

IgE and IgG₄ antibodies from patients with mite allergy recognize different epitopes of *Dermatophagoides pteronyssinus* group II antigen (Der p 2)

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Background: Der p 2, composed of 129 amino acids, is one of the major allergens of *Dermatophagoides pteronyssinus*. Several groups reported epitope mapping of IgE antibody but not of IgG or IgG subclass antibodies in Der p 2.

Objective: The purpose of this study was to compare the epitopes recognized by Der p 2-specific IgE antibodies with those recognized by IgG₄ antibodies.

Methods: Recombinant fusion proteins containing a full-length peptide or overlapping five fragments of Der p 2 were prepared and analyzed for their reactivity with IgE and IgG subclass antibodies in sera from patients with mite allergy by means of Western blot procedure.

Results: IgE antibodies in 12 of 13 sera tested bound to full-length Der p 2 (1-129). In 11 of these 12 sera, IgE antibodies bound to one or more of fragments 41-80, 64-105, or 81-129 but not to those of 1-40 or 20-63. Of the 11 sera in which the IgE antibodies to the fragments were detected, only four contained detectable levels of IgG₄ antibodies reactive to the fragments. The binding spectra of the IgG₄ antibodies against the fragments were rather wide and differed from those of IgE antibodies.

Conclusion: Results revealed that there are differences in epitope recognition between IgE and IgG₄ antibodies; IgE antibodies recognize restricted parts of Der p 2 antigen compared with IgG₄. (J ALLERGY CLIN IMMUNOL 1996;97:638-45.)

Key words: *Dermatophagoides pteronyssinus*, Der p 2, fusion protein, IgE, IgG₄, epitope mapping, Western blot analysis

Class switch to IgE is enhanced by IL-4 or IL-13 and suppressed by interferon- γ (IFN- γ).¹⁻⁵ IL-4 and IL-13 are also known to enhance production of IgG₄ but not of other subclasses of IgG antibodies.^{1,5} From this point of view, recent studies have focused on whether IgG₄ and IgE antibodies in the sera of patients with allergic disorders recognize identical epitopes of allergens.^{6,7}

Dermatophagoides pteronyssinus and *D. farinae* are major causes of allergy in more than 80% of

Abbreviations used

IFN- γ :	Interferon- γ
PCR:	Polymerase chain reaction
MBP:	Maltose binding protein
SDS:	Sodium dodecylsulfate
TBST:	Tris-buffered saline, pH 7.5, containing 0.1% Tween-20.

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children with bronchial asthma.⁸⁻¹¹ Major allergens in each species have been classified into three groups: group I (Der p 1 and Der f 1), group II (Der p 2 and Der f 2), and group III (Der p 3 and Der f 3). Complementary DNA of group I and group II of both species have already been cloned.¹²⁻¹⁶ Mature Der p 2 peptide derived from cDNA sequence consists of 129 amino acids (molecular weight, 14 kd) and shares high homology with Der f 2.¹⁴⁻¹⁶

Our previous epitope analysis with proteolytically prepared fragments of Der p 2 from mite extracts demonstrated that IgE antibody in the sera of patients with mite allergy preferentially bound to the fragment of N-terminal of 76 amino acids.¹⁷ van't Hof et al.¹⁸ reported that IgE antibodies in 60% of mite-allergic sera bound to synthetic peptide of N-terminal 65-78, although significant reactivity was observed in only a few cases. Little is known about epitopes in Der p 2 recognized by IgG or IgG subclass antibodies, although IgG from most patients with mite allergy is known to react with the native Der p 2 antigen. An epitope mapping study in which synthetic peptides were used failed to determine the specificities of the IgG antibody.¹⁸

In this study we prepared fusion proteins containing full-length peptide or overlapping fragments of Der p 2, using polymerase chain reaction (PCR) and a fusion protein expression vector system to compare the specificities of IgE antibodies with those of IgG₄ antibodies in the sera of patients with mite allergy. Our data revealed that the specificities of IgE antibody in mite-allergic sera were restricted to the C-terminal part of Der p 2 (peptides 41-80, 64-105, and 81-129) and differed among individuals. In contrast, IgG₄ antibodies bound to a more divergent range of fragments.

