

Determinant analysis of IgE and IgG4 antibodies and T cells specific for bovine α_{s1} -casein from the same patients allergic to cow's milk: Existence of α_{s1} -casein-specific B cells and T cells characteristic in cow's-milk allergy

Haruyo Nakajima-Adachi, PhD,^a Satoshi Hachimura, PhD,^a Wataru Ise,^a
Kiri Honma, MD, PhD,^b Shinya Nishiwaki,^a Maiko Hirota,^a
Naoki Shimojo, MD, PhD,^b Toshiyuki Katsuki, MD, PhD,^b Akio Ametani, PhD,^a
Yoichi Kohno, MD, PhD,^b and Shuichi Kaminogawa, PhD^a Chiba and Tokyo, Japan

In an effort to clarify the etiology of milk allergy from the standpoint of allergen-specific immune reactions, we investigated the determinants of IgE, IgG4, and T cells specific for bovine α_{s1} -casein from the same individual patients by using its synthetic peptides and cyanogen bromide-digested fragments. α_{s1} -Casein is a major allergen in cow's milk, and its unique conformation enabled us to investigate the determinants of antibodies without consideration about missing the reactivities because of conformational changes. Nine patients were selected as subjects from among 129 milk-sensitive infants screened by ELISA to assess the anti- α_{s1} -casein IgE levels in their sera. By using ELISA for epitope mapping, a C-terminal region of α_{s1} -casein was identified as a common binding site for IgE from all of these patients, whereas those for anti- α_{s1} -casein IgG4 were located in multiple regions of α_{s1} -casein. We determined the specificities of seven α_{s1} -casein-specific T-cell lines established from peripheral blood mononuclear cells of two of the patients. These T cells have been shown to secrete IL-4. All of the T-cell lines had different specificities to α_{s1} -casein. However, a common amino acid residue use was found among the determinants of various T-cell lines from each patient. The results suggest that patients allergic to cow's milk have characteristic B cells recognizing a limited region of α_{s1} -casein and secreting α_{s1} -casein-specific IgE. These B cells may interact particularly with T cells recognizing determinants with a common structure. (*J Allergy Clin Immunol* 1998;101:660-71.)

Key words: α_{s1} -Casein, cow's-milk allergy, IgE, IgG4, T-cell line, determinant, peptide

IgE is a key molecule in the late phase of the allergic response as well as in cutaneous allergic inflammation.¹ This antibody is synthesized by plasma cells after their interaction with T_{H2}-like T cells, mast cells, and/or basophils in response to stimulation with IL-4, IL-13,

Abbreviations used

APC:	Antigen-presenting cell
CMA:	Cow's-milk allergy
CNBr:	Cyanogen-bromide
EB-B cell:	Epstein-Barr virus-transformed autologous B cell
MMC:	Mitomycin C
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PBS-T:	PBS containing 0.05% Tween-20
PBS-TG:	0.1% gelatin in PBS-T
RT:	Room temperature
TCL:	T-cell line

and CD40 ligand (CD40L, CD154).² It is thought that overproduction of IgE is avoided by negative feedback regulation mediated by IgE itself, whereas breakdown of this regulatory mechanism results in the induction of a severe allergic reaction.³⁻⁵ Recent reports concerning the genetic background of atopic patients have indicated that the clinical appearance of atopy and the total serum levels of IgE in atopic patients are linked to chromosomal loci encoding IL-4, T-cell receptors, and other undefined gene regions relating to costimulation and cytokines.⁶⁻⁸ At present, the mechanisms leading to adverse augmentation of IgE production are being studied from the standpoint of antigen-independent cellular and molecular actions. However, many clinical studies have shown that allergic symptoms are improved when allergens are removed from a patient's surroundings and that allergen-related reactions are essential for the pathogenesis of allergy. These findings point to the importance of fine mapping of antigenic determinants recognized by IgE and T cells. Class switching to IgE or IgG4 from IgM seems to involve a similar pathway induced by stimulation with either IL-4 or IL-13.⁹ Identification of the determinants recognized by both IgE and IgG4 may provide information pertinent to one of the most important problems in allergic disease, that is, whether IgE have characteristic properties different

From ^athe Department of Applied Biological Chemistry, The University of Tokyo, Tokyo; and ^bthe Department of Pediatrics, School of Medicine, Chiba University, Chiba.

Received for publication May 28, 1997; revised Nov. 11, 1997; accepted for publication Dec. 17, 1997.

Reprint requests: Haruyo Nakajima-Adachi, Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan.

Copyright © 1998 by Mosby, Inc.

0091-6749/98 \$5.00 + 0 1/1/88256

from IgG4 with respect to the allergen specificities and mechanisms of synthesis of these antibodies. However, it remains to be clarified in detail how the fine specificities of IgE- or IgG4-producing B cells and those of T cells determined on an allergen relate to the pathogenesis of allergy.

Because a cognate interaction between T and B cells is required for their activation, we attempted to analyze the antigen specificity of both of these types of cells in an individual patient. Although a large number of determinant analyses have been reported with respect to T cells, IgE, and IgG, including those specific for allergens,¹⁰⁻³¹ only a few studies have investigated the antigenic determinants recognized by T and B cells obtained from the same patient.^{12, 14, 31} In these previous experiments, the determinants recognized by IgG were not analyzed, or those recognized by IgE were not fully clarified. It is difficult to analyze the fine specificities of antibodies, even in the case of IgG4, which is present in larger amounts than that of IgE, by using synthetic peptides or natural fragments of an allergen. This is partly because not only IgE but also IgG specific for globular proteins recognize the characteristic three-dimensional structure of the protein.

Bovine α_{s1} -casein is one of the major allergens responsible for cow's-milk allergy (CMA), and many infants and children with CMA display a high titer of IgE specific for this protein. α_{s1} -Casein has a flexible and linear conformation rather than a rigid and compact one because it lacks disulfide bridges and contains a high proportion (17%) of proline residues.³² These characteristic structural features of α_{s1} -casein are advantageous for epitope analysis. Previously, we have elucidated the location of B-cell and T-cell determinants on α_{s1} -casein recognized in several strains of mice by using a set of 13 synthetic peptides spanning the entire length of α_{s1} -casein, which consists of 199 amino acid residues.^{33-39, 42} Therefore we anticipated that the determinants on α_{s1} -casein recognized by human B cells could be mapped by similar means by using this set of thirteen overlapping synthetic peptides, with limited consideration given to failure to detect reactions of immunoglobulins specific for particular three-dimensional structures formed by the antigen. Although patients with CMA usually recover from CMA as they become older, it has been demonstrated that allergy to food can easily lead to development of other forms of hypersensitivity in adults.⁴³ Therefore it is thought that studies focusing on antigen-specific reactions in patients with CMA will be important in the clarification of the etiology of the allergy and the development of effective therapy or prophylactic procedures for other allergies in adults in addition to CMA.

In this report we studied the determinants recognized by IgE, IgG4, and T cells specific for α_{s1} -casein by using synthetic peptides and natural fragments obtained by cyanogen bromide (CNBr)-digestion of this protein. Sera and T cells were isolated from the peripheral blood

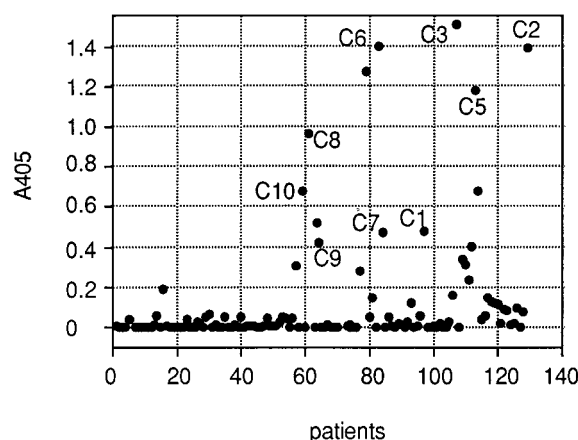


FIG. 1. Screening of allergic patients having high titers of anti- α_{s1} -casein IgE antibodies in their sera. One hundred and twenty-nine allergic infants from the diagnostic department of Chiba University Hospital who manifested severe atopic dermatitis and had positive RAST scores for cow's milk were studied. Binding of their IgE to α_{s1} -casein was analyzed by ELISA, and nine of them (C1 to C3 and C5 to C10) whose sera were available in sufficient quantities for further study were selected.

of nine patients selected from among 129 food-sensitive infants and children screened. Unexpectedly, we found a commonality of regions on α_{s1} -casein recognized by the IgE from each of these nine patients and amino acid sequences common to the determinants on α_{s1} -casein recognized by T cells from each patient.

