

MONOCLONAL ANTIBODIES TO DENATURED RAGWEED POLLEN ALLERGEN *Amb a I*: CHARACTERIZATION, SPECIFICITY FOR THE DENATURED ALLERGEN, AND UTILIZATION FOR THE ISOLATION OF IMMUNOGENIC PEPTIDES OF *Amb a I*

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Abstract—The epitope structure of short ragweed allergen *Amb a I* (formerly antigen E or AgE) has been investigated by characterizing monoclonal antibodies (MAbs) from mice immunized with *Amb a I*. The MAbs to *Amb a I* that we previously produced and characterized (HaAl, HaBl and HaCl) reacted with determinants which were conformationally dependent. Denaturation of *Amb a I*, which is a prerequisite for peptide isolation and hence epitope localization, destroyed these determinants. We report here the isolation of 15 MAbs produced against denatured *Amb a I* and present their reactivities with native *Amb a I*, denatured *Amb a I*, and peptide fragments of *Amb a I*. None of the 15 MAbs to denatured *Amb a I* bind to native *Amb a I*, as judged by their lack of binding to *Amb a I* in immune complex with HaAl and HaCl. All 15 MAbs reacted with the extensively reduced and alkylated form of denatured *Amb a I* and 14 of the 15 were shown by Western blotting techniques and ELISA to have activity against the isolated alpha chain or beta chains of *Amb a I*, but not both. In addition, several MAbs react with tryptic fragments of *Amb a I* which allowed us to isolate and sequence two tryptic peptides of *Amb a I*. Antisera raised against KLH-coupled synthetic analogs of these peptides reacted well with intact, denatured *Amb a I*. Finally, during chromatographic purification of *Amb a I* from fresh pollen extracts, various MAbs began to react with *Amb a I* as a function of stage of isolation, suggesting that some proportion of *Amb a I* spontaneously denatures during purification, converting from the allergenic form possessing two major B cell epitopes to an allergenically inactive form.

INTRODUCTION

Amb a I (formerly Antigen E or AgE) is the major allergenic component of short ragweed (*Ambrosia artemisiifolia* L.) pollen (King *et al.*, 1964), and is a major cause of late summer hayfever in North America (King, 1976). On average, as much as 13% of the total serum IgE in ragweed-sensitive individuals is specific for *Amb a I* (Zeiss *et al.*, 1973). *Amb a I* is an acidic (pI 4–6), 38,000 mol. wt, non-glycosylated protein of the pollen intine (Knox *et al.*, 1970; Knox and Heslop-Harrison, 1970). Although resistant to a

number of proteases, *Amb a I* is cleaved spontaneously during extraction and chromatographic isolation into an alpha chain (26,000 mol. wt) non-covalently associated with a beta chain (12,000 mol. wt) (King *et al.*, 1981). Both the single and two chain forms of *Amb a I* are highly reactive with IgE and are allergenically and antigenically indistinguishable (King *et al.*, 1981). On the other hand, various physical and chemical modifications of *Amb a I* (King *et al.*, 1964, 1974) result in a marked loss of both antigenic and allergenic activity as assessed by immunoassay utilizing polyclonal antisera.

To begin to characterize the epitope structure of *Amb a I*, a series of murine monoclonal antibodies (MAbs) was raised to purified, native *Amb a I* (Olson and Klapper, 1986). Three non-overlapping, non-repeating antigenic sites were defined (sites A, B and C) and MAbs directed to two of these sites (A and B) together inhibited 80% of human IgE binding to *Amb a I* (Olson and Klapper, 1986). As with polyclonal antisera, the reactivity of these MAbs was greatly diminished when *Amb a I* was physically or chemically modified (Olson, 1986). This indicated that sites A and B were conformationally dependent epitopes, i.e. either single structures which lose their conformation during modification or composite

Abbreviations: *Amb a I*, short ragweed pollen allergen *Amb a I*; pp *Amb a I*, partially purified *Amb a I*; r&a *Amb a I*, reduced and alkylated *Amb a I*; DTE, dithioerythritol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline (8.1 mM Na₂HPO₄·7H₂O, 1.47 mM KH₂PO₄, 0.137 M NaCl, 2.68 mM KCl, 0.02% NaN₃, pH 7.4); PBS-Tween, PBS plus 0.05% Tween 20; TBS, Tris buffered saline (50 mM Tris-HCl, 200 mM NaCl, pH 7.4); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

The nomenclature system developed by the IUIS Allergen Nomenclature Sub-Committee (Marsh *et al.*, 1986) will be used throughout this paper.

structures made up of two or more discontinuous peptides which are proximal in the native allergen, but separate after modification. Thus, these MABs have not yet been useful in the determination of epitope structures since modification is necessary to render *Amb a I* susceptible to proteolysis and the generation of peptide fragments (King, 1976).

As an approach to defining sites A and B, and to extend the epitope characterization of *Amb a I*, monoclonal antibodies were raised against extensively reduced and alkylated *Amb a I*. These MABs were screened for ability to bind native *Amb a I*; some MABs which bind to both native and denatured *Amb a I* should inhibit the binding of either human IgE or one of the anti-site A, B, or C MABs to native *Amb a I*. Since such a MAB would be directed against reduced and alkylated *Amb a I*, it could then be used to isolate fragments of *Amb a I*, and part of a native epitope would be defined.

This report describes 15 MABs directed against reduced and alkylated *Amb a I*. These have been characterized with respect to their specificity for *Amb a I*, their binding characteristics to different *Amb a I* preparations, and the spontaneous generation of their epitopes during purification of *Amb a I* from crude pollen extracts. Some of these MABs showed limited binding to native *Amb a I*, but this binding was not sufficient for the detectable inhibition of either human IgE, or site A, B, or C binding to native *Amb a I*. Finally, two immunoreactive peptides of *Amb a I*, defined by five of the fifteen MABs, were isolated and sequenced. These represent the first reported amino acid sequences of fragments of *Amb a I*. Synthetic analogs of these peptides, when coupled to KLH and administered to BALB/c mice, induced the formation of antisera which were specific for denatured *Amb a I*.