METHODS

Production of recombinant fusion proteins

cDNA encoding mature full-length Der p 2 and overlapping five fragments (amino acid residues 1-40, 20-63, 41-80, 64-105, and 81-129) were amplified by PCR.¹⁹ *Eco*RI and *Pst* I sites were attached to the 5' ends of 5'-primers and of 3'-primers, respectively.²⁰ Total RNA was extracted from *D. pteronyssinus* mite bodies by the acid phenol method.²¹ *D. pteronyssinus* mites were kindly supplied by Dr. Ken-ichi Takahashi (Institute of Public Health of Hokkaido). First-strand complementary DNA was prepared from the total RNA with MuMTV reverse transcriptase (Pharmacia, Uppsala, Sweden) and was supplied for the template of PCR. PCR was performed 25 cycles by Thermal Cycler (Perkin Elmer, Brunchburg, N.J.) in the presence of corresponding primers previously described and Taq polymerase (Perkin Elmer). Each cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of polymerization at 72°C. After digestion with *Eco*RI and *Pst* I, amplified cDNA was subcloned at *Eco*RI-*Pst* I site of fusion protein expression vector, pMALc2 (New England Biolabs, Beverly, Mass.). *Escherichia coli* (TB1) was transformed with pMALc2 with or without insert. Because pMALc2 contains the *malE* gene encoding maltose binding protein (MBP) under *tac*-promotor, protein fused with MBP can be induced

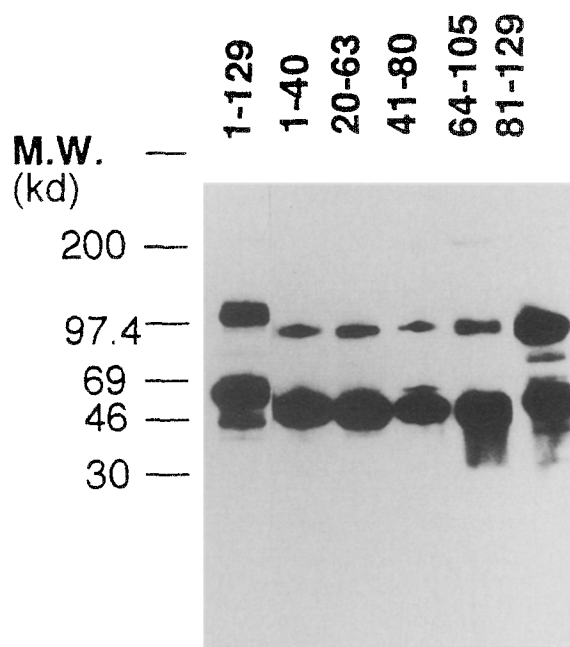


FIG. 1. Western blot demonstrating expression of the fusion proteins. The reactivities of fusion proteins with anti-MBP polyclonal antibody are shown.

by isopropylthiogalactoside. Each construct was examined by DNA sequencing. Transformed *E. coli* was cultured in laked blood medium supplemented with 0.2% glucose and 100 µg/ml aminobenzyl penicillin. Then the expression of the fusion proteins was induced in the medium supplemented with 0.3 mmol/L isopropylthiogalactoside (Sigma Chemical Co., St. Louis, Mo.) for 2 hours. After being solubilized by sonication, each fusion protein was affinity-purified with amylose resin (New England Biolabs), according to the manufacturer's protocol. Protein concentration for fusion proteins was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, Calif.).

E. coli transformed with pMALc2 alone (mock cell) was solubilized in Tris-buffered saline, pH 7.5, containing 0.1% Tween-20 (TBST), and the solution was used as the dilution buffer of sera.

Western blot analysis

Purified fusion proteins were boiled in a sodium dodecylsulfate (SDS) sample buffer containing 2-mercaptoethanol (Sigma).²² 0.5 µg of sample in each lane were applied to SDS-polyacrylamide gel, electrophoresed, and then electrically blotted onto a nitrocellulose membrane (Hybond ECL; Amersham, Amersham, U.K.). After being blocked with 5% nonfat milk in TBST, the membranes were incubated with 1500-fold diluted rabbit anti-native Der p 2 serum or 10,000-fold diluted rabbit anti-MBP polyclonal antibody (New England Biolabs) at room temperature for 1 hour, or five-fold diluted human sera at 4°C for 16 hours. All sera were diluted with TBST containing lysate of *E. coli*

