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CONFORMATIONAL STABILITY OF B CELL EPITOPES ON GROUP I AND GROUP II *Dermatophagoides* spp. ALLERGENS

Effect of Thermal and Chemical Denaturation on the Binding of Murine IgG and Human IgE Antibodies[1]

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The conformational stability of B cell epitopes on the 25-kDa group I and 14-kDa group II mite allergens was compared by using heat-treated or chemically denatured allergens to inhibit the binding of native ^{125}I allergens to murine mAb or to human IgE antibodies. Structural changes after treatment were assessed by SDS-PAGE and circular dichroism spectroscopy. Heating for 1 h at >75°C, treatment at pH 2.0 or pH 12.0, or with 6M guanidine or 6M urea, reduced the binding of the group I allergens to mAb or IgE antibodies by 10- to 1000-fold. The group II allergens were heat stable and even after prolonged heat treatment (5 h at 75°C or 30 min at 100°C) their antibody binding activity was reduced by < twofold. The group II allergens were also resistant to pH and to denaturation with 6M guanidine. However, after reduction and alkylation, antibody binding sites on both the group I and group II allergens were destroyed. Reduction of disulfide bonds with 2-ME caused a marked shift in the molecular mass of group I allergens on SDS-PAGE, from 25 kDa to 28–31 kDa. Reduction and alkylation also generated two high m.w. forms of *Der p* I and *Der f* I. After heating (100° for 30 min), both *Der f* I and *Der f* II retained significant secondary structure, as judged by circular dichroism spectroscopy, but on reduction they showed the typical spectra of fully denatured proteins (>85% random structure). The results show clear differences between the susceptibility of B cell epitopes on the group I and group II allergens to denaturation. Despite these differences in stability, both allergens are equally potent immunogens for IgE antibody responses in man. The results support the view that the physical properties of allergens (low m.w. and solubility), limiting low dose exposure (1 to 10 ng/day), and host genetic and immunoregulatory processes, are more important than gross structural features in the induction and maintenance of IgE antibody responses.

Immediate hypersensitivity reactions to dust mites (*Dermatophagoides* spp.) occur in 10 to 20% of the population and are a common cause of asthma and other allergic diseases (perennial rhinitis and atopic dermatitis) (1, 2). Indeed, on a worldwide basis, there is increasing evidence that the development of IgE antibodies to dust mite allergens is a major risk factor associated with asthma attacks (3–5). Two major groups of mite allergens have been defined: the 25-kDa group I allergens (*Der p* I, *Der f* I, and *Der m* I) and the 14-kDa group II allergens (*Der p* II and *Der f* II). These groups are structurally and antigenically unrelated, however, allergens within each group show extensive cross-reactivity and amino acid sequence homology (1, 6–16). The group I allergens are excreted in mite feces and recent cDNA cloning, and sequence analysis suggests that they are thiol proteases (17). The group II allergens occur in relatively higher proportions in mite bodies, but their physiologic function is unknown. Both groups of allergens are potent immunogens in man. Most mite allergic patients (>80%) give positive immediate skin tests and make serum IgG and IgE antibodies to the group I and group II allergens (1). Proliferative T cell responses and the isolation of CD4$^+$ T cell clones specific for either *Der f* I or *Der f* II have also been reported (18–20).

Current research in several laboratories is aimed at identifying both B and T cell epitopes and their relationship to immunogenicity, and possible use in immunotherapy. An extensive repertoire of B cell epitopes on the group I allergens has been demonstrated by using human IgG and IgE antibodies, rabbit antibodies, and either polyclonal or monoclonal murine antibodies (10, 16, 21–23). Murine mAb define several nonoverlapping, "species specific" epitopes on *Der p* I and *Der f* I, however, human IgE antibodies bind predominantly to group I cross-reactive epitopes. Thus, different sites on the group I allergens appear to be recognized as immunodominant by mice and humans. We previously postulated that this could reflect altered Ag processing or presentation in different hosts (23). Studies of the group II allergens have shown that both murine and human antibodies recognize common epitopes on *Der p* II and *Der f* II and no species-specific sites have yet been demonstrated (15). There is little data

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on the nature of the sites recognized by mAb or human antibodies on either group of allergens (i.e., whether they are sequential or conformational, or which amino acid residues are involved). We have compared the susceptibility of the group I and group II allergens to thermal and chemical denaturation (as judged by binding of mAb and IgE antibodies) and used SDS-PAGE and CD[3] to analyze the structural changes that occur after denaturation. Antigenic determinants on the group I allergens are readily denatured and appear to be highly conformation dependent, whereas those on the group II allergens are resistant to denaturation and only lose their activity after R&A. The results suggest that different strategies may well be required to identify the antibody-binding amino acid residues on each of these groups of mite allergens. Furthermore, comparisons of the stability of mite and other inhalant allergens suggests that structural stability per se does not have a major influence on immunogenicity for IgE antibody responses in Man.

MATERIALS AND METHODS

Allergens

Group I and group II allergens were purified from aqueous extract of *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* culture by monoclonal affinity chromatography as described previously (15, 24). Briefly, *Der p* I, *Der f* I, or *Der m* I were eluted from mAb 4C1 immunosorbent by using 0.005 M glycine/50% ethylene glycol buffer, pH 10.0. *Der p* II and *Der f* II were eluted by using the same buffer, from either mAb CLB@DpX or mAb 7A1 immunosorbents, respectively. A separate immunoaffinity column was used for each allergen and each column was coupled with ~200 mg mAb (i.e., 10 mg mAb/ml cyanogen bromide-activated Sepharose 4B; Pharmacia, Piscataway, NJ). Eluted fractions were pooled on the basis of allergen content (determined by immunoassay), dialyzed extensively against PBS, pH 7.4, and concentrated by ultrafiltration or lyophilization. The group I allergens were used without additional purification. The group II allergens were precipitated with 50 to 80% SAS as a final purification step. Both the group I and group II allergens were >90% pure as judged by SDS-PAGE and showed <0.01% cross-contamination on immunoassay.

