

## MULTIPLE *Amb a* I ALLERGENS DEMONSTRATE SPECIFIC REACTIVITY WITH IgE AND T CELLS FROM RAGWEED-ALLERGIC PATIENTS<sup>1</sup>

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The relationship between the structure and abundance of an inhaled protein and its potential for causing an allergic response is unknown. This study analyzes *Amb a* I, a family of related proteins formerly known as Ag E, that comprise the major allergens of short ragweed (*Ambrosia artemisiifolia*). T cells isolated from ragweed allergic patients were shown to proliferate in response to purified *Amb a* I.1 protein from pollen in in vitro secondary cultures, demonstrating the presence of T cell stimulatory epitopes in *Amb a* I.1. Three recombinant forms of *Amb a* I (*Amb a* I.1, *Amb a* I.2, and *Amb a* I.3) obtained as cDNA derived from pollen mRNA were expressed in bacteria. All three recombinant forms were shown to be specifically recognized by pooled ragweed-allergic human IgE on immunoblots, confirming these gene products are important allergens. An examination of immunoblots probed with sera derived from allergic patients revealed a variation in IgE binding specificity. A minority of patients' IgE exclusively reacted with recombinant *Amb a* I.1, whereas most patients' IgE reacted with *Amb a* I.1 as well as *Amb a* I.2 and *Amb a* I.3 proteins. A detailed examination of the reactivity of T cells derived from 12 allergic patients to these recombinant *Amb a* I forms revealed that these allergens are all capable of stimulating T cell proliferation in in vitro assays. It is concluded that the allergic response to ragweed pollen in most allergic patients is composed of a reaction to multiple related *Amb a* I proteins at both the B and T cell levels.

One of the most important pollen allergens in North America is produced by *Ambrosia artemisiifolia* (short ragweed). Ragweed pollen is composed of a complex mixture of at least 52 Ag (1), 22 of which have been shown to bind specific human IgE (2) using crossed radioimmunoelectrophoresis. The predominant allergen from rag-

weed pollen, designated *Amb a*<sup>3</sup> I or Ag E (3), has been extensively characterized and shown to be a nonglycosylated protein of 38-kDa molecular mass (4, 5). An immunochemically related protein, *Amb a* II (Ag K), has similar properties (4, 6). *Amb a* I can be purified using conventional chromatographic or biochemical techniques (4-6). However, due to cleavage of the 38-kDa single-chain precursors by the action of a trypsin-like pollen protease, purification often results in the isolation of two noncovalently associated chains of 26 and 12 kDa molecular mass, designated  $\alpha$  and  $\beta$ , respectively (5, 7). Biochemically purified *Amb a* I has been used as an immunogen to produce murine mAb as well as rabbit polyclonal antisera (5) reactive with both the native (8) and denatured (9) protein.

Early studies using skin tests with *Amb a* I-depleted pollen extract led to the estimation that at least 90% of the allergenic activity in ragweed pollen can be attributed to *Amb a* I (3, 4). Competition experiments using murine mAb to inhibit the binding of *Amb a* I by human IgE in ELISA assays have confirmed that *Amb a* I binds a substantial proportion of human ragweed allergic IgE (8). The recent cloning of three cDNA encoding proteins with properties of *Amb a* I and the conclusion that *Amb a* I consists of a family of homologous but distinguishable sequences (10) raises the question of which protein or proteins is responsible for the immunogenic activity of what was previously attributed to a single protein. Individual cloned members of the *Amb a* I family, designated *Amb a* I.1, *Amb a* I.2, and *Amb a* I.3, share amino acid sequence homology exceeding 80% (10). The *Amb a* I.1 protein has previously been purified from pollen (10) but the possible role of the *Amb a* I.2 and *Amb a* I.3 gene products in ragweed allergy is unclear.

The regulatory events controlling human IgE synthesis are not well understood, but it has been clearly shown that B cell production of IgE is T cell dependent (11). In addition, T cells isolated from ragweed-allergic patients are capable of proliferating in vitro when exposed both to ragweed pollen extract and partially purified Ag E (12). However, such responses have not been extensively studied.

In this manuscript we analyze the contributions of *Amb a* I.1, I.2, and I.3 to ragweed allergy by 1) examining the IgE binding properties of these three gene products, and 2) defining their ability to induce in vitro proliferation of T cells from ragweed allergic patients.

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<sup>3</sup> Abbreviations used in this paper: *Amb a* I.1, I.2, and I.3, allergens from *Ambrosia artemisiifolia* named according to the IUIS approved nomenclature (*Annals Allergy* 60:499-504, 1988).

