, these recombinant peptides or immunoglobulin heavy chains containing epitopes of these peptides may be biosynthesized by expressing in E. coli or mammalian cells the gene segments containing coding sequences of the peptide.

5. Developing Antibodies to the mb/ec Segment

The α .mb/ec peptide can be used in the immunization of animals to prepare polyclonal and monoclonal antibodies. It can also be used to screen for specific monoclonal antibodies or characterize specific polyclonal antibodies. It can also be used to purify monoclonal and polyclonal antibodies.

In the process of preparing monoclonal antibodies specific for α .mb/ec peptide, it is not necessary to use the synthetic or recombinant α .mb/ec peptides in both immunization and antibody identification. For example, in immunizing mice for preparing immune spleen cells for fusion with myeloma cells, the immunogen may be the membrane-bound IgA isolated from plasma membrane of IgA-bearing myeloma cells, such as

DAKIKI lymphoblastoid cells, or it may be the IgA-bearing myeloma cells themselves. Transfectomas, developed by transfecting mouse myeloma cells with genes of human immunoglobulin heavy chains and light chains and which express on their cell surface membrane-bound immunoglobulins, may also be used as immunogens. For initial monoclonal antibody identification following immunization, the aforementioned synthetic peptide conjugated to bovine serum albumin or ovalbumin with the techniques described below are preferably used.

When using the synthetic or recombinant α .mb/ec peptides as immunogens, it is more effective to conjugate them to a protein carrier, for example, hepatitis B surface antigen, core antigen or preferably keyhole limpet hemocyanin (KLH). If the peptidic segment lacks a Lysine residue or if the Lysine residue is in the middle part of the segment, it is desirable to add a Lysine residue at the C-terminal end. Because the N-terminus already has an α -amino group, the modified synthetic peptidic will have two amino groups for linking.

Multiple molecules of peptides can be conjugated to each molecule of the carrier protein. With KLH, a preferred molar ratio for peptide/KLH is 10. The method of conjugation is well established. Cross-

linkers such as bis (sulfosuccinimidyl) suberate or disulfosuccinimidyl tartarate (Catalogue #21579, 20591, Pierce Chemical Co., Rockford. IL), or preferably glutaraldehyde, can be used.

The immunogen, e.g. the KLH conjugate, can be used to immunize rabbits, goats, rats, or mice to prepare polyclonal antibodies specific for the α .mb/ec peptide. Lymphocytes from the spleen or lymph nodes of immune mice and rats can also be taken to prepare hybridomas secreting monoclonal antibodies specific for the α .mb/ec peptide. A preferred protocol to prepare the monoclonal antibodies is to fuse immune spleen cells of mice with non-secreting mouse myeloma cells, such as NS-1 or SP2/0 cells, using polyethylene glycol.

A preferred immunization procedure for mice is to prime each mouse by injecting 50 μ g of the peptide-KLH conjugate in complete Freund's adjuvant subcutaneously into each mouse. Two and four weeks later, the same amounts of antigen are given subcutaneously in incomplete Freund's adjuvant. After about six weeks, the fourth antigen injection is given intraperitoneally in saline. Mice are sacrificed 4 days after the last injection and the spleens are removed for preparing single cell suspension for fusion with myeloma cells.

A similar protocol can also be used for immunization with other immunogens. For example, a similar protocol can be used where the immunogen is purified native human membrane-bound IgA (having an attached membrane anchoring peptide) isolated from the plasma membrane of IgA-bearing human myeloma cells, such as DAKIKI lymphoblastoid cells, or where the immunogen is recombinant α chain produced by genetically engineered bacteria.

The fusion procedure with polyethylene glycol and other various procedures concerning cloning and hybridoma culturing have been well established. The preferred protocol is the well-known one described, for example, by Hudson, L. and Hay, F.C. (Practical Immunology, 2nd edition, pp. 303-313, 1980, Blackwell Publishing Co., Boston).

