ANAPHYLACTIC REACTIONS IN THE SKIN OF THE GUINEA PIG WITH HIGH AND LOW MOLECULAR WEIGHT ANTIBODIES AND GAMMA GLOBULINS

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It is well known that antibodies from various species can passively sensitize the guinea pig (1). Previous results have indicated that certain human antibodies are able to sensitize the guinea pig for passive cutaneous anaphylaxis (PCA) whereas others such as blood group antibodies give negative results (2, 3). In addition, sera capable of producing positive Prausnitz-Kustner reactions, obtained from patients with various allergies, give inconsistent sensitization of the guinea pig (1). Reverse passive cutaneous anaphylaxis reactions can be provoked in the guinea pig (4–6) if rabbit or human γ-globulins are used as antigen, indicating the ability of these γ-globulins to be fixed to the guinea pig tissues. However, the reason for the discrepancies frequently encountered has not been apparent.

In recent years renewed interest has been focused on variations in the physical properties of antibodies and it has become evident that many antibodies of similar specificity may exist with different molecular weights and sedimentation constants (7). In the present study, a comparison was made between known antibodies and γ-globulins of the 7S and 19S class in respect to their ability to sensitize the guinea pig for PCA. Marked differences were observed, and the high molecular weight 19S group failed to elicit reactions when injected into the skin of guinea pigs.

Materials and Methods

1. Rabbit Antisera.—Two different sera from rabbits immunized with highly purified human macroglobulins were used. The antibody content of these sera was determined by the quantitative precipitin method with the macroglobulin antigen. These sera cross-reacted with normal human 7S γ-globulin as was described previously (8). However, 7S human γ-globulin removed only a portion of the antibodies from these sera and a large fraction of the antibody remained in the supernate which still reacted with the macroglobulin. It was thus possible to absorb out all antibodies which would precipitate with 7S human γ-globulin. From serum Se 2.5 mg. 7S human γ-globulin/ml. removed all antibody which precipitated with 7S human γ-globulin whereas from sera Sa 1 mg./ml. was necessary to obtain a supernate which would react only with the macroglobulin. Absorbed serum Sa precipitated 325 μg. antibody nitrogen

per cc. at equivalence with the isolated 19S protein used in the PCA experiments. Prior to absorption it precipitated 750 μg. of antibody nitrogen.

2. Human Sera from Patients with Acute Infectious Mononucleosis.—These sera were titrated for their antibody content by agglutination using standard agglutination techniques (10) with 2 per cent sheep erythrocytes. The titers were the following: BS 1/3000, VA 1/600, EJ 1/600.

3. Human Sera with Anti-B Antibodies.—The amounts of sera available were insufficient to perform precise quantitative precipitin titrations and therefore these sera were also titrated by standard agglutination techniques, using a 2 per cent suspension of human B cells. The titers were approximately equal (1/1000) for each of these especially selected sera.

Ultracentrifugation:

Separation of 7S and 19S antibodies in these sera was carried out by density gradient ultracentrifugation as described previously (7). Serum (0.2 cc.) was layered over a sucrose density gradient and ultracentrifugation was accomplished in a swinging bucket rotor. Samples were taken by the capillary pipette technique as well as by the drop collection procedure described previously (7). For human serum Vo all anti-B agglutinating activity was located in the upper 7S fraction. This was an unusual serum and was selected from a large number of sera analyzed in a separate study (10). It was the serum of a mother of a baby with erythroblastosis fetalis due to anti-B antibody which passed the placenta. Sera Ro and Wi showed the anti-B activity in the bottom or 19S fraction. These were from individuals injected with B substance.

Antigens:

Human γ-Globulins.—Fraction II obtained from Squibb and Sons (Lot 1776), was used as human γ-globulin with 7S sedimentation rate. In the analytical ultracentrifuge this preparation showed only slight traces of components slightly heavier than 7S and was devoid of 19S material. This γ-globulin preparation was dissolved in 0.15 M NaCl and stored until use at 4°C., or at −20°C. The human γ-globulin with 19S sedimentation rate used was a highly purified preparation containing more than 95 per cent 19S and less than 5 per cent 7S components. This preparation was also dissolved in 0.15 M NaCl and stored until use at 20°C. to prevent precipitation.

B Substance.—A commercial preparation obtained from Knickerbocker Blood Bank was used. A stock solution of 1 mg. per ml., in 0.15 M NaCl was prepared, and stored for a short time at 4°C. or for longer periods at −20°C. From this stock solution appropriate dilutions were made in 0.15 M NaCl.

