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Further Characterization of a Factor from Endotoxin-Treated Serum which Releases Histamine and Heparin from Mast Cells

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

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

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Upon incubation of hamster serum with bacterial endotoxin, a factor is produced which releases histamine and heparin from hamster mast cells and increases capillary permeability in guinea pig skin. The major histamine-releasing activity derived from hamster serum was characterized by gel filtration, found to have a molecular weight of approximately 60,000, and shown by electrophoresis to migrate with alpha-2- or beta-1-globulins. The ability to increase vascular permeability was not reversed by antihistamine. On the basis of these properties, the histamine-liberating factor generated by endotoxin in hamster serum differed significantly from known anaphylatoxins.

The ability of bacterial endotoxin (LPS) to activate a substance in normal hamster serum which released histamine from mast cells has previously been described (6). LPS appeared to indirectly liberate histamine as a result of interaction with the complement system. This was shown by (i) consumption of serum complement components C3 through C9 by the addition of LPS, (ii) inhibition of histamine release by agents such as 0.01 M disodium ethylenediaminetetraacetic acid or by other inhibitors of the LPS-complement reaction, and (iii) the ability of other activators of the complement system, e.g., zymosan or cobra venom factor, to generate histamine-liberating activity (6). Among the known histamine-releasing factors derived from serum, the anaphylatoxins (C3a and C5a) are prominent in that they are generated by incubating antigen-antibody complexes or certain polysaccharides with fresh serum (3). In the present paper the major histamine-liberating factor (HLF) generated in LPS-activated hamster serum is characterized and its relationship to C3a and C5a anaphylatoxins is discussed.

MATERIALS AND METHODS

Serums. Blood was collected from female golden hamsters 10 to 20 weeks of age obtained from the Lakeview Hamster Colony, Newfield, N.J. Animals were anesthetized by carbon dioxide inhalation, and blood was collected by cardiac puncture. The serums were collected, pooled, and stored at −40 C.

Mast cell suspensions. The abdominal washings of normal hamsters were collected as previously described and contained 5 to 10% mast cells (6). For simplicity this is referred to as a mast cell suspension and has been found to be as satisfactory for these experiments as purified mass cells prepared by centrifugation of abdominal washings over bovine serum albumin (6). Mast cells were collected and washed three times by centrifugation at 150 × *g* in gelatin-Veronal-buffered saline (GVB) at *p*H 7.3 (9). Washed peritoneal cells were resuspended in GVB to contain approximately 5 × 10^4 mast cells.

Histamine assay. Histamine release in supernatant fractions of reaction mixtures containing mast cells (6) was measured fluorometrically in a manner similar to the method of Shore et al. (14). All assays were performed in duplicate, and results were recorded as the mean value.

Heparin assay. Heparin release from mast cells was quantitated by spectrophotometrically measuring the binding of Azure A (11). Mast cells sedimented at 150 × *g* for 7 min were washed three times in GVB, fragmented by sonic treatment for 15 sec, mixed with 0.01% Azure A at *p*H 7.2, and the resultant increase of absorbance at 500 nm or decrease at 600 nm was compared to the values obtained with known amounts of sodium heparin. The disappearance of heparin from cell sediments was calculated using the amount of heparin available in cell suspensions to compute the per cent heparin released.

Tests for changes in vascular permeability. These assays were carried out using male, strain 2 guinea pigs weighing 400 to 500 g that had been injected intravenously (iv) with 0.5 ml of 1% Evans blue in saline (3). Immediately thereafter, 0.1-ml samples of activated serum or fractions were injected intradermally (id) at six different sites, and 20 min later

the mean diameter of the blue-colored area of three animals was determined. To evaluate inhibition by antihistamine of the tested solutions, 250 μg of pyrilamine malate in 0.2 ml was given iv 10 min before skin testing (3).

