Immunological Histamine Release from Rat Mast Cells *in vitro*: Effect of Age of Cell Donor.* (30584)

K. Frank Austen,† Kurt J. Bloch,‡ Alan R. Baker and Barry G. Arnason
Departments of Medicine and Neurology, Harvard Medical School at Massachusetts General Hospital, Boston

Recent studies in several mammalian species have delineated differences in the mechanisms of anaphylactic and cytotoxic reactions (1). For example, in the guinea pig one class of antibodies, 7S gamma 1, sensitizes tissues of the guinea pig for anaphylactic reactions *in vivo*(2) as well as *in vitro*(3); while another class of antibodies, 7S gamma 2, fails to mediate this reaction. In contrast, guinea pig 7S gamma 2, but not 7S gamma 1, antibodies fix complement in the presence of antigen and thus participate in complement-dependent cytotoxic injury(4). In addition, experiments performed with a series of organophosphorus compounds have revealed differences in the pattern of inhibition of the esterase involved in anaphylactic release of histamine and that involved in immune hemolysis(5). However, in all these comparisons, anaphylactic and cytotoxic reactions have been studied in different target cells, *e.g.*, mast cells of guinea pig skin or lung and sheep erythrocytes. In the present experiments the same target cell, the normal rat peritoneal mast cell, was sensitized *in vitro* for either anaphylactic or cytotoxic release of histamine. In the course of such experiments it was noted that the age of the cell donor markedly affected the ability of the cell to participate in both reactions.

Materials and methods. Antisera. The rabbit anti-rat gamma globulin antiserum (Ra anti-RGG) used contained antibodies directed against all rat immunoglobulins recognized to date, IgG, IgA, and IgM, and has been described previously(6). The preparation of rat IgG and the absorption of Ra anti-RGG with rat IgG was carried out as described(6). A rabbit antiserum directed against rat IgA globulin(6) was used to absorb IgA from rat sera; serial absorptions were performed until rat IgA could no longer be detected by immunoelectrophoretic analysis.

Rat antisera containing anaphylactic antibody were obtained by injecting rats in each foot pad with 0.25 ml of a mixture consisting of 1.0 mg dinitrophenyl-bovine gamma globulin (DNP-BGG)(7) or 5 times recrystallized egg albumin (Pentex, Inc., Kankakee, Ill.) and 10^9 *Bordetella pertussis* organisms per ml.§ The antisera were collected 7 to 15 days later and were either used intact or fractionated to obtain anti-hapten antibody (8). The rat antisera used had PCA titers ranging from 1:10 to 1:100. The purified antibody preparation had a protein concentration of 1.6 mg per ml, and its PCA titer was 1:30.

Preparation of peritoneal cell suspensions. Peritoneal cell suspensions were obtained from normal Sprague-Dawley or Fisher strain rats and from germ-free Fisher strain rats (Charles River Breeding Laboratories, Wilmington, Mass.) as previously described(9,10). The cells from each animal were sedimented in plastic centrifuge tubes at 400 *g* for 2 minutes at 4°C and resuspended in 2 ml Tyrode's-gelatin (0.1%) buffer. The cell suspensions from 3 to 12 rats were then pooled, centrifuged, resuspended in 6-24 ml Tyrode's-gelain buffer (2 ml per rat), and washed again. The final suspension contained approximately 5% mast cells as determined by phase microscopy, and it is assumed that the mast cells are the source of the histamine liberated in various experimental procedures(1,10).

Sensitization for the cytotoxic reaction. Sensitization was achieved by interacting 6-24 ml of a cell suspension for 5 minutes at 37°C

* Supported by U.S.P.H.S. AI-04536 and AM-3564 and the Charles A. King Trust from Mass. Gen. Hosp.

† Recipient of a Research Career Development Award from Nat. Inst. of Allergy and Infect. Dis.

‡ Senior Investigator, Arthritis Foundation.

§ The *Bordetella pertussis* organisms were generously supplied by Dr. Lee L. Schuchardt, Merck, Sharp & Dohme.

with an equal volume of rabbit anti-rat gamma globulin antiserum (Ra anti-RGG). After sensitization, the cells were washed twice, resuspended in 12-48 ml Tyrode's-gelatin solution and divided into 2.0 ml aliquots. Following preincubation of the aliquots at 37°C, 0.1 ml of normal rabbit serum was added as a source of complement. The reaction mixture was incubated for 7 minutes, then the cells were sedimented by centrifugation at 400 *g* for 5 minutes. The supernatant fluid was decanted and assayed for histamine using the isolated, atropinized guinea pig ileum. Residual histamine in cells was extracted by boiling(10) and results were expressed as percentage of total cell histamine released. Each variable was studied in 3 replicate aliquots: 2 received rabbit serum as a complement source, the third served as a control for spontaneous release of histamine. Duplicate samples gave values for histamine release which were within 3% of one another.