METHODS

Screening of patients with CMA with a high titer of anti- α_{s1} -casein

We studied 129 allergic patients from the diagnostic department of Chiba University Hospital who had positive RAST (Pharmacia Diagnostics, Uppsala, Sweden) scores for milk (>3) and who manifested symptoms of atopic dermatitis. Their IgE titers binding to α_{s1} -casein were assessed by ELISA. According to the results of this analysis, some of the 129 patients had a high serum titer of anti- α_{s1} -casein IgE (i.e., greater than 0.4 absorbance units measured at 405 nm) (Fig. 1). Among them, nine patients whose sera were available in sufficient quantities for further study were selected as the subjects of this investigation. From the results of the milk challenge test described by Sampson et al.,⁴⁰ clinical history, and dietary records, we concluded that each of these patients had CMA. These nine patients ranged in age from 0 to 6 years (mean age, 3 years). α_{s1} -Casein-specific T-cell lines (TCLs) were established from two donors, C1 and C2 (C1: HLA DRB1*11101, 0405, DRB3*0202, DRB4*0101, DQB1*0401, 0301, DPB1*0201, 0402; C2: DRB1*1501, 0403, DRB4*0101, DRB5*0101, DQB1*0602, 0302, DPB1*0501, DPB1*0601), among the nine patients mentioned above. Sera from children without CMA, ranging in age from 2 to 13 years (mean, 6 years), were used as experimental controls in analysis of IgE binding. They had no symptoms in response to ingestion of cow's milk as judged from dietary records. Their RAST scores for milk showed negative results (<0.3).

α_{s1} -Casein, overlapping synthetic peptides, and CNBr-digested fragments

α_{s1} -Casein was isolated from fresh raw milk by the method of Zittle and Custer⁴⁴ and purified by diethylaminoethyl-Sepharcel ion-exchange chromatography (Pharmacia Biotech, Uppsala, Sweden). α_{s1} -Casein-derived overlapping peptides were prepared by a peptide synthesizer 430A (Applied Biosystems, Foster City, Calif.) by using F-moc chemistry. A series of 20-mer peptides was prepared that corresponded to the full length of this protein, with the amino acid sequence of each consecutive peptide overlapping by five residues at both the N- and C-terminals. The peptides were purified by reversed-phase high-performance liquid chromatography with a column of LiChrosorb RP-18 (Merck, Darmstadt, Germany) eluted with a gradient from 0.1% trifluoroacetic acid in 5% CH_3CN to 100% CH_3CN , with UV detection at 230 nm. The sequence of each peptide was checked with a PSQ-1 protein sequencer (Shimadzu, Kyoto, Japan). CNBr-digested fragments (residues 1-54, 61-123, 136-196, and 124-135) of α_{s1} -casein were prepared as described elsewhere.⁴⁵ In short, α_{s1} -casein (211 mg) in 70% vol/vol formic acid was mixed with a solution of CNBr (100-fold molar excess in relation to the number of total moles of methionine in α_{s1} -casein) at room temperature (RT) for 24 hours in the dark. The reaction mixture was diluted with a 10-fold volume of water and lyophilized. The CNBr-digested fragments were isolated by reversed-phase high-performance liquid chromatography with a column of SSC-SC4 (Senshu Kagaku, Tokyo, Japan) in the case of fragments (F) 1 (1-54), F2 (61-123), and F3 (136-196) and with a column of LiChrosorb RP-18 in the case of F4 (124-135). The amino acid composition of each isolated fragment was confirmed by amino acid analysis (L-8500A amino acid analyzer; Hitachi, Tokyo, Japan). A schematic representation of the sequences of α_{s1} -casein, the synthetic peptides prepared, and the CNBr-digested fragments is shown in Fig. 2.

ELISA for epitope mapping of IgE by using synthetic peptides or CNBr-digested fragments

Binding of IgE to α_{s1} -casein and its related peptides was assessed by ELISA with a modification of the method of Honma et al.⁴⁶ The wells of 96-well microplates (Maxi Sorp; Nunc, Roskilde, Denmark) were coated with α_{s1} -casein or peptides (4 $\mu\text{mol/L}$ in phosphate-buffered saline [PBS]) and incubated at 4° C overnight. After washing the plate with PBS, PBS containing 0.3% gelatin was added to each well for blocking, and incubation was continued at RT for 2 hours. After washing with PBS containing 0.05% Tween-20 (PBS-T), the patients' sera, diluted 20 times with 0.1% gelatin in PBS-T (PBS-TG), was added to the wells and the plates were left to stand at 4° C overnight. The plates were washed, and biotin-labeled anti-human IgE (KPL, Gaithersburg, Md.) diluted with PBS-TG was added to the wells and incubated for 2 hours at RT. The wells were washed with PBS-T and further treated with alkaline phosphatase-avidin (Zymed, South San Francisco, Calif.) in PBS-TG for 30 minutes at RT. The plates were then washed, and an enzyme substrate (0.1% *p*-nitrophenylphosphate in 0.1 mol/L diethanolamine buffer, pH 9.8) was added. After incubation for 6 to 12 hours at RT, the absorbance was measured at 405 nm. Positive reactions were determined, comparing the absorbance observed in assays of the binding of patients' sera to α_{s1} -casein or peptide with that of control sera in each instance as follows:

(Absorbance of each of the peptide-coated wells–Absorbance of uncoated wells in the case of serum from each patient)

> (Average of the difference obtained by subtraction of those absorbances in the case of sera from four control donors + 2SD)

IgE from myeloma patients (Chemicon, Temecula, Calif.) was purchased and used to confirm that binding of the IgE to peptide-coated wells was caused by an antigen-specific interaction.

ELISA for epitope mapping of IgG4 with synthetic peptides

In assays of sera from patients and normal individuals, binding of IgG4 was assessed by the same method as mentioned above except that biotin-labeled anti-human IgG4 (Zymed) was used in place of biotin-labeled anti-IgE, and the sera were diluted 40 times for this analysis. Because, in contrast to IgE, some of the sera from either the patients or normal donors had high titers of anti- α_{s1} -casein IgG4 as assessed by ELISA, we could not define standard values for this analysis of binding. Thus we evaluated the positive binding of anti- α_{s1} -casein IgG4 to α_{s1} -casein and each of the α_{s1} -casein-related synthetic peptides as follows. The absorbance observed in the assay to measure binding of the sera diluted with PBS-TG to a peptide was corrected for nonspecific reactions of biotin-labeled anti-human IgG4 to each antigen by subtracting the absorbance of the wells incubated with PBS-TG only. The positive reactions of IgG4 binding to synthetic peptides were further evaluated by comparing the corrected absorbance of the peptide-coated wells with that of uncoated wells.

Inhibition of IgE binding

The ability of IgE to bind to α_{s1} -casein or synthetic peptides was studied also by an inhibition ELISA. Wells of a 96-well microtiter plate were coated with α_{s1} -casein or peptide (P) 13 (181-199) (at 0.12 $\mu\text{mol/L}$), and the plate was incubated at 4° C overnight. Each well was washed with PBS, and then blocking reagent (0.3% gelatin in PBS) was added and incubation was continued at RT for 2 hours. Another plate was incubated with only the blocking reagent in the same manner. Patient sera diluted 20 times with PBS-TG were allowed to react with various concentrations of either α_{s1} -casein, P3 (31-50), or P13 in the liquid phase in the latter plate at RT for 2 hours. These reaction mixtures were added to the former plate after coating and blocking treatment and incubated at 4° C overnight. Binding of free antibodies in the mixtures to α_{s1} -casein or peptides on the solid phase was detected by the same procedures as those described in the section on ELISA for epitope mapping. Analysis of inhibition of IgG4 binding to α_{s1} -casein was performed by a method similar to that for IgE mentioned above.