The screening of hybridomas for monoclonal antibodies (or the identification of polyclonal antibodies) reactive with α .mb/ec peptide can be performed with an enzyme linked immunosorbent assay (ELISA) using the synthetic or recombinant α .mb/ec peptide as the solid phase antigen. An alternative solid phase antigen is the conjugate of α .mb/ec peptide with a carrier protein different from that used as the immunogen, such as ovalbumin or bovine serum albumin.

Further characterization of the monoclonal and polyclonal antibodies are shown in Table 5. The assays employed in these studies are also indicated. The assays have been described in detail in the U.S. Patent Application Serial No. 07/226,421, filed July 29, 1988, and U.S. Patent Application Serial No. 07/140,036, filed December 31, 1987, the teachings of which are incorporated by reference herein.

Table 5. The Reactivity of Antibodies Specific for the α .mb/ec Peptide with Different IgA-Containing Targets

	Reactivity	<u>Assays</u>
Synthetic α .mb/ec peptide	+	ELISA
Soluble IgA	-	ELISA
DAKIKI myeloma cells	+	Immunofluorescence staining
IgA-bearing B cells	+	Immunofluorescence staining
Cells not expressing surface IgA	-	Immunofluorescence staining

6. Experiments with Animal Models

The substances and methods are tested on animal model systems. A number of experiments are designed to investigate whether peptides

representing ec/mb segments of various immunoglobulins and the antibodies specific for epitopes on these ec/mb segments will enhance the production of immunoglobulins of various isotypes in the animals. The peptides and antibodies relating to a particular isotype may affect the synthesis of several isotypes and subclasses. In the discussion below, we will primarily focus on peptides and antibodies relating to α .ec/mb segment. The purpose of the animal studies is to investigate:

- a) Do the peptides and antibodies enhance antibody production?
- b) Do the peptides and antibodies enhance secretory IgA production in the mucosal surface?
- c) Can the peptides and antibodies be used prophylactically in preventing from infectious diseases?
- d) Can the peptides and antibodies be used in patients with immunodeficiency diseases?
- e) Can the peptides and antibodies be used in patients to prevent or alleviate symptoms of allergic diseases?

Two of the most relevant systems are the following.

A. Primate model

The monoclonal antibodies specific for human α .mb/ec peptide and their related substances of this invention are tested to determine whether they react with IgA-bearing cells of rhesus monkeys.

A small portion of rhesus monkeys, which have been infected with the nematode, Ascaris suum, develop sensitivity to extract of ascaris. When these sensitive monkeys are given spray containing ascaris antigen, they develop breathing problems resembling asthma. Patterson, R., J. Clin. Invest. 57:586-593 (1976).

The various compositions of this invention can be examined in the asthma/rhesus monkey model system. The ascaris sensitive monkeys are given the experimental treatment or control treatment and measurements are made to determine:

- (a) Do the asthma symptoms associated with ascaris administration decline?
- (b) Does the circulating IgA increase?
- (c) Does secretory IgA increase in pulmonary lavage?

- (d) Does ascaris antigen-specific IgA increase?
- (e) Do IgA-bearing B cells in the circulation and the mucosal layer increase?

B. Mouse model system

The α .mb/ec segment of the mouse has already been sequenced. Word, C.J. *et al.*, EMBO J. 2:887-898 (1983). The 26 amino acid residue peptide is:

Glu · Arg · Gln · Glu · Pro · Leu · Ser · Tyr · Val · Leu · Leu · Asp · Gln ·
Ser · Gln · Asp · Ile · Leu · Glu · Glu · Glu · Ala · Pro · Gly · Ala · Ser ·

The peptide is synthesized in several forms, including one that has extra Leu-Lys residues at the C-terminus.

