IgE AND IgGa ANTIBODY-MEDIATED RELEASE OF HISTAMINE FROM RAT PERITONEAL CELLS

I. Optimum Conditions for In Vitro Preparation of Target Cells with Antibody and Challenge with Antigen*

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The capacity of homocytotropic antibodies to interact with certain target cells to mediate the release of pharmacologic agents upon contact with antigen has been demonstrated and reviewed for several mammalian species including mouse, rat, guinea pig, rabbit, dog, and man (1). Two major types of homocytotropic antibodies have been recognized. One type is characterized by its low concentration in serum after immunization, heat and sulfhydryl lability, and firm binding to target cells; homocytotropic antibodies of this type have been assigned to the IgE class in man (2) and several other species (3–5). The second type of homocytotropic antibody is characterized by its relatively high concentration in serum after immunization, heat and sulfhydryl stability, and weak binding to target cells; mouse IgG1 (6), guinea pig IgG1 (7), and rat IgGa (8) are examples of this type. Homocytotropic antibodies of both types may occur in a single species; however, it is not known whether both interact with the same target cell receptor and utilize the same biochemical pathways involved in the release of pharmacologic mediators.

In order to study these problems, it is necessary to use well-defined in vitro test systems in which components of the reaction can be controlled and varied. In vitro systems for studying the antigen-induced release of histamine from mixed rat peritoneal cells or purified rat peritoneal mast cells (1, 9, 10) have been described; these systems have used cells either passively or actively prepared with homocytotropic antibodies of the IgE type. Similarly, mixed peritoneal cells and isolated mast cells from mice actively immunized to produce a heat-labile antibody with a long latent period in skin sites have been shown

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to release histamine upon in vitro challenge with the sensitizing antigen (3). In addition, antigen-induced release of histamine has been demonstrated in vitro in a system employing relatively large amounts of mouse IgG1 antibody and a minimal latent period before antigen challenge (6). To date there have been no direct in vitro comparisons of antigen-induced histamine release from target cells prepared with the two types of homocytotropic antibody in a single species of animal; the present studies describe and compare the experimental conditions for the in vitro preparation of mixed rat peritoneal cells, or their mast cell fraction, with rat IgE or IgGa antibodies. A second paper (11) describes the interactions of these two types of antibodies and the target cell.

Materials and Methods

Antigens.—Dinitrophenyl-bovine serum albumin (DNP-BSA),[1] dinitro-phenyl-keyhole limpet hemocyanin (DNP-KLH), and dinitrophenyl-bovine gamma globulin (DNP-BγG) were prepared as described (12). The life cycle of *Nippostrongylus brasiliensis* was maintained in the laboratory as previously described (10). Adult worms were maintained in Eagle's basal medium containing L-glutamine, 20 mM, penicillin, 100 units/ml, and streptomycin 100 μg/ ml, at 37°C. The culture medium was changed after 24 hr and collected daily for a period of up to 5 days (13). Worms maintained in culture have been shown to release antigen which reacts with rat anti-*N. brasiliensis* homocytotropic antibody as tested by passive cutaneous anaphylaxis (PCA) (13). Individual batches of medium were tested for their ability to release histamine from rat peritoneal cells prepared in vitro with the appropriate antibody (see below). Active preparations were pooled, distributed in small portions, and stored at −70°C. These preparative procedures involved three cycles of freezing and thawing before final use of the antigen solution. Since repeated freezing and thawing has deleterious effects on the antigen, antigen solutions remaining at the end of an experiment were discarded.

Antisera.—Sera containing heat-stable IgGa antibodies were obtained from rats 4 wk after the injection into the footpads of 1 ml of emulsion containing 4.0 mg of DNP-BγG in complete Freund's adjuvant (14). The IgGa fraction was obtained by diethylaminoethyl (DEAE[DE-52])-cellulose chromatography (14) and concentrated to the volume of serum applied. These fractions had PCA activity (15) in rats up to a dilution of 1:16; PCA tests were performed after a latent period of 2 hr. All activity was heat stable, and there was no reaction if the latent period was extended to 48 or 72 hr.

Sera containing homocytotropic anti-worm antibodies were obtained 7-10 days after a fourth infection of rats with live *N. brasiliensis* larvae (10). These sera had a PCA titer of 1:400 to 1:1000; PCA tests were carried out after a 48 hr latent period.

Media.—The basic medium employed in all studies was medium RPMI 1634 (Grand Island Biological Co., Grand Island, N. Y.) plus 0.1% gelatin. This medium was modified further for the preparation of cells with IgE antibody and for challenge with antigen on the basis of the findings reported in the Results section.

For the preparation of cells with IgE antibody-rich sera, medium I was used. This medium consisted of RPMI 1634 plus 0.1% gelatin, modified so that it was 0.005 M with respect to *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, Calbiochem Los Angeles, Calif., No. 391338) and 2-(*N*-morpholino) ethanesulfonic acid (MES, Calbiochem, No.

[1] *Abbreviations used in this paper:* DNP-BγG, dinitrophenyl-bovine gamma globulin; DNP-BSA, dinitrophenyl-bovine serum albumin; DNP-KLH, dinitrophenyl-keyhole limpet hemocyanin; PCA, passive cutaneous anaphylaxis.

475893) buffers, 4.6×10^{-3} M with respect to $CaCl_2$, 10^{-7} M with respect to $Al_2(SO_4)_3$, and contained 35 ng/ml of heparin (Mann Research Laboratories, Inc., New York). In order to prepare this medium, solid gelatin (Fisher Scientific Co., Medford, Mass.) was dissolved in medium RPMI 1634 with cautious heating. After chilling to 4°C, 10% by volume of a solution 0.05 M with respect to both MES and HEPES buffers, and 1% by volume of a solution which was 3.8% with respect to $CaCl_2$, 10^{-5} M with respect to $Al_2(SO_4)_3$, and containing 3.5 μg/ml of heparin were added. The pH of the solution was adjusted to 6.60 ± 0.05 at 4°C using 0.1 N HCl. Once prepared, this solution was not stored for more than 1 day.

