ANTIBODIES INVOLVED IN ANTIGEN-INDUCED RELEASE OF SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS-A) IN THE GUINEA PIG AND RAT*

By DANIEL J. STECHSCHULTE,‡ M.D., K. FRANK AUSTEN, M.D., AND KURT J. BLOCH,§ M.D.

(From the Department of Medicine, Harvard Medical School, Robert B. Brigham Hospital, and the Medical Services, Massachusetts General Hospital, Boston)

(Received for publication 26 August 1966)

Certain immediate hypersensitivity reactions depend upon the union of antigen with specific antibody "fixed" on the surface of target cells. This interaction starts an enzymatic sequence leading to release of pharmacologically active substances from the sensitized cells or a fluid phase substrate (1–3). These agents (histamine, serotonin, SRS-A, and kinins) in turn increase vascular permeability and/or constrict smooth muscles, contributing to a clinical phenomenon known as "anaphylaxis." The homologous immunoglobulins which mediate passive cutaneous or systemic anaphylaxis in certain species have been characterized (4, 5), and in the guinea pig and rat the immunoglobulin which sensitizes guinea pig or rat mast cells, respectively, for antigen-induced release of histamine has been identified (6–8).

Slow reacting substance of anaphylaxis is an acidic material of unknown chemical composition (9). Its pharmacological effects include the ability to constrict smooth muscle of the guinea pig ileum, human bronchiole, and rabbit duodenum in the presence of an antihistamine; and the inability to act on the estrous rat uterus preparation (9). SRS-A is released into the incubation fluid following the exposure of sensitized guinea pig, rabbit, or human (10) lung tissue to antigen. Unlike histamine, which is set free from preexistent stores by certain antigen-antibody interactions, SRS-A is detected only after sensitized tissue is challenged with antigen, indicating that it is present in a precursor form prior to antigen-antibody interaction. The term "release" is used in this communication in place of the more accurate but cumbersome "formation and release" of SRS-A.

---

* This investigation was supported by Grant No. AI-04536 from the National Institute of Allergy and Infectious Disease, Grant No. AM-3564 from the National Institute of Arthritis and Metabolic Diseases, and a grant from the American Cancer Society, Inc. (Massachusetts Division).

‡ Recipient of a USPHS Postdoctoral Research Fellowship from the National Institute of Arthritis and Metabolic Diseases.

§ Senior Investigator, Arthritis Foundation.

Limited information is available concerning the mechanism of release of slow reacting substance of anaphylaxis (SRS-A) from guinea pig lung slices (11, 12). Rapp demonstrated that SRS-A was released into the peritoneal cavity of rats injected with hyperimmune rabbit antisera followed, after a latent period, by challenge with antigen (13). In these experiments, little or no release of histamine was observed. Mota has shown that mast cells were not altered by exposure to rabbit antiserum and subsequent challenge with antigen (14). It is also known that the release of histamine from rat mast cells can be effected by antisera containing homocytotropic antibody,[1] but not by hyperimmune rat antisera (14, 15). The possibility therefore existed that different homologous immunoglobulins might mediate the release of different pharmacological agents in a single species of animals. The present studies were undertaken to identify the guinea pig and rat antibodies involved in the release of SRS-A and to compare them with the homologous immunoglobulins mediating histamine release in these species.

Materials and Methods

Preparation of Antigen.—Bovine serum albumin (BSA) and egg albumin (Ea) (5 times recrystallized) were supplied by Pentex, Inc., Kankakee, Ill.

Dinitrophenyl bovine γ-globulin (DNP-BγG), dinitrophenyl bovine fibrinogen (DNP-fibrinogen), and dinitrophenyl bovine serum albumin (DNP-BSA) were prepared as previously described (18); DNP-BγG contained 40 and DNP-BSA 18 haptenic groups/mole of protein.

Nippostrongylus brasiliensis larvae were supplied by Dr. R. J. M. Wilson of the Harvard School of Tropical Medicine. Adult worms were obtained from the small intestine of rats 10 days following a primary infection with 3000–5000 larvae administered subcutaneously (19). The soluble extract obtained by grinding 1000 adult worms in 1.0 ml saline in a tissue grinder was used to challenge rats following intradermal (passive cutaneous anaphylaxis, PCA) or intraperitoneal injections of antiserum or isolated antiserum fractions.

Preparation of Antisera.—Guinea pig anti-DNP antisera were obtained from animals hyperimmunized with DNP-BγG emulsified in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) (20). Antisera were subjected to starch block electrophoresis (20), and γ_1- and γ_2-regions were identified by immunoelectrophoresis and by the serological properties of γ_1- and γ_2-antibodies (4, 21).

Rabbit antibovine serum albumin (Ra anti-BSA) antisera were obtained from albino rabbits injected in the toe pads with 2.5 mg of BSA emulsified in complete Freund's adjuvant. 3 months later, each rabbit received 6 mg of alum-precipitated BSA intravenously every other day for a 1 week period, and serum was obtained 7–10 days after the last injection.

---

[1] The term "homocytotropic antibody" has been proposed (16, 17) as a replacement for "mast cell–sensitizing" (6) or "anaphylactic" antibody (5). Redefinition is neceessry because the systemic or cutaneous reactions termed "anaphylactic" arise by various different immunological mechanisms and do not necessarily involve the mast cell or histamine release. It has therefore been suggested that the term "anaphylactic" be restricted to a description of in vivo reactions. "Homocytotropic antibody" will be used in this paper to describe a unique homologous immunoglobulin capable of sensitizing host mast cells for release of histamine upon contact with antigen; the existence of other homocytotropic antibodies, capable of sensitizing other target cells and/or mediating the release of other mediators, is considered likely.

Sprague-Dawley rats were injected in the footpads and intraperitoneally with an emulsion consisting of 0.5 ml of DNP-BγG (2 mg/ml) and 0.5 ml of complete Freund's adjuvant. 10 and 20 days later the animals were boosted subcutaneously and intraperitoneally with a total of 1 mg of antigen emulsified in complete Freund's adjuvant. On day 27, the rats were injected intradermally at two sites with 0.1 ml of antigen solution (1 mg/ml); these injections were repeated every 7 days until a definite Arthus reaction was obtained. "Hyperimmune" antisera were collected 35–45 days after initial immunization.

Antisera containing homocytotropic antibody were obtained from rats injected in each footpad with 0.3 ml of a mixture containing 3 mg of DNP-BγG and 2×10^9 *Bordetella pertussis* organisms[2] per ml, and the rats were exsanguinated 7–10 days later. Antisera containing high concentrations of homocytotropic antibody directed against worm antigens were obtained from rats 7–10 days after a second injection with 3000–5000 *N. brasiliensis* larvae. The larvae were administered to these animals 20 or 30 days after their primary infection.

Determination of Antibody Concentration.—The antibody concentration of various antisera was determined by precipitin analysis (5, 22). Rabbit anti-BSA antisera contained 4.0–6.5 mg antibody protein per ml; rat antisera contained 0.5 to 1.6 mg antihapten antibody per ml.

Passive hemagglutination, passive immune hemolysis, and complement fixation tests were performed as previously described (21).

Passive Cutaneous Anaphylaxis.—Passive cutaneous anaphylaxis was performed as described (23). Intracutaneous injections of 0.1 ml aliquots of serial dilutions of homologous antiserum or antiserum fractions were made into the shaved backs of albino guinea pigs weighing approximately 300 g. 4 hr later, the animals were injected intravenously with 1 ml of solution containing 1 mg of antigen in 0.5% Evans' blue dye. The reactions were read 30 min later, and the diameters of the blue spots recorded. Titers are expressed as the reciprocal of the final dilution yielding a positive reaction measuring 10 mm in diameter. All reactions were performed in triplicate, and no more than 16 sites were injected per animal.

