SERUM-DEPENDENT PHAGOCYTOSIS OF PARAFFIN OIL EMULSIFIED WITH BACTERIAL LIPOPOLYSACCHARIDE*

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The ability of fresh serum to opsonize microorganisms was recognized nearly 70 yr ago (1). Subsequent studies identified synergistic effects of both antibody and complement on opsonization (2) and demonstrated that particles to which C3 was affixed by the sequential participation of antibody, C1, C4, and C2 became opsonized (3–6). In 1968, a patient was found with marked susceptibility to infection who spontaneously inactivated C3 in vivo. His serum had normal functional levels of antibody, C1, C4, and C2, but failed to opsonize bacteria even when fortified with purified C3 (7). Further studies of his serum revealed a defect in his properdin pathway (8, 9). This patient, and the good health of humans deficient in C2 (10) and of guinea pigs deficient in C4 (11), have emphasized the functional significance of this alternate pathway of C3 activation, first recognized by Pillemer and his associates (12). The properdin system has been shown to participate in bacteriolysis, protozoal inactivation, virus neutralization, and lysis of erythrocytes in paroxysmal nocturnal hemoglobinuria (13–16). Unlike the classical complement pathway, the properdin system can be activated by polysaccharides in the absence of antibody (17).

This report describes quantitative studies of the phagocytosis of paraffin oil droplets coated with bacterial lipopolysaccharide. Opsonization of these particles occurs in the absence of C4 and C2, but is dependent upon C3 and the properdin pathway. Measurement of the opsonization of these particles thus constitutes a quantitative functional assay for this system.

Materials and Methods

Preparation of Phagocytes.—Suspensions containing over 95% polymorphonuclear leukocytes were obtained from guinea pig peritoneal exudates. The exudates were elicited with sodium caseinate, and the cells were collected and washed as previously described (18). Human peripheral blood from normal laboratory personnel or patients with infections and leukocytosis was collected with acid-citrate dextrose, National Institutes of Health formula A; the erythrocytes were removed by dextran sedimentation and lysis with ammonium chloride; and leukocytes were washed as described previously (19). The cells were suspended in Krebs-Ringer phosphate medium, pH 7.4 (medium), at a concentration of 2–5 mg of guinea pig

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granulocyte protein/ml or 1.5–6.0 × 10^7 human leukocytes/ml, of which 70–95% were phagocytes (polymorphonuclear leukocytes, band forms, and monocytes). Cellular protein was determined by the Folin method (20), and cells were enumerated with an electronic counter (Coulter Electronics, Inc., Hialeah, Fla.)

Serum and Purified Serum Proteins.—Guinea pig blood was collected by cardiac puncture and human blood, by venipuncture. The serum was separated and maintained at 0°C until used in experiments or stored at −70°C. Serum of guinea pigs genetically deficient in C4 (11) was a gift of Dr. Leonard Ellman. Serum was obtained from individuals whose serum contained less than 1% of the normal C2 concentration (10), from patients with hereditary angioneurotic edema (HANE)[1] during acute attacks (whose serum contained less than 1% of normal C6 and C2 (21), from a patient with hereditary deficiency of C3 (22), from a patient with type I hypercatabolism of C3 (7), and from cord blood of full-term newborn infants, some of which were deficient in glycine-rich beta-glycoprotein (GBG) (23). In some experiments, human serum was treated with zymosan, 70 mg/ml, or hydrazine, 0.03 M, or heated for 30 min at 56°C. GBG, factor B of the properdin system, or the C3 proactivator (24) was purified from normal human serum as described previously (25). C3 was prepared by the method of Nilsson and Müller-Eberhard (26). C3 was labeled with ^{125}I by the iodine monochloride technique (27). C4, C2, C3, and GBG were measured by electroimmunoassay (28).

Lipopolysaccharide Particles.—Lipopolysaccharide, prepared by extraction with trichloroacetic acid and ethanol precipitation (29) from *Escherichia coli* O26:B6, was purchased from Difco Laboratories, Detroit, Mich. It was used as received or after the treatments described below:

(*a*) 100 mg of the lipopolysaccharide was suspended for 12 h in 40 ml of chloroform-methanol (2:1) at 4°C and centrifuged. After 12 h, the lipopolysaccharide was collected by centrifugation and dried under N_2.

(*b*) 30 mg of the lipopolysaccharide was suspended for 15 min at 37°C in 2 ml of fresh human serum and then washed and collected twice by sedimentation through 10 ml of 2% bovine serum albumin in 0.15 M NaCl and twice through 0.15 M NaCl at 100,000 *g* (max) for 30 min.

(*c*) The lipopolysaccharide was acetylated (30) or deacylated with pyridine-formic acid or BF_3-methanol (31).