Antibodies

The murine IgG mAb used in these studies have been described previously (23). The anti-group I mAb were as follows: 10B9, 5H8 C12, 5H8 D8, and C4.1, that defined four non-overlapping, species-specific epitopes on *Der p* I; 6A8, that recognized a species-specific epitope on *Der f* I; and 4C1 that bound to a common epitope on all group I allergens. The anti-group II mAb (7A1, 6D6, and CLB@DpX) defined three non-overlapping epitopes present on both *Der p* II and *Der f* II (15). Unless otherwise stated, the mAb were used as 50% SAS fractions of ascites. A mAb to cat allergen *Fel d* I (6F9) was used as a negative control.

A pool of human sera from 11 mite allergic patients was used as a source of IgE antibodies. This serum pool (designated UVA 87/01) was derived from patients with a clinical history of mite allergy, positive immediate skin tests to *D. farinae* (Hollister-Stier, Spokane, WA) and IgE anti-mite RAST values of 100 to 3000 U/ml (1 U is ~0.1 ng IgE) (4). The UVA 87/01 pool contained 346 and 57 U/ml of IgG and IgE anti-*Der p* I antibody, respectively.

^{125}I Labeling

Group I allergens, mAb 4C1 and mAb CLB@DpX were radioiodinated by using the Chloramine T technique (25). In each case, 20 μg protein was labeled with 1 mCi ^{125}I (IMS 30, Amersham International, Arlington Heights, IL) and the sp. act. of labeled proteins were 20 to 40 μCi/μg. The mAb used for iodinations had been purified by preparative IEF (19). Group II allergens (10 μg) were labeled with 1 mCi ^{125}I by using Bolton-Hunter reagent (2000 Ci/mmol, ICN Radiochemicals, Irvine, CA), to a sp. act. of 5 to 10 μCi/μg (15).

Thermal and Chemical Denaturation Experiments

For these experiments, 0.2-ml of group I or group II allergens (at concentrations of 1 mg/ml or 0.2 mg/ml, respectively, in PBS) were treated under various conditions and then tested for their ability to competitively inhibit binding of native ^{125}I-labeled allergens to either mAb or human IgG antibodies. The effect of heat was compared by incubating the allergen solutions at room temperature (22°C), 37°C, 56°C, 75°C, or 100°C at intervals ranging from 30 min to 5 h. The solutions were cooled on ice and stored at 4°C. The effect of pH was assessed by diluting allergens to the same concentrations as above in either PBS; 20 mM HCl, pH 2.0; 20 mM NaOH, pH 12.0; or 5 mM glycine/50% ethylene glycol, pH 10.0. After 2 h at room temperature, the samples were neutralized either by dialysis against PBS or by the addition of 0.2 ml of acid (20 mM HCl), or base (20 mM NaOH), and 20× PBS.

To determine of the effect protein denaturants, 40 to 200 μg allergen was incubated in either 6 M guanidine-HCl or 6 M urea in 0.25 M Tris-HCl/1 mM EDTA buffer, pH 8.5, for 4 h and dialyzed overnight against the same buffer. For reduction and alkylation, 40 to 200 μg allergen was incubated in 6 M guanidine-HCl, 0.25 M Tris-HCl/1 mM EDTA/0.5% 2-ME, pH 8.5, for 2 h in the dark and sparged intermittently with nitrogen. Reduced cystine residues were S-alkylated by the addition of 4 μL 4-vinyl pyridine (final concentration 4%) for 2 h and samples were then dialyzed against 0.25 M Tris-HCl, pH 8.5. The final concentrations of allergens used in the denaturation or reduction and alkylation experiments were 1 mg/ml and 0.2 mg/ml of the group I and group II allergens, respectively, in a reaction vol of 0.2 ml.

Competitive Inhibition Studies

Using murine mAb. For group I allergens, plastic microtiter wells (Immulon II Removawells, Dynatech, Alexandria, VA) were coated with 1 μg/well of 50% SAS precipitated mAb in 0.1 M bicarbonate buffer, pH 9.6, overnight at 4°C. The wells were incubated with 0.1 ml 1% BSA in PBS-T for 45 min, washed three times with PBS-T, and incubated with 0.05 ml ^{125}I allergen (2 to 5 ng, ~100,000 cpm), together with 0.05 ml of native or treated allergen at concentrations of 0.01 to 100 μg/ml. After 2 h, wells were washed five times with PBS-T and counted in a gamma counter. Mean binding in the absence of inhibitor (determined by incubating 8 wells with 0.05 ml 1% BSA PBS-T) ranged from 25 to 40% of the added radioactivity, depending on the mAb used on the solid phase. Background binding, using wells coated with anti-*Fel d* I mAb (6F9) was 0.8 to 1.3% of the counts added. The percentage inhibition of binding of ^{125}I allergen to the mAb by native or treated allergens was calculated as:

$$100 - 100 \times \frac{\text{mean cpm inhibitor} - \text{mean cpm background (6F9)}}{\text{mean cpm 0\% inhibition} - \text{mean cpm background (6F9)}}$$

For the group II allergens, the assay was modified by using mAb coupled to cyanogen bromide-activated paper discs as the solid phase. Maximum binding in this assay was 12 to 30% of ^{125}I allergen added, with a background of 1.4 to 1.7%.