## MATERIALS AND METHODS

**Expression in *Escherichia coli* of multiple members of *Amb a 1* multigene family.** Expression vectors containing cDNA inserts encoding *Amb a 1* were constructed in the vector pTrc99A, B or C (13). The expression vectors pTrc99A, B and C were kindly provided by Dr. Egon Amann (Behringwerke AG, Marburg, FRG). The nucleotide sequences and derived amino acid sequences of the *Amb a 1* family members have been described elsewhere (10). Full length and truncated cDNA inserts were cloned in-frame into the appropriate pTrc99 vector and transformed into the JM109 host bacteria. Expression of individual recombinant *Amb a 1* proteins was induced when cultures reached OD<sub>600</sub> 0.6 to 0.7 by 1 mM isopropyl- $\beta$ -thiogalactopyranoside, cells were pelleted, lysozyme treated, sonicated, and insoluble inclusion bodies were recovered by a low speed centrifugation (14). Recombinant *Amb a 1* protein present in the recovered pellet was solubilized in buffer containing 8 M urea, 50 mM Tris HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF. After solubilization, the urea lysate was dialyzed at 4°C against PBS. *Amb a 1* purified from pollen is composed of *Amb a 1.1* (10) and was prepared as previously described (5).

**Antibodies.** Rabbit anti-*Amb a 1* antiserum was kindly provided by Dr. T. P. King (Rockefeller University, New York, NY). Allergic patient plasma was isolated from heparinized whole blood (received from Dr. John Saryan, Lahey Clinic and Dr. Albert Sheffer, Harvard Medical School, Boston, MA) and stored at -20°C, before analysis. Allergic patients were chosen on the basis of a clinical history of ragweed hypersensitivity and positive skin test for ragweed pollen extract. Control plasma was obtained from a cat allergic in-house donor, who was skin test negative for ragweed pollen extract but positive for cat dander extract. Pooled human ragweed-allergic sera was kindly provided by Dr. Lawrence Lichtenstein (Johns Hopkins University, Baltimore, MD).

**Immunoblot analysis.** The gel electrophoresis and Western blot transfer procedures used were essentially as described elsewhere (15). Briefly, SDS-PAGE was performed in 10% acrylamide gels under reducing conditions, 10 mM dithiothreitol, at constant current. The transfer to nitrocellulose (0.1  $\mu$ m, Schleicher & Schuell, Keene, NH) was performed in a Hoeffer apparatus according to the protocol of Towbin et al (15). After transfer, the blots were rinsed in blot solution (25 mM Tris-HCl, pH 7.5, 0.17 M NaCl, and 0.05% Tween 20) and stained for 1 h with 0.1% India ink. All subsequent incubations with antibodies and washes were performed in blot solution at room temperature. The first antibody incubations were performed overnight then rinsed and incubated with the appropriate biotinylated second antibody (Kirkegaard Perry Laboratories, Gaithersburg, MD). The final incubation was performed using <sup>125</sup>I streptavidin (1  $\mu$ Ci/25 ml blot solution) for 1 h followed by removal of unbound labeled material and autoradiography at -80°C with an intensifying screen.

**Isolation of Ag reactive T cell lines.** Patients chosen from routine visitors to an allergy clinic were determined to be ragweed allergic through both prick skin-testing and clinical history. These patients had not undergone immunotherapy and were not taking any medication that would interfere with the immune response. Heparinized peripheral blood samples were taken from these patients and the leukocytes were purified within 24 h of the sampling. Briefly, PBL were separated from the plasma by centrifugation at 150  $\times g$  for 15 min. The plasma was removed and used for antibody analysis. The pelleted cells were restored to their original volume in FCS and the PBL isolated by either centrifugation with Sepacell-MN (Sepacell Corp., Oklahoma City, OK) or lymphocyte separation medium (Or-ganon Teknika, Durham, NC). The cells were washed three times with PBS containing 0.1% BSA, and then resuspended in RPMI 1640 supplemented with 10%, heat-inactivated human AB serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 5  $\times 10^{-5}$  M 2-ME, and 10 mM HEPES. A number of aliquots of the isolated PBL from each patient were either stimulated with Ag, frozen in liquid nitrogen or transformed with EBV.

Long term T cell lines were established by stimulation of 1  $\times 10^6$  PBL/ml in complete medium with aqueous ragweed pollen extract (20  $\mu$ g/ml) for 7 to 10 days at 37°C in a humidified 5% CO<sub>2</sub> incubator. This amount of extract was determined to be optimal for the activation of T cells from most ragweed-allergic patients. The non-adherent T cells were isolated after centrifugation with lymphocyte separation medium, washed, and cultured in complete medium containing IL-2 (5 U/ml) and IL-4 (5 U/ml) (recombinant products, Boehringer-Mannheim, Genzyme, Boston, MA). Cells were cultured until "rested" and no longer responsive to growth factors. At this time the cell line was either set up in a secondary proliferation assay (see below) or restimulated in secondary bulk culture with pollen extract and APC (see below). Cycles of restimulation/rest were continued and the cells used for assay after a rest cycle. Alternatively, the

stimulated cell line was saved by freezing and storage in liquid nitrogen.