For the challenge of cells which had been prepared with IgE antibody and for the combined reaction using the IgGa antibody-mediated system, medium II was used. The difference between medium II and medium RPMI 1634 plus gelatin was a reduction in the concentration of sodium and potassium salts to 0.108 M and a reduction in the concentration of calcium ion to 4.0×10^{-4} M. A stock solution, medium III, was prepared which contained the salts and glucose of medium RPMI 1634, as well as the added buffers and gelatin of medium I, but which was devoid of sodium and potassium salts and had a lowered calcium content. Medium III contained 180 mg of glucose, 100 mg of gelatin, 10 mg of $MgSO_4 \cdot 7H_2O$, and 4.2 mg of $CaCl_2 \cdot 2H_2O$/100 ml of distilled water and was 0.005 M with respect to each HEPES and MES buffers. Medium II was prepared in the same manner as medium I, except that 40% by volume of medium III was added to the RPMI 1634 with gelatin, and the pH was adjusted to 6.80 ± 0.5 with 0.1 N HCl at 4°C.

A stock solution of Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was prepared in Tyrode's buffer (16) modified to contain 0.1% gelatin and to be 0.005 M with respect to each HEPES and MES buffers. The pH of this solution was adjusted to 6.6 with 0.1 N HCl, and the Ficoll concentration of the solution was adjusted to 35% (w/v) by the addition of modified Tyrode's buffer, using as a guide a standard curve relating concentration of Ficoll to refractive index (17).

Cell Suspension.—Unless otherwise stated, all experiments were conducted with the total mixed peritoneal cell population obtained from male Sprague-Dawley rats weighing 120–250 g, as described (18); media I and II were used to wash the cells in place of Tyrode's solution in preparation for the IgE and IgGa antibody-mediated systems, respectively. Mast cells were isolated from the peritoneal cell suspensions as follows: 2 ml of the Ficoll solution was placed into rinsed and dried 13 mm (I.D.) round bottom polycarbonate tubes at room temperature. Cells representing the average yield from one rat were suspended in 1 ml of medium I and were carefully layered over the Ficoll. The tubes were centrifuged for 15 min at room temperature in a clinical tabletop centrifuge (International Equipment Co., Boston, Mass.) at a speed setting of '6'. Except for this operation, all other centrifugations were carried out at 4°C in a refrigerated centrifuge (International Equipment Co., Model PRIL, rotor No. 284). The supernatant medium and the layer of cells at the interface between the medium and Ficoll were carefully aspirated. The Ficoll surface was cautiously rinsed twice with fresh medium I, before the Fincoll solution was diluted with 7 volumes of medium I, and the cells which had initially entered the Ficoll layer were recovered by centrifugation at 1000 rpm for 10 min. The pooled cells from several tubes were washed twice with medium I before use, and the purity of the preparations was assessed by differential cell count under the phase contrast microscope. The total histamine content of a sample of the cell suspension was assessed to obtain an estimate of the histamine content per mast cell. The purity of the preparations ranged from 95 to over 99% mast cells, and the histamine content per cell varied from 25 to 42 pg.

Conditions for Preparation of Cells with IgE Antibodies In Vitro and for Challenge with Antigen.—Experiments in which the release of histamine was initiated by antigen challenge of cells prepared with rat anti-*N. brasiliensis* antiserum rich in homocytotropic antibody are

termed IgE antibody-mediated reactions. This terminology seems permissible in view of the recent demonstration that rat homocytotropic antibody does belong to a unique immunoglobulin class termed IgE (4). The mixed peritoneal cells were sedimented by centrifugation at 500 rpm for 5 min and washed once in medium I. A cell count was made and 6–10 × 10^6 cells were placed in 11 × 75 mm plastic culture tubes (Falcon Plastics Los Angeles, Calif., division of BioQuest, No. 2052). After sedimentation of the cells at 500 rpm for 5 min, the supernatant was aspirated and preparation of the cells initiated with antibody in a total volume of 40 μl of medium I. In experiments involving the preparation of larger batches of cells with antibody, the ratio of the volume of antibody solution to cell number, and of total volume of cell suspension to surface area was maintained by using larger tubes. Cells were incubated for 2 hr at 30°C with intermittent suspension of the cells by gentle shaking. After incubation, a few drops of medium I were added and the cells recovered by centrifugation as above. Thereafter, each cell button was washed once with 0.5 ml of medium II and once with 1 ml of medium II. The contents of each tube were suspended in 2.2 ml of medium II and 0.5 ml was distributed into each of four tubes; of these, two tubes were challenged with antigen and two served as controls. After incubation at 37°C for 15 min, the cells were again sedimented and the histamine released into the supernatant was determined by bioassay on the guinea pig ileum (19). The residual histamine content of the cell pellets which had not been challenged with antigen was determined after extraction by boiling. Antigen-induced release of histamine, corrected for spontaneous release from control cells, was expressed as per cent of net histamine release.

Conditions for Combined Preparation and Challenge in the IgGa Antibody-Mediated System.— For all experiments dealing with IgGa anti-DNP antibody-mediated release of histamine, mixed peritoneal cells or isolated mast cells were treated as described above, except that medium II was used throughout. 3 million mixed peritoneal cells were placed into 1 ml disposable microcentrifuge tubes (Fisher, cat. No. 4-978-145) and sedimented by centrifugation at 500 rpm for 5 min. After removal of the medium by suction, the cells were resuspended in a final volume of 80 μl of medium II containing IgGa antibody fraction, antigen, and in certain experiments 5% fresh normal rat serum. The cells were generally incubated at 30°C for 30–45 min and pelleted by centrifugation as above. 50 μl of the supernatant solutions were transferred with disposable capillary pipettes into 0.5 ml of Tyrode's buffer and assayed for release of histamine. The histamine content of the residual cell buttons was determined as above.

RESULTS

In all experiments in this study, the preparation of cells with IgE antiworm antibody and their challenge with worm antigen are treated as two separate events. Cells prepared with IgE antibody retain their capacity to release histamine upon antigen challenge, even if the free IgE antibody is removed from the suspending medium by washing the cells several times. This property of the IgE antibody has afforded the opportunity to examine separately the conditions which are optimal for the preparation and challenge steps. The experiments which determined the optimum experimental conditions are detailed here. As will be shown in the next paper in this series (11), the preparation of cells for the release of histamine in the IgGa anti-DNP antibody-mediated system did not survive washing of the cells. Thus, it was not possible to study preparation with antibody and challenge with antigen as separate steps. Instead, all studies with the IgGa antibody-mediated system, of necessity, involved a combined preparation and challenge reaction.

