

TWO MAJOR HUMAN ALLERGENIC SITES ON RAGWEED POLLEN ALLERGEN ANTIGEN E IDENTIFIED BY USING MONOCLONAL ANTIBODIES¹

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Murine monoclonal antibodies specific for antigen E (AgE), the major allergen isolated from short ragweed pollen, have been produced and characterized. These monoclonal antibodies, when coupled to Sepharose and used as immunoabsorbents, specifically bound AgE when a crude pollen extract was passed through the column. Three antigenic sites (A, B, and C) on AgE were identified by using five of these monoclonal antibodies in both inhibition and double-bind solid-phase ELISA. These three antigenic sites appear to be nonoverlapping and nonrepeated, that is, present only once on each AgE molecule. Site C on AgE could readily be bound by the monoclonal antibody specific for that site, but only when AgE was in solution or "presented" by an anti-site A or anti-site B antibody. Site C appears to be only marginally available for binding when AgE is directly adsorbed to polyvinyl chloride microtiter wells.

The majority of monoclonal antibodies isolated after immunization of BALB/c mice were specific for site A on AgE. In addition, the binding to AgE of pooled BALB/c polyclonal, hyperimmune antisera against AgE was blocked approximately 80% by a monoclonal antibody directed against site A, but was only blocked approximately 20% by an anti-site B monoclonal antibody. This suggests that site A on AgE is the predominant antigenic site in the BALB/c immune response and that site B represents a less dominant site.

The binding of IgE in pooled human serum from ragweed-allergic individuals is blocked approximately 50% by a monoclonal antibody directed to site A on AgE and also approximately 50% by a monoclonal antibody directed against site B. A series of individual human short ragweed allergic antisera also showed significant, although varied, inhibition of IgE binding to AgE by both anti-site A and anti-site B monoclonal antibodies. Simultaneous addition of anti-site A and anti-site B was somewhat additive and inhibited up to 80% of the binding of human IgE specific for AgE. The conclusion from these data is that site A and site B defined by two murine monoclonal antibodies represent two very major allergenic sites in the human response to this molecule.

Antigen E (AgE)³ is the major allergen found in short ragweed (*Ambrosia elatior*) pollen. This pollen, and AgE in particular, is the chief cause of late summer hay fever in North America (1). Immunotherapy with purified antigen E leads to alleviation of symptoms in ragweed-allergic individuals comparable to that obtained with whole pollen extract (2). In addition, approximately 13% of the total serum IgE in ragweed-allergic individuals is specific for AgE, although different individuals vary from less than 1% to greater than 40% (3). Both because it is such a major human allergen and because it represents approximately 6% of the protein content of ragweed pollen, AgE has been studied with regard to a number of characteristics. AgE has been isolated and found to be an acidic single-chain polypeptide (no detectable carbohydrate) with a m.w. of 38,000 (1). After chromatographic isolation (molecular sieve and ion-exchange procedures) AgE is isolated as a two-polypeptide chain structure with an α -chain of 26,000 m.w. and a β -chain of 12,000 m.w. This two-chain structure, which represents nearly 100% of the isolate, appears to be antigenically and allergenically indistinguishable from the single-chain form (4). On the other hand, virtually any modification of either the single or the two-chain form of AgE renders that molecule essentially totally nonreactive with human IgE and incapable of eliciting a positive allergic reaction, such as histamine release from sensitized basophils. The modifications of AgE that have been studied include reduction and alkylation of disulfide bonds, urea denaturation and renaturation, and succinylation of lysine residues (1, 5). Any one of these procedures renders AgE nonallergenic. Urea-denatured AgE, although no longer reactive with B cells, can still be used to prime T cells for a subsequent response to native AgE (6). The relatively large size and the extraordinary B cell conformational dependence of this allergen suggested that it would be of interest to study its epitope structure.

Protein allergens usually have m.w. that range from 5000 to 60,000 and in general do not seem to possess any obvious physicochemical differences from other protein antigens (1). Little information is available regarding whether or not allergenic epitopes share some unique property that distinguishes them from antigenic sites, although there is no *a priori* reason to suggest there is a difference. The only allergen in which both allergenic and antigenic peptide determinants have been localized, although not completely, is the food allergen, cod allergen

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³Abbreviations used in this paper: AgE, short ragweed pollen allergen antigen E; 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.

M (7). Using numerous overlapping peptides of allergen M, Elsayed and Apold (7) have obtained results consistent with the following three conclusions: 1) allergenic and antigenic determinants appear to occupy the same sites on allergen M, 2) these determinants are linear as opposed to conformational, and 3) several determinants are cross-reactive or repeated.

In this report, five monoclonal antibodies specific for AgE of short ragweed are described. These antibodies were used to identify three nonoverlapping antigenic sites (A, B, and C) on AgE. All three antigenic sites are nonrepeated on a given molecule of AgE, and two of these sites are apparently major human allergenic sites, for monoclonal antibodies to each inhibit the binding of significant amounts of IgE from allergic sera. These allergenic sites are conformation dependent in contrast to the sites on cod allergen M.

This identification and characterization of the antigenic and allergenic epitope structure of AgE will help in the understanding of the role of this molecule in allergic sensitization and will be useful in designing more efficacious modes of desensitization to this widespread allergen.