Emulsions of paraffin oil containing oil red O were prepared as previously described (32). 30 mg of the lipopolysaccharide was suspended in 3 ml of medium and dispersed by sonication for 5 s. 1 ml of heavy paraffin oil containing oil red O (32) was layered over the lipopolysaccharide suspension and emulsified by sonication for 45 s.

Lipopolysaccharide Immune Serum.—Antibody to lipopolysaccharide was prepared by injecting 0.2 ml of lipopolysaccharide particles into rabbits intravenously every other day for 7 days. 3 wk later another injection was administered, and 7 days thereafter the rabbits were bled. This serum and serum from untreated rabbits were heated at 56°C for 30 min. The immune serum, but not the control serum, yielded precipitin lines with the lipopolysaccharide on double diffusion in agarose gel. It contained 941 μg of antibody protein/ml.

Opsonization.—Unless indicated otherwise, the particles were opsonized before being added to the cells. Thus, the measured rate of ingestion was not influenced by the rate of opsonization. In all instances, homologous serum was used. In most experiments with guinea pig granulocytes, serum was incubated with lipopolysaccharide particles (0.3 ml serum/0.7 ml of particles) for 15 min at 37°C. In most experiments with human leukocytes, 0.5 ml of human serum was incubated with an equal volume of particles. In some experiments, the particles were washed after opsonization. The washing was accomplished by centrifugation

[1] *Abbreviations used in this paper:* GBG, glycine-rich beta-glycoprotein; HANE, hereditary angioneurotic edema.

at 100,000 *g* (max) for 30 min, aspiration of the infranatant fluid from the pellicle of particles, and resuspension of the particles in fresh medium. For investigation of the effect of divalent cations on ingestion, the particles were dialyzed after opsonization, first against 1 mM EDTA, pH 7.0, in 0.15 M NaCl, and then against 0.13 M NaCl and 15 mM sodium phosphate, pH 7.4.

Fixation of [^{125}I]C3 to Lipopolysaccharide and Lipopolysaccharide Particles.—^{125}I-labeled C3, specific activity 6×10^4–3×10^5 cpm/μg, was added to fresh human serum or heat-inactivated human serum (final activity 2.1×10^5 cpm/ml). Lipopolysaccharide or lipopolysaccharide particles were incubated with the serum so that the final lipopolysaccharide concentration was 15 mg/ml of serum, and then washed with albumin and NaCl as described above. Radioactivity of the lipopolysaccharide or lipopolysaccharide particles was determined with a Packard Auto-Gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Incubations.—The rate of ingestion of paraffin oil red O particles by leukocytes was measured as previously described (32). Briefly, lipopolysaccharide particles were incubated with polymorphonuclear leukocytes in medium at 37°C. At zero time and appropriate intervals thereafter, uningested particles were removed from the cells by differential centrifugation in 0.15 M NaCl containing 1 mM *N*-ethyl-maleimide at 150 *g*, and the oil red O content of dioxane extracts of the cell pellets was measured spectrophotometrically at 525 nm as previously described (32). Zero time values were zero in this system, and differences between duplicate samples at all times were less than 5%.

RESULTS

Assay of Ingestion of Lipopolysaccharide Particles by Guinea Pig Granulocytes.—As shown in Fig. 1, when opsonized particles in the usual concentration were added to granulocytes, ingestion began immediately and continued at a constant rate for about 6 min. The rate of ingestion was not increased when the particle concentration was doubled. Particles not treated with serum were ingested at a rate less than 10% of that of opsonized particles. When cells, unopsonized particles, and guinea pig serum were incubated simultaneously, rapid ingestion was achieved only after 4 min, presumably the time required for opsonization. No detectable ingestion of opsonized particles occurred during incubation with cells for 10 min in the presence of 1 mM *N*-ethyl-maleimide or at 0°C. Similar results were obtained with human serum (19).

Maximal opsonization was produced with 0.03 ml of guinea pig serum/mg of lipopolysaccharide (Fig. 2, inset). As shown in Fig. 2, the stimulatory effect of serum on the initial rate of ingestion was not significantly decreased by prolonged dialysis against 0.15 M NaCl at 4°C. It was abolished if the serum was heated (56°C for 30 min), incubated with zymosan, hydrazine, or EDTA (2 mM). Serum, hydrazine, and EDTA were removed from the particles by washing in these experiments.

Effect of Divalent Cations on the Ingestion of Lipopolysaccharide Particles by Guinea Pig Granulocytes.—Fig. 3 shows that opsonized and dialyzed particles were ingested by guinea pig granulocytes in the absence of added divalent cations. Addition of Ca^{++} or Mg^{++} to the incubation medium at concentrations of less than 1 mM caused a small but reproducible increase in the rates of ingestion, whereas concentrations greater than 1 mM were slightly inhibitory. In medium without added divalent cations, 1 mM EDTA completely inhibited particle uptake (Table I).

