

Identification of human reagins isolated with immunosorbent as IgE

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An immunosorbent prepared by attaching covalently the constituents of the aqueous extract of ragweed pollen to aminocellulose was used to remove reagins from a fraction of allergic serum enriched with respect to skin-sensitizing activity. The adsorbed antibodies were eluted with a solution of 2M NaI, pH 9.1, or of glycine-HCl, pH 2.5. The presence of ragweed-binding IgE antibodies in these eluates was revealed by radioimmuno-electrophoresis and radio-Ouchterlony analysis. Guinea pigs, which had been rendered tolerant to normal human cord serum proteins, were immunized with these eluates and produced anti-IgE antibodies, as demonstrated by radioimmunodiffusion and by reversed PCA in monkeys. These results confirm that human reagins elicited to the allergens of ragweed pollen belong to the IgE class of immunoglobulins.

In earlier studies it had been shown that reagins could be isolated from sera of ragweed-allergic individuals with immunosorbents.^{1, 2} For this purpose, the nondialyzable constituents of the aqueous extract of ragweed pollen (DWSR) were attached by stable covalent bonds to polyamino-polystyrene¹ or amino-benzene cellulose,^{2, 3} and the resulting immunosorbent was used to isolate reagins from ragweed-allergic sera. Skin-sensitizing antibodies were eluted with glycine-HCl buffer, pH 2.5, or 6M urea at neutral pH (7.4), or a solution of 2M NaI at pH 9.1.⁴ However, the elution of the adsorbed reaginic antibodies was not complete with either the acid or the NaI buffer alone, i.e., for maximal recovery of reagins from the immunosorbent both eluting agents had to be employed in succession. Moreover, incorporation of a "stabilizing protein" such as human or rabbit serum albumin in the eluting fluid at a concentration of 0.7 to 7 mg per milliliter was shown to be required for the preservation of reaginic activity.

The effectiveness of the immunochemical methods developed for the isolation of reagins, as measured in terms of their biologic activity, suggested that similar procedures might also be useful in attempts to characterize these antibodies. Hence, the present study was undertaken with a view to isolating reagins from sera of ragweed-allergic individuals and identifying them by immunochemical procedures. It was concluded that these reagins were immunoglobulins of the IgE class, in accord with the previous demonstration of Ishizaka and Ishizaka.⁵

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TABLE I. Reaginic activity of eluates of "fraction 35-45" of allergic serum Kh following chromatography on DEAE-Sephadex A-50

Eluate	Molarity of Tris-HCl Buffer*	P-K titer
I	0.1	10
II†	0.2	200
III	0.3	100
IV	0.4	50
VI	0.6	0

*Tris-HCl buffers, pH 8.0, containing 0.5M sucrose.

†This fraction containing highest reaginic activity was used for isolation of reaginic antibodies with the immunosorbent; this fraction is also referred to as preparation II.

Special abbreviations used

DEAE	= diethylaminoethylcellulose
DWSR	= nondialyzable fraction of the water-soluble extract of ragweed pollen
HSA	= human serum albumin
NHCS	= normal human cord serum
PBS	= phosphate-buffered saline
PCA	= passive cutaneous anaphylaxis
PK	= Prausnitz-Küstner

MATERIALS AND METHODS

Fractionation of allergic sera

Sera were obtained from a ragweed-allergic patient, Kh, who had received hyposensitization treatments, and from a ragweed-allergic individual, Gr, who had not undergone treatment.