MATERIALS AND METHODS

Immunization. Female BALB/c mice (Charles River) were immunized i.p. with 0.15 ml (approximately 2 mg) of alum-precipitated partially purified AgE and were boosted 2 wk later with the same material. A third injection (AgE without alum) was administered 3 days before hybridization.

Cell fusion and cloning. The general procedure of Kennett et al. (8) was used throughout these studies. Spleen cells from an immunized mouse were mixed with either 45.6TG1.7 BALB/c plasmacytoma cells or with Sp2/0 clone E-5 cells at one of several ratios from 10:1 to 33:1 and were fused with 33% (w/v) polyethylene glycol 1500 (lot 795760; Fisher Scientific, Pittsburgh, PA). These cells were placed into microtiter plate wells in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) plus 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (2.5 mg/ml), 50 µM 2-mercaptoethanol, 15% horse serum, and HAT (10 mM hypoxanthine, 1 mM aminopterin, and 3 mM thymidine). Hybridomas were cloned in soft agar (8).

Screening for hybridomas specific for AgE. Hybridoma-containing wells were tested by using an enzyme-linked immunosorbent assay (ELISA) based on the method of Voller et al. (9). Polyvinyl chloride microtiter plate wells (Dynatech, Alexandria, VA) were coated with 3 µg of pure AgE (from the Research Resources Branch of the NIH) in 0.2 ml of carbonate buffer at pH 9.6 and incubated overnight at 4°C. The wells were washed three times with PBS Tween²⁰ and incubated overnight at 4°C with 0.2 ml of a dilution of hybridoma tissue culture supernatant in PBS Tween. The wells were again washed three times with PBS Tween and incubated at room temperature for 3 hr with 0.2 ml of a 1/1000 dilution of heavy and light chain-specific alkaline phosphatase-labeled anti-mouse IgG (Sigma, St. Louis, MO). After three more washings with PBS Tween, 0.2 ml of the enzyme substrate p-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer was added. Absorbance at 405 nm was determined by using an automated ELISA reader (Dynatech).

Isotype determination. The ELISA was modified in order to type the hybridoma antibodies by replacing the alkaline phosphatase-labeled anti-mouse IgG with alkaline phosphatase-labeled rabbit antisera specific for each of six mouse isotypes (IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA). Isotype-specific antisera were purchased from Litton Bionetics (Kensington, MD) and labeled with alkaline phosphatase in this laboratory by using dilute glutaraldehyde coupling (9).

Ascitic fluid. BALB/c mice received approximately 1 million viable, cloned cells i.p. several days after being pretreated with 0.5 ml pristane (10).

Affinity purification of antibodies. Tissue culture supernatants containing hybridoma antibodies or antisera containing antibodies against AgE were passed through an immunoadsorbent column consisting of partially purified AgE bound to Sepharose CL4B (Pharmacia, Piscataway, NJ) by cyanogen bromide coupling (11). The column was washed with 1.0 M NaCl in PBS Tween, after which the

antibody was eluted with 0.5 M acetic acid and immediately neutralized with Tris (Sigma) base.

Preparation of immobilized hybridoma antibody columns. Ascites fluid rich in hybridoma antibody was passed through a DEAE-Biogel (Bio-Rad, Richmond, CA) column equilibrated in 0.015 M Tris, pH 8.2, and eluted with a sodium chloride gradient. The γ-globulin fraction containing approximately 20 mg of monoclonal antibody was coupled to 10 ml of Sepharose CL4B by using cyanogen bromide (11). The immunoadsorbent was equilibrated in PBS.

ELISA. The general procedure of Voller et al. (9) was followed. Coupling of various reagents to alkaline phosphatase type VII (Sigma) was performed in 0.2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) at room temperature for 2 hr, followed by exhaustive dialysis against PBS to remove the excess glutaraldehyde. Alkaline phosphatase-labeled avidin was purchased from Zymed Laboratories (S. San Francisco, CA) and alkaline phosphatase-labeled goat anti-human IgE was purchased from Sigma.

Biotin labeling. Following the method of Bayer et al. (12), 25 µl of BnHS (biotinyl-N-hydroxysuccinimide ester) dissolved in dimethyl formamide were mixed with 500 µl of either affinity-purified antiserum or AgE in borate-buffered saline (BBS) and then incubated at room temperature for 7 hr. After dialysis at 4°C overnight against BBS, 0.1% sodium azide was added as a preservative.

Crude short ragweed pollen extract. The method of King et al. (13) was followed with some modifications. Briefly, defatted pollen (Greer Laboratories, Lenoir, NC) was soaked overnight in 0.015 M Tris, pH 8.2, filtered, and precipitated overnight at 4°C with 62% (w/v) ammonium sulfate. The resuspended precipitate was dialyzed against water and then passed over a G25 fine Sephadex (Pharmacia, Piscataway, NJ) column equilibrated in 0.015 M Tris, pH 8.2, to remove low m.w. materials.