Using human IgE antibodies. 0.1 ml of diluted mite allergic serum pool (UVA 87/01) was incubated with 0.05 ml ^{125}I allergen (2 to 5 ng) and serial 10-fold dilutions of native/treated allergen (0.002 to 20 μg/ml) for 4 h. After the addition of 0.1 ml 1/200 dilution of IgE PS myeloma serum, the bound ^{125}I allergen was immunoprecipiated overnight, at 4°C, with 0.1 ml monospecific goat anti-human IgE (diluted 1/4). The precipitates were washed three times with BBS, pH 8.0, and counted in a gamma counter. The serum pool was used either undiluted or at 1/3 or 1/4 dilution, depending on the allergen tested. Maximum binding, determined by incubating *Fel d* I (30 μg/ml) with ^{125}I allergen was 15% of the added radioactivity and the background binding using a serum with no specific IgE anti-mite antibody was 0.4 to 0.7%. In all competitive inhibition experiments, assays were carried out in duplicate and the assay diluent was 1% BSA/PBS-T.

Two-site Immunoassays

Group I and group II allergen concentrations were measured by two-site mAb RIA, as described previously (15, 26). Values for group I were interpolated from a control curve using reference preparations, *D. pteronyssinus* UVA 87/03 and *D. farinae* UVA 87/02, that had been sub-standardized from the WHO/IUIS International Reference Preparation of *D. pteronyssinus* (code NIBSC 82/518; 12.5

[3] Abbreviations used in this paper: CD, circular dichroism; group I allergen, the 25-kDa mite allergens (*Der p* I, *Der f* I, and *Der m* I); group II allergen, the 14-kDa mite allergens (*Der p* II and *Der f* II); SAS, saturated ammonium sulfate; BBS, borate buffered saline, pH 8.0; PBS-T, PBS, pH 7.4, containing 0.05% Tween 20; R&A, reduced and alkylated.

μg *Der p* I per ampoule) (27). The reference for the group II assays was affinity-purified *Der f* II (12).

CD Spectroscopy

The CD spectra of *Der f* I and *Der f* II were taken with a JASCO J600C spectropolarimeter (Jasco, Easton, Md) calibrated with 0.06% ammonium d-10 camphorsulfonate. *Der f* I (800 μg/ml, 3.0×10^{-4} M) and *Der f* II (300 μg/ml, 4.6×10^{-2} M) in BBS, pH 8.0, were scanned a minimum of 10 times at room temperature and the resultant spectra averaged. The band width was 1.0 nm; scan speed 5 nm/min; time constant, 8 s; and step resolution 0.1 nm. Estimates of structural content were made according to the method of Chen et al. (28). After taking the CD spectra of the native allergens, the solutions were heated at 100°C for 30 min and reanalyzed. The solutions were then reduced by the addition of 2-ME (final concentration 0.5%) and CD spectra of the heated and reduced allergens were taken.

RESULTS

Thermal and pH stability of B cell epitopes on group I and group II allergens. The effect of thermal denaturation was studied using heated group I or group II allergens to inhibit binding of the respective ^{125}I-labeled native allergens to murine mAb or human IgE antibodies. Heating *Der p* I reduced binding of the allergen to both IgE antibodies and to a panel of 5 mAb directed against non-overlapping epitopes on the molecule. At 37°C for 1 h, there was no effect on binding, whereas at 56°C (1 h) there was a 5- to 10-fold reduction in binding, and at 75°C (1 h) and at 100°C for 10 min, allergen binding was reduced by >100-fold (Fig. 1, *A–D*). Similar results were obtained on heating *Der f* I (Fig. 2, *A* and *B*) and *Der m* I (data not shown). In contrast, the group II allergens were heat stable and even after prolonged heating (5 h at 75°C or 30 min at 100°C) there was a less than twofold loss in antigenic activity (Fig. 1, *E* and *F* and Fig. 2, *C* and *D*). Differences in the heat stability of the group I and group II allergens were confirmed by comparing the two-site mAb RIA binding curves for each allergen in *D. pteronyssinus* and *D. farinae* extracts before and after heating at 100°C. Both extracts completely lost their group I allergen, but 50 to 60% of the group II allergen was detectable, even after heating at 100°C for 20 min (Fig. 3). To exclude the possibility that heating had activated the enzymic activity of the group I allergens, leading to "autolytic" digestion of the molecules, six samples of *Der p* I that had been heated at 56°C for 2 to 24 h were analyzed on SDS-PAGE. These samples all showed a single 25-kDa band indistinguishable from that of native allergen, indicating that the molecule remained intact after heating (data not shown).

There were also differences in the susceptibility of the group I and group II allergens to pH. The group I allergens were extremely sensitive to acid pH and after 2 h treatment at pH 2.0, the binding of both mAb and IgE antibodies to *Der p* I was reduced by 100- to 1000-fold as compared to allergen kept at pH 7.4 (Fig. 4). *Der f* I and *Der m* I showed similar susceptibility to acid pH (Table I). Treatment of the Group I allergens at pH 12.0 reduced mAb and IgE antibody binding by 10- to 100-fold, however, mild alkaline treatment (pH 10.0), in the presence of ethylene glycol, had no effect on antibody binding (Fig. 4). The group II allergens were resistant to changes in pH and when they were treated at either pH 2.0 or pH 12.0 they showed the same degree of inhibition of mAb or IgE antibody binding as the native allergens (Table I).