For the isolation of the fractions of allergic serum enriched with respect to reaginic activity, blocking antibodies that are normally present in sera of both treated and nontreated patients and that have the properties of IgG^{6,7} were first removed by precipitation with ammonium sulfate at a concentration corresponding to 35 per cent saturation at 22° C.; this precipitate was removed by centrifugation. Ammonium sulfate was then added to the supernatant to increase its concentration to 45 per cent saturation at 22° C. This second fraction of precipitated serum proteins was recovered by centrifugation, dissolved in distilled water, and desalted by gel filtration on Sephadex G-25, which had been previously equilibrated with 0.1M Tris-HCl buffer, pH 8.0; this serum fraction will be referred to as "fraction 35-45." The proteins in this fraction were further separated by ion-exchange chromatography on DEAE-Sephadex A-50, utilizing a batch-wise procedure with Tris-HCl buffers, pH 8.0, at molarities of 0.1, 0.2, 0.3, 0.4, and 0.6; each buffer also contained sucrose at 0.5M. For easy identification of the corresponding chromatographic fractions, these will be referred to by the Roman numerals I, II, III, IV, and VI, respectively. The eluted proteins were first concentrated by "dialysis" against dry sucrose, then dialyzed against PBS, pH 7.4, and adjusted to the original serum volume. Reaginic activity was evaluated by the P-K test.⁸

Isolation of reagins by immunosorbent

The constituents of DWSR were coupled covalently to aminocellulose as described previously.^{3,4} Before addition of the immunosorbent to the fraction II of the allergic serum possessing the highest reaginic activity (Table I), the former was pretreated successively with 0.1M glycine-HCl at pH 2.5 (gly-HCl); saline, 0.1M Tris-HCl, pH 9.1 (Tris-HCl); 2M NaI in the Tris-HCl buffer (referred to as NaI), and finally with saline. The skin-sensitizing antibodies were absorbed from fraction II (in a ratio of the serum fraction equivalent to

1.66 ml. of the original serum to 1 mg. of immunosorbent) by stirring with the immunosorbent at room temperature for a period of 2 hours. To remove any nonspecifically adsorbed proteins, the immunosorbent-antibody complex was thoroughly washed by successive centrifugation and resuspension in saline. The adsorbed antibodies were eluted by treatment with the NaI solution for 15 minutes at room temperature (in a ratio of 1 ml. of NaI containing 5 mg. of HSA for 8 mg. of immunosorbent-antibody complexes). The suspension was centrifuged at $5,000 \times g$ for 10 minutes, the supernatant recovered, and a fresh portion of NaI-HSA solution was used to resuspend the immunosorbent. This procedure was repeated 3 more times. The eluates (supernatants) were dialyzed first against Tris-HCl buffer and then against PBS. After washing the immunosorbent with saline, elution was continued at 0° C. with three portions of gly-HCl buffer containing HSA (5 mg. per milliliter). The supernatants obtained after each centrifugation were immediately neutralized with sodium bicarbonate and dialyzed against PBS. After dialysis, all eluates were concentrated to small volumes by "dialysis" against dry sucrose and finally dialyzed against PBS.

To establish if the antibodies eluted off the ragweed immunosorbent belonged to immunoglobulins of the IgE class,⁵ radio-Ouchterlony analysis was performed in agarose (1 per cent w/v) using an antiserum specific to IgE (kindly provided by Dr. K. Ishizaka), and the preparation of DWSR was labeled with ¹³¹I according to the method developed by Yagi and associates.⁹ Radioimmunoelectrophoresis was performed in agarose gel (1 per cent w/v) in veronal buffer (0.033M, pH 8.6); a myeloma IgE serum (provided also by Dr. K. Ishizaka) was added to the sample wells prior to electrophoresis. After thorough washing, the agarose plates were dried and placed in contact with Kodak Industrial x-ray film, type KK, exposed for a suitable time (6 to 24 hours), and the films were developed with Diafine developer (Acufine Inc., Chicago, Illinois). To eliminate the bulk of HSA from the eluates containing IgE antibodies to the constituents of ragweed pollen, these eluates were pooled and fractionated by salting-out with ammonium sulfate at a concentration corresponding to 47 per cent saturation at 22° C.

