

The characterization of human antibody to heterologous serum protein

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Human allergic and nonallergic sera containing antibody to horse serum albumin were characterized and compared as to their capacity to bind I_{131} antigen, to passively hemagglutinate coated sheep cells, and to sensitize skin. They were also compared as to the presence of specific immunoglobulins in each fraction. Localization of the immunological activity was determined in partially purified fractions, and compared to that of the whole serum.

The definition of the antigen-antibody systems involved in human allergic disease has been retarded by the lack of reliable in vitro methods of detection of allergic antibody, as well as by the complex nature of the antigens. Various models of the immediate type of hypersensitivity have been developed in an attempt to overcome these difficulties. These include the diphtheria toxin-antitoxin system,¹ the model of spontaneous canine pollen allergy resembling human pollen allergy,² and the passive transfer of human hypersensitivity to monkeys.^{3, 4}

The in vivo methods of direct skin testing and of passive transfer still remain the standard techniques for demonstrating the presence of and estimating the quantity of the allergic antibody. These tests, though sensitive, are semiquantitative at best. For this reason, certain in vitro techniques have been developed as a means of measuring antibodies in the sera of allergic humans. The techniques used include the hemagglutination of tanned sheep erythrocytes coated with antigen by sera from allergic humans,⁵ coprecipitation of serum from

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allergic humans with isotope-labeled antigen by the ammonium sulfate⁶ or anti-globulin techniques,^{7, 8} and autoradiographic immunoelectrophoresis.⁹ These methods, however, may detect and measure both allergic and nonallergic antibodies. In this report, allergic antibodies are considered those responsible for immediate type, wheal-and-erythema cutaneous reactivity and, presumably, the clinical manifestations of atopic disease.

Since relatively pure and well-defined antigens, such as heterologous serum proteins, are readily available, the application of the *in vitro* methods provides a model of study of single antigen-antibody reactions occurring in allergic individuals sensitive to these proteins. Reactions of the immediate type occurring in these patients are probably the result of the same type of antibody present in patients with pollen hypersensitivity, so comparisons between the systems appear justifiable.

The present study was designed to use various methods of characterizing the antibodies present in individuals with and without anaphylactic sensitivity to a major antigenic component of horse serum, horse serum albumin. In addition, fractionation of the human serum was carried out and antibody activities of the fractions were determined and compared to the activity of the whole serum by means of certain of the *in vivo* and *in vitro* techniques.

METHODS AND MATERIALS

Human antisera

Serum containing allergic or skin-sensitizing antibody (SSA) was obtained from an individual with a history of severe asthma following exposure to horse dander and no history of previous foreign serum injection. The patient's sensitivity was such that the accidental instillation of horse serum into the conjunctiva had resulted in systemic anaphylaxis. Wheal-and-erythema skin reactivity to horse serum albumin (HSA) was determined by the cutaneous method, using tenfold dilutions of antigen ranging in concentration from 8 to 0.0008 $\mu\text{g N}$ per milliliter.

Human serum without SSA was obtained from an individual with anti-HSA antibody detectable by *in vitro* techniques but with negative intracutaneous and passive transfer skin tests to as much as 50 μg of HSA N.

I¹³¹ labeling of antigen

HSA was obtained commercially as Cohn Fraction V (Pentex Corporation, Kankakee, Illinois), and the quantity of HSA used was expressed as micrograms nitrogen as determined by the Markham modification of the micro-Kjeldahl technique.¹⁰ The HSA was labeled with I¹³¹ (I*) by the method of Talmage, Baker, and Akeson,¹¹ and radioactivity was measured in a NaI scintillation counter.

