THE OPSONIC FRAGMENT OF THE THIRD COMPONENT OF HUMAN COMPLEMENT (C3)*

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Fresh serum opsonizes certain microorganisms, conferring upon them the property of being recognized and rapidly ingested by phagocytes (1). Studies in several laboratories during the past decade have established that erythrocytes, bacteria, and other particles can be opsonized by the sequential reactions of antibody and classical complement (C) components C1, C4, C2, and C3 or by the properdin system and C3 (2–10). In either case, the fixation of C3 in some form to the surface of the particles seems to be critical for rapid ingestion by phagocytes (3,10). Erythrocytes or bacteria opsonized by C action subsequently lose ingestibility when incubated for prolonged intervals in serum (2,8). One enzyme of the blood, the C3b inactivator (or conglutinogen-activating factor [KAF][1]) which attacks C3 bound to erythrocytes, has been isolated from the serum of various species (11–15) and is capable of modifying the opsonic expression of bound C3 (2).

No information exists concerning the molecular nature of the C3-derived product that confers opsonic expression. C3 is an incompletely characterized and multifunctional molecule which when activated by the classical or by the properdin pathways liberates an anaphylatoxic fragment, C3a, while the rest of the molecule, C3b, is somehow involved in the activation of the terminal C molecules, C5-C9, and acts as a cofactor in the properdin system (16,17). C3b is cleaved by the C3b inactivator to yield a product, $C3b^i$, which is no longer capable of activating the properdin pathway. $C3b^i$ is sensitive to various proteolytic enzymes which further degrade the molecule to C3c (18, 19). Two other fragments, called C3c and C3d, appear during activation of C3 (16, 20). Their detailed structures and biological significance are unknown.

Analysis of opsonically active C3 requires an approach which provides for a strict correlation between the structure of bound C3 and its opsonic expression. We have demonstrated that the initial rate of ingestion by human peripheral

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[1] *Abbreviations used in this paper:* HSA, human serum albumin; KAF, conglutinogen-activating factor; KRPM, Krebs-Ringer phosphate medium; LPS, lipopolysaccharide; PAS, periodic acid Schiff stain.

blood phagocytes of paraffin oil droplets containing Oil red O and stabilized with bacterial lipopolysaccharide (LPS) or with human serum albumin (HSA) increases as C3 is deposited from fresh serum onto the surfaces of the particles (9, 10). The properdin system mediates the deposition.

This report describes the effect of various physical and chemical agents and of proteolytic enzymes on the ingestibility and bound radioactivity of LPS- and HSA-coated particles opsonized with fresh serum containing [^{125}I]C3. Ingestibility and bound radioactive C3 on the particles resisted treatment with strong dissociating agents. Therefore, it was possible to undertake immunochemical and structural analysis of opsonized particles after removal of many contaminating serum proteins. The findings indicated that opsonically active C3 is a molecule composed of two 70,000 mol wt subunits linked by disulfide bonds. Reduction and alkylation of these linkages impairs opsonic function as does alkylation of reactive sulfhydryl groups on the molecule. Comparison of the opsonically active fragment of C3 with purified C3 indicated that one of the 70,000 mol wt polypeptides is also a 70,000 mol wt subunit of intact C3; and that the other is a fragment of a 115,000 mol wt heavy chain in intact C3. Summaries of this work have been published (21, 22).

Materials and Methods

LPS and HSA. LPS prepared by the procedure of Westphal and Jann (23) from *Escherichia coli* 026:B6 or else *Salmonella enteritidis* was purchased from Difco Laboratories, Detroit, Mich. The LPS as received, when dissolved in 1% sodium dodecyl sulfate, 8 M urea, and 2-mercaptoethanol solution sometimes revealed bands of variable molecular weights after electrophoresis on polyacrylamide gels. Attempts to diminish the protein content of the LPS by additional cycles of extraction with phenol-water (23) were effective, but repurified LPS with low protein content or prepared by phenol-water extraction from *E. coli* 026:B6 (acquired from the American Type Cell Culture Collection, Rockville, Md.) had identical properties to the crude commercial LPS in the biological system under study. [^{14}C]LPS with a sp act of 4.63 μCi/mg, prepared from *S. enteritidis* grown in [^{14}C]glucose, was a gift from Dr. Jon Rudbach of the University of Montana, Missoula, Mont. HSA was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

LPS- and HSA-Coated Particles. Heavy paraffin oil containing Oil red O was emulsified with LPS suspended in modified Krebs-Ringer phosphate medium (KRPM: 119 mM NaCl, 4.8 mM KCl, 0.9 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 9.3 mM sodium phosphate buffer, pH 7.4) at a concentration of 10 mg/ml or emulsified with HSA dissolved in modified KRPM at a concentration of 20 mg/ml, as previously described (9, 10). Oil red O was dissolved in the paraffin oil by mixing 5 g of the dye with 50 ml of oil in a mortar with a pestle. The solution was centrifuged at 20,000 *g* for 30 min to remove undissolved dye. The process was repeated, mixing the oil with dye that sedimented during centrifugation. By this technique, 0.01 ml of Oil red O in paraffin oil diluted to 10 ml with *p*-dioxane had an absorbance of 0.4–0.9 at 525 nm which was higher than that achieved with methods previously described (9, 10). For experiments in which ingestion rates of the particles were not correlated with C3 binding or elution, particles were prepared with fluorochemical liquid (Minnesota Mining & Manufacturing Co., St. Paul, Minn.) as previously described (10). Because of the higher density of these particles (1.74), washing was accomplished more easily than with the paraffin oil particles. The biological properties of HSA-coated fluorochemical particles with respect to recognition by phagocytes before and after exposure to serum were previously shown to correlate with those of HSA-coated paraffin oil particles (10). [^{14}C]LPS-coated particles were prepared by adding 5.26 μCi of [^{14}C]LPS from *S. enteritidis* to 30 mg of carrier *S. enteritidis* LPS, and the mixture was used to emulsify paraffin oil. The final particle preparation had a specific radioactivity of 2.44 μCi/ml of particle suspension. All particles were washed twice with modified KRPM to remove LPS in free suspension or HSA in solution after emulsification. Paraffin oil particles were centrifuged at 20,000 *g* for 10 min, and infranatant solutions were removed from the particle pellicles. Coated fluorochemical

particles were centrifuged at 100 *g* for 5 min, and supernatant fluids were removed between washes. The particles were finally suspended in modified KRPM such that 1 ml of fluorochemical liquid or paraffin oil was contained in 3 ml of aqueous medium.

Reaction of Coated Particles with Serum plus [^{125}I]C3 (Opsonization). Purified human C3 (24) labeled with ^{125}I (sp act 1–5 × 10^5 cpm/μg) (25) was added to serum prepared from freshly drawn human blood or stored at −70°C (fresh serum) or to serum heated for 30 min at 56°C (heated serum) to yield a final sp act of 10^5–10^6 cpm/ml of serum. The C3 concentration of fresh serum samples was determined by an automated nephelometric method (26). Suspensions of coated particles were added to equal volumes of fresh or heated serum plus [^{125}I]C3, the amounts of particles and serum being determined by the volumes of particles required for a given experiment. In time-course experiments, particles and sera were heated to 37°C before mixing. Samples were removed at zero time and at intervals thereafter, and cooled immediately by being added to 15 volumes of ice-cold solutions of bovine serum albumin, 20 mg/ml in 0.15 M NaCl (albumin solution). The particles were washed twice in albumin solution and then twice in 0.13 M NaCl-20 mM sodium phosphate buffer, pH 7.4 (isotonic buffer). Particles treated as described were designated "opsonized washed particles."

