INTERACTIONS OF THE COMPLEMENT SYSTEM WITH ENDOTOXIC LIPOPOLYSACCHARIDE

Generation of a Factor Chemotactic for Polymorphonuclear Leukocytes

By R. SNYDERMAN, M.D., H. GEWURZ, M.D., and S. E. MERGENHAGEN, Ph.D.

(From the Immunology Section, Laboratory of Microbiology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 25 March 1968)

Endotoxic lipopolysaccharide (LPS) is a potent inflammatory agent, and thus can serve as a valuable tool in studying the inflammatory response. Recent morphological studies showed that LPS, as well as the bacterial cell from which it was derived, when incubated in fresh serum, developed defects or "lesions" similar to those seen on erythrocytes undergoing immune hemolysis (1, 2). LPS, like the sensitized red cell, developed these lesions by means of complement (C′) system activation (2). Furthermore, it was recently shown that during the LPS-C′ interaction, all of the terminal C′ components (C′3, C′5, C′6, C′7, C′8, and C′9) are "fixed" or utilized (3). Utilization of those components associated with the biological effects subserved by the C′ system (4, 5) suggested that certain aspects of endotoxin-induced inflammation might be mediated by C′ (3).

Accumulation of polymorphonuclear leukocytes (PMN's) at the site of injection is a prominent characteristic of inflammation induced by endotoxin (6, 7). Information relating to the mechanism by which endotoxin mediates this phenomenon would be useful in further understanding the inflammatory response in general.

In 1962, Boyden developed a technique for the study of chemotaxis in vitro (8). He found that a factor (referred to in the singular for convenience, though multiple factors may be involved) chemotactic for PMN's was generated when immune precipitates were incubated with fresh rabbit serum, and speculated that a serum system like C′ might be involved. One group using Boyden's technique showed that aggregated human gamma globulins (AHGG) in the presence of fresh serum generated a chemotactic factor, but presented evidence against C′ being associated with its generation (9). Subsequently, it was shown that a chemotactic factor could be generated from purified human and guinea pig C′ components or whole rabbit serum upon interaction with immune precipitates or sensitized sheep erythrocytes. Furthermore, the chemo-

tactic activity was associated with a trimolecular complex of C'5, C'6, and C'7 (10, 11). More recently it was found that LPS, in the presence of fresh serum, generated a chemotactic factor for PMN's, but the relationship of the C' system to this chemotactic factor was not established (12).

This report presents evidence that LPS in the presence of fresh serum generates neutrophil chemotactic factor in vitro by means of C' system activation. This chemotactic factor is a heat-stable, low molecular weight (15,000–30,000) product and as such is different from previously described chemotactic factors derived from the C' system (10, 11, 13).

Materials and Methods

Endotoxic Lipopolysaccharide (LPS).—The LPS used in most of the experiments was derived from *Serratia marcescens* by the trichloroacetic acid extraction procedure as previously described (14).[1] A detoxified derivative of this preparation ("endotoxoid") was prepared by deacylation with potassium methylate (15).[1] LPS was also prepared from *Veillonella alcalescens* by the phenol-water extraction procedure of Westphal and Lüderitz (16).

Anti-S. marcescens Antibody.—Rabbit anti-*S. marcescens* serum was chromatographed on Sephadex G-200. The first peak eluted, which contained 2-mercaptoethanol-sensitive hemagglutinating activity for erythrocytes sensitized with *S. marcescens* LPS, was used as the antibody source.[1]

Zymosan.—Zymosan was obtained from General Biochemicals, Chagrin Falls, Ohio.

Immune Complexes of Bovine Serum Albumin (BSA) and Rabbit Antiserum (Rabbit Anti-BSA).—Crystalline BSA was obtained from Pentex Inc., Kankakee, Ill. Rabbit anti-BSA was obtained from Hyland Laboratories, Los Angeles, Calif., and contained 240 μg of antibody nitrogen/ml.[2] BSA and rabbit anti-BSA were reacted at equivalence for 24 hr at 4°C in the presence of 0.01 M EDTA. The precipitate was washed twice in EDTA-saline, twice in saline, and resuspended in saline. Final protein concentrations were determined by the assay of Lowry et al. (17).

Sources of Various Mammalian Sera.—Guinea pig serum was obtained from Texas Biologicals, Inc., Fort Worth, Texas. Normal human serum was provided by young adult male laboratory personnel and normal NIH blood bank donors. Rabbit serum was collected from adult New Zealand white rabbits. Sow serum was obtained from Minnesota miniature pigs.[3] Mouse serum was collected from strains B10.D2/Sn "new" (C'-normal) and B10.D2/Sn "old" (C'5-deficient) (18) obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine; the hemolytic C' deficiency in the old line serum was confirmed by hemolytic assay.

Rabbit Peritoneal PMN's.—Rabbit PMN's were collected as described by Hirsch and Church (19) with the following modifications: 120 ml of 0.1% glycogen in normal saline containing 50 mg/100 ml neomycin was injected intraperitoneally in adult New Zealand rabbits 6 hr before the exudate was withdrawn. The exudate was collected by injecting 60 ml of heparinized saline (2 units heparin/ml) intraperitoneally; the fluid was allowed to drain through a perforated No. 15 needle into sterile conical centrifuge tubes. The cells were collected immediately prior to use and suspended in Gey's medium with 2% BSA at pH 7.2 containing added penicillin and streptomycin (Gey's medium).

Incubation Mixtures for Chemotactic Factor Generation.—Unless stated otherwise, all incubation mixtures contained 0.1 ml serum and 0.1 ml test reagent diluted to 1 ml with

[1] Generously provided by Dr. A. Nowotny.

[2] Kindly determined by Dr. L. Lichtenstein.

[3] Generously provided by Dr. Y. B. Kim and Dr. D. W. Watson.