MATERIALS AND METHODS

Purification of *Amb a I*. Defatted short ragweed pollen was purchased from Greer Laboratories (Lenoir, NC), proteins were extracted in 0.015 M Tris (pH 8.4), and *Amb a I*, was isolated with a modification of King *et al.* (1964). This involved ammonium sulfate precipitation of the crude pollen extract proteins, passage of the reconstituted pellet through Sephadex G-25, anion exchange fractionation of the high mol. wt peak (DEAE Fractogel), Sephadex G-100 fractionation of the retained material (acidics), and finally a second DEAE Fractogel chromatography step. By SDS-PAGE analysis, this *Amb a I* preparation consists of the single chain form of *Amb a I* (38,000 mol. wt) as well as alpha chains (26,000 mol. wt) and beta chains (12,000 mol. wt) indicative of cleavage to the two chain form of *Amb a I*. As these preparations are processed very rapidly to preserve as much of the single chain form of *Amb a I* as possible, there are invariably a few minor con-

taminant bands on the gel; these preparations of *Amb a I* will be referred to as "partially purified" to distinguish them from very highly purified *Amb a I* (consists mainly of two chain form).

Reduction and alkylation of *Amb a I*. Partially purified *Amb a I* was reduced (0.05 M dithioerythritol) in Tris-buffered 7 M guanidine-HCl at 37°C for 30 min and alkylated with 0.11 M iodoacetic acid in the dark at room temp for 1 hr. The mixture was either dialysed extensively against water and then 0.05% ammonium bicarbonate or desalted on a Sephadex G-25 (fine) column equilibrated in 0.05% ammonium bicarbonate. This material will be referred to as r&a *Amb a I* and on SDS-PAGE is indistinguishable from partially purified *Amb a I*.

Isolation of alpha and beta chains. Reduced and alkylated *Amb a I* was fractionated on AcA 34 (2.6 × 90 cm; LKB) equilibrated in 5 M guanidine-HCl. Individual fractions from the eluate were examined by SDS-PAGE and pools representing alpha and beta chains were made. Subsequent analysis of more concentrated aliquots of those pools showed that the alpha chain preparation was slightly contaminated with beta chains while the beta chain preparation was free of alpha chains (not shown).

Isolation of tryptic fragments of *Amb a I*. Reduced and alkylated *Amb a I* was digested with trypsin-TPCK for 4 hr at 37°C in 1% ammonium bicarbonate (enzyme:substrate ratio of 1:50). The digest was initially fractionated on Sephadex G-50 (2.6 × 30 cm) equilibrated in 0.1 M ammonium hydroxide. Five pools were collected, lyophilized, resuspended in 0.1% TFA and fractionated by HPLC using a Polymer Laboratories reverse phase column (4.6 × 15 cm) and a linear gradient of 0.025% TFA and acetonitrile from 5 to 30% over a 30 min period. The eluate from this column was monitored at 214 and 280 nm and fractions were collected. Major peaks from this fractionation were lyophilized and used for ELISA, amino acid analysis, and protein sequence analysis.

Analytical techniques. Amino acid analysis was performed using the PICO-TAG PITC prederivatization method (Bidlemeier *et al.*, 1984), after hydrolysis of peptides *in vacuo* with 6 N HCl containing 0.01% phenol for 1 hr at 150°C. Protein sequence analysis was performed with an Applied Biosystems model 470A gas phase sequencer and PTH amino acids were identified by HPLC using a NOVA-PAK radial compression cartridge. Peptide synthesis was performed on a Bioscience model 9500 peptide synthesizer using tBoc chemistry and methylbenzhydrylamine resin. A capping step (acetylimidazole) was performed after each coupling cycle to prevent internal amino acid deletions. Synthetic peptides were purified by HPLC using either C4 or C18 reverse phase resins and 0.025% TFA/acetonitrile gradients.

Immunization of mice. Synthetic peptides were coupled to KLH by a slightly modified glutaraldehyde

procedure (Avrameus, 1969) and emulsified in complete Freund's adjuvant for subcutaneous injection. Three injections were given (0, 14 and 28 days) and mice were bled at days 0, 14, 28, and 42. For MAb production, mice were immunized with either reduced and alkylated *Amb a I* alone or with reduced and alkylated *Amb a I* and then partially purified *Amb a I* (a single dose 3 days before sacrifice). Some injections were with material precipitated with alum and some injections were simply in saline. Immunization for MAb production was performed both subcutaneously and intraperitoneally.

Cell fusion and cloning. Spleens were removed 3 days after the last immunization and cell suspensions from those spleens were fused with PEG to one of two parent cell lines, either 45.6TG1.7 (M45) or X63-Ag8.653 (P3X). M45 secretes IgG2b, κ immunoglobulin while P3X is a non-secretor. Antibody producing wells were selected by direct-bind ELISA: PVC ELISA plate wells were coated with reduced and alkylated *Amb a I* (0.3 μ g) and *Amb a I* reactive murine antibodies were identified with alkaline phosphate labeled goat anti-mouse IgG (whole molecule specific; Sigma A5153) and *p*-nitrophenyl phosphate. Cells from positive wells were cloned by limiting dilution or in soft agar.

MAb production. Either tissue culture supernatants or ascites fluids were used as the source of MAbs. Large amounts of tissue culture supernatants were processed to recover MAb using methods described by Klapper *et al.* (1986). BALB/c mice were pre-treated with pristane (Potter, 1972) and infused with approx. 10^6 viable, cloned cells for production of ascites fluids. These fluids were processed in an analogous manner to recover the highly purified MAb.

Preparation of $F(ab')_2$ antibody fragments. The method of Parham (1983) was used. Whole ascites fluid was digested for 36 hr at pH 3.5. The digest was fractionated by gel filtration on Sephadex S200 equilibrated in 0.015 M Tris (pH 8.4) containing 0.15 M NaCl. Digestion was verified by SDS-PAGE and activity assessed by ELISA.