Sheep Erythrocytes.—Fresh sheep whole blood was mixed with an equal volume of Alsever's solution (9) and kept in sterile condition at 4°C. for a week before use. The erythrocytes were carefully washed three times and suspended in 0.15 M NaCl to a final suspension of 2 or 5 per cent.

Human B Cells.—These were obtained from group B patients and carefully washed in 50-fold volumes of isotonic saline and suspended to a final suspension of 2 per cent in 0.15 M NaCl.

The volume of the intradermal injections was 0.1 ml.; that of the intravenous injections, 1.5 ml. The diluent that was used was 0.15 M NaCl.

Evans blue (Eastman Organic Chemicals), 1 per cent solution was used as the dye.

PCA Techniques:

The technique of PCA has been extensively described previously (1, 11). It consists of an intradermal injection of a solution of antibody and, after a suitable latent period, the challenge by an intravenous injection of the corresponding antigen mixed with 0.5 ml. of dye. The

animals are killed 30 minutes after the challenge, skinned, and the diameter of the reactions measured on the internal side of the skin.

Albino guinea pigs of the Hartley strain, weighing approximately 250 gm., were used for passive cutaneous anaphylaxis (PCA) or for the reverse passive cutaneous anaphylaxis experiments.

TABLE I

PCA Reactions with Unabsorbed and Absorbed Rabbit Anti-Macroglobulin Sera

Intradermal injections of serum dilutions	Diameter of reactions, *mm.*											
	Challenging antigen											
	750 μg. 7S				400 g. 19S				375 μg. 19S			
Unabs. Sa												
1/100	20	25	22	15	25	25	30	23	—	—	—	—
1/300	10	18	16	10	20	20	25	15	22	17	20	19
1/900	tr	tr	0	tr	15	16	20	22	—	—	—	—
1/1800	—	—	—	—	—	—	—	—	12	0	tr	tr
1/2700	—	—	—	—	—	—	—	—	0	0	0	0
Abs. Sa												
1/100	0	0	0	0	25	22	30	22	—	—	—	—
1/300	0	0	0	0	20	20	25	18	20	17	20	18
1/900	0	0	0	0	16	16	20	22	—	—	—	—
1/1800	—	—	—	—	—	—	—	—	tr	tr	tr	10
1/2700	—	—	—	—	—	—	—	—	0	0	0	0
	240 μg. 7S				1250 μg. 19S							
Unabs. Se												
1/100	20	20	25	25	14	12	25	18				
1/300	20	15	25	16	12	12	18	10				
1/900	8	0	22	0	0	0	0	0				
Abs. Se												
1/100	0	0	0	0	12	16	25	20				
1/300	0	0	0	0	12	12	17	16				
1/900	0	0	0	0	0	0	0	0				

Unabs., unabsorbed; abs., absorbed (see text); 7S, human γ-globulin with 7S sedimentation rate; 19S, human γ-globulin with 19S sedimentation rate; —, not done, latent period 3 hours every vertical row represents results obtained on the same animal; tr, trace.

RESULTS

PCA Experiments with Unabsorbed and Absorbed Rabbit Antisera.—

Guinea pigs were given three intradermal injections on either side of the back. On one side three different dilutions of the unabsorbed antiserum were injected, on the other side the same dilutions of the absorbed antiserum. This procedure was used to compare on the same animal and at symmetric sites the reactions with unabsorbed and absorbed antisera and thus to minimize individual variations in the strength of the reactions. After a latent period of 3 hours, one group of four animals was challenged with 500 μg. 7S human γ-globulin and the

other with 800 μg. 19S macroglobulin. It is known that small amounts of antigen are sufficient to provoke PCA reactions (11); however, as a large excess of antigen is not inhibitory (1), and since threshold amounts of antibody react faster with excess of antigen, it was decided to use a large excess of antigens.

The results are shown in Table I. With the unabsorbed antiserum reactions were obtained when either 7S or 19S γ-globulins were used as antigens. However, with the absorbed sera, PCA reactions were obtained only if 19S material was used as antigen.

TABLE II

Reversed PCA Experiments with Unabsorbed Rabbit Antisera

Intradermal injections in μg. protein	Diameter of reaction, *mm.*							
	Challenging antiserum							
	Sa 0.5 ml./animal				Se 0.1 ml./animal			
7S								
5	30	20	20	18	20	20	16	20
2.5	25	18	19	18	20	20	15	20
1.25	—	—	—	—	—	—	—	—
0.62	—	—	—	—	5	10	0	0
19S								
50	0	0	0	tr	0	0	0	0
5	0	0	0	0	0	0	0	0

For remarks see Table I.

