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Announcement

The National Council of Jewish Women Center for the Child, in collaboration with the Society for Research in Child Development, has been awarded the contract by The Administration on Children, Youth and Families to hold the second national Head Start research conference, entitled "Translating Research into Practice: Implications for Serving Families with Young Children," on November 4-7, 1993 in Washington, DC. Following the success of the first Head Start research conference, "New Directions in Child and Family Research: Shaping Head Start in the Nineties," the major goals of the second conference are to identify and disseminate current research in early childhood and families, discuss child and family issues, and identify new research and program needs. A special focus will be on translating research into practice in an effort to help practitioners serving low-income families gain a better understanding of effective early childhood interventions and programming. For additional information, contact Faith Lamb Parker, Ph.D., Project Director, Head Start Research Projects, NCJW Center for the Child, 53 West 23rd Street, New York, NY 10010, (212) 645-4048.

0031-3998/93/3302-0209\$03.00/0

PEDIATRIC RESEARCH

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Vol. 33, No. 2, 1993
Printed in U.S.A.

A Study of the Binding of Immunoglobulin G and Immunoglobulin E from Children with Bronchial Asthma to Peptides Derived from Group II Antigen of *Dermatophagoides pteronyssinus*

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ABSTRACT. The peptides that were produced by the treatment of the purified group II antigen of *Dermatophagoides pteronyssinus* with cyanogen bromide, lysylendopeptidase, *Staphylococcus aureus* V8 protease, or *N*-tosyl-L-phenylalanylchloromethyl ketone-treated trypsin were examined for their binding with IgG and IgE from children with bronchial asthma by immunoblotting analysis. Both IgG and IgE bound to the peptides from the amino terminus to the 76th residue and the carboxyl terminal side from the 13th or 15th residue; on the other hand, both Ig failed to bind to the carboxyl terminal side from the 39th, 56th, or 77th residue. The data demonstrated that peptides lacking residues 1 to 15 possessed the ability to bind Ig, whereas those lacking residues 1 to 39 had lost it. The present study indicates that the amino terminal half of the group II antigen of *Dermatophagoides pteronyssinus* is more important for the binding of IgG and IgE than the carboxyl terminal half and suggests that the residues 15 to 39 may be directly related to the binding of both IgG and IgE. (*Pediatr Res* 33: 209-213, 1993)

Abbreviations

Der p I (or II), the group I (or II) antigen of *Dermatophagoides pteronyssinus*
Der f I (or II), the group I (or II) antigen of *Dermatophagoides farinae*
 TPCK, *N*-tosyl-L-phenylalanylchloromethyl ketone
 PVDF, polyvinylidene difluoride
 CNBr, cyanogen bromide
 V8 protease, *Staphylococcus aureus* V8 protease

The allergens from *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* have been shown to be important factors in the development of bronchial asthma and allergic rhinitis. Group I and group II antigens with apparent molecular masses of 24 kD (*Der p* I and *Der f* I) and 15 kD (*Der p* II and *Der f* II), respectively, have been purified (1, 2). Most mite-allergic patients have measurable amounts of IgE antibodies to these allergens (3).

The cDNA clone encoding *Der p* II has been isolated by IgE plaque RIA (4). The deduced amino acid sequences revealed that

it consisted of 129 amino acid residues containing six cysteine residues. IgE from 90% of mite-allergic patients reacted with *Der p* II (5-8). Recent studies from this (9) and other laboratories (10, 11) have also revealed that IgG from patients with bronchial asthma strongly recognized *Der p* II and *Der f* II. We measured the antibodies of IgG subclasses to the purified *Der p* II (9). The levels of antibodies to total IgG and in all IgG subclasses in asthmatic patients were higher than those of nonatopic healthy individuals.