Effect of protein denaturants and reduction and alkylation. Antigenic sites on the group I allergens were susceptible to treatment with 6 M guanidine and, to a lesser extent, 6 M urea. Guanidine treatment completely denatured *Der p* I or *Der f* I so that they showed no significant inhibition of either mAb or IgE antibody binding to ^{125}I native allergen (<15% and <5%, respectively) (Table II). Urea-treated *Der p* I had little effect on the binding of two mAb (10B9 and 5H8D8, <15% inhibition), and partially inhibited binding of three other anti-*Der p* I mAb to the native allergen (5H8 C12, C4.1, and 4C1, 39 to 59% inhibition). Similar partial inhibition was obtained by using urea denatured *Der f* I (Table II), suggesting that 6 M urea was less effective than 6 M guanidine at unfolding these proteins. When the group II allergens were treated with guanidine, they retained their ability

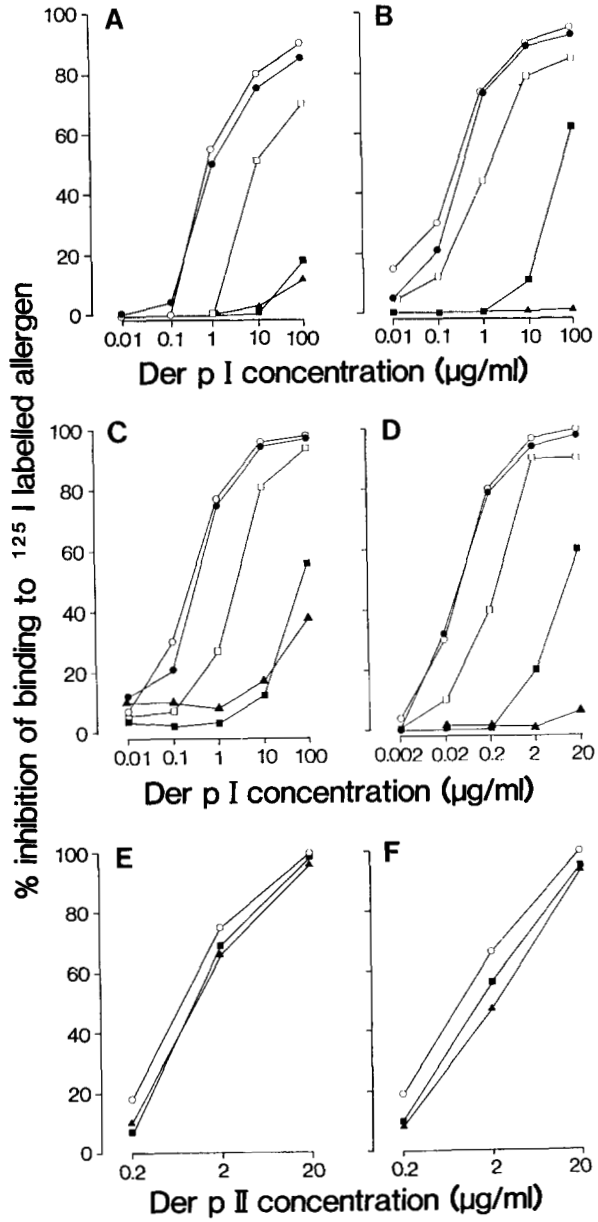

Figure 1. Thermal stability of *Der p* I and *Der p* II. Binding of either mAb or human IgE antibodies to either *Der p* I or *Der p* II was inhibited by using 0.1 ml of dilutions of native allergen (○) or allergens that had been heat treated for 1 h at 37°C (●), 56°C (□), 75°C (■), or for 10 min at 100°C (▲). The panels show results obtained by using anti-*Der p* I mAb, clones 10B9, (*A*); 5H8 C12, (*B*); and 4C1, (*C*); human IgE antibodies in serum pool UVA 87/01, (*D*); anti-*Der p* II mAb, clone CLB@DpX, (*E*); and anti-*Der f* II clone 7A1, (*F*).

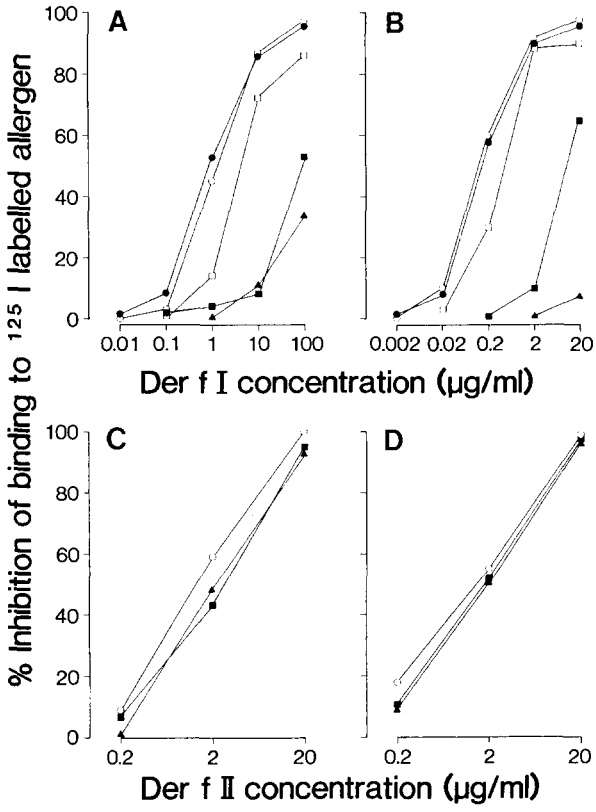

Figure 2. Thermal stability of *Der f* I and *Der f* II. Allergens were heated as described in the legend to Figure 1 and tested for inhibition of the binding of native allergen to mAb 4C1, (*A*); human IgE antibodies, (*B*); mAb 7A1, (*C*); or 6D6, (*D*).