Partially purified AgE. A modification of methods described by King et al. (4, 13) was used. The crude ragweed pollen extract was separated into basic, neutral, and acidic fractions by passage through a DEAE-Biogel (Bio-Rad) column equilibrated in 0.015 M Tris, pH 8.2. Cationic materials (such as Ra3, Ra4, Ra5, and Ra6) are excluded from this column and appear in the void volume. Sodium chloride (0.25 M) was added to the column, and essentially all of the acidic and neutral materials (including AgE) were eluted. The AgE-containing fraction was concentrated by positive pressure on a PM10 membrane (Amicon, Lexington, MA) and passed through a Sephadex G100 (Pharmacia) column equilibrated in 0.015 M Tris, pH 8.2. The AgE-containing fractions were monitored by double diffusion in agar by using a monospecific rabbit antiserum to AgE. The AgE-containing effluent was applied to a second DEAE-Biogel column and eluted with a linear gradient of sodium chloride. The early fractions from this gradient consisted of greater than 90% purified AgE and are comparable to the A form of AgE (4). For assays in which AgE is directly bound to an ELISA plate, highly purified AgE obtained from the Research Resources Branch of the NIH was used.

Quantitation of AgE. The amount of AgE present in partially purified AgE-containing extracts was determined by an inhibition ELISA in which microtiter plate wells were coated with an affinity-purified hybridoma antibody (HaA1) and incubated overnight at 4°C. The wells were washed three times with PBS Tween, and biotin-labeled partially purified AgE was added and incubated at room temperature for 3 hr. The wells were again washed three times with PBS Tween, after which alkaline phosphatase-labeled avidin was added and incubated at room temperature for 90 min. After three more washings with PBS Tween, the enzyme substrate was added and the OD₄₀₅ was measured. A standard inhibition curve was obtained by using purified AgE (Research Resources Branch of the NIH) as an inhibitor of the binding of the biotin-labeled partially purified AgE to the hybridoma antibody HaA1. The inhibition caused by several dilutions of a given preparation of partially purified AgE was measured and compared with the inhibition caused by known amounts of pure AgE in order to determine the amount of AgE in the partially purified AgE preparation. This assay assumes that AgE is the only antigen in crude pollen extract that reacts with this hybridoma, and there is evidence to suggest that is the case (see Fig. 1).

Affinity purification of AgE. Crude ragweed pollen extract was passed through a column of hybridoma antibodies bound to Sepharose CL4B. The column was washed with PBS and then with 1.0 M sodium chloride in PBS Tween. AgE was eluted with 0.5 M acetic acid, and the eluate was immediately neutralized with Tris (high molarity, pH 9.6), dialyzed extensively against several changes of water for several days, and lyophilized. This material (no longer immunoreactive due to the lyophilization step) (13) was then passed through an Aca44 (LKB, Gaithersburg, MD) column equilibrated in 1% ammonium bicarbonate. A small peak in the void volume, which contained heterogeneous aggregates, was obtained, followed by the

peak containing AgE, which had no visible contaminants on SDS-PAGE (see Fig. 1), performed by using a modified Laemmli technique (14).

RESULTS

Hybridomas specific for AgE. Cell fusion between spleen cells obtained from female BALB/c mice immunized with partially purified AgE and either of two fusion partners (45.6TG1.7 or Sp2/0) resulted in a series of hybridomas specific for AgE. After cloning in soft agar (8), five monoclonal antibody-producing lines, originating in five different microtiter wells during the initial plating, were chosen for further study. All five hybridoma antibodies were shown to bind specifically to AgE by their ability to bind purified AgE (obtained from the Research Resources Branch of the NIH), which had been coated directly onto a polyvinyl microtiter plate, although the binding of one of these antibodies, HaC1, was poor. Four of the five hybridoma antibodies were isolated and immobilized on Sepharose CL4B by using cyanogen bromide-activated resin. Figure 1 depicts the pattern obtained when crude short ragweed pollen extract is passed down such an immunoadsorbent and the material that is bound by the column is subsequently eluted. Although only two of the four results are depicted here, the data for HaA1 and HaA2 are essentially identical. Nearly all of the affinity-purified AgE from each of the four different hybridoma antibody columns consists of a single polypeptide chain, with only traces of the α and β subunits. In contrast, almost all of the NIH AgE, which was obtained by multi-step chromatographic purification, is in the two-chain form. This is consistent with the results of King et al. (4) and supports the hypothesis that the two-chain form of AgE arises during purification due to a specific proteolytic event.