Reaction of Opsonized Washed Particles with Various Reagents. Opsonized washed fluorochemical liquid particle pellets or paraffin oil particle pellicles were suspended in five volumes of solutions containing test agents and incubated at concentrations and times described in the Results section. Of the test materials, NaCl, glucose, $MgCl_2$, sodium metaperiodate, guanidine, urea, Triton X-100, Brij-35, EDTA, deoxycholate, sodium dodecyl sulfate, sodium decyl sulfate, 2-mercaptoethanol, chloroform, and butanol were reagent-grade chemicals. Phospholipase A and phospholipase C were purchased from Calbiochem, San Diego, Calif., and trypsin from Worthington Biochemical Corp., Freehold, N. J. KAF-deficient human serum was obtained from a patient with genetically determined total deficiency of that enzyme (27). Purified KAF was generously provided by Dr. Peter Lachmann. Anti-C3 $F(ab')_2$ fragments were prepared by pepsin digestion of goat antihuman C3 (28). Particles reacted with the test substances were subsequently washed twice with isotonic buffer and once with distilled water.

Preparation of Particles for Elution. Of packed, washed, opsonized LPS-coated or HSA-coated particles, 1 ml was suspended in 5 ml of 2 M NaCl and boiled for 15 min. The particles were packed by centrifugation, washed once with deionized distilled water, and packed again. The emulsions were broken by addition of 5 ml of *n*-butanol and centrifuged at 40,000 *g* for 20 min. The butanol was evaporated under a stream of nitrogen, and the paraffin oil or fluorocarbon removed by aspiration. The dried residue, containing about 6,000 cpm of ^{125}I radioactivity, was suspended in 0.3 ml of a solution containing 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol (or 50 mM dithiothreitol), 8 M urea, 0.01 mg/ml eosin Y, 100 mg/ml sucrose, and 20 mM Tris-HCl buffer of pH 7.0 (gel sample solution), and incubated for 1 h at 37°C. Dithiothreitol or 2-mercaptoethanol were omitted from solutions used for preparation of unreduced eluates. Some eluates of opsonized washed LPS-coated particles contained material that precipitated during electrophoresis on polyacrylamide gels with dodecyl sulfate. This material, which was not identified, was removed when eluates were passed over 1 × 60 cm columns of Sephadex G-100 equilibrated with dodecyl sulfate, 2-mercaptoethanol, and 20 mM Tris-acetate buffer of pH 7.0. The radioactive peaks eluted yielded the same results when subjected to electrophoresis on polyacrylamide gels with dodecyl sulfate as did eluates for which gel filtration was not required.

Alkylation of Opsonized Washed Particles. Of opsonized, washed, packed paraffin oil particles, 1 ml was suspended in 3 ml of 0.03 mM iodoacetic acid and incubated at 25°C for 30 min. The particles were washed five times with isotonic buffer and prepared for assessment of ingestibility and ^{125}I radioactivity as described below. Control alkylation experiments were performed by adding iodoacetic acid at the end of the 30-min incubation to ensure that any detrimental effects of iodoacetate on ingestibility were not due to toxicity exerted by residual-free iodoacetate against test cells. Reduction and alkylation of particles were performed by incubating opsonized, washed paraffin oil particles with 0.025 mM 2-mercaptoethanol for 15 min at 25°C before adding the iodoacetate.

C3 Fragments and Subunits. C3b was prepared by brief tryptic digestion of C3 (29). The C3 fragments C3c and C3d were isolated from aged human serum and identified by their electrophoretic mobilities in agarose gels and reactivities with anti-C3 (A determinant) and anti-α_2D. C3 and [^{125}I]C3 were denatured by boiling for 15 min in 1% dodecyl sulfate, 2-mercaptoethanol (or 50 mM dithiotreitol), 8 M urea, and 20 mM Tris-acetate buffer of pH 7.0. Approximately 100 μg of denatured protein

and 5,000–10,000 cpm were applied to polyacrylamide gels and electrophoresed as described below. Peptides in the gels were located by scanning at 280 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a scanning and recording device, and protein-containing portions of the gels were eluted overnight with a solution of dodecyl sulfate. In other experiments, 2 mg of denatured C3 plus 2–5 $\times$ 10^4 cpm of [^{125}I]C3 were applied to 102 x 0.8 cm columns of Sephadex G-100 and eluted with a solution containing 1 M proprionic acid, 6 M urea, 0.5% dodecyl sulfate, and 1% 2-mercaptoethanol. [^{125}I]C3 was also chromatographed on a 2 x 89 cm column of Bio-Rad A15 M (Bio-Rad Laboratories, Richmond, Calif.), 200–400 mesh (4% agarose), equilibrated and eluted with 0.6 M KCl and 20 mM Tris-maleate buffer of pH 7.0. Eluted column or gel fractions were assayed for ^{125}I radioactivity, concentrated by vacuum dialysis, and analyzed for purity by polyacrylamide gel electrophoresis in dodecyl sulfate.

Antisera. Opsonized, washed, LPS-coated fluorochemical particles were washed with 2 M NaCl, boiled for 15 min, and washed and suspended in sterile distilled water. Samples of 0.1 ml were injected every other day for a total of seven injections into rabbits. Injections were repeated thereafter at monthly intervals, and blood samples were taken 1 wk after the last monthly injection. Antisera to C3 and C3d were generated in rabbits. The antiserum to C3 reacted in immunoelectrophoresis only with native C3, C3b, and C3c but not C3d. The antiserum to C3d reacted with native C3, C3b, and C3d but not C3c.

Analytical Procedures

ASSAY OF INGESTIBILITY. Opsonized, washed paraffin oil particles or particles exposed to other reagents were suspended in modified KRPM such that 0.3 ml of paraffin oil was suspended in 1 ml. Of the particle suspension, 0.2 ml was added to 2–6 $\times$ 10^7 human peripheral blood leukocytes in 0.8 ml of modified KRPM. The leukocytes, prepared as described (28), were composed of 70–90% "phagocytes" (neutrophils plus monocytes). The initial rate of ingestion of the paraffin oil particles by the phagocytes was spectrophotometrically assayed as previously described (9, 10, 30).

ASSAY OF RADIOACTIVITY. ^{125}I of labeled C3, of serum to which radioactive C3 was added, of opsonized washed particles, of samples eluted from Sephadex columns, and of slices cut from polyacrylamide gels was measured directly with an autogamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples (0.2 ml) of [^{14}C]LPS, of opsonized washed particles prepared with [^{14}C]LPS, and of [^{14}C]LPS were dissolved in 0.8 ml of Protosol (New England Nuclear, Boston, Mass.) overnight at 25°C. The digested samples were neutralized with 0.25 ml of glacial acetic acid and added to 10 ml of Aquasol (New England Nuclear). Radioactivity of the samples in the scintillation fluid was determined with a Packard scintillation spectrometer (Packard Instrument Co., Inc.). Polyacrylamide gel slices containing ^{14}C were dissolved in 0.2 ml of 30% hydrogen peroxide before addition of Protosol.