Gey's medium. The reaction mixtures were incubated in water baths at 37°C for 30 min followed by 56°C for 30 min. The reaction mixtures were then diluted to 3.0 ml with Gey's medium. Test reagents were diluted in gelatin-Veronal-buffered saline supplemented with Mg^{++} and Ca^{++} (GVB^{++}).

Quantitation of Chemotaxis.—The assay system used was as described by Boyden (8) with the following modifications: 1.2 μ Millipore discs were used (obtained from Millipore Filter Corp., New Bedford, Mass.). The number of cells per high power field was determined by inverting the Millipore disc after staining and clearing, and placing it bottom side up on a standard microscope slide. Any cells seen on the surface of the disc had to migrate through its entire width. Only cells on the surface of the disc were counted using the 40× objective with the aid of a microgrid. The average number of cells in 10 random fields was determined for each disc.

Sagittal Views of Millipore Discs.—Millipore discs incubated with PMN's and with normal guinea pig serum, or with a guinea pig–LPS incubation mixture were prepared and stained as previously described (8). The Millipore discs were then imbedded in paraffin and 10 μ sections were prepared. The sections were counterstained with eosin and fixed using standard methods. Photomicrographs were taken of the sections to illustrate the migration of cells through the filter disc.

Complement Fixation.—The ability of LPS, zymosan, and BSA–anti-BSA complexes to fix C′ was determined in reaction mixtures identical with those used in testing for their ability to generate the factor chemotactic for PMN's. In this experiment, 0.1 ml of test reagent was reacted with 0.9 ml of guinea pig serum for 1 hr at 37°C. Residual total C′ activity was measured by the method of Osler et al. (20), and residual classical C′3 (C′-EDTA) activity was assayed with EA C′4, 2a by minor modifications (21) of the methods of Mayer (22). This classical C′3 activity is now known to consist of at least six separate proteins termed C′3, C′5, C′6, C′7, C′8, and C′9 (4, 5).

The effect of antibody upon the ability of "endotoxoid" to fix C′ also was tested in parallel with tests of its effect on the ability of endotoxoid to induce generation of the chemotactic factor. In this experiment, 0.4 ml anti-*S. marcescens* antibody, 0.1 ml containing 50 μg of endotoxoid and 0.5 ml of a 1:5 dilution of guinea pig serum (containing 15 $C'H_{50}$) were incubated at 37°C for 60 min. Residual total C′ activity was determined by the method of Osler et al. (20).

To assay C′ activity in small volumes of mouse serum, washed chicken erythrocytes were presensitized with rabbit anti-chicken erythrocyte serum in the presence of 0.01 M EDTA. After two washings, the sensitized cells (2×10^8/ml) were suspended in GVB^{++}. Serial dilutions of mouse serum (0.025 ml) were incubated with 0.025 ml of GVB^{++} and 0.025 ml of sensitized cells for 60 min at 37°C in Microtiter plates (Cook Engineering Co., Alexandria, Va.); the plates were centrifuged and hemolysis was scored according to Nelson et al. (23).

Chromatography.—Guinea pig serum (5.4 ml) was incubated with 0.6 ml GVB^{++} containing 125 μg *S. marcescens* LPS for 45 min at 37°C followed by 30 min at 56°C. A 2.0 ml aliquot was chromatographed on a G-100 Sephadex column (38 cm × 2.5 cm). Phosphate-buffered saline (PBS) at pH 7.1 (0.02 M phosphate, 0.85% NaCl) was used as the eluent. Blue dextran, hemoglobin, and cytochrome *c* were used as molecular markers. 1 ml aliquots of eluent fractions were diluted with 2.0 ml Gey's medium and tested for chemotactic activity as described.

Sucrose Density Gradient Separation.—A linear density gradient of 10%–40% sucrose in PBS was prepared as previously described (24). Samples to be separated by sucrose density gradient ultracentrifugation were prepared as follows: molecular markers consisting of catalase, bovine serum albumin, and cytochrome *c* (0.4 mg of each in 0.2 ml of PBS); pooled lyophilized fractions containing the peak of chemotactic factor activity after Sephadex

chromatography reconstituted to one-fourth the original volume with distilled water; and chemotactic factor generated in whole fresh guinea pig serum with *S. marcescens* LPS. Samples containing 0.6 ml of molecular markers, or pooled lyophilized chemotactic factor or whole serum in which chemotactic factor was generated, were layered on top of individual sucrose density gradients. The tubes were centrifuged at 35,000 rpm using a SW39 head in a model L Spinco ultracentrifuge for 16 to 18 hr at 5°C. Fractions containing approximately 0.4 ml were collected by puncturing the bottom of the centrifuge tube. The fractions were analyzed in a Beckman DB spectrophotometer at 280 mμ, and 410 mμ, and also tested for chemotactic activity.

RESULTS

Quantitation of Chemotaxis Generated in Guinea Pig Serum by LPS.—Various concentrations of LPS preparations were incubated in guinea pig serum and

TABLE I

Chemotactic Factor Generation by LPS in Guinea Pig Serum

Amount of LPS in reaction mixture*	Cells per high power field			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4‡
(μg)				
20	2000	2000	—	—
5	2000	2000	1536	1776
0.5	1608	888	1056	836
0.05	420	600	240	246
0.005	96	400	228	108
Serum alone	36	216	72	36
20 *S. marcescens* LPS without serum	96	36	0	—
5 *V. alcalescens* LPS without serum	—	—	—	48

* Reaction mixture: 0.1 ml guinea pig serum and 0.1 ml of various concentrations of LPS diluted to 1.0 ml with Gey's medium. Incubation at 37°C for 30 min followed by 56°C for 30 min. Controls consisted of 0.1 ml serum or LPS incubated with 0.9 ml Gey's medium. In Experiments 1–3, *S. marcescens* LPS was used, whereas *V. alcalescens* LPS was used in Experiment 4.

