THE ROLE OF NON-PRECIPITATING ANTIBODIES IN THE PASSIVE SENSITIZATION OF HUMAN SKIN BY RABBIT ANTI-OVALBUMIN

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Since local passive sensitization of normal human skin by the sera of allergic individuals was described by Prausnitz and Kuestner (1) many investigators have attempted to produce similar passive sensitization of human skin with the antisera of rabbits and guinea pigs, which had been actively sensitized to such antigens as egg albumin, horse serum, and ragweed pollen. Earlier studies gave both positive and negative results (2, 3), but later reports (4–8) have agreed that many but not all rabbit and guinea pig antisera passively sensitize normal human skin. The reactions resulting from the injection of specific antigen into such passively sensitized sites are immediate urticarial wheals indistinguishable from the Prausnitz-Kuestner reaction produced with sera of allergic men, and they lack the delayed hemorrhagic characteristics of the passive Arthus reaction produced by rabbit antisera in rabbits and guinea pigs. Chase (9, 10) has described similar immediate urticarial reactions in the skin of guinea pigs passively sensitized with guinea pig antisera.

The activity of various rabbit antisera in passively sensitizing human skin was found to bear no relationship to the precipitin titer as measured by the serum dilution method (6, 8), but studies using well defined, purified antigens, which permit the utilization of quantitative immunochemical methods, including controlled partial or total absorption of the precipitins present in the sera, have not been reported.

In the following we present studies of the relationship of precipitating antibody content of rabbit anti-ovalbumin sera to the passive sensitization of human skin: (1) after sensitization of rabbits with dissolved and with alum-precipitated crystalline egg alubmin; (2) with rabbit antisera from which a considerable portion of precipitating antibody had been removed by a single addition of antigen; and (3) with rabbit antisera from which all precipitable antibody had been removed either by one single addition or by several fractional additions of antigen.

EXPERIMENTAL

Methods.—Crystalline egg albumin was prepared by the method of Heidelberger (11), recrystallized three times, and freed from ammonium sulfate by dialysis against distilled

water in the cold until the dialysate gave a negative Nessler reaction. Analyses for total nitrogen and for protein nitrogen (phosphotungstic acid precipitation method) coincided within the limits of the micro-Kjeldahl method. Sterilization was performed by filtration through Seitz filters. Saline solutions containing 5.0 mg. egg albumin per ml. were prepared and likewise alum-precipitated suspensions of the same albumin content by adapting the directions and applying the proportions as recommended by Kendall (12) to the egg albumin concentration used. Rabbits of mixed breeds were injected subcutaneously with either of these antigens three times a week, the animals receiving eight to thirteen injections of usually 10 mg. of egg albumin per injection. The rabbits were exsanguinated about 1 week after the last injection and the sera, sterilized by filtration through Seitz filters, were used without addition of any preservative.

The precipitin content of the sera was determined according to the method of Heidelberger and Kendall (13–15), by adding to 2.00 ml. samples of serum measured amounts of antigen contained in 2.00 ml. saline at three antigen levels (excepting sera 1, 16, 135, 164, and 165, where only two different antigen levels were used) in the region of antibody excess. The mixtures were incubated at 37°C. for 2 hours and kept in the refrigerator for 7 days prior to performing the final stage of the determinations. Blank determinations in which 2.00 ml. of saline was added to the sera instead of the antigen solutions, were run simultaneously and the nitrogen values (micro-Kjeldahl method) of the blanks were deducted from those of the actual precipitates. All determinations were carried out in duplicate. The precipitin nitrogen content of a serum was taken as the maximum antibody nitrogen value calculated in the usual manner (16) by evaluating the constants a and b in the equation $y = ax - bx^2$, where y represents precipitated antibody nitrogen in milligrams and x antigen nitrogen added in the individual experiments. In every instance more than one-third of the calculated total antibody nitrogen was precipitated at the lowest antigen level used. The greatest deviation of any determined nitrogen value from the theoretical nitrogen value (calculated on basis of the above mentioned equation in the instances in which determinations were performed at three different antigen levels) was 6 per cent, 5 per cent, and 4 per cent in the cases of sera 132, 11, and 15 respectively; in all other instances the maximal deviations were 3 per cent or less, in the instances of sera 3, 5, 9, 10, and 130 even less than 1 per cent. Where analyses were performed at two antigen levels only, such calculations naturally could not be carried out. The supernatant liquids of corresponding duplicate determinations were pooled and so were the supernatant liquids of the serum blank determinations, the latter pools representing dilutions 1:2 of unabsorbed sera, the former like dilutions of absorbed sera. Percentage of precipitin removal was evaluated from the precipitin nitrogen content as calculated and the antibody nitrogen precipitated in the individual determinations; the estimated maximal deviations of the values of percentage precipitin removal are given in the instances of sera 132, 11, and 15 in Table II, from which it becomes evident that not even in these extreme cases was the interpretation of the results of the passive transfer experiments affected by this factor of uncertainty. Strictly aseptic technique was observed throughout.