POLYACRYLAMIDE GEL ELECTROPHORESIS. Samples for electrophoresis were dissolved (gel sample buffer), boiled for 15 min, centrifuged for 5 min in a Beckman microcentrifuge apparatus (Beckman Instruments, Inc., Fullerton, Calif.), and applied in 40 to 80-μl quantities to 5% polyacrylamide gels with 0.13% cross-linker. The electrophoretic procedure was that of Fairbanks et al. (31). After electrophoresis, the gels were stained with Coomassie Brilliant Blue and destained with 10% acetic acid in a Hoefer destaining apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) or else frozen and cut into 2-mm slices with a razor blade. Some gels were treated with periodic acid-Schiff (PAS) stain (32). The molecular weights of unknown proteins were determined by comparing their mobilities with those of known molecular weights including rabbit muscle myosin (33), beta-galactosidase (Sigma Chemical Co., St. Louis, Mo.), bovine serum albumin (Nutritional Biochemicals Corporation), rabbit muscle actin (34), chymotrypsinogen A, ovalbumin, and cytochrome C (Worthington Biochemical Corp.).

IMMUNOCHEMICAL ANALYSIS. Reactions of antisera with purified proteins were analyzed by double diffusion in agar gel and by immunoelectrophoresis (35).

TRYPTIC PEPTIDE MAPS. Of [^{125}I]C3, 0.2 μg was heated in a boiling water bath for 40 min, reduced and alkylated with 2.5 mM dithiothreitol and 5 mM ethyleneimine, dialyzed against distilled water, and lyophilized. The residue was dissolved in 5 ml of 0.1 M ammonium bicarbonate containing trypsin, 0.02 μg/μg of C3, and was incubated for 14 h at 37°C. The sample was repeatedly lyophilized with 5% acetic acid and finally dissolved in distilled water at a concentration of 50 μg/ml. Of the sample, 20 μl were electrophoresed on Whatman 3M paper for 38 min at 3,000 V in water:pyridine:acetic acid,

90.6:9.1:0.3, pH 6.5. Chromatography was performed in the descending direction in butanol:acetic acid:pyridine:water, 37.5:7.5:25:30. The maps were stained with ninhydrin or else autoradiographed with Kodak Royal X-Omat RP/R54 X-ray film (Eastman Kodak Co., Rochester, N. Y.). C3 fragments were eluted from polyacrylamide gels by incubating gel slices in 0.05% sodium dodecyl sulfate, 5 mM sodium bicarbonate at 37°C for 12 h. The procedure was repeated twice and the three eluates were pooled and concentrated to 1-ml vol in collodion sacs under vacuum. After reduction and alkylation, the samples were dialyzed against three changes of 1 liter of absolute methanol to remove the dodecyl sulfate. The samples were dialyzed against distilled water, lyophilized, and digested with trypsin as described above.

Results

Ingestibility and Bound C3 Radioactivity of LPS-Coated Particles

CORRELATION DURING INCUBATION IN FRESH SERUM. Human neutrophils ingested LPS-coated paraffin oil particles at a very slow rate, but this rate was markedly accelerated by prior incubation of the particles in fresh human serum containing [^{125}I]C3 at 37°C, and they bound radioactivity (Fig. 1). The increase in

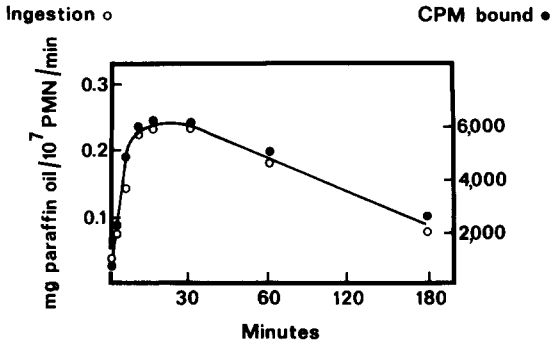

FIG. 1. Ingestibility and bound C3 radioactivity of LPS-coated paraffin oil particles. Particles were incubated with serum containing [^{125}I]C3 at 37°C. At the indicated times, samples were removed, washed, and tested for bound radioactivity and ingestibility by human blood phagocytes.

ingestibility and radioactivity of the particles with time of incubation in serum correlated precisely, and both properties were maximal at 25–30 min of incubation. When serum-treated LPS-coated particles were washed with isotonic buffer and incubated a second time with fresh serum containing [^{125}I]C3, the ingestion rate and bound radioactivity increased by 25% at most, indicating that the maximal opsonic expression and uptake of radioactivity reflected saturation of available sites on the particles rather than depletion of reagents in the serum required for deposition of C3.

However, decay of opsonic expression and of radioactivity was in part responsible for the lack of a greater increment during incubation of the particles in serum. As shown in Fig. 1, radioactivity and ingestibility decreased in parallel at a constant rate for at least 90 min after maximal activity and radioactivity were achieved; the rate of decline in activity was slower than the rate of opsonization and binding of radioactivity. The disappearance of radioactivity and loss of ingestibility from the particles occurred in serum, in heated serum,

and in serum containing 1 mM EDTA, but not in buffer (Table I). The rate of decay of radioactivity was diminished but not abolished by treatment of the serum with 3 mM sodium *m*-periodate, an inhibitor of KAF, or by employing serum totally lacking KAF activity. Purified KAF and trypsin removed radioactivity and diminished the ingestibility of particles. Phospholipases A and C had no effect on either property.

EFFECTS OF PHYSICAL AND CHEMICAL AGENTS. The particles retained ingestibility and radioactivity after being washed in isotonic buffer and albumin solutions. To determine the stability of opsonically active C3, particles were maximally opsonized (for 30 min) in fresh serum containing [^{125}I]C3, washed with isotonic buffer and albumin, and then exposed to the treatments summarized in Table II. Extremes of ionic strength and of pH, various dissociating agents, repeated freezing and thawing, sonication, certain nonionic or ionic detergents either alone or in combination, and even with boiling caused less than 25% of bound radioactivity and less than 15% of ingestibility to be lost from the serum-treated particles. Treatment of unopsonized particles with the various reagents alone did not improve their poor ingestibility nor did it prevent subsequent opsonization by fresh serum. As shown in Table III, the paraffin oil plus Oil red O could be extracted from opsonized and washed particles with butanol. Radioactivity remained associated with the LPS residue which, when re-emulsified in fresh paraffin oil containing Oil red O, yielded fully ingestible particles, indicating that the C3 was bound to the LPS and not to the hydrocarbon. The table reveals that treatment of the unopsonized particles with butanol did not alone render them

TABLE I

Effect of Various Treatments of the Bound C3 Radioactivity and Ingestibility of Opsonized, Washed, LPS-Coated Paraffin Oil Particles

Treatment	Time of incubation	Radioactivity	Ingestibility
	min	*% of value at zero time**	
Modified KRPM	120	100	100
+ KAF, 50 μg/ml	120	74	75
+ Trypsin, 1 mg/ml‡	30	20	20
+ Phospholipase A, 200 μg/ml + 5 mM $CaCl_2$	30	100	100
+ Phospholipase C, 200 μg/ml + 3 mM $CaCl_2$	30	100	100
Fresh serum	120	54	55
+ 1 mM EDTA	120	55	50
+ 3 mM sodium *m*-periodate	120	66	Not tested
Heated serum	120	55	52
KAF-deficient serum	120	76	Not tested

* The zero time values were determined using particles opsonized for 30 min and washed before incubations with the indicated reagents. The zero time radioactivity was 10,700 cpm/ml of particle suspension; the particles were ingested at a rate of 0.226 mg paraffin oil/10^7 phagocytes/min.

‡ The incubation was terminated by addition of soybean trypsin inhibitor at a final concentration of 1 mg/ml before washing.

