INFECTION AND IMMUNITY, Oct. 1970, p. 462-467
Vol. 2, No. 4

Histamine-Releasing Factor Generated by the Interaction of Endotoxin with Hamster Serum

WILLIAM A. HOOK, RALPH SNYDERMAN, AND STEPHAN E. MERGENHAGEN

Laboratory of Microbiology, Immunology Section, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 9 June 1970

Injection of hamsters with endotoxin (LPS) resulted, 4 to 14 days later, in a marked decline in the mast cell count and in a reduction of the available histamine content of cells obtained by peritoneal lavage. The release of histamine from isolated peritoneal hamster mast cells in vitro could be achieved by incubating LPS with fresh hamster serum followed by addition of mast cells and a second incubation. The generation of a histamine-releasing factor from serum could be inhibited by conditions which inhibit the consumption of complement by LPS. Several substances could be substituted for LPS in the generation of histamine-releasing activity including heat-aggregated human gamma globulin, zymosan, purified cobra venom factor, and washed antigen-antibody precipitates. With all of these agents, the generation of a histamine-releasing substance from hamster serum was accompanied by consumption of hemolytic complement. The results suggest that LPS indirectly leads to the release of histamine from mast cells as a result of interactions with the complement system.

The numerous physiopathological effects produced with bacterial endotoxin (LPS) have been reviewed by Zweifach and Janoff (22). Among the systemic, pyrogenic, vascular, and immunological responses to LPS mentioned, it is noteworthy that some investigators believe that histamine release resulting from LPS injection is a major factor in the irreversible phase of endotoxic shock (22). Of particular interest is the similarity between the vascular response toward LPS and the histamine-releasing compound 48/80 which further suggests a role for histamine release in endotoxin shock (8).

LPS incubated in vitro with diluted, fresh, heparinized normal rat plasma was shown by Greisman to activate a factor capable of contracting the isolated guinea pig ileum (6). Similarly, Lichtenstein et al. (12) described the interaction of LPS with guinea pig serum to generate a substance which induced tachyphylaxis when repeatedly added to the isolated guinea pig ileum and thus resembled an anaphylatoxin. Anaphylatoxins are also formed as a result of the interaction of antigen-antibody complexes with complement and are defined by their ability to cause changes in the capillary permeability of guinea pig skin (16), to show chemotaxis of rabbit polymorphonuclear leukocytes (10), to contract the guinea pig ileum (16), or to cause histamine release from rat mast cells (16). In contrast to work with antigen-antibody complexes, LPS had not been shown until the present report to cause release of histamine from mast cells. Gustafson (7) demonstrated that LPS alone, or in the presence of serum without incubation, had no effect on hamster mast cells as judged by electron microscopy.

Mast cells are a particularly abundant source of histamine as well as serotonin and other pharmacologically active substances which are released into surrounding tissues upon alteration of the mast cell surface. Keller studied the efficacy of polypeptides from lysosomes of rabbit leukocytes and of compound 48/80 in rupturing mast cells and found that hamster mast cells are only slightly less susceptible than rat cells (11). In the present work, a factor capable of releasing histamine from hamster mast cells was generated with a concomitant consumption of complement activity, by incubating normal hamster serum with LPS.

MATERIALS AND METHODS

Buffer. The diluent for all experiments, unless otherwise specified, was gelatin-Veronal-buffered saline (GVB) of *p*H 7.3 to 7.4 and containing calcium and magnesium salts in concentrations optimal for immune lysis by human and guinea pig complement (14). For the collection and washing of mast cells, calcium and magnesium were omitted from the buffer.

Endotoxins. LPS was purified from the cell walls of *Veillonella alcalescens* by the phenol-water method of Westphal and Lüderitz (20). LPS prepared in a similar manner from *Salmonella typhosa* 0901 was purchased from Difco Laboratories, Detroit, Mich. (no. 3124–25), and was used where specified. *V. alcalescens* LPS (4 mg/ml) was used to inject 5- to 7-week-old hamsters intraperitoneally with a dose of 4 mg/kg.