Male Sprague-Dawley rats weighing 150–200 g were injected in the skin of the back with 0.1 ml volumes of various dilutions of antiserum or antiserum fractions. After a latent period of 4 *or* 48 hr, they were injected intravenously with 1 ml of a solution containing 1 mg of antigen or 0.2 ml of worm extract in 0.5% Evans' blue dye. 45 min after the injection of antigen, the rats were killed and skinned, and the underside of the skin was examined for the presence and extent of blueing. Results are recorded as described above.

Purification and Fractionation of Rat Antisera.—Rat anti-DNP antibodies were isolated according to method II of Eisen (24) with slight modifications. After decomplementing rat antiserum with a heterologous antibody-antigen system at equivalence, antibodies were precipitated with DNP-fibrinogen; the resulting antigen-antibody complexes were dissociated with DNP-ε-L-lysine. Excess hapten was subsequently removed from the antibody solution by prolonged dialysis against buffered saline.

Preparative starch block electrophoresis was performed as described (20, 25). The starting material consisted of the globulin fraction obtained by 50% saturation with $(NH_4)_2SO_4$ of 30 ml of rat anti-DNP antiserum (pool D, containing 1.1 mg of antibody protein per ml). Following electrophoresis, the starch block was cut into 1 cm strips, and the proteins contained in each strip were eluted by displacement filtration. After dialysis against phosphate buffer at 0.001 M, pH 7.4, fractions were lyophilized and subsequently resuspended in 1.0 ml saline. Protein determinations were made by a modified Folin-Ciocalteu method (26). Prior to intraperitoneal injection, each fraction was dialyzed against Tyrode's solution (glucose-free) for 3 hr.

Chromatography on DEAE-cellulose columns was performed using either sequential (27) or

---

[2] We are indebted to Dr. Irving Millman of Merck Sharp and Dohme for the heat-killed *B. pertussis* cultures.

gradient (28) elution with slight modifications. 4–8 ml of antiserum or the globulin fraction from 14 ml of antiserum was applied to columns 2.2 cm in diameter and 35 cm in length; sequential elution was performed with three phosphate buffers: 0.01 M, pH 7.5; 0.02 M, pH 6.2; and 0.05 M, pH 5.0. The amount of each buffer applied was equal to 1.5 times the void volume of the column. 4 ml samples were collected at the rate of approximately 0.7 ml per min. The protein concentration of the eluates was determined by spectrophotometry at 2800 A; protein peaks were combined and concentrated by ultrafiltration at 4°C. The concentrated samples were tested by immunoelectrophoresis and PCA in the rat, as well as for their ability to release SRS-A in the rat. Almost all of the rat γG-globulin was present in the first peak, labeled *a*, but trace amounts were distributed throughout the subsequent eluates. The rat globulin identified as γA (29) was not detected until phosphate buffer 0.05 M, pH 5.0, was applied; this buffer also eluted homocytotropic antibody. γM-globulin was not eluted with these buffers.

Gradient elution was performed by gradually combining 500 ml of phosphate buffer 0.01 M, pH 7.5, with 500 ml of phosphate buffer 0.1 M, pH 5, in 500 ml Erlenmeyer flasks. The rate of elution, pattern obtained, and processing of eluates were the same as described above.

Immunoelectrophoresis.—Immunoelectrophoretic analysis was performed according to a modification (4) of the method of Scheidegger (30). Lantern slides (3¼ × 4 inches) were coated with 1% agar in a barbital buffer, pH 8.6, ionic strength 0.075. Whole antiserum or antiserum fractions were subjected to electrophoresis at 15 ma and 200 v/slide for 120 min. Precipitin lines were developed with rabbit antisera to guinea pig or rat immunoglobulins. These antisera were obtained from rabbits immunized with antigen-antibody complexes according to the method of Arnason, de Vaux St.-Cyr, and Relyveld (29). Antiserum specific for rat IgA was prepared as described (29). By using constant amounts of antisera in both the center wells and troughs of the immunoelectrophoresis plate, it was possible to grade arbitrarily on a scale of 0–4+ the relative immunoglobulin content of a sample.

Bioassay of Histamine and SRS-A.—Histamine was assayed on the standard isolated guinea pig ileum preparation in the presence of 10^{-7} M atropine. Recorded values, representing the average quantity of histamine released in at least two replicate experiments, were determined by the closed-bracket assay technique comparing unknown samples with standard histamine base solutions. Values are corrected for spontaneous histamine release observed in control samples.

Slow reacting substance of anaphylaxis was assayed on the standard guinea pig ileum preparation which had been treated with atropine and mepyramine maleate[3] at a concentration of 10^{-6} M in order to abolish the histamine response. Recorded values represent the average number of SRS-A units released in two or more replicate experiments. The SRS-A assay was calibrated using a standard SRS-A sample supplied by Dr. W. E. Brocklehurst; 1 unit refers to a characteristic contraction of the guinea pig ileum equal in amplitude to approximately 5 mμg of histamine. Since the peritoneal fluid (obtained as described below) must be used for several different biological assays, it is necessary to dilute the original sample 5-fold. As a result, more than 15 units of SRS-A (5 units/ml) must be present in the original sample in order to be detected.

All samples of peritoneal fluid were assayed for the presence of kinins and serotonin using the estrous rat uterus (31, 32).

Sensitization of Perfused, Sliced Guinea Pig Lung.—Guinea pigs weighing approximately 300 g served as a source of lung tissue. The tissue was perfused, washed, sliced, and passively sensitized in vitro with 7Sγ_1 and 7Sγ_2 guinea pig antibody as previously described (7).

Preparation of Rats for SRS-A Release.—Male Sprague-Dawley rats weighing 150–400 g were injected intraperitoneally with antiserum or antiserum fractions. Only rats of approxi-

---

[3] Kindly supplied by Mr. R. C. Anderson of Merck Sharp and Dohme.

mately equal weight were used in a single experiment. 4–6 hr later, antigen, together with 250 μg of heparin in 5 ml of Tyrode's solution, was introduced by the same route. Exactly 5 min later, the rats were killed by a blow to the head, and peritoneal fluid was collected. The peritoneal cells were separated by centrifugation at 175 *g* for 10 min at 4°C. The supernatant fluid was immediately assayed for its SRS-A, histamine, serotonin, and kinin content or was stored at −70°C. The histamine content of peritoneal cells was determined after extraction of histamine by boiling in sealed test tubes for 10 min in 3 ml of physiological saline solution.

RESULTS

In Vitro Release of SRS-A from Guinea Pig Lung

Preliminary experiments indicated that guinea pig lung tissue exposed in vitro to guinea pig anti-DNP antisera and later challenged with antigen released both histamine and SRS-A. In order to identify the class of immunoglobulin responsible for mediating this reaction, guinea pig antisera were subjected to starch block electrophoresis. Both the immunoelectrophoretic and serological properties of eluates obtained from the starch block were used to identify the γ_1- and γ_2-regions. Thus, the ability of eluted fractions to sensitize guinea pigs for PCA reactions was used to locate the γ_1-antibody region, and the ability to sensitize antigen-coated, tannic acid–treated erythrocytes for lysis in the presence of complement, or to fix complement in the presence of antigen (in the Ea–anti-Ea system), was used to locate the γ_2-antibody region.

Results obtained with a fractionated guinea pig anti-DNP antiserum are presented in Fig. 1. Fractions 17–21, containing primarily γ_1-antibodies, were combined, as were fractions 9–13, containing primarily γ_2-antibodies. Guinea pig lung tissue exposed to γ_1-anti-DNP antibodies and challenged with antigen after a 2 hr interval released 3 μg of histamine (18% of total histamine available in the lung slices) and 600 units of SRS-A per g of wet lung. Tissue exposed to γ_2-antibodies failed to release either histamine or SRS-A. Similar results were obtained with isolated guinea pig γ_1- and γ_2-anti-Ea antibody fractions.