‡ Note photomicrographs taken of chemotactic response in Experiment 4 (Fig. 3).

tested for their chemotaxis-generating properties. Decreasing the concentration of LPS in serum resulted in decreased chemotaxis. The results of three different experiments using *S. marcescens* LPS are shown in Table I. It should be noted that significant chemotaxis was generated with as little as 0.05 μg of LPS. This sensitivity has not been previously reported with other chemotaxis-generating agents employing the Boyden chamber technique or modifications thereof. The highest readings were approximate as the number of cells which migrated through the filter were densely packed and sometimes difficult to distinguish with certainty.

Photomicrographs were taken of a typical dose-response experiment using

V. alcalescens LPS and guinea pig serum (Table I, Fig. 3). The cells which appear to be out of focus are those which have not quite migrated to the lower layer of the filter. The gradation of cells in each field as a reflection of the amount of LPS employed to generate chemotaxis can be appreciated in the photomicrographs.

In addition, sagittal sections through the Millipore filter show the migration of cells through the disc (Fig. 4). The migration of cells in a control preparation (serum alone) is compared with the migration of cells incubated with chemotactic factor generated by a serum-LPS reaction mixture. One can see the length (approximately 150 μ) of the path through which the PMN's must migrate to reach the counting surface. As can be seen in the control disc, during the 3 hr incubation many PMN's have penetrated the upper portion

TABLE II

Effects of Complement System Inhibition or Inactivation on Chemotaxis Generation by LPS

Reaction mixture*	Chemotaxis (cells per high power field)	C′ available for fixation ($C'H_{50}$)
1. No inhibition	1830	15
2. Incubation at 0°C	340	<2
3. Incubation in EDTA	108	<2
4. Preheated serum	65	<2

* Reaction mixtures: 1. As described in Materials and Methods using 0.1 ml guinea pig serum and 5 μg LPS. 2. Initial incubation at 0°C for 30 min, then 56°C for 30 min. 3. Incubation in the presence of 0.02 M EDTA. 4. Serum source preheated at 56°C for 30 min prior to incubation.

of the disc but few have migrated to the counting surface. In the test disc, a massive migration of PMN's to the counting surface is obvious. Sectioning of the disc in this manner might provide alternate methods of quantitating chemotaxis as well as studying kinetics of PMN migration.

Effects of Inhibiting Hemolytic C′ on Chemotactic Factor Generation.—Preheating serum at 56°C for 30 min, incubating at 0°C rather than the usual 37°C, and incubating in the presence of 0.02 M EDTA, all effectively diminished C′ fixation (22). Incubation of LPS in such sera generated little or no chemotactic factor (Table II). Chemotaxis generation was fully restored when a 37°C incubation followed the 0°C incubation.

Detoxified preparations of LPS ("endotoxoids") are deficient in certain biologic properties characteristic of LPS and are also deficient in C′-fixing properties which can be restored when anti-endotoxin serum is incubated with the endotoxoid (15, 25). Endotoxoid serves as a useful control to determine whether a non-C′-fixing endotoxin-derived preparation generates chemotaxis in

fresh serum. The endotoxoid was found to be deficient in chemotactic generating properties. However, in the presence of rabbit anti-*S. marcescens* antibody, the endotoxoid regained a capacity to generate chemotactic factor concomitant with the ability to fix hemolytic complement (Table III).

The ability of LPS to generate chemotaxis was tested in sera of mice con-

TABLE III

C′ Fixation and Chemotactic Factor Generation Using Endotoxoid with and without Antibody

Incubation mixture*	C′ fixation‡	Chemotactic factor generation (cells per high power field)
	%	
Endotoxoid alone	<10	48
Endotoxoid with "heavy" fraction anti-*S. marcescens* antibody	81	228

* 50 μg endotoxoid incubated with 0.1 ml guinea pig serum alone or with addition of 0.4 ml pooled anti-*S. marcescens* antibody (see Materials and Methods).

‡ Expressed as per cent of total available units ("fixed") (15 $C'H_{50}$ available).

TABLE IV

Chemotactic Factor Generation by LPS in C′5-Deficient Compared to Normal Mouse Serum

Reaction mixture*	Cells per high power field		
	Exp. 1	Exp. 2	Exp. 3
B10.D2/Sn "old" and 10 μg LPS	36	24	36
B10.D2/Sn "new" and 10 μg LPS	264	228	396
B10.D2/Sn "old" serum control without LPS	18	24	12
B10.D2/Sn "new" serum control without LPS	48	3	48
10 μg LPS without serum	12	18	12

* 0.2 ml pooled mouse serum incubated with 10 μg LPS for 30 min at 37°C followed by 30 min at 56°C. Controls incubated similarly.

genitally C′-deficient. Different strains of inbred mice vary greatly with respect to their hemolytic C′ titers (26, 27). Mice of the strains B10.D2/Sn "old" and B10.D2/Sn "new" differ in that the old strain is completely devoid of C′ measured by the hemolytic assay while the new strain has normal mouse C′ titers (27). Furthermore, the C′ deficiency in the old strain was found to be due to a complete lack of a serum protein analogous to human C′5 (18). The strains are otherwise coisogenic. While chemotactic factor was generated by LPS in the new strain serum, no significant chemotactic factor generation is detectable using old strain serum (Table IV).

Comparison of Chemotactic Factor Generation, Hemolytic C′ Fixation, and Classical C′3 Fixation by V. alcalescens LPS, BSA–anti-BSA, and Zymosan in Guinea Pig Serum.—In this experiment, 0.9 ml of fresh guinea pig serum was incubated with various quantities of *V. alcalescens* LPS, BSA–anti-BSA, or zymosan. Chemotactic assays were otherwise the same as previously described. C′ fixation assays were performed to determine total hemolytic $C'H_{50}$ units fixed and classical C′3 fixed (Table V).