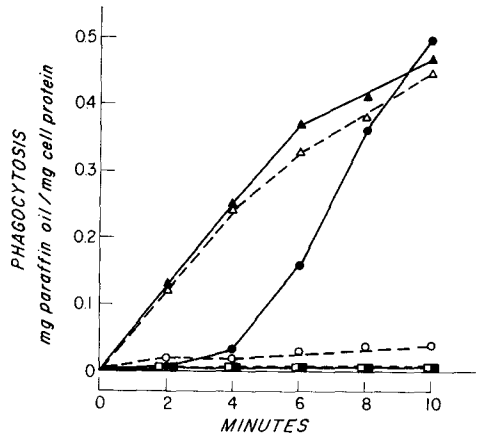

FIG. 1. Ingestion of *E. coli* lipopolysaccharide particles by guinea pig granulocytes. The experiment was performed with six flasks, each containing 15.6 mg of cell protein. All flasks were incubated for 10 min with gentle shaking before addition of particles. Flask 1 (□) contained cells in 4 ml of medium and was maintained at ice bath temperature. 1 ml of particles opsonized with fresh serum was added at zero time. All other flasks were kept at 37°C. Flask 2 (■) contained cells in 4 ml of medium with 1 mM *N*-ethyl-maleimide. Opsonized particles (1 ml) were added at zero time. Flask 3 (○) contained cells in 4 ml of medium. 1 ml of particles previously treated with medium rather than serum was added at zero time. Flask 4 (●) contained cells in 4 ml of medium fortified with fresh guinea pig serum (20% by volume). Particles (1 ml) treated with medium rather than serum were added at zero time. Flask 5 (△) contained cells in 4 ml of medium. 1 ml of particles opsonized with fresh serum was added at zero time. Flask 6 (▲) contained cells in 3 ml of medium. Opsonized particles (2 ml) were added at zero time. Samples were taken from all flasks for washing and oil red O analysis at zero time and at the other times indicated.

Treatment of Lipopolysaccharide Particles after Opsonization by Guinea Pig Serum.—After opsonization, the lipopolysaccharide particles could be washed repeatedly with salt solutions of different concentrations, heated (56°C, 30 min), and even boiled for 15 min without significant loss of ingestibility (Fig. 4). They could also be frozen (−20°C), thawed, and resonicated without diminution of the rate with which they were ingested.

Effect of C4-Deficient Guinea Pig Serum on the Ingestion of Lipopolysaccharide Particles by Guinea Pig Granulocytes.—Lipopolysaccharide particles treated for 15 min at 37°C with C4-deficient guinea pig serum were ingested as rapidly as those incubated with normal guinea pig serum (Table II). The rate, as well as the extent, of opsonization by the C4-deficient serum was normal (Fig. 5).

Effect of Divalent Cations on the Opsonic Activity of Human Serum.—Human serum did not opsonize lipopolysaccharide particles after dialysis against EDTA and NaCl (Table III). Opsonic activity was restored by addition of divalent cations. Magnesium was more effective than calcium, but both magnesium and calcium were required to obtain opsonic activity equal to that of undialyzed serum. If the particles were not dialyzed against EDTA and NaCl