In Vivo Release of SRS-A from Rat Tissue

Experiments Involving Rabbit Antiserum.—Rats weighing 250–300 g were injected intraperitoneally with an aliquot of rabbit anti-BSA antiserum containing 2.5 mg of antibody protein. 4 hr later, 2 mg of BSA was injected intraperitoneally. Under these conditions, 200–300 units of SRS-A were found in the peritoneal fluid obtained 5 min after challenge with antigen. Doubling the quantity of antigen or antibody injected did not increase the yield of SRS-A.

In subsequent experiments, antigen was injected 15 min and 1, 3, 6, 9, 15, 24, and 48 hr after the administration of antiserum. Three or four rats were injected at each time interval. Maximum release of SRS-A was obtained after an interval of 3 hr and was maintained up to 9 hr; gradual decline in the yield of SRS-A occurred when the interval exceeded 9 hr. After a 48 hr interval, the release of SRS-A was approximately 30% of the maximum value obtained. A

4-fold increase in the quantity of either antigen or antibody failed to shorten the 3 hr interval required to obtain maximum release of SRS-A. The histamine content of the peritoneal fluids in these experiments was minimal, 0.25 μg or less.[4] These results indicated that, under the conditions employed, hyperimmune

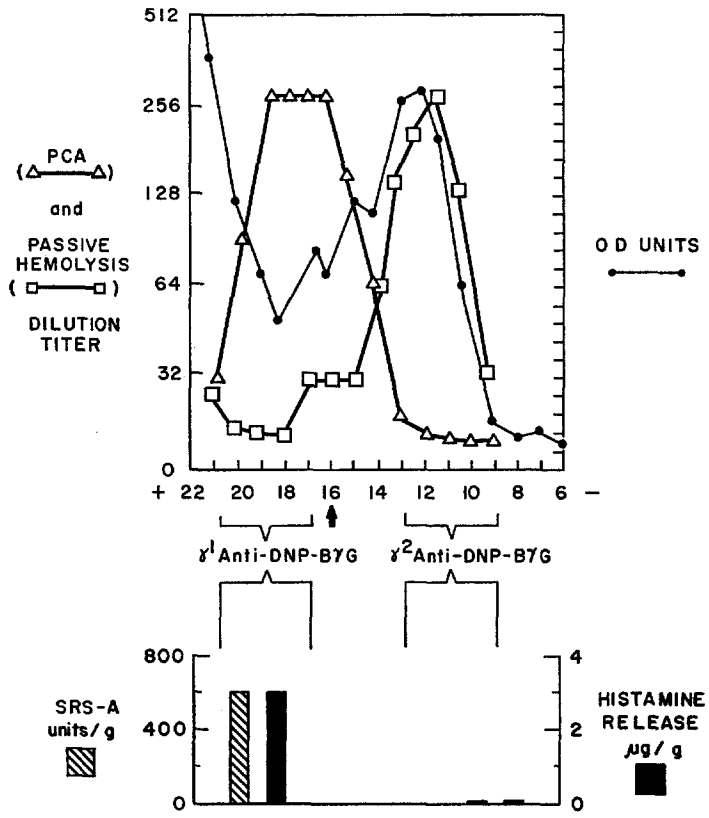


FIG. 1. Comparison of SRS-A and histamine release from sliced guinea pig lung sensitized with either $7S\gamma_1$ or $7S\gamma_2$-guinea pig antibodies separated by starch block electrophoresis. Each division of the right ordinate is equal to 0.02 OD unit (26).

rabbit antiserum prepared rat tissue for the release of SRS-A, rather than histamine.

[4] Although the histamine content of cells in the peritoneal fluid was determined in all experiments and ranged from 15 to 45 μg (increasing with the size of rats used), the amount of histamine released in the fluid is not expressed as a precentage of this value, since other sources of histamine exist in the peritoneal cavity. In each experiment the total cell histamine in control and experimental animals was similar, indicating that antiserum administration per se had not depleted cell histamine.

Experiments Involving Hyperimmune Rat Antiserum.—

Effect on SRS-A release of varying the dose of rat antibody: Rats were injected intraperitoneally with aliquots of rat anti-DNP antiserum containing 0.2–3.5 mg of antihapten antibody protein. 4 hr later, 2 mg of antigen, DNP-BSA, was administered, and 5 min later, peritoneal fluid was recovered and assayed for SRS-A. It was found that at least 0.5 mg of antibody protein was required to release measurable quantities of SRS-A in some animals; 1 mg of antibody protein consistently yielded measurable amounts of SRS-A in all animals tested. A 2.5 mg dose of antibody protein increased the release of SRS-A by approximately 40%; a further increase of antibody protein to 3.5 mg did not enhance the release of SRS-A. In order to conserve antibody, subsequent experiments

TABLE I

*Release of SRS-A from Rat Tissue Exposed to Different Pools of Hyperimmune Rat Antiserum**

Rat anti-DNP-BγG antiserum	PCA titer‡	Histamine release§	SRS-A release§
		μg/rat	*units/rat*
Pool A	0	0.15 ± 0.09	320 ± 120
Pool B	0	0.30 ± 0.1	1250 ± 600
Pool C	0	0.10 ± 0.07	450 ± 120

* 1 mg of precipitating antibody protein was injected intraperitoneally, and 4 hr later 2 mg of DNP-BSA was administered by the same route.

‡ Rats challenged after a 48 hr latent period.

§ Mean value and 1 SD from seven or more experiments.

were performed using 1.0 mg precipitating rat antibody protein per test animal.

Three different pools of hyperimmune rat anti-DNP antisera were compared at constant antibody doses for their ability to prepare rats for SRS-A release. These antisera were free of homocytotropic antibody as determined by a negative PCA reaction in the rat after a 48 hr latent period. The results of at least seven separate experiments with each pool are summarized in Table I. The average number of SRS-A units released per rat varied from 320 units for pool A to 1250 units for pool B. Histamine release was negligible in all instances, and only trace amounts of kinin (less than 0.001 μg/ml) or serotonin (less than 0.005 μg/ml) were detected. These results indicated that SRS-A was the major pharmacological agent released under the experimental conditions tested.

Effect on SRS-A release of varying the dose of antigen: Antiserum pools B and C were used to study the effect of various doses of antigen on the release of SRS-A from rat tissues. Four to six rats were injected intraperitoneally with 1.0 mg antihapten antibody protein, followed, after an interval of 4–5 hr, by

selected amounts of antigen. Rats injected with antibody from pool B received 0.5–4.0 mg DNP-BSA antigen; those injected with antibody from pool C received 0.1–4.0 mg antigen. As indicated in Fig. 2, 2.0 mg of antigen produced optimal release of SRS-A from tissue treated with either antiserum pool B or C, although the total amount of SRS-A released by antiserum pool B was considerably greater.