Sensitization for the anaphylactic reaction. Six-24 ml of a rat peritoneal cell suspension were sedimented and resuspended in a small volume of Tyrode's gelatin, usually 0.3-1.2 ml. Sensitization was achieved by incubating the cells at 37°C for 2½ hours with an equal volume of undiluted rat antiserum or for ½ hour with a purified antihapten antibody solution containing 0.27 mg antibody per ml. Restriction of volume enhanced the rate of sensitization, but it was necessary to agitate the mixture every 5-10 minutes to maintain a uniform suspension. At the end of the sensitization period, 0.15 ml aliquots were removed and added to 1.0 ml Tyrode's-gelatin buffer. The cells were sedimented by centrifugation, resuspended, washed twice more, and resuspended in 1.0 ml Tyrode's-gelatin buffer. Following preincubation at 37°C, 1.0 ml of specific antigen, dinitrophenyl-bovine serum albumin (DNP-BSA) or egg albumin, was added. The reaction mixture was incubated for 5 minutes, and the cells were then sedimented by centrifugation. The supernatant fluid and cells were analyzed for histamine and results expressed as the percentage of total cell histamine released. Each variable was studied in 3 replicate aliquots: 2 received specific antigen, the third served as a control for spontaneous release. Unsensitized cells from the same cell pool were used to rule out any effect of specific antigen alone.

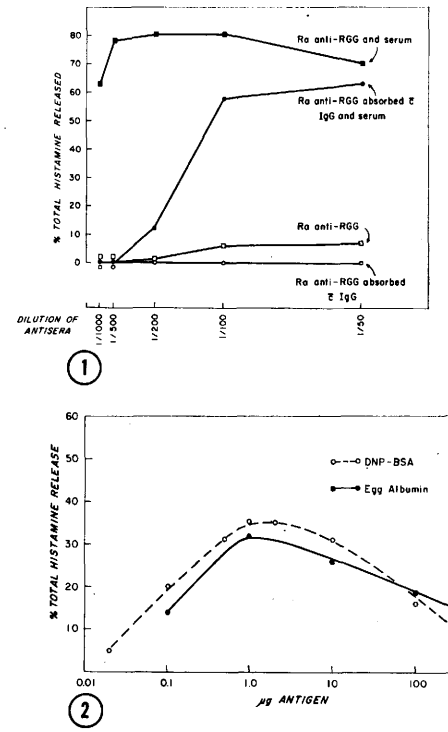

FIG. 1. Cells sensitized with rabbit anti-rat gamma globulin (Ra anti-RGG) before and after absorption of the antiserum with rat IgG; histamine released by subsequent addition of normal rabbit serum.

FIG. 2. Effect of antigen concentration on per cent histamine release from cells passively sensitized *in vitro* with whole rat antiserum against 5 × recrystallized egg albumin or with specifically purified rat anti-DNP antibodies. Cells challenged with egg albumin and DNP-BSA, respectively. The cell donors were 3½-week-old rats.

Passive cutaneous anaphylaxis in the rat was performed as described by Binaghi and Benacerraf(8).

Results. Cytotoxic reaction. In this reaction, rabbit antibodies directed against rat immunoglobulins apparently combine with rat immune-globulins on the surface of mast cells. The addition of fresh serum as a source of complement leads to release of histamine. Fig. 1 compares the sensitizing capacity of Ra anti-RGG before and after specific absorption with rat IgG. Unabsorbed Ra anti-RGG had virtually full sensitizing activity

TABLE I. *In vitro* Sensitization of Peritoneal Mast Cells from Rats of Different Ages.

	% of total histamine released		
	Cytotoxic*	Anaphylactic†	
Age in wk	Exp A	Exp B	Exp C
2½			29
3½	.5		
5		44	
10	66	25	
20	70	2	5

* Histamine was released by addition of normal rabbit serum to cells previously sensitized with Ra anti-RGG.