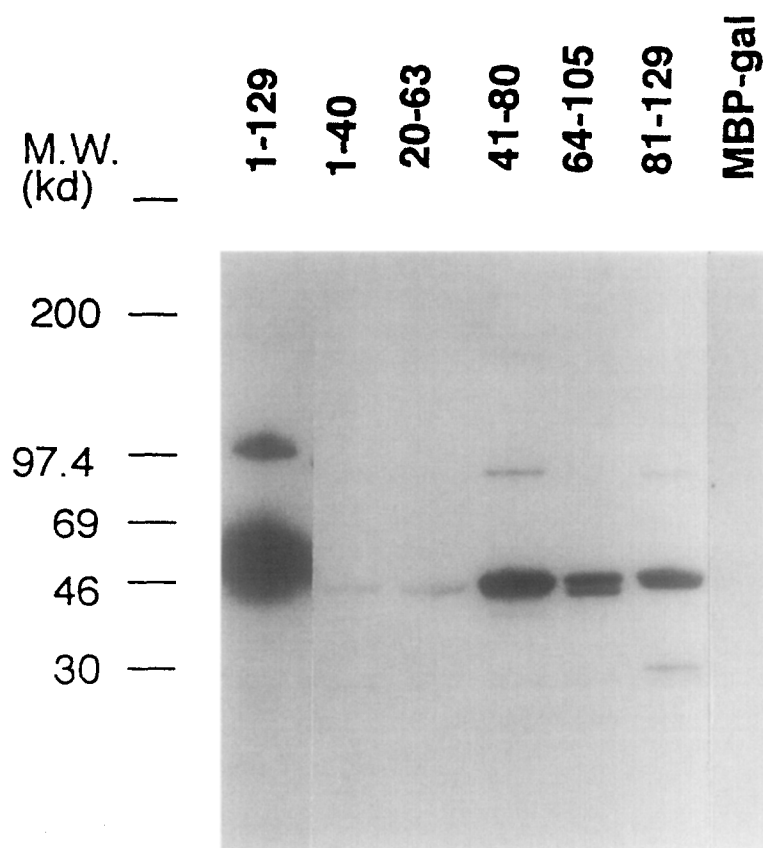


FIG. 2. The reactivities of the fusion proteins with rabbit anti-native Der p 2 serum.

transformed with pMALc2 mock vector to absorb non-specific reaction of antibody with *E. coli* components and/or fusion proteins. After washing four times with TBST, the membranes were incubated with 3000-fold diluted peroxidase-conjugated goat anti-rabbit IgG antibody (Tago, Burlingame, Calif.) or 1500-fold diluted peroxidase-conjugated goat anti-human IgE (CALTAG, San Francisco, Calif.) at 37° C for 90 minutes.

For the analysis of IgG₄ antibody, the membrane was incubated with 400-fold diluted mouse anti-IgG₄ (HP6011) monoclonal antibody (OXOID, Hampshire, U.K.) as the second antibody at 37° C for 90 minutes, followed by washing four times with TBST, and then incubated with 2500-fold diluted peroxidase-conjugated goat anti-mouse IgG (Amersham) at 37° C for 90 minutes. In all experiments signals were visualized by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham).

Sera

A polyclonal rabbit anti-native Der p 2 serum was obtained as previously described.⁸ Sera were obtained from 13 patients with bronchial asthma; three female and 10 male patients, aged 5 to 16 years. None of them had received immunotherapy. All sera were highly positive for mite IgE RAST (RAST score $\geq 3+$). Serum from a patient with hyper-IgE syndrome in which the

IgE level was 12,000 IU/ml and RAST for mite was negative was used as a negative control serum. Another control serum was obtained from an adult volunteer.

RESULTS

Production of the fusion proteins

Coomassie staining of SDS-polyacrylamide gel showed expression of each recombinant protein at deduced molecular weights 57 kD for peptide 1-129 and around 48 kD for other fusion proteins (data not shown). As shown in Fig. 1, these proteins reacted with anti-MBP antibody with two bands: one at around 90-110 kD and the other at around 48-57 kD. The former was considered to be a dimer of the fusion proteins. None of these recombinant proteins reacted with normal rabbit control sera (data not shown). With a rabbit anti-Der p 2 serum, every fusion protein showed reactivity, although the reactivity was variable among fragments; reactivity to fragments 1-129, 41-80, 64-105, and 80-129 was strong (Fig. 2). Nevertheless, these results indicate that each fusion protein contains Der p 2 fragments that are available for epitope mapping studies. Fusion protein without insert, MBP-gal, was not reactive with the rabbit antiserum.

