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THE PRODUCTION OF ANAPHYLACTIC ANTIBODY IN THE RAT[1]

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Immunization of rats with a mixture of antigen and *Bordetella pertussis* organisms generally results in a state of active sensitization characterized by very severe anaphylactic shock, usually fatal, and, upon challenge with the specific antigen (1), by extensive damage to the mast cells with release of histamine and 5-hydroxytryptamine. However, attempts to reproduce this condition in passively sensitized animals had consistently failed (2). The anaphylactic shock observed in animals passively sensitized even with large amounts of rat or rabbit antibodies did not result in mast cell damage or release of histamine and 5-hydroxytryptamine, but other substances, such as slow reacting substance (SRS), have been implicated in this reaction (3).

The hypothesis was advanced by Mota that actively sensitized rats produce a special type of antibody which is responsible for the mast cell damage but not present in the serum, or present in such small quantity as not to allow passive transfer to normal animals (4).

Studies on the antibodies produced by rats sensitized intraperitoneally with antigens mixed with Pertussis organisms or with Freund's complete adjuvant have failed to show any correlation between the level of serum antibody and the induction of mast cell damage by antigen. In addition, it was found that when tissues from actively sensitized animals were incubated with labeled specific antigen, the uptake was much higher for tissues from animals in which mast cell damage could be induced than for tissues from sensitized animals not showing mast cell damage or from normal untreated animals. These observations (5) were interpreted as evidence that some animals were able to produce an anaphylactic antibody that remained bound to the tissues but whose concentration in the serum was too small to mediate passive sensitization of normal animals. In a more recent report, Mota observed that some sera from rats immunized with protein antigens and Pertussis organisms could transfer local anaphylactic reactivity (6).

The present studies were undertaken to explore whether, by modifying the route and schedule of immunization, one could enhance the immune response to such a degree that the serum concentration of the anaphylactic antibody would attain the necessary level to allow studies of the production and properties of this antibody to be made. These experiments demonstrate that the rat produces at least two distinct populations of antibodies, only one of which is able to mediate anaphylactic reactions in this species.

MATERIALS AND METHODS

Animals. Wistar strain rats of both sexes weighing 200 to 400 g were used for immunization and for passive cutaneous anaphylaxis.

Antigens. Hen egg albumin (EA) 2× recrystallized, Worthington Biochemical Corporation, New Jersey; human γ-globulin (HGG), Lederle Laboratories; conjugated antigens were prepared according to the technique described (7): 2,4-dinitrophenyl bovine γ-globulin (DNP-BGG); 2,4-dinitrophenyl bovine serum albumin (DNP-BSA); 2,4-dinitrophenyl fibrinogen.

All the antigens were highly conjugated.

Immunization. The animals were injected in all four footpads, usually with 0.25 ml of the antigen mixture in each one. The antigenic mixture was prepared in such a way as to contain 1 mg/ml of protein antigen (unless otherwise indicated) and either 0.5 ml of complete Freund's adjuvant (Difco Laboratory, Detroit, Mich.) or 7 billion organisms of *Bordetella pertussis*.[2] Boosters, when

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[2] *Bordetella pertussis* whole culture, Lot no. 91453, was kindly supplied by Merck Sharp and Dohme, West Point, Pa.

required, were made intradermally in four sites on the back. Each site was injected with 0.1 ml of the adequate solution of the protein antigen in saline, without adjuvant or Pertussis organisms. Bleedings were performed from the heart, under ether anesthesia.

Antibody determination. The amount of precipitating antibody was determined following essentially the method of Heidelberger *et al.* (8). Varying amounts of antigen, in 0.2 ml, were added to 0.5 ml of the serum aliquots and the tubes incubated 1 hr at 37°C and then 48 hr at 4°C. The precipitates were centrifuged, washed twice with 2 ml of cold saline, finally dissolved in 1 ml of N/10 NaOH and their optical density determined at 2800 Å and, in the case of DNP-conjugated antigens, also at 3600 Å.