TABLE II
Effect of Various Treatments on Ingestibility and Radioactivity of Opsonized, Washed, LPS-Coated Particles

Treatment	Radioactivity		Ingestibility	
	*% of control**			
Boiling	−	+	−	+
Distilled water	100	100	100	100
2 M NaCl	85	80	100	100
1 M glucose	98	95	100	100
0.5 M $MgCl_2$	96	85	100	95
8 M urea‡	86	75	98	95
6 M guanidine	90	80	96	90
2% Triton X-100	79	75	88	85
0.2% Brij 35	85	80	97	94
2% deoxycholate	90	83	96	93
1% sodium decyl sulfate	90	80	95	90
1% 2-mercaptoethanol	94	79	100	99

* Control refers to radioactivity and ingestibility of opsonized, washed particles not treated with any of the indicated reagents but with 0.13 M NaCl-15 mM phosphate buffer, pH 7.4, for 60 min at 37°C. Boiling was performed for 15 min in a water bath.

‡ Similar values were obtained when particles were exposed to 8 M urea dissolved in acetate, citrate, phosphate, or Tris-HCl buffers to provide a pH range of 3–11 or when 8 M urea was combined with 2 M NaCl or with 6 M guanidine.

TABLE III
Effect of Solvent Extraction on the Ingestibility and Radioactivity of Opsonized, Washed, LPS Particles

Initial treatment	Treatment after reconstitution	Radioactivity	Rate of uptake (mg paraffin oil/ 10^7 phagocytes min)
		cpm	
Fresh serum	Heated serum	1,498	0.108
Fresh serum	Fresh serum	1,427	0.124
Heated serum	Heated serum	75	0.010
Heated serum	Fresh serum	57	0.141

LPS particles were treated with fresh or heated serum containing [^{125}I]C3, washed, boiled, extracted with butanol, reconstituted from fresh buffer and paraffin oil Oil red O, and divided into two portions. One was treated with heated serum and the other with fresh serum (without added [^{125}I]C3). Rates of uptake of the particles by human leukocytes and bound radioactivity were assayed.

ingestible nor did it interfere with their ability to be opsonized with fresh serum. Identical results were obtained from chloroform.

Table IV illustrates that some manipulations did impair ingestibility or radioactivity of particles. Reactions of opsonized LPS-coated particles with

TABLE IV
Effect of Various Treatments on Ingestibility and Radioactivity of Opsonized, Washed, LPS Particles

Treatment	Radioactivity	Ingestibility
	*% of control**	
Anti-C3 $(Fab')_2$	100	15
5×10^{-5} M iodoacetate		
without prior reduction	100	45
with prior reduction	100	20
1% Sodium dodecyl sulfate	10	5

* Radioactivity and ingestibility of opsonized washed particles treated with 0.13 M NaCl-15 mM phosphate buffer, pH 7.4, alone for 60 min at 37°C.

anti-C3 $F(ab')_2$ fragments markedly reduced ingestibility without altering radioactivity. Alkylation of opsonized particles with iodoacetate decreased ingestibility without diminishing radioactivity; and, if reduction preceded alkylation, the inhibitory effect was greater. The reducing agent alone had no major effect on either radioactivity or ingestibility (Table II). Sodium dodecyl sulfate at concentrations greater than 0.5% removed over 85% of the bound radioactivity and abolished ingestibility of the opsonized particles.

The Structure of Opsonically Active C3 After Elution with Dodecyl Sulfate. Since ingestibility and radioactivity of the particles withstood boiling in 2 M NaCl solution followed by washing with distilled water, the opsonized particles were treated in this way in order to remove as many loosely bound proteins and salts as possible before elution with dodecyl sulfate. By extracting the hydrocarbon from the particles with butanol and drying the LPS residue to constant weight, it was determined that up to 2.5 μg of C3 was bound/mg of LPS, assuming that the radioactive C3 was equivalent to the serum C3 in biologic activity.

The radioactivity of dodecyl sulfate eluates of opsonized particles was soluble in the detergent. Less than 25% of the radioactivity sedimented after ultrancentrifugation for 16 h at 500,000 *g* or failed to traverse 0.2-μm pore Millipore filters. However, the eluate became insoluble when the detergent was removed by dialysis against 50% methanol or passage over a column of Dowex AG 1×2, 200–400 mesh (Dow Chemical Co., Midland, Mich.).

The dodecyl sulfate eluates of particles treated with fresh or heated (56°C, 30 min) serum were analyzed by electrophoresis on polyacrylamide gels (Fig. 2). Exposure of LPS-coated particles to fresh serum yielded different stainable protein patterns in reduced eluates than exposure on heated serum. More bands were resolved in eluates of particles incubated in fresh serum which migrated with mobilities corresponding to mol wt greater than 100,000. A major difference between eluates of opsonized particles and particles treated with heated serum was the appearance of a Coomassie Blue stainable band in reduced eluates of opsonized particles which had a mobility consistent with a mol wt of approxi-

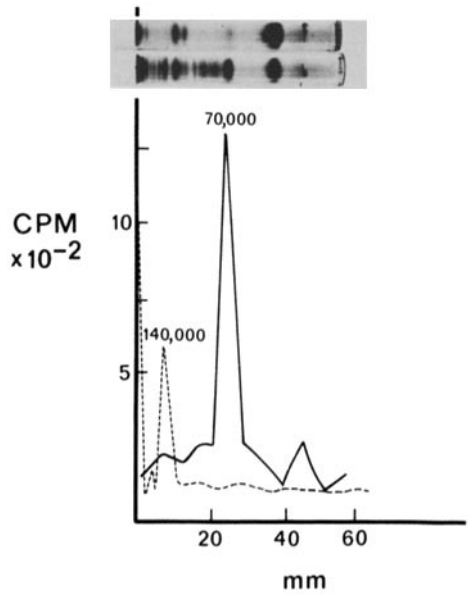

FIG. 2. Electrophoresis of LPS-coated particle eluates on polyacrylamide gels with dodecyl sulfate. A reduced eluate from LPS-coated particles treated with heated serum was applied to the top gel. A reduced eluate from LPS-coated particles treated with fresh serum was applied to the lower gel, and the graph depicts ^{125}I radioactivity of that gel (—) and of a gel to which unreduced eluate from LPS-coated particles treated with fresh serum was applied (---). The radioactive peak in the reduced eluate corresponds with the stained band directly above the radioactive peak. The molecular weights of the radioactive peaks are indicated. An eluate of particles incubated with buffer rather than serum yielded no stainable protein after electrophoresis.

mately 70,000 and which constituted up to 10% of the total stainable protein. Almost all of the radioactivity of reduced eluates prepared from particles incubated in fresh serum with [^{125}I]C3 was invariably found within this band in spite of some variation in the stainable protein profile of nonradioactive bands from one preparation to another. The radioactive band did not stain with PAS. Particles incubated with heat serum containing [^{125}I]C3 contained negligible radioactivity after preparation for elution, and the eluates contained only traces of the 70,000 mol wt band. In unreduced eluates of opsonized particles, the 70,000 mol wt peptide did not appear. Instead, a radioactive peak migrated with a mobility corresponding to a mol wt of 140,000. However, considerable radioactivity remained at the origin of these gels. If particles incubated in fresh serum were washed in isotonic buffers before elution instead of first being boiled and washed with 2 M NaCl, radioactive peaks were found in the reduced eluates with mol wt of 115,000 and 30,000, as well as the major 70,000 mol wt band.