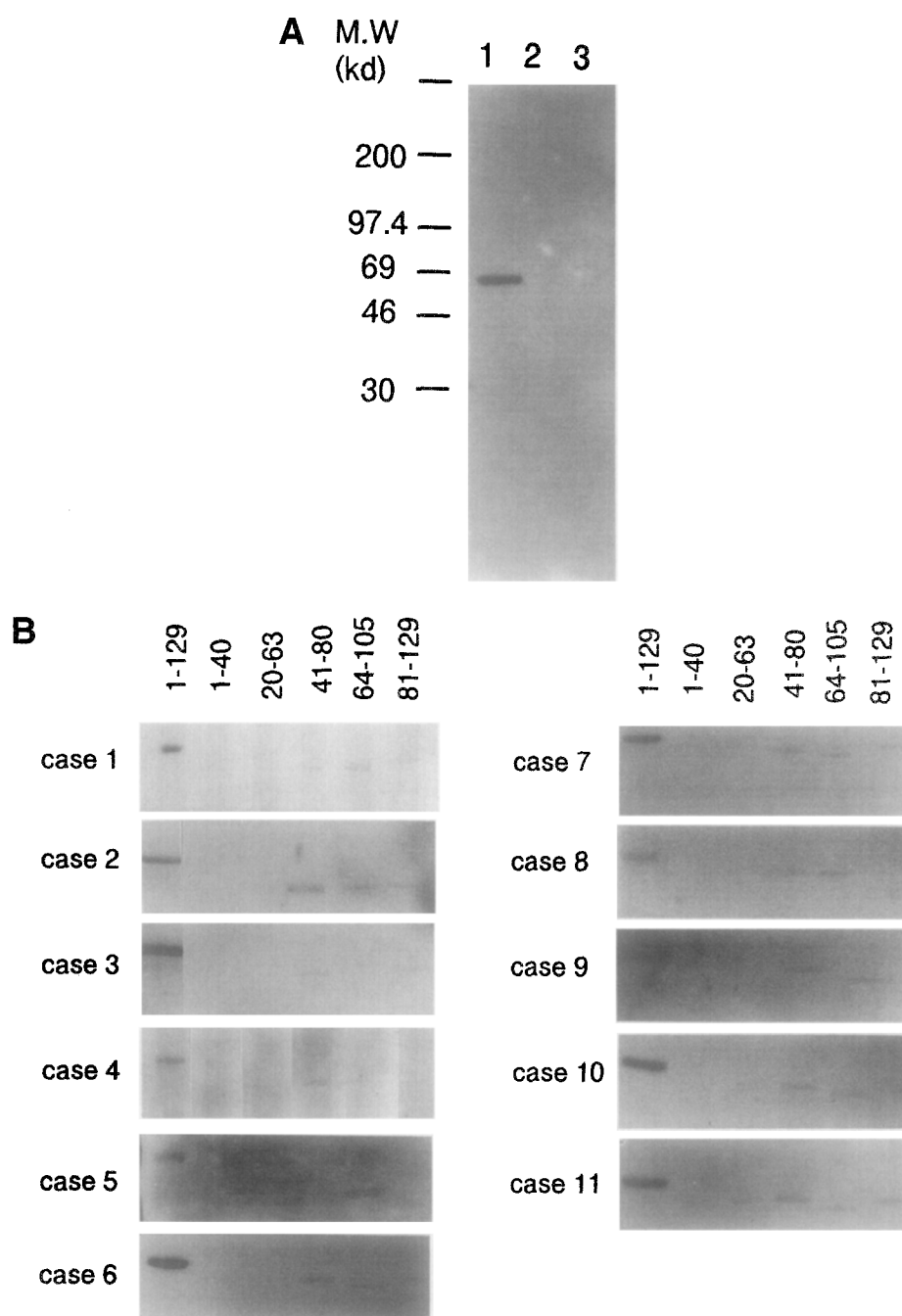


FIG. 3. Reactivities of the fusion proteins to IgE antibody. **A**, lanes 1 and 2 are, respectively, the reactivities of peptide 1-129 and MBP-gal with IgE antibody in the pooled sera from 13 patients allergic to mites. Lane 3 is the reactivity of peptide 1-129 with IgE from a patient with hyper-IgE. **B**, Each panel shows the binding of IgE antibody in individual serum with indicated fusion proteins.