Effect on SRS-A release of varying the time of exposure of rat tisssue to rat anti-

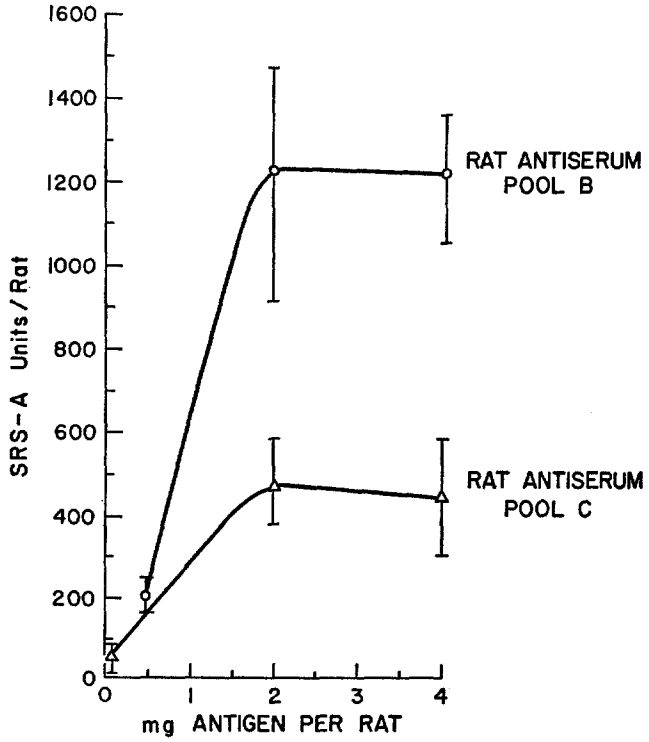


FIG. 2. Effect on SRS-A release of varying the dose of antigen.

serum: 2 mg of antigen was injected intraperitoneally 15 min to 20 hr after the administration of 1.0 mg antibody protein from antiserum pool A (Fig. 3). Four rats were tested at each time interval. Optimal release of SRS-A was obtained by injecting antigen 1–9 hr after administration of antiserum. Injection of antigen at 15 min or 20 hr yielded considerably less SRS-A: 18% and 30%, respectively, of the optimal values obtained. In contrast to rabbit antiserum, homologous antiserum prepared rat tissue for optimal release of SRS-A during a 1 hr rather than a 3 hr interval.

The relationship between release of histamine and SRS-A in the course of

these experiments is presented in Fig. 4. The release of histamine was 9 times greater after an interval of 15 min than after an interval of 1 hr, whereas the release of SRS-A was greater (330 units) after 1 hr compared to the release at 15 min (60 units). These results suggest that different mechanisms are involved in the release of these two pharmacological agents from rat tissues by hyperimmune rat antiserum and specific antigen.

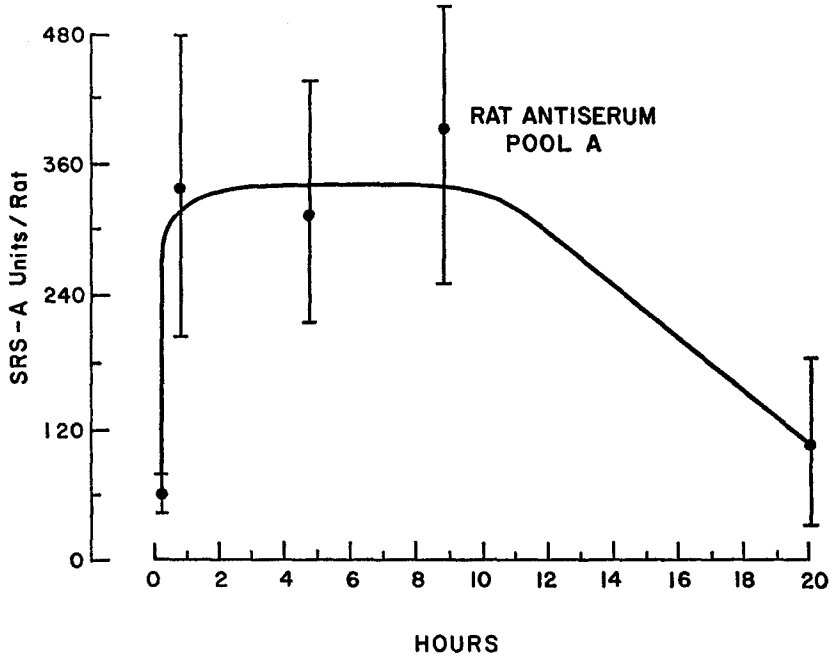


FIG. 3. Effect on SRS-A release of varying the time of exposure of rat peritoneal tissue to rat anti-DNP antiserum. 2 mg of antigen was used in the experiments depicted in Figs. 3–8.

Experiments Involving Fractionated Rat Antiserum.—

Electrophoretic separation of rat immunoglobulins: The globulin fraction of rat antiserum pool D was subjected to electrophoresis on a starch block; eluted fractions were tested as illustrated in Fig. 5. The relative amounts of the three rat immunoglobulins were estimated by immunoelectrophoresis; the peak concentration of rat γG-globulin was present in fractions 14–17, the peak of γA was present in fractions 18–22, and the peak concentration of γM-globulin was distributed about the zone of application of protein to the starch block. Aliquots of each concentrated fraction (0.7 ml) were tested for their ability to prepare rats for the release of SRS-A. Rats injected with fractions 14–20 released SRS-A upon challenge with antigen; the peak activity, 1200–2400 units/rat, was found

in fractions 16–18. Thus, SRS-A release did not coincide with the peak concentration of any of the three known rat immunoglobulins. Although trace amounts of histamine were apparently released by antibodies present in several fractions, somewhat greater release (as much as 0.4 μg/rat) was obtained with those fractions which also produced release of SRS-A.

Chromatographic separation of rat immunoglobulins on DEAE-cellulose columns: Chromatography of rat antiserum on DEAE-cellulose columns resulted in a more complete separation of rat immunoglobulins G and A. Columns 1 and 2 were each loaded with aliquots of antiserum pool D containing approximately 4.0 mg anti-DNP antibody protein. The profiles of protein concentration

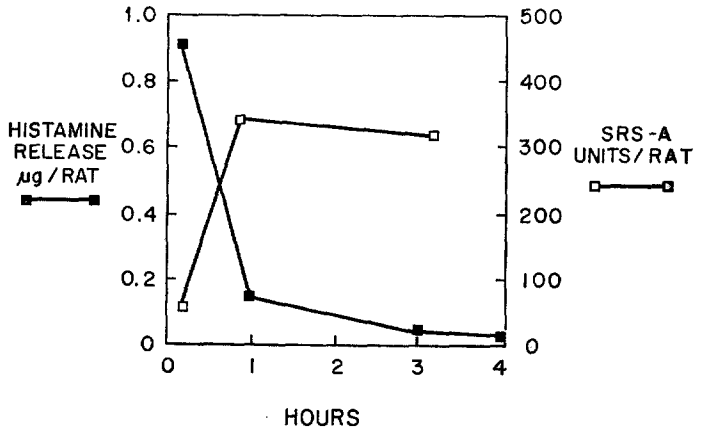


FIG. 4. Effect on SRS-A and histamine release of varying the time of exposure of rat peritoneal tissue to rat anti-DNP antiserum.

in eluates from these columns were very similar; for this reason an average of the two curves is shown in Fig. 6. The peaks of protein eluted with phosphate buffer 0.01 M, pH 7.5, were combined, as were the peaks obtained with phosphate buffer 0.05 M, pH 5.0. On immunoelectrophoresis, the former contained only γG, and the latter contained primarily γA-globulins. Rats were injected intraperitoneally with aliquots of these two peaks and subsequently challenged with antigen. A moderate amount of SRS-A (58 units/rat) was released in rats treated with the γG-containing peak; no SRS-A was released in rats treated with the γA-containing peak. Histamine release was negligible with both fractions.

Purified anti-DNP antibody obtained from 30 ml of pool D antiserum was applied to DEAE-cellulose column 6; two small peaks of protein concentration, *a* and *b*, were eluted (Fig. 7). On immunoelectrophoresis, pooled fractions *a* contained γG-antibodies, and pooled fractions *b* contained γA-antibodies.

Passive hemagglutination revealed comparable antibody titers in *a* and *b*: 1/10,240 and 1/5,120. Aliquots of *a* released 300 units of SRS-A and 3.8 μg of histamine per rat; aliquots of *b* failed to prepare rats for the release of either SRS-A or histamine.