Less than 20% of [^{14}C]LPS eluted with ^{125}I-labeled protein in dodecyl sulfate. The ^{14}C migrated much more rapidly than the ^{125}I on the polyacrylamide gels (Fig. 3). Dodecyl sulfate eluates of HSA-coated paraffin oil particles which were opsonized with fresh serum containing [^{125}I]C3, washed with 2 M NaCl, and extracted with butanol had a stainable peptide of mol wt 70,000 when reduced

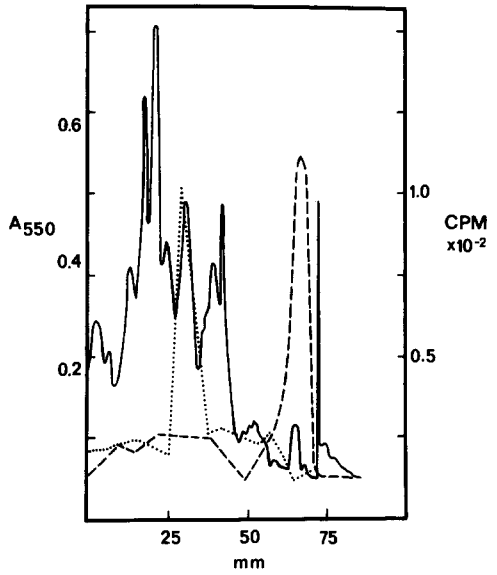

FIG. 3. Electrophoresis of a reduced LPS-coated particle eluate on a polyacrylamide gel with dodecyl sulfate. The eluate was prepared from [^{14}C]-LPS-coated particles opsonized with fresh serum containing [^{125}I]C3. The graph depicts the absorbance of Coomassie Blue-stained protein (—), ^{125}I radioactivity (· · ·), and ^{14}C radioactivity (---).

and subjected to electrophoresis on polyacrylamide gels. All radioactivity migrated in the region of this band.

Immunochemical Reactivity of Opsonized Particles. Opsonized, washed particles boiled in 2 M NaCl and injected repeatedly into rabbits elicited antibodies strongly reactive against C3 in human serum (Fig. 4). Weak reactions with IgG and IgM were detectable. The antiserum reacted against the immunochemically defined C3 fragments, C3b and C3c, but not against C3d (Fig. 5). Conversely, undiluted anti-C3 antiserum but not anti-C3d antiserum agglutinated opsonized, washed LPS-coated particles boiled in 2 M NaCl. Although anti-C3 $F(ab')_2$ fragments blocked the ingestibility of opsonized, washed LPS-coated particles boiled with 2 M NaCl, anti-C3d $F(ab')_2$ fragments did not have this effect. However, if the opsonized, washed particles were not boiled with 2 M NaCl, anti-C3d $F(ab')_2$ fragments did have an inhibitory action (Table V).

Subunit Structure of C3: Correlation with Opsonically Active C3. Purified C3, homogeneous by immunochemical and electrophoretic criteria, eluted from an agarose column in a position corresponding to a globular protein of mol wt 180,000. It could be resolved into subunits by vigorous denaturation and reduction. Scission of the molecule into its constituents was reliably achieved by boiling for 15 min in 1% dodecyl sulfate with 50 mM dithiothreitol or 1% 2-mercaptoethanol. The major peptides of C3 when analyzed by electrophoresis on polyacrylamide gels or chromatography on Sephadex G-100 with dodecyl sulfate had mol wt of 115,000 and 70,000, respectively (Fig. 6). However, additional peptides with mol wt of 80,000 and 35,000 were present in variable amounts and eluted with the 70,000 mol wt peptide during chromatography. A

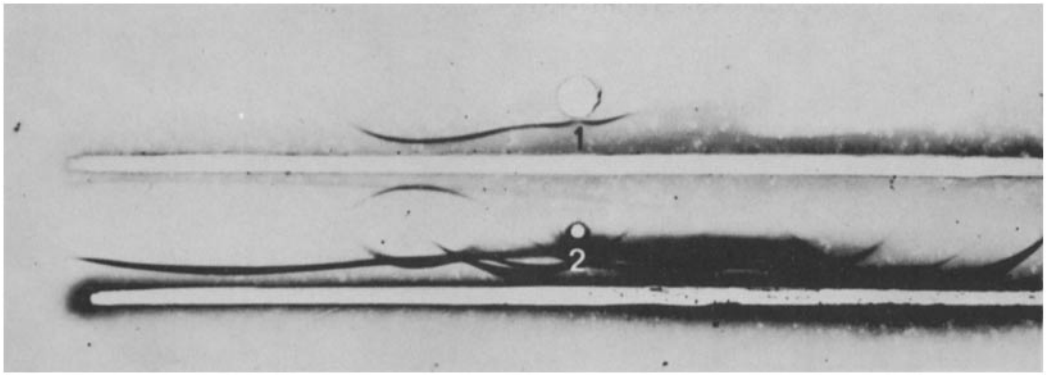

FIG. 4. Reactions of rabbit antiserum to opsonized, washed, LPS-coated particles boiled in 2 M NaCl (upper trough) against purified C3 (1) and whole serum (2), after electrophoresis in agar gel. Rabbit antiserum to whole human serum was in the lower trough.

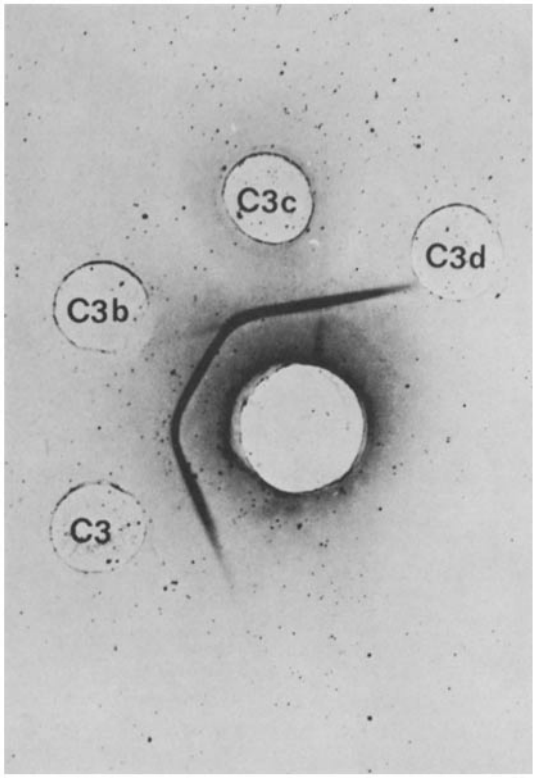

FIG. 5. Reactions of rabbit antiserum to opsonized, washed, LPS-coated particles boiled in 2 M NaCl (center well) against purified C3 and its conversion products, C3b, C3c, and C3d, by double diffusion in agar gel.

number of findings indicated that the 80,000 mol wt peptide is a fragment of the 115,000 mol wt subunit of C3. Tryptic peptides of the 80,000 mol wt band after elution from the polyacrylamide gels corresponded to tryptic peptides found in the 115,000 mol wt subunit purified either by elution from polyacrylamide gels or

TABLE V

Effect of Anti-C3d on the Ingestibility of Opsonized, Washed, LPS-coated Paraffin Oil Particles by Human Peripheral Blood Phagocytes: Influence of Boiling the Particles with 2 M NaCl

	Rate of ingestion (mg paraffin oil/10^7 phagocytes/min)	
	Not boiled with 2 M NaCl	Boiled with 2 M NaCl
Control	0.21	0.20
Anti-C3d	0.04	0.19

Opsonized, washed, LPS-coated particles were divided into two portions, one of which was boiled with 2 M NaCl. Boiled and unboiled particles were reacted with heated (56°C, 30 min) goat serum (control) or else goat antihuman C3d antiserum for 60 min at 25°C, washed with albumin solution, and assayed for ingestibility by human blood phagocytes.

