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REDUCTION IN IgE BINDING TO ALLERGEN VARIANTS GENERATED BY SITE-DIRECTED MUTAGENESIS: CONTRIBUTION OF DISULFIDE BONDS TO THE ANTIGENIC STRUCTURE OF THE MAJOR HOUSE DUST MITE ALLERGEN Der p 2

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Abstract—Site-directed mutagenesis was used to investigate the contribution of disulfide bonds to the antigenic structure of Der p 2. Single amino acid variants were generated at cysteine residues, preventing the formation of disulfide bonds at positions 21–27, 73–78, and 8–119. The variants were tested for binding to murine monoclonal antibodies (mAb) and human IgE antibodies (Ab) in an inhibition enzyme immunoassay. Removal of the disulfide linking the amino–carboxy termini (C8–C119) had no effect on mAb binding, however, IgE Ab binding was reduced by up to 10-fold. The other two disulfides form small loops and disruption of these bonds gave different binding patterns. The variant lacking the C21–C27 bond showed up to a 40-fold reduction in antibody binding, while the variant lacking the C73–C78 bond showed more than a 100-fold reduction in IgE Ab binding and failed to bind 3 of 4 mAb. Intradermal skin testing with the C73–C78 variant supported the *in vitro* findings; the variant was 10 to 100-fold less reactive than rDer p 2. These two bonds thus make markedly different contributions to stabilizing the antigenic determinants of Der p 2. The results suggest that the C73–C78 bond plays a critical role in stabilizing the antigenic structure of this major mite allergen. Copyright © 1996 Elsevier Science Ltd.

Key words: IgE, antigenic structure, allergy.

INTRODUCTION

Inhalant allergens are an important group of antigens: approximately 15–25% of humans are naturally sensitized to common environmental antigens and produce specific IgE Ab and IgG Ab, and T cell responses to these proteins (Platts-Mills *et al.*, 1995; Rawle *et al.*, 1984; O'Brien *et al.*, 1992). The Ab response to allergens can now be studied in detail as many allergens have been purified to homogeneity, biochemically characterized, cloned and sequenced (Chua *et al.*, 1990a, 1990b; Yuuki *et al.*, 1991; Nishyama *et al.*, 1993). Our studies have focused on the 14 kD Group 2 allergens of the house dust mite, *Dermatophagoides pteronyssinus*, which are potent immunogens and elicit humoral and cellular responses in 80–90% of mite allergic individuals (Platts-Mills and Chapman, 1987; Lanzavecchia *et al.*, 1983; O'Hehir *et al.*, 1993; van Neerven *et al.*, 1993). Previous studies have shown that the B cell epitopes are heat and pH resistant, but are destroyed upon reduction and alkylation, suggesting that these determinants are conformational, and dependent on the tertiary structure of the protein (Lombardero *et al.*, 1990). This conclusion is supported by studies using polypeptide fragments produced from truncated Der p 2 cDNA that showed a low prevalence of IgE Ab binding (Chua *et al.*, 1991). Synthetic peptides spanning the entire Der p 2 sequence have also been used to map Ab binding regions, however, only one peptide, amino acids 65–78, retained IgE Ab binding, confirming that the majority of epitopes are conformational (van't Hof *et al.*, 1991). Taken together, these studies suggested that an alternative approach was required to investigate the conformational determinants on Group 2 allergens.

Site-directed mutagenesis has been used successfully for epitope mapping of antigens of known three dimensional structure (Smith *et al.*, 1991; Smith and Benjamin, 1991; Dudler *et al.*, 1994). The tertiary structure of Der p 2 is not known, however, this structure is stabilized by three disulfide bonds and the reduction and alkylation experiments suggested that these bonds are critical to the antigenic structure (Nishyama *et al.*, 1993; Lombardero *et al.*, 1990). We have systematically disrupted each of the three disulfide bonds (C8–C119, C21–C27 and C73–C78) to evaluate the contribution of each bond to the

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Abbreviations: mAb, monoclonal antibody (ies); Ab, antibody (ies); Ag, antigen; GST, glutathione-S-transferase; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

antigenic structure of the protein. Comparisons of the binding of human IgE Ab, *in vitro* and *in vivo*, as well as murine mAb binding, showed that each of the three disulfide bonds of Der p 2 is important to the antigenic structure, but the C73–C78 bond makes the major contribution to stabilizing the antigenic determinants of Der p 2. Furthermore, using site-directed mutagenesis it may be possible to develop new approaches to allergen immunotherapy using variants that lack IgE Ab binding determinants.

