DETECTION OF "NON-PRECIPITATING" ANTIBODIES IN SERA OF INDIVIDUALS ALLERGIC TO RAGWEED POLLEN BY AN IN VITRO METHOD*

By J. GORDON,‡ B. ROSE, M.D., and A. H. SEHON, Ph.D.

(From the Allergy Research Division, McGill University Clinic, Royal Victoria Hospital, and the Department of Chemistry, McGill University, Montreal)

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Specific antibodies to a great variety of soluble and particulate antigens can be demonstrated *in vitro* by the classical immunological techniques, such as precipitation, agglutination, complement fixation and/or lysis. In all these methods, combination of antibodies with their appropriate antigens leads to a readily detectable product of reaction, which can be easily measured. As in all chemical reactions, the sensitivity and the accuracy of any test for antibodies will be enhanced if the amount of the product resulting from antigen-antibody combination can be increased. This is exemplified by methods using antigen-coated erythrocytes or collodion particles. Furthermore, very small concentrations of antigen-antibody complexes which would escape detection by the ordinary immunological methods might be detected by the use of more sensitive methods, such as light scattering. (1) Numerous attempts to demonstrate the presence of skin-sensitizing and blocking antibodies in sera of treated or non-treated[1] allergic individuals by the above methods have met with little success.

In 1925 Coca and Grove (2) reported that no precipitate could be detected on adding ragweed pollen extract to sera from allergic individuals, which contained skin-sensitizing antibodies as demonstrated by the passive transfer (Prausnitz-Küstner)[2] test. This observation has since been confirmed repeatedly.

On immunization of humans with diphtheria toxoid, Kuhns and Pappenheimer (3, 4) found that in some cases skin-sensitizing antibodies were produced in the ab-

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[1] All ragweed-sensitive individuals who had undergone at least one series of injections of ragweed pollen extract are referred to as treated. Sera obtained from such individuals are referred to as treated sera as compared with non-treated sera from non-treated allergic individuals.

[2] Hereafter this will be referred to as P-K test.

sence of precipitins. However, these antibodies could be absorbed out on incubation with toxoid and an appropriate amount of another serum containing precipitating antitoxins. These data may be interpreted as an indication that combination between non-precipitating antibody and antigen may occur *in vitro*.

In terms of the lattice framework theory (5), precipitating or agglutinating antibodies are considered to be bivalent, and by contrast non-precipitating antibodies have been assumed to be univalent or incomplete (6). Theoretically, the addition of non-precipitating antibodies to a system containing precipitating antibodies and a common antigen could result in a decrease or increase of the amount of precipitate. The actual effect may vary from system to system and would depend on the antigen/antibody ratio and on the relative affinities of the different antibodies for the antigen. A decrease in the amount of precipitate was found by Bukantz, Johnson, and Hampton (7) when a serum of an individual allergic to ragweed (pre-incubated with the antigen) was added to a rabbit anti-ragweed serum. Normal human serum by itself did not inhibit the precipitation, but, remarkably, when used along with an allergic serum the extent of the inhibition was greater than with the allergic serum alone. In contrast Hampton, Johnson, Alexander, and Wilson (8) reported an increase in the precipitate for a similar system, when ragweed was pre-incubated with an allergic serum. This increase was obtained for various antigen concentrations. Unfortunately no quantitative data were given. Independently Orlans, Rubinstein, and Marrack (9) used a grass antigen-antibody system with the difference that the allergic serum was decomplemented by an ovalbumin-antiovalbumin precipitating system. Their results were equivocal, as on some occasions an increase, and on others an inhibition of the precipitation occurred. The differences were always within the limits of experimental accuracy. All these results were obtained with sera from both treated and non-treated individuals. Using an ovalbumin-rabbit antiovalbumin system, and sera of egg-sensitive individuals, Miller and Campbell (10) were amongst the first to claim to have obtained a small but consistent increase in the specific precipitate. However, their results were subsequently criticized by Kabat (11) who pointed out that this increase could be accounted for in terms of co-precipitation of the complement from the human serum.