TABLE V

*Comparison of the Effects of V. alcalescens LPS, Zymosan, and Preformed Complexes of BSA-Rabbit–Anti-BSA Upon Classical C′3, Total C′ Hemolytic Activities, and Chemotactic Factor Generation in Undiluted Guinea Pig Serum**

Material tested	Amount	Total C′	Classical C′3 (C′-EDTA)	Cells per high power field
	μg			
LPS	10	<10	19	1044
LPS	25	25	52	840
LPS	50	38	69	1332
LPS	100	43	83	1244
LPS	200	>67	89	1290
Zymosan	25	<10	<10	252
Zymosan	100	<10	22	612
Zymosan	200	21	30	1392
Zymosan	500	41	58	1404
BSA–anti-BSA	33	<10	19	348
BSA–anti-BSA	65	26	43	372
BSA–anti-BSA	130	43	49	408
BSA–anti-BSA	260	63	69	1116
None	—	0	0	36
C′ activity available		126	819	

* C′ fixation expressed as per cent available C′.

Significant chemotaxis could be seen wherever there was significant C′ fixation. Chemotactic factor generation was detectable with lower doses of test reagents than was fixation of total hemolytic C′, or classical C′3. This may indicate that the chemotaxis assay is more sensitive than the hemolytic assay for detection of C′ fixation. Chemotactic factor generation was more closely correlated with fixation of the classical C′3 components than with total C′ fixation.

Comparison of Sera of Various Species as Source of Chemotactic Factor Generated by LPS.—Sera of guinea pig, sow, mouse, rabbit, and human were tested

to determine if all could generate chemotactic factor in the presence of LPS. All were found to do so but not to the same degree. Guinea pig and sow sera were the most potent sources of chemotactic factor after incubation with a constant amount of LPS (Table VI). In mouse, rabbit, and human sera, chemo-

TABLE VI

Chemotactic Factor Generation in Sera of Various Species

Species (serum-LPS incubation mixtures) *	Cells per high power field	
	With LPS	Serum control
Guinea pig (0.1 ml)	1830	120
Sow (0.1 ml)	828	36
Mouse B10.D2/Sn "new" (0.2 ml)	264	3
Rabbit (0.2 ml)	256	3
Human (0.3 ml)	276	12

* The indicated volume of serum was incubated at 37°C for 30 min with 5 μg *S. marcescens* LPS and diluted to a total volume of 1.0 ml with Gey's medium.

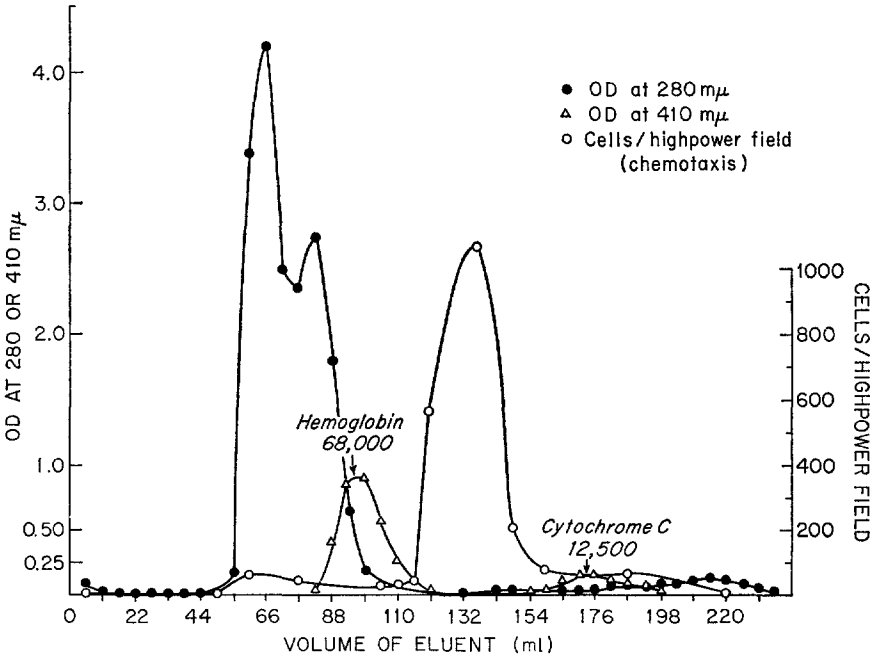

FIG. 1. G-100 Sephadex separation of fresh guinea pig serum-LPS reaction mixture. The peak of chemotactic factor activity lies between the previously calibrated hemoglobin and cytochrome *c* peaks.

tactic factor generation could be reproducibly detected only when 0.2 ml or 0.3 ml of sera was used in the reaction mixture.

Characterization of Chemotactic Factor Generated by LPS in Guinea Pig Serum.—Chemotactic factor generated by interaction of LPS with whole guinea pig serum was found to be heat stable (56°C) and nondialyzable. It furthermore maintained activity during storage at −70°C or 0°C for 96 hr. Maximal generation of chemotactic factor took place within 15 min after incubation of serum and LPS at 37°C.

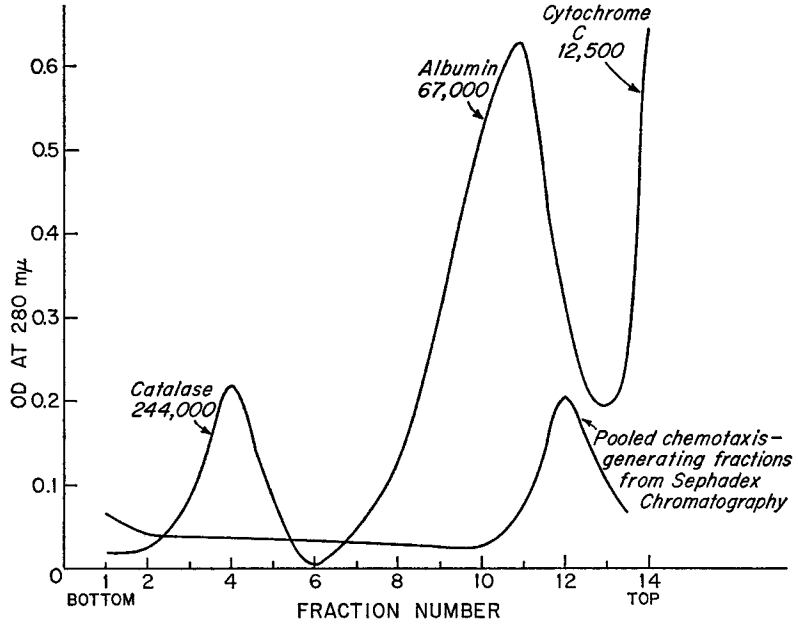

FIG. 2. Sucrose density gradient ultracentrifugation of chemotactic factor obtained by pooling the fraction with chemotactic activity from Sephadex chromatography. The peak of chemotactic factor 280 mμ absorption is compared to molecular weight markers centrifuged simultaneously.