Coprecipitation of I* HSA with human antisera

The *in vitro* binding of I* HSA by the human antisera was determined by a modification of the ammonium sulfate method⁶ applied to the detection of small amounts of antibody.¹² In addition, the coprecipitation of the bound antigen was

also carried out using rabbit antihuman globulin.⁷ These were done in the following manner: Duplicate sets of 0.05 ml. samples of human serum were incubated for one hour with varying concentrations of I* HSA, or mixtures of I* HSA and unlabeled HSA, in a final volume of 1 ml. One set of samples was then brought to a concentration of 50 per cent saturated ammonium sulfate by adding slowly, with mixing at 4° C., 1 ml. of saturated ammonium sulfate, pH 7.0. Rabbit antihuman globulin was added to the other set of serum samples in amounts determined previously as sufficient to precipitate all of the immunoglobulins in the serum.⁷ The samples were then centrifuged at 2,000 r.p.m. for 30 minutes at 4° C. The percentage of counts present in each of the precipitates formed and the total nitrogen added to each aliquot were recorded in all determinations and graphically represented. From this, the maximal amount of antigen bound by equal amounts of each of the sera could be determined, and the capacity to bind antigen compared.

Autoradiographic immunoelectrophoresis

Human antisera were subjected to electrophoresis in agar gel (Ionagar, Oxoid, Consolidated Laboratories, Chicago Heights). Immunoglobulin bands were developed with goat antisera against human γ^G , γ^A , and γ^M (Hyland Laboratories, Los Angeles). After washing with normal saline, pH 7.0, for 72 hours, the slides were incubated for 48 hours at 4° C. with I* HSA, rewashed, dried, and placed in contact with x-ray film.¹² Several films were exposed to each dried agar gel slide for varying periods of time to determine the proper length of exposure.

Hemagglutination of tanned red cells

The hemagglutination of tanned sheep red cells coated with HSA by the human sera was carried out by the method of Stavitsky.¹³

Passive transfer studies

These studies included the determination of the SSA titer, the minimal amount of antigen necessary to elicit the wheal-and-flare reaction, and the reagin neutralization titer.^{14, 15} All recipients of serum were normal nonallergic volunteers and had no skin reactivity to the intracutaneous injection of 10 μ g of HSA N.

Determination of the SSA titer was carried out by the intracutaneous injection of 0.1 ml. of human serum in fivefold dilutions, and challenge 48 hours later with 10 μ g of HSA N. The end point was determined as that site sensitized with the highest dilution of serum which exhibited a greater wheal-and-flare reaction than that of a control site of the same undiluted human serum challenged with 0.1 ml. of 0.15M NaCl.

The determination of the minimal amount of antigen required to elicit the immediate wheal-and-flare reaction was carried out by injecting progressively decreasing twofold amounts of antigen, ranging from 100 μ g N to 0.0001 μ g N, into sites sensitized 48 hours earlier with a 1:5 dilution of serum.¹⁵ The end point and controls were as described above.

The reagin neutralization technique of Levine and Coca¹⁴ was used to deter-

mine the capacity of the human antisera to bind HSA as detected *in vivo*. Equal amounts of unheated serum containing SSA, serum with no detectable SSA to HSA, or serum containing SSA heated to 60° C. for 4 hours, were mixed with equal volumes of decreasing amounts of antigen, ranging from 100 μg N to 0.0001 μg N, and were incubated for 2 hours at 37° C. One-tenth milliliter aliquots of each sample were then injected intracutaneously into sites sensitized 48 hours earlier with unheated serum containing SSA. The end point was determined in each case as the maximal amount of HSA combined with the human antiserum which did not elicit an immediate reaction in the previously sensitized site. Nonreactive controls included mixtures of antigen and serum injected into nonsensitized sites, heated or unheated serum without antigen injected into sensitized sites, and equal volumes of 0.15M NaCl injected into sensitized sites.

In addition to these studies in humans, local and systemic sensitization of monkeys, with the subsequent production of local or systemic anaphylaxis after appropriate challenge, was determined as previously described.⁴

Fractionation of the serum containing SSA

The serum was initially fractionated by the addition of saturated ammonium sulfate, pH 7.0 at 4° C. to a final concentration of 33 per cent. The ammonium sulfate was removed by dialysis against 0.15M NaCl at pH 7.0 at 4° C., and the fractions soluble and insoluble in the 33 per cent saturated ammonium sulfate were then each further separated into distilled water insoluble and soluble fractions by dialysis against distilled water pH 7.0 at 4° C. for 72 hours. All fractions were then reconstituted to the original serum volume in 0.15M NaCl at pH 7.0.