Suggestive evidence that antibodies present in allergic sera combine with the corresponding allergens has been obtained from complement fixation studies. Cavelti (12) showed that complement was frequently bound in the presence of treated sera and that sera of untreated patients were only rarely capable of complement fixation. Furthermore no relation was found between the amount of complement fixed and the P-K titer of the skin-sensitizing antibodies in these sera. Portnoy and Sherman (13) claimed that antibodies in allergic sera could be measured by determining the extent to which these antibodies decreased the amount of complement fixed by a known precipitating system. The amount of complement fixed by a constant quantity of ragweed and rabbit anti-ragweed serum was determined in the presence or absence of an allergic serum. Inhibition was obtained only by sera containing a high level of blocking antibodies and rarely by sera containing only skin-sensitizing antibodies.

A number of workers showed that collodion particles or red blood cells coated with the allergen were clumped when suspended in allergic serum. In 1941, Cohen and

Weller (14) stated in a preliminary report that collodion particles coated with an adsorbed layer of ragweed pollen extract, were clumped when suspended in allergic sera. The clumping was observed regularly with sera of treated allergic individuals, whereas most of the sera of non-treated patients gave negative results. However, in 1947 a systematic reinvestigation of this technique by Swineford and Houlihan (15) failed to confirm the earlier results. These workers were able to obtain clumping of ragweed-coated collodion particles only with rabbit anti-ragweed serum but not with allergic human serum, and concluded that the clumping had been due to non-specific factors. Unfortunately, it is difficult to assess Cohen and Weller's experiments since they used only one normal serum as a control throughout their work.

In an attempt to demonstrate antibodies in allergic sera, Orlans, Rubinstein, and Marrack (9) and more recently Feinberg, Davidson, and Flick (16) used Boyden's hemagglutination technique (17). Grass and ragweed pollen antigens were adsorbed to tannic acid treated red blood cells. The former group obtained positive results in only 22 out of 49 cases and then only in relatively low serum dilutions (from 1:6 to 1:96). The results of the second team are comparable; *i.e.*, only 21 per cent of the untreated and 67 per cent of the treated sera gave positive results. The disconcerting feature of this technique is that the antigen is not firmly bound to the red cells and may be eluted during the test (18). This would obviously lead to a decrease in the amount of clumping or to complete inhibition of the test.

In terms of the lattice framework theory, specific clumping of antigen-coated particles may be attributed to bivalent antibodies, and one would not expect clumping to occur with univalent antibodies. Nevertheless, clumping of antigen-coated red cells which had adsorbed univalent antibodies, could, in principle, be brought about by a modified Coombs test (19). Britton and Coombs (20), in 1955 tried to adapt their method for the detection of antibodies in sera of hay fever patients. For this test they used bovine erythrocytes and a homologous rabbit antiserum which did not agglutinate the cells. Grass antigens were coupled by diazotization to the rabbit anti-bovine red cell serum and the latter complex was then adsorbed onto the oxen red cells. The sensitized cells were subsequently suspended in the allergic serum with the expectation that the antibodies present in allergic serum, would in turn be adsorbed out. The cells were again resuspended and rabbit anti-human gamma globulin was added. Clumping was expected to take place at this stage.[3] These workers (20) were able to demonstrate antibodies to grass in only seven out of eleven sera of non-treated sensitive individuals in serum dilutions not higher than 1:4. On the other hand, antibodies were demonstrated more regularly and in higher titers in sera of treated individuals.[4] In view of the low titers obtained with non-treated sera, the authors suggested without any further evidence that agglutination was not caused by the antibodies responsible for the P-K reaction. This technique has a number of disadvantages: (*a*) it is too involved, (*b*) its sensitivity is low, (*c*) it requires preparation of a number of antisera, and (*d*) it assumes that all antibodies in allergic sera are gamma globulins.

[3] If no clumping took place human gamma globulin followed by rabbit anti-human gamma globulin serum was added, thus lengthening the antigen-antibody chains that could link the sensitized red cells.

[4] These workers reported also that by applying the Boyden technique all non-treated sera gave negative results, whereas positive results were obtained with some treated sera.