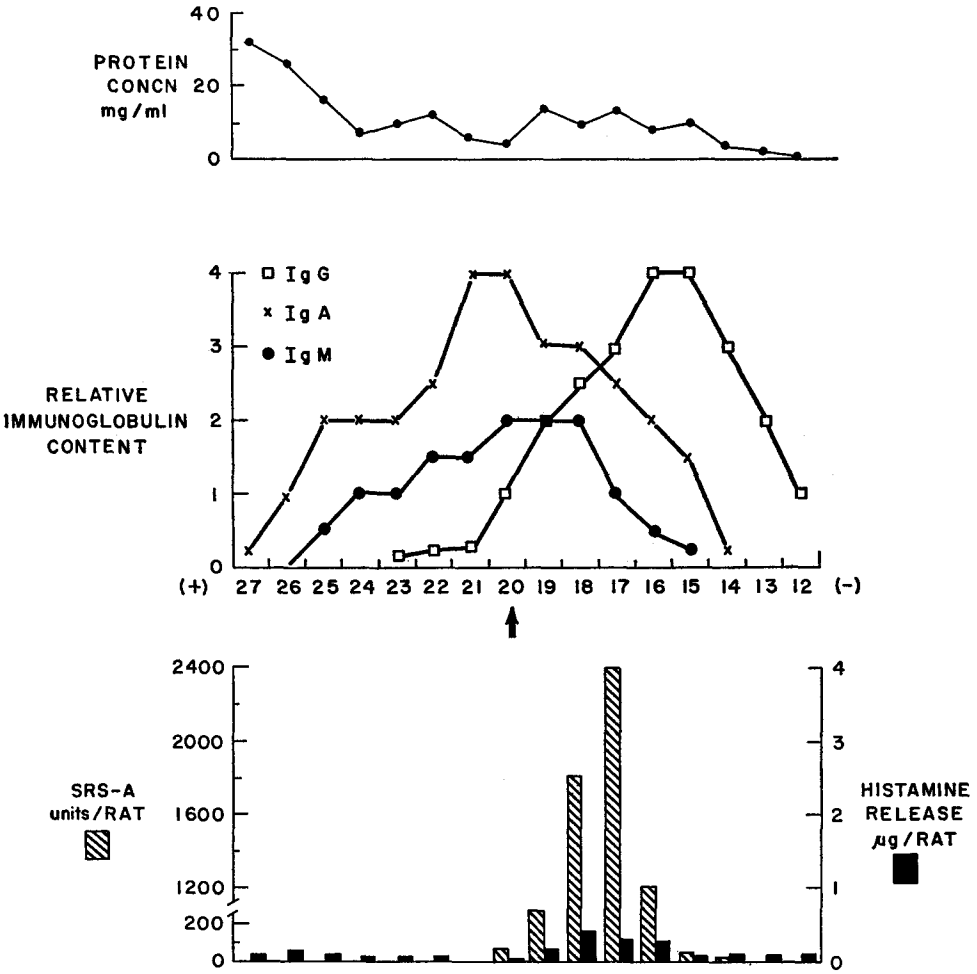


FIG. 5. SRS-A and histamine release with fractions of rat anti-DNP antiserum pool D (PCA negative at 48 hr) separated by starch block electrophoresis.

The experiments presented in Figs. 6 and 7 indicate that under the conditions tested, rat γG-, but not γA-, antibodies mediate the release of SRS-A in the rat.

Chromatographic separation of pools of antisera containing homocytotropic antibody: Pool E, consisting of a mixture of 4 ml of rat anti-DNP pool D (PCA

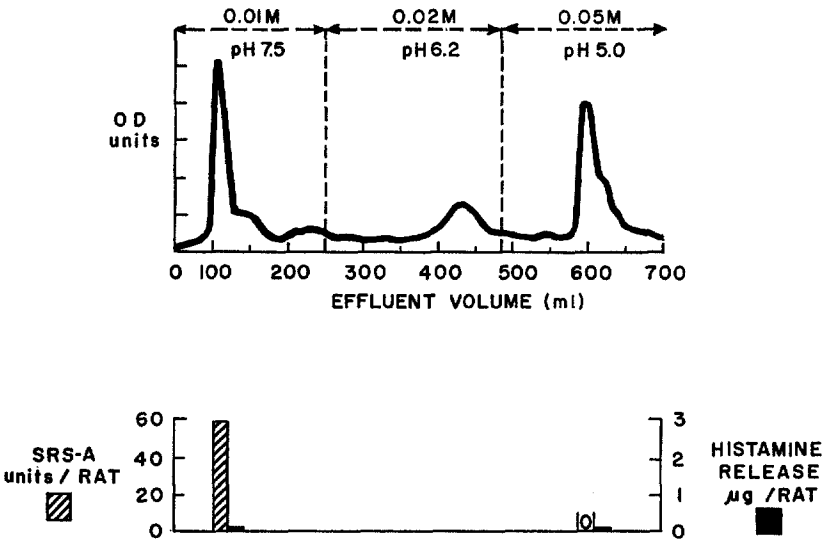


FIG. 6. SRS-A and histamine release with pooled eluates containing γG- or γA-globulins obtained by DEAE-cellulose chromatography of rat anti-DNP antiserum (pool D) on columns 1 and 2. Each division of the left ordinate is equal to 0.2 OD unit.

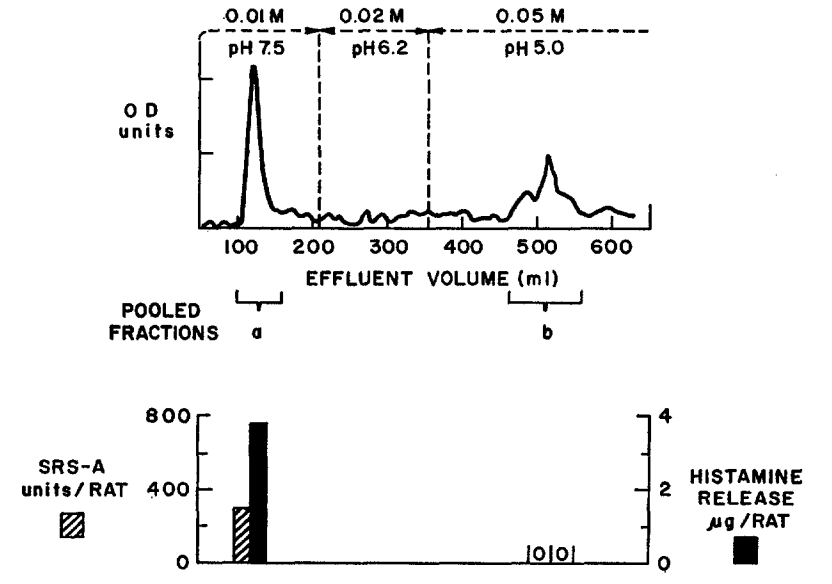
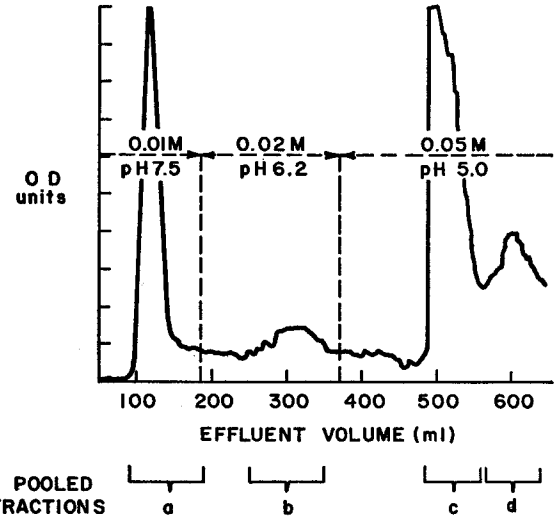


FIG. 7. SRS-A and histamine release with pooled eluates containing γG- or γA-antibodies obtained by DEAE-cellulose chromatography of rat anti-DNP antibodies (isolated from pool D) on column 6.

