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Standardization of antigen E in ragweed pollen extracts using a monoclonal antibody-based enzyme immunoassay

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A hybridoma-derived monoclonal IgG antibody specific for ragweed AgE was used to develop a competitive binding enzyme immunoassay suitable for quantitation of antigen E levels in ragweed pollen extracts. The assay was capable of detecting as little as 30 ng/ml AgE in crude pollen extracts. The monoclonal antibody was shown to react with AgE present in commercial pooled pollen extracts from a number of ragweed species as well as a laboratory extract from a single species. In contrast to previous conventional xenoantisera, it could distinguish true ragweed (Ambrosia sp) from false ragweed (Franseria sp). The use of monoclonal antibodies in assay systems such as this offers a reproducible and widely applicable method for allergen standardization. (J ALLERGY CLIN IMMUNOL 71:261, 1983.)

The use of unstandardized allergen extracts for immunotherapy and diagnosis of IgE-mediated allergic reactions remains an anachronism in an age in which quality control of therapeutic and diagnostic agents is considered mandatory. Although the need for stan-

dardization of allergenic preparations is widely recognized,¹⁻³ progress has been slow because of the complex nature of many allergen sources and the need to combine quantitative procedures with assays of biologic potency. The recent development of the technology for production of monoclonal antibodies⁴ offers a unified approach to both isolation and standardization of clinically important allergens.⁵ To demonstrate the potential value of this technology, a monoclonal antibody was prepared against the purified major allergen, AgE, from ragweed pollen.⁶ When used in an enzyme immunoassay the antibody proved to be a suitable primary standard for single-step quantitation of AgE levels in crude ragweed pollen extracts.

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Abbreviations used

PBS: Phosphate-buffered saline
RAST: Radioallergosorbent test

MATERIALS AND METHODS**Preparation of ragweed AgE-specific monoclonal antibodies**

Immunization and cell fusion. Somatic cell hybrids secreting specific antibody to AgE were prepared by the method of Kohler and Milstein.⁴ Female BALB/c mice were injected intraperitoneally with 100 μ g of ragweed pollen AgE (lot No. A-5138; Sigma Chemical Co., St. Louis, Mo.) on three occasions at monthly intervals. The first two injections were given in complete and incomplete Freund's adjuvant, respectively, and the third was dissolved in saline. The day after the third injection and 3 days before fusion of spleen cells with the murine plasmacytoma NS-1, 300 μ g of AgE were administered intraperitoneally. Cell fusion was achieved in the presence of polyethylene glycol (mol. wt. 4000). Antibody-secreting hybrid cells were cloned twice by limit dilution as described elsewhere.⁵

Screening of culture supernatants for AgE-specific antibodies. Culture supernatants were assayed for the presence of AgE-specific antibodies by solid-phase radioimmunoassay. Wells of flexible polyvinyl microtiter plates (Cooke Laboratory Products, Alexandria, N.J.) were coated with AgE by the addition of 100 μ l of a 10 μ g/ml solution in PBS, pH 7.4. After incubation overnight at room temperature and three washes with PBS, bovine serum albumin 1% in PBS (400 μ l) was added to each well and left for 1 hr at 37° C. Plates were rewashed with PBS before addition of 50 μ l of culture supernatant to each well. Plates were incubated for 2 hr at 37° C and washed twice with PBS, and 50 μ l of ¹²⁵I-labeled polyvalent rabbit IgG anti-mouse immunoglobulin were added to each well and left for 1 hr at 37° C. After washing with PBS, wells were cut out and bound radioactivity was measured in a gamma spectrometer.

Isolation of AgE-specific IgG. A cell hybrid-secreting immunoglobulin with specificity for AgE was grown in mass culture. Culture supernatant (300 ml) was concentrated 10 times by ultrafiltration using a YM10 membrane filter (Amicon) before being applied to a protein A-Sepharose CL-4B (Pharmacia, Piscataway, N.J.) column previously equilibrated with 0.14M phosphate buffer, pH 8.⁷ The column was extensively washed with the equilibration buffer until the eluate showed no absorbance at 280 nm. Bound IgG was eluted with 0.1M glycine-HCL buffer, pH 2.5, in 0.15M NaCl, immediately neutralized with 2M Tris, pH 8, and dialyzed against PBS.

Isoelectric focusing

The homogeneity of the monoclonal antibody eluted from the protein A column was checked by isoelectric focusing in a thin-layer polyacrylamide gel over the pH range 3.5 to 10, as described by Awdeh et al.⁸

Enzyme labeling of antibodies

Alkaline phosphatase (lot No. 60F-0351; Sigma) was conjugated to affinity-purified anti-ragweed AgE monoclonal antibody by means of glutaraldehyde.⁹ Equal volumes (200 μ l) of a stock solution of monoclonal antibody (1 mg/ml in PBS, pH 7.4) and enzyme (5 mg/ml in 3.2M ammonium sulfate solution, pH 7, containing 10⁻³M magnesium chloride and 10⁻⁴M zinc chloride) were mixed and dialyzed overnight against PBS at room temperature before sequential dialysis against multiple changes of PBS and Tris-HCL buffer, pH 8.6. The solution was then made up to 5 ml with Tris-HCL buffer containing 1% bovine serum albumin, 10⁻⁴M magnesium chloride, and 0.02% sodium azide to give a final antibody concentration of 40 μ g/ml. It was stored at 4° C.