Chemotactic factor was generated in whole guinea pig serum by incubation with *S. marcescens* LPS and was separated on a G-100 Sephadex column. The elution volumes of blue dextran, hemoglobin, and cytochrome *c* were determined and used as molecular weight markers. The peak of chemotactic factor generation was eluted at a volume falling between that of hemoglobin and cytochrome *c* (Fig. 1).

Sucrose density gradient separation was performed using catalase, albumin, and cytochrome *c* as molecular weight markers. An aliquot of the pooled peak of chemotactic factor activity isolated by Sephadex chromatography was placed

on another sucrose density gradient and centrifuged simultaneously with the molecular weight markers. A sharp peak of the chemotactic factor protein was localized in the lighter fractions of the gradient. The relationship of the molecular markers to the chemotactic factor is seen in Fig. 2. In addition, the chemotactic factor in whole serum was isolated by sucrose density gradient separation. The peak of chemotactic factor activity was again localized in the lighter fractions of the gradient. This supported the previous size characterization of the chemotactic factor by Sephadex chromatography.

These two physical methods of isolating chemotactic factor indicate its molecular weight to be between 12,400 and 68,000. Furthermore, the elution volume (V_e) of the chemotactic factor as well as the void volume (V_o) of the G-100 Sephadex column was determined. These values were used to determine K_{av}[4] of the chemotactic factor. When this was plotted on a K_{av} vs. molecular weight scale for G-100 Sephadex, the molecular weight was estimated to be in the range of 15,000–30,000 (28).

DISCUSSION

There is an increasing awareness of the important role the complement system, and in particular the terminal components, can play as a mediator of the inflammatory response (4, 5).

It has recently been shown that LPS fixes large amounts of those terminal C′ components related to the biologic activity mediated by the complement system (3). It has thus been suggested that the inflammatory response to endotoxin might be at least in part complement dependent (2).

Injection of submicrogram quantities of LPS into the skin of rabbits or man leads to the accumulation of large numbers of PMN's within hours after injection (6, 7). Appreciating the strong reactivity of endotoxin with the complement system, we investigated the possibility that the in vitro generation of factors chemotactic for rabbit PMN's by endotoxin in serum might be dependent upon complement system activation.

The most suitable means for studying in vitro chemotaxis was found to be a minor modification of the method described by Boyden (8). Using a smaller pore size Millipore filter we reduced the background counts of serum controls and still were able to get high and reproducible cell counts in test situations. Significant PMN chemotaxis was recorded when submicrogram levels of LPS were incubated in fresh guinea pig serum. This indicates the assay system has a sensitivity not previously appreciated, and detects chemotaxis generated by quantities of LPS approaching the lower limits which are able to produce inflammation in vivo.

In the present experiments, immune precipitates and zymosan as well as LPS

[4] K_{av} is the partition coefficient between the liquid phase and the gel phase calculated for the particular column used in these experiments.

generated chemotactic factor in fresh guinea pig serum. Chemotaxis generation could be detected using all these agents at dilutions too great to detect C′ fixation employing a standard hemolytic C′ assay (22). Zymosan was less efficient in chemotaxis generation than LPS or immune precipitates when compared on a weight basis. This corresponds closely to what was seen in hemolytic C′ fixation assays.

Three lines of evidence demonstrated the requirement of the C′ system for the generation of chemotactic factor by LPS in serum. First, LPS did not generate chemotaxis in sera so pretreated as to prevent C′ fixation. Preheating serum, incubating at 0°C, and incubating in the presence of EDTA effectively diminished chemotactic factor generation. When incubation was first carried out at 0°C and then at 37°C, activity was restored. These tests suggested that the generation of the chemotactic factor in serum was enzymatic in nature and demonstrated that this generation required a heat-labile, cation-dependent serum system. A second line of evidence implicating C′ was the lack of chemotactic factor generation by the endotoxoid preparation. This preparation was chemically modified so as to lose certain toxicities and C′-fixing abilities which could be restored by adding specific anti-endotoxin serum (15, 25). In the present experiment, when rabbit anti-*S. marcescens* antibody was added to the endotoxoid preparation, both chemotaxis-generating and C′-fixing properties in normal guinea pig serum were restored. The third line of evidence implicating the C′ system was the lack of chemotactic factor generation by LPS in C′-deficient mouse serum while the serum of the coisogenic, non–C′-deficient strain supported chemotactic factor generation by LPS. The "old" strain was found to be deficient in a euglobulin designated Hc^1 which was present in the "new" strain (29). It was furthermore found that the presence of this protein was related to a single gene (30). This euglobulin was found to be analogous to human C′5 (18). New line mouse sera when incubated with endotoxin generated chemotactic factor, but the C′5 deficient old strain serum under the same conditions did not. This finding implicated C′5 and/or components terminal to C′5 as being associated with the LPS-generated chemotactic factor.