titer at 48 hr, 0) and 4 ml of anti-DNP antiserum obtained 10 days after immunization with a DNP-BγG and *B. pertussis* mixture (PCA titer at 48 hr, 1/80), was separated by DEAE-cellulose chromatography on column 14 (Fig. 8). Eluates were combined as indicated. On immunoelectrophoresis pooled frac-



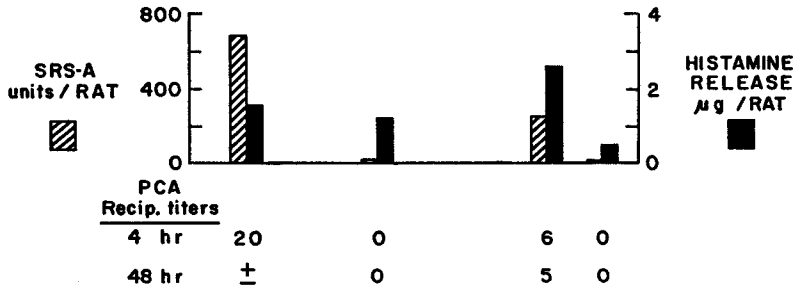


FIG. 8. SRS-A and histamine release with pooled eluates containing γG- and γA-globulins, as well as homocytotropic antibody obtained by DEAE-cellulose chromatography of rat anti-DNP antiserum (pool E) on column 14.

tions *a* and *b* contained γG-globulins, and pooled fractions *c* and *d* contained primarily γA-globulins. Aliquots of each were injected intradermally into a group of rats. Some animals were injected with antigen after 4 hr, and others after 48 hr. Dermal blueing was observed at sites injected with pooled fractions *a* in animals challenged after a 4 hr interval; this reaction was barely elicited

after 48 hr. Positive reactions were also observed at skin sites injected with pooled fractions *c* after a latent period of both 4 and 48 hr. These results indicate that homocytotropic antibody was eluted with proteins constituting peak *c*.

Aliquots were also used to prepare rats for the release of SRS-A. Pooled fractions *a* mediated the release of 675 units of SRS-A and 1.5 μg of histamine,

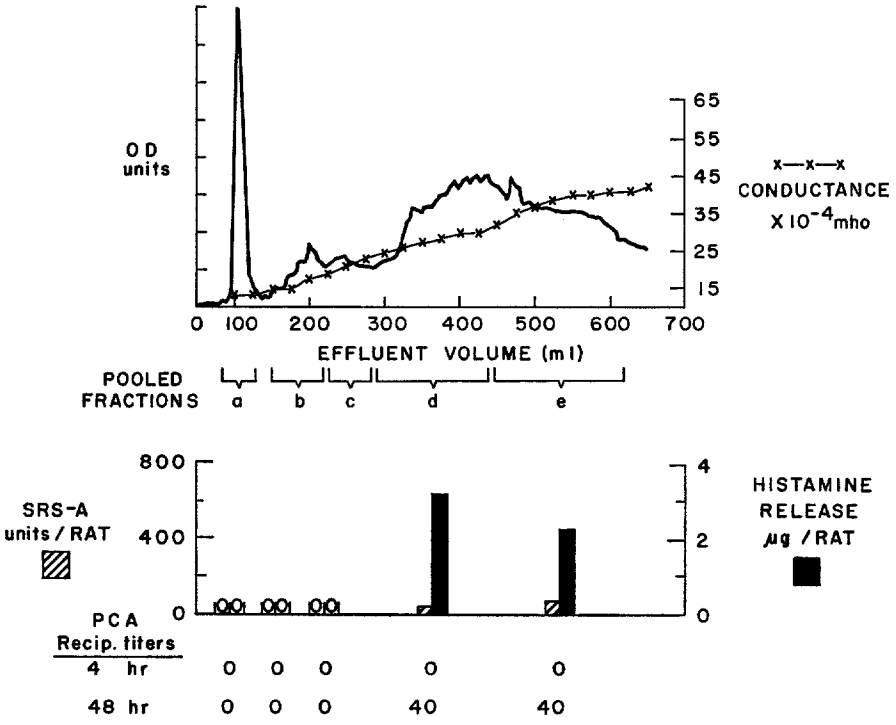


FIG. 9. SRS-A and histamine release with pooled eluates containing γG- and γA-globulins, as well as homocytotropic antibody obtained by DEAE-cellulose chromatography of rat anti-*N. brasiliensis* antiserum (pool 4) on column 12.

pooled fractions *b* mediated the release of 20 units of SRS-A and 1.1 μg of histamine, and pooled fractions *c* produced 249 units of SRS-A and 2.6 μg of histamine per rat. A small amount of both agents was released in rats injected with pooled fractions *d*. The ratio of SRS-A to histamine-releasing activity of *a* was almost 5 times greater than that of *c*. Thus, despite the presence of homocytotropic antibody in the eluates from column 14, the major SRS-A–releasing activity continued to be associated with the γG-containing fractions.

The effect of relatively large amounts of homocytotropic antibody on the release of SRS-A was further tested using eluates from a DEAE-cellulose

column loaded with the globulin fraction obtained from 14 ml of anti-*N. brasiliensis* antiserum pool 4 (PCA titer at 48 hr, 1/300). Eluates were combined as shown in Fig. 9. On immunoelectrophoresis pooled fractions *a*, *b*, and *c* contained γG-globulins; pooled fractions *d* and *e* contained primarily γA-globulins. PCA reactions were elicited only after a 48 hr latent period at sites injected with aliquots of *d* and *e*, indicating the presence of homocytotropic antibody in these fractions. Pooled fractions *a*, *b*, and *c* failed to prepare rat tissue for the release of either SRS-A or histamine, presumably because of the low concentration of γG anti-*N. brasiliensis* antibodies present in the antiserum pool employed. Pooled fractions *c* and *d* mediated the release of 3.3 and 2.4 μg of histamine per rat and only 15 and 30 units of SRS-A, respectively. Serotonin was also released following sensitization with homocytotropic antibody; the ratio of histamine to serotonin released was 10–20:1. The results demonstrate that homocytotropic antibody was capable of sensitizing mast cells for the release of histamine and serotonin. These same fractions mediated the release of small amounts of SRS-A.

DISCUSSION

Earlier studies have established that guinea pig γ_1-, but not γ_2-, antibodies mediate passive cutaneous and systemic anaphylaxis and sensitize guinea pig lung tissue in vitro for antigen-induced release of histamine. The present study has defined an additional function of γ_1-antibodies in the guinea pig, i.e. mediation of the release of SRS-A (Fig. 1). Indirect evidence suggests that guinea pig γ_1-antibodies become attached or "fixed" to host mast cells so that subsequent contact with antigen leads to the release of various pharmacological agents (4, 7). Whether the mast cell is the source of SRS-A in the guinea pig is not established. Release of histamine from certain sensitized guinea pig tissues was always accompanied by the release of SRS-A (10, 11), and Austen and Brocklehurst, using a variety of enhancing and inhibiting substances, never observed changes in yield of histamine or of SRS-A independently of the other (12). Although these data are compatible with the view that both substances arise from the same cell, it is also possible that both are liberated from different cells by the union of antigen with γ_1-antibodies on the surface of the respective cells.

Rabbit anti-BSA antiserum prepared rat tissue for the release of SRS-A, and little or no histamine was released into the peritoneal cavity under experimental conditions leading to optimal release of SRS-A. The immunoglobulin class of the rabbit antibodies involved in this reaction was not identified; however, the immunization schedule used generally results in the production of γG-antibodies.

A latent period of several hours was required between injection of rabbit antiserum and challenge with antigen. This interval may be required for the

diffusion of antiserum to critical sites on tissue, or it may represent the interval needed for antiserum to become "fixed" on a target cell which is the source of SRS-A.