**APC.** Two sources of APC have been used in these studies. Secondary T cell cultures were established using autologous  $\gamma$ -irradiated (3500 rad) PBL as APC. In subsequent stimulations either  $\gamma$ -irradiated PBL (3500 rad) or  $\gamma$ -irradiated autologous EBV transformed cell lines (up to 25,000 rad) were used as APC. The EBV transformed cell lines were made by incubation of 3  $\times 10^6$  PBL with 1 ml of B-59/8 Marmoset cell line (ATCC CRL1612) conditioned media in the presence of 1  $\mu$ g/ml PMA at 37°C for 60 min. The cells were then diluted to 5  $\times 10^5$  cells/ml in medium containing 10% FBS and cultured in 96-well culture plates (Costar, Cambridge, MA) until visible colonies were detected. The EBV cell line was then expanded and could be used for APC.

**T cell proliferation assay.** T cell lines were used for assay after the rest cycle of their growth. The 2  $\times 10^4$  T cells were cultured in 200  $\mu$ l of complete medium with 5  $\times 10^4$   $\gamma$ -irradiated PBL or 2  $\times 10^4$  EBV cells as APC and with various concentrations of a panel of Ag in round bottom 96 well dishes (Costar, Cambridge, MA) for 3 days at 37°C in a humidified 5% CO<sub>2</sub> incubator. The cultures were pulsed with tritiated thymidine (1  $\mu$ Ci/well <sup>3</sup>H-methyl-thymidine, ICN, Irvine, CA) on day 3 and harvested on glass fiber filters on day 4. The amount of incorporated radioactivity was measured by liquid scintillation counting. The results are expressed as mean cpm from duplicate or triplicate cultures. The SEM for the replicates was less than 15% of the mean.

## RESULTS

**Expression of individual recombinant *Amb a 1* proteins.** Although *Amb a 1* possesses allergenic activity as defined by human IgE binding (8), the relative importance of the *Amb a 1.1*, *Amb a 1.2*, and *Amb a 1.3* variants in ragweed allergy was unclear. At present, some members of the *Amb a 1* family (*Amb a 1.2* and *Amb a 1.3* variants) have not been individually purified to homogeneity from pollen extract. To assess the allergic properties of the *Amb a 1.1*, *Amb a 1.2*, and *Amb a 1.3* forms each cDNA was cloned into the *E. coli* expression vector pTrc99. A schematic diagram in Figure 1 depicts each of the three cDNA. The listed amino acid residues are numbered from the N-terminal methionine. The N-terminal 26 residues are hydrophobic and are predicted to be a leader sequence. Consequently, the three recombinant *Amb a 1.1*, *Amb a 1.2*, and *Amb a 1.3* proteins (starting at residues 10, 32, and 32, respectively), constitute full-length or near full-length sequences. The expressed recombinant products have slightly different lengths with *Amb a 1.1* being 391 residues long and *Amb a 1.2* and *Amb a 1.3*, 368 and 370 residues, respectively (see Fig. 1). The *Amb a 1* from pollen has a relative mobility on SDS-PAGE gels consistent with a size of approximately 370–380 amino acids (see Fig. 2, lane 1). However, the exact size is unknown because the amino terminus is blocked (10).

**Reactivity of an anti-*Amb a 1* antibody and human IgE with recombinant *Amb a 1* family members.** Bacteria harboring the cDNA constructs were induced to express recombinant *Amb a 1.1*, *Amb a 1.2*, and *Amb a 1.3* and prepared for antibody binding analysis. Immunoblots were used to determine the antibody binding capacity of the three recombinant proteins in bacterial lysates as compared to pollen extract and a negative control bacterial lysate. Figure 2A, shows the binding pattern of a rabbit anti-*Amb a 1* antibody on one blot section. It is clear that the recombinant *Amb a 1.1*, *Amb a 1.2*, and *Amb a 1.3* proteins react with this antibody preparation, made against native biochemically purified *Amb a 1* preparation composed of *Amb a 1.1* (5, 10). This affinity purified polyclonal antibody preparation is specific for *Amb a 1* and *Amb a 1.2* and degradation products thereof

**Figure 1.** The expressed recombinant proteins from *Amb a 1* cDNA are shown in stick figures that have various lengths as shown by the amino acid numbering above each protein (10). Amino acids at the N-terminus artificially introduced into the gene construct due to cloning into the pTrc99 vector (13) are represented by the single letter code. The predicted recombinant gene product size varies because the cDNA are slightly truncated but includes the region that constitutes the predicted biologic *Amb a 1* gene products in pollen (10).

