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DETECTION OF ANTIBODIES IN THE SERUM OF PATIENTS SUFFERING FROM HAY FEVER

By

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Passive sensitisation of the skin (Prausnitz-Kustner reaction) and the protective effect of "blocking" antibodies (Cooke et al., 1935, 1937) have been the usual methods of demonstrating the presence of specific antibodies in the serum of hay fever patients. These methods involve the injection of serum into the skin of a subject, with the risk of infection. At the suggestion of Dr. R. R. A. Coombs we have tried the method of Boyden (1951), who found that sheep red cells, treated with tannic acid, adsorb proteins on their surfaces from solution; suspensions of these red cells which have adsorbed proteins are agglutinated by the homologous antisera in high dilutions. This method has been used by Hoet et al. (1952) in a serological study of pollen extracts and promised to be a good method of demonstrating the presence of small amounts of antibody.

METHODS

Red Blood Cells. In the earlier experiments sheep red blood cells were used; fresh batches were obtained each week from the Wellcome Research Laboratories. Later human group O cells were used, in order to obviate the need for

preliminary removal from human serum of antibodies that might agglutinate the untreated red cells. Fresh cells were obtained by bleeding a donor on the day on which the cells were used. During a period of transition cells of both species were used; the sheep cells were agglutinated slightly more strongly than the human cells.

Antigen solution was supplied by the Wright-Fleming Institute of Microbiology, St. Mary's Hospital. It was made by extracting Timothy and Cocksfoot grass pollen with Coca's buffer solution. It contained 100,000 Noon units per ml.

Tannic Acid. Fresh solutions of 1 in 300 tannic acid in distilled water were made each week; the solution was kept in the cold. Fresh solutions of 1/30,000 tannic acid were made up for use each day by diluting the 1/300 solution with saline.

Rabbit Serum Albumin. Normal rabbit serum was dialysed against 22 % Na_2SO_4 . The precipitated globulin was filtered off and acetic acid added to the filtrate to bring the pH to approximately 4.6. The precipitated albumin was filtered off, dissolved in water, neutralized and dialysed against saline. The final concentration was approximately 4 %.

Treatment of Red Blood Cells with Tannic Acid. The cells were washed 3 times with saline. A 2 % suspension, in a mixture of 2 parts of 0.9 % NaCl solution with 1 part of M/15 phosphate buffer (pH 7.2), was mixed with an equal volume of a solution of 1 part of tannic acid in 0.9 % NaCl solution. This mixture was kept for 10 minutes at 37°. It was then centrifuged gently, so that the cells did not form clumps. The cells were then mixed with saline-buffer mixture; the mixture was re-centrifuged; the washing was repeated twice and the cells were then suspended in 0.9 % NaCl to make a 2 % suspension.

Adsorption of Pollen Extract. One volume of the pollen extract was diluted with 19 volumes of a mixture of 60 parts of 0.9 % NaCl with 1.8 part of M/15 phosphate buffer, pH 6.4. Equal parts of diluted pollen extract and 2 % cell suspension were kept at room temperature for 30 minutes. The mixture was centrifuged gently and the cells were washed

twice with the 1/50 solution of rabbit serum albumin in buffered saline. They were then suspended in the rabbit serum albumin solution to make a 1 % suspension.

Tests with Sera. 0.4 ml. of progressive dilutions of serum to be tested were mixed with 0.2 ml. of the cell suspension. The mixtures were kept at room temperature for 4 hours and at 4° overnight and the degree of agglutination in the tubes was then read. Little difference was found between the degrees of agglutination after 4 hours at room temperature and after 24 hours at 4°.

The degree of agglutination was assessed by the pattern, which ranged from a crenated deposit of tight clumps, formed when the sera agglutinated strongly, to the uniform small disc which settled from sera that did not agglutinate. The type of agglutination varied with different sera, even when they were investigated with the same red cell suspension at the same time under the same conditions. Thus, serum Sh (Table I) agglutinated strongly, but only in low dilution (1/10), whereas serum P did not agglutinate strongly in any dilution but agglutinated weakly down to a dilution of 1/640. The titre was not, therefore, the sole index of the strength of the serum. As an alternative method of reporting, a system of marking was adopted; sera that agglutinated in high dilutions and sera that agglutinated strongly in intermediate dilutions were marked 3 or 4; sera that agglutinated weakly in lower dilutions or strongly only in the lowest dilutions were given markings 1 or 2. Examples are given in Table I.