The peptide and its KLH conjugate are used as antigens to immunize rabbits and goats. The anti-sera are collected. The antigen-specific antibodies are purified using column of Sepharose 4B conjugated with the peptide (with Leu-Lys addition) or with peptide linked to ovalbumin or bovine serum albumin. Alternatively, the peptide-KLH conjugate is used to immunize rats and monoclonal antibodies specific for

mouse α .mb/ec are developed by procedures described earlier (Section 5).

Normal mice are injected intravenously or intraperitoneally with the purified antibodies or their related substances. The mice may also be given α .ec/mb peptide-conjugated with KLH to induce active immunization and endogenous production of anti- α .ec/mb antibodies. They can also be actively immunized with a viral antigen, such as one from a rotavirus, in combination with antibody or peptide treatment. The questions to be investigated are:

- (a) Does the total IgA in circulation increase?
- (b) Does total secretory IgA increase in the intestinal lumen?
- (c) Does antigen-specific IgA increase?
- (d) Does the number of IgA-bearing B cells in the spleen and Peyer's patches increase?
- (e) Can the mice resist better the challenge with live virus?

7. Application of α .ec/mb Peptide and Antibodies Specific for this Epitope in Infectious Diseases, Allergies, and Immunodeficiency Diseases

The α .ec/mb peptide and antibodies specific for α .mb/ec epitopes can be used to increase total IgA, secretory IgA, or antigen-specific IgA in humans or other animals (e.g. dogs, cats, and horses). The antibodies can be used therapeutically and prophylactically in several ways.

A. Antibodies specific for IgA-bearing cells.

Antibodies of certain IgG subclasses, such as mouse IgG₁ and human IgG₂ and IgG₄, or F(ab')₂ fragments may be used to enhance antibody production. The antibodies can administered as free antibodies (preferably intravenously) to patients in amounts sufficient to induce proliferation of IgA-bearing B cells and thereby increase IgA production.

The antibodies can also be administered nasally. The concentration of IgA-producing B cells is densest on the lining of the nasal channel and respiratory tract. Nasal administration (e.g. by nasal spray) may be used to deliver relatively high concentrations of antibodies into the nasal and respiratory tracts and thus achieve speedier and more effective results. The antibodies can also be administered ocularly.

For therapeutic uses in humans, especially when repeated or long

term administration is necessary, less immunogenic antibodies which are either human or chimeric are preferred. Human antibodies, or fragments thereof, can be readily produced from human genomic expression libraries (Stratagene Corp., La Jolla, California). Chimeric antibodies comprise a variable or antigen binding (hypervariable or complementarity determining) region derived from an animal antibody, and the remaining regions derived from a human antibody. Methods for producing chimeric (e.g. murine/human) antibodies are well established. Chimeric antibodies can be produced in large quantities. Antibody fragments of the human or chimeric antibodies can also be used.

Immunotherapies employing the antibodies of this invention may be used in combination with conventional vaccination and desensitization immunotherapy. For example, desensitization with allergen may be performed in conjunction with the administration of anti- α .mb/ec antibodies.

B. α .mb/ec Peptide Analogues and Active immunization Against α .mb/ec Epitope

Even though human α .mb/ec peptide is probably not immunogenic

in humans, synthetic or recombinant peptides with the same sequence, or immunological equivalents can be linked to carrier proteins, such as hepatitis B surface antigen core antigen, or KLH, and become immunogenic and capable to induce antibodies that cross react with authentic α .mb/ec epitope. The preferred synthetic or recombinant peptides have the amino acid sequence GSCS(or C)V·ADWQM·PPPYV·VLDLP·QETLE·EEPGA·N, or immunological equivalents as described above. These α .mb/ec peptide conjugates can be administered to patients susceptible to infectious diseases or IgE-mediated allergies. The antibodies induced by this active immunization can achieve the same functions as the antibodies described above in section A.