† In Exp B cells were sensitized by incubation for 2½ hr at 37°C with rat anti-egg albumin antiserum; in Exp C cells were sensitized for ½ hr at 37°C with specifically purified rat anti-DNP antibodies. Histamine was released by addition of specific antigen.

at dilutions of 1:500 or even 1:1000. Following absorption with rat IgG, the antiserum continued to react fully with IgA and IgM as determined by immunoelectrophoresis; but its ability to sensitize normal mast cells for histamine release by rabbit complement was markedly reduced at dilutions greater than 1:100. Whether the residual sensitizing activity of the absorbed antiserum is due to trace amounts of anti-rat IgG or due to the unabsorbed anti-IgA or IgM is not known. All studies of the cytotoxic reaction described below were performed with unabsorbed Ra anti-RGG diluted 1:750. It may therefore be assumed that sensitization for cytotoxic histamine release resulted primarily from the interaction of rabbit anti-rat IgG and rat IgG on the surface of the mast cell.

The ability of peritoneal mast cells to be sensitized with Ra anti-RGG increases with the age of the cell donor. As shown in Table I, Exp. A, it was not possible to sensitize cells from rats 3½ weeks old, whereas excellent sensitization was achieved with cells from rats 10 or 20 weeks old.

Anaphylactic reaction. In this reaction, rat peritoneal mast cells are sensitized for antigen-induced release of histamine by exposure to a specialized rat immunoglobulin, anaphylactic (or mast cell-sensitizing(11)) antibody. The effect of antigen concentration on histamine release from cells passively sensitized *in vitro* is depicted in Fig. 2. The optimal concentration of antigen was 1.0 μg antigen protein per ml of reaction mixture in both the rat anti-EA and anti-DNP systems; this concentration of antigen was used in all experiments described below. There was a striking inhibition of histamine release with excess antigen. Antigen-induced release of histamine from cells passively sensitized *in vitro* was not enhanced by addition of fresh normal rabbit, rat, or guinea pig serum.

The capacity of peritoneal mast cells to be passively sensitized with rat anaphylactic antibody decreased with increasing age of the cell donor. In Exp. B, Table I, after passive sensitization *in vitro* with rat anti-egg albumin antiserum, cells obtained from animals 5 weeks old released 44% of their total histamine on exposure to specific antigen, while cells from animals 20 weeks old, sensitized and challenged in an identical manner, released only 2%. In Exp. C, Table I, after passive sensitization with specifically purified rat anti-DNP, cells from rats 2½ weeks old released 29% of their histamine on challenge with antigen, while cells from animals 20 weeks old released only 5% under identical experimental conditions.

Table II shows the effect of a germ-free environment *in vivo* on the capacity for passive sensitization of mast cells *in vitro* by rat anaphylactic antibody. In Exp. D, the capacity of peritoneal mast cells from normal rats to be passively sensitized with rat anti-egg albumin antiserum again diminished with increasing age of the cell donor. In contrast, the capacity of the cells for passive sensitization was not diminished with increasing age when the donors were germ-free rats. Furthermore, as shown in Exp. D, the extent of

TABLE II. *In vitro* Sensitization of Peritoneal Mast Cells from Normal and Germ-Free Rats.

			% of total histamine released by specific antigen	
	Age in wk	Avg wt, g	Normal	Germ-free
D*	2½	45	15	22
	5	150	9	20
E†	2½	50	4	37

* Cells were sensitized for 2½ hr at 37°C with rat anti-egg albumin antiserum.

† Cells were sensitized for ½ hr at 37°C with specifically purified rat anti-DNP antibodies.

TABLE III. Effect of Removing Rat IgA from Rat Anti-Egg Albumin Antiserum on the Anaphylactic Activity of the Antiserum.

Absorption of rat antiserum with:	Rat PCA titer	% of total cell histamine released by specific antigen*
Normal rabbit serum	1:100	13 (16)
Rabbit anti-rat IgA	1:100	10 (11)

* Cells sensitized by incubation with antiserum for 2½ hr at 37°C. Data in parentheses refer to results with same antisera using a second cell preparation.

sensitization achieved with cells from germ-free rats clearly exceeded that obtained with cells from control rats of comparable age. In Exp. E, after passive sensitization with specifically purified rat anti-DNP, cells from germ-free rats released 37% of their total histamine on exposure to antigen, while cells from control rats, sensitized and challenged in an identical fashion, released only 4%.

The relationship of rat anaphylactic antibody to the 3 major classes of rat immunoglobulin has not been clearly defined. Separation of rat antiserum by starch block or agar gel electrophoresis indicated that the sensitizing activity did not migrate with the peak concentration of any of the 3 rat immunoglobulins. Furthermore, absorption of rat antiserum with rabbit anti-rat IgA so as to remove all rat IgA detectable by immunoelectrophoresis had little or no effect on the ability of the antiserum to sensitize rat skin for PCA or peritoneal cells for antigen-induced release of histamine (Table III).