Sera Tested. Most of the hay fever sera were supplied by Dr. Frankland from the Wright-Fleming Institute of Microbiology. A few were obtained from students and acquaintances. They were classed into four groups: (1) *untreated* who had had no treatment with injections of pollen extract, (2) *incompletely treated* who had been treated with less than 10,000 units, (3) *treated* who had been treated with more than 10,000 units, (4) *previously treated*—3 subjects who had received incomplete treatment about a year before the blood was taken.

TABLE I
*Examples of the Agglutination of Treated Red Blood Cells by Various Sera. The First 9
and Second 5 Test were Performed on the Same Day.*

	Dilution of Serum								Marking
	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	
1 Rabbit T	+	+	+	++++	++++	++++	++	++	
2 " B.G.IV	o	o	++	++	++++	++++	++	++	
3 " B.G.V	(+)	(+)	+	(++)	(++)	++	++	(++)	
4 " Cambridge	o	o		+	(++++)	++++	++	(++)	
5 Subject P	+	(++)	++	++	++	++	(++)	+	3
6 " V	±	±	(+)	(+)	+	(+)	±	±	2
7 " Sh	++++	(++++)	o		o	o	o	(++)	2
8 " M	++++	++++	(++)	++++	++++	++++	(++)	(++)	4
9 " O	++++	+	++	(++++)	++++	++++	(++)	+	4
1 " S	+	+	+	(+)	(+)	±	o	±	2
2 " B	++++	++++	++++	++++	++++	(++++)	(++++)	+	4
3 " C	(++)	(+)	(++)	++	(++)	+	±	+	3
4 " J	o		++++	++	++	++		+	4
5 " L	o	++	++++	++	+	++	(++)	(++)	4+

Attempts were made to get precipitating rabbit sera against pollen extract by immunizing rabbits by various methods. Eventually antisera that contained precipitating antibody, in amounts sufficient to allow the use of quantitative methods, were obtained after intraperitoneal injections of pollen extract mixed with freshly precipitated $\text{Al}(\text{OH})_3$. When sheep red cells were used in the agglutination tests both human and rabbit sera were absorbed with untreated sheep cells before use; when human group O cells were used only the rabbit antisera were absorbed with these cells. Serum of a rabbit (Cambridge) that had been immunized by another pollen extract was supplied by Dr. Coombs.

Controls. In each case the following controls were included: (1) The sera tested were tested with untreated red blood cells; (2) 2 sera of subjects who were not liable to hay fever were tested with treated red blood cells; (3) rabbit antisera and the serum of a hay fever patient previously found to agglutinate were tested with treated red blood cells in order to check whether they were agglutinated to the degree expected by the antibodies in these sera; (4) treated red blood cells were tested with the diluting fluid alone, as slight degrees of agglutination might appear in this control tube and in the highest dilutions of otherwise negative sera.

Precipitation Method. Sera were examined by the customary methods for detecting incomplete antibodies—demonstration of an increase or reduction of the amount of protein precipitated from a rabbit antiserum with antigen in the presence of the suspected serum; such a reduction was found by Bukantz et al. (1949).

To remove any residual complement from a serum to be tested by this method, 1 ml. of rabbit antiovalbumin serum, containing about 2.5 mg. of antibody per ml., was mixed with 4 ml. of the serum. 1 ml. of a solution containing 0.2 mg. of ovalbumin was then added (Heidelberger et al., 1946); the fluids were mixed and after flocculation was complete the tube was centrifuged and the supernatant fluid used for the test.

Equal volumes of pollen extract were put in 2 centrifuge tubes; a measured volume of the complement-free human serum to be tested was put in tube A and an equal volume of saline in tube B. Both tubes were incubated overnight; equal volumes of the precipitating rabbit antiserum were added to the two tubes. The mixtures were kept at 4°C. for 2 or 3 days and centrifuged. The precipitates were washed twice with ice-cold saline and measured by the method of Heidelberger and Macpherson (1943) and Heidelberger and Anderson (1944). The amounts of precipitate formed were compared.