Epitope mapping of *Der p* II for IgE binding has recently been studied using peptides expressed from *Der p* II cDNA constructs (12) or synthetic peptides (13). However, there have been no reports of epitope analysis of *Der p* II using the native protein. The antibody binding capacity is extremely resistant to denaturing conditions such as heat treatment or low and high pH. In the present study, we digested the purified *Der p* II with cyanogen bromide and proteases and examined both IgG and IgE binding to the peptides obtained.

MATERIALS AND METHODS

Sera. Sera from 10 children with bronchial asthma (aged 1-15 y old) who had never received immunotherapy were collected and pooled for the immunoblotting analysis of the mite antigen. All of these patients exhibited positive results to IgE radioallergosorbent tests (score >3+) (Pharmacia LKB Biotechnology, Uppsala, Sweden). The severity of the condition of these patients was mild to moderate. Most patients were medicated with theophyllin, disodium cromoglycate, and β_2 -inhalator.

Purification of *Der p* II. *D. pteronyssinus* was cultivated by the method of Miyamoto *et al.* (15). After cultivation, culture media and mite bodies were collected by repipetting flotation on saturated NaCl solution as described by Sasa *et al.* (16). Collected mite bodies were then homogenized with a Polytron homogenizer, and the homogenate was centrifuged at 100,000 × g for 20 min. The supernatant obtained, *i.e.*, crude mite extract, was then dialyzed against 10 mM Tris buffer (pH 7.4) for 2 d. The crude mite extract was applied to a Sephadex G 75 column and eluted with 10 mM Tris buffer (pH 7.4). Each fraction was then analyzed by SDS-PAGE by the method of Laemmli (17). The fractions containing proteins with an apparent molecular mass of 15 kD were collected and applied to a reverse-phase HPLC using a C₈ column (Cosmosil, Nakarai Industrial Co. Ltd., Tokyo, Japan) equilibrated with 0.1% (vol/vol) trifluoroacetic acid. Bound materials were eluted with a linear gradient of 20 to 80% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min and were analyzed by SDS-PAGE. The fractions containing the 15-kD protein were collected. The amino terminal amino acid sequencing of the purified 15-kD protein was carried

Received July 28, 1992; accepted October 8, 1992.
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 Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Japan.

out with an amino acid sequencer (470A; Applied Biosystems, Inc., Foster City, CA) and on-line 120A HPLC. The sequence of the 15-kD protein purified in the present study corresponded to that obtained from cDNA of *Der p* II recently reported by Chua *et al.* (4).

Chemical and enzymatic cleavage of *Der p* II. Cleavage of *Der p* II at methionyl residues by CNBr was performed according to the method of Miller and Lunde (18). The purified *Der p* II (100 μ g) was dissolved in 70% formic acid to give a 1% (wt/vol) protein solution. CNBr was added at a 100-fold molar excess of methionine residues of *Der p* II. The reaction mixture was kept in the dark and incubated at room temperature for 24 h. The mixture was then diluted with distilled water to about 1/10 strength, lyophilized, and stored at -80°C before use.

The protein was reduced and S-pyridylethylated by the method of Hermanson *et al.* (19). Briefly, *Der p* II (100 μ g) was dissolved in 200 μ L of 0.13 M Tris buffer (pH 7.6) containing 6 M guanidine and 1 mM EDTA. DTT was added at 20-fold molar excess of cysteine residues of the *Der p* II molecule and incubated at 37°C for 2 h. 4-Vinylpyridine was then added at 2-fold molar excess of DTT and incubated at room temperature for 2 h. The mixture was finally applied to reverse-phase HPLC to isolate the S-pyridylethylated protein.

Der p II was digested with lysylendopeptidase (EC 3.4.21.50, Achromobacterlites, Wako Pure Chemical Industries, Ltd, Osaka, Japan). The protein was incubated at 37°C overnight with 1/50 molar enzyme in 10 mM Tris buffer (pH 7.4).