MATERIALS AND METHODS

Oligonucleotide mutagenesis

The cDNA for Der p 2 was obtained from Dr Wayne Thomas, Perth, Australia, and subcloned into the bacteriophage M13mp19. Oligonucleotide directed mutagenesis was performed using the method of Kunkel and the Muta-gene Kit (Bio-Rad Laboratories, Richmond, CA), as previously described (Smith and Benjamin, 1991; Kunkel, 1985; Kunkel *et al.*, 1987). Oligonucleotides were synthesized at the University of Virginia Protein and Nucleic Acid Research Facility and were designed to substitute a variety of residues at positions 8, 21, 27, 73, 78 and 119, by providing a mixture of the four nucleotides (N) at the first and second positions of the target codon and dCTP, dGTP at the third position:

Cys8:	5′-C GAT GTC AAA GAT **NNC/G** GCC AAT CAT G-3′
Cys21:	5′-G GTA CCA GGA **NNC/G** CAT GGT TCA GAA CC-3′
Cys27:	5′-GGT TCA GAA CCA **NNC/G** ATC ATT CAT CGT GG-3′
Cys73:	5′-CCA AAT GCA **NNC/G** CAT TAC ATG AAA TGC-3′
Cys78:	5′-GC CAT TAC ATG AAA **NNC/G** CCA TTG GTT AAA GG-3′
Cys119:	5′-GGT GTT TTG GCC **NNC/G** GCT ATT GCT ACT CAT GC-3′

Mutants were identified by DNA sequence analysis, amplified with specific N and C terminal primers in a standard PCR reaction, and subcloned into pGEX2T (Pharmacia Biotech Inc., Piscataway, NJ).

Production and purification of recombinant (r) Der p 2

The plasmid pGEX2TP2 containing the native or mutated sequences, was introduced into the *E. coli* strain TG1, and protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to logarithmic phase cultures. Briefly, an overnight culture was diluted 1:50 into Luria Broth and grown at 37°C with shaking until the OD_{600}nm of the culture reached 1–2. IPTG was added to a final concentration of 1 mM and cultures were continued 4–6 hr. Bacterial cell pellets from 1 l cultures were resuspended in 10 ml PBS (0.01 M Phosphate Buffer, pH7.4, 0.15 M NaCl) and lysed by the addition of lysozyme (1 mg/ml), frozen at −20°C, and then thawed at room temperature. Fifty units of deoxyribonuclease I (DNase I, Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and the slurry was incubated at room temperature for 15 min then centrifuged at 12,000*g* for 15 min. The supernatant was passed over a glutathione–agarose column (Sigma, St Louis, MO) equilibrated in PBS. The column was washed with five volumes of PBS-1% Triton X100, followed by two column volumes of PBS, and eluted with 10 mM glutathione (reduced form) in 50 mM Tris–HCl, pH7.5. Protein concentration was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) and purity was assessed by SDS–PAGE on a PhastSystem (Pharmacia Biotech, Piscataway, NJ) and stained with Coomassie Brilliant Blue. Purified proteins were stored at −20°C. The unmodified glutathione-S-transferase (GST) fusion protein used for Ab binding studies was designated rDer p 2; variants were designated by the single letter amino acid code for cysteine (C), the position in the primary amino acid sequence of that cysteine, followed by the variant amino acid in single letter code, e.g. C21S is the serine (S) substitution at position 21.