RESULTS

None of the sera of 20 subjects who had not had hay fever agglutinated in dilution of 1/6. Fourteen of the sera of 22 subjects liable to hay fever (Table II), who had not been treated, did not agglutinate in this dilution. Of the remainder two agglutinated in relatively high dilutions. Three subjects had had incomplete treatment about 1 year before the blood was taken; the titre of one serum was 1/96 (marking 3+); the other two agglutinated moderately strongly in dilution 1/24 (marking 2+) and were not tested beyond this dilution. The subject whose serum titre was 1/96 had had a general reaction after the last injection. Of the nine subjects who had been treated with less than 10,000 units the only one with a high serum titre had also had a general reaction.

Higher titres were found in the sera of the subjects that had been treated adequately. Fifteen of them were titrated to the end point (Table II); in 11 of these the titres were 1/80 or more; the markings were 2 in 2 sera, 3 in 6 sera and 4 in 7 sera (Table III). Of the 9 sera that were not titrated beyond a dilution of 1/24, 6 agglutinated in this or in a lower dilution and the markings were 2 in 2 sera, 3 in 3 sera and 4 in 1 serum; 3 did not agglutinate in dilutions down to 1/24.

The rabbit sera and some of the human sera with the

TABLE II

Titres of Sera of Hay Fever Subjects Which were Tested up to High Dilutions.

	Number of Sera	Dilution of Serum					
		1/5-1/6	1/10-1/12	1/24	1/48	1/80-1/96	over 100
Untreated	22	2	2	2	0	2	0
Partially treated	9	5	0	1	0	0	1
Fully treated ...	15	0	1	1	2	1	10

highest titres agglutinated weakly or not at all in low dilutions (Table I). It is possible that the sera that did not agglutinate or agglutinated weakly in dilutions down to 1/24 were actually strong sera with a prozone.

The strength of the agglutinin had little or no relation to the effects of treatment. Thus subject M, in whose serum the titre was 1/320 with marking 4+, was little improved by the treatment; whereas the serum of subject Sh, who had had severe hay fever before treatment and had only very slight hay fever after, had a titre of 1/10 and marking 2+. Case O also (serum titre 1/320, marking 4+) was little improved by treatment. Nor did the strength of the antibody depend on the number of previous years of treatment.

The results of attempts to demonstrate antibodies by the method of Bukantz (1949) were equivocal. In one experiment the complement was not removed from the human serum used. The amount of precipitate formed by a constant volume of pollen extract with constant volume of antiserum in the presence of variable volumes of unheated human serum increased with the amount of human serum added. The maximum increase was approximately equivalent to the amount attributable to complement (Heidelberger and Mayer, 1942). In 3 experiments hay fever sera, from which complement had been removed by absorption on ovalbumin-antiovalbumin precipitate, were incubated with pollen extract before this was added to the rabbit antiserum. In one experiment the amount of precipitate formed in the presence of agglutinating hay fever serum was slightly more than that found in its absence and in two experiments it was slightly less. In one of the

TABLE III
Markings of Sera of Hay Fever Patients.

	Number of Sera	Marking				
		0	1	2	3	4
Untreated	22	13	4	2	1	0
Treated in previous years ...	3	0	0	2	1	0
Partially treated	9	3	4	1		1
Fully treated	24	3 ²	0	4	9	8

latter, a slight precipitate formed during the incubation of the hay fever serum with pollen extract. This precipitate was supposed to be ovalbumin-antiovalbumin complex which had not been fully precipitated and it was removed; it is possible, however, that it was a pollen-antipollen precipitate. The differences between the amounts of precipitate formed in the presence and in the absence of the hay fever serum were little above the errors of the method. In no case did we find a great reduction of the amount of precipitate similar to that described by Bukantz.

DISCUSSION

Pollen extracts contain a variety of substances and such work as has been done on the isolation of the active allergen shows that it forms only a small fraction of the total extract. Other substances in the extract that are not allergens may give rise to antibodies.