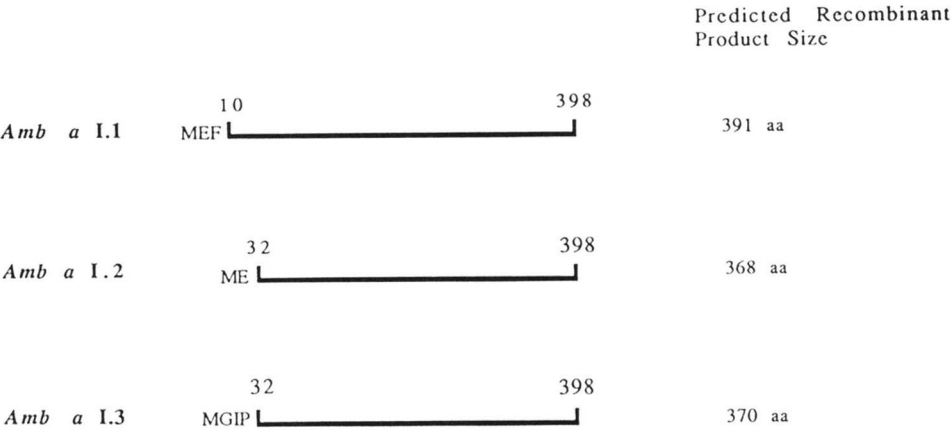
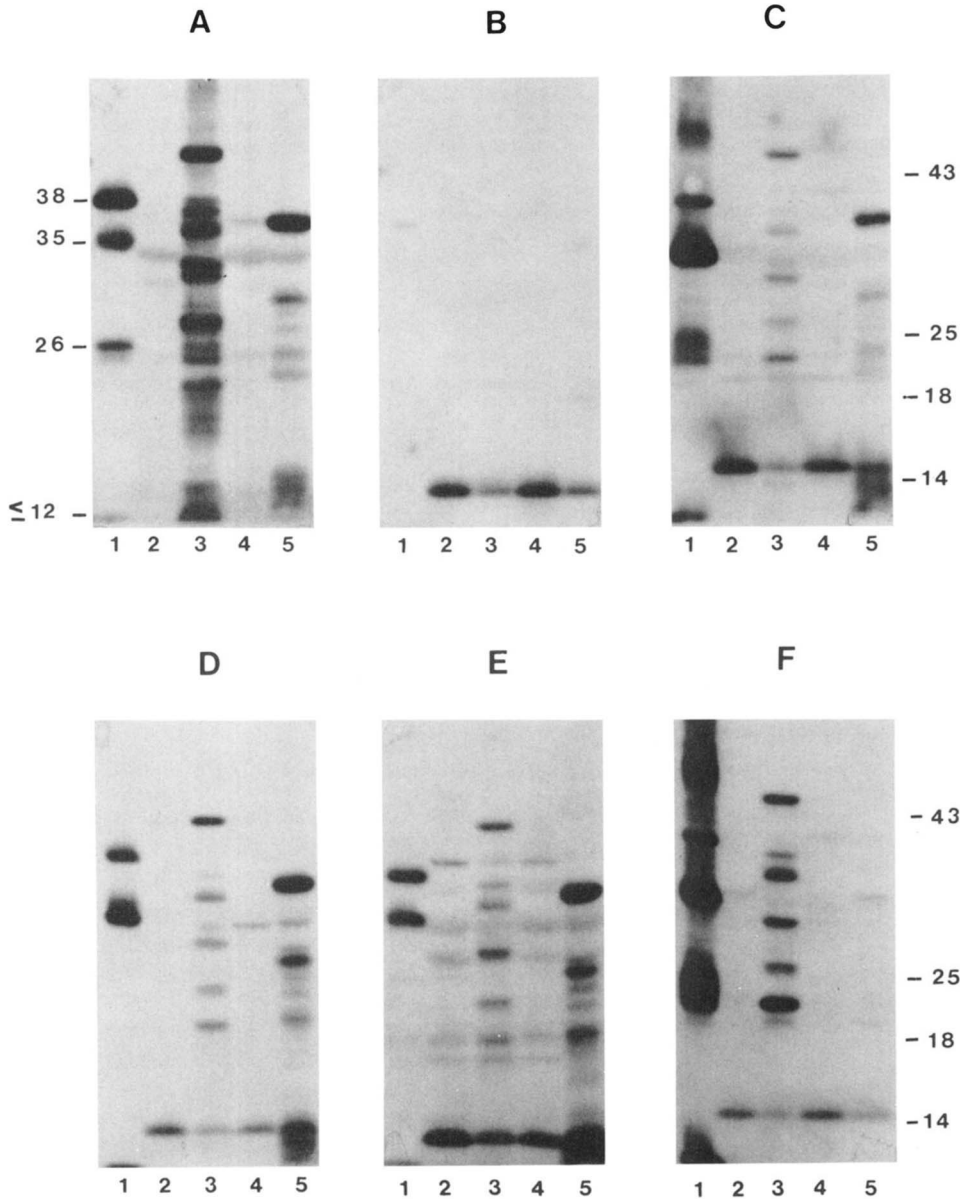