Immunization of guinea pigs with eluates containing reaginic antibodies

For the induction of immune paralysis to serum proteins other than IgE, adult guinea pigs were injected intravenously with 10 mg. of a fraction of NHCS that had been precipitated by salting-out with ammonium sulfate at a concentration corresponding to 50 per cent saturation at 22° C. These animals were then immunized by 3 intramuscular injections of 0.2 ml. each of an emulsion consisting of the concentrated reaginic eluate (0.1 ml.) and complete Freund's adjuvant (0.1 ml.) at intervals of one month. Blood was collected by intracardiac puncture 2 weeks following the last immunizing injection. Although it was expected that these animals had been "tolerogenized" to all normal human immunoglobulins present in cord serum, to ensure elimination of any antibodies that might have been formed against the light chains of human immunoglobulins, 1 mg. of κ and λ chains in the form of the corresponding Bence-Jones proteins was added to 2 ml. of the immune sera prior to their examination by immunoelectrophoresis and by radioimmunodiffusion. For the sake of a control and for reference purposes, in the latter method, myeloma IgE was added to 2 of the allergic sera, and the precipitin arcs were developed with a rabbit antiserum specific to IgE. Ragweed antigen E (provided by Dr. M. W. Chase), labeled with ¹²⁵I, was added to all wells in the Ouchterlony agarose plates. The myeloma IgE used in this experiment was isolated from a myeloma IgE serum (provided by Dr. O. McIntyre, Dartmouth Medical School, Hanover, New Hampshire) by preparative starch block electrophoresis, followed by chromatography on Sephadex G-200. The anti-IgE antibodies were produced in a rabbit by immunization with this myeloma IgE, and the antiserum was absorbed with normal human serum.

Since monkeys possess an immunoglobulin that cross-reacts with human IgE, the antisera produced by immunization with the eluates containing reagins were examined by the reversed PCA test in monkeys.¹⁰ In this procedure, 0.025 ml. of the antiserum, in tenfold serial dilutions, was injected intradermally, and the reactions were made visible by intravenous injection of a volume of 5 ml. of a 1 per cent solution of Evans blue in PBS. Normal guinea pig serum similarly injected in identical dilutions served as control.