ELISA protocols. Direct-bind ELISA was performed according to Voller *et al.* (1976) by coating polyvinyl chloride microtiter plate wells with 0.1–1.0 μ g antigen, and then incubating successively with tissue culture supernatants diluted 1:10, alkaline-phosphatase labeled goat anti-mouse IgG (Sigma A5153), and then *p*-nitrophenyl phosphate. Appropriate washing steps and the use of Tween 20 in the washing buffer (PBS) ensured specificity and low non-specific background reactivity. For subclass determinations, isotype specific rabbit antimouse immunoglobulin (Litton) was used. Double-bind ELISA was performed by coating the microtiter plate wells with anti-site A or anti-site C $F(ab')_2$ antibody fragments, and then incubating with antigen; the subsequent assay procedure was identical to the direct-bind ELISA except that alkaline-phosphatase

labeled goat anti-mouse Fc (Cappel Laboratories, Cochranville, PA) was used as the second antibody.

Western blotting. After SDS-PAGE (but before staining with Coomassie blue) proteins were transferred to nitrocellulose at a constant 50 V for 60–90 min using Tris/glycine/MeOH buffer as described by Towbin and Gordon (1984). The nitrocellulose was incubated with MAb supernatants diluted 1:10 or polyclonal anti-sera diluted 1:100 using every other lane in a decaprobe apparatus (Hoefer), and binding was detected using alkaline-phosphatase labeled goat anti-mouse IgG or IgA (Southern Biotechnology, Birmingham, AL) diluted 1:500 followed by incubation with NBT/BCIP (Promega Biotec, Madison, WI).

RESULTS

Fusions. Monoclonal antibodies were raised against *Amb a I* in three separate fusions employing separate immunization protocols in BALB/c mice. In fusion 1 (MAbs with prefix I), the animal was immunized first with alum precipitated r&a *Amb a I* at day 1 and again at day 15. On day 106, the same amount (50 μ g) of r&a *Amb a I*, but this time in saline, was administered. The spleen was removed at day 109, and 138 of 417 wells were tested for binding to *Amb a I*. Of these, three were selected for cloning and establishing cell lines. In fusion 2 (MAbs with prefix J), the animal was immunized as in fusion 1, but was not sacrificed after the saline-*Amb a I* immunization. Instead, on day 261 the animal was immunized with partially purified *Amb a I* (50 μ in saline) and the spleen was taken at day 264. For this fusion, 219 of 358 wells were screened and 11 selected for establishing cell lines. The third fusion utilized partially purified *Amb a I* in alum for immunization on days 1 and 15 and partially purified *Amb a I* in saline was administered on day 27. The spleen was taken on day 30 and a single cell line (2D8/E6) cloned and propagated.

Binding to *Amb a I*. All 15 MAbs produced reacted well with r&a *Amb a I* presented by a polyvinyl chloride surface in a direct-bind ELISA, while the three antibodies HaA1, HaB1 and HaC1, previously described (Olson and Klapper, 1986) and specific for native *Amb a I* showed negligible binding [Fig. 1(a)]. All MAbs except two (JB1F11-5 and JB4G5-4) bound pp *Amb a I* in the direct-bind ELISA [Fig. 1(b)], and seven of these 13 reacted better with pp *Amb a I* than HaA1 and HaB1 [the anomalous binding characteristics of HaC1 have previously been described (Olson and Klapper, 1986)]. However, when pp *Amb a I* was "presented" to the MAb panel by anti-site C or anti-site A $F(ab')_2$, virtually all binding of the 13 MAbs which reacted with pp *Amb a I* in the direct-bind assay was lost, while the binding pattern of HaA1, HaB1 and HaC1 was unaffected [Fig. 1(c)].

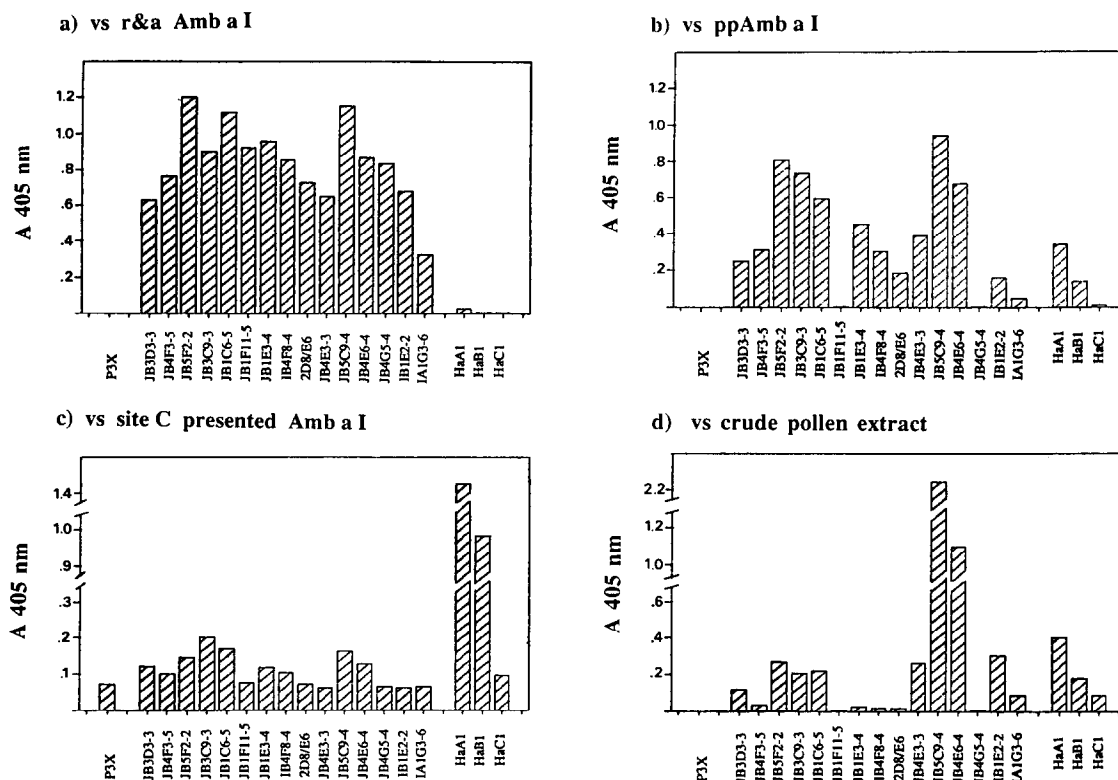


Fig. 1. Binding of anti-*Amb a I* MAbs to different preparations of *Amb a I*. Panel (a) r&a *Amb a I*; (b) pp *Amb a I*; (c) HaCl F(ab')₂ presented pp *Amb a I* (double bind); and (d) crude pollen extract. In each panel, the binding of 15 MAbs against denatured *Amb a I* is compared to the binding of three MAbs raised against native *Amb a I*. P3X supernatant was used as a negative control. ELISA was performed as described in Materials and Methods using MAb tissue culture supernatants diluted 1:10. Note that HaCl did not bind to HaCl-presented pp *Amb a I* (panel c). When HaA1 was used to present *Amb a I*, HaA1 did not bind, while HaCl did; the results with the anti-denatured *Amb a I* MAbs remained the same.