Establishment of α_{s1} -casein-specific TCLs

α_{s1} -Casein-specific TCLs were prepared from peripheral blood mononuclear cells (PBMCs) isolated from two patients with CMA from the nine patients whose serum showed a high titer of IgE specific for α_{s1} -casein in this study. The method has been described in detail elsewhere.⁴¹ Briefly, PBMCs were separated by density-gradient centrifugation by using Ficoll/Paque (Pharmacia Biotech) and suspended in RPMI 1640 (GIBCO, Paisley, U.K.) supplemented with 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 10% heat-inactivated human serum. PBMCs at 2×10^5 cells/well were cultured in a 96-well round-bottomed plate (Costar, Cambridge, Mass.) in medium supplemented with 3.2 $\mu\text{mol/L}$ α_{s1} -casein for 4 days. Then recombinant (r)IL-2 at 25 U/ml was added, and incubation of

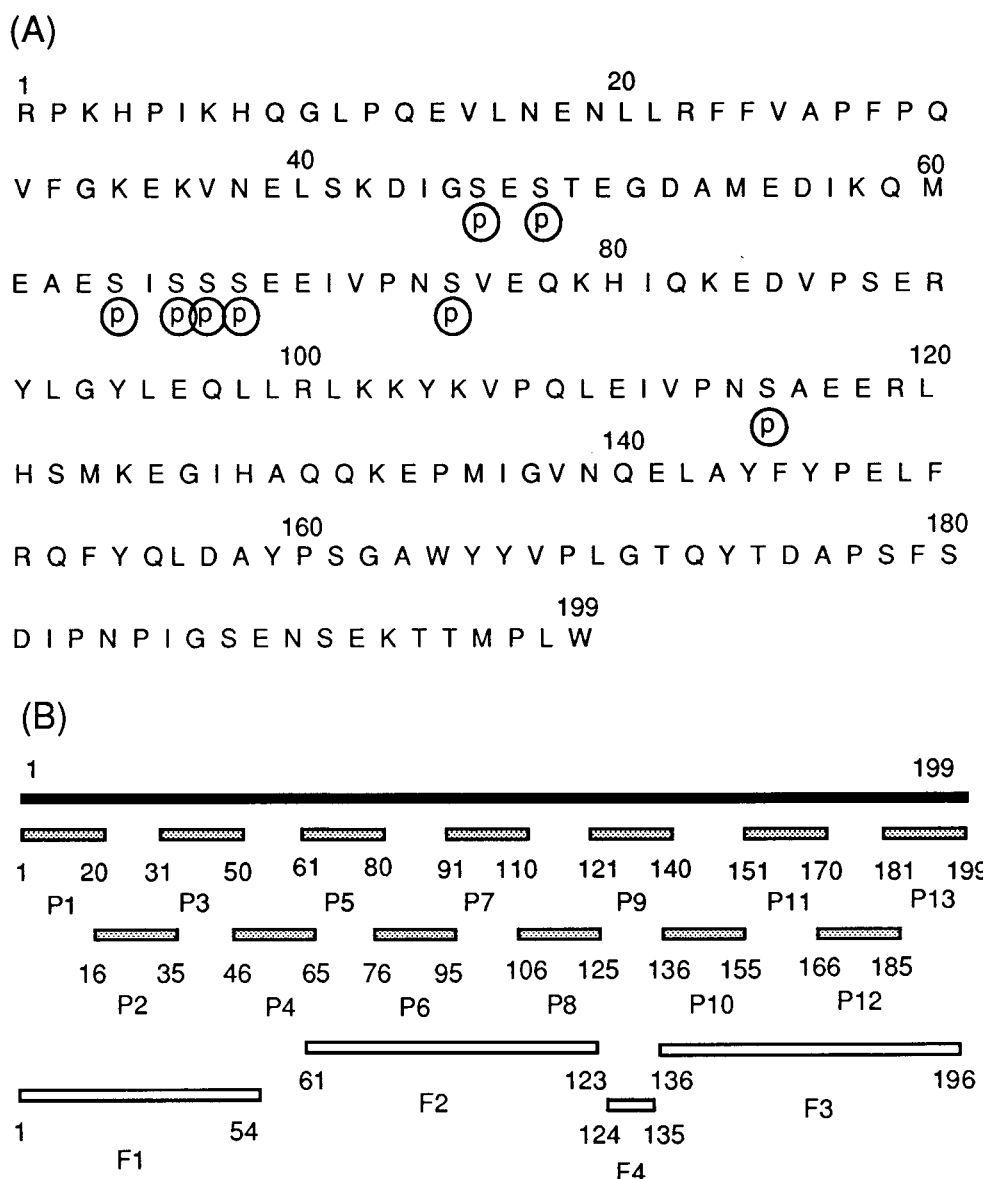


FIG. 2. Amino acid sequence of α_{s1} -casein (A) and scheme of overlapping synthetic peptides and CNBr-digested fragments (B). Circled p, phosphoserine; filled bar, α_{s1} -casein; hatched bars, synthetic peptides corresponding to regions of α_{s1} -casein (P1 to P13); open bars, CNBr-digested fragments corresponding to regions of α_{s1} -casein (F1 to F4).

the cultures was continued for a total period of 18 to 20 days. The population of T cells expanded in each well was divided into two separate wells. One part of the divided culture was stimulated with the antigen and mitomycin C-treated Epstein-Barr virus-transformed autologous B cells (EB-B cells), and the other was stimulated with EB-B cells alone. Seven to 10 days later, the T cells that appeared obviously to respond to α_{s1} -casein compared with T cells incubated without antigen were further transferred to the wells of another 96-well plate. These cells were restimulated with α_{s1} -casein and antigen-presenting cells (APCs) from the culture of mitomycin C-treated EB-B cells. One day after this stimulation, 25 U/ml of rIL-2 was

added, and the expansion of TCLs was repeated similarly every 10 days.

Assay of proliferation of α_{s1} -casein-specific TCLs

Ten days after the last stimulation of α_{s1} -casein-specific TCLs with the antigen and APCs, proliferation of the TCLs in response to α_{s1} -casein, the synthetic peptides, and the CNBr-digested fragments of α_{s1} -casein was assessed according to the methods described elsewhere with minor modifications.⁴¹ In short, TCLs were harvested, washed, and resuspended in the culture medium mentioned above at a concentration of 1 to 2×10^4 cells/well. Mitomycin C-treated EB-B cells (1.5 to 3×10^4

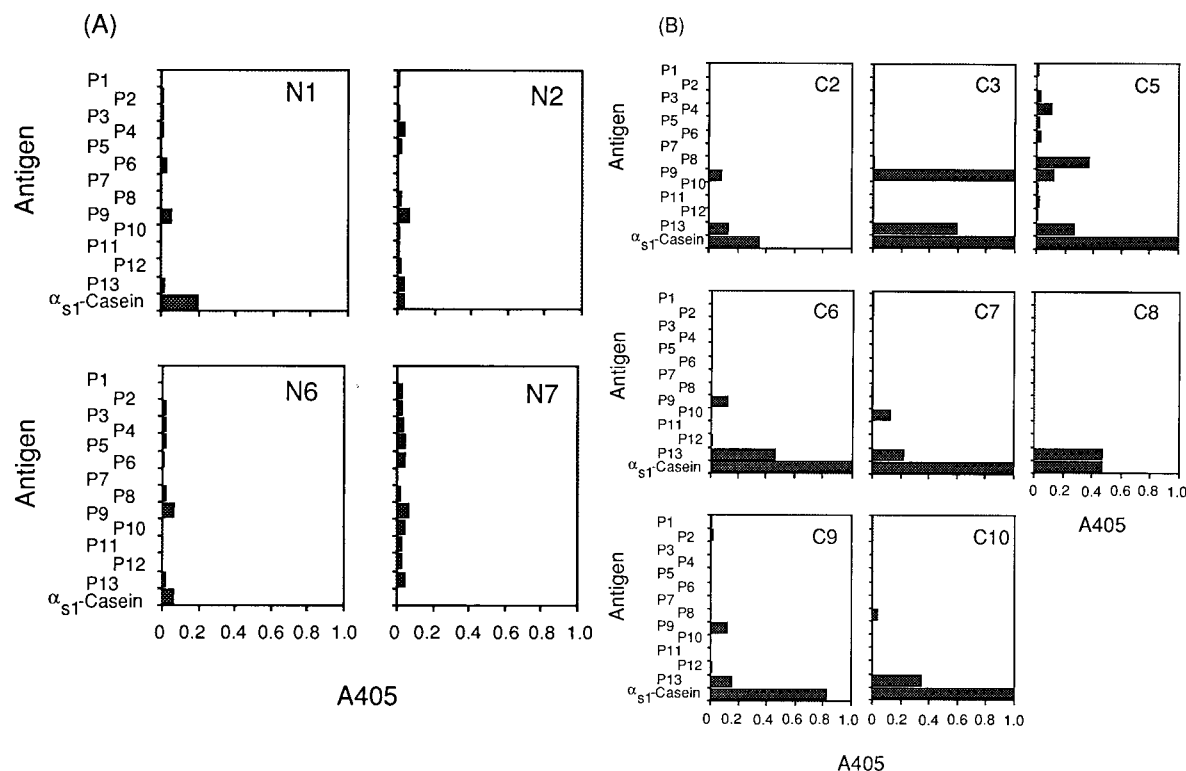


FIG. 3. Mapping of epitopes on α_{s1} -casein recognized by anti- α_{s1} -casein IgE in sera obtained from normal donors (A) and patients with CMA (B) by using synthetic peptides. Synthetic peptides with sequences located throughout an entire α_{s1} -casein molecule are shown as P1 to P13. A405 (absorbance at 405 nm) obtained at the last step of ELISA indicates binding of serum IgE to each of these synthetic peptides. Values shown indicate titers obtained according to the following formula: Absorbance of each of the peptide-coated wells – Absorbance of uncoated wells in the case of serum from each patient. Binding of serum from donor C1 was assessed in another experiment.

cells/well) were added to the wells of 96-well flat-bottomed culture plates. The cultures were incubated with α_{s1} -casein or peptides and, after 72 hours of incubation, tritiated-thymidine was pulsed at 1 μ Ci/well. After further incubation for 16 hours, incorporation of tritiated-thymidine into DNA was assayed by liquid scintillation counting. Proliferation was expressed as the mean cpm of duplicate cultures. This epitope mapping was repeatedly performed.