Inhibitors of the LPS-serum reaction. The following conditions were tested for their ability to influence the generation of a histamine-liberating factor from hamster serum: serum was heated to 56 C for 30 min before addition of LPS; LPS was detoxified with 0.01 N NaOH (5); reaction mixtures were diluted in an equal volume of GVB without added calcium and magnesium and containing 0.02 M disodium ethylenediaminetraacetic acid (EDTA); or the reaction mixture of LPS with serum was incubated at 0 C for 20 min rather than at 37 C.

Other activators of the complement system. Human gamma globulin, purchased from Hyland Laboratories, Los Angeles, Calif., containing 16.5 μg of N/ml was aggregated at 63 C for 40 min. Zymosan (lot 59844) was purchased from General Biochemicals Corp., Chagrin Falls, Ohio, and was suspended to 4 mg/ml in GVB. Cobra venom factor was purified by fractionation on a diethylaminoethyl-cellulose column (18) and contained 100 μg/ml. To provide immune precipitates, New Zealand white rabbits were hyperimmunized with bovine serum albumin (BSA) and their sera were collected, pooled, and heated to 56 C to 30 min. Precipitates were formed at 4 C for 24 hr by incubating 3-ml amounts of undiluted serum with 15 ml of a BSA solution (16.7 μg of N/ml). Precipitates were washed three times with 0.85% NaCl and resuspended to 3 ml with GVB. The aforementioned activators were used in 0.1-ml amounts with 1.0 ml of hamster serum diluted 1:2 to demonstrate histamine-liberating and complement-consuming activities.

Normal sera. Blood was collected from female golden hamsters 7 to 17 weeks of age obtained from the Animal Production Unit, National Institutes of Health. Animals were anesthetized by carbon dioxide inhalation, and blood collected by cardiac puncture was allowed to clot at room temperature for 1 hr followed by storage at 4 C for 5 hr and centrifugation at 2 C to remove cells. Sera were pooled and stored at −100 C.

Mast cell suspensions. Hamsters 5 to 7 weeks of age were anesthetized with carbon dioxide and were injected intraperitoneally in the center of the abdomen with 22 ml of GVB containing 2 units of sodium heparin per ml. The abdomen was gently massaged for 1 min after which time approximately 20 ml of fluid could be collected by removing the syringe and collecting drops into a siliconized, conical centrifuge tube. Cells were sedimented by centrifugation for 7 min at 150 × *g* at 2 C, washed in GVB three times by centrifugation at the same speed, and finally resuspended to original volume with GVB. These suspensions generally had an available histamine content of 0.5 to 1 μg/ml. Cell counts by microscopy revealed that such suspensions contained approximately 2.5 × 10^4 mast cells/ml and that mast cells accounted for 5 to 10% of the total number of cells present. Mast cell counts were made by staining slides with Lillie's Azure A, Eosin B (13). Suspensions containing nearly 100% mast cells could be obtained by layering a concentrated suspension of mixed peritoneal cells over an equal volume of 40% BSA and centrifuging for 5 min at 200 × *g*. The albumin layer containing purified mast cells was utilized to determine that the peritoneal cell histamine was derived almost exclusively from mast cells. For this reason, a mixed peritoneal cell population was used in the following experiments and the cells are referred to for simplicity as "mast cells." For the determination of histamine content of peritoneal washings after LPS injection, the cells were centrifuged once, resuspended to the original volume, and assayed for histamine content.