C. Antiidiotypic Antibodies and Methods of Active Immunization
Against α .mb/ec Epitope

The α .mb/ec-specific antibodies described thus far can be used to generate paratope-specific, anti-idiotypic antibodies which offer another mode of stimulating IgA production. Antibodies against the paratope of the α .mb/ec-specific antibodies conformationally resemble the epitope for which the anti-IgA antibody is specific, that is, they resemble an α .mb/ec

epitope. These anti-idiotypic antibodies (preferably in the form of chimeric or human anti-idiotypes, or fragments thereof, or peptides containing the antigen combining portions thereof) can be administered in an immunogenic quantity to actively immunize against α .mb/ec and induce the endogenous formation of antibodies against the α .mb/ec epitope. The induced antibodies will mediate the various prophylactic and therapeutic effects of α .mb/ec-specific antibodies.

8. Diagnostic Uses

Antibodies against α .mb/ec epitopes can be used for determining numbers and relative proportions of IgA-bearing lymphocytes in mixed leukocyte populations. The α .mb/ec-specific antibodies will not react with cells which bear secreted immunoglobulins on the cells' Fc receptors. Such cells include macrophages and activated T cells. The profile of the IgA-bearing B cells may indicate the immune status of the individual. The same information can indicate how much antibody should be administered to cause proliferation of IgA-expressing B cells. For this purpose, antibodies can be used in standard assay formats which are used to

determine cell surface antigens. In general, the antibody is contacted with a sample of the leukocytes to be tested under conditions which allow the antibody to bind IgA-bearing cells in the sample. The cells are then examined for binding of antibody. This can be accomplished by conventional cell staining procedures, for example, a fluorescently labeled second antibody can be used to detect binding of the anti-IgA antibody.

EXAMPLES: DETERMINING THE SEQUENCES OF
 α .mb/ec Peptide

MATERIALS AND METHODS

DNA library and probe. The human genomic DNA library was purchased from Stratagene (La Jolla, CA). This library was constructed using genomic DNA from human lung fibroblast line, WI38, packaged in phage FIX. A 30-base oligonucleotide 5'GCGAGAAGTACCTGACTTGGGCATCCCGGC3' (Oligo. #1), which corresponds to a segment located in the CH3 coding region of immunoglobulin allotype α 1 and α 2, was synthesized and used as a probe to screen phage clones containing either α 1 and α 2 gene segments using in

situ hybridization.

Polymerase chain reaction (PCR). To amplify genomic DNA segments, the purified DNA from the positive clones was used as the templates. One primer was a 17-base oligonucleotide, 5'CTGCCTGGCCAAGTCTC3' (Oligo. #2), located in the intron about 1kb down-stream from CH3 exon, and the other primer was a 21-base oligonucleotide, 5'GAACAAGCTCAGTAGGAAGAG3' (Oligo. #3), which is a very conservative segment in the published mouse α membrane exon (Word *et al.* EMBO J. 2:887, 1983). To amplify cDNA spanning human CH3 and the membrane exon, purified cDNA reverse transcribed from mRNA from a surface IgA-expressing cell line (see below) was used as the template. One primer was the same 30-base oligonucleotide (#1) located in CH3 exon used in genomic library screening, and the other primer was an 18-base oligonucleotide, 5'GCTCCCGCTCAGTACTGG3' (Oligo. #4), which is located at the junction of human α membrane exon and the 3' untranslated region. The PCR was carried out in DNA Thermal Cycler (Perkin Elmer Cetus) and the reaction conditions were: denaturing at 94° C for 1 minute, annealing at 50° C for 2 minutes, reacting with Taq

polymerase (Perkin Elmer Cetus) at 72°C for 5 minutes with genomic DNA, or 45 seconds for cDNA. The reaction cycles were 30 for genomic DNA and 40 for cDNA.