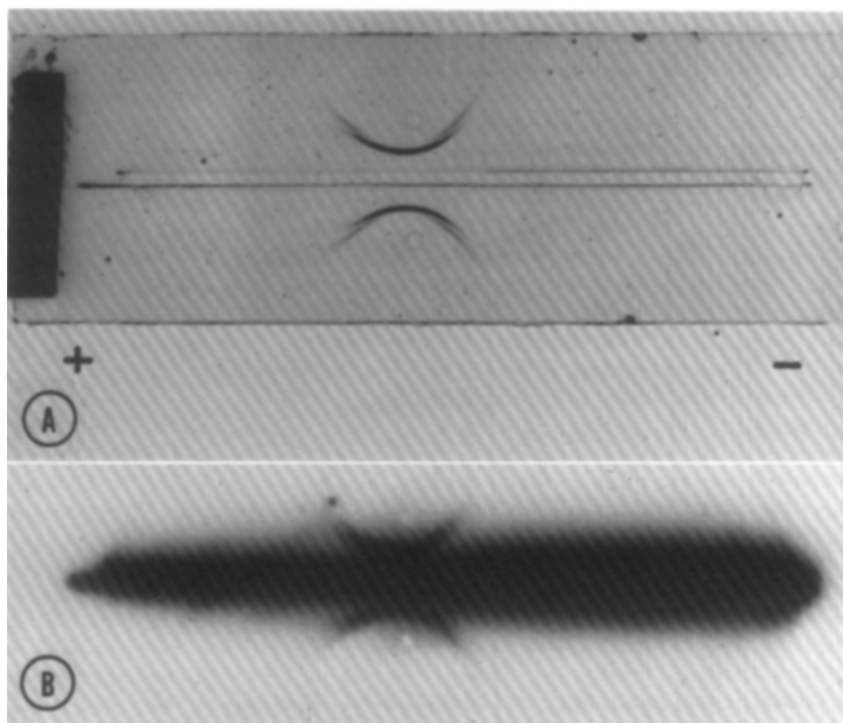


FIG. 1. Immunoelectrophoretic pattern of eluates recovered from immunosorbent saturated with reaginic serum fraction II (see Table I). The upper well contained the eluate obtained with 2M NaI in 0.1M Tris-HCl buffer, pH 9.1; the lower well contained the eluate obtained with 0.1M gly-HCl buffer at pH 2.5. IgE myeloma serum was also added to both sample wells prior to electrophoresis. The immunoelectrophoretic analysis was performed in agarose with veronal buffer, pH 8.6, $I_{1/2} = 0.033$, for 40 minutes at 9.25 V. per centimeter. An antiserum specific to IgE was added to the antibody trough after electrophoresis. ^{131}I -DWSR was also added to the antibody trough after immunodiffusion had been carried out for 48 hours and the slides had been washed to remove unreacted proteins. **A**, Stained slide; **B**, Radioautograph.

RESULTS

Characterization of antibodies recovered from immunosorbents

On immunoelectrophoresis the mobility of the myeloma IgE was anodal to the point of application (Fig. 1, A). In these experiments, the IgE myeloma serum was added to the sample wells prior to the addition of the eluates and before electrophoresis. In a control experiment, it was determined that the ^{131}I -DWSR did not react or bind with the myeloma IgE alone. The presence of radioactively labeled DWSR in the IgE—anti-IgE precipitin arcs was revealed by radioautographic analysis (Fig. 1, B). This procedure demonstrated that the eluted IgE antibodies were still capable of reacting with the ^{131}I -DWSR. Data as to the amount of skin-sensitizing activity recovered from immunosorbent-antibody complex by multiple elutions with 2M NaI, pH 9.1, followed by multiple elutions with gly-HCl, pH 2.5, was presented in another article.⁴

Although the NaI and gly-HCl eluates, isolated from immunosorbents saturated with fraction II of the original "fraction 35-45" of the reaginic serum

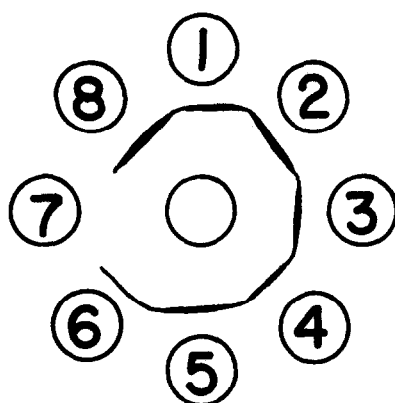


FIG. 2. Radio-autographic pattern of eluates recovered from immunosorbent saturated with preparation II of "fraction 35-45" of allergic serum Kh. The center well was filled with an antiserum specific to IgE (provided by Dr. K. Ishizaka). Wells 1, 2, 3, and 4 contained the first 4 eluates obtained by elution with 2M NaI in Tris-HCl buffer, pH 9.1; well 5, 6, and 7 contained the first 3 eluates obtained with 0.1M gly-HCl buffer, pH 2.5. Well 8 contained the allergic serum preparation II. ^{131}I -labeled DWSR was added to the center well 48 hours after immunodiffusion had taken place and the Ouchterlony slides had been thoroughly washed.

Kh, did not reveal any visible precipitin bands (in the absence of carrier myeloma IgE serum) on Ouchterlony analysis using rabbit antisera to IgE, evidence for the presence in these eluates of ragweed-binding antibodies of the IgE class was obtained by radioautography (Fig. 2). On the other hand, the labeled components of DWSR were not bound by either IgA or IgM on immunodiffusion of these eluates; for this part of the experiment specific rabbit antisera to human IgA and IgM (Hyland Laboratories, Los Angeles, California) were substituted in the appropriate wells.