Hyperimmune rat antiserum also prepared rat tissue for the release of SRS-A, accompanied by minimal release of histamine (Table I and Figs. 2–4). Experiments performed with antiserum fractions obtained by DEAE-cellulose chromatography indicated that antibodies with properties of γG-globulins were primarily involved in the release of SRS-A (Figs. 6–8). On starch block electrophoresis (Fig. 5), the peak of SRS-A–releasing activity migrated faster than the bulk of γG-globulins. The latter observation may indicate that SRS-A–releasing activity is a function of electrophoretically "fast" migrating γG-antibodies or that specific rat γG–anti-DNP antibodies migrate "faster" than the bulk of nonspecific γG-globulins (33).

Rats injected with constant amounts of precipitating antibody from different antiserum pools were found to differ considerably in the quantity of SRS-A released (Table I). This finding suggests the possibility that SRS-A–releasing activity is not a function of the precipitating antibody but may instead be a function of an unidentified subclass of γG-globulins. However, the three antiserum pools were not tested simultaneously on a single group of rats, and it is therefore possible that differences in sensitivity of the animals may account for the results observed. It is also possible that the antiserum pools differed in their avidity for antigen.

A latent period was required for the optimal release of SRS-A by hyperimmune rat antiserum. The latent period with homologous antiserum was shorter than that required with heterologous antiserum; the reason for this disparity is unknown. The demonstration of a latent period (Fig. 3) again suggests the possibility that rat γG-antibodies may be "sensitizing" a target cell for the release of SRS-A upon contact with antigen. It is unlikely that the target cell involved is the rat mast cell, since there is considerable evidence that hyperimmune rabbit and rat antisera do not sensitize rat mast cells. Rats actively immunized so as to achieve high titers of circulating antibody consistently became prostrate following intravenous injections of specific antigen; yet, when the mesenteries of these animals were incubated with specific antigen, there was no detectable histamine release (15, 34). Rats injected intravenously or intraperitoneally with rabbit or rat hyperimmune antiserum and subsequently challenged with antigen administered intravenously also undergo a shock reaction; however, examination of their tissues revealed their mast cells to be intact, and no histamine was released into the plasma during the course of the reaction (14). Rat mesentery or diaphragm exposed in vitro to rabbit antiserum failed to show mast cell damage or histamine release on exposure to antigen (14). In view of these observations, it is unlikely that rabbit or rat hyperimmune antisera mediate the release of SRS-A via sensitization of rat mast cells.

In the present experiments antiserum fractions from the γA-region, containing homocytotropic antibody, sensitized the peritoneal tissues for histamine release, accompanied by minimal (Fig. 9) to moderate (Fig. 8) release of SRS-A. In the experiment shown in Fig. 8, fractions from the γA-region mediated considerable histamine release and only moderate SRS-A release, while fractions from the γG-region directed against the same antigenic determinant prepared rat tissue for the release of large amounts of SRS-A and moderate amounts of histamine. The association of histamine release, in the relative absence of SRS-A release, with material from the γA-region is particularly well illustrated by Fig. 9. Although the homocytotropic antibodies which sensitize for histamine release are associated with the γA-region, absorption experiments with rabbit anti-rat γA have indicated that homocytotropic antibody is not a γA-globulin (8).

It is evident that the rat γG-globulin or a subfraction thereof, which prepares an unidentified target cell or tissue in the rat peritoneal cavity for release of SRS-A upon contact with antigen, is distinctly different from the rat immunoglobulin, homocytotropic antibody, which sensitizes rat mast cells for histamine release. In preparing rat tissue for SRS-A release, the rat γG-globulin or subfraction thereof may be acting as a second type of homocytotropic antibody.

Although histamine release was not observed in rats prepared with whole hyperimmune rabbit or rat antiserum and challenged with antigen after several hours, histamine release was observed if the latent period was brief (Fig. 4). On the other hand, varying histamine release did occur under routine conditions when the rats were prepared with γG-fractions (Figs. 5–8). Since γG-antibodies do not sensitize mast cells for the release of histamine, other mechanisms must be responsible for the release of histamine observed. One such mechanism may involve the formation of histamine-releasing agents by rat γG-antibody–antigen–complement aggregates (35).

In conclusion, it now appears to be possible to distinguish at least two different mechanisms capable of producing the immediate hypersensitivity lesion, termed PCA, in the rat. Homocytotropic antibody provokes passive cutaneous anaphylaxis by sensitizing rat mast cells for release of histamine and serotonin upon contact with antigen. PCA reactions elicited in this manner may be inhibited by antihistamine and antiserotonin agents (36); the optimal latent period for this PCA reaction is 24–72 hr (5, 36).

A clinically identical reaction may be elicited in the rat following intradermal injection of rabbit or rat hyperimmune antiserum, or γG-fractions, followed by intravenous injection of antigen in 4 but *not* 24 hr. This reaction is not suppressed by antihistamine or antiserotonin agents (37) and does not involve mast cell degranulation (14). Whether it represents a single phenomenon or several different ones is uncertain; at least three associated factors have now been recognized. First, the intensity of the lesion can be correlated to some

extent with the level of serum complement activity (38). Second, the reaction can be suppressed by rendering rats leukopenic (39). The latter observation prompted Lovett and Movat (39) to speculate that formation of microprecipitates of antigen and antibody, followed by phagocytosis of the aggregates by polymorphonuclear leukocytes and release of their lysosomal enzymes, was responsible for the change in capillary permeability. Finally, homologous hyperimmune antiserum or its γG-fraction has been shown to mediate release of SRS-A from rat tissue. The interrelationship of these three factors and their role in producing PCA lesions provoked with hyperimmune serum in the rat remains to be further assessed.

Preliminary studies were performed to evaluate the possible effect of SRS-A on vascular permeability. Peritoneal fluid containing large amounts of SRS-A and negligible concentrations of histamine, serotonin, and kinin were injected into the shaved dorsal skin of albino guinea pigs just prior to intravenous administration of Evans' blue dye. Blueing was observed in a dose-response fashion and could not be attributed to the cumulative action of trace amounts of other mediators; however, the contribution of soluble complexes could not be assessed. Any definite conclusion as to the role of SRS-A in increasing vascular permeability must be deferred until the pure material is available for study.

SUMMARY

The antigen-induced release of SRS-A and histamine was studied in the guinea pig and rat using whole and fractionated antiserum preparations.

Guinea pig 7Sγ_1-antibody sensitized sliced guinea pig lung tissue for antigen-induced release of both SRS-A and histamine; neither substance was released from lung tissue prepared with 7Sγ_2-antibody.

Rats injected intraperitoneally with hyperimmune rabbit or rat antiserum released only SRS-A in significant amounts when challenged with antigen by the same route. A definite time interval between the injection of antiserum and challenge with antigen was required for optimal release of SRS-A.

Fractionation of rat antiserum demonstrated that the immunoglobulin responsible for most of the SRS-A release from rat peritoneal tissue was a γG-antibody or fraction thereof. Acting in this capacity, the γG-antibody or its subfraction may be considered a second type of homocytotropic antibody. Fractions of rat antisera containing the first type of homocytotropic antibody, i.e. antibody mediating release of histamine and serotonin, prepared peritoneal tissues for the release of large amounts of these pharmacological agents and only small amounts of SRS-A.

Two different mechanisms for the production of PCA lesions in the rat were considered. One of these involves the antigen-induced release of histamine and serotonin from mast cells sensitized by homocytotropic antibody. This reaction has an optimal latent period of 24–72 hr. The second mechanism involves the

local combination of antigen with "hyperimmune" heterologous or homologous antisera. This reaction can be elicited after a latent period of 4 but not 24 hr; host complement and leukocyte lysosomal enzymes, as well as SRS-A, may be involved.

We acknowledge with gratitude the expert, untiring technical assistance given by Miss Barbara Moyer.