Generation of histamine-releasing factor. Reaction mixtures were made in polystyrene tubes and consisted of 0.1 ml of LPS (100 μg) plus 1.0 ml of hamster serum (1:2). Tubes were incubated at 37 C for 20 min followed by the addition of 1.0 ml of the mast cell suspension and sufficient GVB to bring the contents of each tube to a final volume of 4.0 ml. Tubes were incubated at 37 C for 1 hr, mixing after 30 min, followed by centrifugation at 500 × *g* for 15 min. The supernatant fluids were decanted and assayed for histamine content. For each experiment, appropriate controls containing buffer, mast cells, LPS plus mast cells, serum plus mast cells, serum alone, histamine standards, and total available mast cell histamine were included. Available histamine controls were not centrifuged before assay. Mast cells did not undergo significant spontaneous histamine release during the incubation period. The histamine content of the test serum alone amounted to less than 5% of the total available histamine and could be compensated for by appropriate controls to zero the fluorometer.

Histamine assay. Histamine was measured fluorometrically after removal of proteins by precipitation with perchloric acid and extraction into butanol in a manner similar to the method of Shore et al. (19). A model 111 fluorometer (Turner Instruments, Palo Alto, Calif.) was employed to estimate the fluorescent product formed upon condensation of histamine with *o*-phthaldialdehyde. The fluorometer dial reading was converted to micrograms of histamine by interpolation on a standard curve. Per cent available histamine release by each reaction mixture was calculated by using data from the available histamine control tubes. All histamine assays were performed on duplicate samples, and results were recorded as the mean value. For the assay of histamine in hamster whole blood, animals were bled by cardiac puncture into tubes containing sodium heparin (100 units/ml).

Complement assay. Total hemolytic complement in hamster serum was assayed in a manner similar to that described by Mayer (14). The terminal complement components (C3–C9) were measured in 0.01 M EDTA with sheep erythrocytes sensitized with the first, fourth, and second components of guinea pig complement (14).

RESULTS

In vivo. Injection of hamsters with *V. alcalescens* LPS resulted in a marked decline in the number of mast cells and in the amount of histamine available in the sediment of peritoneal washings (Fig. 1). The maximum depletion of mast cells and cell histamine was observed during the period 4 to 14 days after LPS injection. The dose of LPS given was lethal for approximately 10% of the hamsters injected, and most of these deaths occurred between days 4 and 14. No detectable histamine was found in the supernatant fraction of mast cell suspensions, indicating that the histamine loss was not due to increased fragility of the collected cells. Blood histamine levels were relatively unchanged during the course of the experiment (Fig. 1). Additional samples of blood collected 1, 5, and 10 min after LPS injection also showed no significant changes in blood histamine levels.

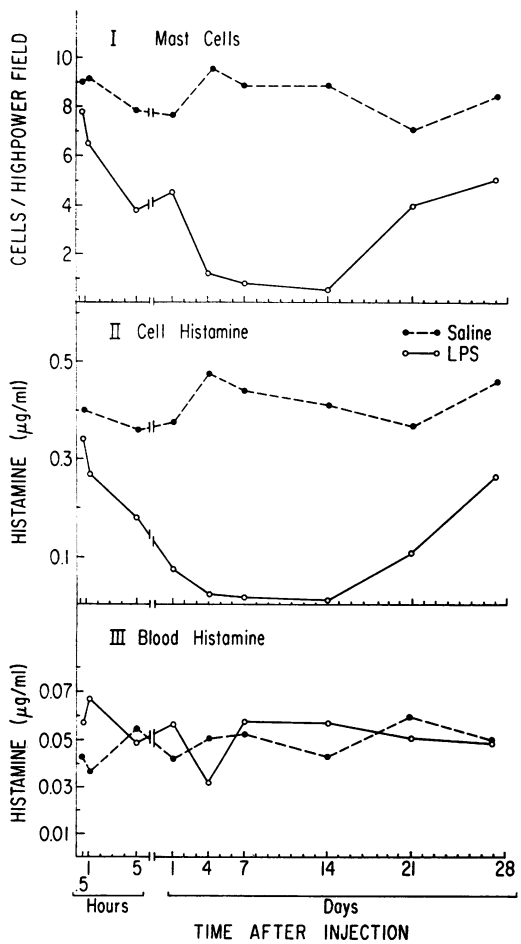

FIG. 1. *Influence of intraperitoneal injection of Veillonella alcalescens endotoxin (LPS) on mast cell count, on available histamine in sedimented cells from peritoneal washings, and on blood histamine levels in hamsters. LPS dose was 4 mg/kg. Symbols: ○, LPS-injected; ●, controls injected with saline.*