Murine and human Ab to mite allergens

The specificity of the murine mAb used in this study has been described previously (Heymann *et al.*, 1989; Ovsyannikova *et al.*, 1994; Akagawa *et al.*, 1991). The mAb αDpX (provided by Dr Rob Aalberse, Amsterdam The Netherlands) and 7A1 define non-overlapping epitopes on Der p 2. The mAb 15E11 and 13A4, which define overlapping epitopes with αDpX and 7A1, respectively, were raised against Der f 2 and kindly provided by Dr Hirokazu Okudaira, Tokyo, Japan. The antibodies were used as 50% ammonium sulfate fractions of ascites. Sera from 40 patients with asthma and 40 patients with atopic dermatitis were selected as a source of IgE Ab. Collection of sera used in this study was approved by the Human Investigation Committee of the University of Virginia. Patients were skin test positive to *D. pteronyssinus*, and/or RAST positive to Group 2 allergens (Duff *et al.*, 1993; Gelber *et al.*, 1993; Sporik *et al.*, 1990). None of the patients were receiving immunotherapy at the time the serum was collected.

Monoclonal antibody modified RAST assay

The anti-Der p 2 mAb 7A1 was coupled to cyanogen bromide activated filter paper discs. After drying, the discs were incubated for 4 hr at room temperature with 100 μl *D. pteronyssinus* extract diluted to contain 1 μg/ml Der p 2, washed, and incubated with human serum diluted 1/10 and 1/50 in horse serum. After incubation for 18 hr at room temperature, discs were washed and incubated with ^{125}I-goat anti-human IgE (10^5 cpm/100 μl) for 6 hr. Discs were washed, transferred to assay tubes and counted. A standard curve was established with a serum pool from mite allergic patients that had been previously assayed against the WHO/IUIS *D. pteronyssinus* reference serum (NIBSC82/528) and was assigned 1000 units/ml IgE Ab to Der p 2 (Pollart *et al.*, 1988).

Direct binding enzyme immunoassay

The reactivity of rDer p 2 with mAb and IgE Ab (serum pool described above) was evaluated in a direct binding ELISA. Antigen was applied to a microtiter plate using 50 μl/well of a 20 μg/ml solution in PBS. After blocking non-reacted sites with PBS containing 0.05 Tween20 and 1% BSA (PBS–Tween, 1% BSA), the mAb or serum pool were added using serial two-fold dilutions. Ab binding was detected using peroxidase conjugated goat anti-mouse IgG or goat anti-human IgE Ab, along with 1 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS, Sigma A-1888) in 70 mM citrate-phosphate buffer, pH4.2 containing 0.03% H_2O_2 (Ovsyannikova *et al.* 1994). The results were evaluated at OD_{405}nm using a Titertek Multiscan Plus plate reader.

Inhibition enzyme immunoassay

The relative ability of variant antigen to interact with mAb and IgE Ab was determined by competitive inhibition in a modified ELISA assay as follows: antigen was mixed with Ab to give a final Ag concentration of 0.01–100 μg/ml. The Ab concentration was predetermined to give an endpoint OD_{405} of 1 in the ELISA in the absence of any inhibitor. All Ag and Ab solutions were prepared in PBS–Tween, 1% BSA. The Ag–Ab mixes were allowed to equilibrate overnight at 4°C and then were pipetted, in duplicate, into wells of a plastic microtiter plate that had been coated with rDer p 2 at 20 μg/ml. The plates were incubated at room temperature for 2 hr and then processed as a standard ELISA (described above) using peroxidase conjugated goat anti-mouse IgG or goat anti-human IgE as the secondary antibody. The positive control was rDer p 2 as inhibitor and the negative controls were GST and PBS-Tween, 1% BSA. Results were expressed as the percent inhibition of the reaction of Ab in the absence of any inhibitor.

Quantitative intradermal skin testing

Skin testing of patients using recombinant allergens was approved by the University of Virginia Human Investigation Committee (protocol 6440). rDer p 2 and C78G were prepared as described above, filtered over a 0.2 μm filter, and diluted to 5 μg/ml in sterile 0.4% phenol/1% human serum albumin saline. Subjects were skin prick tested with these solutions and then injected intradermally with 0.03 ml of dilutions of rDer p 2 and C78G, starting at 10^{-4} μg/ml and increasing the concentration (10-fold dilutions) until the reaction wheal reached 8 × 8 mm.