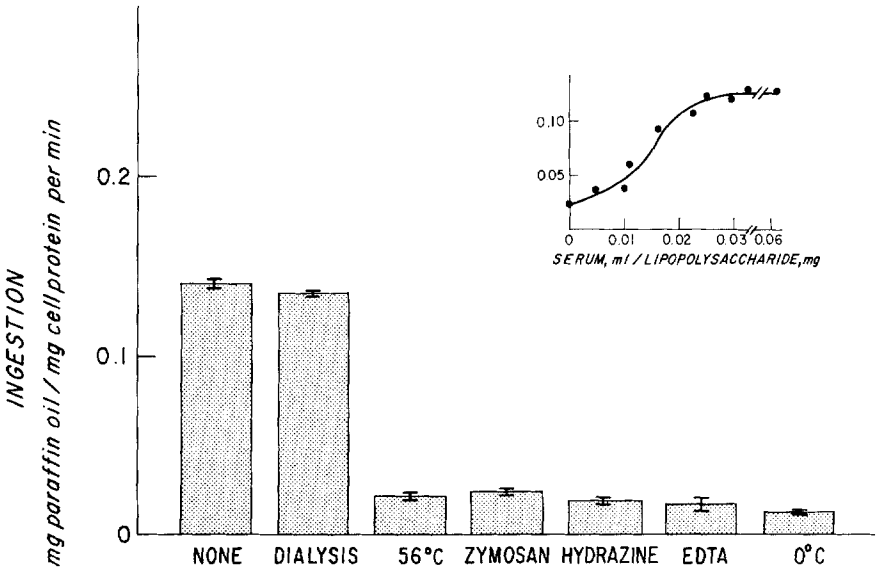

FIG. 2. Effect of guinea pig serum concentration on the rates of ingestion of *E. coli* lipopolysaccharide particles by guinea pig polymorphonuclear leukocytes. The bars indicate the initial rate of ingestion by guinea pig granulocytes of *E. coli* lipopolysaccharide particles opsonized with serum treated as indicated and designate the mean and range of duplicate determinations. All flasks contained 11.2 mg of cell protein in 4 ml of medium. Cells were incubated for 10 min before addition of 1 ml of particles as described in Fig. 1. Samples were taken for washing and oil red O analysis at zero time and at 4 min. (Inset) This emulsion was prepared as described in the text, except that 50 mg of lipopolysaccharide was suspended in 3 ml of medium. *E. coli* lipopolysaccharide emulsion, 0.7 ml, was opsonized with 0.3 ml of serum or serum diluted with medium. The points are means of duplicate determinations.

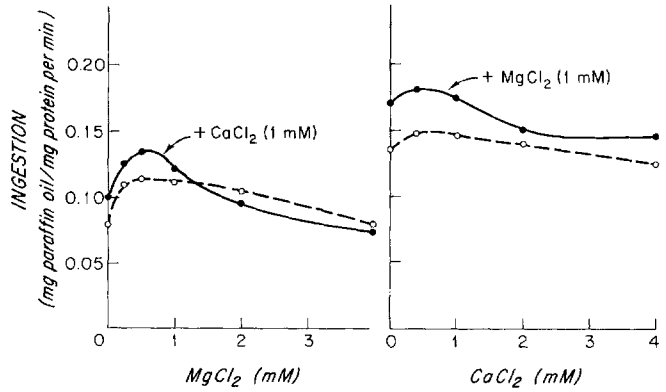

FIG. 3. Effect of Mg^{++} and Ca^{++} on the initial rate of ingestion of opsonized *E. coli* ipopolysaccharide particles by guinea pig granulocytes. The cells were washed twice with 0.15 M NaCl and suspended in 0.13 M NaCl containing 15 mM sodium phosphate, pH 7.4, and divalent cations as indicated. Opsonized particles, dialyzed as described in the text, were added at zero time, and the initial rate of ingestion was determined as described in Fig. 2.

TABLE I

Effect of Divalent Cations and EDTA on Ingestion of Opsonized Lipopolysaccharide Particles by Guinea Pig Granulocytes

Divalent cation composition of buffer	Initial rate of ingestion
No divalent cations	0.090
$CaCl_2$ (1 mM)	0.110
$MgCl_2$ (1 mM)	0.121
$MgCl_2$ (1 mM) + $CaCl_2$ (1 mM)	0.116
No divalent cations + EDTA (1 mM)	0.000

Cells were washed twice with 0.15 M NaCl and suspended in 0.13 M NaCl containing 15 mM sodium phosphate, pH 7.4. The particles were dialyzed against EDTA and NaCl as described in the text. All reagents were prepared with deionized water. Particles (1 ml) were added to granulocytes in 4 ml of medium at zero time. Samples (1 ml) were taken at zero time and at 4 min from duplicate incubations to assay the initial rate of ingestion. The ingestion rate is expressed as milligrams paraffin oil per milligram protein per minute.

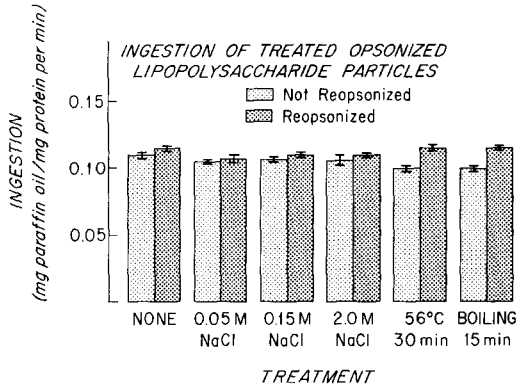

FIG. 4. Effect of heat and NaCl on the ingestibility of opsonized *E. coli* lipopolysaccharide particles by guinea pig granulocytes. The particles were opsonized, washed, or heated, and then divided into two portions. One portion (darker bars) was opsonized again with fresh serum, and the other was incubated with medium alone (lighter bars). The particles (1 ml) were added to flasks containing cells (12 mg protein) in 4 ml of medium. The initial rate of ingestion of particles was determined as described in Fig. 2. The bars represent the mean; and the lines, the range of values from duplicate incubations.

before opsonization, no apparent requirement for divalent cations by dialyzed serum was detectable.