TABLE 1. *Release of histamine and consumption of complement by various combinations of endotoxin (LPS), normal hamster serum, and mast cells (MC)*[a]

First incubation (20 min, 37 C)	Added before second incubation (60 min, 37 C)	Per cent histamine released	Per cent complement consumed[b]
LPS	MC	2	—[c]
Serum	MC	9	0
Serum + LPS	MC	39	>95
LPS + MC	Serum	6	>95
Serum + MC	LPS	17	>95
Serum + LPS + MC	Buffer	15	>95

[a] Amount of LPS used was 100 µg, serum was diluted 1:2, and MC suspension contained 0.62 µg of available histamine.

[b] Complement available was 200 50% hemolytic units.

[c] No complement in test.

Optimal in vitro release of histamine from mast cells. To achieve the maximum release of histamine in vitro, it was found that a preliminary incubation of LPS with serum followed by the addition of mast cells and a second incubation of the mixture were necessary (Table 1). Other sequences of combining reactants, e.g., LPS plus mast cells and then serum, serum plus mast cells and then LPS, or simultaneous incubation of serum, LPS, and mast cells, resulted in the release of less histamine. The reaction mixtures were also studied for consumption of total complement, and it was found that all reaction mixtures containing LPS resulted in consumption of more than 95% of the available complement without regard to the sequence of addition and incubation (Table 1).

When LPS and serum were incubated together for increasing periods of time before the addition of mast cells, it was noted that some histamine-liberating activity existed in unincubated serum and in serum incubated in the absence of LPS (Fig. 2). The most active histamine-liberating substance appeared to be formed with LPS and serum incubated at 37 C for 20 to 60 min. It was noted that the "background" value obtained without LPS also increased as a function of time. A similar experiment revealed that 1 hr was optimal for histamine release to

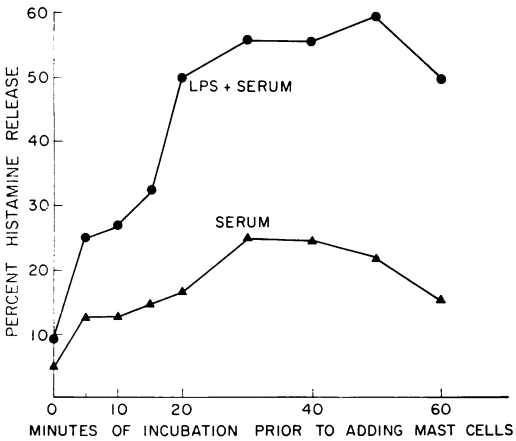

FIG. 2. *Effect of increasing the time Veillonella alcalescens endotoxin (LPS) was incubated with serum on the formation of histamine-liberating substance. LPS (100 μg) was mixed with normal hamster serum (1:2) and incubated at 37 C for the times indicated before adding mast cell suspension and incubating a second time at 37 C for 1 hr.*

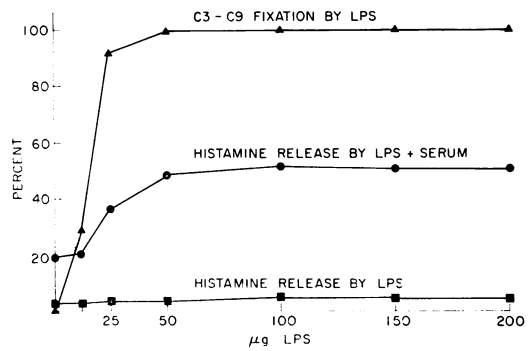

FIG. 3. *Release of histamine and consumption of classical third component of complement (C3-C9) by increasing amounts of V. alcalescens endotoxin (LPS). Mixtures were incubated for 20 min at 37 C with normal hamster serum (1:2).*

occur during the second incubation, after adding mast cells.