Unexpected was a finding that the fusion fragment 64-105 appeared as double bands, each closely adjacent at approximately 48 kd (Fig. 2). However, the fragment 64-105 was proved to be derived from a single clone by DNA sequencing.

Reactivities of the fusion proteins to human IgE antibodies

The reactivity of fusion proteins with human IgE antibody was first assessed by using pooled serum from 13 patients involved in this study. As shown in

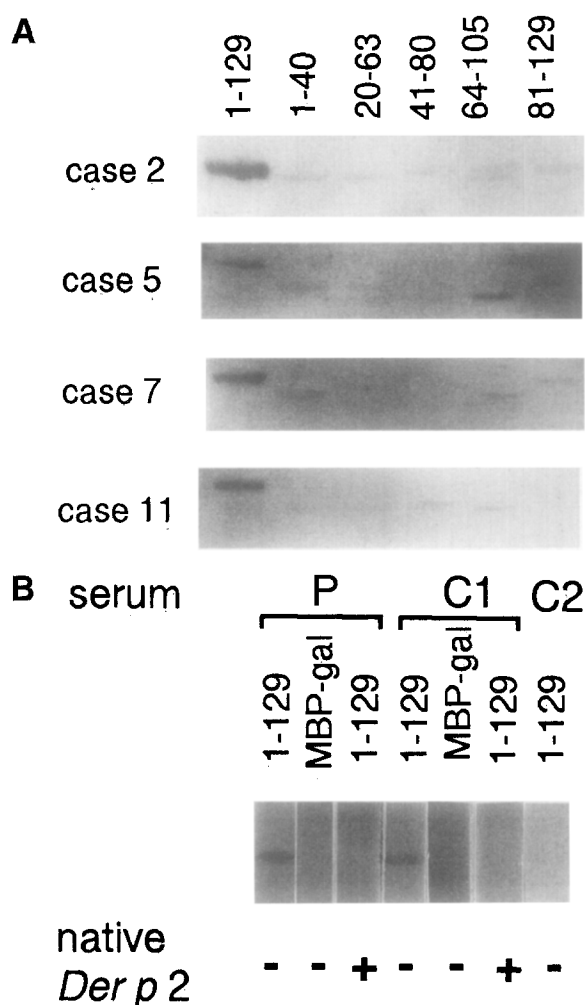


FIG. 4. Reactivities of the fusion proteins with IgG₄ antibodies. **A**, Each panel demonstrates the binding of IgG₄ antibody in four individuals with indicated fusion proteins. **B**, The specificity of IgG₄ binding was tested. Reactivities of peptide 1-129 and MBP-gal with IgG₄ antibodies in the pooled sera (P) and control sera (C1 and C2) as indicated on the top of the panel are demonstrated. The sera were preincubated with or without an excess amount (200 μ g/ml) of native Der p 2 as indicated at the bottom of the panel.

Fig. 3, *A*, a distinct IgE binding was observed against the peptide 1-129, but not against the MBP-gal. The negative control serum from a patient with hyper-IgE syndrome did not show any IgE binding to the fusion proteins. These results indicated that IgE bindings were specific for Der p 2 peptides. Thirteen individual sera were tested for the IgE binding to peptide 1-129. Although IgE antibody bound to peptide 1-129 in 12 of 13 sera, reactivity to either fragment was detected in 11 sera. Fig. 3, *B*, shows reactivities of the fusion proteins with IgE antibodies from 11 indi-

vidual sera. In 10 of these 11 sera, IgE antibodies bound to peptide 41-80. Additionally, nine sera (cases 1, 2, 5, 6, 7, 8, 9, 10, and 11) showed binding to peptide 64-105, and seven sera (cases 1, 2, 3, 6, 7, 10, and 11) showed binding to peptide 81-129. None of the sera showed IgE binding to peptides 1-40 and 20-63.