**Figure 2.** Immunoblot analysis of recombinant *Amb a 1* proteins. SDS-PAGE immunoblot sections were generated with the following Ag: lane 1: pollen extract (10 µg/lane), lane 2: JM109 negative control bacterial lysate (40 µg/lane), lanes 3, 4, and 5 had 40 µg/lane of bacterial lysate containing recombinant protein *Amb a 1.1*, *1.2*, and *1.3*, respectively. These blot sections were probed with: A: rabbit anti-*Amb a 1* (1/1000). B through F were probed with 1/10 dilutions of human plasma.: B: nonragweed allergic patient 70, C: pooled human plasma, D: patient 295, E: patient 296, F: patient 303.



(5). The appearance of a previously unreported strongly binding protein of 35 kDa was identified here by use of lower percentage (10%) acrylamide gels. The first feature of these patterns is the marked variation in signal intensity between the different lysate samples. This may reflect the different expression levels of the recombinant

proteins within the various lysates because the loading of the gels was based on equal amounts of total bacterial protein per lane. For example, the *Amb a 1.2* recombinant is obviously under represented in its respective lysate (lane 4 in Fig. 2 panels). The number of discrete bands in each lane represents both the full length expressed

protein and a number of degradation products. The next five panels in Figure 2, B through F, demonstrate the binding patterns of human IgE on these immunoblots. These plasma samples from ragweed allergic patients, were screened for detection of IgE binding to pollen proteins under the conditions of SDS-PAGE immunoblot analysis (i.e., with denatured Ag). Approximately 40% of skin test positive patient samples show specific binding under these conditions (Table I). It should be noted that IgE from some patients are unable to bind the denatured Ag under these conditions (e.g., patients 222, 280, 308, 336, and 339; Table I). The binding patterns of recombinant *Amb a 1* proteins observed when pooled human IgE and three individual allergic patients' IgE (patients 295, 296, and 303) were analyzed by this method are shown in Figure 2, (C, D, E, and F, respectively). Differences in both the number of protein products recognized and the relative signal intensities are apparent. In one example, human allergic IgE (from patient 303) binds exclusively to recombinant *Amb a 1.1*. The IgE from cat allergic patient 70, who is skin test negative for ragweed pollen extract, binds a common band in all lanes with *E. coli* proteins (Fig. 2B). This band of approximately 14 kDa is observed in all of the panels when IgE binding to recombinant proteins in *E. coli* lysate is examined. This background binding to *E. coli* proteins may have been exacerbated by the use of a high concentration of plasma (10%) (see Fig. 2E, patient 296). To broaden the scope of these analyses we are currently working to isolate highly purified recombinants to allow a comparison of solid phase and fluid phase binding to allergic sera of these recombinant molecules.

These IgE binding data demonstrate that ragweed allergic patient IgE specifically recognizes all three of the recombinant *Amb a 1* proteins. Additional individual ragweed allergic patients whose IgE recognizes pollen proteins of 38 kDa also bind at least one recombinant *Amb a 1* form (data not shown). One common feature of the IgE binding patterns on immunoblots is reactivity with the recombinant *Amb a 1.1* form. A minority of patients' IgE reacts with only the recombinant *Amb a 1.1* (e.g., patient 303, Fig. 2F), whereas most patients' IgE reacts with the recombinant *Amb a 1.1* as well as with the *Amb a 1.2* and *Amb a 1.3* proteins (Fig. 2, patients 295 and 296; data not shown). Further, it is evident that most of

the recombinant protein bands that are recognized by human IgE correspond to protein forms that bind the rabbit anti-*Amb a 1* antibodies.