Cloning and sequencing. The products from PCR were extracted with phenol. The newly synthesized DNA segments were blunted by Mung Bean nuclease (United States Biochemicals) and their 5' ends were phosphorylated by polynucleotide kinase (New England Biolabs). The amplified DNA fragments of interest were isolated by agarose gel electrophoresis and ligated into plasmid pUC19 (United States Biochemicals) at the restriction site of Sma 1. After transforming into E. coli DH5 α (Bethesda Research Laboratories), the amplified plasmids were purified using CIRCLEPREP kit (BIO101). DNA sequences of the inserts were determined by the method of dideoxy sequencing on double stranded DNA with T7 Sequencing Kit (Pharmacia). The membrane exon regions were sequenced on both strands of DNA to minimize errors. An additional step was performed for identifying clones containing inserts of α gene segment amplified by PCR from cDNA. The colonies were hybridized with an oligonucleotide probe of 22 nucleotides, 5'CCTCCCTATGTGGTGCTGGACT3' (Oligo. #5, segment #1774-1795 in

Figure 1), located between the two primers used in PCR.

Southern blot and subclone. As described above, the first genomic DNA segment used for sequencing was obtained by PCR amplification using two primers, the 3'-end primer of which was in the middle of the membrane exon. In order to obtain sequences for the remaining 3'-end of the membrane exon and the 3' untranslated region, gene segments containing these regions were prepared. Purified genomic DNA's from clones containing human $\alpha 1$ and $\alpha 2$ segments were digested with restriction enzyme Ava 1, electrophoresed on 1% agarose gel, and blotted onto nitrocellulose filter according to the standard Southern blot method. A ^{32}P labelled oligonucleotide located in the membrane exon mentioned above (Oligo. #5) was used as a probe to identify DNA fragments containing segments neighboring the oligonucleotide probe. The positive segments were then isolated and subcloned into pUC 19 at the restriction site Ava 1 and sequenced downstream using the same oligonucleotide used in Southern blot analysis.

RNA and cDNA preparation. A mIgA-expressing cell line, DAKIKI (ATCC TIB206), was used as the source of mRNA. About 5×10^7 cells were harvested for isolation of total RNA using guanidinium procedure.

With the purified RNA as the template, an oligonucleotide at the end of membrane exon as the primer (Oligo #4), the first strand cDNA was polymerized by the reverse transcriptase AMV (Life Science, Inc.) according to the procedure in the provided instruction manual.

Results:

PCR Amplification of $\alpha 1$ and $\alpha 2$ Gene Segments from Genomic DNA and Their Nucleotide Sequences.

Nine phage lambda clones containing human $\alpha 1$ and $\alpha 2$ heavy chain gene segments were identified from the human genomic library. These clones were used directly as the template for PCR with Oligo. #3 and Oligo. #2 as primers. The 5' end primer (Oligo. #2) for the PCR was selected from a segment identical between human $\alpha 1$ and $\alpha 2$ genes located near the 3' end of the published genomic DNA sequences, which ends in an intron about 1.1 kb downstream from CH3. Flanagan, J.G., et al. Cell 36:681-688 (1984). Whether the genes belong to $\alpha 1$ and $\alpha 2$ subclasses can be distinguished by subclass-specific sequences immediately downstream

from the Oligo. #2 primer. Both $\alpha 1$ and $\alpha 2$ membrane gene segments were identified among our nine lambda genomic clones. Through agarose gel electrophoresis, the products of PCR were separated into 3 bands of DNA segments, 2 major bands of 1.8 kb and 300 bp, respectively, and a minor band of 2.2 kb. The 1.8 kb segment was thought the segment of interest judging by the size of the corresponding segment in the already sequenced murine α gene. This DNA segment was purified from the agarose gel, subcloned, and sequenced.

The sequence of this 1.8 kb fragment indicated that on its 3' end there was a segment of about 120 bp with a sequence which was very homologous with that of a murine α membrane exon. A possible splicing acceptor sequence, 5'TTGCAGA3', was identified near the 5' end of this 120 bp segment. Since the 1.8 kb fragment ends in the middle of the membrane exon, the sequences for the remaining membrane exon and the 3' untranslated region were obtained by subcloning and sequencing of additional lambda clones containing segments flanking the membrane exons. These clones were identified by Southern blot analysis using a probe (oligo. 5) from the membrane exon.