The optical density per μg N/ml at 2800 Å for the rat antibody was 0.0091. It was obtained from a preparation of rat γ-globulin isolated by electrophoresis on a polyvinyl chloride resin block (9) and analyzed by Kjeldahl. The antigen content of the specific precipitate was calculated, in the case of conjugated antigens, from the absorption at 3600 Å.

Sensitizing antibody. The sensitizing activity of the sera was established in the rat by passive cutaneous anaphylaxis and by passive sensitization (and degranulation) of the mast cells *in vitro*.

Passive cutaneous anaphylaxis (PCA). One-tenth milliliter of appropriate saline dilutions of the antibody to be tested was injected intradermally on the shaved dorsal skin of normal rats. The animals were injected intravenously 16 to 24 hr later with 1 mg of antigen dissolved in 1 ml of 0.5% Evans blue in saline. The animals were killed 1 hr later and the reactions on the inner side of the skin examined.

Mast cell degranulation. Normal rats were killed and bled and the mesentery was carefully dissected. After being washed in Tyrode solution, pieces of it were incubated in dilutions of antibody in Tyrode, usually for 1 hr at 37°C. Thereafter, the tissue was immersed for 15 min at 37°C in 1 ml of a solution containing 0.6 mg of the specific antigen in Tyrode. The tissue was then fixed and stained for 10 min in a 10% formaldehyde solution containing 1% toluidine blue and 0.1% acetic acid before examination.

RESULTS

Immunologic response

Different routes of immunization were tried in order to enhance the production of antibodies. It was found that inoculation of the antigen mixed with adjuvants, into the footpad, resulted in higher titers of antibody and more constant results than other routes. Consequently, this method of immunization was adopted throughout. Two kinds of adjuvant were employed: the usual complete Freund's emulsion and *Bordetella pertussis* organisms. The animals were bled periodically at different stages of the immunization, and the sera were analyzed for their precipitating antibody content and their capacity to transfer anaphylactic sensitivity to the skin of normal rats. Table I shows the results of an experiment in which the initial sensitizing dose was 1 mg of DNP-BGG mixed with either 1 ml of 50% Freund's complete adjuvant or 7 billion Pertussis organisms. The booster dose was in all cases 0.1 mg of DNP-BGG/animal, without adjuvants.

It can be seen that both groups of animals pro-

TABLE I

Appearance of skin sensitizing activity and precipitating antibody in rat sera following immunization with 1 mg DNP-BGG[a] and Bordetella pertussis or Freund's adjuvant

Days after Immunization	*B. pertussis*		Freund's	
	Ab[a] protein[b]	PCA[a, c]	Ab protein	PCA
	mg/ml		*mg/ml*	
7	<0.1	21/25	<0.1	1/15
	Ring test		Ring test	
	+		+	
11	1.370 (65)	11/18	1.590 (18)	12/26
5[d]	1.200 (8)	8/16	2.170 (10)	10/16
7[e]	0.690 (7)	0/7	0.730 (7)	0/7

[a] DNP-BGG = 2,4-dinitrophenyl bovine γ-globulin; Ab = antibody; PCA = passive cutaneous anaphylaxis; DNP-BSA = 2,4-dinitrophenyl bovine serum albumin.

[b] Analysis performed in pools. Numbers in parentheses indicate the number of sera pooled. Antigen used: DNP-BSA.[a]

[c] Number of animals whose sera showed PCA activity/number of animals tested. Animals were challenged with 1 mg DNP-BSA. Incubation period 16 hr.

[d] After first booster given on day 12.

[e] After second booster given on day 24.

duced antibodies with anaphylactic properties. However, striking differences existed at the beginning of the immunization. In the Pertussis group most of the animals produced antibody with PCA activity by the 7th day of immunization, while in the Freund's adjuvant group this was only exceptionally the case. By the 11th day the anaphylactic activity was present in the serum in about the same percentage in both groups. At about 1 month after the immunization the sensitizing antibody disappeared in all animals of both groups.