Opsonic Activity of Human Serum Deficient in Various Complement Proteins.— The opsonic activities of sera from two patients with hereditary C2 deficiency and from five patients with HANE without demonstrable hemolytic C2 or C4 were indistinguishable from those of normals (Table IV and Fig. 6).

Some human cord sera have subnormal GBG levels. Although most cord sera tested with the lipopolysaccharide-paraffin oil system had decreased op-

TABLE II

Effect of Normal Guinea Pig Serum and C4-Deficient Guinea Pig Serum on the Initial Rate of Ingestion of Lipopolysaccharide Particles by Guinea Pig Granulocytes

Serum source	Initial rate of ingestion		
	Exp. 1	Exp. 2	Exp. 3
Normal guinea pig	0.112	0.124	0.096
C4-deficient guinea pig	0.115	0.125	0.094

E. coli lipopolysaccharide particles were opsonized with serum as described in the text. The conditions of incubation and analysis of ingestion were essentially as described in Table I. The means of values from duplicate incubations in three experiments are shown.

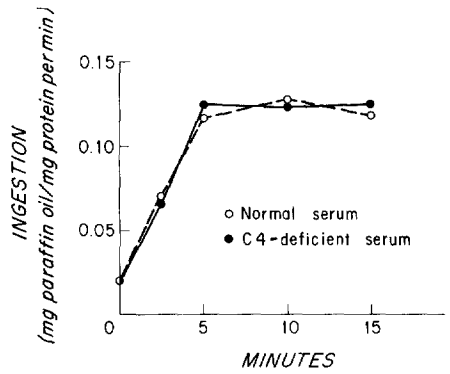

FIG. 5. Rate of opsonization of *E. coli* lipopolysaccharide particles by normal and by C4-deficient guinea pig serum. Normal guinea pig serum (○) or C4-deficient guinea pig serum (●) was incubated with lipopolysaccharide particles at 37°C for the time indicated. The incubations were terminated by addition of 10 vol of ice-cold 0.15 M NaCl containing 1 mM EDTA. The particles were washed and added in 1 ml volumes to flasks containing guinea pig granulocytes (13.2 mg of cell protein) in 4 ml of medium. The initial rate of ingestion was determined from samples taken at zero time and at 4 min. Each point designates the mean of determinations from duplicate flasks.

sonic activity, those with diminished amounts of GBG had even less opsonic capacity than those with normal GBG. The addition of purified GBG to GBG-deficient cord serum increased its opsonic activity (Fig. 7). This cord serum had a C3 concentration of 1,200 mg/liter, i.e., within the normal range. The serum of a child with hereditary C3 deficiency failed to opsonize the lipopolysaccharide emulsion. As shown in Fig. 8, addition of purified C3 to the serum of this patient restored opsonic capacity. However, addition of purified C3 to the serum of a patient with hypercatabolism of C3 was without effect.

The initial rate of ingestion of lipopolysaccharide particles by human leukocytes was reproducibly increased 15–30% by incubating the particles with heated lipopolysaccharide immune serum (0.4 mg antibody protein) before opsonization with fresh normal serum, C2-deficient serum, or cord serum deficient in GBG (Table V).

TABLE III

Effect of Divalent Cations on the Opsonization of Lipopolysaccharide Particles by Fresh Human Serum

Divalent cations added to dialyzed serum	Initial rate of ingestion
None	0.029
$CaCl_2$, 5 mM	0.284
$MgCl_2$, 5 mM	0.345
$CaCl_2$, 5 mM + $MgCl_2$, 5 mM	0.444
Undialyzed Serum	0.455

Human serum was dialyzed against 0.15 M NaCl-10 mM phosphate buffer, pH 7.4, and used to opsonize lipopolysaccharide particles. The particles were dialyzed against EDTA and NaCl as described in the text before opsonization. After opsonization, the particles (0.4 ml) were washed free of serum and then suspended in Krebs-Ringer phosphate medium and added to 2.4×10^7 human leukocytes in 1.6 ml of medium. Samples (0.5 ml) were taken at zero time and at 4 min from duplicate incubations to assay the initial rate of ingestion. The ingestion rate is expressed as milligrams paraffin oil per 10^7 phagocytes per minute.

TABLE IV

Effect of Normal Human Serum, C2-Deficient Human Serum, and Hereditary Angioneurotic Edema Serum on the Initial Rate of Ingestion of Lipopolysaccharide Particles by Human Leukocytes

Serum source	Initial rate of ingestion
Normal	0.204 ± 0.044*
C2-deficient	
Patient 1	0.196
Patient 2	0.215
Hereditary angioneurotic edema	
Patient 1	0.211
Patient 2	0.178
Patient 3	0.192
Patient 4	0.169

Lipopolysaccharide particles were opsonized with equal volumes of the indicated sera. The conditions of incubation and analysis of ingestion were those described in Table III.