The S-pyridylethylated *Der p* II was digested with V8 protease (EC 3.4.21.19) or TPCK-treated trypsin (EC 3.4.21.4). The reduced and S-pyridylethylated protein was incubated at 37°C overnight with 1/50 molar V8 protease (Wako) in 50 mM NH_4HCO_3 (pH 7.8) or with 1/50 weight of TPCK-treated trypsin (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris buffer (pH 7.4).

Sequence analysis of peptides derived from *Der p* II. The peptides obtained by the treatments of *Der p* II with CNBr, lysylendopeptidase, V8 protease, and TPCK-treated trypsin were electrophoresed on a 15% polyacrylamide gel. The peptides were then electrically transferred to a Millipore PVDF membrane in 10 mM CAPS buffer [3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.0] containing 10% methanol. The PVDF membrane was rinsed with deionized water for 5 min and stained with Ponceau-S (Sigma). The peptide stained on the PVDF membrane was cut and applied to the amino acid sequencer described above (20, 21).

Immunoblotting analysis. *Der p* II treated with CNBr or proteases was subjected to SDS-PAGE and electrotransferred to a nitrocellulose sheet by the method of Towbin *et al.* (22). The nitrocellulose sheet was incubated with 5% (wt/vol) skim milk containing 0.2% (vol/vol) Triton X-100 in 10 mM PBS, pH 7.4 (buffer A), at room temperature for 30 min to block nonspecific binding. The sheet was then incubated with the pooled sera (1:50 dilution) from patients with bronchial asthma in buffer A at 4°C overnight and washed with 0.2% (wt/vol) Triton X-100 in PBS for 60 min with three changes. Next, the sheet was incubated with horseradish peroxidase-conjugated goat anti-human IgG (Tago Inc., Burlingame, CA) or horseradish peroxidase-conjugated anti-human IgE (Tago Inc.) at 37°C for 90 min. After washing with 0.2% (vol/vol) Triton X-100 in PBS, the sheet was substrated with diaminobenzidine tetrachloride (Katayama Chemical Co. Ltd., Tokyo, Japan).

RESULTS

Der p II was digested with lysylendopeptidase and analyzed by SDS-PAGE. The enzyme treatment produced two major peptides (Fig. 1A). Lys-1 and Lys-2, with apparent molecular masses of 6.5 kD and 4 kD, respectively, both of whose amino terminal sequence began at the 56th residue (Ala⁵⁶) of the *Der p* II. Because of the enzyme specificity and the molecular size on the electrophoretic gel, it is likely that the peptide Lys-1 is the carboxyl

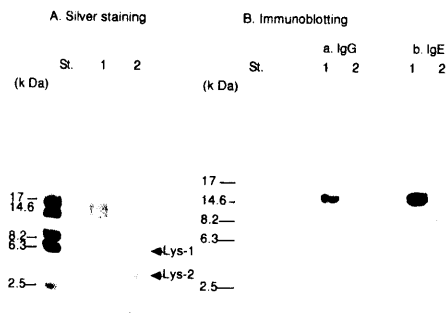


Fig. 1. Immunoblotting analysis of *Der p* II peptides obtained by the treatment with lysylendopeptidase for IgG and IgE binding. *Der p* II (7 μ g) was treated with lysylendopeptidase and was electrophoresed on 12.5% polyacrylamide gel in the presence of 8 M urea (26), and the proteins were stained with silver (panel A). The proteins were also transferred to a nitrocellulose sheet and immunostained with pooled sera from bronchial asthma for IgG (panel B, a) and IgE binding (panel B, b). St., molecular weight standard; lane 1, control *Der p* II; lane 2, *Der p* II treated with lysylendopeptidase.