It is evident from the data that some animals in the Pertussis group had anaphylactic activity in their sera on the 7th day of immunization, which subsequently disappeared. When these animals were followed individually, anaphylactic activity was never observed to reappear in their sera.

The presence of small quantities of precipitating antibody could already be detected on the 7th day in all animals, and a sharp increase took place over the following few days. Following a booster injection, a further increase in total antibody production was observed only in the animals immunized with Freund's adjuvant, but not in the Pertussis group.

Some experiments were performed in which the animals were not boosted, and it was found that the antibody titer started to diminish after 2 weeks of immunization. On the other hand, repeated boosters every week not only did not result in a significant increase over the levels of antibody obtained after a single booster, but a marked decrease in the antibody concentration was observed.

As can be inferred from Table I, no correlation was found between the level of precipitating antibody and the capacity to transfer anaphylactic activity. Although the data on precipitating antibody presented in the table refers to pools of sera, this lack of correlation was verified by analyzing both properties in individual sera. In general, the anaphylactic activity appears in the serum of the animals at the beginning of the immunization. In the animals injected with Pertussis organisms this occurred at a time when the total quantity of precipitating antibody was small. Later on, and even though the precipitating antibody increased considerably, the anaphylactic activity diminished and eventually disappeared.

These results strongly suggest that two different types of antibodies are produced in the rat: the anaphylactic type and another, nonanaphylactic, that probably constitutes the bulk of the precipitating antibody.

The titer of PCA activity found in the sera was never very high, and no significant difference was detected whether the animals were injected with Pertussis or Freund's adjuvant. The PCA titer of 101 sera taken 11 days after immunization with 1 mg of DNP-BGG and Pertussis are presented in Table II.

TABLE II

PCA[a] titer of sera from rats after 11 days of immunization with 1 mg DNP-BGG[a] and Pertussis[b]

PCA[a] Titer[b]	No. of Animals
No activity	40
1:1	19
1:9	24
1:27	16
1:81	2
Total	101

[a] PCA = passive cutaneous anaphylaxis; DNP-BGG = 2,4-dinitrophenyl bovine γ-globulin; DNP-BSA = 2,4-dinitrophenyl bovine serum albumin.

[b] Animals challenged with 1 mg DNP-BSA.[a] Incubation period 16 hr.

Other experiments were made employing various other protein antigens: human γ-globulin, hen egg-albumin and human serum albumin at a dosage of 1 mg/animal. Anaphylactic antibody was produced in the animals immunized either with Pertussis or with Freund's adjuvant and followed the same general pattern observed after immunization with DNP-BGG. Also, the animals immunized with Freund's adjuvant produced large amounts of antibody in a short period of time, usually of the order of 1 mg/ml by the 11th day and very frequently over 2 mg/ml 5 days after a booster given on the 12th day.

A few experiments were performed to investigate the influence of the dosage of antigen on the production of anaphylactic antibody. Groups of animals were inoculated in the footpads with different amounts of DNP-BGG and the same quantity (7 billion) of Pertussis organisms. Boosters were given intradermally on the back, and the same dose as in the initial inoculation distributed in four sites was used, without Pertussis.

TABLE III

Appearance of skin sensitizing activity and precipitating antibody in rat sera following immunization with different doses of DNP-BGG[a] and Bordetella pertussis

Dosage of DNP-BGG[a]/ Animal	Days after Immunization					
	6		10		7[b]	
	Ab[a] protein[c]	PCA[a, d]	Ab protein	PCA	Ab protein	PCA
	mg/ml		*mg/ml*		*mg/ml*	
10γ	<0.1 (10)	1/10	0.785 (8)	1/8	0.250 (7)	3/7
100γ	<0.1 (10)	2/10	1.860 (8)	3/8	1.210 (8)	7/8
1000γ	<0.1 (8)	8/8	1.320 (8)	5/8	1.080 (8)	5/8

[a] DNP-BGG = 2,4-dinitrophenyl bovine γ-globulin; Ab = antibody; PCA = passive cutaneous anaphylaxis; DNP-BSA = 2,4-dinitrophenyl bovine serum albumin.