The presence of immunoglobulins in each fraction was determined by paper electrophoresis in addition to double gel diffusion using specific goat antihuman immunoglobulin sera. The immunologic activities of each fraction were determined and compared to that of the whole serum by certain of the above techniques.

RESULTS

The results of the immunologic studies are summarized in Table I.

Skin reactivity

The major difference between the two anti-HSA sera, as determined by direct and passive transfer, was the presence of SSA in the serum from the allergic individual, and its absence in the serum from the nonallergic individual. The SSA activity could be passively transferred to human skin at a dilution of 1:1,250, which is the order of reactivity of the sera of some ragweed-sensitive individuals.⁴ The minimal concentration of HSA required to evoke a reaction in the allergic individual by the direct cutaneous (scratch) method was 0.08 μg N per milliliter. Skin reactivity of this individual's serum, when evaluated by the passive transfer technique, was such that the wheal-and-flare reaction could be elicited by the intracutaneous challenge with as little as 0.0002 μg of HSA N. The skin reactivity of the HSA-sensitive individual was not evaluated by intracutaneous testing because of the risk of anaphylaxis.

Table I. *Summary of immunologic studies*

	<i>Serum from allergic individual</i>	<i>Serum from non-allergic individual</i>
<i>In vivo studies</i>		
Skin-sensitizing antibody activity—direct cutaneous test*	0.08 μ g HSA N/ml.	No reaction to 50 μ g HSA N
SSA activity by intracutaneous passive transfer test*	0.0002 μ g HSA N	No reaction to 50 μ g HSA N
SSA transfer—human†	1,250	None
SSA transfer—monkey†	125	None
Transfer of anaphylaxis to monkey	Positive	Negative
Maximal amount of antigen bound by 1 ml. unheated serum‡	0.01 μ g HSA N	< 0.0002 HSA N (see text)
Maximal amount of antigen bound by 1 ml. heated serum‡	0.01 μ g HSA N	Not done
<i>In vitro studies</i>		
Hemagglutinating titer§	640	80
Maximal amount of I*HSA bound by 1 milliliter serum	0.04 μ g N	0.008 μ g N
Immunoglobulin binding I*HSA by autoradiographic immunoelectrophoresis	γ^G , γ^A	γ^G

*SSA activity is measured as the lowest dose of antigen eliciting a reaction.

†Expressed as the reciprocal of the highest dilution of serum giving a positive test to intracutaneous challenge with 5 μ g of HSA N.

‡As detected by challenge of sites passively sensitized with unheated allergic serum. (See text.)

§Expressed as the reciprocal of the highest dilution giving a positive test.

In vitro binding of I* HSA by the antisera

Both sera specifically and consistently bound antigen, as demonstrated by means of either the ammonium sulfate or antihuman globulin coprecipitation methods (Fig. 1). Furthermore, heating the serum containing SSA to destroy skin-sensitizing activity did not alter the capacity to bind antigen. The serum from the allergic individual maximally bound 0.04 μ g of HSA N per milliliter, about five times the binding capacity of the serum from the nonallergic individual. Sera from other nonallergic individuals with no known exposure to HSA failed to bind significant amounts of I* HSA.⁷

Autoradiographic immunoelectrophoresis

This technique demonstrated the binding of I* HSA by two immunoglobulins in the serum from the allergic individual, consistent with γ^G and γ^A , but by only one band, γ^G , in the serum from the nonallergic individual. Binding of antigen by γ^M was not detected in either serum by this method in these studies. In addition, serum without SSA which did not bind I* HSA, as determined by the coprecipitation technique, did not bind antigen by this method.