This suggested that the 15 MAbs did not react with the same molecule as HaA1, HaB1 and HaC1. Therefore, experiments were designed to determine if the 15 MAbs react with *Amb a I* or some unrelated contaminant in the pp *Amb a I* mixture.

Amb a I is characterized by its component alpha chains (26,000 mol. wt) and beta chains (12,000 mol. wt), which are formed by enzymatic cleavage (King *et al.*, 1981) of the parent molecule (38,000 mol. wt). This two-chain form can retain its native antigenic and allergenic activity but gives rise to distinct 26,000 mol. wt and 12,000 mol. wt bands (in addition to the 38,000 mol. wt band) on SDS-PAGE. Fourteen of the 15 MAbs in question bound to both the 38,000 mol. wt band and to either the alpha chain or beta chain (Fig. 2) when assayed against Western blots of pp *Amb a I*. Nine MAbs were specific for the alpha chain [Fig. 2(b-j)] and five were specific for the beta chain [Fig. 2(l-p)]. Similar results were obtained when we tested MAb binding to isolated alpha and beta chain preparations in a direct-bind ELISA (Table 1). Finally, four representative MAbs (2D8/E6, JB3C9-3, JB4E6-4 and IB1E2-2) bound very well to affinity purified *Amb a*

I (HaCl immunoadsorbent) as determined by Western blots (not shown). We therefore concluded that 14 of the 15 MAbs bind specifically to denatured *Amb a I*, and show no reactivity with the native *Amb a I* molecule. Only JB4E3-3 did not bind to isolated alpha or beta chains and gave a Western blot which left doubt about its binding to *Amb a I* (Fig. 2(k)). However, *Amb a I* bound to JB4E3-3 coupled to Sepharose CL-4B and JB4E3-3 reacted with the eluate from affinity columns constructed with MAbs 2D8/E6, JB4E6-4 and JB3C9-3. This indicated that JB4E3-3 does indeed bind to *Amb a I*.

Mab subdivisions based on binding characteristics. The alpha chain binding MAbs and the beta chain binding MAbs can both be subdivided into three groups based on (i) Western blot profiles; (ii) binding to pp *Amb a I* vs r&a *Amb a I*; (iii) binding to isolated alpha and beta chains; (iv) binding to tryptic fragments of *Amb a I*. The relative binding of a given MAb to pp *Amb a I* vs r&a *Amb a I* was obtained by dividing the values obtained in the pp *Amb a I* direct-bind ELISA [pp; Fig. 1(b)] by those from the r&a *Amb a I* direct-bind ELISA [r&a; Fig. 1(a)]. These values (Table 1, column b) ranged from 0.01



Fig. 2. Binding patterns of 15 anti-denatured *Amb a* I MAbs to "Western" blots of pp *Amb a* I after SDS-PAGE show their specificities for *Amb a* I (38,000 mol. wt) and their localizations to the alpha chain (26,000 mol. wt) or beta chain (12,000 mol. wt) of *Amb a* I. Fifty μ l of pp *Amb a* I (150 μ g) were separated on SDS-PAGE using a modified Laemmli technique (Laemmli, 1970) with a 20% acrylamide resolving gel and a 3% acrylamide stacking gel. After blotting to nitrocellulose, immunochemical specificities of individual MAbs were determined by subsequent incubations with MAb supernatants diluted 1:10, the appropriate subclass specific AP-labeled goat anti-mouse Ig, and an NBT/BCIP substrate system. Lane a was probed with polyclonal serum (1:100 dilution) from a mouse immunized with r&a *Amb a* I and boosted with pp *Amb a* I. The MAbs in lanes b-j are alpha chain specific while those in lanes l-p are beta chain specific. Lane q was probed with polyclonal serum from a mouse immunized with KLH-sTs-4 and shows the alpha chain specificity of this antiserum.

Table 1. Binding characteristics of 15 anti-denatured *Amb a* I MAb

Mab	Class/ subclass ^a	pp/r&a ratio ^b	A405 nm vs alpha ^c	A405 nm vs beta ^d	α/β ratio ^e	Western blot ^f
JB3D3-3	G1, G2b	0.40	0.967	0.072	13.4	Alpha
JB4F3-5	G2b	0.41	1.134	0.125	9.1	Alpha
JB5F2-2	G1, G2b	0.67	2.241	0.269	8.3	Alpha
JB3C9-3	G1, G2b	0.80	1.904	0.298	6.4	Alpha
JB1C6-5	G1, G2b	0.52	1.761	0.324	5.4	Alpha
JB1F11-5	G1, G2b	0.01	1.813	0.260	7.0	Alpha
JB1E3-4	G1	0.47	1.784	0.035	51.0	Alpha
IB4F8-4	G1	0.36	1.501	0.059	25.4	Alpha
2D8/E6	G1	0.26	0.787	0.044	17.9	Alpha
JB4E3-3	G1	0.61	0.011	0.007		
JB5C9-4	G2b	0.82	1.095	1.559	0.7	Beta
JB4E6-4	G2b	0.78	0.772	1.315	0.6	Beta
JB4G5-4	G1	0.01	0.240	1.080	0.2	Beta
IB1E2-2	G1	0.24	0.276	0.799	0.3	Beta
IA1G3-6	A	0.14	0.074	0.236	0.3	Beta

^aAssay as described in Materials and Methods.^bELISA values of Fig. 1(b) divided by those of Fig. 1(a).^{c,d}A405 after 40 min; MAb supernatants diluted 1:10, 1.5 μ g alpha or beta chain preparation coated.^eColumn *c* values divided by column *d* values.^fChain specificities as determined by Western blot (Fig. 2).