BIBLIOGRAPHY

1. Mongar, J. L., and H. O. Schild. 1957. Inhibition of the anaphylactic reaction. *J. Physiol., (London).* **135**:301.
2. Austen, K. F., and W. E. Brocklehurst. 1961. Anaphylaxis in chopped guinea pig lung. I. Effect of peptidase substrates and inhibitors. *J. Exptl. Med.* **113**:521.
3. Becker, E. L., and K. F. Austen. 1964. A comparison of inhibition by phosphonate esters of the first component of complement and the antigen-induced release of histamine from guinea pig lung. *J. Exptl. Med.* **120**:491.
4. Ovary, Z., B. Benacerraf, and K. J. Bloch. 1963. Properties of guinea pig 7S antibodies. II. Identification of antibodies involved in passive cutaneous and systemic anaphylaxis. *J. Exptl. Med.* **117**:951.
5. Binaghi, R. A., and B. Benacerraf. 1964. The production of anaphylactic antibody in the rat. *J. Immunol.* **92**:920.
6. Mota, I. 1963. Biologic characterization of "mast cell sensitizing" antibodies. *Life Sci.* **7**:465.
7. Baker, A. R., K. J. Bloch, and K. F. Austen. 1964. *In vitro* passive sensitization of chopped guinea pig lung by guinea pig 7S antibodies. *J. Immunol.* **93**:525.
8. Austen, K. F., K. J. Bloch, A. R. Baker, and B. G. Arnason. 1965. Immunological histamine release from rat mast cells *in vitro*: Effect of age of cell donor. *Proc. Soc. Exptl. Med.* **120**:542.
9. Brocklehurst, W. E. 1962. Slow reacting substance and related compounds. *Progr. Allergy.* **6**:539.
10. Brocklehurst, W. E. 1960. The release of histamine and formation of a slow-reacting substance (SRS-A) during anaphylactic shock. *J. Physiol., (London).* **151**:416.
11. Chakravarty, N., and B. Uvnas. 1960. Histamine and a lipid-soluble smooth-muscle stimulating principle ("SRS") in anaphylactic reaction. *Acta Physiol. Scand.* **48**:302.
12. Austen, K. F., and W. E. Brocklehurst. 1961. Anaphylaxis in chopped guinea pig lung. II. Enhancement of the anaphylactic release of histamine and slow-reacting substance by certain dibasic aliphatic acids and inhibition by monobasic fatty acids, *J. Exptl. Med.* **113**:541.
13. Rapp, H. J. 1961. The release of a slow-reacting substance (SRS) in the peritoneal cavity of rats by antigen-antibody interaction, *J. Physiol., (London).* **158**:35P.
14. Mota, I. 1962. Failure of rat and rabbit antiserum to passively sensitize normal and pertussis-treated rats and mice so as to induce mast cell damage and histamine release on later contact with antigen. *Immunology.* **5**:11.
15. Austen, K. F., and J. H. Humphrey. 1962. Mechanism of the anaphylactic re-

lease of histamine and slow reacting substance. *Immunopathology, Intern. Symp., 2nd, Brooks Lodge.* 88.

16. Becker, E. L., and K. F. Austen. 1966. Mechanisms of immunologic injury of rat peritoneal mast cells. I. The effect of phosphonate inhibitors on the homocytotropic antibody–mediated histamine release and the first component of rat complement. *J. Exptl. Med.* **124**:379.
17. Austen, K. F., and E. L. Becker. 1966. Mechanisms of immunologic injury of rat peritoneal mast cells. II. Complement requirement and phosphonate ester inhibition of histamine release by rabbit anti-rat gamma globulin. *J. Exptl. Med.* **124**:397.
18. Benacerraf, B., and B. Levine. 1962. Immunologic specificity of delayed and immediate hypersensitivity reactions, *J. Exptl. Med.* **115**:1023.
19. Wilson, R. J. M., J. Bienenstock, and K. J. Bloch. 1966. Anaphylactic antibody response to *Nippostrongylus brasiliensis* infection in the rat. *Federation Proc.* **25**:681.
20. Benacerraf, B., Z. Ovary, K. J. Bloch, and E. C. Franklin. 1963. Properties of guinea pig 7S antibodies. I. Electrophoretic separation of two types of guinea pig 7S antibodies. *J. Exptl. Med.*, **117**:937.
21. Bloch, K. J., F. H. Kourilsky, Z. Ovary, and B. Bencerraf. 1963. Properties of guinea pig 7S antibodies. III. Identification of antibodies involved in complement fixation and hemolysis. *J. Exptl. Med.*, **117**:965.
22. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. 2nd edition. Charles C Thomas, Springfield, Ill. 22.
23. Ovary, Z. 1958. Immediate reactions in the skin of experimental animals provoked by antigen-antibody interaction. *Progr. Allergy.* **5**:460.
24. Eisen, H. N. 1965. Preparation of purified anti-2,4-dinitrophenyl antibodies. *Methods Med. Res.* **10**:94.
25. Kunkel, H. G. 1954. Zone electrophoresis. *Methods Biochem. Anal.* **1**:141.
26. Lowery, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
27. Askonas, B. A., C. P. Farthing, and J. H. Humphrey. 1960. The significance of multiple antibody components in serum of immunized rabbits. *Immunology.* **3**:336.
28. Fahey, J. L., P. F. McCoy, and M. Goullian. 1958. Chromatography of serum proteins in normal and pathologic sera: The distribution of protein-bound carbohydrate and cholesterol, siderophilin, thyroxin-binding protein, B_{12}-binding protein, alkaline and acid phosphatases, radio-iodinated albumin and myeloma proteins. *J. Clin. Invest.* **37**:272.
29. Arnason, B. G., C. de Vaux St.-Cyr, and E. H. Relyveld. 1965. Role of the thymus in immune reactions in rats. *Intern. Arch. Allergy Appl. Immunol.* **25**:206.
30. Scheidegger, J. J. 1955. Une micro-méthode de l'immunoéléctrophorèse. *Intern. Arch. Allergy Appl. Immunol.* **7**:103.
31. Gaddum, J. H. 1959. Pharmacology. 5th edition. Oxford University Press, N.Y. 245.
32. Spragg, J., K. F. Austen, and E. Haber. 1966. Production of antibody against

bradykinin: Demonstration of specificity by complement fixation and radioimmunoassay. *J. Immunol.* **96**:865.

33. Nussenzweig, V., and B. Benacerraf. 1964. Differences in the electrophoretic mobilities of guinea pig 7S antibodies of different specificities. *J. Exptl. Med.* **119**: 409.
34. Humphrey, J. H., K. F. Austen, and H. J. Rapp. 1963. *In vitro* studies of reversed anaphylaxis with rat cells. *Immunology.* **6**:226.
35. Archer, G. T. 1960. Release of histamine from mast cells by antigen-antibody reactions *in vitro*. *Australian J. Exptl. Biol. Med. Sci.* **38**:147.
36. Mota, I. 1964. The mechanism of anaphylaxis. I. Production and biologic properties of "mast cell sensitizing" antibody. *Immunology.* **7**:681.
37. Brocklehurst, W. E., J. H. Humphrey, and W. L. M. Perry. 1955. The role of histamine in cutaneous antigen-antibody reactions in the rat. *J. Physiol.*, (*London*). **129**:205.
38. Osler, A. G., M. M. Hawrisiak, Z. Ovary, M. Siqueira, and O. G. Bier. 1957. Studies on the mechanism of hypersensitivity phenomena. II. The participation of complement in passive cutaneous anaphylaxis of the albino rat. *J. Exptl. Med.* **106**:811.
39. Lovett, C. A., and H. Z. Movat. The role of PMN-leukocyte lysosomes in tissue injury inflammation and hypersensitivity. III. On passive cutaneous anaphylaxis induced in the rat with homologous and heterologous hyperimmune antibody. *Proc. Soc. Exptl. Biol. Med.* In press.