Composition of the Incubation Media

pH Optima.—In order to assure viability of the peritoneal cells, experiments were conducted in medium RPMI 1634, one of a series of media which had been specifically developed for the culture of mammalian cells (20). For optimum control of pH, two sulfonic acid buffers, HEPES, pK 7.55, and MES, pK 6.15, were added at final concentrations of 0.005 M each. At these concentrations, the buffers had no deleterious effects on the cells. There was no change

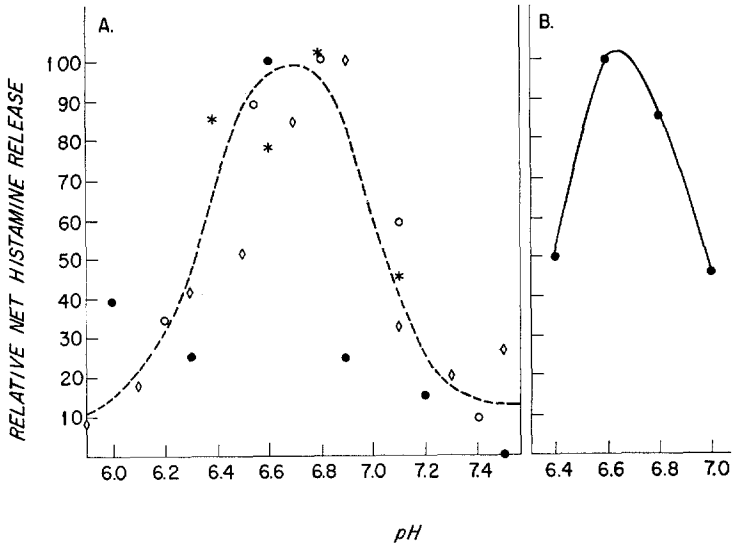

FIG. 1. Effect of pH of the incubation medium on the IgE (A) and IgGa (B) antibody-mediated release of histamine. A: 10 million mixed rat peritoneal cells were incubated with a 1:8 dilution of rat anti-*N. brasiliensis* IgE-rich antiserum (PCA titer 1:400 after a 72 hr latent period) in a volume of 40 μl of medium RPMI 1634 which was 0.05 M with respect to each HEPES and MES buffers and contained 0.1% gelatin; pH as shown was measured at 4°C. After 2 hr at 30°C, cells were diluted with 0.2 ml of the same medium and sedimented (500 rpm for 5 min). Cell buttons were washed with 0.5 ml and 1.0 ml of the same medium and were then suspended in 2.2 ml of the same medium. Duplicate samples (0.5 ml) were incubated (37°C for 15 min) with or without added *N. brasiliensis* antigen (1:17 dilution). Results represent four individual experiments (○, ●, ◇, *) in which the highest net per cent of release of histamine ranged from 11 to 25%, the spontaneous release ranged from 2.5 to 5%, and the residual cells in each tube contained 650–800 ng of histamine. For purposes of comparison, the highest net per cent of release of histamine in each experiment was designated as 100% and the remaining values normalized accordingly. B: 3 million mixed rat peritoneal cells in sextuplicate tubes were incubated with a 1:16 dilution of the IgGa antibody fraction of a rat anti-DNP antiserum reconstituted to the original volume of serum from which it had been isolated. (PCA titer of this fraction after a 2 hr latent period was 1:8.) DNP-BSA at a concentration of 0, 250, and 500 ng/ml was added to duplicate tubes, the final volumes adjusted to 90 μl with the same medium used in A, and the tubes incubated at 30°C for 15 min. The highest release of histamine was 5%, while the spontaneous release was 0%. The cells in each tube contained 2400 ng of histamine.

in pH of the solutions when cells were incubated for 2–3 hr in media containing these buffers at initial pH values within ±0.5 pH units of the pK values. Beyond this range, control of pH was less satisfactory and thus a combination of the two buffers was required to cover the complete range of pH values employed in the present experiments. The effect of the pH of the medium on the two step reaction (preparation with IgE antibody and challenge with antigen and the combined preparation and challenge in the IgGa antibody-mediated system), is shown in Fig. 1. In both the IgE and the IgGa antibody-mediated systems, a narrow range of pH values yielded optimum release of histamine.

The optima for the preparation and challenge steps in the IgE antibody-mediated reaction were also examined separately. The results, shown in Table I, indicate a narrow region of pH in which conditions are optimal for preparation and challenge. The pH conditions employed in the preparation step appeared to influence the pH found to be optimal for the challenge step. Based on these findings, the pH selected for preparation of cells by antibody was 6.60 ± 0.05, and for challenge with antigen, 6.80 ± 0.05. As already mentioned, the preparation and

TABLE I

*Effect of Varying the pH during either Preparation with Antiserum Rich in IgE Antibody or during Challenge with Antigen on Release of Histamine**

pH during challenge with antigen	pH during preparation of cells with antibody			
	6.4	6.6	6.8	7.0
6.4	21‡	13	14	13
6.6	24	20	13	9
6.8	19	20	25	10
7.0	10	12	11	11
7.2	10	11	6	4

* Experimental conditions same as in Fig. 1.

‡ Results are expressed as per cent net histamine released. The cells suspended in each tube contained 500 ng of histamine at the time of challenge with antigen; spontaneous release of histamine in control tubes was 2–6%.

antigen challenge steps in the IgGa antibody-mediated system could not be studied separately because of the failure of the preparation of cells with this antibody to persist through washing. Therefore, only a combination of the two steps was studied.

The preparation of 10^7 or fewer peritoneal cells in a totel reaction volume of only 40 μl minimized the spontaneous release of histamine and yielded greater antigen-induced release than was obtained with larger reaction volumes. Preparation of cells was therefore performed in a total reaction volume of 40 μl.