The sequence of the membrane exon (194 bp) and about 1,700 bp

of 5' flanking sequence and about 500 bp in the 3' untranslated region of α 1 subclass is shown in Figure 1. The sequence indicates that the stop codon TGA is at exactly the same site as that of a murine α membrane exon, indicating that human and mouse α membrane exons are both of 194 nucleotides in length. At about 400 bp downstream from the membrane exon, there is a possible mRNA termination and polyadenylation signal sequence, 5'AATAAA3' (the underlined nucleotides in Fig. 1). Like the murine α gene, the human α genes have only one membrane exon, while all other classes of human or murine heavy chain genes with known sequences have two membrane exons. The intron between CH3 and the membrane exon has 2,579 bp, somewhat longer than the 2,350 bp of murine. In this intron, there is a region of about 630 bp (the portion in brackets in Fig. 1) in which only a few C bases and more than 20 repeated sequences of 5'GGATGGA3' and other repeated sequences exist. The significance of this is unknown.

PCR and DNA sequencing on cDNA. As mentioned above, the human α membrane exon was located by comparing the sequence of the segment amplified from genomic DNA with that of murine α membrane exon as well as by searching for the splicing acceptor consensus sequence.

A segment of 194 nucleotides (from nucleotide #1760 to #1953 in Figure 1) was originally thought to be the membrane exon. To confirm this, we isolated the total RNA from a human mIgA-expressing cell line, DAKIKI, and prepared its cDNA. With this cDNA as template, a segment spanning CH3 and the membrane exon was amplified by PCR. Although we increased the PCR cycles from 30 to 40, the efficiency of amplification was still not as good as that of PCR on genomic DNA, probably because of the relative lower proportion of the specific template of interest in the cDNA prepared from the total RNA. On agarose gel electrophoresis, the PCR products displayed a weak band of the right size with a heavy smear around it. This band was cut out and subcloned. To help in identifying the specific clones, in situ colony hybridization was performed using a probe located between the two primers of PCR. Positive clones were picked up, purified and sequenced, using the same method as that used for the genomic DNA sequencing.

The results showed that there were clones containing DNA inserts which correspond to two species of mRNA of human membrane-bound $\alpha 1$. The two mRNA species resulted from the use of two different splicing acceptor sites: one from the predicted site, 5' TTGCAGA3' which

corresponding site existed in the murine α gene; and one from an acceptor site, 5'TGGCAGG3' 18 nucleotides upstream in the same reading frame, for which there is not a corresponding site in the murine α gene (the slashes indicate the cleavage/splicing sites). The two mRNA species would yield two membrane-bound $\alpha 1$ polypeptides, one with 65 and the other with 71 amino acid residues in the membrane anchoring peptide region (Figures 1 and 2B). These two mRNA species and their corresponding $\alpha 1$ peptides are referred to as isoforms. As shown in Figures 1 and 2B, the 6 extra amino acid residues in isoform 2 (the longer) are indicated by lower case letters.

The proposed extracellular segments of the membrane anchoring peptides of these two isoforms are respectively either 25 or 31 amino acid residues in length (designated α .mb/ec). These segments are proposed to be extracellular based on the fact that they contain high proportions of acidic residues. These mb/ec- $\alpha 1$ segments are the target antigenic epitopes for antibody-based treatments. Figure 2A shows a comparison of the nucleotide sequence of the membrane exon of human $\alpha 1$ and $\alpha 2$ compared with murine α . Figure 2B shows the deduced amino acid sequences of the membrane exons of human $\alpha 1$ and $\alpha 2$ and their comparisons with the

murine α . It can be seen that human $\alpha 1$ and $\alpha 2$ are highly homologous to murine α . The mb/ec- α peptides are identical between $\alpha 1$ and $\alpha 2$ for isoform 1 and are only one amino acid residue different for isoform 2.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described above. Such equivalents are encompassed by the following claims.