Discussion. In the present experiments, histamine was released from rat peritoneal mast cells *via* 2 different mechanisms. Sensitization of normal mast cells for the cytotoxic reaction resulted from the interaction of a rabbit antiserum (anti-RGG) with a surface constituent of the mast cell. Absorption of the rabbit anti-RGG with rat IgG led to a marked reduction in sensitizing activity, (Fig. 1), suggesting that the surface constituent involved is adsorbed rat IgG. To serve as a substrate for the cytotoxic reaction, the mast cell must be obtained from a donor rat older than 3½ weeks (Table I). Presumably, mast cells obtained from young rats are insufficiently coated with rat IgG. As a result, interaction with rabbit anti-RGG does not lead to formation of an immune complex capable of activating the rabbit complement system so as to lyse the rat mast cell. These results were not expected because the serum concentration of IgG (determined by quantitative gel diffusion(12)) in a 3½-week-old rat is approximately two-thirds that of 10- and 20-week-old rats. Thus, factors in addition to the serum concentration of IgG appear to be involved in modifying the peritoneal mast cell surface so that sensitization can occur with rabbit antibody to rat immunoglobulins.

The evidence that it is the complement system in normal rabbit serum which releases histamine from cells sensitized with rabbit anti-RGG includes: heat lability at 56°C, inhibition of the serum effect by ethylenediaminetetraacetate, and removal by a preformed antigen-antibody aggregate(1).

The development of a procedure for studying the anaphylactic release of histamine from rat peritoneal cells passively sensitized *in vitro* yielded information on the optimal antigen concentration required (Fig. 2), the nature of the sensitizing antibody involved, the absence of a complement requirement, and the blocking of sensitization sites, presumably by immunoglobulins acquired *in vivo*. The antibody which sensitized the cells for anaphylactic histamine release was not associated with any of the 3 known rat immunoglobulins, IgG, IgA, and IgM, and most likely represents a fourth type of immunoglobulin. This view(13) is consistent with recent studies in man which also indicate that human skin sensitizing antibody may not be an IgA globulin(14-16). The release of histamine from normal peritoneal mast cells, passively sensitized *in vitro* with specifically purified rat anti-DNP, was not enhanced by addition of normal serum as a source of complement. Thus, a cell type capable of undergoing complement-dependent, cytotoxic release of histamine, did not require complement in order to release histamine by the anaphylactic route.

Sensitization for the anaphylactic release of histamine is thought to involve "fixation" of a specialized host immunoglobulin, anaphylactic antibody, to certain sites on target cells. The results reported herein (Tables I

and II) suggest that these sites have been preempted in older rats by anaphylactic antibody directed against indifferent, naturally occurring antigens. This is consistent with the demonstration that anaphylactic antibodies, acquired by active immunization, interfere with sensitization by antibodies of another specificity in the PCA reaction of the guinea pig(17). Alternatively, another immunoglobulin, such as rat IgG, may block access to sensitization sites by steric hindrance. In either event these studies demonstrate that there is a normal mechanism by which a host may develop some resistance to anaphylactic sensitization.

Summary. Suspensions of rat peritoneal cells were sensitized for either anaphylactic or complement-dependent cytotoxic release of histamine. In both cases the reactivity of the mast cells *in vitro* appeared to be influenced by the coating of rat immunoglobulin previously acquired *in vivo*. Sensitization for the complement-dependent release of histamine resulted from the interaction of rabbit anti-rat immunoglobulin antiserum with host IgG on the mast cell. Mast cells from young rats failed to participate in this reaction presumably because of an insufficient coating of IgG. In contrast, cells from young or germ-free rats had a much greater capacity for *in vitro* passive sensitization by anaphylactic antibody than cells of older rats. In older rats the sensitization sites may have been preempted by anaphylactic antibody directed against indifferent, naturally occurring antigens, or another immunoglobulin may block access to sensitization sites by steric hindrance.

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Received July 21, 1965. P.S.E.B.M., 1965, v120.

Xanthurenic Acid Dehydroxylation by Fecal Microflora. (30585)

A. N. BOOTH, D. J. ROBBINS, F. T. JONES, O. H. EMERSON AND M. S. MASRI
Western Regional Research Laboratory, Albany, Calif.*

Takahashi and Price reported formation of 8-hydroxyquinaldic acid from xanthurenic acid *in vivo* in 1958(1). Subsequently, Kaihara and Price concluded that this reaction depends on the gut microflora since 8-hydroxyquinaldic acid was not excreted in the urine of rabbits pretreated with the intestinal antibiotic neomycin(2).

In vitro dehydroxylation of caffeic acid to

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Dept. Agri.