Generation of histamine-liberating activity. LPS from *Salmonella typhosa* strain 0901, purchased from Difco Laboratories, Detroit, Mich., was suspended to a concentration of 1 mg/ml in GVB. Reaction mixtures contained 100 μg of LPS/ml normal hamster serum diluted with an equal volume of GVB. To generate the histamine-liberating substance, mixtures were incubated at 37 C for 20 min in polystyrene tubes (12 by 75 mm). Activated serum was tested by adding 1 ml of cell suspension containing approximately 5×10^4 mast cells and sufficient GVB to bring the reaction mixture to 4 ml. Tubes were incubated at 37 C in a stationary water bath for 1 hr followed by centrifugation to sediment mast cells. Supernatant fluids were assayed for histamine released.

Gel filtration. A 2-ml volume of LPS-activated hamster serum was heated to 56 C for 5 min, cooled, and passed through a column (35 by 2.5 cm) of Sephadex G-200 dextran gel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) at 4 C. The eluent used was 0.02 M phosphate-buffered 0.85% saline (PBS) at a *p*H of 7.2. Fractions of 4 ml each were collected and promptly tested for ability to release histamine. The relative protein content of the eluates was determined by optical density measurements at 280 nm. An estimate of the molecular weight of the histamine-liberating substance was made using marker proteins of known molecular weight (1).

Density gradient ultracentrifugation. Sucrose density gradient ultracentrifugation was also used to fractionate HLF. LPS-activated serum was concentrated fourfold with a Diaflow UM-2 ultrafilter (Amicon Corp., Lexington, Mass.), and 0.9-ml portions were layered on a 10 to 30% linear gradient of sucrose in PBS and centrifuged for 20 hr at 200,000 × *g* in a SW-41 rotor. Fractions of approximately 1 ml were collected and tested for their ability to release histamine from mast cells.

Electrophoresis. Immunoelectrophoresis was performed with *p*H 8.4 buffer containing 8.5 g of sodium barbital and 11.5 ml of N HCl/liter. Samples were electrophoresed at 250 v for 1.5 hr. Rabbit anti-hamster serum (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the center wells, and slides were allowed to stand for 24 hr at 4 C. HLF fractions of LPS-activated serum were obtained by continuous-flow preparative paper electrophoresis with a Spinco model CP apparatus (Beckman Instruments, Palo Alto, Calif.) with *p*H 8.4 barbital buffer. A sample volume of 11 ml was applied and subjected to electrophoresis for 4 hr at a constant 550 v. Fractions of 11 ml each were collected and tested for the ability to release histamine.

RESULTS

Release of histamine and heparin from mast cells and increased capillary permeability by LPS-treated serum. Incubation of LPS with normal hamster serum at 37 C for 20 min followed by the addition of mast cells and a second incubation at 37 C for 60 min was shown to concomitantly release histamine and heparin (Table 1). Optimal conditions for the in vitro release of histamine by LPS-treated serum have been described previously (6). Although endotoxin alone released less than 2% of the available histamine or heparin from mast cells, untreated serum alone caused release of 6.7% of the available histamine and 12.5% of the heparin. The reason for the effect of serum alone is unclear. However, when LPS was incubated with serum, histamine release was increased to 28.2% and 59.7% of the heparin was released. Insignificant histamine release resulted from incubation of mast cells with LPS or with buffer alone (Table 1).

The ability of mixtures of LPS and serum alone to cause increased permeability of guinea pig skin is also shown in Table 1. Injection (id) of guinea pigs with LPS-treated serum produced a marked area of skin bluing (10-mm diameter zone), whereas control sites prepared with untreated serum, LPS alone, or buffer showed less than 5 mm of bluing.