Ward has recently described two different chemotactic factors derived from the C′ system. The first of these was a trimolecular complex of C′5, C′6, and C′7 (10, 11). The second factor described was a "split" product of C′3, generated upon interaction of this component with plasmin (13). The C′3 split product was of low molecular weight (6000) and heat labile, while the trimolecular complex was described as being of high molecular weight (>300,000) and heat stable. The LPS-generated chemotactic factor was heat stable, nondialyzable, and dependent on at least the C′5 component of the C′ system, and on this basis we initially thought it might be analogous to the trimolecular complex. We found, however, that when the chemotactic factor generated by LPS in whole guinea pig serum was chromatographed on a G-100 Sephadex column, its elu-

tion volume was between that of hemoglobin and cytochrome *c*. This suggested a molecular weight between 15,000 and 30,000 when plotted on a K_{av} vs. molecular weight scale for G-100 Sephadex (28). Sucrose density gradient separation of LPS-induced chemotactic factor using similar molecular markers supported the chromatographic findings. On the basis of molecular weight, the LPS-induced chemotactic factor appears different from previously described chemotactic factors derived from the C′ system. Whether this is the result of species difference, mode of activation, or other factors is under investigation.

LPS-induced chemotactic factor could well be a product derived from one of the C′ components terminal to C′3, perhaps C′5. This view is supported by the finding of an inability to generate chemotactic factor by LPS in C′5-deficient mouse serum. Further insights will be gained by the use of purified C′ components to study the interaction of LPS with the C′ system. The mechanism of generation of a low molecular weight chemotactic factor might be analogous to recently proposed mechanisms of anaphylatoxin generation (31–33). Two products with anaphylatoxin activities have been shown to be "split" products of C′ system components. One was a derivative of C′3, while the other was derived from C′5.

During activation of the C′ system by immune precipitates, endotoxins, or products liberated during hemostasis (13), a number of biologically active products might be liberated from the terminal components of complement. Endotoxin-induced chemotactic factor could be among these C′-derived products. Anaphylatoxin generation by LPS in fresh serum has recently also been shown to be C′ dependent (34) and similarly might be released along with the chemotactic factor upon endotoxin-serum interaction. Such products could initiate certain of the inflammatory processes with which endotoxin has been associated.

Thus, the present study supports the hypothesis that the C′ system mediates certain aspects of the inflammatory process induced by endotoxin by releasing biologically active products.

SUMMARY

Endotoxic lipopolysaccharide has recently been shown to fix large amounts of the complement components related to the biologic activities mediated by that system. The present study sought to determine whether the generation of chemotactic factor by endotoxin in serum was dependent upon complement system activation.

Preheating serum, incubating at 0°C, or incubating in the presence of EDTA, all prevented chemotactic factor generation as well as complement fixation by endotoxin. "Endotoxoids" deficient in complement-fixing activity were also deficient in chemotactic factor generation. Chemotactic factor could not be generated by endotoxin in sera of mice congenitally deficient in the C′5 component of complement, while chemotactic factor was generated by endotoxin in the sera of coisogenic mice with normal complement levels for that species.

The chemotactic factor induced by endotoxin was heat stable and nondialyzable. Molecular sieve chromatography and sucrose density gradient ultracentrifugation demonstrated that the chemotactic factor was a relatively low molecular weight product (15,000–30,000) and as such different from previously scribed C′ system–derived chemotactic factors.

These experiments demonstrate that generation of chemotactic factor by endotoxin in serum is dependent upon C′ system activation involving at least C′5. Furthermore, the relatively low molecular weight of this factor suggests that it might be derived from activation of a single complement component rather than from complexing of multiple complement components.

The authors gratefully acknowledge the technical assistance of Mrs. Jean K. Phillips and Mrs. Suanne F. Dougherty. We would also like to thank Miss Cheryl Burg for preparation of the sagittal sections of Millipore discs.

BIBLIOGRAPHY

1. Bladen, H. A., R. T. Evans, and S. E. Mergenhagen. 1966. Lesions in *Escherichia coli* membranes after action of antibody and complement. *J. Bacteriol.* **91**:2377.
2. Bladen, H. A., H. Gewurz, and S. E. Mergenhagen. 1967. Interactions of the complement system with the surface and endotoxic lipopolysaccharide of *Veillonella alcalescens*. *J. Exptl. Med.* **125**:767.
3. Gewurz, H., H. S. Shin, M. M. Mayer, and S. E. Mergenhagen. 1968. Further studies of the interaction between the complement (C′) system and endotoxic lipopolysaccharide (LPS). *Federation Proc.* **27**:479.
4. Nelson, R. A., Jr. 1965. The role of complement in immune phenomena. *In* The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McClusky, editors. Academic Press Inc., New York. 819.
5. Müller-Eberhard, H. J., U. R. Nilsson, A. P. Dalmasso, M. J. Polley, and M. A. Calcott. 1966. A molecular concept of immune cytolysis. *Arch. Pathol.* **82**:205.
6. Thomas, L. 1954. The physiological disturbances produced by endotoxins. *Ann. Rev. Physiol.* **16**:467.
7. Jensen, S. B. 1967. The influence of endotoxin from oral bacteria on host resistance. Doctoral Thesis. University of Aarhus, Aarhus, Denmark.
8. Boyden, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J. Exptl. Med.* **115**:453.
9. Keller, H. U., and E. Sorkin. 1965. I. On the chemotactic and complement-fixing activity of γ-globulins. *Immunology.* **9**:241.
10. Ward, P. A., C. G. Cochrane, and H. J. Müller-Eberhard. 1965. The role of serum complement in chemotaxis of leukocytes *in vitro*. *J. Exptl. Med.* **122**:327.
11. Ward, P. A., C. G. Cochrane, and H. J. Müller-Eberhard, 1966. Further studies on the chemotactic factor of complement and its formation *in vivo*. *Immunology.* **11**:141.
12. Keller, H. U., and E. Sorkin. 1967. Studies on chemotaxis. V. On the chemotactic effect of bacteria. *Intern. Arch. Allergy Appl. Immunol.* **31**:505.
13. Ward, P. A. 1967. A plasmin-split fragment of C′3 as a new chemotactic factor. *J. Exptl. Med.* **126**:189.