Passive sensitization was carried on the skin of the backs or arms of healthy men who showed no immediate urticarial reaction to intracutaneous injections of approximately 0.02 ml. of rabbit serum diluted 1:10 or a similar quantity of crystalline egg albumin solution containing 2.02 mg. per ml. Skin sites were sensitized by injecting into each 0.1 ml. of serial dilutions of rabbit anti-ovalbumin sera. The test subject was instructed to abstain from eating eggs during the experiment. Twenty-four to 48 hours later each site was tested by injection of 0.02 ml. of crystalline egg albumin solution containing 2.02 mg. per ml. The reactions were observed after 10 to 15 minutes. Reactions showing wheals more than 2 cm. in diameter were recorded as ++++, 1.5 to 2.0 cm. +++, 1.0 to 1.5 cm. ++, distinct wheals less than 1.0 cm. +. In certain tests subjects who showed no immediate reaction to the preliminary tests with rabbit serum and ovalbumin, the sensitized sites developed delayed urticarial or

inflammatory reactions before testing with antigen. In such cases subsequent injections of antigen produced no further reaction and experiments on such subjects were of no value. These reactions were presumed to be due to minor degrees of sensitivity to rabbit serum, although in some instances the possibility of inadvertent ingestion of egg albumin in mixed foods was not excluded. Among test subjects showing unequivocal reactions when antigen was injected into sensitized sites, there was a great variation in susceptibility to sensitization by a given sample of rabbit antiserum, so that only comparisons between serum sites made simultaneously in the same test subject were significant.

Skin Sensitization with Unabsorbed Sera.—All of fourteen sera from rabbits injected subcutaneously with alum-precipitated (crystalline) egg albumin produced passive sensitization of normal human skin. Ten of these reacted when diluted 1:10 and six in dilutions of 1:100.[1] The precipitin nitrogen content of these fourteen sera varied from 0.047 to 0.604 mg. per ml. (average 0.299).

On the other hand, none of the nine sera from rabbits injected subcutaneously with dissolved (recrystallized) egg albumin produced definite sensitization of human skin, one giving a questionable (±) reaction when tested undiluted.[2] The precipitin nitrogen content of five of these sera varied from 0.088 to 0.446 per ml. Qualitative tests on the other four sera indicated precipitin nitrogen contents of less than 0.05 mg. per ml. The average precipitin content of these nine sera appeared to be less than 0.189 mg. per ml., considerably lower than the average for rabbits injected with alum-precipitated antigen; but two sera of the soluble antigen group had precipitin nitrogen contents higher than the average of the precipitated antigen group, and the five that have been analyzed fell in the same range. It was apparent that the precipitin content was no measure of activity in passively sensitizing human skin (Table I).

It seems worthy of mention, that in the five sera analyzed from the group injected with dissolved egg albumin, R values (*i.e.* the ratio of antibody nitrogen to antigen nitrogen at the calculated point of maximal antibody precipitation) ranging from 6.2 to 8.8 (average 7.5) were encountered, while the R values in the group injected with alum-precipitated ovalbumin ranged from 6.1 to 10.6 (average 8.8). Therefore, the range of R values for the sera sensitizing human skin was not strikingly different from that of sera lacking such activity; furthermore sera of approximately equal skin-sensitizing qualities and of very divergent R values (6.1 and 10.0 respectively) were encountered; accordingly it was evident, that the skin-sensitizing property could not be correlated with a characteristic range of the antibody-antigen ratio at the calculated point of equivalence (Table I).