The amount of histamine released from mast cells by reaction mixtures containing LPS and serum was also shown to be influenced by the amount of LPS added (Fig. 3). LPS in the range from 50 to 100 μg of serum per ml was satisfactory for maximum histamine release. Incubation of mast cells with as much as 200 μg of LPS in the absence of serum did not result in a significant amount of histamine release. Another observation made during the course of this experiment was that classical C3 was consumed by LPS-serum mixtures. It was apparent that a

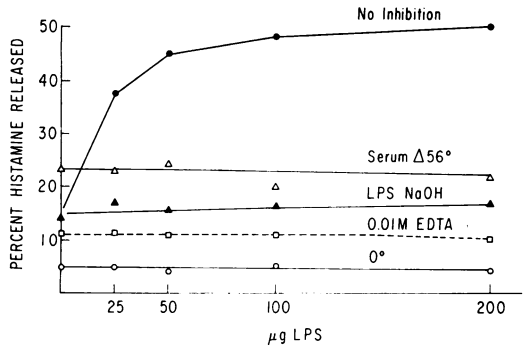

FIG. 4. *Inhibition of generation of histamine-releasing activity. Specified amounts of Veillonella alcalescens endotoxin (LPS) were incubated for 20 min with normal hamster serum (1:2) under the inhibitory conditions described.*

parallelism existed between the consumption of C3–C9 and the ability of reaction mixtures to liberate histamine (Fig. 3).

Inhibitors. The reaction of LPS with hamster serum to form a histamine-liberating substance was inhibited by conditions known to interfere with the consumption of complement by LPS (Fig. 4). For example, incubating reaction mixtures at 0 C rather than at 37 C, using serum containing 0.01 M EDTA, or using serum preheated to 56 C for 30 min failed to result in the generation of histamine-releasing activity. LPS detoxified with 0.1 N NaOH so as to render the preparation deficient in complement-fixing properties (5) also blocked its histamine-releasing effects. It was noteworthy that the background histamine release by heated serum without LPS seemed significantly higher (23%) than the release by unheated serum in the absence of LPS (Fig. 4). Conversely, serum without LPS incubated at 0 C, or in the presence of 0.01 M EDTA, showed less histamine release than the control serum.

Generation of histamine-liberating activity by other activators of complement. The experiments described above suggested that the interaction of *V. alcalescens* LPS with hamster serum to form a histamine-liberating substance involved the complement system. Accordingly, other substances known to react with complement were tested for this ability, including *S. typhosa* LPS, heat-aggregated human gamma globulin, zymosan, purified cobra venom factor, and washed antigen-antibody precipitates. Table 2 indicates that the aforementioned activators were capable of forming a substance which released histamine from hamster mast cells and which consumed hamster complement. In addition, all of these substances were tested for their ability to release histamine

TABLE 2. *Formation of histamine-liberating activity in hamster serum by activators of the complement system*

Activator added to hamster serum[a]	Per cent histamine released from mast cells[b]	Per cent complement consumed[c]
None	10	0
LPS (*V. alcalescens*)	36	80
LPS (*S. typhosa*)	50	88
Aggregated gamma globulin	43	91
Zymosan	34	30
Cobra venom factor	36	85
Antigen-antibody precipitate	23	25

[a] Amount of endotoxin (LPS) used was 100 μg, heated (63 C, 40 min) human gamma globulin contained 1.65 μg of N, zymosan concentration was 400 μg, purified cobra venom factor contained 10 μg of N, and the washed, antigen-antibody precipitate contained 210 μg of N. The normal hamster serum was diluted 1:2. Mixtures were incubated at 37 C for 20 min.

[b] Mast cell suspension contained 1.08 μg of available histamine.