(JB1F11-5 and JB4G5-4, the two which do not bind pp *Amb a* I) to 0.82 (JB5C9-4). Alpha/beta ratios for MAb (Table 1, column e) were calculated by dividing the values obtained in the alpha chain direct-bind ELISA (Table 1, column c) by those obtained in the beta chain direct-bind ELISA (Table 1, column d). These values ranged from 51.0 (JB1E3-4) to 0.2 (JB4G5-4).

Group alpha-1 consists of JB3D3-3, JB4F3-5, JB5F2-2, JB3C9-3, and JB1C6-5 which have in common the ability to bind to a subset of tryptic fragments of *Amb a* I (see below). Group alpha-2 consists of a single MAb (JB1F11-5) which binds to r&a *Amb a* I but not pp *Amb a* I, and gives a very faint Western blot profile with pp *Amb a* I suggesting that its epitope depends upon reduction and alkylation. Group alpha-3 consists of JB1E3-4, IB4F8-4 and 2D8/E6, which have similar Western blots but higher alpha/beta binding ratios than the alpha-1 MAb, and do not bind to tryptic peptides of *Amb a* I. MAb 2D8/E6, although having similar characteristics to the other alpha-3 MAb, is unique since it was obtained from the fusion in which the mice were immunized only with partially purified *Amb a* I.

Group beta-1 consists of JB5C9-4 and JB4E6-4, characterized by strong, diffuse binding to the 38,000 mol. wt "parent" *Amb a* I band and the beta chain on Western blots. JB4G5-4 is the sole member of beta-2 and like JB1F11-5 (alpha-2) binds to r&a *Amb a* I but not to pp *Amb a* I. IB1E2-2 and IA1G3-6 both bind to the 38,000 mol. wt *Amb a* I band and the beta chain but not with the diffuseness of the beta-1 MAb, and the beta-3 MAb also have the lowest alpha/beta binding ratios.

Emergence of alpha and beta chain epitopes of pp *Amb a* I. The anti-denatured *Amb a* I MAb specific for the alpha chain did not react with crude pollen extracts as well as they reacted with pp *Amb a* I [compare Fig. 1(b) and (d)]. On the other hand, the

beta chain specific MAb showed much the same binding pattern to both crude pollen extracts and partially purified *Amb a* I. To understand this phenomenon, the activities of HaA1 (specific for native *Amb a* I), JB3C9-3 (alpha specific) and JB4E6-4 (beta specific) were monitored by direct-bind ELISA in different column fractions during the preparation of pp *Amb a* I from crude pollen extracts.

Most JB4E6-4 (beta) activity of the crude extract partitioned into the column fall-through of the initial DEAE Fractogel column in the pp *Amb a* I preparation protocol (Fig. 3; see Materials and Methods for protocol), separating from the HaA1 activity which was eluted from this column with salt. In the fractions

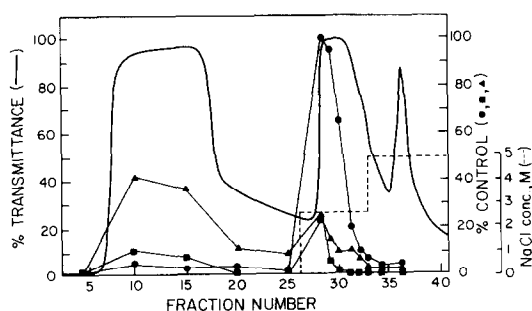


Fig. 3. Elution of *Amb a* I from DEAE Fractogel during pp *Amb a* I isolation. *Amb a* I-containing fractions were eluted from the column with 0.25 M NaCl. The column eluate was monitored at 280 nm and aliquots of selected fraction were diluted 1:1000 for coating ELISA wells. After coating, the wells were incubated with MAb supernatants diluted 1:100, and the remainder of the direct-bind ELISA was as described in Materials and Methods. The activities of three MAb were measured: HaA1 [specific for native *Amb a* I (●—●)]; JB3C9-3 [specific for the alpha chain (■—■)]; and JB4E6-4 [specific for the beta chain (▲—▲)]. The activity of a given MAb in a given fraction is expressed as the percentage of the value obtained with the pp *Amb a* I control, normalized to the *Amb a* I concn.

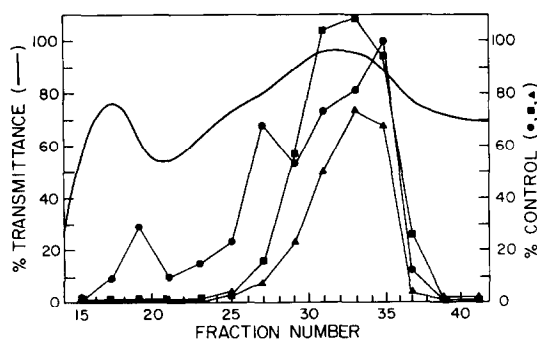


Fig. 4. Sephadex G-100 size fractionation of *Amb a I*-containing fractions from DEAE Fractogel. The column eluate was monitored at 280 nm and aliquots of every other fraction from a void vol (fraction 15) through the Ouchterlony positive fractions (fractions 28–35) were diluted 1:1000 for ELISA, which was performed as in Fig. 3. The activities of three MABs were measured: HaA1 [specific for native *Amb a I* (●—●)]; JB3C9-3 [specific for the alpha chain (■—■)] and JB4E6-4 [specific for the beta chain (▲—▲)]. The activity of a given MAB in a given fraction is expressed as the percentage of the value obtained with the pp *Amb a I* control, normalized to the *Amb a I* concn.

which contained HaA1 activity, the JB4E6-4 and JB3C9-3 activities were much lower than in the pp *Amb a I* control. This suggests that the *Amb a I* is somehow “more native” at this stage than in pp *Amb a I*; conversely, something in the mixture might be inhibiting the binding of the anti-denatured MABs.

A dramatic change occurred when the HaA1-active fractions from the initial DEAE column were size fractionated on Sephadex G-100 (Fig. 4); the anti-alpha chain activity (JB3C9-3), very slight in the crude pollen extract and less than half the HaA1 activity before G-100 separation, rose to greater than the HaA1 activity in most of the Ouchterlony positive fractions after G-100 separation (Fig. 4). The same was true for the JB4E6-4 activity, which was low in association with *Amb a I* in the starting material but high in association with *Amb a I* in the G-100 column fractions.