[1] The serum of one of two rabbits injected intramuscularly with alum-precipitated egg albumin and one of two injected intraperitoneally with the same antigen sensitized human skin. Serum of the second rabbit by each route lacked skin-sensitizing activity.

[2] Likewise sera of two rabbits injected intravenously and one rabbit injected intramuscularly with dissolved egg albumin lacked skin-sensitizing activity.

TABLE I

Analytical and Immunological Data on Rabbit Antisera

Serum No.	Sera of rabbits immunized with alum-precipitated antigen				
	Precipitin N	Value of R calculated	Reactivity on passive transfer to human serum diluted:		
			Undiluted	1:10	1:100
	mg. per ml.				
9	0.258	7.5	+++	++	±
10	0.381	6.1	++	+	−
11	0.358	9.0	+	+	−
12	0.595	9.3	+	+	−
13	0.531	10.2	+	+	−
14	0.604	10.0	++	+	−
15	0.338	9.0	+++	+++	+++
16*	0.553	8.9	+	−	−
130	0.170	8.4		++	+±
132	0.047	7.5		+++	++
135*	0.071	10.0		++	++
164*	0.102	10.6	+±	±	±
165*	0.087	7.5	++	+	+
191	0.083	9.1	+		
Serum No.	Sera of rabbits immunized with dissolved antigen				
	Precipitin N	Value of R calculated	Reactivity on passive transfer to human serum diluted:		
			Undiluted	1:10	1:100
	mg. per ml.				
1*	0.088	6.7	−		
3	0.420	7.4	−		
4	0.446	8.8	−		
5	0.193	6.2	−		
8	0.359	8.4	±		
2	0.05‡		−		
6	0.05‡		−		
154	0.05‡		−		
155	0.05‡		−		

* Analysis at two antigen levels only.

‡ No analysis performed on these sera; values estimated from qualitative sera.

Skin Sensitization with Partially Absorbed Sera.—Further experiments on skin-sensitizing sera were carried out by comparing their reactivity on human skin in dilution tests with samples of the same antisera from which in the course of the quantitative precipitin determination 68 to 90 per cent of the total calculated precipitable antibody had been absorbed. In such comparisons with

antisera from eight rabbits, the partially absorbed sera in every instance passively sensitized human skin in the same dilutions as did the unabsorbed sera, although the calculated precipitable antibody content was only 10 to 32 per cent as great. Typical examples are shown in Table II. It was apparent that a major portion of the precipitable antibody could be removed without affecting the skin-sensitizing activity of active sera. The absorption technique undoubtedly resulted in the precipitation of a small amount of incomplete antibody with the precipitin, but the comparative dilution tests using successive twofold dilutions of serum were not sufficiently sensitive to reveal this loss.

TABLE II

Comparison of Reactivity of Unabsorbed and Absorbed Sera on Passive Transfer to Human Skin

Serum No.	Precipitin N removed	Reactivity on passive transfer to human skin diluted:			
	per cent				
		1:200	1:400	1:800	
9	0	+	±	−	
	89	+	±	−	
		1:2	1:10	1:20	1:40
11	0	+	+	±	−
	94 ± 4	+	+	±	−
		1:2	1:4	1:8	1:16
14	0	+	+	±	−
	66	+	+	±	−
		1:80	1:160	1:320	
15	0	+	±	−	
	92 ± 6	+	±	−	
		1:10	1:100	1:200	
130	0	+++	±	−	
	68	+++	±	−	
		1:100	1:1000	1:2000	
132	0	++	±	−	
	82 ± 5	++	±	−	
		1:10	1:100	1:1000	
135	0	++	±	−	
	75	++	+	±	
		1:20	1:40	1:80	
164	0	+	±	−	
	79	+	±	−	

Skin Sensitization with Totally Absorbed Sera.—In the case of serum 14 all of the precipitable antibody was removed by one addition of the calculated amount of antigen; qualitative tests on the supernatant revealed no detectable antigen

or antibody. While the original antiserum had shown moderate activity in sensitizing human skin, the sample of serum from which all precipitable antibody had been removed by one single addition of antigen failed to produce skin sensitization in either of two subjects tested. It therefore appeared that the antibody responsible for the passive sensitization of human skin was com-