TABLE 1. *Ability of normal hamster serum treated with endotoxin (LPS) to release histamine and heparin from mast cells (MC) and to increase vascular permeability*

Reaction mixture[a]	Histamine released from added MC		Heparin released from added MC		Diameter[b] of skin response (mm)
	μg	%	μg	%	
LPS + serum	0.381	28.2	12.5	58.7	10.0
Buffer + serum	0.090	6.7	2.7	12.7	4.5
Buffer only	0.015	1.1	0.02	0.9	1.3
LPS + buffer	0.012	0.9	0.03	1.4	3.3
Available histamine or heparin in MC	1.35	(100)	21.3	(100)	

[a] Reaction mixtures contained 100 μg of LPS and 1 ml of normal hamster serum (diluted 1:2) in a volume of 2 ml. Tubes were incubated at 37 C for 20 min. For histamine and heparin release experiments approximately 5×10^4 MC were added and tubes were incubated additionally at 37 C for 60 min. Gelatin-Veronal-buffered saline was used as diluent.

[b] Mean diameter of skin bluing in guinea pigs determined 20 min after intradermal injection of 0.1 ml of reaction mixtures (no mast cells were added).

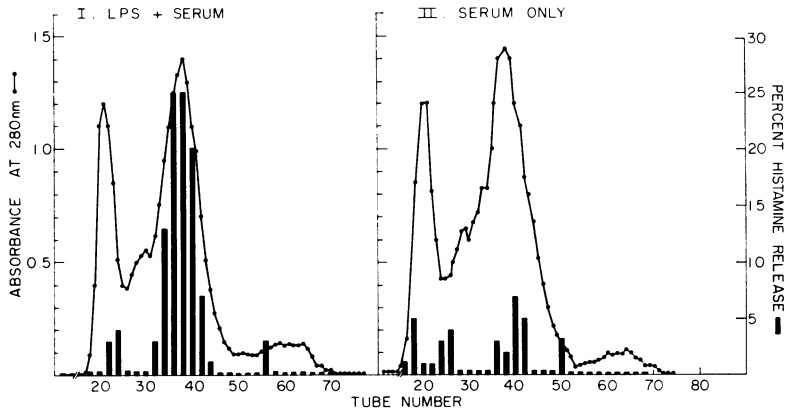

FIG. 1. *Gel filtration elution patterns obtained using Sephadex G-200 to fractionate LPS-treated hamster serum and untreated serum.*

The histamine-liberating activity of LPS-treated serum was not destroyed by heating to 56 C for 5 to 10 min after the initial incubation at 37 C for 20 min. This treatment was capable of decreasing total complement levels in the absence of LPS to less than five 50% hemolytic units of complement. The background histamine-releasing activity of serum incubated without LPS was not significantly reduced by heating serum for as long as 50 min, whereas the activity induced by LPS markedly declined after 20 min at 56 C. Accordingly, sera were routinely heated to 56 C for 5 min prior to fractionations.

Isolation of HLF. Gel filtration using Sephadex G-200 was used to partially purify and to estimate the molecular weight of the major histamine-releasing substance derived from LPS-treated serum. Maximal HLF activity appeared in the area of fractions 36 to 38, with lesser activity seen with fractions of higher or lower molecular weight (Fig. 1). The small amount of histamine (molecular weight 111) normally present in hamster serum eluted in fraction 72 and therefore was excluded from HLF. Application of molecular weight markers to the same column (Fig. 2) revealed that maximal HLF activity had eluted at a volume corresponding to a molecular weight of approximately 60,000.

Sucrose gradient ultracentrifugation was also utilized to fractionate LPS-activated serum (Fig. 3). The greatest HLF activity was seen in fractions obtained from the upper third of the gradient, with another area of activity found near the bottom of the tube. Maximal HLF activity was detected in the gradient at a point corresponding to the tube where an ovalbumin marker was detectable. Serum alone showed slight histamine-liberating activity among the heavier fractions in the bottom third of the tube. However, little or no activity was demonstrable with the lighter fractions of untreated serum.