More recently Hoigné, Storck, and Grossman (21, 22) have shown in a series of papers that antibodies in allergic sera could be detected by the agglutination of thrombocytes suspended in the allergic sera to which the appropriate allergen was added. This agglutination was shown to be due to a specific dialyzable factor and a non-specific non-dialyzable factor present in normal human, horse, cow, and guinea pig sera. These authors also claimed that combination of the specific factors with a variety of allergens could be demonstrated by a light-scattering technique (23). Equal volumes of the allergen solution were added successively to a given volume of serum, and a dilution curve was plotted. If the serum contained a specific antibody, a break in the curve was obtained which was absent with normal serum. This break occurred at an "optimal" antigen concentration and was interpreted to be due to the formation of antibody-antigen aggregates. However, attempts to reproduce these results by a number of other workers (24) have so far met with failure and the original conclusions of the authors ought to be regarded with some doubt.

Another avenue of approach to detect antibodies in allergic sera has been explored by Noah and Brand (25), who compared the amounts of histamine liberated *in vitro* from allergic and normal bloods in presence of ragweed extract. The histamine liberated in bloods of allergic individuals was significantly higher than that in normal bloods indicating thus that a specific interaction took place between the ragweed and the antibodies present in allergic serum. Recently, Black (26) reported that reactions between allergens and the corresponding antibodies in presence of normal polymorphonuclear leukocytes and platelets result in the destruction of these cells. Although Black claimed that this technique could be used diagnostically, his results could not be confirmed (27).

From this survey, it would appear that combination between some antibody-like factors in allergic sera and their corresponding antigens might take place *in vitro*. The present study was undertaken with a view to developing a simple, specific, quantitative and sensitive *in vitro* method for the demonstration of the combination between these antibodies and their allergens, and with a view to elucidating the nature of these "non-precipitating" antibodies. On the assumption that these antibodies were divalent and that their failure to give a visible "precipitin" reaction was due only to their being present in an exceedingly low concentration, it was felt that the method of Pressman, Campbell, and Pauling (28)—as recently modified by Stavitsky and Arquilla (29) for the demonstration of antibodies to insulin—could be profitably adapted for our purpose.[5]

EXPERIMENTAL

The essential steps of the method are: (*a*) coupling of the antigen to rabbit red cells through chemically stable covalent azo bonds, and (*b*) clumping of these "sensitized" red cells by a homologous antibody.

[5] We also tried the method of Cole and Farrell (30) with precipitating and non-precipitating systems but all our attempts met with failure.

Sensitization of red cells.—*Bis*-diazotized benzidine (B.D.B.)[6] was used to couple the antigen to red cells. It was prepared by dissolving 0.23 gm. of benzidine in 45 ml. of 0.2 N HCl, and adding 0.175 gm. of $NaNO_2$ in 5 ml. of distilled water to this solution at 0°C. The reaction was allowed to proceed for 30 minutes with intermittent stirring. Aliquots of the solution were then placed into 2 ml. vials, quick frozen at −78°C. in a dry ice–acetone bath, and stored at −20°C. until required. Some of the batches were used over periods as long as 5 months without detectable deterioration. For each experiment the content of a vial immediately after thawing, was diluted fifteenfold with 0.15 M phosphate buffer[7] at pH 7.3 and was used for coupling the antigen to the red cells.

Blood was collected from the marginal ear vein of a rabbit into an equal volume of Alsever's solution (31) and stored at 4°C. for as long as 10 days. The red blood cells when needed were separated by centrifugation and were washed 3 times with chilled saline.

Normal rabbit serum (NRS)[8] was collected from the same rabbit which supplied the erythrocytes. The serum was heated at 56°C. for 30 minutes to deactivate the complement and was stored at 4°C. for 7 to 10 days. The "diluent solution" consisted of NRS diluted hundredfold with phosphate buffer.