* Mean ± SD; $N = 82$.

Treatment of Lipopolysaccharide before Preparing Particles.—Table VI shows that particles made from lipopolysaccharide that had been extracted with chloroform-methanol fixed more [^{125}I]C3 in the presence of fresh human serum and were ingested more rapidly after opsonization than native lipopolysaccharide particles. Deacylation or acetylation of the lipopolysaccharide rendered particles prepared from it unsuitable for opsonization. To assess whether the lipopolysaccharide could be opsonized before preparation of the particles, lipopolysaccharide was incubated with serum containing [^{125}I]C3 and washed by sedimentation in albumin and NaCl solutions. The lipopolysaccharide

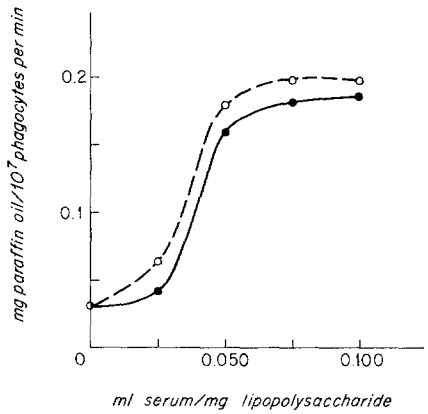

FIG. 6. Effect of serum concentration on the opsonic activity of normal (●) and hereditary angioneurotic edema (○) serum. *E. coli* lipopolysaccharide particles were opsonized with the indicated concentrations of serum. The initial rate of ingestion of these particles by human leukocytes was assayed as described in Table III.

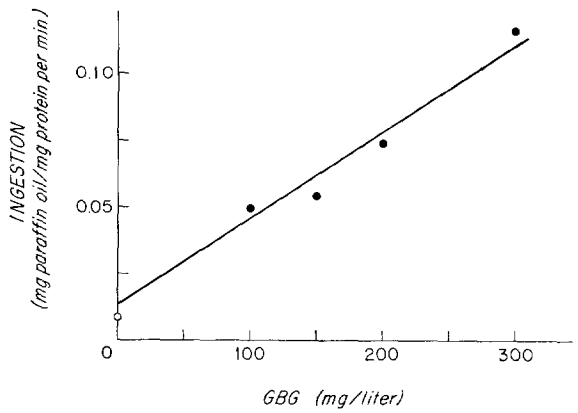

FIG. 7. Effect of GBG on the opsonic activity of cord serum. *E. coli* lipopolysaccharide particles were incubated with heat-inactivated cord serum (○) and with fresh cord serum (●) to which purified GBG or appropriate amounts of 0.15 M NaCl diluent were added. The GBG concentration of the serum without additions was 100 mg/liter. The opsonized particles (0.2 ml) were added to 3.4×10^7 human phagocytes in 0.8 ml of Krebs-Ringer phosphate medium. The initial rate of ingestion was determined as described in Table III.

suspended in serum did not bind significant quantities of [^{125}I]C3. When particles were prepared from this pretreated material, they were poorly ingested unless incubated with serum.

DISCUSSION

Lipopolysaccharide constitutes the outer cell wall of gram-negative bacteria (33) and forms a membranous structure when suspended in aqueous medium

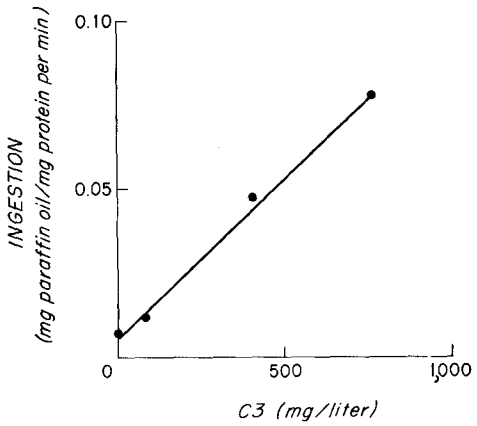

FIG. 8. Effect of C3 on the opsonic activity of serum of a patient with hereditary deficiency of C3. *E. coli* lipopolysaccharide particles were incubated with the patient's serum to which purified C3 or appropriate amounts of 0.15 M NaCl diluent were added. The rate of engulfment of the opsonized particles was assayed as described in Table III.