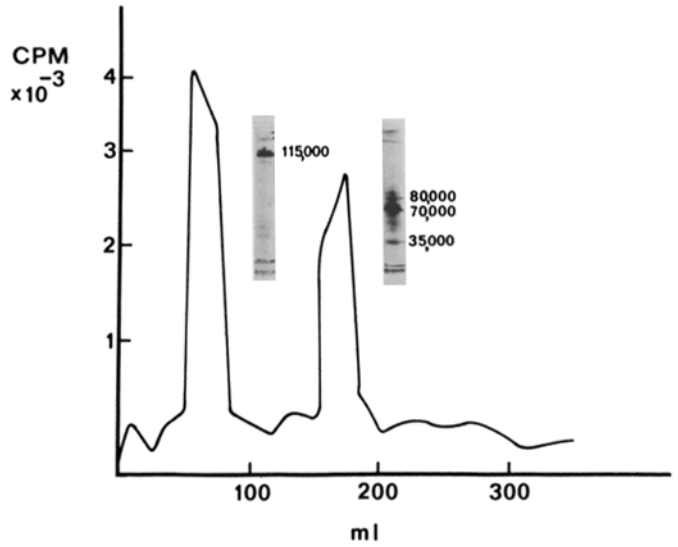

FIG. 6. Chromatography of purified, reduced, denatured C3 + [^{125}I]C3 on Sephadex G-100. The elution profile of ^{125}I is indicated. The polyacrylamide gels were prepared from the pooled radioactive peaks, and the molecular weights of the stainable peptides are indicated.

chromatography on Sephadex G-100 with dodecyl sulfate. Furthermore, when the areas under the 80,000 and 35,000 mol wt peaks were added to that of the 115,000 component on densitometric scans of stained polyacrylamide gels, total areas accounted for an equimolar amount of protein relative to the 70,000 mol wt peptide. Some of the tryptic peptides of the 70,000 mol wt component comigrated with those of the 115,000 subunit, both as determined by ninhydrin staining or autoradiography of ^{125}I-labeled peptides. However, as shown in Fig. 7, the 115,000 and 70,000 mol wt chains did have distinct peptides. The findings suggest

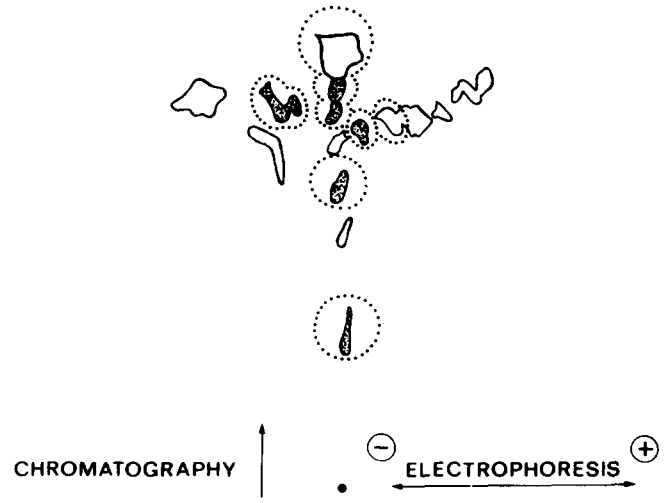

FIG. 7. Composite tryptic peptide map autoradiogram indicating radioactive peptides found only in the isolated 115,000 mol wt component of C3 (open spots), spots found only in the isolated 70,000 mol wt subunit of C3 (enclosed by dotted circles).

that C3 has 115,000 and 70,000 mol wt subunits but that the heavier peptide chain undergoes some decomposition during the vigorous denaturation required for separation of the C3 subunits.

Tryptic peptide map autoradiograms of the 70,000 mol wt radioactive band eluted from polyacrylamide gels to which dodecyl sulfate eluates of opsonized LPS-coated particles were applied revealed spots corresponding to all of the distinct radioactive peptides of the 70,000 mol wt chain of C3 and to some of the distinct radioactive peptides of the 115,000 mol wt chain (Fig. 7). Opsonically active C3 is, therefore, composed of the 70,000 mol wt subunit of C3 and a 70,000 mol wt fragment of the heavy chain.

Discussion

The Opsonically Active Fragment of C3. Considerable evidence points to a form of bound C3 as an agent that elicits recognition by phagocytic cells. Erythrocytes or pneumococci coated sequentially with antibody and classic C components C1, C4, and C2 become opsonized only when C3 is added. Subsequent proteins of the C system have little or no influence on opsonic expression (2–4). Fresh serum opsonized LPS- or HSA-coated paraffin oil particles by reactions involving the properdin system alone. C3-deficient serum has no opsonic activity for these particles, and addition of C3 to the deficient serum restores opsonic activity in proportion to the quantity of C3 added (9, 10). During opsonization of particles by either classic or properdin pathways, C3 becomes fixed to the particle surface (3, 9, 10). Anti-C3 $F(ab')_2$ fragments block the opsonic expression of C3 bound either to erythrocytes by antibody and the classic pathway (36) or, as demonstrated in this report, LPS-coated paraffin oil particles opsonized by the properdin pathway. Although proteins other than C3 become attached to LPS-coated particles and to membranes of antibody-coated

erythrocytes during incubation in fresh serum (37, 38), it appears to be C3 in its activated and bound form that affords opsonic expression.

Binding and Stability of C3 on Opsonized Particles. Early studies with bacteria suggested moderate thermal and physical stability of the opsonized state, but the fragility of the test particles precluded adequate functional analysis (39, 40). An investigation of [^{125}I]C3 bound to erythrocyte membranes revealed firm binding of the radioactive C3, although opsonic function of the bound protein was not assayed (41).

The experiments described in this report demonstrated that C3 is strongly bound to HSA- and LPS-coated paraffin oil or fluorochemical liquid particles and that its opsonic expression is strikingly resistant to physical and chemical perturbation. Bound radioactivity and opsonic expression withstood treatment with extremes of pH, temperature, and ionic strength, organic solvents, and a number of strong protein perturbants. Only the detergent sodium dodecyl sulfate efficiently removed radioactivity. A closely related detergent, sodium decyl sulfate, was ineffective. From these facts in can be inferred that C3 is attached to the LPS by a hydrophobic bond. It is possible, however, that this linkage is complex and that the dodecyl sulfate acts on neighboring molecules, as well as on the binding site to effect removal. The possibility that C3 is covalently bound to the LPS and that dodecyl sulfate removes a peripheral LPS-C3 complex is unlikely because [^{14}C]LPS did not comigrate with [^{125}I]C3 when subjected to electrophoresis on polyacrylamide gels in dodecyl sulfate. That the opsonic fragment of C3 might be covalently bound to protein in the LPS is also improbable, since the molecular weight of the eluted, reduced radioactive fragment was the same whether LPS-coated or HSA-coated particles were employed.

Serum also removed C3 radioactivity and opsonic expression from particles. This serum activity was heat resistant, did not require divalent cations, and was diminished in KAF-deficient or sodium metaperiodate-treated sera. The serum activity is, therefore, partly attributable to the presence of KAF, which is sensitive to sodium metaperiodate but not to heat or EDTA (13).

Structure of Opsonically Active C3. Opsonic expressions of C3 was resistant to phospholipases A and C and to organic solvents suggesting that it is not a lipoprotein. The eluted polypeptide was PAS negative, consistent with a lack of abundant carbohydrate. The sensitivity of opsonic expression to alkylation and greater sensitivity to reduction followed by alkylation indicate the influence of both sulfhydryl and disulfide groups on the function of the molecule. The presence of inter- and intrachain disulfide bridges could explain the remarkable stability of particle-bound C3, the covalent bonds protecting the molecular structure from thermal and chemical denaturation.