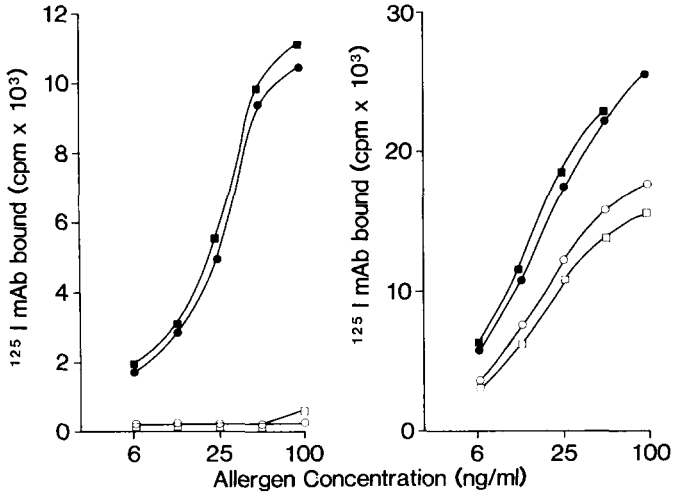

Figure 3. Effect of heat on the group I and group II content of mite extracts. *D. pteronyssinus* (●) or *D. farinae* (■) extracts were assayed for group I (*left panel*) or group II allergens (*right panel*) by monoclonal RIA before (*solid symbols*) and after (*open symbols*) heating at 100°C for 20 min.

to bind both mAb and IgE antibodies and inhibited antibody binding to the same extent as native allergen (70 to 87%, Table II). However, after reduction and alkylation by using 2-ME and 4-vinyl pyridine, both the group I and group II allergens showed complete loss of antigenicity (Table II).

Reduction of the disulfide bonds in the group I allergens with 2-ME or reduction and alkylation (as above) appeared to cause conformational changes resulting in an increase in the apparent molecular mass of these allergens, from 25 kDa to 28–31 kDa, on SDS-PAGE (Fig. 5, *A* and *B*). The magnitude of this shift in molecular mass was greatest for *Der f* I, suggesting that there may be some differences between the denatured structures of *Der f* I and the other group I allergens. After reduction and alkylation, both *Der p* I and *Der f* I showed two higher molecular mass species (28 to 33 kDa) derived from homogeneous preparations of the native allergens (Fig. 5*B*). Changes in apparent molecular mass were less marked for group II allergens, though a slight increase of ~1 kDa was seen in both *Der p* II and *Der f* II (Fig. 5*B*).

CD analyses. Changes in the secondary structure of *Der f* I and *Der f* II after thermal denaturation and reduction were analyzed by comparison of their CD spectra at 200 to 240 nm (Fig. 6, *A–C*). Based upon the CD spectrum of native *Der f* I, the molecule was estimated to contain

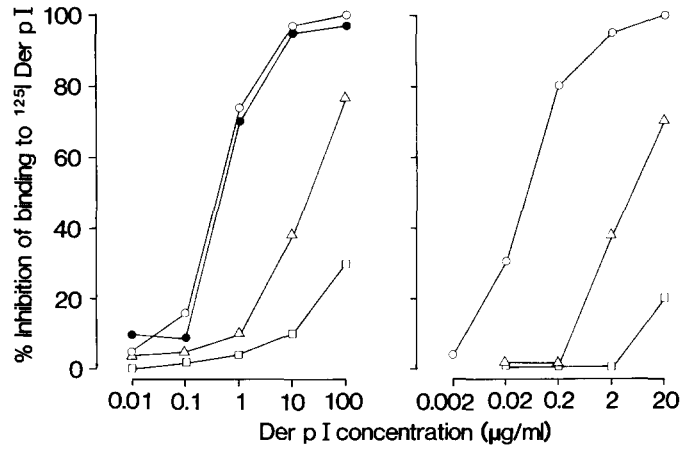

Figure 4. pH stability of *Der p* I. The allergen was treated at pH 7.4 (○); at pH 2 (□); at pH 12 (△); or at pH 10 in the presence of 50% ethylene glycol (●); as described in *Materials and Methods*, and used to inhibit binding of either mAb 4C1 (*left panel*) or human IgE antibodies (*right panel*) to native ^{125}I allergen.

TABLE I

pH treatment of group I and group II allergens—effect on antibody binding[a]

Inhibitor[b]		% inhibition of ^{125}I allergen binding		
		IgE	4C1	6A8
Group I				
Der f I	native	94	94	95
	pH 2	<5	15	<5
	pH 12	64	45	75
Der m I	native	90	94	NT
	pH 2	10	8	NT
	pH 12	70	61	NT

Inhibitor[c]		% inhibition of ^{125}I allergen binding		
		IgE	@DpX	7A1
Group II				
Der p II	native	88	75	70
	pH 2	86	75	63
	pH 12	85	70	68
Der f II	native	87	71	72
	pH 2	87	92	77
	pH 12	85	70	50

[a] Group I allergens (at 1 mg/ml) or group II allergens (at 0.2 mg/ml) were treated for 2 h at either pH 2.0 or pH 12.0, neutralized, dialyzed overnight against PBS, and used to inhibit binding of ^{125}I allergen to mAb or IgE antibodies (mite allergic serum pool UVA 87/01). NT = not tested.

[b] Results obtained by using 2 µg/ml inhibitor in IgE assays and 10 µg/ml inhibitor in assays using mAb. The results obtained by using *Der f* I or *Der m* I at these concentrations were very similar to those obtained for *Der p* I (Fig. 4).

[c] Percent inhibition obtained by using inhibitor concentrations of 0.2 µg/ml and 2.0 µg/ml in assays using IgE antibodies and mAb, respectively.

TABLE II

Effect of protein denaturation and reduction and alkylation on antibody binding to group I and group II allergens[a]

Allergen	Treatment	% inhibition of ^{125}I allergen binding to	
		IgE[b]	mAb[c]
Group I			
Der p I	native	100	98 (92–98)
	6 M guanidine	<5	10 (0–15)
	6 M urea[d]	ND	39 (10–59)
	R & A	<5	<5
Der f I	native	96	91
	6 M guanidine	<5	<5
	6 M urea[d]	ND	45
	R & A	<5	<5
Group II			
Der p II	native	83	70
	6 M guanidine	81	68
	R & A	<5	<5
Der f II	native	87	72
	6 M guanidine	86	78
	R & A	<5	<5

[a] 40 to 200 μg allergen was treated with 6 M guanidine or 6 M urea, or reduced and alkylated with 2-ME and 4-vinyl pyridine (R & A). After dialysis, samples were diluted and used to inhibit binding of ^{125}I allergen to either mAb or IgE antibodies. For group I assays, allergens were used at 10 μg/ml and for group II assays, at 0.2 μg/ml using IgE antibodies and at 2 μg/ml using mAb.