**Response of ragweed pollen-specific human T cells to recombinant *Amb a 1* proteins.** Inasmuch as IgE from different patients varies widely in its binding patterns to various expressed recombinant *Amb a 1* proteins, it was of interest to explore the extent to which T cells from the same patients react to these recombinant preparations. T cells from patients allergic to ragweed, previously stimulated with pollen extract, can recognize and proliferate in response to pollen extract, biochemically purified *Amb a 1* protein and bacterial lysates containing recombinant *Amb a 1* gene products 1.1, 1.2, and 1.3 (Fig. 3; Table I). For example, ragweed-specific T cell lines derived from patients 295 and 296 (Fig. 3, A and B, respectively) responded in a dose-dependent manner to *Amb a 1.1*, 1.2, and *Amb a 1.3*, although the responses to *Amb a 1.2* were the weakest. This proliferation was specific, because an equivalent amount of control bacterial lysate (JM109) did not induce measurable T cell proliferation under the same conditions. As a specificity control, at the levels used in these experiments the recombinant *Amb a 1* proteins showed no stimulation of *Fel d 1* (cat dander allergen) specific T cell lines derived from the same patients (data not shown). For comparison, the same patients (295, 296, and 303) were chosen for individual IgE immunoblot reactivity with the three recombinant *Amb a 1* proteins (Fig. 2). Pollen-primed T cell lines derived from patient 303 proliferated in response to control bacterial proteins, and thus could not be included in the T cell analysis. As shown in Figure 2, *Amb a 1.1* and *Amb a 1.3* also appear to be the major IgE-binding forms of *Amb a 1* in patients 295 and 296. However, in an examination of T cell responses in a larger panel of patients (Table I), *Amb a 1.1* and *Amb a 1.3* are not always the dominant stimulatory forms. These results demonstrate that in general, ragweed pollen-specific T cells from allergic patients can respond to all three forms of recombinant *Amb a 1*, although the reactivities to each form are variable from patient to patient. Thus, no single form of the *Amb a 1* family of proteins appears to predominate in stimulating T cell reactivity. This is not necessarily the case with IgE binding where greater variability in the recognition of the different forms on immunoblots can

TABLE I  
Secondary human T cell proliferative responses to purified and recombinant ragweed allergens

| Patient | Ragweed Pollen Extract | Biochemically Purified <i>Amb a 1</i> | Recombinant Lysate |                  |                  | Control Lysate | IgE Reactivity on Western Blot <sup>a</sup> |
|---------|------------------------|---------------------------------------|--------------------|------------------|------------------|----------------|---------------------------------------------|
|         |                        |                                       | <i>Amb a 1.1</i>   | <i>Amb a 1.2</i> | <i>Amb a 1.3</i> |                |                                             |
| 151     | 16.9 <sup>b</sup>      | 4.1                                   | 2.0                | 3.0              | 2.7              | 1.4            | +                                           |
| 222     | 25.9                   | 12.4                                  | 6.9                | 1.3              | 4.9              | 1.1            | -                                           |
| 280     | 116.3                  | 77.6                                  | 80.0               | 25.3             | 11.5             | 1.9            | -                                           |
| 295     | 13.6                   | 8.6                                   | 4.1                | 3.5              | 5.4              | 0.9            | +                                           |
| 296     | 15.1                   | 11.4                                  | 7.9                | 2.5              | 14.1             | 1.4            | +                                           |
| 308     | 18.2                   | 2.0                                   | 22.9               | 6.2              | 23.6             | 1.2            | -                                           |
| 331     | 23.3                   | 5.7                                   | 3.1                | 5.1              | 15.9             | 1.4            | ND                                          |
| 333     | 9.6                    | 3.4                                   | 5.0                | 2.8              | 5.0              | 2.0            | -                                           |
| 335     | 90.3                   | 34.9                                  | 2.7                | 1.5              | 6.0              | 1.2            | ND                                          |
| 336     | 15.9                   | 7.1                                   | 6.8                | 3.7              | 8.7              | 1.1            | -                                           |
| 339     | 4.2                    | 3.5                                   | 2.6                | 1.6              | 5.2              | 1.0            | -                                           |
| 340     | 487.0                  | 84.3                                  | 40.1               | 1.4              | 14.2             | 1.3            | +                                           |

<sup>a</sup> +, indicates specific IgE binding to recombinant *Amb a 1* Ag, -, indicates a lack of IgE binding.

<sup>b</sup> Ragweed pollen extract-primed T cells were tested in secondary T cell proliferation assays as described in *Materials and Methods* with three dilutions of the antigens indicated. The highest stimulation index (with T cells, APC, and medium only as the negative control) seen in each titration is shown. Medium control cpm values showed a range of 86 to 3352 in this group of patients. Sera from each patient was used to probe Western blots of recombinant antigens as described in *Materials and Methods* and Figure 2.