Characterization of antibodies in the guinea pig antisera to reaginic eluates

On analysis of the sera of the two treated ragweed-allergic patients, C.B. and G.C. (to which nonreaginic IgE had been added), by the Ouchterlony procedure with the guinea pig antiserum to the reaginic eluates, weak precipitin bands were observed (Fig. 3, A); this evidence was considered further proof that these eluates used for immunization of guinea pigs had contained IgE. Moreover, these precipitin bands merged with those produced with the rabbit antiserum specific to myeloma IgE. Furthermore, by radioautography, it was revealed that these precipitin bands representing IgE—anti-IgE complexes incorporated ^{125}I -labeled antigen E (Fig. 3, B). An additional set of precipitin bands, closer to the central antibody well, was obtained with these allergic sera on reaction with the guinea pig antiserum only (Fig. 3, A). However, these precipitin bands did not bind the ^{125}I -labeled antigen E.

On analysis of whole allergic sera Kh and Gr by immunoelectrophoresis with guinea pig antiserum to the reaginic eluates, it was found that antibodies had not been produced to the HSA that had originally been used for the stabilization

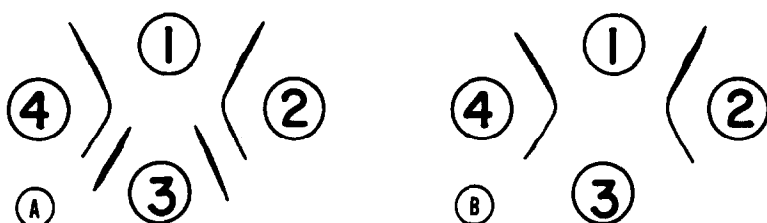


FIG. 3. Ouchterlony patterns obtained with guinea pig antiserum to reagenic eluates. The guinea pig antiserum (well 3) was produced to the reagenic fractions eluted from the immunosorbent, which had been saturated with preparation II of "fraction 35-45" of allergic serum Kh. Well 1 contained rabbit antiserum specific to IgE. Myeloma IgE was also added to wells 2 and 4 containing sera C.B. and G.C., respectively. Ragweed antigen E labeled with ^{125} I was added to all wells. **A**, Stained slide. **B**, Radioautograph.

of reagins during elution. Thus, it was concluded that immune tolerance to this protein had been indeed induced by the intravenous pretreatment of the guinea pigs with the NHCS preparation. However, antibodies to component(s) with the mobility of α globulins were also present in the guinea pig antiserum.

Demonstration of antibodies to IgE by the reversed PCA test in monkey

The presence of antibodies to IgE in the guinea pig antiserum, produced by immunization with the reagenic eluates, was also demonstrated by the reversed PCA test in a *Macaca irus* monkey since the antiserum elicited a positive cutaneous reaction, whereas normal guinea pig serum did not (Table II).

DISCUSSION

The differential solubility of blocking and reagenic antibodies in ammonium sulfate solutions of different concentrations was exploited for their separation from each other using a procedure established in an earlier study.¹¹ Further purification of reagenic antibodies, measured in terms of enrichment of serum fractions with respect to reagenic activity, was accomplished by ion-exchange chromatography on DEAE-Sephadex A-50 (i.e., preparation II of Table I).

To ensure maximal saturation of the immunosorbent with the homologous antibodies, it was incubated with an excess of preparation II of reagenic serum "fraction 35-45," as revealed by the P-K titer of the supernatant approaching that of the preparation II prior to exposure to the immunosorbent. For maximum recovery of adsorbed reagins, the elutions were performed sequentially with NaI and gly-HCl buffers.⁴ The presence of IgE in the eluates isolated from the immunosorbent was demonstrated in 2 ways: (1) IgE was detected in the reagenic fractions, eluted with NaI and gly-HCl, with the help of the antiserum specific to IgE (obtained from Dr. Ishizaka), and (2) production of specific anti-IgE antibodies was elicited in guinea pigs on immunization with these eluates.