[b] Mite allergic serum pool (UVA 87/01).

[c] Values for group I and group II allergens were obtained by using mAb 4C1 or 7A1, respectively. Values in parenthesis indicate the range of results obtained by using four other anti-*Der p* I mAb: 10B9, 5H8 C12, 5H8 D8, and C4.1.

[d] Used at 1 μg/ml.

10% helix, 50% β-sheet, 20% β-turn, and 20% nonregular structure (see Table III). This is in good agreement with the CD spectrum of *Der p* I reported by Stewart and colleagues (29). Upon heat treatment, there was an observed change in the secondary structure of *Der f* I, however, the molecule was not totally denatured. When the heated allergen was reduced, there was an overall loss of structure, with the molecule shifting to 85% nonregular structure (Fig. 6C and Table III). Native *Der f* II appears to have a different secondary structural composition to *Der f* I, as would be expected (Fig. 6*B* and Table III). Heat treatment appeared to have less of an effect on the secondary structure of *Der f* II than *Der f* I. However, as in the case of *Der f* I, *Der f* II showed almost complete loss of secondary structure after reduction, essentially giving the CD spectrum of a completely denatured molecule.

Biologic effects of heat denaturation. To investigate whether heat treatment of the group I and group II allergens also affected their activity in vivo, the immediate skin test reactivity of native and heat-treated *Der f* I and *Der f* II was compared by quantitative intradermal skin testing (Table IV). There was a 3 to 4 log reduction in the skin test reactivity of *Der f* I after heating at 100°C for 5 min, and complete loss of reactivity after heating for 20 min. In contrast, heat treatment of *Der f* II had no effect on the end point concentration giving a positive skin test in any of the four patients tested. The skin test results were in good agreement with mAb immunoassay results comparing allergen levels in the native and heat-treated allergens (Table IV), thus showing a good correlation between the effects of heat denaturation both in vitro and in vivo. Similar data on the heat susceptibility of *Der f* I and *Der f* II were obtained by Yasueda et al using the histamine release assay (13).

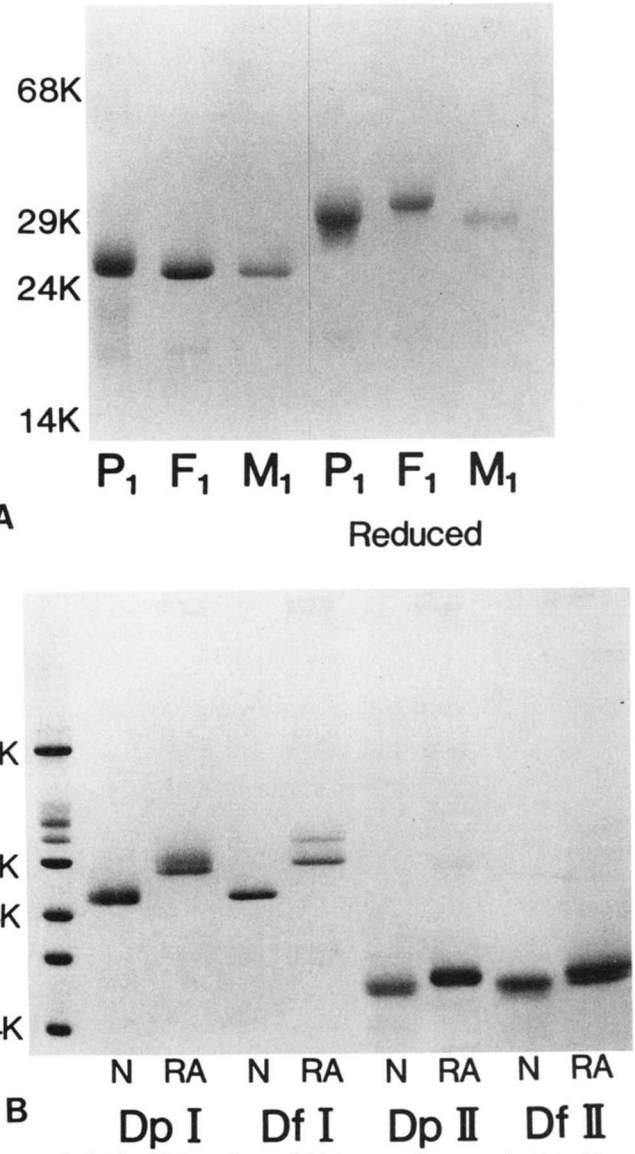

Figure 5. SDS-PAGE analysis of R&A mite allergens. *A*, 10 to 20 μg *Der p* I, *Der f* I, or *Der m* I was analyzed on a 15% acrylamide gel with a 5% stacking gel under nonreducing conditions (*left panel*), or under reducing conditions (5% 2-ME) (*right panel*). *B*, analysis of 10 to 20 μg either *Der p* I/*Der f* I or *Der p* II/*Der f* II under nonreducing conditions. N = native allergen. RA = allergen treated by reduction and alkylation with 2-ME and 4-vinyl pyridine.