Hemagglutination of tanned red cells

The hemagglutination titer of the serum containing SSA, using tanned sheep red cells coated with HSA, was eight times that of the serum without SSA. This is consistent with the binding data in the order of magnitude of reactions with each serum.

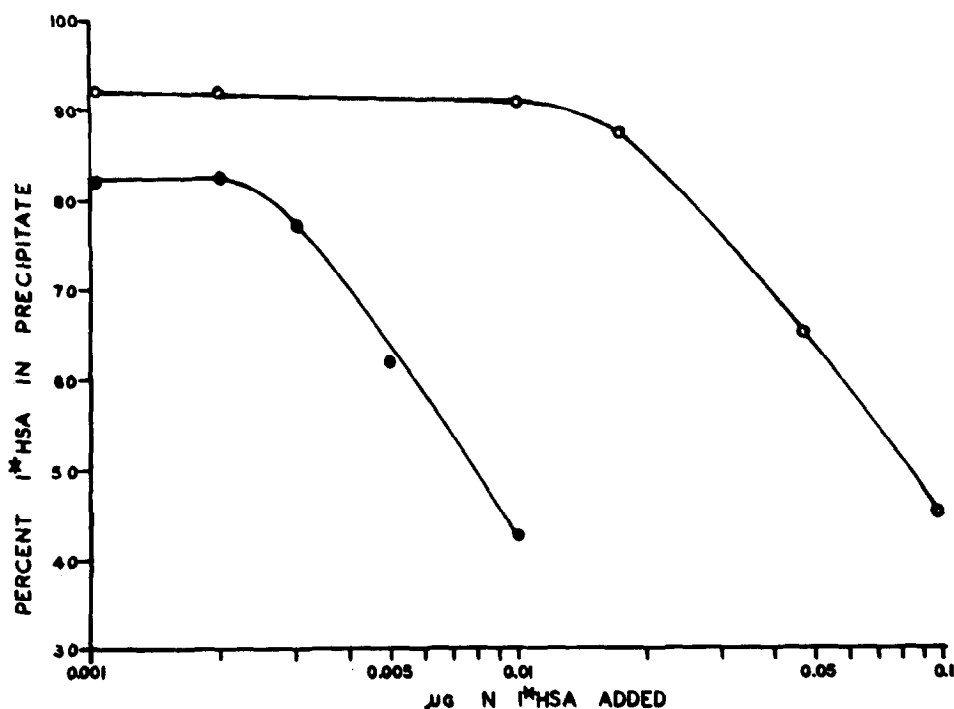


Fig. 1

Binding of I¹³¹ HSA by 0.25 ml. aliquots of human serum. ○—○, Serum from allergic individual. ●—●, Serum from nonallergic individual.

The determination of binding by reagin neutralization

Serum from the allergic individual, either unheated or heated to destroy SSA activity, was capable of maximally combining with 0.01 µg of HSA N per milliliter, as determined by challenge of passively sensitized sites following in vitro incubation of antigen with serum. This value is probably comparable to that determined by in vitro coprecipitation, considering the experimental error of the in vivo tests. The ability of the serum from the nonallergic individual to bind HSA could not be determined by this method since as little as 0.0002 µg of antigen N was in excess of the antibody in 0.5 ml. of serum and thus elicited a wheal-and-flare reaction in the passively sensitized sites.

Localization of antibody reactivity

The localization of the various immunological properties of the fractions of the serum from the allergic individual is shown in Table II. It appears that the SSA activity could be separated from the I* antigen binding and hemagglutinating (TRBC) activities of the whole serum by the methods of fractionation employed. The SSA activity was present mainly in the 33 per cent ammonium sulfate soluble fractions where most of the γ^A could be detected. On the other hand, the binding of I* HSA and the hemagglutinating activity were maximal in the fractions containing γ^G, but these activities were only partially separable by distilled water fractionation.