Site specificity of the five hybridoma antibodies. Two

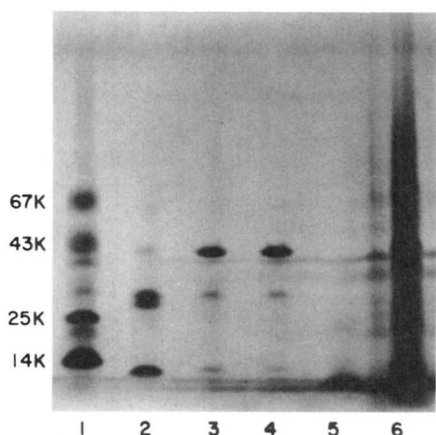


Figure 1. SDS-polyacrylamide gel of affinity-purified ragweed antigens. Crude aqueous ragweed extract was applied to hybridoma antibodies immobilized on Sepharose CL4B and washed extensively with PBS Tween containing 1 M NaCl. The adsorbed allergens were eluted with 0.5 M acetic acid, neutralized with Tris, dialyzed against H₂O, lyophilized, passed over an AcA44 column, and then applied to the SDS-PAGE gel. Affinity-purified AgE (lanes 3 and 4) is a single polypeptide chain of m.w. 38,000, whereas multi-step-purified AgE from NIH (lane 2) consists of two chains. Hybridoma antibody CBC7 does not react with AgE, but reacts with a low m.w. component in ragweed pollen extract (lane 5). Lane 1: Molecular weight standards (BSA, 67,000; ovalbumin, 43,000; chymotrypsinogen, 25,000; ribonuclease, 13,700). Lane 2: AgE obtained from the Research Resources Branch of the NIH. Lane 3: Affinity-purified AgE eluted from hybridoma antibody HaB1. Lane 4: Affinity-purified AgE eluted from hybridoma antibody HaC1. Lane 5: Affinity-purified low m.w. protein isolated from crude ragweed extract using hybridoma antibody CBC7. Lane 6: Crude aqueous ragweed pollen extract (24 hr).

different ELISA blocking assays were used to determine the site specificity on AgE of the five hybridoma antibodies: a) an inhibition ELISA and b) a double-bind ELISA.

In the inhibition ELISA, the HaA3 hybridoma (IgG2b) is used to inhibit the binding to AgE of three different IgG1 hybridoma antibodies. The use of alkaline phosphatase-labeled anti-mouse IgG1 heavy chain-specific antiserum as the second antibody ensures that only IgG1 hybridoma antibodies give a positive ELISA reaction. If a hybridoma antibody binds to the same region of AgE as does HaA3, then HaA3 will inhibit that binding. The results of this inhibition ELISA shown in Figure 2 indicate that HaA1 and HaA2 bind to the same region of AgE (called site A) as does HaA3. HaB1 apparently binds to a different region of AgE (site B), for it is not inhibited by HaA3.

In Table I, results of the double-bind ELISA are presented. In this assay, the microtiter plate well is first coated with a given hybridoma antibody. Then, AgE in PBS Tween is added. After washing with PBS Tween, the only AgE remaining in the well is that bound by the immobilized monoclonal antibody, and the particular binding region by which the AgE molecule is held should be unavailable for reaction with any other monoclonal antibody. Finally, alkaline phosphatase-labeled monoclonal anti-AgE is added, allowed to bind if it is able, then washed with PBS Tween, and the substrate is added. The results of the double-bind ELISA confirm the results of the inhibition ELISA and suggest that HaA1, HaA2, and HaA3 compete for the same (or similar) site on AgE, whereas HaB1 clearly binds to an unrelated site. The double-bind ELISA also allows two additional conclusions. First, HaC1 clearly binds to a different site (site C) than do the other four hybridoma antibodies. Second, when the same hybridoma antibody is used first to coat the well and then as the alkaline phosphatase-labeled hybridoma antibody, the alkaline phosphatase-labeled hybridoma antibody shows negligible binding to the AgE. This suggests that all five hybridoma antibodies bind to antigenic sites that are nonrepeated, i.e., present only once on each AgE molecule. An alternative explanation is that the hybridoma antibody that presents AgE is in such high concentration on the microtiter plate well that

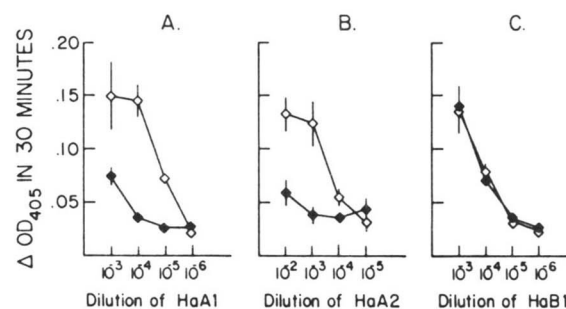


Figure 2. Inhibition ELISA assay showing that two of three hybridoma antibodies tested bind to the same site on AgE as does hybridoma antibody HaA3. Microtiter plate wells were coated with partially purified AgE (3 μ g) and incubated overnight at 4°C. After the wells were washed, dilutions of IgG1 hybridoma ascites fluid were added in the presence (●) or absence (○) of the inhibitor, HaA3 (IgG2b) ascites fluid at a dilution 1/500, and incubated overnight at 4°C. The wells were washed, and alkaline phosphatase-labeled anti-mouse IgG1 was added and incubated at room temperature. After washing, the enzyme substrate was added, and readings were taken at 50 and 80 min. Vertical bars represent $\pm$ SEM. Each point represents triplicate analyses.