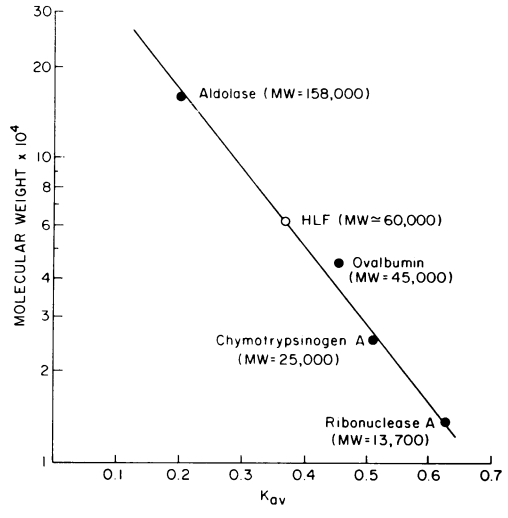

FIG. 2. *Estimation by method of Andrews (1) of molecular weight of major histamine-liberating factor (HLF) obtained as fractions 36 to 38 from Sephadex G-200 gel filtration.*

Results of fractionation by continuous-flow preparative electrophoresis as listed in Table 2 indicated that maximal histamine-liberating activity was associated with the region of alpha-2- and beta-1-globulins, and that little activity was found in the albumin fraction. Fractions from untreated serum demonstrated only minor activity (Table 2). LPS-treated serum and HLF as pooled, Sephadex G-200 fractions 36 to 38 were tested by immunoelectrophoresis with antiserum toward whole hamster serum. The results suggested that HLF contained proteins resembling albumin and beta globulin (Fig. 4).

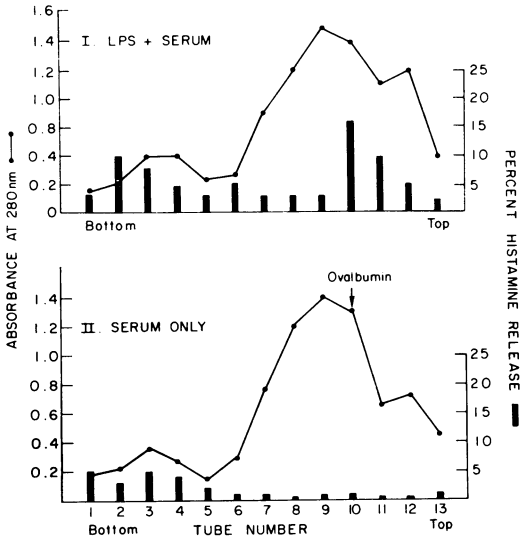

FIG. 3. *Sucrose density gradient ultracentrifugation of histamine-liberating activity generated by LPS in hamster serum and of serum alone.*

TABLE 2. *Histamine release from mast cells by serum fractions separated by continuous-flow electrophoresis*

Fraction[a]	Protein region	Per cent histamine release by:	
		LPS-treated serum	Untreated serum
5	Gamma-2-globulin	3	3
6	Gamma-2-globulin	3	2
7	Gamma-2-globulin	3	3
8	Gamma-2-globulin	1	3
9	Gamma-2-globulin	2	3
10	Gamma-2-globulin	2	3
11	Gamma-1-globulin/ beta-2-globulin	3	3
12	Beta-1-globulin	11	2
13	Alpha-2-globulin	18	4
14	Alpha-2-globulin	11	3
15	Alpha-1-globulin	4	3
16	Alpha-1-globulin	5	3
17	Albumin	4	2
18	Albumin	0	3
19	Prealbumin	2	2

[a] Reaction mixtures containing 1 ml of the designated electrophoretic fraction, 1 ml of mast cells (2.5×10^4) and 2 ml of buffer were incubated for 1 hr at 37 C.

Permeability effect of HLF in guinea pig skin. Sephadex G-200-purified HLF produced a marked increase in capillary permeability when injected id into guinea pigs (Table 3). The mean diameter of skin bluing was greater with the purified HLF (14.2 mm) than was achieved with an analogous fraction from untreated serum (4.7 mm). These fractions were obtained from tube number 38 of LPS-activated and non-activated serum, respectively, as shown in Fig. 1. Pretreatment of guinea pigs with antihistamine revealed that such treatment did not neutralize the increased vascular permeability produced by HLF (Table 3). In contrast, the absence of bluing caused by the prior id injection of 1 μg of histamine was nearly complete. With untreated serum, a reduction in the amount of bluing was achieved (4.7–2.0 mm).