RESULTS

Reactivity of human IgE Ab and murine mAb with rDer p 2

A monoclonal antibody modified RAST was used to compare the binding of IgE Ab to the native and recombinant Der p 2 by 80 sera from patients with asthma or atopic dermatitis. Figure 1 shows a strong quantitative correlation between IgE binding to Der p 2 and rDer p 2 ($r^2 = 0.77$, $p < 0.001$). In a direct binding ELISA, IgE Ab in a serum pool from seven patients gave overlapping binding curves to Der p 2 and rDer p 2 (Fig. 2A). The mAb αDpX, 7A1, 15E11, and 13A4 gave parallel binding curves using rDer p 2 (Fig. 2B).

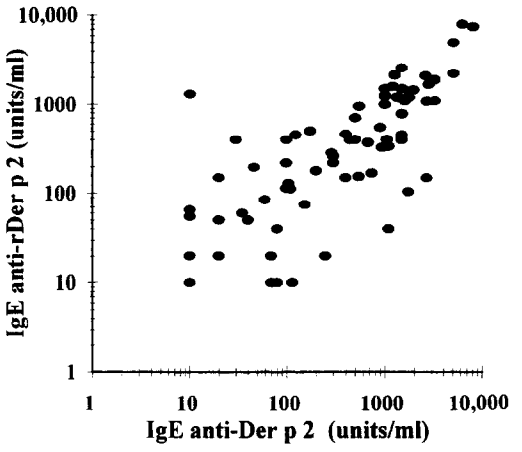

Fig. 1. Correlation between IgE Ab binding to native and recombinant Der p 2. A mAb modified RAST was used to measure IgE Ab binding to Der p 2 and rDer p 2 in sera from 80 patients with asthma or atopic dermatitis. Linear regression analysis showed an excellent correlation between IgE Ab binding to the two proteins ($r = 0.77$, $p < 0.001$).

Generation of rDer p 2 variants

Oligonucleotides were designed to give all 19 amino acid substitutions at each target cysteine and selected variants are shown in Table 1. Bradford Assay and SDS–PAGE analysis of the variants indicated that they were produced in approximately equal amounts from bacterial expression cultures, 1–4 mg/l after affinity purification (Fig. 3).

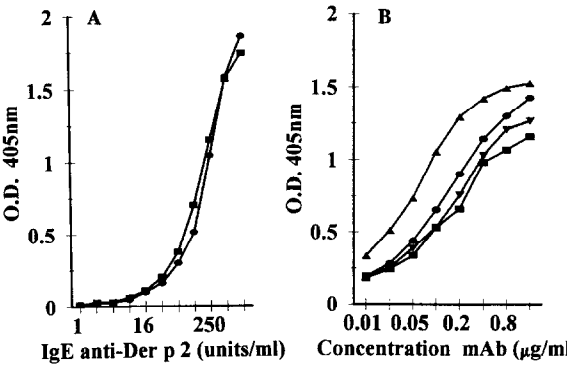

Fig. 2. Comparison of IgE Ab and mAb binding to rDer p 2. Binding curves for IgE Ab and murine mAb were compared by ELISA using plates coated with rDer p 2. Figure 2A compares IgE Ab from a serum pool of seven *D. pteronyssinus* allergic patients binding to Der p 2 (●) and rDer p 2 (■). Figure 2B compares reactivity of 4 mAb with rDer p 2: mAb αDpX (●), mAb 7A1 (▲), mAb 13A4 (▼) and mAb 15E11 (■)

Table 1. Recombinant Der p 2 variants at cysteine residues generated by oligonucleotide-directed mutagenesis

Cysteine Residue[a]	Variant
8	**Gly**[b]
21	**Ser**
21	Arg
21	Leu
27	**Gly**
27	Trp
73	**Arg**
73	Leu
78	**Gly**
119	**Tyr**
119	Pro

[a]Disulfide bonds join cysteine residues 8–119, 21–27 and 73–78 in Group 2 proteins.
[b]Variants shown in bold were evaluated by inhibition ELISA.