RESULTS

Binding of IgE to α_{s1} -casein synthetic peptides

The α_{s1} -casein epitopes recognized by serum IgE from patients with CMA and infants without CMA were assessed by ELISA by using synthetic peptides corresponding to regions of α_{s1} -casein. In previous reports we have shown that binding of antibody to these peptides adsorbed on the surface of the polystyrene plates used in this study can be specifically detected.^{33-39, 42} As shown in Fig. 3, B, IgE from patients with CMA displayed significant binding to α_{s1} -casein, and IgE from infants without CMA did not (Fig. 3, A). The ELISA results indicated that IgE from patients with CMA, but not that from infants without

CMA, interacted with some of the synthetic peptides (P4, P8, P9, and P13). To confirm that a positive reaction was detected only in the case of IgE in sera from patients with CMA, we calculated the superiority values according to the formula described in the Methods section, and the results of this calculation are shown in Fig. 4. Sera from nine patients with CMA (C1 to 3 and C5 to 10) all reacted with a peptide corresponding to a C-terminal region of α_{s1} -casein (P13, consisting of residues 181-199). One of these sera (C1) also reacted with P8 (residues 106-125). Although sera from several patients seemed to react weakly with some of the other peptides tested (P4, P8, and P9 in C5; P9 in C3; P9 in C6; P10 in C7; and P9 in C9), these weak binding reactions were considered to be negative ones according to calculations based on the formula described above.

Human IgE from a patient with myeloma was analyzed for nonspecific binding to α_{s1} -casein and the synthetic peptides. Although myeloma IgE seemed to bind to some of the peptides and α_{s1} -casein at a level above the background, this binding was not dependent on the concentration of this myeloma IgE in the wells, and there was no relation to the titer of total and

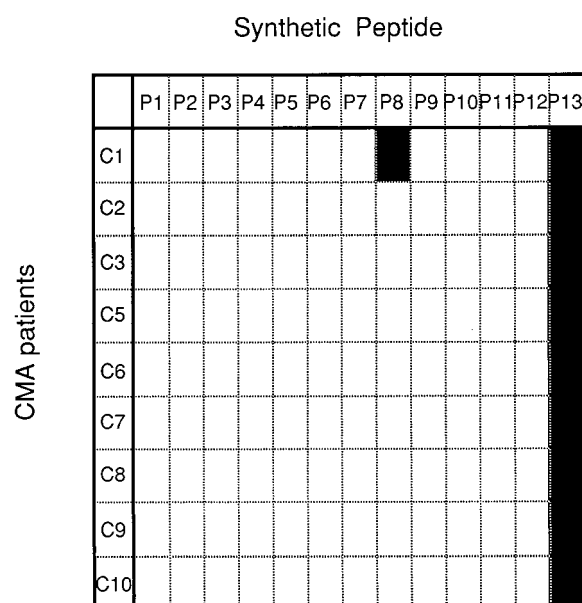


FIG. 4. Schematic representation of binding of IgE from patients with CMA. Positive binding was evaluated by formula described in Methods section. Black squares, positive binding.

specific IgE in the sera from the patients. In addition, no binding to the other peptides was detected (data not shown).

Inhibition analysis of IgE binding to α_{s1} -casein and the synthetic peptides

The specificity of IgE to P13 was confirmed by another binding assay in which inhibition of binding to immobilized α_{s1} -casein by P13 in the liquid phase was tested. As shown in Fig. 5, when the 20-times-diluted sera of two patients with CMA were allowed to react with α_{s1} -casein at the concentration of 20 nmol/ml in the liquid phase, almost 100% inhibition of IgE binding to α_{s1} -casein on the solid phase was observed with the serum of C1, and almost 80% inhibition was observed with the serum of C3. The experiments showed that binding of IgE from C1 to immobilized α_{s1} -casein was 91.5% inhibited by P13 at 20 nmol/ml, and the extent of inhibition was 30% for the C3 serum. Inhibition of binding of IgE to α_{s1} -casein by P3 (with which no sera reacted) did not occur in the case of serum from either of the two patients (Fig. 5). Binding of IgE to P13 on the solid phase was 100% inhibited by either α_{s1} -casein or P13 at each concentration of 0.2 nmol/ml (data not shown).

Binding of IgE from patients with CMA to CNBr-digested fragments of α_{s1} -casein

We studied the binding of IgE to CNBr-digested fragments of α_{s1} -casein, comparing the IgE from patients with CMA with that of infants without CMA. IgE from patients with CMA bound more strongly to CNBr-digested F3 (residues 136-196), which is located in a

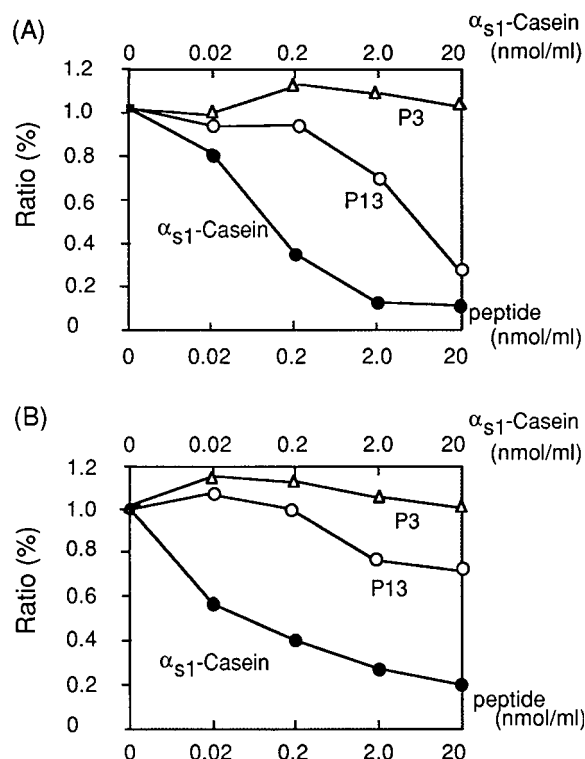


FIG. 5. Inhibition assay of binding of IgE from patients with CMA to α_{s1} -casein. Sera from two patients with CMA, C1 (A) and C3 (B), were preincubated with various concentrations of α_{s1} -casein (filled circles), P13 (the common binding peptide for IgE from nine selected patients with CMA) (open circles), or P3 (the peptide with which no sera reacted) (open triangles). These sera were then tested for their binding to plate-bound α_{s1} -casein.

C-terminal region of α_{s1} -casein, than to other fragments (residues 1-54, 61-123, and 124-135). On the other hand, no significant binding activity to any of these fragments was detected in the case of sera from infants without CMA (Fig. 6).

Binding of IgG4 to α_{s1} -casein synthetic peptides

To study whether anti- α_{s1} -casein IgG4 recognized a limited region of this protein, we analyzed the ability of IgG4 to bind to α_{s1} -casein and synthetic peptides related to this protein. No difference in the binding pattern of anti- α_{s1} -casein IgG4 was found between normal donors and patients with CMA as determined by ELISA (Fig. 7, A and B). In both groups, regardless of the titers of anti- α_{s1} -casein IgE in their sera, some donors had only a small amount of anti- α_{s1} -casein IgG4. In the case of the donors who showed higher titers of this IgG4 in their sera, these antibodies interacted significantly with peptides corresponding to both N-terminal (peptides P1 to P6; residues 1-95) and C-terminal regions (peptide P13; residues 181-199) of α_{s1} -casein, whereas these antibodies exhibited weak binding to peptides P8 to P12 (residues 106-185). The results of an inhibition ELISA indicated that binding of IgG4 to immobilized α_{s1} -casein

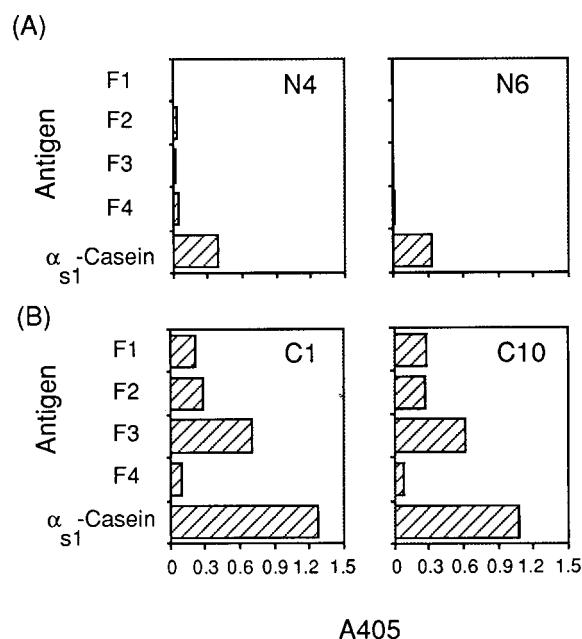


FIG. 6. Binding of anti- α_{s1} -casein IgE from sera of normal donors and patients with CMA to CNBr-digested fragments of α_{s1} -casein. Sera from normal donors interacted with none of four fragments, although we observed a slight reaction to α_{s1} -casein (A). Sera from patients with CMA exhibited significant interaction with α_{s1} -casein and F3 (amino acid residues 136-196) compared with other fragments (B).

was inhibited by this protein in the liquid phase (data not shown).