Reactivity of the fusion proteins with IgG₄ antibody

Next, we examined the reactivities of the fusion proteins with IgG₄ antibodies. In nine of 11 sera, all of which were proven to contain IgE antibody reactive with the fragments, we detected binding of IgG₄ antibodies to the peptide 1-129. However, only four sera (cases 2, 5, 7, and 11) contained detectable levels of IgG₄ antibodies reactive to any of the five fragments. Fig. 4, *A*, shows reactivities of the fusion proteins with IgG₄ antibodies in the four individual sera. IgG₄ antibodies in cases 2 and 11 bound to all five fragments—peptides 1-40, 20-63, 41-80, 64-105, and 81-129—each with almost equal intensity. In case 5 IgG₄ antibodies bound to peptides 1-40, 64-105, and 81-129 and weakly to 20-63. In case 7 IgG₄ antibodies bound to peptides 1-40, 64-105, and 81-129 but not to peptides 20-63 and 41-80.

We also found that a serum from a nonallergic control subject (control 1) showed IgG₄ binding to peptide 1-129. To prove the specificity of the IgG₄ bindings seen in the patients and the control 1, the following experiments were performed. A pooled serum from the four patients (positive for IgG₄ binding to peptide 1-129) and the serum of control 1 were each divided in two: one part served as the original serum, and the other was used for absorption with an excess amount of purified native Der p 2⁸; each part was tested for reaction to the peptide 1-129. Both of the sera that were absorbed with the native Der p 2 abolished the IgG₄ binding to peptide 1-129, whereas the original sera showed the binding to the peptide but not to MBP-gal (Fig. 4, *B*). Fig. 5 summarizes the reactivities of the fusion proteins with human IgE and IgG₄ antibodies. A relative reaction intensity of a rabbit anti-Der p 2 with the fusion proteins was also included.

DISCUSSION

In this study we prepared recombinant fusion proteins consisting of MBP and a full-length or 5 fragments of Der p 2 for the analysis of epitopes recognized by IgE and IgG₄ antibodies. Eleven of 12 sera in which IgE antibodies reactive to full-length Der p 2 were detected showed IgE reactivity

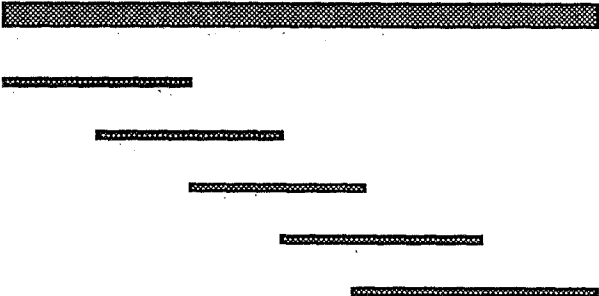
Peptide fragments contained in fusion proteins		Reactivity		
NH2	COOH	Rabbit	Human	
		anti-Der p2	IgE	IgG4
	1-129	+++	12/13	9/11
	1-40	+	0/11	4/9
	20-63	+	0/11	3/9
	41-80	++	10/11	2/9
	64-105	++	9/11	4/9
	81-129	++	7/11	4/9

FIG. 5. Schematic figure of Der p 2 fragments contained in the fusion proteins and summary of rabbit anti-native Der p 2 serum human IgE and IgG₄ binding study.

to the recombinant fragments with different intensity. Chua et al.²³ reported epitope mapping and demonstrated that IgE bindings to fusion proteins consisting of several Der p 2 fragments of more than 60 amino acid residues were weak and detectable only in 15 of 57 sera with dot blot analysis. Despite the use of a similar procedure, we could detect more IgE bindings than reported by Chua et al., even though we used smaller fragments. Reasons for this discrepancy are not known, but two possibilities are considered. First, we used polyclonal instead of monoclonal anti-IgE antibody as the second antibody, thereby leading to the amplification of sensitivity of IgE detection. Second, the sera we used were restricted to those containing high levels of mite-specific IgE (IgE RAST-mite $\geq 3+$).