Reversed PCA Experiments with Unabsorbed and Absorbed Rabbit Antisera.—

The technique of reversed PCA consists of the intradermal injection of the antigen and, after a suitable latent period, the challenge by an intravenous injection of the corresponding antibody mixed with 0.5 ml. of dye (6). The guinea pigs in this series of experiments received intradermal injections of various amounts of 7S or 19S γ-globulins. One group of four animals was challenged 3 hours later with the unabsorbed antiserum, another group of four animals with the absorbed antiserum.

The results with the unabsorbed antisera are given in Table II. The absorbed antiserum did not provoke any reaction and therefore these results are not indicated in the table. It is evident that the unabsorbed antisera provoked good reverse PCA reactions with the intradermally injected 7S γ-globulin, whereas they failed to elicit reactions with the 19S macroglobulin.

PCA Reactions with Sera from Patients with Infectious Mononucleosis.—

Undiluted sera and 1/10 and 1/100 dilutions were injected intradermally, utilizing four guinea pigs for each experiment. Six hours after intradermal injection, 1 ml. of Evans blue dye was injected intravenously. If no reactions of a non-specific type were observed, within 20

minutes after the dye injection, 0.5 ml. of a 5 per cent sheep erythrocyte suspension was injected intravenously. As a control a guinea pig anti-sheep erythrocyte serum was used.

The control serum elicited a strong PCA reaction at a dilution of 1/100, whereas none of the three sera from the patients with infectious mononucleosis provoked any reaction. These results are not tabulated. Previous studies (7) as well as density gradient experiments on these infectious mononucleosis sera indicated that all of the human anti-sheep cell agglutinins were of the 19S class. The guinea pig antiserum contained a high titer of 7S class sheep red cell agglutinins (Table III).

PCA Reactions with Human Anti-B Sera. Varying dilutions of these sera were injected intradermally into guinea pigs and 6 hours later the animals

TABLE III

Distribution of Sheep Red Cell Agglutination Activity in Fractions Taken after Density Gradient Ultracentrifugation for Infectious Mononucleosis Serum VA and Guinea Pig Anti-Sheep Cell Serum

Fraction	Serum	
	Inf. Mon(VA)	G.P. anti-sheep cell
1. Alb. (top)	0	0
2. 7S	0	1280
3. Intermediate	20	80
4. 19S (bottom)	640	0

were challenged with 500 μg. of B substance mixed with 0.5 ml. of dye. The results of these experiments are tabulated in Table IV. It is evident that only serum Vo elicited PCA reactions.

Mention should be made of the reasons for using large amounts of B substance for challenge. Preliminary experiments demonstrated that the 1/100 dilution of the antiserum was ineffective with 100 μg. of B substance, whereas with 500 μg. or more B substance, this dilution was able to produce PCA reactions. The amounts of antigen needed for PCA reactions is inversely proportional to the molecular size of the antigen (12). The B substance has a molecular weight approximately twenty times higher than that of egg albumin. With rabbit anti-egg albumin serum it was seen that about 60 μg. of egg albumin is needed to elicit PCA reactions with 0.02 μg. antibody nitrogen. Moreover, the B substance used in these experiments was prepared from horse stomach. It is known (13) that cross-reacting antigens are less effective in eliciting PCA reactions and hence on a weight basis it is necessary to use higher amounts of challenging antigen.

The possibility arose that the B substance might be removed rapidly from the circulation and not reach the site of the injection of the antibody in a sufficient quantity.

Experiments were therefore carried out to detect circulating B substance by the agglutination inhibition technique. A standard anti-B serum with agglutination titer of 1/500 was used in these experiments. This serum was diluted 1/200 and 0.4 ml. was mixed with 0.1 ml. of the specimen in question. The mixture was allowed to stand at room temperature for 30 minutes, 0.1 ml. of the 2 per cent B cell suspension added, and the agglutination observed macroscopically according to standard techniques (9). In preliminary experiments it was seen that 0.2 μg. of B substance was able to completely inhibit the agglutination. It was also determined that normal guinea pig serum was not inhibitory. When 100 μg. of B substance was injected intravenously into the guinea pig, 4 minutes later less than 25 μg. was present in the peripheral blood. The