Analysis of antigenic determinants recognized by α_{s1} -casein-specific TCLs from patients with CMA

Because T cells in primary cultures of PBMCs from patients with CMA proliferated only weakly in the presence of α_{s1} -casein, we could not investigate T-cell determinants with such primary cultures. Then we prepared α_{s1} -casein-specific TCLs from two patients with CMA. We clarified the locations of antigenic determinants on α_{s1} -casein, which induced significant proliferative responses in the case of seven TCLs by using synthetic peptides corresponding to regions located throughout the entire α_{s1} -casein molecule: C1, P6 (76-95) for 1A3 and P10 (136-155) for 5A7; C2, P3 (31-50) for 1G1, P7 (91-110) for 1C7, and P8 (106-125) for 2C1. We further examined the regions recognized by the other two TCLs with the CNBr-digested fragments. The regions containing TCL determinants were in the CNBr-digested fragments: C2, F1 (1-54) for 3C3 and F4 (124-135) for 4E11 (Fig. 8).

Common structures consisting of two amino acid residues were found in T-cell determinant regions recognized by TCLs from each of the patients. In C1 glutamic acid at position i and leucine at position i+7 (-E-(X)₇-L-) are found in two peptides, and in C2 glutamic acid at position i and lysine at position i+6 (-E-(X)₆-K-) are present in five peptides to which the

TCLs displayed a significant proliferative response (Fig. 9). The cytokine secretion pattern of these TCLs analyzed in detail was presented in our previous report.⁴¹

DISCUSSION

Understanding the mechanisms of allergen-specific immune reactions in allergic disease is expected to offer us the means to establish methods for more efficient immunoprophylaxis or more appropriate treatment of allergic diseases. Thus in this article we have investigated the determinants of α_{s1} -casein recognized by T and B cells in individual patients with CMA by using synthetic peptides and native fragments derived from bovine α_{s1} -casein. In contrast to other allergens, the characteristic structural features of this protein enable us to map almost all of the determinants recognized by B cells with overlapping synthetic peptides and natural fragments. The results of epitope mapping by assays of direct binding demonstrated that a peptide corresponding to a C-terminal region of α_{s1} -casein (P13) showed not only a predominant binding to anti- α_{s1} -casein IgE in the sera of some patients with CMA, but the peptide also contained a common binding region for the IgE from all of the nine patients examined. Although sera from these patients seemed to react weakly with some of the other peptides, these did not have common binding regions. Specific binding of anti- α_{s1} -casein IgE to P13 was verified by inhibition ELISA. The extent of inhibition of IgE binding to α_{s1} -casein on the solid phase by P13 in the liquid phase differed for the two patients analyzed. In the case of C3, the extent of inhibition of anti- α_{s1} -casein IgE binding by P13 was less (30%) compared with the case of the C1 sera (91.5%). This may be due to a difference in fine predominance of the IgE antigen specificity, considering that among the 13 synthetic peptides tested there was a difference between the peptides bound significantly by IgE in the case of C3 (P9) and C1 (P13). The specificity of binding of IgE observed in the direct and indirect ELISA was further confirmed by the lack of nonspecific binding of myeloma IgE to P13 and by the dominant binding of IgE in the sera from two patients (C1 and C10) to a CNBr-digested native fragment of α_{s1} -casein consisting of residues 136-196. These results suggest that, independent of HLA types, the patients with CMA had IgE specific for the common determinant or determinants located within the C-terminal region of residues 181-199 of α_{s1} -casein.

One might still argue that masking of binding of IgE to other regions by other isotype antibodies resulted in the failure to detect IgE binding to N-terminal regions other than residues 181-199 of α_{s1} -casein. However, although a significant proportion of IgG4 bound to P13, we could still detect IgE binding to P13. Thus we consider that even if IgE specific for those other regions is present in the sera of patients with CMA, IgE binding to P13 is characteristic at least in the points of stronger affinity to the C-terminal region of α_{s1} -casein, its excess production compared with IgE specific for those other regions, or both.

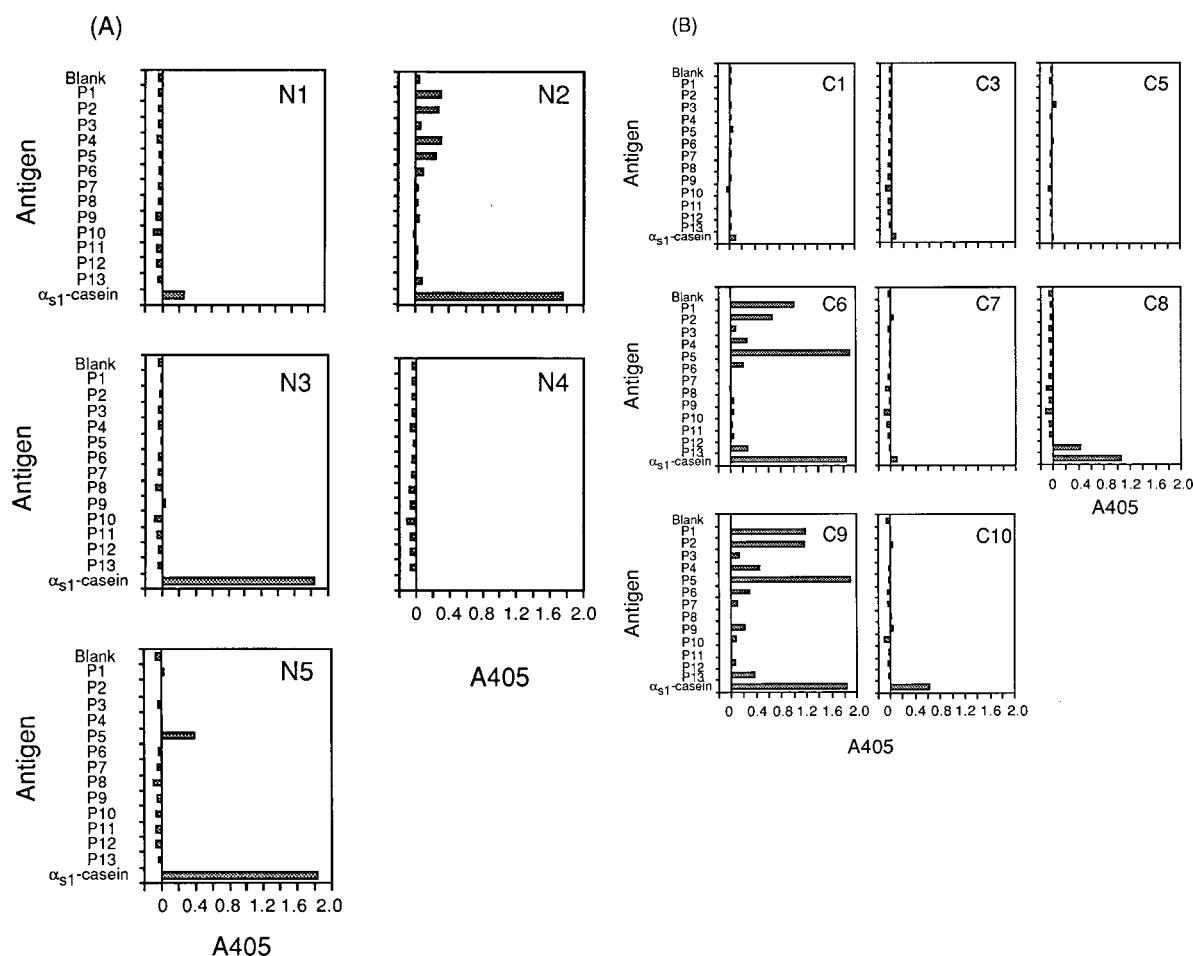


FIG. 7. Mapping of epitopes recognized by anti- α_{s1} -casein IgG4 in sera obtained from normal donors (A) and patients with CMA (B) by using synthetic peptides. A405 (absorbance at 405 nm) obtained at last step of ELISA indicates binding of serum IgE to each synthetic peptide. ELISA values shown indicate titers obtained by correcting for nonspecific binding of biotin-labeled anti-human IgG4 in each patient according to following formula: Absorbance of the antigen-coated wells in which the sera diluted with PBS-TG were added – Absorbance of the antigen-coated wells in which PBS-TG only in the place of the sera were added. Positive reactions of anti- α_{s1} -casein IgG4 binding were further evaluated according to the method described in the Methods section.