[c] Amount of complement available was 167 50% hemolytic units.

from mast cells in the absence of serum, and in all cases these controls caused a release of less than 4% of the available histamine. In general, the ability to produce histamine-releasing activity correlated with the total complement-consuming activity of the substance tested.

DISCUSSION

Injecting animals with bacterial LPS may result in many pathological effects, some of which resemble those produced by antigen-antibody complexes. For example, the agglutination of platelets with release of vasoreactive or thromboplastic factors and the generation of anaphylatoxic and chemotactic activities have been described (15). In rabbits given LPS intravenously, a rapid increase in plasma histamine reportedly occurs 15 sec after injection followed by a return to normal levels after 15 min (3). In the present work, hamsters were injected intraperitoneally with LPS and no important changes were found in blood histamine levels. The disappearance of histamine from the peritoneal washings (Fig. 1) may have occurred as a result of degranulation of mast cells followed by enzymatic inactivation of released histamine.

Much of the histamine in the animal body is located in tissue mast cells, and the mast cell is probably the main source of histamine released during the anaphylactic reaction (17). In addition, histamine may be liberated from platelets and leukocytes by allergic injury (17). Histamine may be released from mast cells by antigen-antibody reactions through at least two mechanisms. In one type, there is a direct action on rat mast cells by homocytotropic antibody which in the presence of specific antigen leads to histamine release independently of complement (2). Alternatively, antigen-antibody complexes can cause the indirect release of histamine by activating the complement sequence to form biologically active products such as anaphylatoxins that have a direct action against histamine-releasing cells (1, 16).

In the present work, histamine release from mast cells by LPS was investigated. The mechanism appeared to be of the indirect type because LPS had no histamine-releasing effect on mast cells in the absence of serum. In addition, the conditions optimal for histamine release appeared to result from generating a substance from serum which was active on mast cells rather than by sensitizing cells with endotoxin or serum components (Table 1). It is noteworthy that histamine-releasing activity was very low (6%) when LPS and mast cells were incubated together before the addition of serum (Fig. 1). This may have been due to inactivation of LPS by the cell suspension. This may also explain the lessened histamine-releasing activity observed when serum, LPS, and mast cells were incubated together in one step.

The data presented showing (i) consumption of C3–C9 by LPS, (ii) inhibition of histamine release by inhibiting the LPS-complement reaction, and (iii) the ability of other activators of the complement system to form histamine-liberating activity indicate a requirement for hamster complement in the release of histamine by LPS. The background histamine-releasing activity of normal serum observed in the absence of LPS may be due to cleavage during the clotting process of active products from the third component of complement (C3) by plasmin or other serum enzymes (21).

Similar systems that have been shown to be complement-dependent include the generation of skin-bluing and ileum-contracting anaphylatoxins from rat and guinea pig serum by interaction with immune aggregates (16). Lichtenstein et al. implicated the complement system in the generation of an ileum-contracting anaphylatoxin from normal guinea pig serum by LPS (12). The use of purified human C1 esterase, C4, C2, and C3 to generate an anaphylatoxin capable of contracting the guinea pig ileum, enhancing vascular permeability in guinea pig skin, degranulating guinea pig mesentery mast cells, and liberating hista-

mine from suspensions of rat peritoneal mast cells also supports the role of the complement system as a source of anaphylatoxin (4). We have recently found that LPS incubated with normal hamster serum consumes complement components C1, C4, and C2 in addition to the classical third component containing C3–C9 (9). The results described in the present paper suggest that a factor resembling an anaphylatoxin may be formed by LPS when reacted with normal hamster serum.