DISCUSSION

The vascular reactions to LPS have been reviewed by Zweifach and Janoff (17). They list the evidence that the release of histamine is a major factor in the hemodynamic effects characteristic of the irreversible phase of endotoxin shock. They suggest that the elaboration of a variety of chemical mediators including active polypeptides and the generation of anaphylatoxins may lead to the release of vasoactive materials such as histamine and serotonin. Histamine release from mast cells may or may not be accompanied by liberation of other intracellular constituents such as serotonin, heparin, or various enzymes (12, 15).

Several substances having the characteristics of anaphylatoxin are generated as a result of incubating fresh serum with activators such as LPS, cobra venom factor, agar, or immune complexes (7, 8). The interaction of purified human complement components was shown by Dias da Silva et al. to produce an anaphylatoxin of relatively low molecular weight which was derived from the third component of complement (C3) and was identified as C3a (4). The C3a anaphylatoxin had the ability to cause histamine release and a marked degranulation of rat peritoneal mast cells as well as a moderate degranulation of guinea pig mesentery mast cells. Contraction of the isolated guinea pig ileum and increased permeability of guinea pig skin was also seen (4). C3a anaphylatoxin from human complement has been described as a basic peptide fragment corresponding to beta-1A-globulin and having an approximate molecular weight of 7,000 (2). At *p*H 8.6, human C3 migrated as a cation.

A different anaphylatoxin (C5a) is formed by the cleavage of the fifth component of guinea pig complement (C5). C5a anaphylatoxin is chemotactic for rabbit polymorphonuclear leukocytes and mammalian leukocytes in addition to its ability to contract the guinea pig ileum (7). No documentation of the effect of guinea pig C5a on

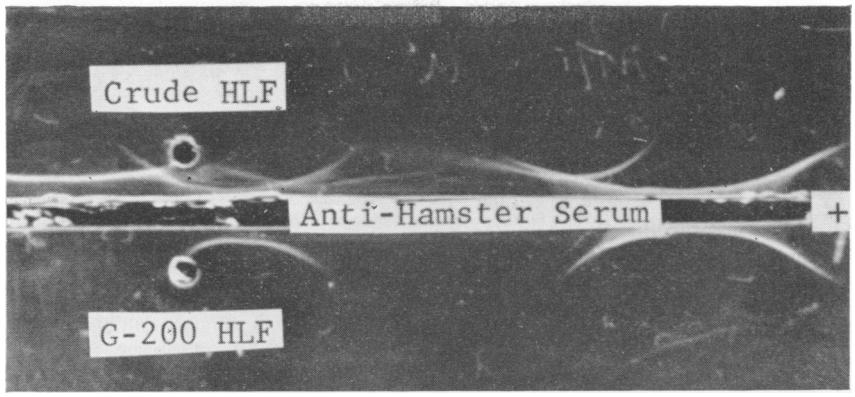

FIG. 4. *Immunoelectrophoretic analysis of whole, LPS-treated hamster serum (upper well) and of histamine liberating factor (HLF) partially purified by gel filtration (lower well). The trough contained antiserum to whole hamster serum.*

TABLE 3. *Influence of antihistamine on ability of the major histamine-releasing factor (HLF) from hamster serum to increase vascular permeability*

Material injected[a]	Mean diameter (mm) of skin response in guinea pigs given:	
	No antihistamine	250 μg of pyrilamine maleate
HLF	14.2	13.7
Fraction from untreated serum	4.7	2.0
1.0 μg of histamine	12.0	2.0
Buffer	1.0	1.0

[a] Partially purified HLF was obtained by gel filtration of endotoxin-treated serum, and 0.1 ml was injected intradermally into three guinea pigs. The corresponding fractions of untreated serum, histamine dihydrochloride or gelatin-Veronal-buffered saline were similarly tested. The same animals were pretreated with antihistamine 10 min before a second intradermal injection. All skin responses were measured 20 min after intradermal challenge.

isolated mast cells is known. C5a anaphylatoxin from guinea pig serum has been described as having a molecular weight of approximately 15,000, a sedimentation constant of 1.5*S*, and exhibiting no substantial electrophoretic migration at alkaline *p*H (13).