The antigens used were bovine serum albumin (BSA),[9] human serum albumin (HSA),[10] human γ-globulins (HGG),[11] and a water-soluble extract of ragweed pollen (WSR).[12] The sera tested were: rabbit anti-BSA, anti-HSA, anti-HGG, anti-WSR obtained from one rabbit (referred to as anti-WSR-1), and an anti-WSR serum pooled from 5 rabbits (referred to as anti-WSR-5), 22 sera from normal individuals, 16 sera from untreated and 30 from treated ragweed-sensitive individuals, one serum of an individual allergic to both ragweed and grass pollen, three sera from individuals allergic to aspirin and grasses and one serum from a normal volunteer who was immunized by subcutaneous injections of 2,500,000 N units of WSR in Coca's solution.[13]

For sensitization of red cells[14] the ratio of benzidine to antigen was found to be critical. This ratio was established for each batch of B.D.B. (*a*) by varying the B.D.B. concentration and keeping the antigen concentration constant, and (*b*) by varying the antigen concentration and keeping the B.D.B. concentration constant. Each batch of sensitized cells was tested with a ragweed-sensitive serum and a normal human serum. The concentrations of benzidine and ragweed adopted were the ones which gave the highest sensitivity; *i.e.*, the highest titer with the allergic serum and no reaction with the normal human serum.

In the present experiments 3 ml. of 75 mg. per cent WSR solution were mixed with 0.1 ml. of a 50 per cent suspension of washed red blood cells, and 0.5 ml. of the B.D.B.-phosphate was added to this suspension.[15] The reaction mixture was kept at room temperature for 15 minutes with occasional stirring. It was then centrifuged at 500 *G* at 4°C. for 5 minutes, the

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[6] B.D.B., *bis*-diazotized benzidine.

[7] The buffer used in all experiments was prepared by mixing 215 ml. of 0.15 M Na_2HPO_4 with 49 ml. of 0.15 M KH_2PO_4.

[8] NRS, normal rabbit serum.

[9] BSA, bovine serum albumin.

[10] HSA, human serum albumin.

[11] HGG, human γ-globulin.

[12] WSR, water-soluble extract of ragweed pollen. The preparation of WSR is described elsewhere (32). This extract contains multiple antigens as shown in another study (36).

[13] This individual received 12 injections over a period of 6 weeks (2 injections per week).

[14] The cells have to be sensitized for each experiment.

[15] The B.D.B.-phosphate solution was prepared by diluting B.D.B. fifteenfold with the phosphate buffer.

supernatant was discarded, and the cells were washed with 3.5 ml. of the diluent solution (the wash was always colorless). The cells were then redispersed in 2.5 ml. of diluent.