TABLE III

Secondary structure estimates for Der f I and Der f II based on circular dichroism spectroscopy

Protein[a]	Estimated % Structural Content[b]			
	Helix	β-Sheet	β-turn	Nonstructure
Der f I (native)	10	50	20	20
Der f I (heat-treated)	20	65	5	20
Der f I (reduced)	15			85
Der f II (native)	10	50	15	25
Der f II (heat-treated)	5	55	15	25
Der f II (reduced)	5			95

[a] The CD spectra of *Der f* I and *Der f* II were compared by using the native molecules and by using allergens that had been heat-treated (100°C for 30 min) and then reduced with 2-ME.

[b] Based on the method of Chen *et al.* (28).

DISCUSSION

The present results clearly demonstrate that B cell epitopes on the group I allergens are more susceptible to denaturation than those on the group II allergens. The

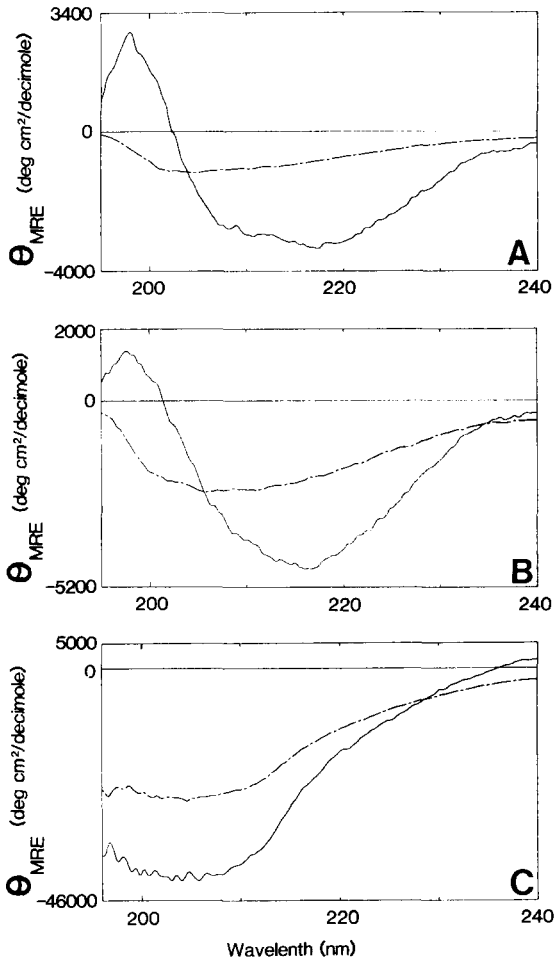

Figure 6. CD spectra of *Der f* I and *Der f* II. *Panels A* and *B* show the CD spectra of *Der f* I and *Der f* II, respectively, under native conditions in BBS, pH 8.0, (———), and after treatment (100°C for 30 min) in the same solution (-—-). *Panel C* shows the spectra obtained when the heated allergens, *Der f* I (—·—) or *Der f* II (———), were analyzed after being reduced with 2-ME for 16 h.

TABLE IV

Effect of heating on immediate skin test reactivity of Der f I and Der f II

	Quantitative intradermal skin test end point (μg/ml)[a]					
	Der f I[b] Heated (100°C)			*Der f* II[b] Heated (100°C)		
	Native	5 min	20 min	Native	5 min	20 min
Patient						
M.H.	10^{-5}	10^{-1}	$>10^{-1}$	10^{-5}	10^{-5}	10^{-5}
P.D.	10^{-4}	10^{-1}	10^{0}	10^{-6}	10^{-6}	10^{-6}
L.G.	10^{1}	NT	NT	10^{-3}	10^{-3}	10^{-3}
J.B.	10^{-3}	10^{1}	$>10^{1}$	$>10^{1}$	NT	NT
M.H.	10^{-4}	10^{0}	$>10^{0}$	10^{-4}	10^{-4}	10^{-4}
Controls ($n = 4$)[c]	$>10^{1}$	$>10^{1}$	$>10^{1}$	$>10^{1}$	$>10^{1}$	$>10^{1}$

[a] Values represent end point dilutions which gave a >8 × 8 mm wheal at 15 min. Patients were injected intradermally with 0.03 ml of dilutions of either native or heat treated allergens. Negative skin tests are recorded as either $>10^{0}$ (i.e., 1 μg/ml) or $>10^{1}$ (10 μg/ml); NT = not tested.

[b] Allergen solutions (at 1 mg/ml) were filtered through 0.22 μ Millipore filters: 200 μL of each allergen was then boiled and 40 μL aliquots were withdrawn at 5 min and at 20 min. Native and heat treated allergens were serially diluted from 10 to 10^{-6} μg/ml (10-fold dilutions) in sterile 0.4% phenol/1% human serum albumin saline. By mAb immunoassay, the 10 μg/ml solution of *Der f* I, heated for 5 min at 100°C, contained 0.01 μg/ml of immunoreactive *Der f* I (i.e., a 1000-fold reduction in activity). The 10 μg/ml *Der f* I solution that had been treated for 20 min showed no reactivity on immunoassay. The heat-treated *Der f* II solutions showed a <two-fold difference in activity on immunoassay, when compared to native *Der f* II.

[c] Four non-atopic individuals with negative skin prick tests to *D. farinae*.

group I allergens were thermolabile and irreversibly denatured by short treatment at acid pH or by protein denaturants. In contrast, antigenic sites on the group II allergens were resistant to most denaturation procedures, with the exception of reduction and alkylation. Our results for the group I allergens are in keeping with previous reports that these allergens were susceptible to heat or acid treatment (6, 10, 13, 16). In the present experiments, there was no evidence for selective loss of specific sites during or after denaturation. Binding of each of the mAb or IgE antibodies to either group of allergens was affected equally by any procedure that caused denaturation. The finding that urea was a less effective denaturant than guanidine for the group I allergens probably reflects differences between the reaction kinetics of these denaturants, although intrinsic differences in the susceptibility of proteins to guanidine or urea have been reported (e.g., using staphylococcal nuclease mutants) (30). The precise mechanisms for denaturation of the group I allergens remain to be established, though the effects of alkaline pH could reflect deamidation and/or disulfide interchanges.