TABLE IV

PCA Reactions with Human Anti-B Sera

Intradermal injections of serum dilutions	Diameter of reactions, *mm.*							
Vo undiluted	30	25	25	20	—	—	—	—
Vo/10	—	—	—	—	30	24	20	20
Vo/100	20	18	15	16	18	18	16	14
Vo/1000	12	12	8	0	10	10	0	0
Ro undiluted	0	0	0	0				
Ro/10	—	—	—	—	0	0	0	0
Ro/100	—	—	—	—	0	0	0	0
Wi undiluted	0	0	0	0	—	—	—	—
Wi/10	—	—	—	—	0	0	0	0
Wi/100	0	0	0	0	—	—	—	—

Latent period 5 hours. Challenge with 500 μg. B substance. For other abbreviations and remarks see Table I and text.

blood volume of the guinea pigs was determined by the intravenously injected Evans blue concentration. The guinea pig erythrocytes did not adsorb the B substance onto their surface as they were not agglutinated by the anti-B serum. The rapid removal of intravenously injected B substance was therefore another reason for use of large amounts of the antigen.

Inhibition of the PCA Reaction of Human 7S Anti-B Antibody by Mixing with Human Anti-B Antibody of 19S Type.—

The serum Vo was diluted 1/25 and the serum Wi 1/10. Then equal amounts of these sera were mixed just prior to injection into the guinea pig. As a control instead of serum Wi a normal anti-A serum was diluted 1/10 and mixed with the 1/25 dilution of Vo. In a third site a 1/50 dilution of Vo was injected. (The final dilution was therefore everywhere 1/50 with respect to Vo.) The animals were challenged with 500 μg. B substance after 3 hours latent period.

The results are tabulated in Table V. It can be seen that Vo mixed with normal serum or with 0.15 M NaCl provokes a good PCA reaction; however, when it is mixed with the serum of Wi no PCA reactions are elicited.

Studies with Thyroglobulin Autoantibodies.—In view of reports that these antibodies may be of 19S type in certain sera, three sera from patients with thyroiditis and good precipitin reactions with thyroglobulin were investigated. All gave good PCA reactions with thyroglobulin and density gradient studies demonstrated that these antibodies were all of the 7S class. No sera were found with the 19S type antibodies.

TABLE V

Inhibition of PCA Reaction of 7S Anti-B by 19S Anti-B

Intradermal injections of serum dilutions, final concentrations	Diameter of reactions,, *mm.*			
Vo 1/50 Wi 1/20	0	0	0	0
Vo 1/50 anti-A 1/20	15	18	14	12
Vo 1/50	16	18	16	15

Latent period 3 hours. Challenge with 500 μg. B substance.

DISCUSSION

It is evident from the results presented that human γ-globulins of both the 7S and the 19S class are good antigens for challenging antisera in PCA reactions. Serum Sa contained less antibody reacting with 7S γ-globulin than with 19S material; at a dilution of 1/900 the 7S antigen elicited only trace reactions whereas the 19S provoked much stronger reactions. When the antisera were absorbed with 7S γ-globulin, they no longer elicited PCA reactions with the 7S antigen; however, their capacity to provoke PCA reactions with the 19S antigen remained strong. When reversed PCA experiments were performed with unabsorbed serum, it was seen that with the 7S human γ-globulin good reactions were obtained. The threshold was about the same as obtained previously (0.10 μg. N (6)). However, even 10 μg. N from the 19S γ-globulin was ineffective. With the absorbed serum no reversed PCA reactions could be provoked even with 1 μg. N of 7S material.

It is possible, therefore, that the 19S human γ-globulin has a different kind of relationship with the guinea pig skin tissues than the 7S human γ-globulin. On the other hand, perhaps the 19S γ-globulin is not as accessible for the antibody in reversed PCA reactions as is the 7S γ-globulin. That the latter is not the case shall be pointed out later when discussing the inhibition experiments.

The sera from three patients with infectious mononucleosis which contained sheep cell agglutinins only of the 19S class all gave negative results when the guinea pigs were challenged with 0.5 ml. of 5 per cent sheep erythrocytes.