There are few reports concerning the detailed analysis of determinants of allergen-specific IgG4 because of difficulties in detection of binding of antibodies to peptides.²⁸⁻³⁰ For the first time, our results demonstrated the specific binding of IgG4 to α_{s1} -casein by both binding and inhibition ELISA, and our findings probably indicate the existence of multiple IgG4 determinants on the allergen of α_{s1} -casein by the detailed analysis with overlapping synthetic peptides. The determinants recognized by anti- α_{s1} -casein IgG4 from the patients with CMA were more widely distributed on α_{s1} -casein compared with those recognized by IgE. Peptides with sequences derived from the N-terminal region (P1, P2, P3, P4, P5, and P6) and P13 were predominantly recognized by the IgG4 antibodies, whereas the profiles of epitope distribution were not different between patients with CMA and nonallergic individuals. The 19-residues

region of P13 may be regarded to contain a common binding site reacting with both IgG4 and IgE in the patients' sera. These results suggest that in the patients with CMA, class switching to IgE occurred more preferentially in the case of B cells producing antibody specific for the P13 region than in the case of those specific for other regions. Thus if the sequential switching of IgM⁺ B cells to IgE-secreting cells through IgG4-secreting cells actually occurs,⁴⁷⁻⁴⁹ we would expect that IgG4 specific for the P13 region may switch to IgE more readily. However, switching from IgM to IgG4 specific for α_{s1} -casein has been shown to occur randomly in B cells recognizing these various determinants. The regulation of antigen-specific IgE and IgG4 synthesis might be also different in CMA.⁵⁰

Epitopes recognized by IgE specific for other allergens (e.g., bovine milk β -lactoglobulin, ant venom Myr p

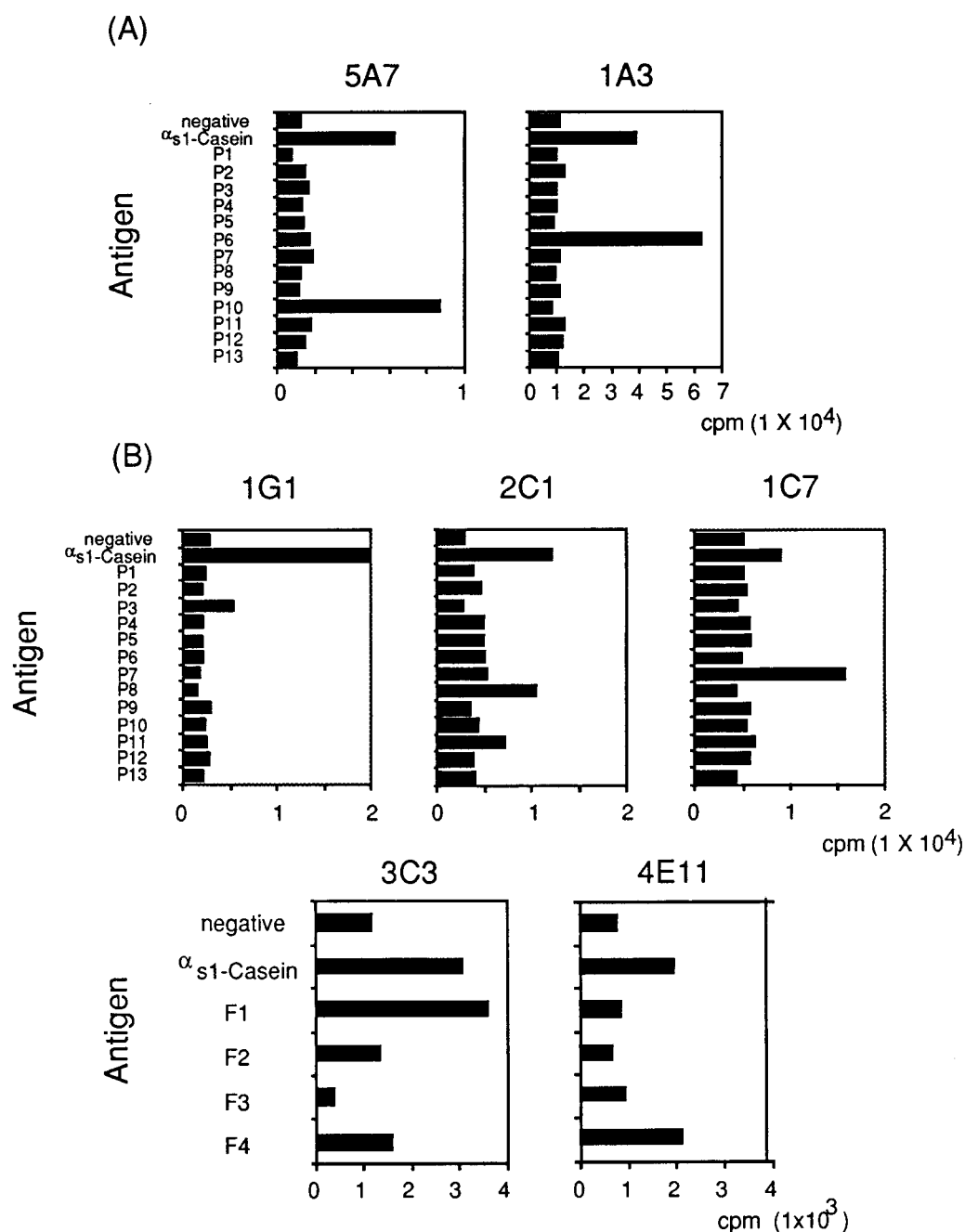


FIG. 8. Determinants recognized by α_{s1} -casein-specific TCLs from two patients with CMA. **A**, Proliferation of TCLs 5A7 and 1A3 established from donor C1 in response to synthetic peptides. **B**, Proliferation of TCLs 1G1, 2C1 and 1C7 established from donor C2 in response to synthetic peptides and that of 3C3 and 4E11 to CNBr-digested fragments of α_{s1} -casein. TCLs (1 to 2×10^4) were incubated in presence of EB-B cells (1.5 to 3×10^4) as APCs and the optimal concentration of antigen (at $4.0 \mu\text{mol/L}$ of either α_{s1} -casein or the synthetic peptides) for 3 days. After this incubation, tritiated-thymidine was added, and its uptake was analyzed.

I, rye-grass pollen Lol p VB, grass pollen Phl p I, house dust mite Der p II, cat Fel d I, and Kentucky Bluegrass pollen *Poa pratensis*) were also found only in particular regions.²²⁻³¹ Immunoglobulin gene analysis has shown that among V_H genes encoding IgE from spleen and peripheral blood B cells of atopic patients, the genes in

the V_H5 family are used preferentially.^{51, 52} These observations suggest that only a particular repertoire of B cells differentiates and seems to produce IgE relevant to inducing allergy. With respect to almost all of the IgE epitopes described above, the ability of these peptides to induce histamine release from human leukocytes has not

been studied yet. It is reported that two C-terminal linear region peptides of ovalbumin comprising residues 347-366 and 357-376 stimulated histamine release from basophils. This 347-376 region is one of the major binding sites recognized by anti-ovalbumin IgE from patients allergic to ovalbumin.⁵³ Similar results have been demonstrated by using synthetic peptides corresponding to residues 52-71 or 117-133 in the sequence of Der p I.⁵⁴ Considering these results, it may be possible that more than one epitope recognized by anti- α_{s1} -casein IgE is located in the sequence of P13 and that this short peptide can induce histamine release from human leukocytes by cross-linking the IgE. In addition, because α_{s1} -casein is a protein easily dimerized through sheet-sheet interactions,³² even if there is only a single determinant on P13 recognized by anti- α_{s1} -casein IgE, dimerized- α_{s1} -casein probably plays a role in inducing inflammatory responses in CMA. In this case P13 itself may function as an immunodominant hapten and inhibit allergic reactions in CMA. Nevertheless, the C-terminal region of α_{s1} -casein may be able to cross-link IgE bound to Fc ϵ receptors and activate human leukocytes in patients with CMA. If this is the case, B cells interacting with a limited region, as well as those displaying a preferential use of receptor genes, may play important roles in the pathogenesis of CMA. It remains unknown whether P13 represents some immunodominant and immunogenic IgE epitopes or an immunodominant IgE hapten. Further studies would provide us with more crucial information about P13 function in CMA. The C-terminal region of the α_{s1} -casein molecule exhibits a high degree of hydrophobicity, and this structural property may influence its interaction with B cells that produce some particular antibodies.³¹

B cells specific for a protein antigen proliferate on stimulation and differentiate into plasma cells after cognate interaction with helper T cells. Thus we also analyzed the determinants of T cells specific for α_{s1} -casein from the same two patients with CMA (C1 and C2) whose sera were analyzed for specificity of antibodies and determined for the locations of the T-cell determinants recognized by seven TCLs. In a previous paper,⁴¹ we assessed surface marker expression and cytokine secretion by α_{s1} -casein-specific TCLs, including these seven TCLs. Most of these seven TCLs were CD4⁺CD8⁻ and predominantly secreted IL-4. Because these TCLs were established from patients with CMA with high titers of anti- α_{s1} -casein IgE in their sera, these cells may display the characteristics of T_{H2}-type T cells, which can help IgE production.