The increased apparent M_r of the group I allergens after reduction suggests that reduction of the disulfide bonds unfolds these proteins, giving a more linear conformation. When the allergens were otherwise denatured, by heat or pH treatment, they did not show this effect. Comparison of the amino acid sequence of *Der p* I with other thiol proteases, suggests that *Der p* I may have three disulfide bonds, and we would expect the other group I allergens to have similar disulfide linkages, based on their extensive amino acid sequence homology (17, 31). However, the numbers and alignments of the disulfide bonds in *Der p* I have not been formally proved, but the *Der p* I cDNA sequence does show eight cystine residues. Although the full sequences of *Der f* I and *Der m* I have not yet been determined, the N-terminal sequences are similar (16) and our protein sequence data on *Der f* I, which is 90% complete, shows ~80% homology with the *Der p* I cDNA sequence (P. Griffin, D. Hunt, J. W. Fox, and M. D. Chapman, unpublished observations). The molecular mass shift that we have demonstrated on SDS-PAGE explains apparent variations in the molecular mass of the group I allergens (from 24 to 30 kDa) reported previously (1, 6–10, 14, 32), and also confirms that detection of these allergens by immunoblotting would vary depending on the conditions used for electrophoresis (32). The rapid loss of antigenic reactivity of the group I allergens caused by heat or reduction must also account for the fact that group I allergens appear to be relatively unimportant when assessed by immunoblotting (32). The generation of two high m.w. bands on SDS-PAGE after reduction and alkylation appears to be a particular feature of *Der f* I and we have demonstrated double bands with similar m.w. by using 2-ME with iodoacetamide as the alkylating agent (data not shown). These double bands may represent differentially alkylated forms of *Der f* I and may also reflect structural differences between *Der f* I and *Der p* I. Additional studies will be required to establish the biochemical basis for the high m.w. species.

In practice, there are several areas where these stability studies are relevant to the clinical investigation of mite allergy: i) in monitoring of mite allergen exposure in the

home; ii) in the development of treatment regimes based on allergen avoidance; and iii) in the preparation and standardization of mite extracts used for diagnosis and treatment. Despite their susceptibility to denaturation, there has been surprisingly little difficulty in using the group I allergens as markers to assess environmental exposure to dust mites and the consistency of *Der p* I and *Der f* I measurements in epidemiologic studies from many parts of the world (reviewed in Ref. 3) suggests that these allergens must be relatively stable in house dust. We recently showed that the ratio of group I:group II allergens in mite body and mite culture extracts can vary by 300-fold, from 0.1:1 to 35:1 (15), and have confirmed those observations in a series of commercially available mite extracts (M. D. Chapman and P. W. Heymann, unpublished observations). The reasons for these differences relate in part to the source material used for extraction, however, our results also raise the possibility that some manufacturing procedures may selectively remove or destroy different mite allergens.

Our results strongly suggest that antigenic determinants on the group I allergens are conformational. The loss of antigenic reactivity and secondary structure of the group II allergens after reduction and alkylation argues against any strictly sequential sites on these molecules. However, this possibility that cannot be excluded. The presence of cystine as part of a sequential epitope, or of conformational changes resulting in the internalization of sequential sites after reduction and alkylation could also explain the results. Although not yet published, the amino acid sequence of *Der p* II has been determined by cDNA cloning and contains six cystine residues which could potentially form disulfide bonds.[4] The group II allergens are clearly more resistant to denaturation than the group I allergens and the present studies suggest that different strategies will be required to identify the amino acid residues involved in antibody binding for each of these groups of allergens. Mapping experiments using peptides either in solution or on a solid phase are unlikely to be useful for the group I allergens, unless the peptides are large enough to assume a functional conformation. This approach may be more worthwhile for the group II allergens that bind well to antibodies under denaturing conditions, such as those used for immunoblotting. Expression systems for producing recombinant mite allergens are currently being developed and the use of site-directed mutagenesis and deletion mutants combined with mAb appears to be the most promising approach to epitope mapping, particularly for the group II allergens.

Parallel situations in susceptibility to denaturation have been observed for several other allergens. Virtually any modification of the ragweed allergen *Amb a* I makes the molecule essentially nonreactive with IgG or IgE antibodies, and mAb raised against denatured *Amb a* I do not bind the native allergen (33–35). In contrast, rye grass allergen *Lol p* I, cod allergen *Gad c* I, and the chironomid hemoglobin allergens are resistant to reduction and alkylation, and sequential antigenic sites on these allergens have been identified by peptide mapping studies (36–40). A striking feature of the present results is that two groups of allergens derived from a single source show great differences in stability and yet are almost equally effective in stimulating IgE antibody responses. This data indirectly supports the view that the physical properties of allergens (low m.w. and rapid solubility in aqueous solution); the route and dose of immunization; and the ability of the host to recognize limiting doses of inhaled Ag are key factors influencing the development of IgE antibody responses in man. Despite increasingly sophisticated molecular analyses, including primary and tertiary structure, there is no evidence for characteristic structures or chemical properties associated with IgE antibody responses. Indeed, it seems increasingly likely that any soluble, low m.w. protein, that is regularly inhaled in the dose range from 1 to 10 ng/day, will cause IgE responses in genetically predisposed individuals.

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Note added in proof. The full paper of the cDNA cloning of *Der p* II, by Chua et al.,[4] is in press in *Int. Archs. Allergy Appl. Immunol.*

[4] **K. Y. Chua et al.** 1988. Paper presented at the XIII International Congress of Allergology and Clinical Immunology, Montreux, Switzerland, October 16–21. *N. Engl. Reg. Allergy Proc. 9:415 (Abstr.).*

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