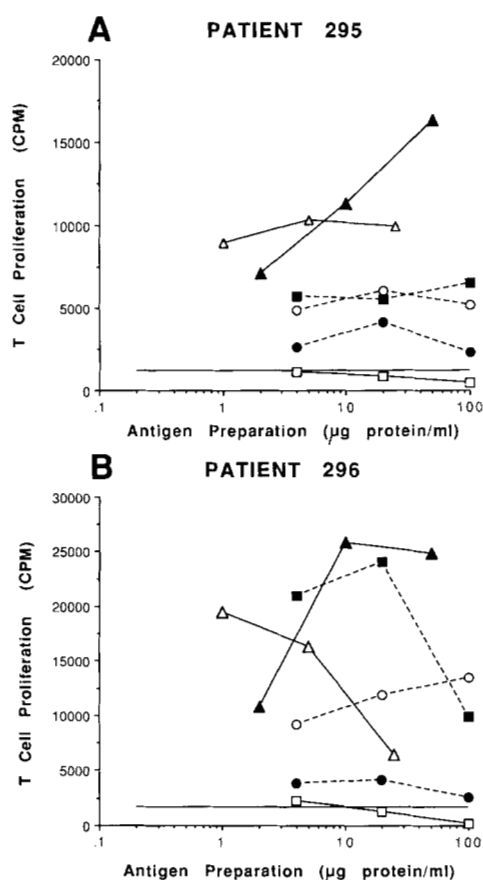


Figure 3. Secondary human T cell responses to recombinant ragweed allergens. PBMC from ragweed-allergic patients 295 and 296 were cultured in the presence of ragweed pollen extract, rested, and restimulated in a secondary assay with the Ag indicated; medium only (no symbol), ragweed pollen extract (▲), biochemically purified *Amb a I* (Δ), bacterial lysates containing recombinant *Amb a I* 1.1 (○), *Amb a I* 1.2 (●) or *Amb a I* 1.3 (■), or, as a negative control, JM109 bacterial lysate containing no recombinant protein (□). The proliferation of the T cells was assessed by uptake of tritiated thymidine in duplicate cultures.

be demonstrated (Fig. 2). The requirement that T cell response and IgE binding be examined from a single bleed of individual patients may be important. Future studies will include comparing both IgE and T cell reactivities serially in patients during and after pollen season and before and after immunotherapy.

#### DISCUSSION

Research on the important allergens of ragweed hay fever has previously led to the identification of two major allergenic 38-kDa preparations, *Amb a I* and *Amb a II* (4–6). In addition, there are a number of minor allergens of lower m.w. that have also been described (*Amb a III* through *Amb a VI*) (4). Allergic reactivity to *Amb a V* and *Amb a VI* has been correlated to specific HLA types although this does not appear to be the case with *Amb a I* and *Amb a II* (16). *Amb a I* and *Amb a II* have been shown to have similar amino acid composition (3, 6) but differ in their isoelectric points. The recent cloning and sequencing of *Amb a I* cDNA derived from pollen and flowerhead mRNA revealed that these proteins constitute products of a multigene family (10). *Amb a II* cDNA has also been recently cloned and sequenced and shares homology at the amino acid level with members of the *Amb a I* family of proteins (unpublished data).

In the present study we have used immunoblots of IgE

binding and human T cell culture techniques to analyze reactivity to *Amb a I* family members. We have applied immunoblot methods to demonstrate strong recognition of multiple recombinant *Amb a I* proteins by IgE from certain ragweed allergic patients. In the future, isolation of purified *Amb a I* recombinants will allow us to more clearly delineate the contribution that each gene product plays in IgE reactivity. Reactivity will be assessed by immunoblot analysis and the more physiologically relevant determination of histamine release from allergic patient basophils in vitro. In a recently published report, an *Amb a I* preparation was shown to have three strongly antigenic sites as defined by mAb recognition (8). Two of these epitopes were demonstrated to be major human allergenic epitopes. The defined sequences and expressed *Amb a I* proteins may allow us to determine these regions and possibly others that make up IgE epitopes.

The use of T cell culture systems has allowed us further insight into the immune mechanism of the allergic response. It is interesting to compare the high level of T cell reactivity and the IgE binding pattern on immunoblots to the individual recombinant *Amb a I* proteins. T cells from most ragweed allergic patients react in secondary proliferation assays with all three recombinant *Amb a I* family members (Table I), yet IgE from individual patients may display biased reactivity to recombinant *Amb a I* proteins (Fig. 2F). It will be interesting to determine if T cell recognition of epitopes shared by all forms can drive B cells to produce IgE that can recognize one form. Expansion of these types of studies with purified recombinant *Amb a I* will allow us to dissect these mechanisms further. The high level of shared sequence between the several *Amb a I* proteins, approximately 80% (10), and the high level of observed T cell reactivity to the three forms examined here suggests the presence of multiple shared T cell epitopes. This hypothesis is consistent with the absence of an observed HLA association with *Amb a I* reactivity (16). We are presently conducting studies aimed at mapping the T cell epitopes of these *Amb a I* proteins using overlapping peptides derived from the known cDNA sequences.