[b] After booster given on day 11.

[c] Analyses performed in pools. Numbers in parentheses indicate number of sera pooled. Antigen used: DNP-BSA.[a]

[d] Numbers of animals whose sera showed PCA activity/number of animals tested. Animals were challenged with 1 mg DNP-BSA. Incubation period 16 hr.

Results are presented in Table III. It can be seen that a dosage of 100 γ of antigen per animal produced essentially the same results as a higher dosage. However, the appearance of the anaphylactic antibody seemed delayed when low dosages were employed.

Biologic properties of the anaphylactic antibody

Since the only criteria employed to detect and evaluate the anaphylactic antibody was, by definition, its capacity to passively confer anaphylactic sensitivity, a series of studies were made to establish the optimal conditions of the passive transfer test.

Passive cutaneous anaphylaxis. After inoculation of sensitizing antibody into the skin of normal rats, a certain time must elapse before passive sensitization becomes established. It was found that the sensitization was maximal after 16 hr and persisted for many days without significant change in the level of sensitivity (Table IV).

The dosage of antigen required for challenge was investigated employing rat anti-DNP-BGG sera and DNP-BSA as antigen. The dose of antigen necessary to elicit a maximal reaction was of the order of 1.5 mg/kg of body weight. A dosage of 500 γ/kg produced definitely reduced reactions and a 4-fold increase in the threshold dose of sensitizing antibody. It may be possible that this large dose of antigen was required because the sera contained, together with the anaphylactic antibody, a considerable amount of nonanaphylactic antibody that competes for antigen. Further investigation is required to clarify this point.

Attempts were made to passively sensitize the skin of guinea pigs using rat antibody. No positive reactions were obtained using sera with or without anaphylactic activity from rats. Experimental conditions included incubation times from 1 to 48 hr.

Passive sensitization of mast cells. When the mesentery from normal rats was sensitized *in vitro* by incubation with anaphylactic antibody, subsequent contact with the antigen produced extensive mast cell damage with extrusion of the granules. Experiments were performed to compare the techniques of passive sensitization of the skin (PCA) and of the mast cells. It was found that both techniques gave similar results and that threshold reactions occurred at about the same dilutions with all sera studied. Because the PCA reaction is technically very simple and demands smaller amounts of serum than the passive sensitization of the mesentery, no further studies were made on the conditions of the later reaction.

Quantitative precipitin reactions

The result of a quantitative precipitin test, performed with a pool of sera taken from 65 rats on the 11th day of immunization with DNP-BGG and Pertussis, is presented in Table V. The antigen employed in this experiment was DNP-BSA

TABLE IV

Latent period and persistence of passive cutaneous sensitization in rats with rat Anti-DNP-BGG[a] antibodies

Interval between Sensitization and Challenge	Diameter of PCA[a] Reactions[b]				
	Dilutions of serum				
	1:3	1:6	1:12	1:24	1:48
	mm				
1 hr	Trace	Trace	Neg.[c]	Neg.	Neg.
	Trace	Trace	Neg.	Neg.	Neg.
	Neg.	Neg.	Neg.	Neg.	Neg.
	Neg.	Neg.	Neg.	Neg.	Neg.
6 hr		Trace	Neg.	Neg.	Neg.
		Neg.	Neg.	Neg.	Neg.
		Neg.	Neg.	Neg.	Neg.
		Neg.	Neg.	Neg.	Neg.
24 hr	14	14	11	5	Neg.
	16	14	6	Neg.	Neg.
3 days	15	12	8	4	Neg.
	12	11	7	4	Neg.
	14	11	12	Neg.	Neg.
	12	14	7	Neg.	Neg.
5 days	16	12	9	Neg.	Neg.
	17	12	6	Neg.	Neg.
11 days		12	7	Neg.	Neg.
		14	10	Neg.	Neg.
		14	7	Neg.	Neg.
		14	Neg.	Neg.	Neg.