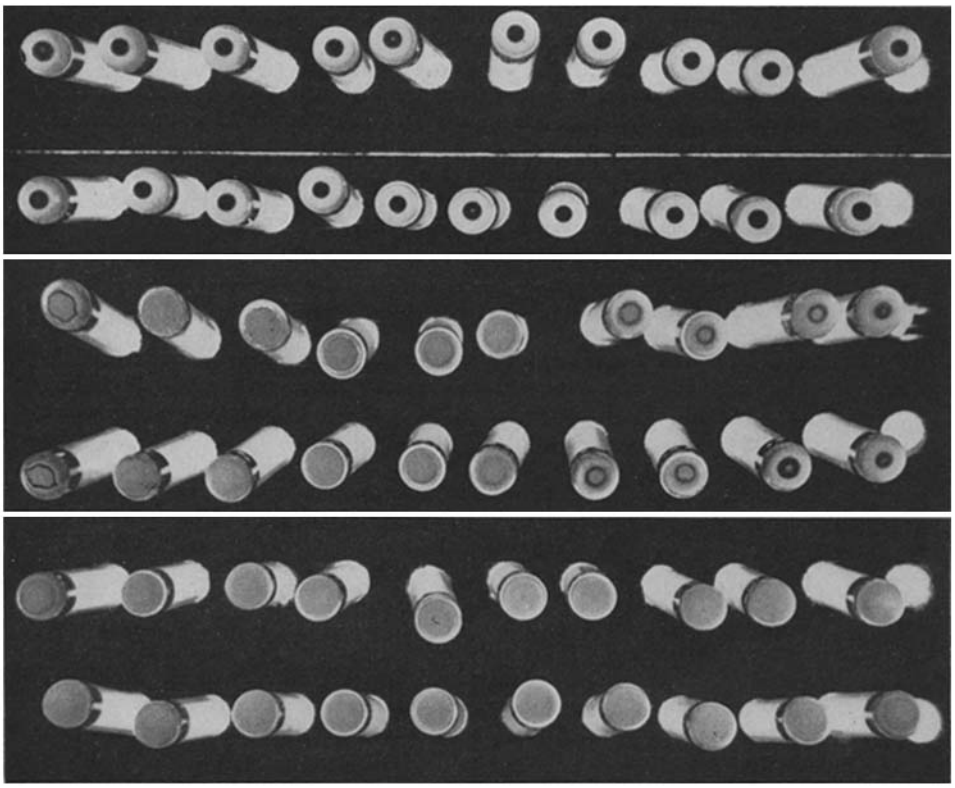

FIG. 1. Hemagglutination patterns. Each row represents tubes containing serum in halving dilutions, the concentration of serum decreasing from left to right. The top two rows represent a control experiment in duplicate with normal human serum: no hemagglutination is observed in any tube. The center two rows represent an experiment in duplicate with allergic serum: the patterns represent a gradual decrease in the extent of hemagglutination. The bottom two rows (in duplicate) represent hemagglutination patterns obtained with rabbit anti-ragweed serum. The patterns in all 10 tubes are positive; the actual titer of this serum was 10.[7]

The "Hemagglutination" Test.[16]—In order to remove non-specific agglutinins, all sera before testing for antibodies were absorbed out at room temperature for 20 minutes with an equal volume of packed, washed, non-sensitized cells. The cells used for the absorption and those for the sensitization were always from the same rabbit. Subsequently, twofold serial dilutions

[16] In the present paper the term hemagglutination refers to the cross-linking of the sensitized red blood cells by the appropriate antibodies; the specific patterns obtained have a gelatinous appearance.

of the serum with the diluent were prepared. The volume in each tube was 0.5 ml.[17] For inhibition studies, the antigen was added at this stage in a volume of 0.1 ml. (the concentration was arbitrarily chosen as 3 mg./ml.). Finally, 0.05 ml. of the "sensitized" cell suspension were dispersed in each tube. For each experiment two controls were used: (*a*) in one series of tubes the serum was replaced by the diluent, (*b*) in another series untreated cells were used instead of sensitized cells. Most experiments were done in duplicate.

The tubes were allowed to stand overnight at room temperature to permit the development of specific patterns. These, once formed, remained unchanged for days. If no antibodies were present, the red cells sedimented to the bottom of the tube to form a compact button; this is a negative pattern (see top two rows, Fig. 1). On the other hand, if antibodies were present, a

TABLE I

Hemagglutination and Inhibition Experiments

Antigen coupled to red cells	Inhibitor	Antiserum*	Titer (reciprocal of dilution)
WSR	None	Anti-WSR No. 1‡	10,000,000
"	"	" " 5	250,000
"	WSR	" " 5	8
"	None	Anti-BSA	No reaction
BSA	"	"	20,000
"	BSA	"	No reaction
"	WSR	"	20,000
HSA	None	Anti-HSA	40,000
HGG	"	Anti-HGG	320,000
"	HGG	"	No reaction
WSR	None	B.P.*	1,024
"	WSR	"	No reaction
"	Grass pollen	"	1,024
"	Wormwood pollen	"	1,024

* All antisera were produced in rabbits. The serum designated B.P. was obtained from a ragweed-sensitive individual.

‡ Repeated intravenous injections of alum-precipitated WSR over a period of 2 to 4 months were used for immunization. The other antigens, dissolved in saline, were injected intravenously.

gelatinous layer spreading across the bottom of the tube was obtained; this corresponds to a positive pattern. The more concentrated the antibody solution the larger is the area over which this layer spreads out (see middle two rows, Fig. 1). The reciprocal of the highest dilution of the test serum which still gave a positive pattern was taken as the titer of the serum. For example, in Fig. 1 the pattern in the 9th tube in each of the center rows is still positive, while that in the 10th tube is negative; therefore, the titer of this serum is considered to be $2^9 = 512$.

RESULTS

The hemagglutination method was evaluated with antisera which contained precipitating antibodies to WSR, BSA, HSA, and HGG. Positive ring tests

[17] The tubes used for this test were 10 × 75 mm. They were suspended through a 1 × 10 × 1/8 inch sheet of masonite provided with 10 mm. holes, and the hemagglutination patterns were observed with the help of a mirror.