Effect of Ionic Strength and Urea.—The ionic strength of the incubation medium was increased by adding different amounts of sodium and potassium salts, at the molar ratio in which they are found in medium RPMI 1634. The ionic strength of the incubation medium was decreased by adding different amounts of medium III which lacked sodium and potassium salts. As shown in Fig. 2 A, there was a narrow range of ionic strength of the medium used during either preparation with antibody or challenge with antigen, which yielded optimum antigen-induced release of histamine. This range differed markedly for the two steps of the reaction; a 40% reduction in ionic strength of the medium yielded optimum conditions for antigen challenge, while the same conditions during preparation of the cells with antibody markedly reduced the release of histamine during the subsequent challenge step. Optimum

conditions for the IgGa antibody-mediated system resembled those for the challenge step in the IgE antibody-mediated system (Fig. 2 B).

Binaghi (21) reported a marked stimulation of the anaphylactic release of histamine from the guinea pig ileum on adding 0.2–0.4 M of urea to the medium. In the present experiments, the addition of 0.2 M of urea during the preparation step of the IgE antibody-mediated system

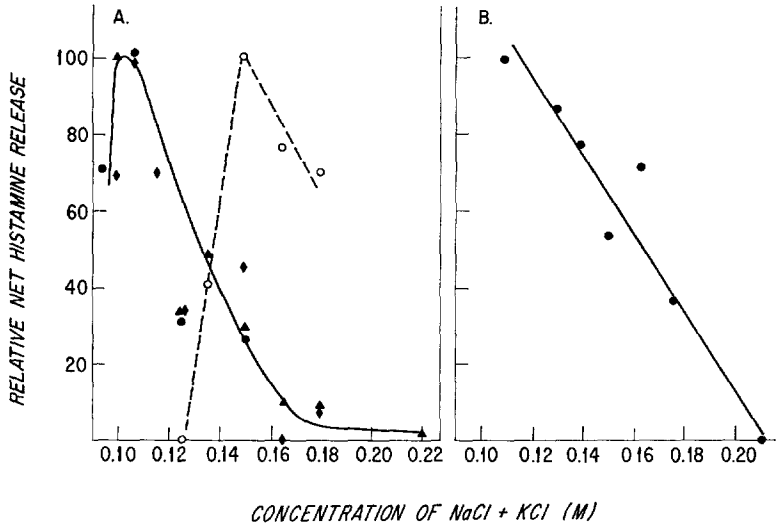

FIG. 2. Effect of ionic strength of the incubation medium on preparation with antibody or challenge with antigen in the IgE antibody-mediated system (A) and on the combined reaction in the IgGa antibody-mediated system (B). The experimental conditions were those described in Fig. 1, except for the pH which was 6.6 for preparation with IgE antibody and 6.8 for challenge with antigen in the IgE antibody-mediated system, as well as for the entire IgGa antibody-mediated system, and except for the variation in ionic strength. The ratio of NaCl to KCl was maintained at 30:1. (A) The ionic strength was varied during preparation of cells with antibody (○, -----). The ionic strength was varied during challenge with antigen in three separate experiments (▲, ●, ◆, ——). The maximum per cent of net release of histamine observed was 10–25%; spontaneous release of histamine was 1–2%; and the cell residues in each tube contained 1000–1200 ng of histamine. (B) Medium as in part A with added 5% fresh normal rat serum, pH 6.8. The 100% value represents 8% net release of histamine. Spontaneous release was 0%, and the cell residues in each tube contained 2400 ng of histamine. Results were normalized as in Fig. 1.

was without effect, while 0.4 M of urea increased the spontaneous release of histamine. When added during the challenge step of the reaction, urea at both concentrations strongly inhibited the net and total release of histamine.

Effect of Calcium and Magnesium Ion.—Medium RPMI 1634 is 0.42 mM with respect to $CaCl_2$, and 0.40 mM with respect to $MgCl_2$. A 10-fold increase in the calcium concentration during the preparation of cells with IgE antibody nearly doubled the subsequent antigen-induced release of histamine (Fig. 3). At higher concentrations, calcium ion became increasingly toxic to the cells, resulting in progressive spontaneous loss of intracellular histamine during

the challenge with antigen. This loss persisted despite washing of the cells after preparation with antibody and resuspension in a medium of lower calcium concentration during the challenge step of the reaction. During challenge with antigen, optimum results were obtained by reducing the calcium concentration. The effects of calcium on the preparation and challenge steps were apparently specific for calcium ion, since increasing the $MgCl_2$ concentration from 0.40 to 0.60 mM at the optimal calcium ion concentration neither stimulated nor inhibited either step of the reaction. It should also be noted that in order to lower the calcium ion concentration of the medium below 0.42 mM, magnesium-ethylenediaminetetra-

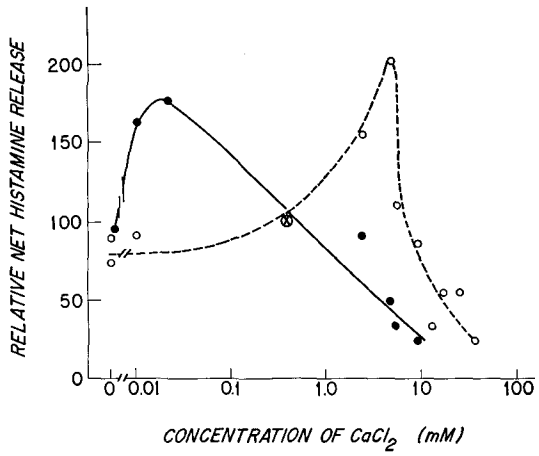

FIG. 3. Effect of calcium ion concentration of the incubation medium on preparation with antibody or challenge with antigen in the IgE antibody-mediated system. Preparation with antibody as described in Fig. 1 except that pH was 6.6, and $CaCl_2$ concentration was varied; challenge as described in Fig. 1 but using medium II (○, ——). Preparation with antibody as described in Fig. 1, except that pH was 6.6; challenge with antigen in medium II, but varying the $CaCl_2$ concentration (●, ——). Results are the averages of three experiments and are normalized by taking the release of histamine in the unaltered medium (0.42 mM $CaCl_2$) (⊗) as 100%. This value varied from 6 to 15% net histamine release in the different experiments. Spontaneous release of histamine was 5%, except at the highest $CaCl_2$ concentrations, where it approached 100%. Cell residues in each tube contained 500–600 ng of histamine.

acetic acid (EDTA) was added, this, in effect, replacing calcium ion by magnesium ion. As shown in Fig. 3, this change, when effected during the preparation step, resulted in a decrease in antigen-induced release of histamine.