[a] DNP-BGG = 2,4-dinitrophenyl bovine γ-globulin; PCA = passive cutaneous anaphylaxis; DNP-BSA = 2,4-dinitrophenyl bovine serum albumin.

[b] Animals challenged with 1 mg DNP-BSA.[a]

[c] Negative.

conjugated to the extent of 46 groups of DNP/molecule of BSA. A precise analysis of the composition of the precipitate could be made from the light absorption of the dissolved precipitate at 2800 Å and 3600 Å.

It can be seen that maximal antibody precipitation occurred only with considerable antigen excess when nearly 20% of the antigen added remained in the supernatant. Consequently, no sharp equivalent zone could be defined in this system, since at no point were the supernatants of the precipitates essentially free of antibody and antigen. The system seemed to be rather insensitive to inhibition of precipitation by excess antigen.

Some analyses were made using the same serum

TABLE V

Quantitative precipitin titration of a pool of rat anti-DNP-BGG[a] with DNP-BSA[a, b]

DNP-BSA[a] Added	DNP-BSA Precipitated	Ab[a] Precipitated	Weight Ratio of Ab/Ag[a] in Precipitate
mg	*mg*	*mg*	
0.010	0.010	0.176	17.6
0.025	0.024	0.346	14.4
0.050	0.048	0.555	11.5
0.075	0.066	0.635	9.6
0.100	0.082	0.685	8.4
0.125	0.091	0.682	7.5
0.150	0.097	0.677	7.0
0.200	0.099	0.640	6.4
0.250	0.101	0.608	6.0
0.300	0.095	0.530	5.6

[a] DNP-BGG = 2,4-dinitrophenyl bovine γ-globulin; DNP-BSA = 2,4-dinitrophenyl bovine serum albumin; Ab = antibody; Ag = antigen.

[b] Analysis performed with 0.5-ml serum aliquots.

and DNP-BGG or DNP-fibrinogen, and in all cases the same amount of antibody was precipitated and the precipitin curves were similar, indicating that most of the antibody specific sites were directed to DNP determinants. Comparable results were also obtained with antibodies against EA and HGG and their homologous antigens.

As it has been reported that the salt concentration of the medium considerably affects the results of the precipitin reaction with rabbit (10) and chicken (11) antibodies, an attempt was made to determine the presence of such an effect. Rat anti-DNP-BGG serum was dialyzed against distilled water, slightly buffered with 42 mg/L of $NaHCO_3$, and the precipitate formed was removed by centrifugation. Precipitin analyses were then carried out at various salt concentrations. The maximum antibody precipitated at 0.001 M, 0.15 M and 1.5 M in NaCl were, respectively, 1.00, 0.95 and 0.82 mg protein/ml, indicating a slight decrease of the precipitation at high salt concentration. Before dialysis, the serum tested contained 1.370 mg/ml of antibody. The loss after dialysis, 0.370 mg/ml, represents the antibody present in the euglobulin fraction, precipitated during the dialysis against distilled water.

DISCUSSION

The study of the properties of sera obtained from rats immunized with a variety of antigens

mixed either with *Bordetella pertussis* organisms or with complete Freund's adjuvant, has shown that two types of antibody are produced in this species of animal. One of these antibodies present in many of the sera was able to passively sensitize the tissues of normal rats. Because this activity appeared early during immunization, at a moment when the level of precipitating antibody of the sera was very low, and never showed any correlation with the total amount of antibody present, it can be concluded that only a minor fraction of the specific antibody possesses anaphylactogenic properties. This is in agreement with the low titers of PCA activity encountered, usually no higher than 1:27. Although it may be justified to establish comparisons with quantitative data obtained in other species, such a low titer suggests that the quantity of antibody involved must be of the order of a few micrograms per milliliter. Similar findings have been recently reported by Mota (6).