WHAT IS CLAIMED IS:

1. An antibody preparation which binds specifically to immunoglobulin A bound to the membrane of B cells but not to the secreted form of immunoglobulin A.
2. An antibody preparation of Claim 1, which comprises an antibody, or an antibody fragment or a peptide containing the antigen combining portion thereof.
3. An antibody preparation which specifically binds to the extracellular segment of the membrane-bound domain of an immunoglobulin A chain.
4. An antibody preparation of Claim 3, which binds to the peptide GSCS (or C) V·ADWQM·PPPYV·VLDLP·QETLE·EEPG·AN or an epitope thereof.
5. Monoclonal antibody which binds to human IgA on the surface of



B cells but does not bind to secreted, soluble IgA.

6. Monoclonal antibody of Claim 5, which binds to the peptide GSCS (or C) V·ADWQM·PPPYV·VLDLP·QETLE·EEPG·AN or an epitope thereof.
7. A chimeric antibody which binds specifically to human IgA on the surface of B-cells, but does not bind to secreted, soluble IgA, the antibody having a murine antigen binding region and a human heavy chain constant region.
8. A chimeric antibody of Claim 7, which binds to the extracellular segment of the membrane-bound domain of IgA.
9. A continuous, stable cell line which produces a monoclonal antibody which binds to human IgA on the surface of B-cells but does not bind to secreted, soluble IgA.
10. A cell line of Claim 9, which is a hybridoma.



11. A cell line of Claim 10, which is a murine hybridoma.
12. Isolated DNA comprising functionally rearranged genes encoding the variable region of an antibody which binds to membrane-bound IgA on B cells but not to secreted, soluble IgA.
13. A DNA construct comprising the DNA of Claim 12 linked to DNA encoding a human constant region.
14. A myeloma cell transfected with the DNA construct of Claim 13, which secretes a chimeric antibody.
15. A method of stimulating the production of IgA in vivo, comprising administering an antibody which binds to membrane-bound IgA on B cells but does not bind to secreted, soluble IgA.
16. A method of stimulating the production of IgA in vivo, comprising administering to an animal an antibody which binds to membrane-bound IgM or IgD on B cells but does not bind to secreted, soluble



IgM or IgD.

17. Monoclonal anti-idiotypic antibody specific for the paratope of an antibody which binds to membrane-bound IgA on B cells but does not bind to the secreted, soluble form of IgA.
18. Monoclonal anti-idiotypic antibody of Claim 17, specific for the paratope of an antibody which binds to the peptide GSCS (or C) V·ADWQM·PPPYV·VLDLP·QETLE·EEPG·AN or an epitope thereof.
19. A paratope-specific, anti-idiotypic antibody of Claim 17, which is a chimeric antibody having an antigen binding region of murine origin and a constant region of human origin.
20. An antigen binding fragment of the anti-idiotypic antibody of Claim 17.
21. A continuous, stable cell line which secretes monoclonal antibody of



Claim 17.

22. A method of stimulating IgA production, comprising administering to a person an anti-idiotypic antibody against the paratope of an antibody which binds to membrane-bound IgA on B cells but does not bind to secreted, soluble IgA.
23. A peptide including an amino acid sequence corresponding to the extracellular segment of the membrane-bound domain of IgA on the surface of B cells, an immunologically equivalent sequence.
24. A peptide including the amino acid sequence GCCS (or C) V·ADWQM·PPPYV·VLDLP·QETLE·EEPG·AN or an epitope thereof or modifications of the peptide or epitope in which amino acids have been added, inserted, deleted or substituted without significantly affecting the immunological activity of the peptide.
25. A peptide of Claim 24, conjugated to a carrier protein.



26. A method of stimulating the production of IgA *in vivo*, comprising administering a peptide having an amino acid sequence corresponding to the extracellular segment of the membrane-bound domain of IgA on the surface of B cells.