Effect of Heparin.—Levy and Osler (22) have reported a stimulatory effect when heparin was added during the preparation of human leukocytes with human IgE antibody for antigen-induced release of histamine. The presence of minute amounts of heparin in the medium during the preparation of rat peritoneal cells with IgE antibody consistently increased the amount of histamine released upon challenge with antigen (Fig. 4). The presence of heparin during the challenge step of this reaction had no effect.

Heparin also did not affect the combined preparation and challenge reaction in the IgGa antibody-mediated system. For this reason, 35 ng/ml of heparin was incorporated into medium I and none was added to medium II.

Effect of Salts of Zinc, Copper, Iron and Aluminum.—The effect of adding various salts to the medium during preparation of peritoneal cells with IgE antibody are summarized in Table II. All of the salts tested enhanced the antigen-induced release of histamine under the conditions tested; aluminum sulfate had the greatest effect. At a concentration of 7×10^{-8} M,

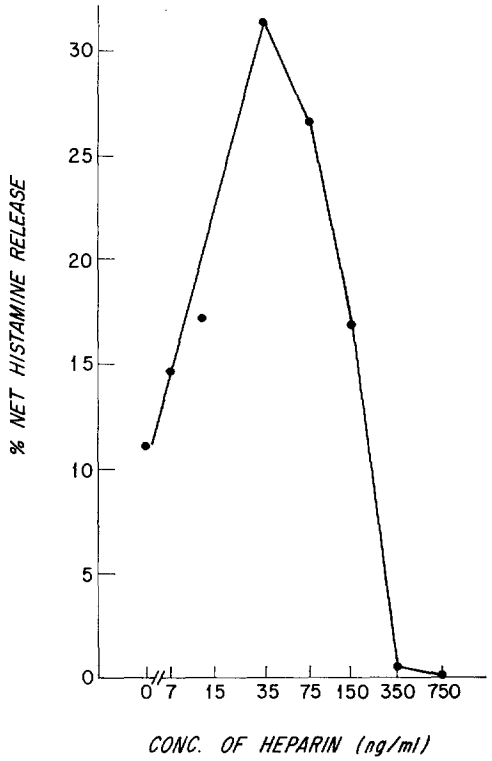

FIG. 4. Effect of the heparin concentration of the incubation medium during preparation of mixed peritoneal cells with IgE antibody-rich serum on the subsequent antigen-induced release of histamine. For preparation, medium I minus $Al_2(SO_4)_3$ and heparin was used, while medium II was used for challenge. Remainder of experimental conditions as in Fig. 1. Spontaneous release of histamine was 4%, and the cell residues in each tube contained 630 ng of histamine.

aluminum sulfate did not significantly enhance the spontaneous release of histamine from control cells, and a concentration of 10^{-7} M was routinely used for preparing cells with IgE antibody. The addition of aluminum sulfate did not affect the antigen-induced release of histamine in the IgGa antibody-mediated system.

The contents of the standard incubation media described in Materials and Methods were based on the results of the experiments cited above. These conditions were consistently confirmed to yield optimum results by incorporating all variables in a single replicate reaction mixture and comparing the resulting release of histamine with that obtained in reaction mixtures in which one optimal condition was not met.

Temperature and Time of Incubation

The optimum time of incubation of cells in the IgE antibody-mediated system was determined at 30°C and 37°C. Preparation of cells for optimum antigen-induced release of histamine was achieved more rapidly at the higher temperature, reaching a peak at 1 hr, but was only briefly maintained, falling off to only 25% of maximum by 2 hr. In contrast, the maximum release of histamine after preparation of cells at 30°C was achieved after 2 hr of incubation with antiserum, but was maintained through 4 hr of incubation. The maximum release of histamine in cells prepared at 30°C was approximately two-

TABLE II

*Effect of Adding Various Salts during Preparation of Cells with Antiserum Rich in IgE Antibody on the Subsequent Release of Histamine during Challenge with Antigen**

Addition	Total histamine release	Net histamine release‡
	(%)	(%)
None	14	11
$ZnCl_2$, 7×10^{-6} M	20	14
$CuSO_4$, 7×10^{-9} M	22	16
7×10^{-8} M	20	14
7×10^{-7} M	32	22
$FeCl_3$, 7×10^{-7} M	24	18
7×10^{-6} M	28	22
$Al_2(SO_4)_3$, 7×10^{-8} M	42	39
7×10^{-7} M	18	15
7×10^{-6} M	20	15

* Preparation with anti-*N. brasiliensis* antiserum was carried out in medium I without heparin or $Al_2(SO_4)_3$; challenge with antigen was performed in medium II. Other conditions as in Fig. 1.

‡ The cells suspended in each tube contained 630 ng of histamine at the time of challenge with antigen.

thirds of that which was achieved briefly at 37°C. There was no appreciable release of histamine with antigen from cells which had been prepared with antibody at 4°C for as long as 18 hr. An incubation period of 2 hr at 30°C was routinely used to prepare cells for the IgE antibody-mediated reaction.

The effect of temperature during challenge with antigen in the IgE antibody-mediated system was tested at 0°, 30°, and 37°C. After the addition of antigen, no release of histamine was observed during 2 hr at 0°C. Histamine was rapidly released after the addition of antigen at 30°C or 37°C; and maximum release, which was the same at both temperatures, was achieved essentially within 5 min. Longer incubation increased the spontaneous release of histamine from control cells and did not enhance specific release. Incubation with antigen was routinely performed at 37°C for 15 min.