LITERATURE CITED

1. 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 release of histamine by rabbit anti-rat gamma globulin. J. Exp. Med. 124:397–416.
2. 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. Exp. Med. 124:379–395.
3. Davis, R. B., W. L. Bailey, and N. P. Hanson. 1963. Modification of serotonin and histamine release after *E. coli* endotoxin administration. Amer. J. Physiol. 205:560–566.
4. Dias da Silva, W., and I. H. Lepow. 1967. Complement as a mediator of inflammation. II. Biological properties of anaphylatoxin prepared with purified components of human complement. J. Exp. Med. 125:921–946.
5. Gewurz, H., S. E. Mergenhagen, A. Nowotny, and J. K. Phillips. 1968. Interactions of the complement system with native and chemically modified endotoxins. J. Bacteriol. 95:397–405.
6. Greisman, S. 1960. Activation of histamine-releasing factor in normal rat plasma by *E. coli* endotoxin. Proc. Soc. Exp. Biol. Med. 103:628–632.
7. Gustafson, G. T. 1968. Ultrastructural vascular and mast cell changes in the local Shwartzman reaction. Acta Pathol. Microbiol. Scand. 74:114–126.
8. Hinshaw, L. B., T. E. Emerson, Jr., P. F. Iampietro, and C. M. Brake. 1962. A comparative study of the hemodynamic actions of histamine and endotoxin. Amer. J. Physiol. 203:600–606.
9. Hook, W. A., R. Snyderman, and S. E. Mergenhagen. 1970. Consumption of hamster complement by bacterial endotoxin. J. Immunol. 105:268–270.
10. Jensen, J. A., R. Snyderman, and S. E. Mergenhagen. 1969. Chemotactic activity; a property of guinea pig C'5 anaphylatoxin, p. 265–278. *In* H. Z. Movat (ed.), Cellular and humoral mechanisms in anaphylaxis and allergy. Kargar, New York.
11. Keller, R. 1968. Interrelations between different types of cells. Int. Arch. Allergy Appl. Immunol. 34:139–144.
12. Lichtenstein, L. M., H. Gewurz, N. F. Adkinson, Jr., H. S. Shin, and S. E. Mergenhagen. 1969. Interactions of the complement system with endotoxic lipopolysaccharide: the generation of an anaphylatoxin. Immunology 16:327–336.
13. Lillie, R. D. 1965. Histopathologic technic and practical histochemistry, 3rd ed., p. 162–165. McGraw Hill, New York.
14. Mayer, M. M. 1961. Complement and complement fixation. p. 133–240. *In* E. A. Kabat and M. M. Mayer (ed.), Experimental immunochemistry, 2nd ed. Charles C Thomas, Springfield, Ill.
15. Mergenhagen, S. E., R. Snyderman, H. Gewurz, and H. S. Shin. 1969. Significance of complement to the mechanism of action of endotoxin. Curr. Top. Microbiol. Immunol. 50:37–77.
16. Osler, A. G., H. G. Randall, B. M. Hill, and Z. Ovary. 1959. Studies on the mechanism of hypersensitivity phenomena. III. The participation of complement in the formation of anaphylatoxin. J. Exp. Med. 110:311–339.
17. Riley, J. F., and G. B. West. 1966. The occurrence of histamine in mast cells, p. 116–135. *In* O. Eichler and A. Farah (ed.), Handbook of experimental pharmacology, vol. 18/1. Springer-Verlag, Berlin.
18. Shin, H. S., H. Gewurz, and R. Snyderman. 1969. Reaction of a cobra venom factor with guinea pig complement and generation of an activity chemotactic for polymorphonuclear leukocytes. Proc. Soc. Exp. Biol. Med. 131:203–207.
19. Shore, P. A., A. Burkhalter, and V. H. Cohn, Jr. 1959. A method for the fluorometric assay of histamine in tissues. J. Pharmacol. Exp. Ther. 127:182–186.
20. Westphal, O., and O. Lüderitz. 1954. Chemische Enforschung von Lipopolysacchariden gramnegativer Bakterien. Angew. Chem. 66:407–417.
21. Ward, P. A. 1967. A plasmin-split fragment of C'3 as a new chemotactic factor. J. Exp. Med. 126:189–206.
22. Zweifach, B. W., and A. Janoff. 1965. Bacterial endotoxemia. Annu. Rev. Med. 16:201–220.