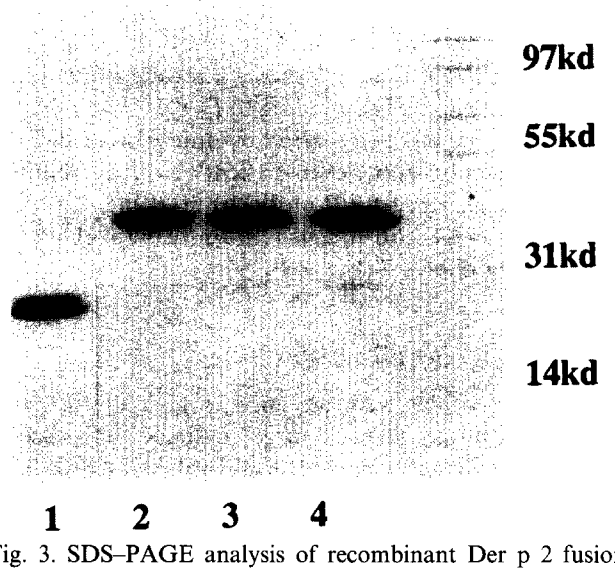

Fig. 3. SDS–PAGE analysis of recombinant Der p 2 fusion proteins. Equal volumes (1 μl) of GST and 3 rDer p 2 variants, affinity purified from 1 liter bacterial expression cultures, were analysed by SDS–PAGE on an 8–25% gel under reducing conditions. Lane 1: GST; Lane 2: C21S; Lane 3: C73R; Lane 4: C119Y. The nomenclature for the rDer p 2 variants is described in Materials and Methods.

Role of disulfide bonds in maintaining antigenic structure

The role of each of the three disulfide bonds in maintaining the antigenic determinants of Der p 2 was evaluated by generating a panel of independent variants and comparing the variants with rDer p 2 in an inhibition ELISA. The ability of the variants to inhibit binding of mAb to rDer p 2 was determined by comparing the amount of variant or rDer p 2 required to give 50% inhibition of Ab binding (Fig. 4). Thus approximately 15 μg/ml of rDer p 2 gave 50% inhibition of mAb αDpX binding (Fig. 4A, 4C, 4E). The C8G variant (shown in Fig. 4A), C21S and C27G variants (Fig. 4C) showed comparable reactivity with αDpX, however, the C73R and C78G variants gave <20% inhibition of αDpX binding at the highest inhibitor concentration tested (Fig. 4E). The C119Y variant showed increased reactivity for both mAb αDpX and 7A1, giving 50% inhibition at 10-fold less antigen, when compared with rDer p 2 (Fig. 4A and 4B). The mAb 7A1 reacted with the other five variants: the C8G variant (Fig. 4B) and the C21S and C27G variants (Fig. 4D) required 0.5 to 10-fold more antigen to give 50% inhibition, the C73R and C78G variants required 10 to 15-fold more antigen to give 50% inhibition (Fig. 4F). The C73R variant also failed to inhibit the binding of two additional mAb, 13A4 and 15E11 (data not shown). As would be expected for mAb with different fine specificities, all 4 mAb gave unique binding profiles, however, the C73R and C78G variants consistently showed the greatest reduction in antigenicity.