The bindings of IgE antibody to peptides 41-80 and 64-105 were detected, respectively, in 10 and 9 of 11 sera. This result is consistent with that reported by van't Hof et al.¹⁸; the peptide 65-78 reacted with IgE antibodies in 60% of mite-allergic sera. However, in this study we identified another IgE binding, the binding to peptide 81-129, a C-terminal fragment of the Der p 2, in seven of 11 sera. These results suggest that the bindings of IgE antibodies to peptides 41-80, 64-105, or 81-129 are different among patients. van't Hof et al.²⁴ have reported that in the study of IgE binding to mutation-induced peptides 65-78, IgE antibodies reacting to the original peptide 65-78 can be divided into at least three subgroups by their reactivity with the mutation-induced peptides and that the balance of these three subgroups was different among patients. It has been indicated that

most of the IgE antibodies specific to Der p 2 are included in conformational antibodies.²⁵ In this study, however, the IgE bindings to peptides were detected by Western blotting under reducing agent, a condition in which the conformation of peptides would be deteriorated. Although we could detect the IgE binding, our assay system required a high concentration of IgE antibody (fivefold diluted serum), supporting the previous reports that the IgE antibodies to Der p 2 are conformational ones. The biologic and clinical significance of the IgE antibodies reactive to the deteriorated antigens remains unknown.

Concerning the IgG₄ antibody against Der p 2, we detected the bindings of IgG₄ antibodies to peptide 1-129 in nine of the 11 sera. Peptide 1-129 was found to react also with IgG₄ from a nonallergic control serum but not with other control sera. These findings are consistent with previous reports, including ours, that some normal sera without IgE antibodies also contain IgG₄ antibody reacting with Der p 2.^{8,11} Of these nine patients' sera, the binding of IgG₄ antibodies to either peptide was detected in only four sera. So far no report is available concerning the epitopes of Der p 2 antigen recognized by IgG₄ antibodies. It was noteworthy that IgG₄ antibodies reacted with peptides 1-40 or 20-63, both of which showed no detectable binding with IgE antibodies, although it is possible that the sera contain IgE antibodies at levels undetectable by our assay system.

Recent studies demonstrated that either IL-4 or IL-13 induced class switch from IgM to IgE and IgG₄.¹⁻⁵ Accordingly, it would be expected that IgE and IgG₄ antibodies had identical specificities. How-

ever, in all four cases we tested, the specificities of IgG₄ antibodies to fragments were different from those of IgE antibodies. Furthermore, the reactivity to five fragments of IgG₄ antibodies in case 2, and possibly in case 11, was almost even among the fragments and completely different from that seen in IgE antibodies. Differences in specificities of IgG or IgG₄ and IgE antibodies have also been reported in other allergens, Der p 1⁶ and cat major allergen Fel d 1.⁷

Because IgE antibodies bind to more restricted peptides than those by IgG, some mechanisms specific to IgE antibody formation must be considered. One possibility concerning the antigen would be that some particular sequences or conformations may preferentially induce IgE antibody production. Apparently, IgE antibodies are produced to certain antigens (allergens) but not to others even when exposed to the patients with allergy. In mice, it has been reported that induction of IgE antibody depends on epitopes of antigen, even in the same strain.²⁶ However, characterization of the epitopes that induce IgE antibody formation has not been clarified. We realized in this study that the rabbit antibody raised against the native Der p 2 preferentially reacted with peptides 41-80, 64-105, and 81-129 (Fig. 2), a reaction spectrum similar to that of human IgE antibodies. In this context, these fragments contain epitopes capable of inducing antibody formation. The difference in epitope specificity between human IgG₄ and rabbit IgG may be due to the conditions of sensitization—inhaled or intramuscularly injected. Another possibility would be that the selection of specificities or the changes in affinities may occur in B cells. Affinity maturation through somatic mutation in the variable region of the immunoglobulin gene may be a candidate mechanism;^{27,28} B cells in which immunoglobulin genes have been mutated and, as a result, produce IgE antibodies specific to particular epitopes with high affinities, which preferentially proliferate and differentiate into plasma cells. However, we have no evidence that these mechanisms are implicated in IgE production.

Nevertheless, we substantiated the presence of differences in epitope specificities to Der p 2 between IgE and IgG₄ antibodies in sera from patients allergic to mites without immunotherapy. Studies of similar epitope mapping for IgE and IgG₄ antibodies from patients after immunotherapy will provide insight into understanding such differences.

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