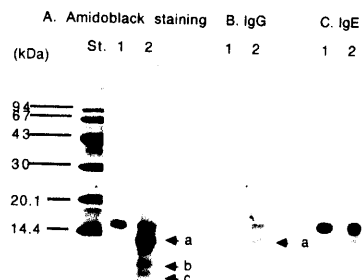


Fig. 2. Immunoblotting analysis of *Der p* II peptides obtained by treatment with CNBr for IgG and IgE binding. *Der p* II (5 μ g) was treated with CNBr and was electrophoresed on 15% polyacrylamide gel, transferred to a nitrocellulose sheet, and stained with amidoblack (panel A) or immunostained with pooled sera from bronchial asthma for IgG (panel B) and IgE binding (panel C). St., molecular weight standard; lane 1, control *Der p* II; lane 2, *Der p* II treated with CNBr.

terminal half of the protein from the 56th residue. The same amount of peptides was analyzed by immunoblotting with the pooled patient sera (Fig. 1B). IgG and IgE from patient's sera clearly recognized control *Der p* II, whereas they bound to neither Lys-1 nor Lys-2. When skin tests were performed on 10 patients with bronchial asthma using Lys-1 and Lys-2 isolated by HPLC, these peptides induced positive skin reaction in none of the patients examined. These results indicate that the binding site or sites for IgG and IgE do not appear to exist in the carboxyl terminal half of *Der p* II.

Next, *Der p* II was digested with CNBr and analyzed by immunoblotting. CNBr treatment produced three major peptides (Fig. 2A, a, b, and c), and IgG and IgE-binding to these peptides was performed. Both IgG and IgE bound exclusively to the peptide a, which extended from the amino terminus to the 76th residue (Met⁷⁶), although the binding of Ig to this peptide was weak in comparison to the binding to undigested *Der p* II. However, IgG and IgE failed to bind to the peptides b (beginning

at Lys⁷⁷) and c (beginning at Pro⁹⁹). The data indicate that both IgG and IgE bind to the amino terminal half of *Der p* II.

The effects of reduction and/or S-pyridylethylation of *Der p* II on IgG and IgE binding were examined. *Der p* II was electrophoresed under nonreducing or reducing condition or after S-pyridylethylation. Nonreduced and reduced proteins showed the same intensity on both the IgG and the IgE bindings. S-pyridylethylated protein also showed positive staining with Ig, though its binding was weak (data not shown). These results indicate that reduction of disulfide bonds in the *Der p* II molecule does not affect the binding of IgG and IgE, but that the formation of S-pyridylethylated cysteine reduced the IgG and IgE binding. However, these data also suggest that S-pyridylethylated protein may be used for further analysis of enzyme-treated protein, because the native form of *Der p* II is resistant to the attack of trypsin and V8 protease. But S-pyridylethylated proteins become susceptible to these proteases.

Next, S-pyridylethylated *Der p* II was digested with V8 protease and the peptides were analyzed by immunoblotting. Both IgG and IgE bound to the major peptide produced (Fig. 3, V8-1) in addition to the undigested protein. V8-1 was identified as the carboxyl terminal region from the 13th residue (Ile¹³). V8-2, which was from the 39th residue (Ala³⁹), exhibited negative staining with IgG and IgE. These data suggest that the 13th to the 39th residues may be responsible for binding IgG and IgE.

Treatment of S-pyridylethylated protein with TPCK-treated trypsin produced the peptide Tr-1, which was the carboxyl terminal side from the 15th residue (Lys¹⁵). Immunoblotting analysis revealed that both IgG and IgE bound to Tr-1 (Fig. 4), indicating that the binding site for IgG and IgE exists in the carboxyl terminal region from the 15th residue. The result corresponds to that obtained from the treatment of *Der p* II with V8 protease.