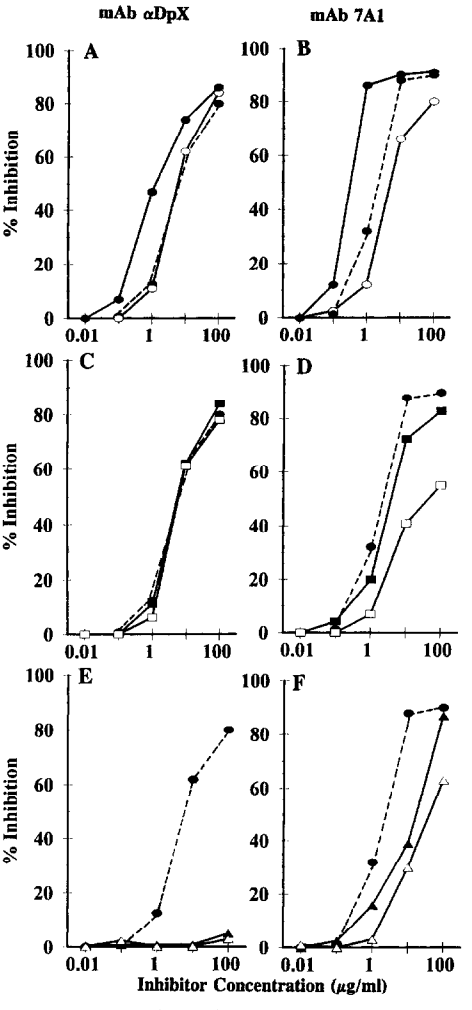

Fig. 4. Monoclonal antibody binding to rDer p 2 cysteine variants. Increasing concentrations of rDer p 2 or rDer p 2 variants were used to block binding of mAb αDpX (Panels A, C and E) and mAb 7A1 (Panels B, D and F) to rDer p 2 in a modified ELISA. Inhibitor antigens were: rDer p 2 (● dashed line), C8G (○) and C119Y (●) (Panels A and B); C21S (■) and C27G (□) (Panels C and D); C73R (▲) and C78G (△) (Panels E and F).

The inhibition of binding of IgE Ab was assessed for 13 individual sera and for the serum pool, from seven additional patients. Using the serum pool, the C8G and C119Y variants gave 50% inhibition of IgE binding at 3-

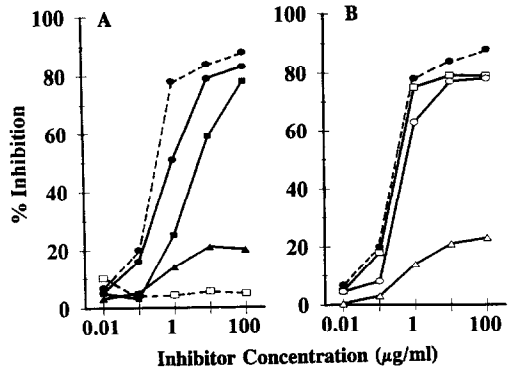

Fig. 5. Inhibition of IgE Ab binding to rDer p 2 using the cysteine variants. rDer p 2 or rDer p 2 variants were used to block binding of IgE Ab from a serum pool as described for Figure 4. The rDer p 2 inhibition curve is shown by (● dashed line). Panel A gives results for inhibitor antigens GST (□ dashed line), C119Y (●), C21S (■), and C73R (▲). Panel B shows results for inhibitor antigens C8G (○), C27G (□) and C78G (△).

to 8-fold higher concentration than rDer p 2; the C21S variant required 35- to 40-fold more antigen, however, the C27G variant gave overlapping inhibition curves with rDer p 2; and the C73R and C78G variants failed to inhibit IgE Ab (Fig. 5). Figure 6 shows the inhibition curves for 4 representative sera of the 13 individual sera