Comparing the sequences of the peptides containing determinants recognized by these TCLs from each patient, we found that all of the peptides inducing significant responses by TCLs commonly share the amino acid sequence -E-(X)₇-L- in the case of patient C1 and -E-(X)₆-K- in the case of patient C2 (Fig. 9). Many studies have identified T-cell determinant regions recognized by distinct allergen-specific T cells from atopic

C 1

(P6) V E Q K H I Q K E D V P S E R Y L G Y L (76-95)
(P10) I G V N Q E L A Y F Y P E L F R Q F Y Q (136-155)

C 2

(F1) V F G K E K V N E L S K D I G S E S T E (1-54)
(P3) V F G K E K V N E L S K D I G S E S T E (31-50)
(P7) Y L G Y L E Q L L R L K K Y K V P Q L E (91-110)
(P8) V P Q L E I V P N S A E E R L H S M K E (106-125)
(F4) K E G I H A Q Q K E P M (124-135)

FIG. 9. Common structures found in peptides recognized by α_{s1} -casein-specific TCLs from each patient with CMA. Although no TCLs from either patient reacted with same peptide, peptides recognized by these T cells had common structure: -E-(X)₇-L- in C1 and -E-(X)₆-K- in C2. -E-(X)₇-L- was found in three different regions, and -E-(X)₆-K- was found in four different regions within amino acid sequence of α_{s1} -casein.

patients by using synthetic peptides and HLA molecules presenting these determinants. Most of the reports indicate that the T-cell determinants are located along the entire length of the allergen molecules.⁵⁵ On the other hand, other studies have indicated a cross-reactivity of T cells among various allergens.^{56, 57} Interestingly, one of these cross-reactive T-cell determinants contains -E-(X)₅-K-, which is similar to one of the common amino acid sequences identified in this study, -E-(X)₆-K- from C2. Two of the peptides corresponding to regions 91-110 and 106-125 of α_{s1} -casein also contain -E-(X)₅-K-.

In this study it is still unknown how the common structure of T-cell determinants observed is related to an essential motif for binding to HLA molecules or particular T-cell receptor gene products.^{58, 59} However, if characteristic B cells specific for limited regions of the allergen exist in CMA and other allergies as described above, it seems likely that only particular T cells would interact specifically with these B cells and induce characteristic antibody production by these B cells. The common structures found in the determinants recognized by our TCLs have a possibility to relate to such a particular interaction. Accordingly, to clarify the etiology of CMA from the standpoint of allergen-specific immune reactions, we need to study how preferential pairing between T and B cells through cognate interaction is related to the pathogenesis of CMA. Further studies focusing on the function of P13-specific IgE-secreting B cells and T cells in the specific response to α_{s1} -casein in patients with CMA will allow us to understand the mechanisms of highly augmented production of specific IgE, which is a rarely circulating isotype in normal humans. Thus clarifying these problems will make it possible to regulate CMA in an allergen-specific manner and will lead to the development of immunoprophylactic procedures or a new immunotherapeutic approach to the disease.

We thank Dr. Chisei Ra of Juntendo University for helpful discussions, Dr. Hidetoshi Inoko and Dr. Taeko Naruse of

Tokai University for typing HLA DP molecules, Dr. Tateo Sakamaki of Sakura National Hospital for typing of HLA DR and DQ molecules, Dr. Osame Shiho at Takeda Chemicals for providing human rIL-2, and the faculty and staff of the Biotechnology Research Center at the University of Tokyo for use of their FACSsort machine.

REFERENCES

- Zweiman B. The late-phase reaction: role of IgE, its receptor and cytokines. *Curr Opin Immunol* 1993;5:950-5.
- Gauchat JF, Henchoz S, Mazzei G, Aubry JP, Brunner T, Blasey H, et al. Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 1993;365:340-3.
- Hewitt CRA, Brown AP, Hart BJ, Pritchard DI. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by anti proteases. *J Exp Med* 1995;182:1537-44.
- Sutton BJ, Gould HJ. The human IgE network. *Nature* 1993;366:421-8.
- Yu P, Kosco-Vilbois M, Richards M, Kohler G, Lamers MC. Negative feedback regulation of IgE synthesis by murine CD23. *Nature* 1994;369:753-5.
- Shirakawa T, Li A, Dubowitz M, Dekker JW, Shaw AE, Faux JA, et al. Association between atopy and variants of the β subunit of the high-affinity immunoglobulin E receptor. *Nat Genet* 1994;7:125-30.
- Moffatt MF, Hill MR, Cornelis F, Schou C, Faux JA, Young RP, et al. Genetic linkage of T-cell receptor α/δ complex to specific IgE responses. *Lancet* 1994;343:1597-600.
- Marsh DG, Neely JD, Breazeale DR, Ghosh B, Freidhoff LR, E-Kautzky E, et al. Linkage analysis of IL-4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 1994;264:1152-6.
- Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, et al. Interleukin-13 induces interleukin-4 independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993;90:3730-4.
- Carballido JM, Carballido-Perrig N, Kagi MK, Meloen RH, Wuthrich B, Heusser CH, et al. T cell epitope specificity in human allergic and nonallergic subjects to bee venom phospholipase A2. *J Immunol* 1993;150:3582-91.
- Dhillon M, Roberts C, Nunn T, Kuo M. Mapping human T cell epitopes on phospholipase A2: the major bee-venom allergen. *J Allergy Clin Immunol* 1992;90:42-51.
- Ebner C, Szepefalusi Z, Ferreira F, Jilek A, Valenta R, Parronchi P, et al. Identification of multiple T cell epitopes on Bet v I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol* 1993;150:1047-54.
- Ebner C, Schenk S, Najafian N, Siemann U, Steiner R, Fischer GW, et al. Nonallergic individuals recognize the same T cell epitopes of Bet v I, the major birch pollen allergen, as atopic patients. *J Immunol* 1995;154:1932-40.
- van Neerven RJJ, van't Hof W, Ringrose JH, Jansen HM, Aalberse RC, Wierenga EA, et al. T cell epitopes of house dust mite major allergen Der p II. *J Immunol* 1993;151:2326-35.
- van Neerven RJJ, van de Pol MM, van Milligen FJ, Jansen HM, Aalberse RC, Kapsenberg ML. Characterization of cat dander-specific T lymphocytes from atopic patients. *J Immunol* 1994;152:4203-10.
- Spiegelberg HL, Beck L, Stevenson DD, Ishioka GY. Recognition of T cell epitopes and lymphokine secretion by rye grass allergen *Lolium perenne* I-specific human T cell clones. *J Immunol* 1994;152:4706-11.
- Yssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R, et al. T cell activation-inducing epitopes of the house dust mite allergen Der p I: proliferation and lymphokine production patterns by Der p I-specific CD4⁺ T cell clones. *J Immunol* 1992;148:738-45.
- Blaher B, Suphiogu C, Knox RB, Singh MB, McCluskey J, Rolland JM. Identification of T-cell epitopes of Lol p 9, a major allergen of ryegrass (*Lolium perenne*) pollen. *J Allergy Clin Immunol* 1996;98:124-32.
- Katsuki T, Shimojo N, Honma K, Tsunoo H, Kohno Y, Niimi H. Establishment and characterization of ovalbumin-specific T cell lines from patients with egg allergy. *Int Arch Allergy Immunol* 1996;109:344-51.
- Colloff MJ, Howe CW, McSharry C, Smith HV. Characterization of IgE antibody binding profiles of sera from patients with atopic dermatitis to allergens of the domestic mites *Dermatophagoides pteronyssinus* and *Euroglyphus maynei*, using enhanced chemiluminescent immunoblotting. *Int Arch Allergy Immunol* 1992;97:44-9.
- Atassi H, Atassi MZ. Antibody recognition of ragweed allergen Ra3: localization of the full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain. *Eur J Immunol* 1986;16:229-35.
- Ball G, Shelton MJ, Walsh BJ, Hill DJ, Hosking CS, Howden MEH. A major continuous allergenic epitope of bovine β -lactoglobulin recognized by human IgE binding. *Clin Exp Allergy* 1994;24:758-64.
- Donovan GR, Street MD, Baldo BA, Alewood D, Alewood P, Sutherland S. Identification of an IgE-binding determinant of the major allergen Myr p I from the venom of the Australian jumper ant *Myrmecia pilosula*. *Biochem Biophys Acta* 1994;1204:48-52.
- Ong EK, Knox RB, Singh MB. Mapping of the antigenic and allergenic epitopes of Lol p VB using gene fragmentation. *Mol Immunol* 1995;32:295-302.
- Ball T, Vrtala S, Sperr WR, Valent P, Susani M, Kraft D, Valenta R. Isolation of an immunodominant IgE hapten from an epitope expression cDNA library. *J Biol Chem* 1994;269:28323-8.
- van't Hof W, Driedijk PC, van den Berg M, Beck-Sickinger AG, Jung G, Aalberse RC. Epitope mapping of the *Dermatophagoides pteronyssinus* house dust mite major allergen Der p II using overlapping synthetic peptides. *Mol Immunol* 1991;28:1225-31.
- van Milligen FJ, van't Hof W, van den Berg M, Aalberse RC. IgE epitope on the cat (*Felis domesticus*) major allergen *Fel d I*: a study with overlapping synthetic peptides. *J Allergy Clin Immunol* 1994;93:34-43.
- Oshika E, Kuroki Y, Sakiyama Y, Matsumoto S. Measurement of IgG subclass antibodies to the group II antigen of *Dermatophagoides pteronyssinus* (Der p II) in sera from children with bronchial asthma. *Ann Allergy* 1992;69:427-32.
- van Milligen FJ, van't Hof W, Aalberse RC. IgE and IgG4 binding to synthetic peptides of the cat (*Felis domesticus*) major allergen *Fel d I*. *Int Arch Allergy Immunol* 1994;103:274-9.
- Kobayashi I, Sakiyama Y, Tame A, Kobayashi K, Matsumoto S. IgE and IgG4 antibodies from patients with mite allergy recognize different epitopes of *Dermatophagoides pteronyssinus* group II antigen (Der p 2). *J Allergy Clin Immunol* 1996;97:638-45.
- Zhang L, Yang M, Chong P, Mohapatra SS. Multiple B- and T-cell epitopes on a major allergen of Kentucky Bluegrass pollen. *Immunology* 1996;87:283-90.
- Kumosinski TF, Brown EM, Farrel HM Jr. Three-dimensional molecular modeling of bovine caseins: α_{s1} -casein. *J Dairy Sci* 1991;74:2889-95.
- Ametani A, Kaminogawa S, Shimizu M, Yamauchi K. Rapid screening of antigenically reactive fragments of α_{s1} -casein using HPLC and ELISA. *J Biochem (Tokyo)* 1987;102:421-5.
- Ametani A, Kim SM, Kaminogawa S, Yamauchi K. Antibody response of three different strains of mice to α_{s1} -casein analyzed by using synthetic peptides. *Biochem Biophys Res Commun* 1988;154:876-82.
- Enomoto A, Shon DH, Aoki Y, Yamauchi K, Kaminogawa S. Antibodies raised against peptide fragments of bovine α_{s1} -casein cross-react with the intact protein only when the peptides contain both B and T cell determinants. *Mol Immunol* 1990;27:581-6.
- Shon DH, Enomoto A, Yamauchi K, Kaminogawa S. Antibodies raised against peptide fragments of bovine α_{s1} -casein cross-react with the native protein, but recognize sites distinct from the determinants on the protein. *Eur J Immunol* 1991;21:1475-80.
- Hachimura S, Takahashi Y, Fujikawa Y, Tsumori C, Enomoto A, Yoshino U, et al. Suppression of the systemic immune response to casein by oral administration of a tryptic digest of casein. *Biosci Biotech Biochem* 1993;57:1674-7.
- Hachimura S, Fujikawa Y, Enomoto A, Kim SM, Ametani A, Kaminogawa S. Differential inhibition of T and B cell responses to