The anaphylactic antibody appeared in the serum of rats during the first week of immunization in practically all the animals inoculated in the footpads with a mixture of 1 mg protein and 7 billion Pertussis organisms, and its appearance was delayed by a few days in the animals receiving Freund's adjuvant, of which only about 50% produced it. The anaphylactic antibody appeared to have a rather short life span in the serum. By the 11th day after immunization it had already disappeared in some of the animals in the Pertussis group, and after 1 month its presence could no longer be detected in any of the sera tested. This could not be modified by changes in the immunization schedule.

The use of the footpads as the route of immunization proved to be of great importance in these experiments. It resulted, apparently, in a strong stimulation of the antibody-forming mechanism, as evidenced not only by the production of significant quantities of anaphylactic antibody, but also by the production of high concentrations of precipitating antibody within a relatively short period of time.

It is interesting to note in this respect that following inoculation with one single dose of 100 γ of antigen per animal, the rats produced in 10 days an average concentration of antibody of 1860 γ/ml. This strong and accelerated antibody production may prove of interest for producing precipitating rat antibody with materials available only in limited quantities.

The fact that the anaphylactic antibody appeared in the serum in response to strong antigenic stimulation and even then only in very low concentration, together with its very short life in the circulation, may explain the failure of its demonstration by previous workers who, in most cases, employed intraperitoneal initial antigen injections followed by repeated intramuscular boosters. The very low serum titer of the anaphylactic antibody cannot be taken as an indication of the level of its synthesis if one considers that this antibody probably has a high affinity for certain rat tissues. It can be reasoned that significant serum concentrations, high enough to mediate passive sensitization, can be attained only after the tissues are saturated with anaphylactic antibody.

It must be stressed that although the use of Pertussis produced better results in the earlier stage of the immunization, anaphylactic antibody was also produced in the animals inoculated with Freund's adjuvant which, in addition, showed higher amounts of total precipitating antibody.

The mechanism by which the Pertussis organisms enhance particularly the production of anaphylactic antibody is not known at the present time. However, the demonstration that passive transfer of the sensitivity is possible with serum or with purified antibody preparations (12) rules out the possibility that the Pertussis organisms were only modifying the physiologic response of the animals to the mediators involved in anaphylaxis.

The characteristics of the PCA reaction in rats with rat anaphylactic antibody are interesting to compare with those observed in other species. The long incubation period necessary to attain maximal sensitization and the persistence of the antibody at the skin site for many days are in marked contrast with the PCA performed in guinea pigs with guinea pig or rabbit antibodies where the sensitization is maximal 3 or 4 hr after the injection of antibody and begins to diminish soon afterwards. On the other hand, they are quite similar to what is observed in the Prausnitz-Kustner reaction. In this respect, the rat anaphylactic antibody behaves as the human reagin, an analogy further extended by other properties of both antibodies (12).

The persistence of the sensitization at the skin site is a direct reflection of the affinity of the antibody for the tissues. It is reasonable to expect that in those species where the passive skin

sensitization lasts for many days, as in man and rat, the level of serum antibody in actively sensitized animals must be very low, which is actually the case.

The study of the precipitating reaction revealed some features of the rat antibody not usually found with rabbit antibodies. Maximal antibody precipitation occurred at considerable excess antigen, probably indicating a high dissociation constant of the antigen-antibody complexes. The salt concentration of the medium did not markedly influence the course of the precipitin reaction.

SUMMARY

The immune response of rats injected in the footpads with protein antigens mixed with *Bordetella pertussis* organisms or with complete Freund's adjuvant has been investigated.

All the animals produced a high concentration of precipitating antibody in a short period of time. A different type of antibody, able to transfer passive cutaneous anaphylaxis and mast cell damage in normal rats, could also be demonstrated in many of the sera of these animals. This anaphylactic antibody appeared early during immunization, was present in low titers, and disappeared from the serum in about 4 weeks. The presence and titer of the anaphylactic antibody did not show any correlation with the concentration of total precipitating antibody.

The conditions for passive cutaneous anaphylaxis in rats, with rat anaphylactic antibody, were investigated. The optimal sensitization required a latent period of 16 hr and persisted at the skin site for many days.

The significance of these findings is discussed.

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