In the present work, hamster serum activated by LPS was tested for its ability to liberate histamine and heparin from hamster peritoneal mast cells. Heparin may be an important mediator of certain types of inflammatory tissue damage since heparin has been shown to synergistically enhance the resorption of bone in vitro (5). For example, alveolar bone loss is a prominent feature in inflammatory periodontal disease. Moreover, mast cells can be found in high numbers in this condition. Because the mast cell is likely to be the only source of heparin in tissue (15), it therefore may play an important role in physiological and pathological bone resorption (5).

Serum and mast cells were obtained from the same animal species in these experiments to avoid possible imcompatibilities between reagents from differing species. For studies of capillary permeability, it was necessary to utilize guinea pigs because hamster skin was not suitable for skin-bluing experiments. The major histamine-releasing activity derived from hamster serum as described here was found to have a molecular weight of approximately 60,000 and migrated electrophoretically to an area corresponding to the alpha-2- or beta-1-globulin fraction of serum. These characteristics do not correspond to the published descriptions of C3a and C5a anaphylatoxins. However, the hamster HLF resembled a component of human globulin that has been briefly described by Wolters et al. (16). This vasoactive fragment, derived from C3, was characterized as an alpha-2-globulin with a molecular weight of from 50,000 to 60,000 and caused increased permeability of guinea pig skin. HLF from hamster serum obtained in the present experiments was similar to the aforementioned anaphylatoxin-like substance in that it was capable of causing a marked increase in capillary permeability of guinea pig skin. However, HLF activity was not neutralized by pretreatment of animals with antihistamine as was the case with C3a and C5a anaphylatoxins (3).

The plasma kinins have the ability to induce a slow contraction of the smooth muscle of isolated ileum and to increase the permeability of venules to large molecules. Bradykinin (2.2×10^{8} M) was not inhibited by antihistamine in its ability to

induce contraction of the guinea pig ileum (3). The substrate in plasma which yields kinins is present in all mammals and is an alpha-2-globulin. An alpha-2-globulin with permeability-enhancing properties is also formed when plasma from certain animal species is diluted with saline or brought into contact with glass and is known as PF dil (10). The relationship of the aforementioned mediators of inflammation to anaphylatoxins and HLF is not clear since histamine release from isolated mast cells has generally not been studied concomitantly with skin permeability changes.

The finding by immunoelectrophoresis that HLF obtained by gel filtration contained proteins resembling albumin and beta-2-globulin (Fig. 4) may not necessarily conflict with the observation that the major histamine-liberating activity obtained by continuous-flow electrophoresis was found closer to the alpha-globulin area (Table 2). HLF may be present in a small quantity and may not be detectable by precipitation with antiserum to normal hamster serum.

The major histamine-liberating activity derived from hamster serum in the present experiments resembles anaphylatoxin in (i) the manner by which it is generated, (ii) a dependence upon an intact complement system, and (iii) its biological activity in releasing histamine from mast cells and increasing skin permeability. However, HLF differs from the C3a and C5a anaphylatoxins in its molecular weight as determined by gel filtration (approximately 60,000) and its ability to increase vascular permeability in guinea pigs pretreated with antihistamine. It seems likely that HLF obtained in these experiments may contain more than a single vasoactive substance and one of these may have properties in addition to that attributable to histamine release in view of its ability to increase capillary permeability in antihistamine-treated animals. This activity could be due to a direct effect of HLF in blood vessels or to the elaboration of chemical mediators such as the kinins or of serotonin.

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