14. Nowotny, A. M., S. Thomas, O. S. Duron, and A. Nowotny. 1963. Relation of structure to function in bacterial O antigens. I. Isolation methods. *J. Bacteriol.* **85**:418.
15. Tripodi, D., and A. Nowotny. 1966. Relation of structure to function in bacterial O antigens. V. Nature of active sites in endotoxic lipopolysaccharides of *Serratia marcescens*. *Ann. N. Y. Acad. Sci.* **133**:604.
16. Westphal, O., and O. Lüderitz. 1954. Chemische erforschung von lipopolysacchariden gram negativer bakterien. *Angew. Chem.* **66**:407.
17. Lowry, 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.
18. Nilsson, U. R., and H. J. Müller-Eberhard. 1967. Deficiency of the fifth component of complement in mice with an inherited complement defect. *J. Exptl. Med.* **125**:1.
19. Hirsch, J. G., and A. B. Church. 1960. Studies of phagocytosis of group A streptococci by polymorphonuclear leukocytes *in vitro*. *J. Exptl. Med.* **111**:309.
20. Osler, A. G., J. H. Strauss, and M. M. Mayer. 1952. Diagnostic complement fixation. I. A method. *Am. J. Syph. Gonor. Ven. Diseases.* **36**:140.
21. Gewurz, H., A. R. Page, R. J. Pickering, and R. A. Good. 1967. Complement activity and inflammatory neutrophil exudation in man. Studies in patients with glomerulonephritis, essential hypocomplementemia and agammaglobulinemia. *Intern. Arch. Appl. Immunol.* **32**:64.
22. Mayer, M. M. 1961. Complement and complement fixation. *In* Kabat and Mayer's Experimental Immunochemistry. Charles C Thomas, Springfield, Ill. 2nd edition. 133.
23. Nelson, R. A., Jr., J. Jensen, I. Gigli, and N. Tamura. 1966. Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry.* **3**:111.
24. Kunkel, H. G. 1960. *In* The Plasma Proteins. F. W. Putnam, editor. Academic Press, New York. **1**:279.
25. 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.
26. Rosenberg, L. T., and D. K. Tachibana. 1962. Activity of mouse complement. *J. Immunol.* **89**:861.
27. Terry, W. C., T. Borsos, and H. J. Rapp. 1964. Differences in serum complement activity among inbred strains of mice. *J. Immunol.* **92**:576.
28. Andrews, P. 1964. The gel filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* **91**:222.
29. Herzenberg, L., D. K. Tachibana, and L. T. Rosenberg. 1963. A gene locus concerned with hemolytic complement in *Mus musculus*. *Genetics.* **48**:711.
30. Erickson, R. P., D. K. Tachibana, L. Herzenberg, and L. T. Rosenberg. 1964. A single gene controlling hemolytic complement and a serum antigen in the mouse. *J. Immunol.* **92**:611.
31. Jensen, J. 1967. Anaphylatoxin in its relation to the complement system. *Science.* **155**:1222.
32. Dias da Silva, W., J. W. Eisele, and I. H. Lepow. 1967. Complement as a mediator