We have initiated experiments with T cell clones and lines derived from ragweed allergic patients aimed at determining the regions of the three forms of *Amb a I* that contain cross-reactive T cell epitopes. Examination of the spectrum of cytokines produced by T cells reactive with distinct *Amb a I* epitopes may provide important information concerning the regions of the molecules responsible for providing T cell help for IgE production. These experiments may be particularly illuminating because it is known that IL-4 plays an important role in the IgE response in vivo (17) and has been implicated in B cell Ig class switching to IgE production (18).

An important study relating the clinical relevance of an *Amb a I* preparation in ragweed allergy was reported in 1968 (19). Norman and colleagues compared purified *Amb a I* preparation to whole ragweed extract and placebo in a 4-yr immunotherapy study. The purified *Amb a I* preparation was as effective as the whole extract in reducing the symptoms of ragweed hay fever in the allergic patients of this study (19). We aim to define which portions of the *Amb a I* family of proteins play a role in its allergenic properties and those which presumably abrogate this response. These studies will allow the ra-

tional design of an immunotherapeutic agent with improved efficacy for the treatment of ragweed allergy.

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## REFERENCES

1. Lowenstein, H., and D. G. Marsh. 1981. Antigens of Ambrosia elatior (short ragweed) pollen. I. Crossed Immunoelectrophoretic analysis. *J. Immunol.* 126:943.
2. Lowenstein, H., and D. G. Marsh. 1983. Antigens of Ambrosia elatior (short ragweed) pollen III. Crossed radioimmuno-electrophoresis of ragweed-allergic patients' sera with special attention to quantification of IgE responses. *J. Immunol.* 130:727.
3. King, T. P., P. S. Norman, and J. T. Connell. 1964. Isolation and characterization of allergens from ragweed pollen. II. *Biochemistry* 3:458.
4. King, T. P. 1976. Chemical and biological properties of some atopic allergens. *Adv. Immunol.* 23:77.
5. King, T. P., A. Alagon, L. Kochoumain, J. Kuan, A. K. Sobotka, and L. M. Lichtenstein. 1981. Limited proteolysis of antigens E and K from ragweed pollen. *Arch. Biochem. Biophys.* 212:127.
6. King, T. P. 1972. Separation of proteins by ammonium sulfate gradient solubilization. *Biochemistry* 11:367.
7. King, T. P., P. S. Norman, and N. Tao. 1974. Chemical modification of the major allergen of ragweed pollen, antigen E. *Immunochemistry* 11:83.
8. Olson, J. R., and D. G. Klapper. 1986. Two major human allergenic sites on ragweed pollen allergen antigen E identified by using monoclonal antibodies. *J. Immunol.* 136:2109.
9. Smith, J. J., J. R. Olson, and D. G. Klapper. 1988. 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. *Molec. Immunol.* 25:355.
10. Rafnar, T., I. G. Griffith, M. C. Kuo, J. F. Bond, B. L. Rogers, and D. G. Klapper. 1991. Cloning of Amb a I (Antigen E), the major allergen family of short ragweed pollen. *J. Biol. Chem.* 266:1229.
11. Vercellii, D., and R. S. Geha. 1989. Regulation of IgE synthesis in humans. *J. Clin. Immunol.* 9:75.
12. Phillips, L., C. Chambers, B. J. Underdown, and B. Zimmerman. 1987. Lymphocyte proliferation to antigen E: demonstration of the restriction of antigen E-specific T cells to ragweed-allergic donors. *J. Allergy Clin. Immunol.* 79:933.
13. Amann, E., B. Ochs, and K.-J. Abel. 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* 69:301.
14. Marston, F. A. O. 1987. The purification of eukaryotic polypeptides expressed in *Escherichia coli*. In *DNA Cloning: A Practical Approach*, Vol. III. D. M. Glover, ed. IRL Press, Oxford, p. 59.
15. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350.
16. Marsh, D. G. 1988. Immunogenetics of allergic disease. In *Allergy. Principles and Practice*. E. Middleton, C. E. Reed, E. F. Ellis, N. F. Adkinson, and J. W. Yunginger, eds. C. V. Mosby Company, MO, p. 94.
17. Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, M. P. Beckman, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303.
18. Bergstedt-Lindqvist, S., H.-M. Moon, U. Persson, G. Moller, C. Heusser, and E. Severinson. 1988. Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG<sub>1</sub> and IgE. *Eur. J. Immunol.* 18:1073.
19. Norman, P. S., W. L. Winkenwerder, and L. M. Lichtenstein. 1968. Immunotherapy of hay fever with ragweed antigen E: comparisons with whole pollen extract and placebos. *J. Allergy*. 42:93.