TABLE II

*Hemagglutination and Prausnitz-Küstner Tests**

No. of serum	Donor	Hemagglutination titer‡	P – K titer‡
1–22	Normal	No reaction	No reaction
	Sera from individuals sensitive to other allergens		
23	Sensitive to aspirin	No reaction	No reaction
24	" " grass	" "	" "
25	" " "	" "	" "
	Sera from ragweed-sensitive individuals‡		
26	Non-treated	64	10
27	"	128	100
28	"	64	500
29	"	128	100
30	"	256	100
31	"	1024	1000
32	"	16	Positive§
33	"	32	" §
34	"	256	Not tested
35	"	64	(*)
36	"	64	100
37a	"	4	(*)
37b	Treated	128	10
38a	Non-treated	32	10
38b	Treated	128	(*)
39*a*	Non-treated	512	250
39*b*	Treated	1024	250
40*a*	Non-treated	128	Not tested
40*b*	Treated	512	" "
41*a*	Non-treated	128	250
41*b*	Treated	2048	500
42	"	256	1000
43	"	512	1000
44	"	32	10
45	"	128	1000
46	"	64	100
47	"	128	500
48	"	256	100
49	"	256	100
50	"	512	100
51	"	32	(*)
52	"	16	(*)
53	"	8	(*)
54	"	128	500
55	"	16	100

* WSR was used for all these experiments.

‡ Expressed as reciprocal of the highest dilution still giving a positive reaction. In most cases the samples were evaluated in terms of twofold serial dilutions of the whole serum for hemagglutination, and tenfold serial dilutions for P-K test.

§ These sera were not diluted for P-K tests.

(*), the P-K test was negative for a tenfold dilution of the serum. The undiluted serum was not tested.

Sera obtained from the same individual before treatment are referred to by letter (*a*), and after treatment by letter (*b*).

TABLE II—*Concluded*

No. of serum	Donor	Hemagglutination titer‡	P – K titer‡
Sera from ragweed-sensitive individuals‡—*concluded*			
56	"	64	100
57	"	32	100
58	"	32	100
59	"	1024	1000
60	"	2048	1000
61	"	1024	Positive§
62	"	512	" §
63	"	128	Not tested
64	"	1024	" "
65	"	1024	" "
66	"	512	500

were obtained with dilutions only as high as 1:64, whereas the hemagglutination titers for the same sera were 10^4 to 10^7. (Table I)

The immunological specificity of the hemagglutination reaction was demonstrated by the fact that positive patterns were obtained only with the antisera containing antibodies to the antigens coupled to red blood cells. Furthermore, the reaction could be inhibited by addition of soluble antigen. Thus, addition of a solution of ragweed antigens to a system containing BSA-sensitized red cells and anti-BSA serum had no effect on the extent of hemagglutination, whereas addition of a solution of BSA completely inhibited the test. Conversely, addition of a solution of WSR to systems containing WSR-sensitized red cells and rabbit anti-WSR serum or serum from ragweed-sensitive patients resulted in complete inhibition of the test. However, addition of BSA or pollen extracts of other plants, such as grass and wormwood did not have any effect on the patterns obtained with the ragweed systems. All these results are listed in Table I.

For inhibition tests the antigen solution could be added at the beginning of the experiment, or after the positive patterns were obtained. In the latter case the gelatinous patterns were dislocated by gentle tapping; on re-incubation[18] negative patterns were obtained. On the other hand, dislocation of patterns without addition of the appropriate soluble antigen did not interfere with the reproduction of the positive patterns.

The results obtained with all the human sera mentioned in the experimental section are recorded in Table II. As can be seen, none of the normal sera used as controls, nor the sera of grass- and aspirin-sensitive patients gave any positive results with ragweed-sensitized red cells. In contrast, all the

[18] In these tests the original supernatant was removed before WSR in saline, or saline alone was added.

other sera from individuals allergic to ragweed gave positive results, the titer varying from 200 to 2000. On the whole the titers obtained with sera of treated individuals did not appear to be significantly higher than those obtained with untreated sera; however, the highest titers were predominantly due to treated sera. The hemagglutination titers are compared with the skin-sensitizing titers for a number of allergic sera in Table II. Generally the two titers are closely