- individual antigenic determinants in orally tolerized mice. *Int Immunol* 1994;6:1791-7.
39. Kim SM, Enomoto A, Hachimura S, Yamauchi K, Kaminogawa S. Serum antibody response elicited by a casein diet is directed to only limited determinants of α_{s1} -casein. *Int Arch Allergy Immunol* 1993;101:260-5.
40. Sampson HA. Role of immediate food hypersensitivity in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* 1983;71:473-80.
41. Nakajima H, Hachimura S, Nishiwaki S, Katsuki T, Shimojo N, Ametani A, et al. Establishment and characterization of α_{s1} -casein-specific T cell lines from cow's milk allergic patients: unexpected higher frequency of CD8⁺ T cell lines. *J Allergy Clin Immunol* 1996;97:1342-9.
42. Kaminogawa S, Ametani A. Milk proteins. In: Van Regenmortel MHV, editor. *Structure of antigen*. Vol. 3. Boca Raton (FL): CRC Press; 1996. p. 387-411.
43. Baba M, Yamaguchi K. "The allergy march": Can it be prevented? *Allergy Clin Immunol News* 1989;1:71-3.
44. Zittle CA, Custer JH. Purification and some of the properties of α_{s1} -casein and κ -casein. *J Dairy Sci* 1963;46:1183-8.
45. Gross E. The cyanogen bromide reaction. In: Hirs CHW, editor. *Methods in enzymology*. Vol. 11. New York: Academic Press; 1967. p. 238-55.
46. Honma K, Kohno Y, Saito K, Shimojo N, Tsunoo H, Niimi H. Specificities of IgE, IgG, and IgA antibodies to ovalbumin. *Int Arch Allergy Immunol* 1994;103:28-35.
47. Gascan H, Gauchat JF, Roncarolo MG, Yssel H, Spits H, de Vries JE. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4⁺ T cell clones. *J Exp Med* 1991;173:747-50.
48. Jabara HH, Loh R, Ramesh N, Vercelli D, Geha RS. Sequential switching from μ to ϵ via $\gamma 4$ in human B cells stimulated with IL-4 and hydrocortisone. *J Immunol* 1993;151:4528-33.
49. van der Stoep N, Korver W, Logtenberg T. In vivo and in vitro IgE isotype switching in human B lymphocytes: evidence for a predominantly direct IgM to IgE class switch program. *Eur J Immunol* 1994;24:1307-11.
50. Akdis CA, Blesken T, Akdis M, Alkan SS, Wüthrich B, Heusser CH, et al. Induction and differential regulation of bee venom phospholipase A2-specific human IgE and IgG4 antibodies in vitro requires allergen-specific and nonspecific activation of T and B cells. *J Allergy Clin Immunol* 1997;99:345-53.
51. Snow RE, Chapman CJ, Frew AJ, Holgate ST, Stevenson FK. Analysis of Ig V_H region genes encoding IgE antibodies in splenic B lymphocytes of a patient with asthma. *J Immunol* 1995;154:5576-81.
52. van-der Stoep N, van-der Linden J, Logtenberg T. Molecular evolution of the human immunoglobulin E response: high incidence of shared mutations and clonal relatedness among ϵ V_H5 transcripts from three unrelated patients with atopic dermatitis. *J Exp Med* 1993;177:99-107.
53. Honma K, Kohno Y, Saito K, Shimojo N, Horiuchi T, Hayashi H, et al. Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: inhibition of basophil histamine release by haptenic ovalbumin peptide. *Clin Exp Immunol* 1996;103:446-53.
54. Jeannin P, Didierlaurent A, Gras-Masse H, Ellass AA, Delneste Y, Cardot E, et al. Specific histamine release capacity of peptides selected from the modeled *Der p 1* protein, a major allergen of *Dermatophagoides pteronyssinus*. *Mol Immunol* 1992;29:739-49.
55. van Neerven RJJ, Ebner C, Yssel H, Kapsenberg ML, Lamb JR. T-cell responses to allergens: epitope-specificity and clinical relevance. *Immunol Today* 1996;17:526-33.
56. Schenk S, Breiteneder H, Susani M, Najafian N, Laffer S, Duchene M, et al. T-cell epitopes of Phl p 1, major pollen allergen of timothy grass (*Phleum pratense*): evidence for crossreacting and non-cross-reacting T-cell epitopes within grass group I allergens. *J Allergy Clin Immunol* 1995;96:986-96.
57. Mohapatra SS. Determinant spreading: implications in allergic disorders. *Immunol Today* 1994;15:596-7.
58. Huang SK, Yi M, Palmer E, Marsh DG. A dominant T cell receptor β -chain in response to a short ragweed allergen, Amb a 5. *J Immunol* 1995;154:6157-62.
59. Rentz H, Bradley K, Saloga J, Loader J, Larsen GL, Gelfand EW. T cells expressing specific V β elements regulate immunoglobulin E production and airways responsiveness in vivo. *J Exp Med* 1993;177:1175-80.