Table II. *Localization of the immunologic activity to fractions of human allergic sera*

<i>Fraction</i>	<i>Major immuno- globulins present</i>	<i>TRBC</i>	<i>SSA titer</i>	<i>Binding of I*HSA N</i>
Whole serum		1:640	1:1250	0.04 $\mu\text{g/ml}$.
Sp Wp*	γ^G, γ^M	1:320	Undiluted only	0.0005 $\mu\text{g/ml}$.
Sp Ws	γ^G	1:640	1:10	0.02 $\mu\text{g/ml}$.
Ss Wp	γ^A	1:80	1:100	Control serum value
Ss Ws	γ^A	1:20	1:100	Control serum value

*S, 33% saturated ammonium sulfate.

W, distilled water.

p, precipitable.

s, soluble.

DISCUSSION

The major difficulties in the characterization of allergic antibody have been the problems encountered in demonstrating this antibody by *in vitro* techniques, the complex nature of the major allergens, and the lack of sufficient purified antigen for extensive study. The availability of human antibody to heterologous serum proteins provides a means of studying an immunological system using well-defined antigens readily available in purified form. Individuals may become sensitive to these proteins by receiving injections of foreign serum, by inhalation of foreign protein, and, possibly, by ingestion. In addition, as suggested by the presence of antibody with specific binding and hemagglutinating capacity but without skin-sensitizing activity in a nonallergic individual, the response to contact with antigen may not be detectable by *in vivo* methods. This may be similar to the findings of Reisman and co-workers,¹⁶ in which the finding of an elevated preantitoxin hemagglutination titer in individuals without known exposure to horse serum proteins was used to predict the likelihood of the development of serum sickness in these patients following the injection of antitoxin. It is possible that these patients' sera would also bind HSA, as demonstrated by the coprecipitation techniques, to a degree significantly greater than those without elevated hemagglutination titers.

Skin-sensitizing antibody activity has been localized to the γ^A fraction of human serum.¹⁷ However, recent studies have suggested that skin-sensitizing activity may also be found in fractions containing γ^M or γ^G without skin-sensitizing activity in fractions containing γ^A .¹⁸⁻²⁰ Studies performed in this laboratory, in the present experiments as well as those utilizing the model of spontaneous pollen hypersensitivity occurring in dogs,²¹ have demonstrated that skin-sensitizing antibody is present in the 33 per cent ammonium sulfate soluble fraction containing the immunoglobulin migrating electrophoretically as γ^A . In addition, *in vitro* antibody activity has been demonstrated in the canine model to be present in fractions migrating electrophoretically as γ^A but without any skin-sensitizing antibody activities.²²

The immunological activities of the allergic antibody, aside from its skin-sensitizing activity, have not yet been fully clarified. In the present study,

skin-sensitizing antibody did not appear to bind I* antigen, or result in passive hemagglutination of coated tanned sheep cells, since the activities could be partially separated by fractionation. In addition, no difference in binding of heated or unheated serum could be detected by passive transfer techniques. These findings may be compared to those of Arbesman,²³ in which there was no correlation of ragweed reagin and hemagglutinating antibody titers. Furthermore, specific absorption studies have also failed to indicate that hemagglutinating activity present in the sera of ragweed-sensitive individuals is due to skin-sensitizing antibodies.²⁴

Recent studies of fractionating of serum from allergic individuals by gel filtration have also indicated that skin-sensitizing activity localized to the γ^A containing fraction could be separated from the I* antigen binding activity of the serum localized to the γ^G and γ^M fractions.²⁵ These findings are in contrast to those of Reid,²⁰ in which fractions of serum containing skin-sensitizing antibody, associated with γ^G , had binding and hemagglutinating activity. It may be that, in our system, skin-sensitizing antibody is present in such small amounts that any binding activity, if present, cannot be detected by the experimental methods. Further purification and separation of the immunoglobulins, as well as the development of more sensitive methods of detection, may be necessary to clarify these problems, in addition to those of the definition of the specific biologic activities of the allergic antibody.

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