TABLE V

Effect of Antibody on the Opsonic Activity of Fresh Homologous Serum for Lipopolysaccharide Particles

Source of human serum	Rabbit Serum		Initial rate of ingestion
	Immune	Normal	
Normal	−	+	0.180
Normal	+	−	0.230
Normal, heated 56°C, 30 min	+	−	0.011
C2-deficient	−	+	0.169
C2-deficient	+	−	0.230
Cord	−	+	0.102
Cord	+	−	0.122

Lipopolysaccharide particles, 0.4 ml, were incubated with 0.4 ml of heat-inactivated normal or immune rabbit serum (containing 0.4 mg of antibody protein) for 15 min at 37°C and were then washed and resuspended to a volume of 0.4 ml with Krebs-Ringer phosphate medium, pH 7.4. Of the indicated human serum, 0.4 ml was added and the particles incubated for 15 min at 37°C. The opsonized particles were added to phagocytes, and the rate of ingestion was assayed as described in Table III.

(34). The molecule is amphipathic, which presumably accounts for its effectiveness in maintaining a stable emulsion of paraffin oil in an electrolyte solution. Ingestion of paraffin oil emulsified with *E. coli* lipopolysaccharide by polymorphonuclear leukocytes simulated the ingestion of the parent bacteria insofar as the rate of engulfment of the particles was markedly stimulated by fresh homologous serum. The opsonic effect of the serum was found to be exerted on the particles and to be resistant to dialysis, labile to heat, hydrazine, and zymo-

TABLE VI

[^{125}I]C3 Uptake by Lipopolysaccharide Particles

Treatment of lipopolysaccharide	Treatment of serum	Initial rate of ingestion of the particles by human leukocytes	[^{125}I]C3 bound to the particles
			%
None	56°C, 30 min	0.011	1.3
None	None	0.266	6.1
Chloroform-methanol-extracted	56°C, 30 min	0.015	1.0
Chloroform-methanol-extracted	None	0.452	9.5

Particles were prepared with untreated or chloroform-methanol-extracted lipopolysaccharide, suspended in the indicated human sera, washed, assayed for radioactivity and then for ingestibility by human leukocytes as described in the text.

san, and dependent upon temperature and divalent cations. These findings were consistent with observations implicating complement or complement-like agents as the heat-labile opsonins of serum (2–6, 35). Further pursuit of the nature of the opsonic process delineated the role of C3 and the properdin system, but not of the classical complement pathway in the opsonization of lipopolysaccharide particles. First, C4- and C2-deficient serum from humans and guinea pigs had normal opsonic activity for the lipopolysaccharide particles. Similar findings were previously reported with respect to the opsonization of various bacterial species by C4- and C2-deficient HANE serum by Stollerman and co-workers (36) and by genetically C2-deficient serum by Johnston and his colleagues (4). These observations were recently confirmed with sera deficient in C1q, C4, or C2 (37–39). Secondly, the opsonic activity of cord serum deficient in GBG or factor B of the properdin system was subnormal and was enhanced by addition of purified GBG (40). Finally, addition of purified C3 to the serum of a patient with hereditary absence of C3 but with normal GBG restored opsonic activity to that serum in proportion to the amount of C3 added. On the other hand, addition of C3 to the serum of the patient with type I essential hypercatabolism of C3 did not have this effect. This patient has normal properdin but no properdin factor B activity and absent GBG, and has normal levels of C2 and C4 (6, 7). Absence of an inhibitor of the properdin system, the C3 inactivator, has been shown to be responsible for a lack of GBG in this patient's serum, which secondarily results in deficient complement-mediated functions (41). However, enrichment of this patient's serum with purified C3 inactivator, GBG, and C3 does not enable it to opsonize, which indicates that this serum is deficient in yet other constituents of the properdin system which are incompletely understood. Nevertheless, all of these findings indicate that the lipopolysaccharide particles activate C3 in normal serum by means of the properdin system. Therefore, measurement of the opsonization of these particles comprises a functional assay for this pathway. With intact

bacteria as the test particle, bactericidal activity of serum may be exerted by reactions of the properdin system, the classical complement sequence, or both, the latter predominating if an excess of antibody is present (10). This fact probably explains why it was possible to observe normal opsonization of pneumococci by sufficient quantities of C2-deficient serum. Even in the presence of immune serum to lipopolysaccharide, C2- and C4-deficient sera promoted rates of ingestion of lipopolysaccharide particles equivalent to that provided by normal sera. Furthermore, GBG-deficient cord serum containing normal concentrations of C2 and C4 did not acquire normal opsonic activity by the addition of antibody to lipopolysaccharide. The lipopolysaccharide particles thus appear to have an absolute dependence on the properdin system for opsonization.

Some previous studies of the properdin system indicated that magnesium but not calcium was required for the activity of this pathway (15). In the present investigation, both ions were necessary for optimal opsonic activity of dialyzed serum, although magnesium alone was more effective than calcium alone. It is noteworthy that no divalent cation requirement could be demonstrated unless the lipopolysaccharide particles were first dialyzed against EDTA and NaCl, which presumably removed loosely bound divalent cations. The apparent absence of a divalent cation requirement for the heat-labile opsonization of intact bacteria described in an earlier report (35) may have resulted from such contamination.