TABLE I
Double-bind ELISA showing three nonoverlapping and nonrepeated antigenic sites on AgE

Coating ^a	Change in OD ₄₀₅ in 20 min				
	AP-HaA1	AP-HaA2	AP-HaA3	AP-HaB1	AP-HaC1
None	0.005 ± 0.000	0.002 ± 0.002	0.008 ± 0.002	0.011 ± 0.001	0.019 ± 0.007
HaA1	0.007 ± 0.002	0.005 ± 0.002	0.012 ± 0.001	0.212 ± 0.009	0.956 ± 0.060
HaA2	0.006 ± 0.000	0.004 ± 0.002	0.007 ± 0.001	0.195 ± 0.003	0.621 ± 0.029
HaA3 ^b	0.007 ± 0.001	0.004 ± 0.002	0.009 ± 0.001	0.020 ± 0.001	0.044 ± 0.003
HaB1	0.343 ± 0.018	0.257 ± 0.011	0.224 ± 0.006	0.011 ± 0.001	0.597 ± 0.002
HaC1	0.780 ± 0.002	0.356 ± 0.007	0.494 ± 0.017	0.302 ± 0.005	0.010 ± 0.003
αAgE ^c	0.037 ± 0.000	0.014 ± 0.001	0.028 ± 0.003	0.073 ± 0.004	0.246 ± 0.009
Summary:					
	When wells are initially coated with hybridoma antibody	Hybridoma antibodies able to bind AgE effectively		Hybridoma antibodies unable to bind AgE effectively	
	HaA1	HaB1, HaC1		HaA1, HaA2, HaA3	
	HaA2	HaB1, HaC1		HaA1, HaA2, HaA3	
	HaA3	HaB1, HaC1		HaA1, HaA2, HaA3	
	HaB1	HaA1, HaA2, HaA3, HaC1		HaB1	
	HaC1	HaA1, HaA2, HaA3, HaB1		HaC1	

^a Microtiter plate wells were coated with affinity-purified hybridoma antibody and incubated overnight at 4°C. After the wells were washed, partially purified AgE (15 µg/ml) was added and incubated overnight at 4°C. The wells were washed, and alkaline phosphatase-labeled hybridoma antibody was added and incubated at room temperature. After washing, the enzyme substrate was added and readings were taken at 20 and 40 min. Each point represents duplicate analyses.

^b HaA3 lost considerable activity during affinity purification, but the results are reproducible and consistent with the inhibition ELISA depicted in Figure 2.

^c Affinity-purified polyclonal antibody from a pool of sera from BALB/c mice immunized with AgE in Freund's complete adjuvant.

even if, for example, site A was present more than once on AgE, every site A would be bound by the presenting hybridoma antibody. Dilutions of HaA1 (spanning over 4 orders of magnitude) used to coat the plate and present AgE to alkaline phosphatase-labeled HaA1 also gave rise to no binding of the alkaline phosphatase-labeled HaA1 (data not shown). It appears, therefore, that site A is indeed a single, nonrepeated site. By this same reasoning, it can be concluded that all five hybridoma antibodies are monospecific, i.e., each binds to only one antigenic site on AgE.

Polyvinyl chloride interferes with the reactivity of the anti-site C monoclonal antibody. When performing solid-phase immunoassays in which antigen is coated onto polyvinyl chloride, it is usually assumed that the antigen coats the plate "randomly," i.e., in enough different orientations so that all the antigenic determinants that are present on the molecule in solution are still available for binding on at least some fraction of the bound antigen. This assumption does not appear to be valid in the case of AgE. In Table II, binding of hybridoma antibodies to AgE presented by either polyvinyl chloride or by a hybridoma antibody is compared. The anti-site A hybridoma antibody, HaA1, binds quite well to both polyvinyl chloride-presented AgE and AgE presented by either anti-site B or anti-site C monoclonal antibody. The anti-site B monoclonal antibody does not bind quite as well to AgE presented by polyvinyl chloride as it does to

AgE presented by an anti-site A or anti-site C antibody. In marked contrast, the anti-site C monoclonal antibody, HaC1, binds extremely poorly when AgE is coated directly on polyvinyl chloride, but binds very well to AgE when the antigen is presented off the surface of the plate by an anti-site A or anti-site B monoclonal antibody. This high binding is not the consequence of an antibody-induced conformational change in AgE as a result of binding by anti-site A or anti-site B, for HaC1 is able to bind AgE in solution quite well (see Table I and Fig. 1).

Monoclonal antibodies inhibit the binding of polyclonal antisera to AgE. The reactivity of polyclonal antisera to sites A and B was measured by using ascites fluid containing hybridoma antibodies specific for each site to inhibit the binding of the antisera to AgE. Control ascites fluid contained a monoclonal antibody, which does not bind to AgE. The binding of biotin-labeled, affinity-purified antibodies from a rabbit hyperimmunized against AgE was inhibited at least 50% by the addition of HaA1, the anti-site A monoclonal antibody, and at least 50% by the anti-site B monoclonal antibody, HaB1. The inhibition is only partially additive, for inhibition by HaA1 and HaB1 together in the microtiter plate well resulted in only 75 to 80% inhibition of the binding of the rabbit antibodies (Fig. 3). Antisera were titrated and used at the linear part of the reaction curve so that, for example, a 50% decrease in antisera concentration resulted in a 50% decrease in OD₄₀₅.