DISCUSSION

In the present study, immunoblotting analysis was used to study the binding of IgG and IgE from children with bronchial asthma to the peptides that were produced by the treatments of native *Der p* II with CNBr and proteases. Schematic figures of *Der p* II peptides and summary of the IgG and IgE binding studies are shown in Figure 5. Ig bound to the peptide from the amino terminus to the 76th residue and to the carboxyl terminal side from the 13th or the 15th residue. However, IgG and IgE failed to bind to the carboxyl terminal side from the 39th, 56th, or 77th residue. The present study indicates that the amino terminal half of *Der p* II is more important for the binding of

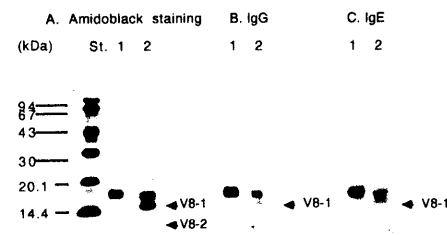


Fig. 3. Immunoblotting analysis of *Der p* II peptides obtained by treatment with *Staphylococcus aureus* V8 protease for IgG and IgE binding. *Der p* II (5 μ g) was treated with V8 protease and was electrophoresed on 15% polyacrylamide gel, transferred to a nitrocellulose sheet, and stained with amidoblack (panel A) or immunostained with pooled sera from bronchial asthma for IgG (panel B) and IgE binding (panel C). St., molecular weight standard; lane 1, control *Der p* II; lane 2, *Der p* II treated with V8 protease.

Fig. 4. Immunoblotting analysis of *Der p* II peptides obtained by the treatment with TPCK-treated trypsin for IgG and IgE binding. *Der p* II (5 μ g) was treated with TPCK-treated trypsin and was electrophoresed on 15% polyacrylamide gel, transferred to a nitrocellulose sheet, and stained with amidoblack (panel A) or immunostained with pooled sera from bronchial asthma for IgG (panel B) and IgE binding (panel C). St., molecular weight standard; lane 1, control *Der p* II; lane 2, *Der p* II treated with TPCK-treated trypsin.

IgG and IgE than the carboxyl terminal half and suggests that the 15th to 39th residues may be directly related to the binding of Ig.

We have determined the amino terminal sequences of the peptides obtained and deduced the residues that make them up using the specificities of CNBr and proteases and molecular masses on an electrophoretic gel. Thus, there is a possibility that the peptide lost its ability to bind with Ig due to the cleavage of the carboxyl terminus with the enzymes. In the present study, it was clearly demonstrated that the peptide lacking the residues 1 to 15 (Tyr-1) could bind with Ig, whereas the one lacking the residues 1 to 39 (V8-2) showed no such activity. We have not determined whether human IgG and IgE bind directly to the peptide comprising residues 15 to 39, but it is possible to conclude that these residues are required for the binding of IgG and IgE to *Der p* II.

Der p II has been shown to be heat stable and resistant to treatments at pH 2.0 or pH 12.0 and to denaturation with 6 M guanidine in terms of binding by murine MAb and human IgE to this protein (14). However, after reduction and alkylation, antibody binding was reduced (14). Our result from the study of binding by human IgG and IgE to reduced and S-pyridylethylated *Der p* II corresponds to their result. Native *Der p* II is protease resistant, but we found that reduced and S-pyridylethylated *Der p* II became susceptible to the attack of proteases such as V8 protease and trypsin, although the modified protein itself decreased binding by IgG and IgE. The only protease that was able to digest the native *Der p* II among various enzymes we have tested was lysylendopeptidase. Isolated peptides digested with lysylendopeptidase failed to induce positive skin reaction in patients with bronchial asthma; this result is consistent with that obtained by immunoblotting analysis with human IgE. When reductive methylated *Der p* II with sodium cyanoborohydride and formaldehyde (23) was analyzed by ELISA, IgG and IgE binding was decreased by 40 to 50% (data not shown), suggesting that lysine residues may be involved in the binding of Ig to *Der p* II.