The differences between the types of agglutination by different sera, which has already been noted, suggest that reactions between different antigens and the corresponding antibodies are involved; two such antigen-antibody reactions may account for the double zones found with sera M, O and L (Table I). Also, the shape of the precipitation curve obtained with rabbit antiserum (Fig. 1) suggests that at least two antigens are involved in the precipitating system. There is no reason to suppose that the antibodies detected in the serum of subjects who had had a course of treatment was antibody against the actual allergen. The absence of any re-

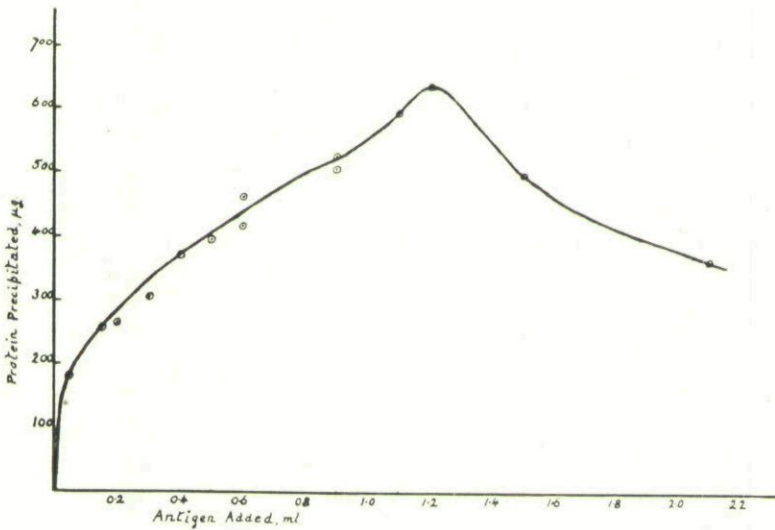


Fig. 1.

Precipitation curve of rabbit anti-pollen serum T, 0.3 ml.

lation between the strength of the antibody and the clinical results of treatment suggests that the antibody detected has no relation to the allergen.

This criticism applies to all *in vitro* methods of detecting antibodies in the sera of allergic subjects before or after treatment unless the antigen used is the actual allergen and is pure. The antibody detected by Bukantz et al. (1949) by a precipitation method may be unrelated to the actual ragweed allergen. Even if antibodies against the allergen are found it would be uncertain whether they were actually either reagin or "blocking" antibody. The only satisfactory criteria seem still to be "*in vivo*" tests. The fact that antibodies were found in the sera of untreated hay fever patients, with a moderately high titre in one case, whereas none were found in the sera of controls, suggests that these subjects may be specially liable to form antibodies to inhaled pollen.

Borduas and Grabar (1952) found that antibody in a concentration as low as 0.006 μg . per ml. could be detected by this agglutination reaction. A serum that agglutinates in a

dilution of 1/640 may, therefore, contain no more than 4 μ g. of antibody per ml. This would not be detectable by precipitation methods. Cohn and Pappenheimer (1949) have found that heated normal human serum may greatly delay the formation of precipitates by human antitoxin with diphtheria toxin. It is possible that the reduction of the amount of precipitate found by Bukantz may be due to this inhibitory effect.

SUMMARY

1. The sera of hay fever subjects and control subjects were tested for antibodies against constituents of grass pollen extract by Boyden's (1951) agglutination method.

2. Antibodies were not found in the sera of control subjects. They were found in relatively low titre in about 1/3 of the sera of hay fever subjects and in all the subjects who had received a full course of treatment with injections of pollen extract. The titres found in the sera of treated subjects were, as a rule, considerably higher than those in the sera of untreated subjects.

3. The amounts of antibody in the sera of treated subjects bore no relation to the results of treatment.

4. It is suggested that the antibodies detected were not antibodies against the allergen of the pollen.

This investigation was made under the auspices of the Asthma Research Council which made a grant to one of us (E. S. O.).

Our thanks are due to Dr. A. W. Frankland for his help and for a supply of pollen extracts and sera, and to Dr. R. R. A. Coombs for his advice.

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