TABLE II
Comparison of AgE presented by hybridoma antibodies and AgE presented by polyvinyl chloride

Step 1 ^a	Step 2	Change in OD ₄₀₅ in 30 min								
		AP-HaA1 diluted			AP-HaB1 diluted			AP-HaC1 diluted		
		1/300	1/1000	1/3000	1/100	1/300	1/1000	1/100	1/300	1/1000
HaA1	AgE	-0.001			0.264	0.104	0.042	0.630	0.294	0.112
HaB1	AgE	0.191	0.089	0.035	0.001			0.469	0.246	0.100
HaC1	AgE	0.047	0.033	0.013	0.054	0.024	0.008	0.001		
—	AgE	-0.002			-0.003			0.000		
AgE	—	0.135	0.071	0.030	0.026	0.006	0.000	0.003	-0.001	-0.002
—	—	-0.003			-0.002			-0.001		

^a Protocol is that described in Table I. Values shown are average of duplicate wells, in all cases ± less than 10% or 0.005.

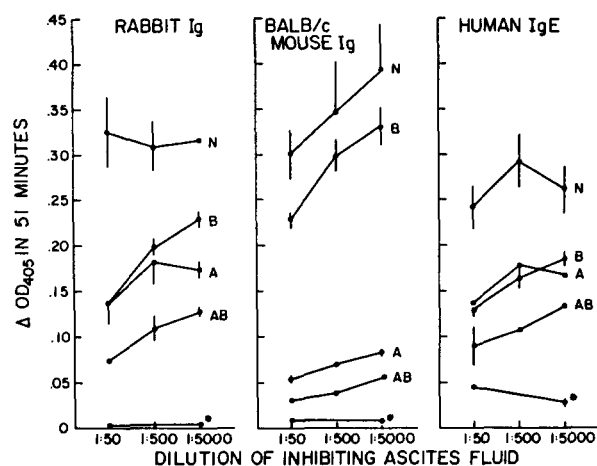


Figure 3. ELISA showing inhibition of binding of polyclonal antisera to AgE by monoclonal antibodies HaA1 and HaB1. Microtiter plate wells were coated with purified AgE (from the Research Resources Branch of the NIH, 500 ng) and incubated overnight at 4°C. The solution was aspirated from the wells, and 0.5% bovine serum albumin was added and incubated at room temperature for 1 hr. The wells were washed, and polyclonal antisera was added along with either one or two ascites fluids: (N) The negative control inhibitor, 45.6TG1.7 (which does not bind AgE) diluted 1/50, 1/500 or 1/5000. (A) Both HaA1 (anti-site A monoclonal antibody) and 45.6TG1.7, each diluted 1/100, 1/1000 or 1/10,000. (B) Both HaB1 (anti-site B monoclonal antibody) and 45.6TG1.7, each diluted 1/100, 1/1000 or 1/10,000. (AB) Both HaA1 and HaB1, each diluted 1/100, 1/1000 or 1/10,000. (*) Background control: no AgE added. Inhibitor is 45.6TG1.7 diluted 1/50 or 1/5000. *Left panel.* The polyclonal antiserum is an affinity-purified, biotin-labeled rabbit anti-AgE. After a 4-hr room temperature incubation, followed by washing, alkaline phosphatase-labeled avidin was added and incubated overnight at 4°C. After washing, the enzyme substrate was added. Duplicate wells were tested. *Middle panel.* The polyclonal antiserum is pooled affinity-purified, biotin-labeled BALB/c anti-AgE. The amount of biotin-labeled antibody binding to AgE was quantitated by using alkaline phosphatase-labeled avidin, as described for left panel. *Right panel.* The polyclonal antiserum is pooled human anti-short ragweed sera (Bureau of Biologics) diluted 1/20. After a 4-hr room temperature incubation, followed by washing, alkaline phosphatase-labeled goat anti-human IgE was added to determine the amount of IgE bound to AgE.

The binding of biotin-labeled, affinity-purified antibodies from BALB/c mice hyperimmunized with AgE was inhibited with a very different pattern by the anti-site A and anti-site B monoclonal antibodies. In this situation, anti-site A inhibited at least 80% of the binding, whereas anti-site B only inhibited approximately 20% of the binding. These results suggest that although both site A and site B are major antigenic sites in rabbit antisera, site A is the predominant antigenic site in BALB/c antisera, with site B reflecting a much less vigorous response by these highly inbred mice.

Figure 3 also depicts an attempt to extend these findings to human sera, and more specifically to examine the question of allergenic determinants defined by reactivity of AgE with human IgE antibodies. Monoclonal anti-site A and anti-site B antibodies were used to inhibit the binding of pooled human serum collected from short-ragweed-allergic individuals. A goat monospecific antiserum with activity against human IgE was labeled with alkaline phosphatase and used as the quantitative detection reagent. It can be seen that the inhibition pattern is quite similar to that seen with the rabbit antisera, i.e., both anti-site A and anti-site B inhibit the binding of human IgE approximately 50%, and a mixture of the two monoclonal antibodies resulted in partially additive inhibition.