Immunoreactivities and synthetic peptides. Tryptic fragments of r&a *Amb a I* separated via Sephadex G-50 yielded five pools (T1–T5; Fig. 5). Five MABs (JB1C6-5, JB3C9-3, JB3D3-3, JB4F3-5, and JB5F2-2) reacted with pools T2 and T3 as measured by both direct-bind and inhibition ELISA [Table 2(a)]. Further fractionation of peak T3 via HPLC yielded seven peaks (T3-1 through T3-7; Fig. 6). When tested for immunoreactivity [inhibition ELISA, Table 2(b)], T3-4 bound three MABs (JB3C9-3, JB3D3-3, and JB5F2-2) while T3-7 bound four MABs (JB1C6-5, JB3D3-3, JB4F3-5, and JB5F2-2).

Peptides T3-4 and T3-7 were isolated after HPLC

and sequenced by automated Edman degradation giving the following sequences:

T3-4 WGSYAIGGSASPWIL(TQTN)* and

T3-7 GMLATVAFNTFTDNVDQR.

Analogues of T3-4 (sT3-4) and T3-7 (sT3-7) were synthesized as described in Materials and Methods with structures as follows:

sT3-4 RWGSYAIGGSASPWILTQTN and

sT3-7 GMLATVAFNTFTDNVDQR.

Peptides were coupled to KLH as described in Materials and Methods. That more sT3-4 than sT3-7 bound to KLH is evidenced by ELISA with KLH conjugates coating the plates (Table 3). Four MABs (JB1C6-5, JB3C9-3, JB3D3-3, and JB5F2-2) reacted very well with KLH-sT3-4 when compared with KLH-nil (Table 3); JB4F3-5 reacts with KLH-sT3-4, with an order of magnitude less than the other four. These results are different from those obtained using the peptide T3-4; JB1C6-5 did not detectably bind T3-4 (Table 2) but bound KLH-sT3-4 very well (Table 3).

BALB/c mice immunized with KLH-sT3-4 or KLH-sT3-7 yielded antisera reacting with r&a *Amb a I* while mice immunized with KLH-nil did not (Fig. 7). Similar results were obtained when antisera were tested against pp *Amb a I* (not shown). The specificities of the anti-KLH-sT3-4 for *Amb a I* and the alpha chain were shown by a Western blot [Fig. 2(q)]. Mice injected with KLH-sT3-4 produced antibodies only after the third injection. Neither antiserum reacted with native *Amb a I*, as determined by ELISA with pp *Amb a I* “presented” by anti-site A (HaA1) or anti-site C (HaCl) F(ab')₂ (not shown).

DISCUSSION

The immunochemical definition of *Amb a I* ultimately will require the determination of the structure of *Amb a I* and the characterization of both its B cell

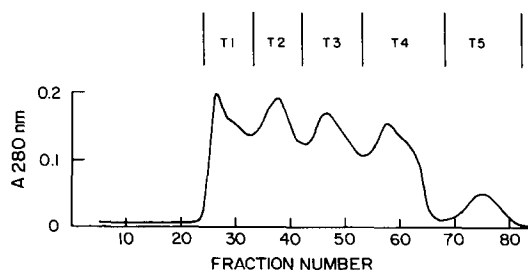


Fig. 5. Separation of tryptic fragments of r&a *Amb a I* by Sephadex G-50 gel filtration. Approximately 30 mg of trypsinized r&a *Amb a I* were lyophilized, resuspended in 0.1 M NH₄, and fractionated on a 29 × 2.9 cm column of Sephadex G-50. Absorbance was monitored at 280 nm, fractions were collected, and five pools were made (T1–T5).

*Sequence in parentheses ambiguous.

Table 2. Reactivities of selected MAbs with trypsinized r&a *Amb a* I after fractionation on Sephadex G-50 and HPLC

(a) Sephadex G50 fractions (Fig. 5)									
mAb	% Inhibition ^a					Times background ^b			
	T1	T2	T3	T4	T5	T1	T2	T3	4
JB1C6-5	— ^c	94	95	—	—	29	23	45	3
JB1F11-5	—	26	26	—	—	—	—	—	—
JB3C9-5	—	77	87	86	—	30	30	36	16
JB3D3-3	48	86	88	—	—	4	13	16	—
JB4F3-5	—	69	81	—	—	10	4	12	—
JB5F2-2	86	94	94	—	—	23	13	28	—

(b) HPLC fractions (Fig. 6)							
MAb	% Inhibition						
	T3-1	T3-2	T3-3	T3-4	T3-5	T3-6	T3-7
JB1C6-5	—	—	—	—	—	—	78
JB3C9-3	—	75	—	93	88	33	—
JB3D3-3	—	—	—	34	29	21	60
JB4F3-5	—	—	24	—	—	—	47
JB5F2-2	—	—	—	76	44	56	94

^aInhibition ELISA using 100-fold weight excess of fragments to inhibit direct-bind ELISA described in Materials and Methods.
^bDirect-bind ELISA using fragments to coat ELISA plates.
^cLess than 20% inhibition or less than twice background.

and T cell epitopes. Previous work has shown that *Amb a* I is a 38,000 mol. wt, non-glycosylated, single polypeptide (A form) which is cleaved proteolytically during chromatographic isolation into a two polypeptide chain structure (King *et al.*, 1981). This two chain form consists of a 26,000 mol. wt alpha chain and a 12,000 mol. wt beta chain which are not covalently attached (Griffiths, 1973), but tightly associated in a non-covalent manner. In its native state, the two chain form is allergenically and antigenically indistinguishable from the single polypeptide structure (King *et al.*, 1981).

The antigenicity and allergenicity of *Amb a* I are conformational-dependent properties (King *et al.*, 1967). Physical or chemical modification of the one chain or two chain form of *Amb a* I results in the loss of allergic reactivity and a marked decrease in antigenic potential. Reversal of modification tends to produce an inactive, aggregated product (King *et al.*, 1974), although Paull *et al.* (1978) have reported that citraconylation of native *Amb a* I, followed by gel filtration and decitraconylation, produced allergenically active alpha and beta chains.