The production of antibodies to proteins other than reagins (IgE) was suppressed in guinea pigs by inducing immune paralysis in these animals to most, if not to all, normal serum proteins by pretreating them intravenously with the NHCS preparation containing most of the globulins; this preparation was used

TABLE II. Demonstration by the reversed PCA test in monkey of the presence of antibodies to IgE in guinea pig antiserum* to reaginic eluates

Reciprocal of dilution of antiserum	Cutaneous reactions† observed with	
	Immune serum	Normal serum
100	+	—
1,000	+	—
10,000	—	—

*Obtained after the second course of immunization with the reaginic eluates.

†To render the cutaneous reactions easily visible, a volume of 5 ml. of Evans blue (1 per cent) was injected intravenously 10 minutes after the intradermal injections of the guinea pig antiserum. (A slight reaction ($\pm$) was also observed with both the immune and normal sera in a dilution of 1:10, which is probably attributable to nonspecific irritation due to the relatively high serum protein concentrations at this dilution.)

since it is known that NHCS is virtually free of reagins. However, since the NHCS preparation also lacked IgA, these animals could not have been rendered tolerant to IgA. Nevertheless, it must be pointed out that antibodies to IgA were not produced on immunization with the reaginic serum fractions, which is considered as further proof that reagins are not associated with the IgA class of immunoglobulins. It was expected, in accord with the report of Henney and Ishizaka,¹² that the NHCS preparation would induce immune paralysis in guinea pigs to the antigenic determinants of the light chains of human immunoglobulins as well as to the antigenic determinants of the Fc portions of the different heavy immunoglobulin chains present in normal serum. As a further precautionary measure, both κ and λ Bence-Jones proteins were used in the present study to absorb the guinea pig antisera so as to ensure removal of any antibodies that may have been produced against the antigenic determinants of these light chains.

As additional evidence for the effectiveness of the method used for induction of immune paralysis, it is to be pointed out that antibodies to neither HSA nor to the other normal human globulins were detected in the sera of these animals. However, immunization of these animals with reaginic fractions eluted off the specific immunosorbents led to the production of antibodies not only to IgE, but also surprisingly to a protein possessing electrophoretic mobility of alpha globulins.

Similar results were obtained with antisera prepared in normal guinea pigs (which had not been rendered tolerant to any serum proteins) on immunization with reaginic fractions of the second allergic serum Gr eluted with NaI and gly-HCl in the presence of rabbit serum albumin (and not HSA). Although the relationship of "alpha globulin" to the reagin-ragweed allergen system was not elucidated, it is obvious that this protein had been bound to the immunosorbent and was later recovered during elution. However, from the Ouchterlony radio-immunodiffusion analysis of 2 allergic sera (Fig. 3), it appears that these globulins had no capacity of combining specifically with ragweed antigens, and one might therefore presume that these globulins were adsorbed nonspecifically to the immunosorbent. Thus the guinea pig antiserum gave only one set of bands on radioautography with ¹²⁵I-labeled ragweed antigen E, which corresponded to the precipitin arcs due to the IgE—anti-IgE system; the second

set of precipitin arcs are, therefore, considered to be due to antibodies to an "alpha globulin," which probably had no relation to antibodies to ragweed pollen.

Further evidence for the production of anti-IgE antibodies in the guinea pig was obtained in the present study by recourse to an indirect, biologic procedure involving reversed PCA in monkey. Thus, the guinea pig antiserum had the ability of eliciting cutaneous reactions on intradermal injection into the monkey; this reverse PCA reaction had been previously shown by Henney and Ishizaka¹² to be due to anti-IgE antibodies and not to antibodies to human IgG, IgA, or IgM.

In summary, reagins to ragweed pollen were identified as immunoglobulins of the IgE type, following their direct isolation with a specific immunosorbent.

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