TABLE III

Titers for Hemagglutination and Skin Tests after Exposure of Sera to Erythrocytes Sensitized with WSR

Donor	Absorbed with cells	Hemagglutination titer*	Blocking titer*	P-K titer to WSR
Treated: sensitive to WSR	Non-sensitized	1024	Not tested	1000
	Sensitized	No reaction	" "	No reaction
Non-treated: sensitive to WSR	Non-sensitized	1024	No reaction	1000
	Sensitized	10	Not tested	10
	Sensitized (2nd absorption)	No reaction	" "	No reaction
Treated: sensitive to WSR	Non-sensitized	2048	32	1000
	Sensitized	10‡	No reaction	10
Non-allergic volunteer immunized with ragweed	Non-sensitized	8192	64	No reaction
	Sensitized	No reaction	No reaction	" "
Sensitive to WSR and to grass	Non-sensitized	512	Not tested	500§
	Sensitized	No reaction	" "	No reaction§

* Expressed as reciprocal of the highest dilution still giving a positive reaction.

‡ This serum was not absorbed out with a sufficient amount of sensitized erythrocytes.

§ An identical P-K titer of 500 to grass extract was obtained with this serum before and after exposure to WSR-sensitized erythrocytes.

related; the higher the skin-sensitizing ability of the serum, the higher is also the hemagglutinating titer.

The hemagglutination titer was found to be identical for any duplicate experiments done with the same serum and the same batch of sensitized red blood cells, as can be seen in Fig. 1. On the other hand, the titers obtained for one and the same serum with different batches of sensitized erythrocytes differed slightly and were reproducible within ± 2 tubes. Since in all these experiments twofold serial dilutions of the antiserum was used the titers are considered to be accurate within a factor of 16.

If the hemagglutination represents a true antibody-antigen reaction, it would be reasonable to expect that an antiserum could be depleted of its spe-

cific antibodies by adsorption of the latter on antigen-coated red cells. For this purpose allergic sera were absorbed out by an equal volume of packed sensitized red cells. The supernatants were tested by passive transfer for skin-sensitizing and/or blocking antibodies,[19] as well as for further hemagglutination. It was shown that all three titers, *i.e.* skin-sensitizing, blocking, and hemagglutinating titers, were reduced on incubation of the sera with sensitized red cells. Complete depletion of antibodies could be achieved by absorption of the sera with a sufficiently large quantity of sensitized red cells. In this case no antibody in the supernatant could be demonstrated either by skin or hemagglutination tests (see Table III). In no instance was activity found by one of the skin tests and not by the *in vitro* method. To ascertain that absorption of antibodies was an immunologically specific reaction, a serum[20] from an individual allergic to both ragweed pollen and grass was absorbed out with ragweed-sensitized red cells. The supernatant was found to contain no antibody to ragweed by both P-K and hemagglutination tests. On the other hand, the skin-sensitizing ability of the serum with respect to grass pollen had not been impaired.[21]

DISCUSSION

The hemagglutination reaction as described here is simple, specific, and highly sensitive. It is a one step reaction, in contrast to the complex multistage test of Coombs (19, 20). The antigen is firmly bound to the red cells by stable covalent azo bonds and cannot be eluted.[22] This is a distinct advantage over the Boyden technique in which the antigen is linked to tannic acid–treated red cells by weak adsorption forces and may be easily washed off as exemplified by the experiments of Feinberg and Flick (18).

The specificity of the method is borne out by the inhibition experiments, and also by the fact that positive results were obtained with all sera of treated and non-treated ragweed-sensitive individuals when ragweed antigens were used for sensitization of red cells. In comparison with results obtained by other methods (see introduction), this method seems to be the only one which detects antibodies in all allergic sera that give rise to P-K tests. The specificity of the method is also demonstrated by the absorption experiments. Red cells sensitized with WSR had the ability to deplete allergic sera of their skin-sensitizing and blocking antibodies as well as of their hemagglutination

[19] The test for blocking antibodies was performed according to the procedure described earlier (33).

[20] This serum contained skin-sensitizing antibodies to both ragweed pollen and grass antigens.

[21] The additional experiment with grass pollen–sensitized red blood cells was not done since we did not have a sufficient quantity of grass pollen to couple to red blood cells.