In the IgGa antibody-mediated system, release of histamine did not reach peak values until 30–90 min after the addition of antigen at 30°C (Fig. 5 A). The continued release after 15 min to achieve a maximal value at 30–90 min was observed repeatedly. It was of interest to establish whether this continued release of histamine was related to the low ionic strength of medium II. As shown in Fig. 5 B, release of histamine in medium I was lower than that

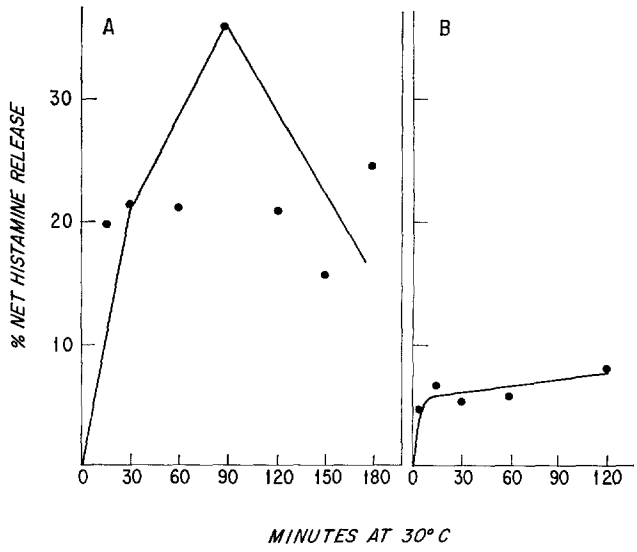

FIG. 5. Effect of the duration of the challenge period on the release of histamine in the combined IgGa antibody-mediated system. (A) Reaction carried out in medium II. (B) Reaction carried out in medium I (isotonic). Remaining conditions as in Fig. 1, but using an IgGa antibody fraction diluted 1:25. Optimal release of histamine was achieved with an antigen concentration of 500 ng of DNP-BSA/ml. Spontaneous release of histamine increased from an initial 1% to 5–8% in the course of the incubation. The cell residues in each tube contained 1200 and 600 ng of histamine in A and B, respectively.

achieved in medium II. The reaction was more rapid in medium I, reaching maximum value in less than 5 min. The combined preparation and challenge step in the IgGa antibody-mediated reaction was routinely performed at 30°C for 45 min.

Concentration of Reactants

Effect of Cell Concentration.—In the IgE antibody-mediated system, there was a linear relationship between target cell concentration and the net per cent of antigen-induced release of histamine (Fig. 6). In contrast, in the IgGa antibody-mediated system, there was an initial increase in histamine release

with increasing cell concentration, followed by a decrease at higher target cell concentrations (Fig. 6). The per cent of spontaneous release of histamine did not increase with increasing cell concentration.

Effect of Antibody and Antigen Concentration.—In both the IgE and IgGa

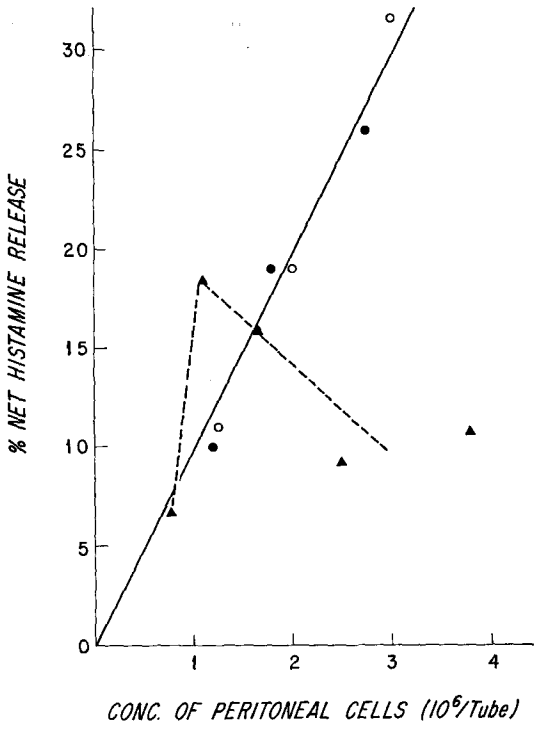

FIG. 6. Effect of the number of mixed peritoneal cells added per tube on the release of histamine in the IgE (——) and IgGa (– – –) antibody-mediated systems. Cells from animals of different weights were used. Cells from animals averaging 305 g (○) contained 300 ng of histamine/10^6 cells. Cells from animals averaging 260 g (●, ▲) contained 500 ng of histamine/10^6 cells. Spontaneous release of histamine was about 3%. Incubation conditions were as described in Fig. 1, except that medium I and II were used, and that a 1:80 dilution of an IgGa antibody fraction and 150 ng/ml of DNP-BSA were used in the IgGa antibody-mediated system.

antibody-mediated systems, an increased antibody concentration resulted in an increase in the net per cent of histamine released up to an optimal concentration (Fig. 7). Above this concentration, an inhibition of histamine release was observed. While the results in part A of this figure (IgE antibody-mediated system) were obtained with a fixed antigen dose, the results in part B (the combined preparation and challenge in the IgGa antibody-mediated system)

represent the maximum per cent of net histamine release which was observed with any of several different antigen concentrations used with each dilution of IgGa antibody fraction. The antigen concentration spanned five threefold dilutions varying from 5 to 405 ng of DNP-BSA/ml; maximum release occurred with 135 or 405 ng in all cases and was not appreciably different at these two antigen concentrations. On the basis of these experiments, the anti-*N. brasiliensis* antiserum (PCA titer 1:400) was used routinely in vitro at a dilution of