Because these data were obtained with pooled allergic human serum, it was of interest to test several individual

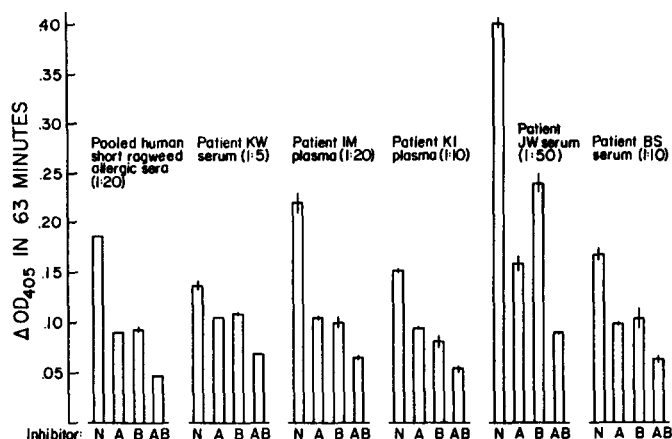


Figure 4. ELISA showing inhibition of binding of IgE in five individual ragweed-allergic sera to AgE by monoclonal antibodies HaA1 and HaB1. The protocol is that given for Figure 3 (right panel), except that the inhibiting ascites fluids were replaced by tissue culture supernatants containing the same monoclonal antibodies. The supernatants were each diluted 1/5 when two supernatants were added, and 1/2.5 when only one supernatant (45.6TG1.7) was added.

sera obtained from short-ragweed-allergic individuals to see if there was individual variation in the IgE response to AgE. To this end, the results with five ragweed-allergic sera are presented in Figure 4. All five showed similar inhibition patterns with 25 to 60% inhibition by both anti-site A and anti-site B and partially additive inhibition when anti-site A and anti-site B are mixed together. Patient KW shows a less typical pattern, with only 20 to 25% inhibition by either anti-site A or anti-site B and additive inhibition much closer to the theoretical level (40 to 50%) than is usually observed. Thus, in all five individual ragweed-allergic antisera examined, HaA1 and HaB1 were able to inhibit a significant, although varied, percentage of the binding of IgE to AgE.

Because there is evidence that AgE interacts with polyvinyl chloride in a nonrandom way, there remained the question of whether the inhibition data represent some artifact of that interaction. HaC1, the monoclonal antibody that reacts with site C on AgE in solution, was used to coat microtiter plate wells and AgE was allowed to bind to those antibodies. Both the pooled human antiserum and the five individual antisera were allowed to bind to the "presented" AgE in the presence or absence of monoclonal anti-site A or anti-site B antibodies. Again, under these conditions, both HaA1 and HaB1 were significant inhibitors, with HaB1 inhibiting better than HaA1 in every case except the JW serum, where both HaA1 and HaB1 inhibited to the same degree (data not shown).

DISCUSSION

AgE, the major allergen of short ragweed, has been used as a model system by a number of investigators interested in various aspects of immediate hypersensitivity (2, 15–20). Among the reasons this antigen enjoys such widespread popularity for this purpose is the fact that AgE is indeed responsible for a significant clinical problem in the United States. In addition, AgE represents approximately 6% of the protein content of ragweed pollen, has been purified in gram quantities, and is available as purified material from the Research Resources Branch of the NIH for investigational use. Much of the physicochemical characterization of AgE has been carried out by

King (1), who established its m.w., described the charge heterogeneity of the two-chain form, and proved that native, freshly isolated AgE is a single polypeptide chain. This latter point is of particular interest because most of the studies performed with AgE utilize the two-polypeptide chain structure of the molecule, which arises during chromatographic purification, a process that takes from days to weeks to perform. Virtually 100% of the NIH supplied AgE is in the two-chain form, having been cleaved at a very specific site by a trypsin-like enzyme that copurifies during the early chromatographic stages along with AgE. The antigenicity and allergenicity of both forms of AgE as measured by such criteria as immunoassay, induction of antibody production, and histamine release are identical. On the other hand, King clearly showed some years ago that modifications of AgE such as urea denaturation (and subsequent renaturation), reduction and alkylation of disulfide bonds, and succinylation of lysine residues, destroy the ability of AgE to react with antibodies or induce an immune response. Although urea-denatured AgE can be used to prime T cells in mice, it appears that B cell determinants are extremely conformationally dependent.

The present study represents the first published attempt to begin to understand the epitope structure of this interesting and significant allergen. The approach has been to immunize mice with purified AgE and produce a series of monoclonal antibodies that specifically react with that allergen.

For some antigens, large panels of monoclonal antibodies have been used to construct detailed antigenic maps. Schroer et al. (21) examined 18 monoclonal antibodies to insulin by a competitive binding assay and concluded that each reacted to a unique antigenic site, although some of these sites are overlapping. Another protein, influenza virus hemagglutinin, has also been extensively studied. In early work, four nonoverlapping sites on the hemagglutinin were defined by a competitive binding assay performed with nine monoclonal antibodies (22). A much more detailed antigenic map of the hemagglutinin from five closely related strains of influenza was constructed by another research group using 166 monoclonal antibodies. By determining the binding of each of the monoclonal antibodies to 16 strains of the same influenza virus subtype, it was demonstrated that these antibodies showed 138 different specificities, which were clustered in the regions of the previously defined antigenic sites (23). A very general conclusion from these and other studies is that proteins have numerous epitopes usually clustered at major antigenic sites, and monoclonal antibodies can recognize very subtle differences in these epitopes. Perhaps a much larger panel of monoclonal antibodies will lead to a much finer discrimination between the specificities of the different monoclonal antibodies to AgE, particularly in the major antigenic region of site A. Assaying the binding of the monoclonal antibodies to extracts from pollens closely related to short ragweed (such as giant, western, false, slender, and southern ragweed) (24) would help to reveal the extent of the heterogeneity of the epitopes that constitute site A.