The major B cell epitopes of *Amb a* I are appar-

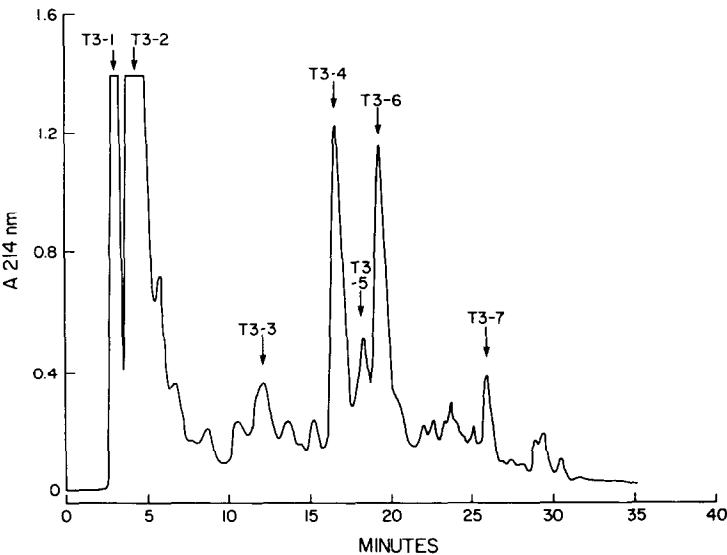


Fig. 6. HPLC profile of Sephadex G-50 fraction T3. Fraction T3 was lyophilized, resuspended in 0.5 ml of 0.025% TFA, and spun in a microfuge. The supernatant was injected onto a reverse phase column (W-Porex 10 C4, PHENOMENEX, Palo Verde, CA) and fractionated as described in Materials and Methods. Fractions, corresponding to each of the seven peaks indicated (T3-1 through T3-7), were pooled and lyophilized for further analysis.

Table 3. Reactivity of five MAbs with KLH-sT3-4 and KLH-sT3-7

MAb	KLH-sT3-4 ^a	KLH-sT3-7 ^b	KLH-nil ^b
JB1C6-5	1.004 ± 0.000	0.060 ± 0.003	0.045 ± 0.001
JB3C9-3	> 2.5 ^c	0.141 ± 0.040	0.068 ± 0.002
JB3D3-3	> 2.5 ^c	0.169 ± 0.030	0.087 ± 0.004
JB4F3-5	0.134 ± 0.004	0.128 ± 0.004	0.055 ± 0.000
JB5F2-2	0.710 ± 0.021	0.052 ± 0.003	0.036 ± 0.000

^aA405 nm after 2 hr reaction in a direct-bind ELISA coating well with KLH-sT-3-4; numbers are the average of duplicate wells.

^bA405 nm after 5 hr reaction in direct-bind ELISA coating wells with KLH-sT3-7 or KLH-nil.

^cJB3C9-3 and JB3D3-3 had A405 values after 15 min reaction of 0.227 ± 0.027 and 0.334 ± 0.044 respectively.

ently few in number. Monoclonal antibodies raised in BALB/c mice primarily recognized a single epitope on native *Amb a* I (site A), while IgE from the serum of allergic humans primarily recognized two epitopes (sites A and B). Surprisingly, neither of two murine IgE MAbs, raised using highly purified *Amb a* I as immunogen, seemed to react with *Amb a* I in a Western blot assay (Liu and Katz, 1984). The MAbs reported here, raised against denatured *Amb a* I, probably bind to very minor B cell epitopes which induce a small proportion of the total antibody formed in response to *Amb a* I stimulation. On the other hand, denatured *Amb a* I was successfully used to prime mice for a secondary response to native *Amb a* I (Ishizaka *et al.*, 1974, 1975), suggesting that at least some T cell epitopes on *Amb a* I are not as conformation-dependent as are the major B cell epitopes.

The structural characterization of sites A and B of *Amb a* I (the two major human B cell epitopes) is not straightforward. First, *Amb a* I is very difficult to obtain in pure form (containing only the 38,000 species)—even *Amb a* I affinity purified directly from fresh pollen extract showed some alpha and beta chain contamination (King *et al.*, 1981; Olson and Klapper, 1986). Partially purified preparations of *Amb a* I, in addition to alpha and beta chains, contain a number of polypeptides cross-reactive with *Amb a* I (Fig. 2, lane a). These may be proteolytic degradation products of *Amb a* I (King *et al.*, 1981), or separate, related proteins (Lowenstein *et al.*, 1981). In either case, these contaminants apparently are not generated during purification of *Amb a* I, since pollen extracted under reducing and denaturing conditions yielded a similar set of proteins as detected by SDS-PAGE (data not shown).

In addition to the very real problem of working with highly purified, native material, the complete primary structure of *Amb a* I has not yet been determined. The amino acid sequences of the peptides T3-4 and T3-7 represent the first published sequences from *Amb a* I.

Finally, antibody binding to sites A and B, in particular the MAbs used for the initial characterization of these sites, depend upon maintaining the conformation of *Amb a* I. This precludes the use of these antibodies to detect peptides derived from *Amb*

a I, as denaturation or chemical modification of the antigen is necessary for the production of peptides (King, 1976). With the exception of the cleavage which generates the two polypeptide form of this allergen, *Amb a* I is normally quite resistant to proteolytic enzymes.

It was anticipated that some MAbs produced against denatured *Amb a* I would react with both denatured and native *Amb a* I, and that a subset of these would block the binding to native *Amb a* I of anti-site A or anti-site B MAbs. However, the anti-denatured *Amb a* I MAbs, reported here, bind to native *Amb a* I very poorly, if at all [Fig. 1(c)]. In the double-bind assays used to measure this binding, an ELISA plate was coated with a MAb directed against either site A or C and *Amb a* I was presented by that antibody. By definition, the *Amb a* I presented by an anti-site A or anti-site C MAb is "native" and in this assay, the anti-denatured MAbs bind most poorly. Furthermore, in no instance was an anti-denatured *Amb a* I MAb able to inhibit an anti-site A or B antibody from binding to immobilized *Amb a* I.