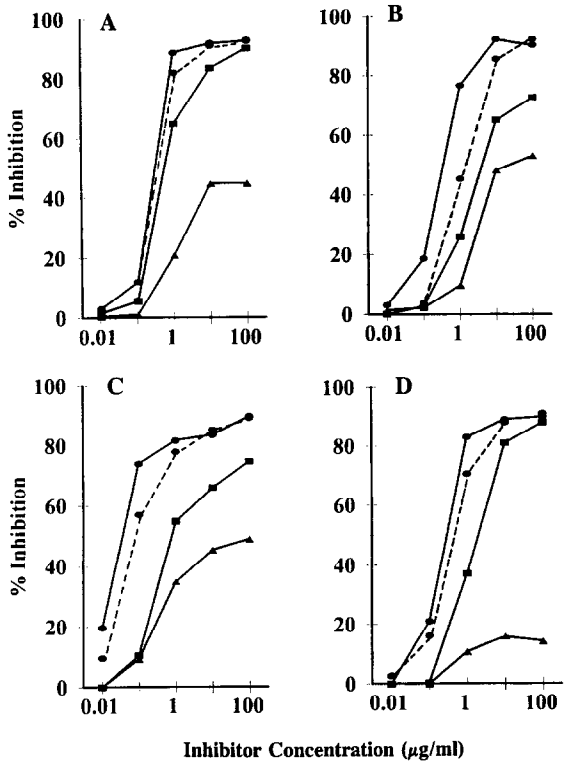

Fig. 6. Inhibition of IgE Ab binding from patients with atopic dermatitis or asthma. Binding of IgE Ab to rDer p 2 was inhibited using rDer p 2 (● dashed line), C119Y (●); C21S (■); C73R (▲). Results are shown for four selected sera: patients J.E. and L.W., shown in Panels A and B, respectively, have atopic dermatitis. Patients K.D. and K.S., shown in Panels C and D, respectively, have asthma. In the absence of inhibitor, using PBS as a control, maximum binding in the ELISA system was OD_{405} = 1.4–1.6, using 1/4–1/16 serum dilutions.

Table 2. Effect of cysteine variants on skin test reactivity

Patient	Quantitative intradermal skin test endpoint (μg/ml) rDer p 2	C78G
M.H.	10^{-1}	10^{0}
M.C.	10^{-2}	10^{-1}
G.R.	10^{-2}	10^{0}
J.L.	10^{-2}	10^{-1}
Control		
T.P.	$>10^{0}$	$>10^{0}$
J.S.	$>10^{0}$	$>10^{0}$
A.S.	$>10^{0}$	$>10^{0}$

Values represent end point dilutions which gave a >8 × 8 mm wheal at 15 min. Negative skin tests are recorded as $>10^{0}$.

tested; Panels 6A and 6B are patients with atopic dermatitis and Panels 6C and 6D are patients with asthma. All of the individual sera were inhibited by C119Y at concentrations equivalent or less than rDer p2. The C21S variant gave overlapping inhibition curves with rDer p2 for two sera, but required 2- to 11-fold more antigen for the other 11 sera. Maximum inhibition by C73R ranged from 11% to 48% for 12 sera and gave 50% inhibition of binding of only one sample at 15 μg/ml, a 12.5-fold concentration increase over rDer p 2. Six sera were from patients with atopic dermatitis and seven were from asthmatics, however, no significant differences in inhibition patterns by the variants were seen among these patients. All 13 sera tested gave the same pattern as seen with the mAb binding to the disulfide variants: the C73R showed the greatest reduction in antigenicity, the C21S variant showed slightly reduced antigenicity, and the C119Y variant showed reactivity comparable to rDer p 2.

Effect of cysteine variants on biological activity of Der p 2

To evaluate *in vivo* reactivity of the variants, quantitative intradermal skin testing was performed using rDer p 2 and the C78G variant. Patients with allergic rhinitis or asthma and Der p 2 specific IgE Ab titers of 40–450 U/ml were tested by prick test using allergen solution of 5 μg/ml. The four patients gave positive prick tests and three non-allergic controls did not react (data not shown). Subjects were injected intradermally with 0.03ml of dilutions of rDer p 2 and C78G. Reactivity to C78G was 10-fold less for three patients and 100-fold less for one patient (Table 2).

DISCUSSION

In 1990, Chua and co-workers cloned the cDNA for Der p 2 and demonstrated that the recombinant protein retained IgE Ab binding reactivity using a solid phase plaque immunoassay (Chua *et al.*, 1990a, 1990b). In the present study, we confirmed that rDer p 2 retains the antigenic structure of the native allergen, by quantitative IgE Ab binding studies using a large panel of sera from atopic dermatitis and asthma patients. In addition, rDer p 2 was recognized by murine mAb raised to the native

allergen. These results indicate that the recombinant allergen is an appropriate model system for investigating the antigen structure of Der p 2.