Enzyme immunoassay

Quantitation of AgE was achieved with an inhibition assay.¹⁰ Polyvinyl chloride microtiter plates (Dynatech Laboratories Inc., Alexandria, Va.) were protein-coated by the addition of 1% bovine serum albumin in PBS (400 μ l) to each well. After incubation for 1 hr at 37° C and two washes with PBS, 60 μ l of allergen extract or purified ragweed AgE at different concentrations were added to the wells and incubated for 1 hr at 37° C with 15 μ l of a 1:40 dilution of enzyme-labeled monoclonal antibody. Incubation mixtures (60 μ l) were then transferred to wells of a second tissue culture plate previously coated with ragweed AgE (100 μ g/ml) and bovine serum albumin (1% w/v) in PBS. After a further incubation for 1 hr at 37° C, the plates were washed three times with PBS, once with distilled water, and once with carbonate-bicarbonate buffer, pH 9.6. The enzyme substrate *p*-nitrophenylphosphate (1 mg/ml) in carbonate-bicarbonate buffer, pH 9.6 (100 μ l), was then added and the mixture was left at 37° C for 1 hr and then at 4° C. Color change was detected by measurement of the absorbance at 405 nm on a Titertek Multiskan (Flow Laboratories, McLean, Va.).

RESULTS**Monoclonal antibodies to ragweed AgE**

Ten hybrid clones secreting antibody to ragweed AgE were isolated from a single fusion. One of these clones, shown to be secreting IgG by reaction with rabbit anti-mouse IgG in immunodiffusion, was grown in mass culture and antibody purified by absorption and elution from protein A-Sepharose. The purified ragweed AgE-specific IgG (2 mg) reacted with AgE bound to microtiter plates and showed a restricted banding pattern in polyacrylamide gel after isoelectric focusing, consistent with a molecule of monoclonal origin.

Quantitation of AgE in ragweed pollen extracts with enzyme-labeled monoclonal antibody

A standard curve was constructed with results from experiments that measured the reaction of enzyme-

labeled monoclonal anti-ragweed AgE IgG (detected by absorbance values at 405 nm) with increasing quantities of AgE. The standard curve was made linear by the logit transformation of absorbance readings after allotting the maximum absorbance a value of 11 units (Fig. 1). Ragweed AgE concentrations were expressed as $\log_3$ dilutions of the standard AgE reference solution (100 $\mu\text{g}/\text{ml}$). The assay permitted the detection of nanogram quantities of AgE per milliliter.

Mixed ragweed pollen prick-test solutions from the same manufacturer, but from two different batches, were examined and found to contain almost the same concentration of AgE, 38 to 40 $\mu\text{g}/\text{ml}$ (Table I). On repeated testing of individual batches there was little variation in AgE level ($\pm 12\%$), which confirmed the reliability of the assay. Its specificity was checked with allergen extracts from rye grass, timothy grass, some dicot pollens, and the house dust mite, *Der-matophagoides pteronyssinus*. AgE was detected in only one of the extracts—an extract of short ragweed pollen prepared by aqueous extraction, ultrafiltration, and lyophilization (Table I). It should be noted in particular that there was no reaction between the monoclonal antibody and a false ragweed extract. When commercial prick-test solutions were used, glycerol was first removed by dialysis, since this component was found to interfere with the enzyme assay.

DISCUSSION

The aim of the present study was to demonstrate that hybridoma-derived, allergen-specific monoclonal antibodies have the potential to serve as valuable tools for the quantitation and thus standardization of biologically characterized allergens used for diagnosis and therapy. In view of the clinical importance of ragweed allergens,^{6, 11} AgE, the major allergen, was selected for study. A single fusion after immunization with AgE yielded 10 clones secreting allergen-specific monoclonal antibodies, one of which was subjected to further analysis. The antibody was shown to be of IgG class and capable of detecting down to approximately 100 ng/ml AgE in crude pollen extracts when used in a competitive binding enzyme immunoassay (Fig. 1). Labeled antibody was preferred to labeled antigen, since this approach has greater potential sensitivity.^{12, 13} The specificity of the remaining antibodies is currently under investigation.