This lack of cross-reactivity between the anti-native *Amb a* I MAbs and anti-denatured *Amb a* I MAbs parallels earlier observations of low cross-

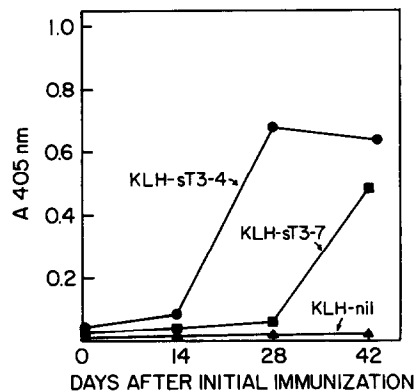


Fig. 7. Reactivities with denatured *Amb a* I of antisera raised in BALB/c mice immunized with *Amb a* I-derived synthetic peptides. ELISA wells were coated with 300 ng r&a *Amb a* I, incubated with antisera diluted 1:100, and then with AP labeled goat anti-mouse IgG (Fc specific) and *p*-nitrophenyl phosphate. Reaction times reported are 50 min for KLH-sT3-4, 120 min for KLH-sT3-7, and 100 min for KLH-nil.

reactivity between polyclonal anti-sera raised against native and denatured *Amb a I* (King *et al.*, 1974). Similarly, it is known that antisera to denatured lysozyme react poorly with native lysozyme and vice versa (Atassi, 1978). Why the immune response to these proteins in their native state is predominantly to these "discontinuous" types of epitopes is not clear. It is equally unclear why antibodies against denatured forms of these antigens react so poorly with the native forms.

Denaturation of *Amb a I*. Most of the anti-denatured *Amb a I* MAbs reacted with preparations of pp *Amb a I* even though they did not react with native *Amb a I*. This implied that some proportion of *Amb a I* denatures during the processing of crude extracts to yield pp *Amb a I*. An anti-alpha chain specific (JB3C9-3) and an anti-beta chain specific (JB4E6-4) MAb had significantly lower reactivities with *Amb a I* eluted from the initial DEAE column of the purification protocol (see Materials and Methods) than with pp *Amb a I* (Fig. 3). Thus, *Amb a I* is apparently "more native" at this stage than in pp *Amb a I* (conversely, something in the mixture may inhibit the binding to *Amb a I* of these anti-denatured MAbs at this stage). Subsequent Sephadex G-100 size fractionation of *Amb a I* eluted from the initial DEAE column yielded *Amb a I* with considerably more anti-alpha chain activity and anti-beta chain activity (Fig. 4). It is not clear why size fractionation at this stage causes the sudden appearance of epitopes which are associated with denatured *Amb a I*, although the sudden removal of salt upon injection onto G-100 may induce some conformational change in *Amb a I*.

Not all anti-denatured *Amb a I* MAbs reacted with pp *Amb a I* and r&a *Amb a I* to the same extent [Fig. 1(a) and (b)]. For each MAb, a ratio of binding to pp *Amb a I* vs binding to r&a *Amb a I* was calculated (Table 1, column b) which provided a mechanism to differentiate between MAbs. In addition, these ratios suggest a reproducible alteration in the structure of *Amb a I* during pp *Amb a I* preparation: MAbs with high ratios (JB4E6-4 and JB3C9-3) most likely bind to epitopes which appear first and are thus near the surface of the native molecule, while MAbs with low ratios (2D8/E6 and IB1E2-2) may bind to epitopes which are not exposed until the molecule is at a more advanced stage of denaturation. These binding characteristics are not unprecedented, as some antibodies raised against influenza virus hemagglutinin peptides do not react with the hemagglutinin until an acid-induced conformational change makes their epitopes accessible (White and Wilson, 1988).

Therefore, the three *Amb a I* preparations discussed here, r&a *Amb a I*, pp *Amb a I*, and *Amb a I* "presented" by anti-native MAbs, are very different preparations which contain different conformers of *Amb a I*. The first preparation, r&a *Amb a I*, is a completely denatured sample prepared in the laboratory. The second preparation, pp *Amb a I*, is appar-

ently a population of *Amb a I* molecules at different stages of denaturation as evidenced by the observed range of pp/r&a ratios (Table 1, column b). The third preparation, *Amb a I* complexed with anti-site A or anti-site C MAbs, may be the only pure preparation of allergenically active *Amb a I*, both in terms of molecular species and conformational state. Disruption of these immune complexes has thus far required conditions which initiate the denaturation process; native *Amb a I* has not yet been isolated completely free of denatured *Amb a I* activity.

Epitope mapping and synthetic peptides. Determination of the B cell and T cell determinants of *Amb a I* will allow the study of immune responses to ragweed allergens using synthetic peptides. These studies have several potential uses including the development of synthetic peptide immunogens for the desensitization of allergic individuals and stimulation of T cells.

The epitopes recognized by the 15 anti-denatured *Amb a I* MAbs have been partially characterized. Sephadex G-50 fractionation of tryptic peptides of *Amb a I* yielded five pools (fractions T1-T5; Fig. 5) which were assayed for immunoreactivity [Table 2(a)]. HPLC fractionation of T3 (Fig. 6) yielded the immunoreactive peptides T3-4 and T3-7 (Table 2(b)). After sequence determination, synthetic analogs of T3-4 and T3-7 were prepared (sT3-4 and sT3-7 respectively) for use in immunization experiments.

Peptides were coupled to KLH for immunization since preliminary immunization using free sT3-4 or free sT3-7 did not give rise to antibodies. Mice immunized with KLH-sT3-4 or KLH-sT3-7 produced antisera which reacted with denatured *Amb a I* (Fig. 7) but not with native *Amb a I* presented by anti-site A or anti-site C F(ab')₂ (data not shown). Antisera raised against KLH-sT3-4 reacted with *Amb a I* and were alpha chain specific as shown by Western blotting (Fig. 2, lane q). Thus, the discrete peptides of *Amb a I* tested so far will not induce antibodies to the conformationally dependent IgE binding sites (A and B) of *Amb a I*. An immunogen for this purpose may be impossible to produce from linear structural information and may require the design and synthesis of peptides based upon molecular modeling studies as discussed by Atassi (1978),

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