The tertiary structure of Der p 2 has not yet been determined and previous studies in our laboratory suggested that the intact structure, stabilized by three disulfide bonds, was essential for antibody binding (Lombardero *et al.*, 1990). The three disulfide bonds of Der p 2 are similar to disulfide bonds in other proteins. The C21–C27 bond and the C73–C78 bond stabilize small loops that, by analogy to loops in proteins of known structure such as hen egg white lysozyme, are predicted to occur on the protein surface. The C8–C119 bond of Der p 2 is a non-local disulfide bond, separated by 110 residues. Such bonds link the N and C termini in approximately one third of known structures, suggesting this is a good mechanism for stabilizing the folded protein (Thornton, 1981). Using our mutagenesis strategy, one cysteine from each pair was replaced by another amino acid, preventing the disulfide from forming at that position. For this analysis, a variant at each disulfide bonding pair was compared to control for the influence of the side chain substitution.

As would be expected with mAb to independent epitopes, the cysteine variants showed different patterns of reactivity with each mAb. The C73R variant showed the least reactivity with all mAb suggesting that the changes in antigenic structure induced by this variant are more global rather than local to a particular epitope. However, the mAb 7A1 recognized the C73R and C78G variants but failed to bind reduced and alkylated Ag, indicating that these variants are not completely denatured.

Polyclonal IgE Ab binding to the cysteine variants showed a different pattern of reactivity with each protein, however, even in this case, the C73–C78 bond made the greatest contribution to maintaining the antigenic structure of Der p 2, with loss of this bond shifting the inhibition curves by more than 100-fold, illustrated in Figs 5 and 6, and reducing *in vivo* reactivity by 10 to 100-fold, as shown in Table 2. The disruption of the N–C-terminal disulfide shifted the inhibition curve for IgE Ab by 8–10 fold using the serum pool (Fig. 5), but had little effect on the majority of sera and 3 of 4 mAb (Figs 6 and 4, respectively), indicating that the overall conformation of the protein is largely intact. It has been shown that the N–C disulfide of lysozyme is exposed and can be reduced without destroying the structure of the protein (Thornton, 1981). The striking difference between disruption of the C21–C27 and C73–C78 loops may be explained by the C73–C78 bond making the greatest overall contribution to the stability of the protein. Additional changes in antigenicity may reflect the actual substitutions at these positions. However, the reduction in antigenicity for both the C73R and the C78G variants suggests that the loss in activity is directly related to the loss of the bond rather than the amino acid substitutions. The importance of the C73–C78 bond is also supported by studies using synthetic peptides, which showed that the only peptide that bound IgE Ab encompassed residues 73–78 (peptide 65–78) (van't Hof *et al.*, 1991). This peptide 65–78 may assume a conformation with the intact disulfide bond, thus mimicking the conformation of this sequence in the native protein. An additional study using serine and methionine substitutions for cysteine in the peptide 65–78 suggests heterogeneity of binding specificities even from an individual donor (van't Hof *et al.*, 1993)

In summary, this study demonstrates the contribution of the three disulfide bonds of Der p 2 to the antigenic structure of the protein. In addition, the bonds make different contributions to maintaining this structure as shown by the IgE Ab binding curves for each variant and the reductions seen *in vivo* on quantitative skin testing. The 10- to 100-fold loss in skin test reactivity observed in these preliminary studies is remarkable for a single amino acid substitution and suggests that targeted substitutions (of Cys or other residues) could result in further reduction of IgE antibody binding. Variants with reduced IgE binding, such as C73R and C78G, may provide an alternative strategy for immunotherapy. Current clinical trials of peptide based immunotherapy use a limited number of allergen specific T cell epitopes to induce T cell unresponsiveness and have yet to establish whether this approach will be sufficient to down regulate T cell responses to the complete allergen and reduce symptoms (O'Hehir *et al.*, 1991; Wallner and Geftner, 1994; van Neerven *et al.*, 1994). The advantage to peptide based therapy is that peptides can be chemically produced in gram quantities and they would be predicted to cause a lower frequency of adverse reactions (through lack of binding to IgE Ab). The disadvantages include rapid metabolism of peptides, the difficulty in designing peptides to bind all HLA alleles, and to cover all immunodominant regions. The use of entire recombinant proteins with reduced IgE Ab binding may offer an alternative approach to immunotherapy, which could potentially be applicable to any cloned allergen.

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