TABLE III

Comparison of the Dilution Titers of Unabsorbed and Step-Wise Completely Absorbed Transferring Sera

Dilution 1:	Reaction on test with crystalline ovalbumin of skin sites sensitized with 0.1 ml. of:					
	Serum 9*			Serum 14		
	Unabsorbed 9	Absorbed 9A‡	Absorbed 9B§	Unabsorbed 14	Absorbed 14A‖	Absorbed 14B¶
5.5				+	+	+
8	+++	+++	+++	+	+	+
16	+++	+++	+++	±	±	±
32	+++	+++	++	0	0	0
64	++	++	++			
128	±	+	+			
256	±	±	±			
512	0	0	0			

* A repeat experiment with these three No. 9 sera performed on a different test subject gave essentially identical results.

‡ Serum 9 from which 66 per cent of precipitable antibody had been removed by one antigen addition, further absorbed by six subsequent antigen additions. Dilutions based on an estimated serum dilution of 1:4.9.

§ Serum 9 from which 66 per cent of precipitable antibody had been removed by one antigen addition, further absorbed by five subsequent antigen additions. Dilutions based on an estimated serum dilution of 1:4.3.

‖ Serum 14 from which 66 per cent of precipitable antibody had been removed by one antigen addition, further absorbed by seven antigen additions. Dilutions based on an estimated serum dilution of 1:5.5.

¶ Serum 14 from which 66 per cent of precipitable antibody had been removed by one antigen addition, further absorbed by six antigen (and one saline) additions. Dilutions based on estimated serum dilution 1:5.5.

pletely removed with the precipitate, when the antigen-antibody reaction was carried out at the range of the calculated point of equivalence.

As this behavior suggested that the skin-sensitizing antibody had the properties of a "univalent" antibody, the precipitating antibody of two skin-sensitizing sera was removed completely by repeated fractional additions to antigen. One 2.00 ml. sample of 66 per cent absorbed serum 9 in a saline dilution of 1:2, representing 0.087 mg. unabsorbed precipitin nitrogen, was treated with 0.4 ml. saline containing 1.67 micrograms of egg albumin nitrogen; after in-

cubation for 2 hours at 37°C. and keeping in the refrigerator for 2 days, the precipitate was removed by centrifugation for 2 hours at approximately 1100 times gravity and the supernatant fluid decanted. The supernatant fluid and also a second 2.00 ml. sample of the same serum were simultaneously subjected to the same procedure of partial absorption with 0.4 ml. of egg albumin solution, incubation, and centrifugation. The same cycle of procedure was repeated on both samples of serum until no precipitate was observed in the first sample after centrifuging, a slight precipitate being present in the second sample. This stage was reached after six and five additions of antigen respectively; the two samples of absorbed serum were designated as 9A and 9B. Similarly 2.00 ml. samples of 66 per cent absorbed serum 14 in a saline dilution of 1:2, representing 0.206 mg. of unabsorbed precipitin nitrogen, were brought to the same end-point of absorption by seven and six additions respectively of 0.4 ml. saline containing 3.06 micrograms egg albumin nitrogen (sera 14A and 14B). In all these operations strict aseptic technique was observed, for the sake of which quantitative exactitude was sacrificed. Accordingly the estimations of the final dilutions of these step-wise absorbed sera were subject to a certain error not significant compared to that inherent in the dilution test on human skin.

The samples of serum from which the precipitin had been completely removed by step-wise absorption were compared with the corresponding unabsorbed antisera in the passive sensitization of human skin. In both instances, the absorbed sera sensitized skin in the same dilutions as the unabsorbed sera (Table III). Since the two samples of absorbed serum in each case gave similar results in the comparative dilution tests, it was apparent that the excess of antigen in one and of antibody in the other were within the limits of error of the method.