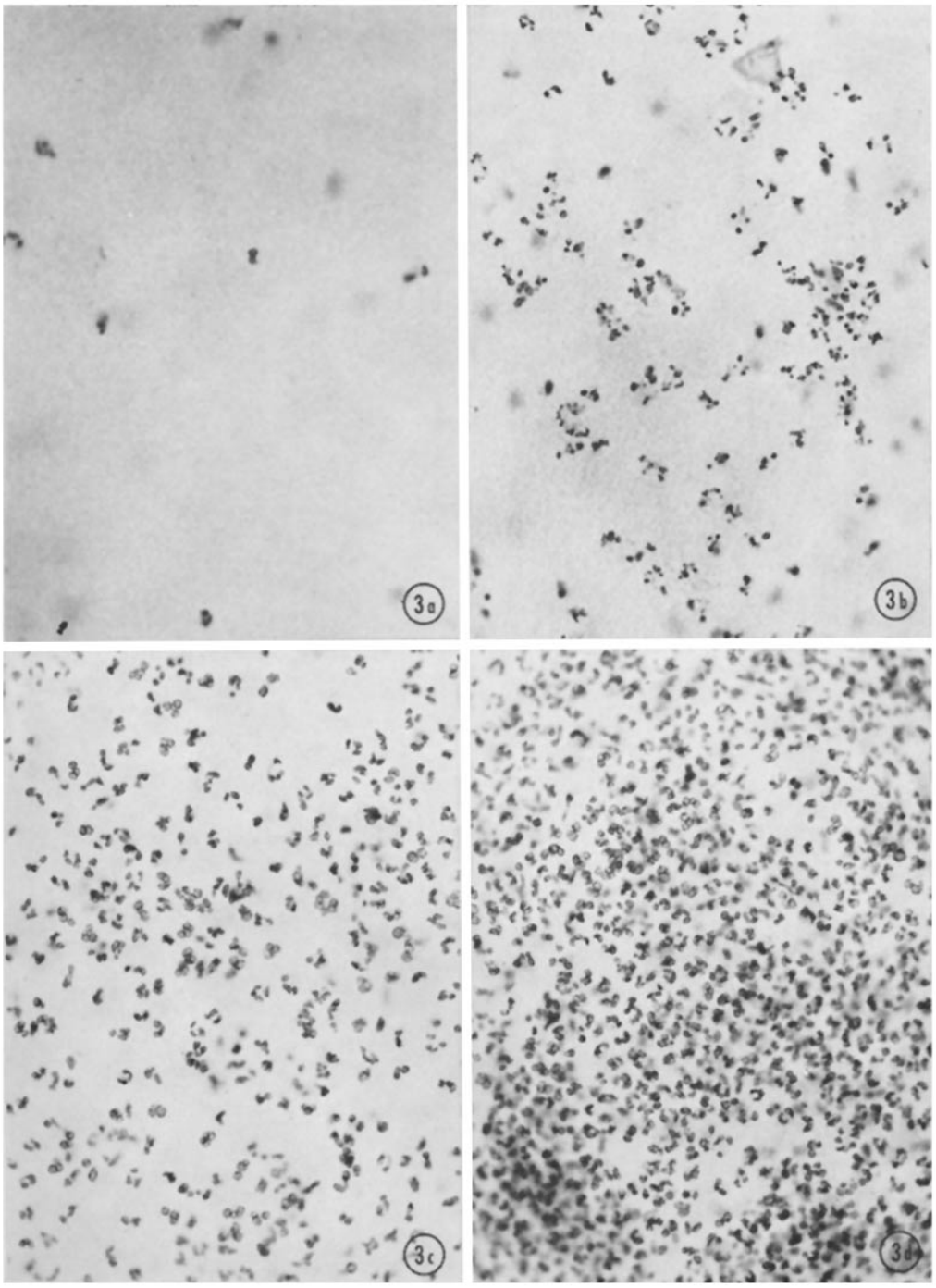

FIG. 3. Neutrophil response to chemotactic factor generated by graded doses of *V. alcalescens* LPS incubated in guinea pig serum. *a*. Serum alone. *b*. Serum plus 0.05 μg LPS. *c*. Serum plus 0.5 μg LPS. *d*. Serum plus 5.0 μg LPS. × 325.

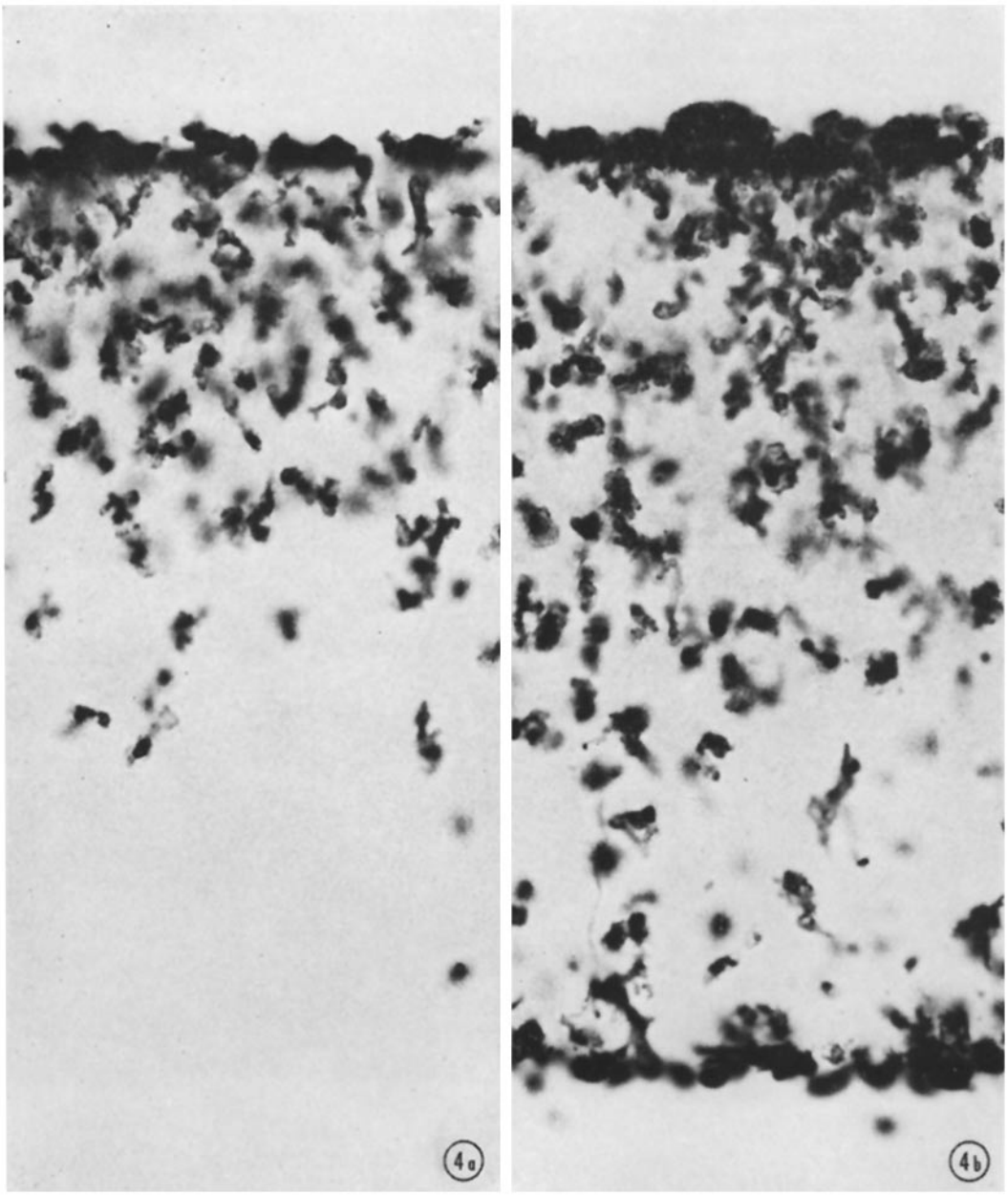

FIG. 4. Sagittal section of Millipore disc after incubation of neutrophils. *a*. Serum alone. *b*. Chemotactic factor generated by LPS and serum. Top of figure represents surface on which neutrophils were applied while bottom of figure represents counting surface. × 770.

of inflammation. III. Purification of the activity with anaphylatoxin properties generated by interaction with the first four components of complement and its identification as a cleavage product of C'3. *J. Exptl. Med.* **126**:1027.

33. Cochrane, C. G., and H. J. Müller-Eberhard. 1968. The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement. *J. Exptl. Med.* **127**:37.
34. Adkinson, N. F., L. M. Lichtenstein, H. Gewurz, and S. E. Mergenhagen. 1968. The generation of anaphylatoxin by interaction of endotoxin and guinea pig serum. *Federation Proc.* **27**:316.