[22] If *bis*-diazotized benzidine is omitted during the coupling of the antigen no hemagglutination reaction is obtained.

capacity. On the other hand using red cells sensitized with WSR and a serum containing skin-sensitizing antibodies to ragweed and grass pollen, the antibodies to ragweed only were removed without affecting the antibodies to grass.

The removal of skin-sensitizing and blocking antibodies by ragweed-coated red cells is interpreted as being due to a firm combination between these antibodies and the ragweed pollen constituents attached to the red cells. Similar results have been obtained in this laboratory[23] by absorption of sera containing skin-sensitizing and/or blocking antibodies with an insoluble ragweed-polystyrene conjugate. On the other hand, these antibodies were not removed by heterologous antigen-polystyrene conjugates.

The great sensitivity of the method is demonstrated by the high titers of the order of 10^6 to 10^7 obtained with the precipitating antisera to WSR. In contrast, the same sera when tested by the ordinary ring test and by the Oudin method (34) gave titers not higher than 64 and 128 respectively.[24] With the hemagglutination technique, antibodies to ragweed can be detected in allergic sera in dilutions only as high as 10^3 which means that already in the undiluted allergic sera the concentration of antibody is extremely small and is less than the minimum concentration that can be detected by the precipitin methods. Therefore, it is not surprising that antibodies in allergic sera have not been detected by precipitin techniques, and it is felt that there is no reason to postulate that these antibodies are "incomplete" or "univalent" (6). We believe that they have evaded detection *in vitro* only on account of their very low concentrations. Although the absolute amount of material present in allergic sera which is responsible for skin-sensitizing and blocking activity has not been determined, there is indirect evidence that it is extremely small. Thus, in a previous study (35) from this laboratory dealing with the localization of skin-sensitizing antibodies in allergic sera by zone electrophoresis, maximum skin-sensitizing activity was found to be associated with some fractions containing hardly any serum proteins.

The exact nature of the hemagglutinating factor present in allergic sera still remains to be elucidated. In terms of our results we conclude that it behaves like a proper divalent antibody, in that it is capable of cross-linking antigen-coated erythrocytes into a three dimensional gel and its agglutinating capacity may be blocked by free, soluble antigen. At the present one cannot categorically state whether hemagglutination is brought about by the skin-sensitizing antibodies and/or by blocking antibodies, or whether it is due to a third antibody-like factor which has no distinct properties demonstrable by skin tests, but which leads to aggregation of the sensitized red cells. On the

[23] The procedure used in these experiments has been described briefly elsewhere (37).

[24] Considering that ragweed is a poor antigen, it is remarkable that the highest titers were obtained with anti-WSR sera (see Table I).

basis of these experiments it may be suggested that the hemagglutination reaction has the characteristics of a precipitin test, except that here the bulk of the "precipitate" is made up of the large antigen-coated erythrocytes. The fact that the titers of the skin-sensitizing and blocking antibodies run somewhat parallel to the hemagglutination titers, and that these antibodies may be completely absorbed out by sensitized red cells could be taken as circumstantial evidence that these antibodies may be identical with the factor(s) responsible for hemagglutination. Further experiments are being designed to provide a more unequivocal answer to this problem and to elucidate the mechanism of the hemagglutination reaction.

SUMMARY

A hemagglutination test capable of demonstrating antibodies in sera of ragweed-sensitive individuals has been described in detail. This test involves coupling of the antigen to rabbit erythrocytes *via* stable azo bonds. The antigen-coated cells are then suspended in the serum which is suspected to contain the homologous antibodies. The test has been shown to be specific and highly sensitive. Precipitating antibodies to ragweed pollen extract produced in experimental animals can be demonstrated in dilutions as high as 10^5 to 10^7, while antibodies to ragweed in allergic sera are detected only in dilutions of the order of 10^3. Positive results were obtained with all sera from treated or non-treated allergic individuals containing skin-sensitizing and/or blocking antibodies. Absorption of these sera with antigen-coated erythrocytes yielded a supernatant devoid of either skin-sensitizing, blocking, or hemagglutinating capacity. It is concluded that the hemagglutinating factor has the properties of a divalent antibody.

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