The stability of C3 facilitated analysis of its structure. By vigorous washing of opsonized particles, including boiling with 2 M NaCl, irrelevant peptides derived from C3 and other proteins could be removed. After such treatment, elution of opsonized particles with sodium dodecyl sulfate and a reducing agent yielded a 70,000 mol wt radioactive polypeptide or a 140,000 mol wt protein in the absence of the reducing agent. The findings suggested the opsonically active moiety of C3 has a mol wt of 140,000 and is composed of two 70,000 mol wt subunits linked

by disulfide bonds. Comparison of this molecule with the subunit composition of C3 revealed the origin of the opsonically active molecule.

During gel filtration of C3 in a nondenaturing solvent and at high ionic strength to prevent adsorption, C3 eluted as a globular protein with a mol wt of approximately 180,000. This value is in agreement with estimates based on other techniques (29). Nilsson and Mapes reported that reduced and denatured C3 could be resolved into 110,000 and 70,000 mol wt subunits (42). Our findings are in general agreement with this formulation although complicated by the fact that other polypeptides, possibly representing partial degradation of the major subunits, were invariably observed. Tryptic peptide mapping and densitometric analysis of C3 fragments resolved by reduction, denaturation, and electrophoresis on polyacrylamide gels with dodecyl sulfate suggested that the isolated 70,000 mol wt polypeptide was contaminated by variable amounts of the 115,000 mol wt component. Nevertheless, the tryptic maps showed that the isolated 70,000 mol wt polypeptide was sufficiently distinct from the 115,000 mol wt chain to permit the conclusion that one of the 70,000 mol wt subunits of opsonically active C3 was, in fact, the 70,000 mol wt piece of C3. The presence of tryptic peptides from the 115,000 mol wt component in opsonically active C3 indicated that a fragment of 115,000 mol wt chain also comprises the opsonically active molecule.

If the opsonically active portion of C3 has a mol wt of 140,000, then a 45,000 mol wt fragment or fragments must be broken from C3 during opsonization. In fact, a 30,000 mol wt radioactive fragment was found in eluates of particles not boiled in 2 M NaCl. This finding suggests that the 30,000 mol wt fragment, or 16% of C3, is removed by 2 M NaCl. Particles treated with 2 M NaCl lost 15–20% of their ^{125}I radioactivity but little or no ingestibility, a discrimination possible because of the quantitative resolution of the ingestion assay.

C3 attacked briefly by $C\overline{142}$ or by trypsin is cleaved into two fragments, C3a and C3b. Since C3a is a small peptide at the amino terminal of the 115,000 mol wt chain of C3, C3b would have to be almost as large a molecule as C3 (29). C3 attacked by more prolonged incubation with trypsin or aged in serum yields at least two electrophoretically distinct fragments, C3c and C3d, estimated to have mol wt of 150,000 and 27,000, respectively (16,20). However, the structure of these fragments is not precisely understood. Recent studies have shown that C3b is cleaved by KAF. This enzyme, which allows bovine serum to conglutinate bound C3, rapidly cleaves the 110,000 mol wt chain of C3b to a smaller size of 85,000 mol wt (19). The molecule, after KAF action, then has an intact polypeptide chain of 70,000 mol wt and another of 85,000 mol wt. It has been designated $C3b^i$ to indicate that the enzymatic property of C3b to activate the properdin system has been inactivated. $C3b^i$ is exquisitely sensitive not only to further KAF action but to a variety of proteolytic enzymes such as trypsin to which C3b is relatively resistant. These agents cleave the 85,000 mol wt chain down to a mol wt of 70,000. Neither the first cleavage product, which has a mol wt of about 25,000, nor the second cleavage product, which as a mol wt of about 15,000, has been well characterized. Furthermore, it is not known which of these fragments is C3d.

Antisera to some of these C3 fragments reacted differently with opsonized, washed, LPS-coated particles depending on whether the particles were washed

with 2 M NaCl. The ingestibility of all opsonized particles was inhibited by anti-C3 $F(ab')_2$ fragments. Anti-C3d $F(ab')_2$ only inhibited uptake of opsonized particles not boiled with 2 M NaCl; they did not inhibit ingestion of opsonized particles treated with 2 M NaCl. Conversely, opsonized, washed particles boiled in 2 M NaCl elicited antibodies that reacted with C3, C3b, and C3c but not with C3d. The results suggest that the fragment which is removed from the opsonized particles with 2 M NaCl is similar not only in molecular size but in antigenicity to C3d and is not relevant for opsonic expression. All of the immunochemical and chemical observations show that the entity defined as C3b itself is not the opsonically active fragment, although all of its parts may be bound to particles during opsonization.

Erythrocytes sensitized with C3b are not ingested (18). KAF treatment of EAC1423b cells results in the release of C3c into the fluid phase with continued binding of C3d on the erythrocyte membrane (15,18). This observation has been taken by some as indirect evidence for opsonic activity of C3b. It was concluded that C3b was constituted only of C3c and C3d and that release of C3c resulted in the loss of the opsonic effector site, whereas C3d contained the erythrocyte-binding site. Evidence presented in this paper showed that C3d is irrelevant to the opsonic process and that C3d could be removed without concomitant loss of opsonic activity. The complexity of the interaction between KAF and C3b precludes simple conclusions concerning binding and effector sites under the conditions of previously reported experiments. It is possible that the conglutinable form of C3, namely $C3b^i$, is also the opsonically active moiety of the molecule.

Summary

Human peripheral blood phagocytes ingest *Escherichia coli* 026:B6 lipopolysaccharide (LPS)-coated paraffin oil droplets containing Oil red O only if fresh serum deposits C3 on the surfaces of the particles (opsonizes them), by reactions involving the properdin system. The rate of binding of purified [^{125}I]C3 in serum to LPS-coated particles correlated precisely with the rate of acquisition of ingestibility assayed spectrophotometrically. Once opsonized, LPS-coated particles remained fully ingestible and retained fixed [^{125}I]C3 radioactivity even after exposure to extremes of temperature, pH, ionic strength, phospholipases, urea or guanidine, some nonionic and ionic detergents, and organic solvents. Trypsin, human conglutinogen-activating factor, another heat-stable activity found in human serum, and sodium dodecyl sulfate removed radioactivity and diminished ingestibility of the opsonized particles. Alkylation, reduction plus alkylation and $F(ab')_2$ from anti-C3 blocked ingestibility but did not alter particle-bound radioactivity.

Electrophoretic and tryptic peptide autoradiographic analysis of dodecyl sulfate eluates of opsonized particles, cleansed of many contaminating proteins by boiling with 2 M NaCl (yet still opsonized), revealed that the polypeptide with C3-derived radioactivity had a mol wt of approximately 140,000 and was composed of 70,000 mol wt subunits linked by disulfide bonds. Immunochemical analysis and comparison of the peptide structure of the eluate with that of C3

indicated that the opsonic fragment is not the fragment defined as C3b but a smaller derivative of C3.