With this background, the results of this study are of interest in several respects. AgE, with a m.w. of 38,000, might be considered to be an ideal model protein for

production of monoclonal antibodies to a large number of antigenic determinants. The results of attempts to produce monoclonal antibodies by using native AgE as the immunogen suggest that there are only a limited number of antigenic determinants that are recognized by BALB/c mice. The entire set of monoclonals isolated from several independent fusions after immunization with alum-precipitated native AgE recognize only three unique determinants. They appear to be non-overlapping and nonrepeated. Of the five monoclonal antibodies extensively studied in this report, three react with site A, and each of the others defines a unique site (B and C, respectively). In three independent fusions after immunization of BALB/c mice with native AgE, by far the predominant isolates are monoclonal antibodies recognizing site A (19 out of 29, data not shown). In rabbits and BALB/c mice hyperimmunized with native AgE, sites A and B account for greater than 75% of the immunoreactivity. As expected, most of the activity in hyperimmunized BALB/c is inhibited by anti-site A antibody. More importantly, in allergic individuals, either before or after ragweed desensitization therapy, site A and site B together account for greater than 75% of the IgE antibody response. The conclusion, therefore, is that in spite of the high m.w. of AgE, there appear to be only a limited number of major antigenic epitopes. This observation goes a long way toward explaining why even minor chemical or physical modifications of AgE result in the complete loss of B cell and antibody reactivity. The major antigenic epitopes presumably are extraordinarily conformation dependent. Lack of success in preserving the major antigenic and allergenic determinants of AgE when the molecule is modified either enzymatically or chemically has made identification and localization of the determinants of AgE difficult, although allergenically active α - and β -chains have been isolated (20). The native conformation of AgE is not cleaved by trypsin (except into α - and β -chains), chymotrypsin, or papain (1), and urea denaturation or reduction and alkylation, which render AgE susceptible to enzymatic hydrolysis, each greatly reduce or abolish the antigenic and allergenic reactivity of AgE (1). The present use of hybridoma antibodies specific for native AgE has allowed the identification of specific antigenic sites on AgE.

One of the three antigenic determinants identified in this study, site C, is almost completely unavailable for binding when AgE is presented by polyvinyl chloride. A similar phenomenon has been reported for monoclonal antibodies to various antigens, including tobacco mosaic virus protein (25), the beta 2 subunit of tryptophan synthetase (26), and interferon- α (27). Investigators working with these antigens have found monoclonal antibodies that bind well to solution-phase antigen, but poorly or not at all to plastic-adsorbed antigen—there are also examples from the same laboratories of monoclonal antibodies that bind well to plastic-adsorbed antigen but poorly to the soluble form. Because the antigenic structure of AgE, as well as that of at least these other proteins, is altered by adsorption to polyvinyl chloride, this type of immunoassay may not always be optimal for quantitating antibody activity in sera or for screening hybridoma antibodies. It is unclear at present whether the unavailability of site C on AgE presented by polyvinyl chloride is due to a restricted orientation of binding of AgE to polyvinyl

chloride or to a conformational alteration of AgE induced by the conditions used for coating the plate.

There are other reports of the use of monoclonal antibodies to study the reactivity of human IgE and specific allergens. In an inhibition assay, Chapman et al. (28) found that monoclonal antibodies to two distinct epitopes on allergen P1 from the dust mite failed to inhibit the binding of human IgE in 14 sera from individuals allergic to mite P1 allergen. Some of the sera, however, were able to inhibit the binding of certain monoclonal antibodies to the P1 allergen. In an unrelated system, both Kahn and Marsh (29) and Esch and Klapper (30) report the ability of murine monoclonal antibodies raised against grass Group 1 antigen to inhibit the binding of human IgE from grass-allergic individuals. In the latter studies, each monoclonal antibody inhibited only a maximum of about 25% of the binding of human IgE. Thus, the extremely high inhibition values reported in this study have not been seen with two other major allergens examined in the same way.

There are other possible explanations for the observation that two monoclonal antibodies inhibit at least 80% of the binding of human IgE to ragweed allergen AgE. The possibility that AgE consists of a large number of repeating determinants at this time can only be eliminated by experiments in which monoclonal antibody bound to the surface of a polyvinyl chloride plate is diluted over a 10,000-fold range. Even under these conditions, monoclonal antibody of the same specificity (another anti-site A) fails to bind to AgE bound to the antibody on the plate. More direct insight into this possibility will come as the structure of AgE becomes known. An alternative explanation is that both anti-site A and anti-site B monoclonal antibodies cause an allosteric alteration in AgE such that the true epitope structure of AgE is lost. This seems unlikely because AgE bound by anti-site A is still recognized by anti-site B and anti-site C (and vice versa).

The explanation that remains for these data is that native AgE contains three major antigenic sites, two of which are significant targets of the human allergic response. The fact that outbred rabbits hyperimmunized against AgE also respond by making IgG antibody directed almost solely to these same sites suggests that the unique aspect of this system resides in the allergen.

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