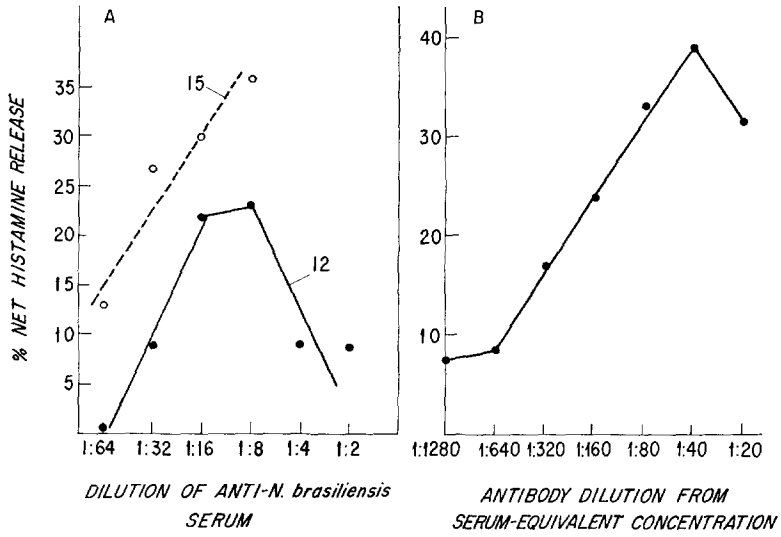

FIG. 7. Effect of the concentration of antibody on the antigen-induced release of histamine in the IgE (A) and the IgGa (B) antibody-mediated systems. Results in A are given for two different rat anti-*N. brasiliensis* antisera, Nos. 12 and 15; the same antigen concentration was used throughout. Results in B are the maximum per cent of net release of histamine at each dilution of the IgGa fraction of rat anti-DNP antiserum. Remaining conditions were as described in Fig. 1, using media I and II. The spontaneous release of histamine was 1–19% and 17–26% in A and B, respectively, increasing with antibody concentration. The cell residues in each tube contained 500–650 and 2350 ng of histamine in parts A and B, respectively.

1:8. The IgGa anti-DNP fraction (PCA titer 1:8) was used routinely in vitro at a dilution of 1:40.

The effect of antigen concentration on the release of histamine from peritoneal cells prepared in vitro with antiserum rich in IgE antibody is shown in Fig. 8 A. Increasing concentrations of antigen yielded increased per cent of net histamine release up to a 1:10 dilution of the *N. brasiliensis* culture medium. Higher concentrations of antigen were not tested because the volume of antigen required would have appreciably altered the composition of the reaction medium. *N. brasiliensis* culture medium did not release histamine from

normal rat peritoneal cells or cells prepared in vivo with IgE anti-DNP–KLH antibodies. The effect of antigen concentration on the IgGa antibody-mediated reaction is shown in Fig. 8 B. Again, it was observed that increasing concentrations of antigen produced increased per cent of net histamine release; maximal release was maintained over a wide range of antigen concentration followed by a decline at high antigen levels. In general, a 1:17 dilution of *N. brasiliensis* culture medium was used for the IgE antibody-mediated system

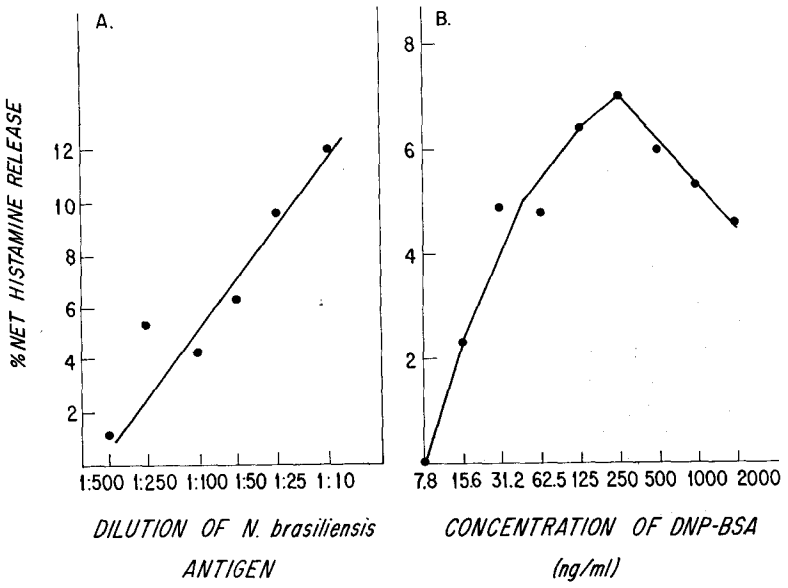

FIG. 8. Effect of the concentration of antigen on the release of histamine in the IgE(A) and IgGa (B) antibody-mediated systems. Experimental conditions, other than the concentration of antigen, were as described in Fig. 1, except that medium I and II and a 1:80 dilution of the IgGa antibody fraction were used. The spontaneous release of histamine was 1 and 1.5% in A and B, respectively. The cell residues in each tube contained 280 and 1600 ng of histamine in A and B, respectively.

and a concentration of 125 and 250 ng of DNP-BSA/ml was used for the IgGa antibody-mediated reaction.

Effect of Isolating Mast Cells.—Mast cells were isolated from the mixed peritoneal cell suspension by centrifugation with Ficoll. A mixed peritoneal cell suspension, a suspension of isolated and recombined cells, and a suspension of isolated mast cells were tested for the ability to release histamine after combined preparation and challenge in the IgGa antibody-mediated system (Table III). Isolated and recombined peritoneal cells were as active in this

system as the initial cell suspension, whereas the net per cent of release of histamine from isolated mast cells was considerably reduced.

Attempts were made to prepare isolated mast cells with antiserum rich in IgE antibodies. Variable results were obtained on challenge with antigen: In some experiments no antigen-induced release of histamine was observed; in others, the net release ranged up to 20%.

TABLE III

*Effect of Isolation of Mast Cells from Mixed Peritoneal Cells by Centrifugation into Solutions of Ficoll on the Release of Histamine in the IgGa Antibody-Mediated System**

Cells used‡	Total histamine release	Net histamine release
	(%)	(%)
6×10^6 mixed peritoneal cells/tube	15	8
3×10^6 isolated and recombined cells/tube	20	10
3×10^5 isolated mast cells/tube	9	4

* Reaction conditions consisted of a 1:80 dilution of IgGa antibody fraction, 125 and 250 ng of DNP-BSA/ml, and 5% fresh normal rat serum in medium II for a challenge period of 30 min at 30°C. Remaining conditions as described in Fig. 1. Results shown were obtained with 250 ng DNP-BSA/ml.

‡ Isolated and recombined cells were cells which were subjected to the entire mast cell isolation procedure (see Materials and Methods) but, rather than discarding the cellular interface between medium I and Ficoll, the total tube contents were diluted with medium I and the cells recovered. The purity of the mast cells used (3rd line) was 97%. The cells suspended in each tube contained 5800, 2400, and 6000 ng of histamine for the mixed cells, isolated and recombined cells, and isolated mast cells, respectively.