Chua *et al.* (12) studied IgE binding with peptides expressed from *Der p* II cDNA constructed by dot-blot analysis. Fusion proteins comprising residues 1 to 69, 69 to 129, and 42 to 117 had weak binding activity with IgE in only 26% of allergic sera, suggesting that the antigenic determinants of *Der p* II are highly conformational and restricted. The epitope for IgE was dependent upon the complete molecule of *Der p* II. Van't Hof *et al.* (13) also examined the epitope mapping of *Der p* II using synthetic peptides. The peptides were coupled to CNBr-activated

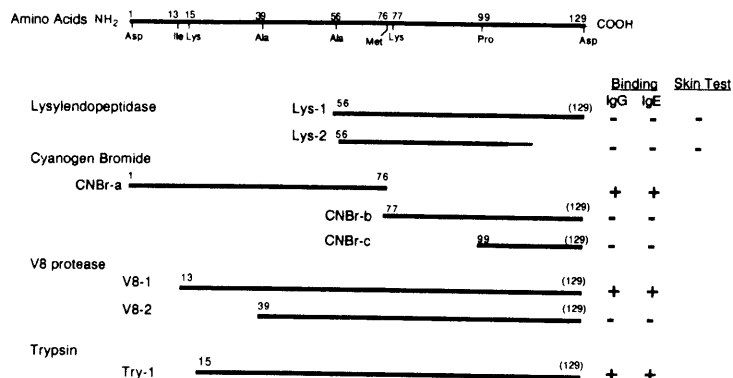


Fig. 5. Schematic figures of *Der p II* peptides and summary of the IgG and IgE binding studies. Peptides were obtained from the treatments of *Der p II* with lysylendopeptidase (Lys-1 and -2), CNBr (CNBr-a, -b, and -c), *S. aureus* V8 protease (V8-1 and -2) and TPCK-treated trypsin (Try-1) as described under Materials and Methods and Results. The amino acid residues are based on the amino terminal sequences, the specificities of CNBr and proteases, and molecular masses on electrophoretic gel.

Sephacrose 4B and used as solid-phase antigens to the binding with human IgE. Although 90% of the sera from mite-allergic patients reacted with *Der p II*, only 60% of the sera tested in their study bound to Sepharose-coupled peptides 65 to 78. We studied the epitope mapping of *Der p II* with IgE, using the purified *Der p II*. Our results suggest that residues 15 to 39 may be important; this is different from the result obtained by Van't Hof *et al.* (13). This discrepancy may be due to the differences of antigens and assays used; we used native protein and immunoblotting assay. IgE binding to residues 65 to 78 was low in their study; only very few patients had appreciable amounts of IgE-anti-mite directed against peptides 65 to 78.

Tsai *et al.* (24) reported that the titer of mite-specific IgG₄ in the asthmatics, which was determined by ELISA using crude mite extract, was 2.23 times higher than that in the control group. Chapman and Platts-Mills (25) also measured antibodies to partially purified mite antigens containing *Der p I* and *II*. IgG antibody to the mite antigens was detected in sera from 94% of mite-allergic patients and from 30% of nonallergic controls. In our recent study (9), the levels of antibodies of total IgG and of all IgG subclasses to the purified *Der p II* in patients with bronchial asthma, who had never received immunotherapy, were higher than those of nonatopic healthy individuals. The levels of *Der p II*-specific IgG₁ and IgG₄ in asthmatic patients were 1.97 and 2.20 times higher, respectively, than those in the control group. Because the epitope mapping for IgG antibody to *Der p II* has not been well documented, we examined the IgG binding site on the *Der p II* molecule as well as IgE. The present results indicate that there have been no differences between IgG and IgE binding to the peptides obtained by the treatments of *Der p II* with CNBr and proteases. Although it is unclear whether both IgG and IgE recognize the same epitope, we conclude that the epitopes for IgG and IgE binding may be located at the same peptides obtained in this study. It would be meaningful to study the epitope mapping of *Der p II* for each subclass of IgG antibody to understand the significance of the appearance of IgG antibody in asthmatic patients.

Acknowledgments. The authors thank Drs. Kenichi Takahashi, Kenji Uruguchi, and Kinpei Yagi (Hokkaido Institute of Public Health) for providing *Dermatophagoides pteronyssinus*.

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