DISCUSSION

The passive sensitization of human skin by rabbit anti-ovalbumin sera was found to be effected by antibody remaining in solution after all precipitable antibody had been removed by fractional additions of antigens. However, when precipitin removal was carried out by a single addition of antigen at the calculated point of antigen-antibody equivalence, as was done in the case of serum 14, the skin-sensitizing activity of the serum was also removed. The antibody sensitizing human skin thus fulfilled the usual criteria of a "univalent" antibody, the true precipitin playing no detectable part in this type of skin sensitization. This is in striking contrast to the findings of Kabat and Benacerraf (17, 18) in studies of passive sensitization of the Arthus type in rabbits and guinea pigs with rabbit antisera. They reported that while the precipitating and non-precipitating antibodies of rabbit anti-ovalbumin sera were equally active in producing anaphylaxis in the guinea pig (17), precipitin was much

more active than "univalent" antibody in producing the passive Arthus phenomenon (18). Thus the phenomena of passive local sensitization produced in human skin and in rabbit skin by rabbit antisera differ not only in pathologic and physiologic features but also in their immunologic mechanisms. The rabbit antibody which passively sensitized human skin resembles the skin-sensitizing antibody of sera of human atopy in the absence of precipitin activity, giving indirect support to the point of view that the sensitizing antibody of human atopic sera differs qualitatively from precipitating antibody and that the failure to detect precipitins in such sera is due to the nature of the antibody rather than to the amount present.

It is premature to state that all precipitating antibodies are devoid of this type of skin-sensitizing property although no evidence of such activity was noted in this experiment. While the skin-sensitizing activity of these rabbit sera was found to be associated with antibody of the "univalent" type, there was no evidence that all "univalent" antibody has skin-sensitizing properties. No attempts were made to determine the quantity of "univalent" antibody in the sera which sensitized human skin or the presence of such antibody in the sera from rabbits, injected with dissolved egg albumin, which lacked skin-sensitizing activity. Further studies on the relationship of precipitins and of "univalent" antibodies to skin sensitization appear desirable.

The antibody nitrogen–antigen nitrogen ratio at the calculated point of equivalence also seemed to have no bearing upon skin sensitization. R values as calculated from the empirical equations given above (where the constant $a = 2R$) represent characteristics which are related to the totality of antibodies entering the precipitin reaction with homologous antigen. Heidelberger (15, 19) reported two instances in which in egg albumin–antiegg albumin systems the value of R increased with progressive immunization of the rabbits. These progressive changes of the values of R were believed to result from the formation of more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule. From this point of view, it would be conceivable that skin sensitization might become manifest only after enough antibody of broadened reactivity has been formed, which then should be reflected in a certain parallelism between skin sensitization and the range of calculated antibody nitrogen–antigen nitrogen ratio in which this property is encountered. As stated before such parallelism was not observed in our experience, since we have encountered transferring antisera within the range of calculated R values as low as 6.1 to R values as high as 10.6. Transferring qualities, therefore, do not seem to be restricted to a definable range of calculated R values. However, our experiments do not exclude the possibility that increases of R values subsequent to progressive immunization of the same animal might be paralleled by changes in the transferring properties of the different bleedings.

The comparison of the antisera from rabbits injected with alum-precipitated antigen with those prepared with dissolved ovalbumin indicated that the form of antigen used was a factor in determining the relative facility with which the skin-sensitizing antibody was formed in addition to precipitin. However, the studies of Cooke and Spain (2) demonstrated that in some cases adequate amounts of skin-sensitizing antibody may be stimulated by the use of dissolved egg albumin (not prepared in crystalline form).

SUMMARY

Sera of fourteen rabbits injected with alum-precipitated recrystallized ovalbumin, containing 0.046 to 0.604 mg. of precipitable antibody nitrogen per ml. (average 0.299 mg.), passively sensitized human skin, while the sera of nine rabbits injected with dissolved recrystallized ovalbumin, containing from less than 0.05 to 0.420 of antibody nitrogen per ml. (average 0.176 mg. or less), were inactive in human skin. The skin-sensitizing activity of the sera bore no relation to the precipitin content.

Removal of 68 to 90 per cent of the precipitin nitrogen by a single addition of antigen did not affect the activity of the sera in sensitizing human skin.

Removal of all precipitable antibody nitrogen in one serum by a single addition of antigen removed the skin-sensitizing activity.

The "univalent" antibody remaining after complete removal of precipitin by fractional addition of antigen showed the same activity in passive sensitization of human skin as the original serum.

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