With one-tenth this amount it was possible to elicit PCA reactions with guinea pig anti-sheep erythrocyte sera or sera from patients with serum sickness (3). Only one of the isoagglutinin sera, namely Vo, was effective in eliciting PCA reactions. This serum contained anti-B antibody which had a sedimentation rate of 7S, whereas the others were shown to have a sedimentation rate of approximately 19S. Here again it appears likely that the 19S antibody must have a different kind of relationship to the cells of the guinea pig than the 7S antibody, although from this experiment alone it could be supposed that the 19S antibody might not be accessible for the antigen when this antibody is injected intradermally. However, the competition experiment described above, in which simultaneous injection of 19S anti-B intradermally blocked the reaction with 7S anti-B, shows this not to be the case. The 19S antibody is clearly accessible to the antigen. Similar inhibition experiments were performed previously with horse and rabbit anti-egg albumin sera (5). It was shown that horse antibody cannot be fixed to the guinea pig cells (6), and that the horse antibody competes with the rabbit antibody for the antigen. This was shown by increasing the amount of egg albumin used for challenge, with enough antigen available, the horse antibody was unable to inhibit the PCA reaction of the rabbit antibody. Similar competition was shown also by the use of papain-digested antibody fragments containing the antibody-combining sites (15). The latter cannot be fixed to the cells of the guinea pig but are able to block the intact antibody from reacting with the antigen. Finally, with papain-digested anti-horse α-globulin antibody (from a serum sickness patient) a similar competition was obtained (16). The papain-digested antibody fragments must be readily accessible to the antigen if they can inhibit the reaction between the antigen with the intact antibody. The same reasoning applies to the inhibition by the 19S anti-B antibody of the PCA reactions given by the 7S anti-B antibody.

SUMMARY

High molecular weight (19S) γ-globulin produces passive cutaneous anaphylaxis (PCA) reactions with its specific antiserum only when it is used as intravenous antigen. Reversed PCA reactions cannot be produced when the 19S protein is injected intradermally in contradistinction to the results with 7S γ-globulin.

Antibodies of the high molecular weight class, when injected intradermally, also failed to give PCA reactions following antigen injection. Heterophile antibodies from the sera of patients with infectious mononucleosis, demonstrated to be entirely of the 19S type, gave negative reactions, while 7S heterophile antibodies from guinea pig did give reactions following intravenous injection of sheep cells. Anti-B isoagglutinins of the 19S class failed to react, while those of the 7S class of similar titer gave clear reactions following the injection of B substance.

Evidence was obtained that 19S antibodies were capable of inhibiting the PCA reactions obtained with the 7S type, indicating that interaction with antigen occurred. The failure to elicit PCA reactions appeared to be due to an inability to fix to guinea pig tissues in a manner similar to that known for 7S antibodies and γ-globulin.

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BIBLIOGRAPHY

1. Ovary, Z., Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. *Progr. Allergy*, 1958, **5,** 459.
2. Ovary, Z., and Biozzi, G., Passive sensitization of the skin of the guinea pig with human antibody, *Internat. Arch. Allergy*, 1954, **5,** 241.
3. Ovary Z., and Ruggieri, P., *Boll. Ital. Ematol.* 1954, **2,** 280.
4. Bier, O. G., and Siqueira, M., Passive reversed cutaneous anaphylaxis to protein antigens, *Internat. Arch. Allergy*, 1955, **6,** 391.
5. Ovary, Z., Anaphylactic reactions in the guinea pig skin with horse, sheep, or chicken antibodies, *Fed. Proc.*, 1959, **18,** 590.
6. Ovary, Z., Reverse passive cutaneous anaphylaxis in the guinea pig with horse, sheep or hen antibodies, *Immunology*, 1960, **3,** 19.
7. Kunkel, H. G., Macroglobulins and high molecular weight antibodies, *in* The Plasma Proteins, Chapter 8, (Putnam, editor), New York, Academic Press, Inc., 1960, **1,** 300.
8. Franklin, E. C., and Kunkel, H. G., Immunologic differences between 19S and 7S components of normal human γ-globulin, *J. Immunol.*, 1957, **78,** 11.
9. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.
10. Fudenberg, H. H., and Kunkel, H. G., High molecular weight antibodies, *in* International Society of Blood Transfusion, 7th Congress, Rome, 1958, Holländer, (editor), Basel, 1959.
11. Ovary, Z., Passive cutaneous anaphylaxis in the guinea pig: degree of reaction as a function of the quantity of antigen and antibody, *Internat. Arch. Allergy*, 1959, **14,** 18.
12. Ovary, Z., and Leskowitz, S., data to be published.
13. Osler, A. G., Hawrisiak, M. M., Ovary, Z., Siqueira, M., and Bier, O. G., Studies on the mechanism of hypersensitivity phenomena. II. The participation of complement in passive cutaneous anaphylaxis of the albino rat, *J. Exp. Med.*, 1957, **106,** 811.
14. Gregersen, I. M., and Rawson, R. A., Blood volume, *Physiol. Rev.*, 1959, **39,** 307.
15. Ovary, Z., and Karush, F., paper submitted to *J. Immunol.*
16. Ovary, Z., and Franklin, E. C., data to be published.