Opsonization by means of the classical complement sequence has an absolute dependence on antibody (3–5). The requirement for antibody in the activation of C3 by means of the properdin pathway remains to be determined. Agammaglobulinemic serum has heat-labile opsonic activity (42); although relative to normal serum, this activity may be slightly diminished (19). Antibody to lipopolysaccharide increased the opsonic capacity of fresh human serum in this study. Antibody may enhance, but not be essential for, opsonic activity mediated by the properdin mechanism.

Complex polysaccharides, including lipopolysaccharides, activate the properdin system (17); and, therefore, it is not surprising that the lipopolysaccharide particles become opsonized by means of that pathway. The way in which lipopolysaccharide particles initiate properdin activation is not known, although the importance of primary structure for the biologic activity of lipopolysaccharide has been illuminated by studies in which chemical modifications of lipopolysaccharide resulted in loss or diminution in endotoxic potency. Diverse treatments, such as acetylation (30), alkaline hydrolysis (43), and deacylation (31), have been shown to diminish mouse lethality and complement-converting ability of lipopolysaccharide without destroying antigenicity. Such treatment also appears to compromise the capacity of lipopolysaccharide to activate the properdin system. Particles prepared from lipopolysaccharide extracted with chloroform-methanol had normal resistance to ingestion before

opsonization, but fixed greater amounts of [^{125}I]C3 and were engulfed more rapidly after opsonization than particles of native lipopolysaccharide. Possibly, removal of noncovalently bound, interfering lipid by chloroform-methanol exposed saccharide groups which interact with properdin.

In many experiments it was necessary to wash the particles, and this procedure did not alter the opsonized state of the particles. Early studies with intact bacteria showed that although serum opsonins were heat labile, the organisms, once treated with serum, remained opsonized after heating (2, 44) or washing in salt solutions up to 0.67 M (2, 5, 34, 36, 45). The studies presented here revealed that the opsonized state of the lipopolysaccharide particles is remarkably stable, withstanding even boiling. But particles prepared from lipopolysaccharide that had been suspended in fresh serum and then washed or repurified were not ingestible without further serum treatment. Although there is evidence that lipopolysaccharide suspended in fresh serum converts C3 (46, 47) and that serum produces morphologic changes in lipopolysaccharide discernible with the electron microscope (47), it does not fix [^{125}I]C3. Therefore, it appears likely that the lipopolysaccharide must be in a particular conformation in order for C3 fixation and hence the opsonic interaction to occur. The paraffin oil may provide a stability and configuration to the lipopolysaccharide that is similar to that of lipopolysaccharide on the bacterial surface and is required for opsonization.

The mechanism by which opsonization accelerates the rate of ingestion of particles by phagocytes remains to be determined. Earlier studies showed that there was an absolute requirement for added divalent cations in order for albumin-coated paraffin oil particles to elicit ingestion by guinea pig granulocytes (32). In this study, added Ca^{++} or Mg^{++} had relatively little influence on the rate of ingestion of opsonized lipopolysaccharide particles. EDTA, however, abolished the uptake of the particles, suggesting that divalent cations released from the washed cells or bound to the plasma membrane may have been sufficient to permit ingestion. Evidence that heat-labile opsonin may act by lowering the concentration of divalent cations required for ingestion is presented in another report (48).

SUMMARY

Paraffin oil containing oil red O and emulsified with lipopolysaccharide obtained from *Escherichia coli* was ingested rapidly by guinea pig polymorphonuclear leukocytes or human peripheral blood granulocytes and monocytes after opsonization by fresh homologous serum. The initial rate of engulfment of the particles was spectrophotometrically assayed by determination of cell-associated oil red O and reflected the opsonic activity of the serum. This activity was resistant to dialysis but labile to heat, hydrazine, and zymosan, required divalent cations, and was maximal in the presence of Ca^{++} and Mg^{++}. It was associated with the fixation of [^{125}I]C3 to the lipopolysaccharide parti-

cles. Genetically C3-deficient serum had no opsonic activity, and this activity was restored by the addition of purified C3. Normal and C4-deficient guinea pig serum and normal, C2-, and C4-deficient human sera were equally effective in opsonizing lipopolysaccharide particles and lipopolysaccharide particles sensitized with heat-inactivated lipopolysaccharide immune serum. Cord serum deficient in glycine-rich beta-glycoprotein (GBG) (properdin factor B) had diminished opsonic activity which was improved by addition of purified GBG. Thus, C3 fixation to lipopolysaccharide particles occurs by means of the properdin system, and the opsonization and ingestion of lipopolysaccharide particles constitutes a quantitative functional assay of this pathway.

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