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References

1. Wright, A. E., and S. R. Douglas. 1903. An experimental investigation of the role of blood fluids in connection with phagocytosis. *Proc. Roy. Soc. Lond. B Biol. Sci.* **72**:357.
2. Gigli, I., and R. A. Nelson, J. 1968. Complement-dependent immune phagocytosis. I. Requirments for C'1, C'4, C'2, C'3. *Exp. Cell Res.* **51**:45.
3. Johnston, R. B., Jr., M. R. Klemperer, C. A. Alper, and F. S. Rosen. 1969. The enhancement of bacterial phagocytosis by serum: the role of complement components and two cofactors. *J. Exp. Med.***129**:1275.
4. Shin, H. S., M. R. Smith, and W. B. Wood, Jr. 1969. Heat labile opsonins to pneumococcus. II. Involvement of C3 and C5. *J. Exp. Med.* **130**:1229.
5. Jasin, H. E. 1972. Human heat-labile opsonins: evidence for their mediation via the alternate pathway of complement activation. *J. Immunol.* **109**:26.
6. Diamond, R. D., J. E. May, M. A. Kane, M. M. Frank, and J. E. Bennet. 1974. The role of the classical and alternate complement pathways in host defenses against *Cryptococcus neoformans* infection. *J. Immunol.* **112**:2260.
7. Winkelstein, J. A., H. S. Shin, and W. B. Wood, Jr. 1972. Heat-labile opsonins to pneumococcus. III. The participation of immunoglobulin and of the alternate pathway of C3 activation. *J. Immunol.* **108**:1681.
8. Root, R. K., L. Ellman, and M. M. Frank. 1972. Bactericidal and opsonic properties of C4-deficient guinea pig serum. *J. Immunol.* **109**:477.
9. Stossel, T. P., C. A. Alper, and F. S. Rosen. 1973. Serum-dependent phagocytosis on paraffin oil emulsified with bacterial lipopolysaccharide. *J. Exp. Med.* **137**:690.
10. Stossel, T. P. 1973. Quantitative studies of phagocytosis. Kinetic effects of cations and heat-labile opsonin. *J. Cell. Biol.* **58**:346.
11. Tamura, N., and R. A. Nelson, Jr. 1967. Three naturally occurring inhibitors of components of complement in guinea pig and rabbit serum. *J. Immunol.* **99**:582.
12. Lachmann, P. J., and R. Liske. 1966. The preparation and properties of alexinated intermediates that react with conglutinin. *Immunology.* **11**:243.
13. Lachmann, P. J., and H. J. Müller-Eberhard. 1968. The demonstration in human serum of "conglutinogen-activating factor" and its effect on the third component of complement. *J. Immunol.* **100**:691.
14. Ruddy, S., and K. F. Austen. 1969. C3 inactivator of man. I. Hemolytic measurement by the inactivation of cell-bound C3. *J. Immunol.* **102**:533.
15. Ruddy, S., and K. F. Austen. 1971. C3b inactivator of man. II. Fragments produced by C3b inactivator cleavage of cell-bound or fluid phase C3b. *J. Immunol.* **107**:742.
16. Bokisch, V. A., H. J. Müller-Eberhard, and C. G. Cochrane. 1969. Isolation of a fragment of (C3a) the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med.* **129**:1109.
17. Müller-Eberhard, H. J., and O. Götze. 1972. C3 proactivator convertase and its mode of action. *J. Exp. Med.* **135**:1003.
18. Abramson, N., C. A. Alper, P. J. Lachmann, F. S. Rosen, and J. H. Jandl. 1971. Deficiency of C3 inactivator in man. *J. Immunol.* **107**:19.
19. Gitlin, J. D., F. S. Rosen, and P. J. Lachmann. 1975. The mechanism of action of the C3b inactivator (conglutinogen-activating factor) on its naturally occurring substrate, the third component of complement (C3). *J. Exp. Med.* **141**:1221.

20. Spitzer, R. E., A. E. Stitzel, V. S. Pauling, N. C. Davis, and C. D. West. 1971. The antigenic and molecular alterations of the fluid phase during an immune reaction in normal human serum. *J. Exp. Med.* **134**:656.
21. Stossel, T. P., R. J. Field, C. A. Alper, and F. S. Rosen. 1974. The opsonically active fragment of C3. *J. Clin. Invest.* **53**:78 *a* (Abstr.)
22. Alper, C. A., T. P. Stossel, and F. S. Rosen. 1975. Genetic defects affecting complement and host resistance to infection. *In* The Phagocytic Cell and Host Resistance. D. H. Dayton and J. A. Bellanti, editors. Raven Press, New York. 127–138.
23. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. *In* Methods in Carbohydrate Chemistry, Volume 5. R. L. Whistler, editor. Academic Press, Inc., New York. **5**:83.
24. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of β_{1F}-globulin from human serum and its characterization as the fifth component of complement. *J. Exp. Med.* **122**:277.
25. McFarlane, A. S. 1964. Metabolism of plasma proteins. *Mammalian Protein Metab.* **1**:331.
26. Ritchie, R. F., C. A. Alper, J. Graves, N. Pearson, and C. Larson. 1973. Automated quantitation of proteins in serum and other biological fluids. *Am. J. Clin. Pathol.* **59**:151.
27. Alper, C. A., F. S. Rosen, and P. J. Lachmann. 1972. Inactivator of the third component of complement as an inhibitor in the properdin pathway. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2910.
28. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestion products. *Methods Med. Res.* **10**:134.
29. Budzko, D. B., V. A. Bokisch, and H. J. Müller-Eberhard. 1971. A fragment of the third component of human complement with anaphylatoxin activity. *Biochemistry*, **10**:1166.
30. Stossel, T. P. 1973. Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. *Blood J. Hematol.* **42**:121.
31. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**:2606.
32. Segrest, J. P., and R. L. Jackson. 1972. Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *Methods Enzymol.* **28B**:54.
33. Richards, E. G., C. S. Chung, D. B. Menzel, and H. S. Olcott. 1967. Chromatography of myosin on diethylaminoethyl-Sephadex A50. *Biochemistry*. **6**:528.
34. Spudich, J. A., and S. Watt, 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **245**:4866.
35. Ouchterlony, O. 1970. Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology. D. M. Weir, editor. F. A. Davis Co., Philadelphia, Pa. 655.
36. Mantovani, B., M. Rabinovitch, and V. Nussenzweig. 1972. Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor sites for complement (C3) and for immunoglobulins (IgG). *J. Exp. Med.* **135**:780.
37. Knüfermann, H., H. Fischer, and D. F. H. Wallach. 1971. Peptide cleavage in sheep erythrocyte membranes during the action of complement. *FEBS* (*Fed. Eur. Biochem. Soc.*) Lett. **16**:167.
38. Bhakdi, S., H. Knüfermann, R. Schmidt-Ullrich, H. Fischer, and D. F. H. Wallach. 1974. Interaction between erythrocyte membrane proteins and complement compo-

nents. I. The role of -S-S linkages as revealed by two-dimensional sodium dodecyl sulfate-polyacrylamide electrophoresis. *Biochim. Biophys. Acta.* **363**:39.

39. Sellards, A. W. 1908. Note on the nature of opsonic immunity. *J. Infect. Dis.* **5**:308.
40. Hirsch, J. G., and B. Strauss. 1964. Studies on heat-labile opsonin in rabbit serum. *J. Immunol.* **92**:145.
41. Dalmasso, A. P., and H. J. Müller-Eberhard. 1967. Physicochemical characteristics of the third and fourth component of complement after dissociation from complement-cell complexes. *Immunology.* **13**:293.
42. Nilsson, U. R., R. J. Mandle, Jr., and J. A. McConnel-Mapes. 1975. Human C3 and C5: subunit structure and modifications by trypsin and $C\overline{42}$-$C\overline{423}$. *J. Immunol.* **114**: 815.