AgE has previously been quantitated in ragweed pollen extracts by RAST inhibition,^{14, 15} immunodiffusion,^{16–18} double-antibody radioimmunoassay,¹⁹ and histamine release from peripheral leukocytes.²² Although these methods can all be used successfully, the application of monoclonal antibodies for qualita-

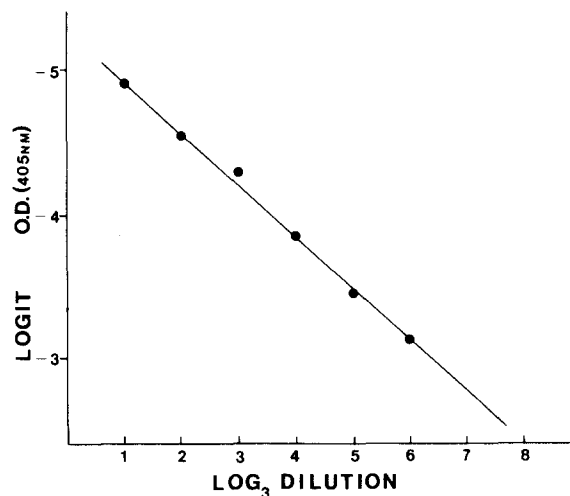


FIG. 1. Logit transformation of the AgE enzyme immunoassay standard curve. Values were obtained by threefold dilutions of a reference solution (ragweed AgE, 100 $\mu\text{g}/\text{ml}$). The standard curve was made linear by the logit transformation. O.D., Optical density.

tive and quantitative studies of allergen extracts has some obvious advantages, particularly when compared with heterogeneous xenoantisera. The first advantage is their specificity for individual allergenic determinants. This is illustrated here by the finding that the monoclonal antibody reacted only with the two commercial AgE-containing ragweed extracts and the single aqueous extract of *Ambrosia elatior*, whereas it did not bind to other allergen mixtures nor for that matter to false ragweed (Table I). The latter finding is interesting, since previous RAST inhibition studies with conventional reagents had revealed cross-reactivity between short (*A. elatior*) and false (*Franseria acanthicarpa*) ragweed but did not establish whether this was due to the presence of AgE in false as well as short ragweed.^{20, 21} The exquisite specificity of monoclonal antibodies therefore makes them ideal tools for precise analysis and standardization of cross-reactive determinants, which is of considerable importance in the case of the ragweed species.^{22–24} Work currently in progress with the antibody described here and with additional antibodies from the remaining clones is being directed toward analyzing cross-reactivity between AgE and other ragweed determinants.

The second advantage of hybridoma technology is its capacity to provide antibodies of high affinity for development of sensitive immunoassays. Instead of having to rely on xenoantibodies from species like the rabbit, which vary greatly in the quality and quantity of their responses, multiple fusions can be undertaken until one or more antibodies of suitable affinity have been generated. Once cloned, the hybridomas then

TABLE I. Quantitation of ragweed AgE in allergen extracts with enzyme-labeled monoclonal ragweed AgE-specific IgG

Allergen extracts	Genus and species	AgE concentration ($\mu\text{g/ml}$)*
Commercial extracts†		
Ragweed pollen mix	<i>Ambrosia elatior</i> <i>Ambrosia trifida</i> <i>Ambrosia psilostachya</i> <i>Franseria acanthicarpa</i>	(batch 1)‡ (batch 2)‡
Perennial ryegrass	<i>Lolium perenne</i>	—
Timothy grass	<i>Phleum pratense</i>	—
Plantain	<i>Plantago lanceolata</i>	—
House dust mite	<i>Dermatophagoides pteronyssinus</i>	—
Extracts of dicot pollens§		
Short ragweed	<i>A. elatior</i>	5
False ragweed	<i>F. acanthicarpa</i>	—
Common mugwort	<i>Artemisia vulgaris</i>	—
Golden rod	<i>Solidago vigaurea</i>	—
Paterson's curse	<i>Echium plantagineum</i>	—

— = <100 ng/ml.

*Concentration of AgE obtained from standard curve.

†Extracts dialyzed to remove glycerol and tested at 1:2 and 1:10 dilutions.

‡Two different batches of extract from the same manufacturer containing a mixture of the four species shown.

§Prepared as described by Katerlaris et al.²⁷ and tested up to a concentration of 100 $\mu\text{g/ml}$.

have the additional advantage of being amenable to long term storage and subsequent passage in mice or tissue culture. The homogeneity of the product, coupled with the potential immortality of the cell lines, could well serve as the basis for developing a panel of primary international standards for biologically characterized allergens.^{5, 25}

Monoclonal antibodies are also of potential value in the identification, isolation, and ultimately standardization of allergens in complex mixtures. This is illustrated by recent experiments carried out in our laboratory in which high-affinity antibody to a clinically important allergen from the house dust mite, *D. pteronyssinus*, was obtained by immunization with a crude aqueous extract.^{5, 7, 26} When coupled to a solid-phase support, the antibody could be used for single-step purification of biologically active allergen as judged by skin prick testing, RAST, and histamine release. Furthermore, radiolabeling of the antibody yielded a reagent that served as a primary standard for quantitating the level of allergen in commercial extracts. In the short term, monoclonal antibodies will not replace conventional standardization techniques for complex allergen mixtures such as dilution prick testing and RAST inhibition but can serve as an adjunct to them. Ultimately it is theoretically possible to develop a panel of monoclonal antibodies capable of reacting with all the major allergens in such complex mixtures.

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