DISCUSSION

The Tyrode's solution, which had been used previously for in vitro studies involving peritoneal cells from rats (9, 10), is not suitable for a detailed analysis of the effect of pH on the reaction because it depends on bicarbonate ion for buffering. In the open system routinely used this buffer does not provide a constant pH. None of the buffers which have been traditionally used for pH stabilization in the physiologic range is free of objectionable properties. Thus, phosphate markedly affects the availability of calcium and magnesium ions, and cacodylate, barbiturate, and tris(hydroxymethyl)aminomethane (Tris) buffers are toxic to many cells (23). It is for these reasons that the sulfonic acid buffers were developed as a series of compounds which are free of the shortcomings cited (24, 25). HEPES and MES are members of this family of buffers. It is noteworthy that the pH optimum for both the preparation and the challenge steps of the IgE antibody-mediated system was distinctly lower than that of blood (Fig. 1, Table I) and that the optimum region was very narrow. This pH optimum (6.6–6.8) is also considerably lower than the

pH optimum (7.2–7.4) which has previously been reported for the human leukocyte system (26, 27) and for the guinea pig ileum (21). On the other hand, it is close to the pH of normal peritoneal fluid in the rat, which is about 6.8. A requirement for calcium ion has not been reported previously in the preparation of cells (or tissues) with IgE or IgGl antibody for the subsequent antigen-induced release of histamine (26, 28, 29). The narrow range of calcium concentration over which potentiation was observed in the present studies (Fig. 3) may account for the failure of other investigators to detect such enhancement (26). The toxicity of higher calcium concentrations on the target cells is in accord with previous observations (28).

The function of the other components of the medium used in the preparation step of the IgE antibody-mediated reaction is not clear. The stimulatory activity of low concentrations of zinc, copper, and iron (Table II) may be nonspecific and due to displacement of another essential metal ion which is bound to serum proteins. Both aluminum (Table II) and heparin (Fig. 4) may act by neutralizing certain interfering components in the IgE antibody-rich serum. Aluminum salts are effective adsorbents, and heparin can inhibit a large number of enzymatic reactions by virtue of its high negative charge (30). Another site of action of heparin may be at the receptor for IgE antibody on the target cell. This possibility seems unlikely since, at least initially, target cells are exposed to a higher concentration of heparin during the collection process.

Under the in vitro conditions employed, the extent of preparation of the target cells with IgE antibody, as determined by the subsequent release of histamine upon challenge with antigen, appears to be defined by the rates of at least two opposing reactions: the attachment of IgE antibody to receptor sites and the decay in the ability of prepared cells to release histamine upon challenge with antigen. At 37°C, the capacity of cells prepared with IgE antibodies to release histamine upon antigen challenge declines sharply by 2 hr in vitro; whereas in vivo, in the skin, the capacity of IgE antibodies to mediate PCA reactions increases for many hours, reaching peak value at 48–72 hr (10). Furthermore, the concentration of IgE antibody required for such in vitro reactions is several times greater than that required for PCA. These differences in persistence of a prepared cell and in the concentration of antibody required to prepare the cells may be explained in part by adverse alterations of the target cell in vitro. In addition, in vitro, competing immunoglobulins will be present in a fixed concentration, whereas, in vivo, those immunoglobulins with a lesser capacity to fix to target tissue may gradually diminish in concentration.

Other adverse effects were encountered whenever cells were washed in large volumes of medium, for example, during the removal of Ficoll after isolation of mast cells. Under these circumstances, the spontaneous release of histamine increased markedly, while the net release after antigen challenge was

diminished. The dependence of the net per cent of histamine release on the number of cells present (Fig. 6) probably also reflects the effect of target cell dilution. A similar dependence of net release of histamine on cell number has been reported by Henson (31). Levy and Osler also employed small volumes of medium during washing of cells in the human leukocyte test system (26).

The optimum conditions employed during the challenge step in the IgE antibody-mediated reaction and for both preparation and challenge in the IgGa antibody-mediated reaction were found to be identical and different from the conditions found to yield optimum preparation in the IgE antibody system. The demonstration that the conditions which are optimal for the IgGa antibody-mediated reaction are virtually the same as those for the challenge step in the IgE antibody-mediated reaction suggests that the release of histamine takes place by a very similar mechanism, even though it is initiated by the combination of antigen with two distinctly different immunoglobulins. The ionic strength of the medium used in these procedures, medium II, is so low that any red cells which contaminate the peritoneal cell preparations were promptly lysed upon suspending the cells in this medium. In the absence of an understanding of the mechanism of histamine release at the molecular level, the reason for the enhancing effect of low ionic strength, which was also seen qualitatively in other systems (32), is a matter of conjecture. Although anaphylactic release of histamine is not a cytotoxic reaction (33), the reaction may be enhanced by inflicting an incipient osmotic insult on the cells. The obligatory requirement for calcium and for conditions which permit the generation of energy from respiration during the release reaction, are in complete agreement with all previous work which has differentiated the anaphylactic release of histamine from cytotoxic reactions (34).

The capacity of the IgE antibody to bind to cells with enough tenacity to resist washing is well known. The transient nature of the binding of IgGa antibody to target cells as evidenced by the apparent failure to survive washing of the cells will be discussed in the subsequent paper (11). Further, although the optimum conditions for release of histamine are identical for the two systems, the two reactions differ in the time course of release of histamine. In contrast to the IgE antibody-mediated reaction, where histamine release upon antigen challenge is complete within a few minutes, the IgGa antibody-mediated reaction continues for as much as 90 min (Fig. 5). It is possible that the IgGa antibody–antigen complex is capable of triggering the release of histamine from many receptor sites because of a relatively transient interaction with any one site.

SUMMARY

The optimum conditions for antigen-induced release of histamine in the rat IgE and IgGa antibody-mediated systems were studied in vitro. The IgE antibody-mediated reaction could be separated into two steps: preparation of

target cells with antibody and challenge with antigen. The optimal conditions for these two steps were distinctly different. Release of histamine by IgGa antibody and antigen could not be separated into two steps, and the optimal conditions for the total reaction were identical to those of the antigen challenge step of the IgE antibody-mediated system.

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