THE ENHANCEMENT OF BACTERIAL PHAGOCYTOSIS BY SERUM

The Role of Complement Components and Two Cofactors*

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When Metchnikoff defined the process of phagocytosis in 1884 (1, 2), he conceded to serum the minor role of an element that merely modified the phagocyte. However, in 1903 Wright and Douglas clearly established that phagocytosis of pathogenic bacteria could not occur without serum; that this effect was achieved by modification of the microorganism rather than the phagocyte; and that immunity developed in the serum, not the leukocyte (3, 4). Whether this serum effect was accomplished by specific antibody alone or by antibody acting in conjunction with a nonspecific, heat-labile serum activity, namely complement, was not clear until the experiments of Ward and Enders in 1933. They demonstrated that ingestion of pneumococci could occur slowly in the presence of heated immune serum; however, the addition of small amounts of fresh, nonimmune serum markedly enhanced the rate of ingestion (5).

The heat-labile factor or factors necessary for optimal phagocytosis of bacteria have not been precisely identified. On the basis of their own and other available experimental data, Boyden et al. found it impossible to define the role of thermolabile factors as opsonins and pointed out the need for a means of accurately measuring phagocytosis of bacteria (6). The present report describes a spectrophotometric method of measuring bacterial phagocytosis and its application to demonstrate that the first four components of human complement and two serum cofactors are necessary for the optimal phagocytosis of pneumococci by human leukocytes.

Materials and Methods

Pneumococci.—Diplococcus pneumoniae, type II, was obtained from the Department of Microbiology, Harvard Medical School, through the kindness of Miss Marjorie Jewell. It

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was maintained in virulent form by passage through white laboratory mice which were frozen at −20°C upon death. Fresh cultures were prepared by thawing the mice and inoculating heart blood into Todd-Hewitt broth and onto blood agar plates.

Preparation of Immune Antibody and Sensitization of Pneumococci.—Antiserum to type II pneumococci was raised in rabbits by repeated injections of formalinized, heat-killed organisms. Gamma G globulin (γG) was separated from the antiserum by single-step elution from diethylaminoethyl (DEAE) cellulose with 0.007 M sodium phosphate buffer, pH 6.3. The eluate was dialyzed against 0.15 M NaCl before use.

Overnight cultures of pneumococci were formalinized (final volume 1%), heated at 65°C for 30 min, centrifuged at 8000 *g* for 15 min, washed three times with 0.15 M saline, and re suspended in a 17 mm diameter tube to an optical density (OD) of 0.500 at 620 mμ in a Coleman Junior spectrophotometer. Two volumes of the pneumococcal suspension were mixed with one volume of antibody and incubated at 37°C for 30 min. The sensitized bacteria were centrifuged at 8000 *g* for 15 min, washed twice, and resuspended in sucrose-saline veronal buffer, μ 0.065, pH 7.35 (7) (hereafter termed veronal buffer), and the OD was adjusted to 0.150 at 620 mμ in a Coleman Junior spectrophotometer. This corresponded to a concentration of approximately 9×10^8 sensitized bacteria/ml.

Preparation of Leukocyte Suspensions.—Human venous blood was collected in heparinized plastic syringes and one-fourth volume of 6% dextran in saline was added. The syringe tip was fitted with a curved 18 gauge needle, the syringe was rested on its plunger, and the blood was allowed to sediment at room temperature for 60–90 min. With the syringe still upright, leukocyte-rich plasma was separated by pressure on the plunger and centrifuged in plastic conical tubes at 200 *g* for 15 min. The cell pellet was resuspended and agitated in approximately 50 volumes of 0.87% NH_4Cl to lyse red cells, centrifuged for 4 min, and washed twice in Krebs-Henseleit bicarbonate buffer (8), pH 7.35, containing 200 mg/100 ml glucose. The final cell pellet was suspended in 10–20 volumes of this buffer, and the volume of this suspension which contained 2.5×10^6 phagocytic cells (monocytes, juvenile and adult polymorphonuclears) was determined. Ordinarily, this number of phagocytes was contained in approximately 0.1 ml.

Normal and Complement-Deficient Sera.—Normal serum was separated from freshly clotted blood, frozen in small aliquots at −65°C, and thawed as required. Complement activity was destroyed by heating to 56°C for 30 min, or by treatment with 0.01 M Na_3H EDTA[1], 0.025 M hydrazine anhydride, or 1 μg/ml serum of Naja naja venom (Ross Allen's Reptile Institute, Inc., Silver Springs, Fla.). Sera specifically deficient in C2 were obtained from two healthy males homozygous for an hereditary deficiency of this component and from relatives heterozygous for the deficiency (9, 10). Sera specifically deficient in C3 were obtained from two children with progressive glomerulonephritis (11), an adult male with acute glomerulonephritis (12), and three individuals who are heterozygous for hereditary C3 deficiency (13). Serum from DBA strain mice with an hereditary deficiency of C5 (14) was kindly donated by Dr. Henry Winn. Serum from rabbits deficient in C6 (15, 16) was generously given by Dr. Shaun Ruddy and Dr. Carlos Biro.

Purification of Human Complement Components.—The C1 macromolecule was purified from human serum by the method of Nelson (17). C4 was isolated by a modification of the method of Müller-Eberhard and Biro (18), C2 by the method of Lepow et al. (19), and C3 by the method of Nilsson and Müller-Eberhard (20). C2 was iodinated by the method of Polley and Müller-Eberhard (21). C3 and iodinated C2 were dialyzed against veronal buffer at 4°C for 4 hr before use.

Peptide Solutions.—Glycyl-L-tyrosine, glycyl-L-leucyl-L-tyrosine, glycyl-L-leucine, glycyl-L-serine, and glycyl-L-valine were obtained from Mann Research Laboratories, New York,

[1] Trisodium hydrogen ethylene diamine tetraacetate.

N.Y. Peptides were dissolved in veronal buffer to a concentration of 0.04–0.2 M, adjusted to pH 7.35 with 2 N NaOH, and stored at −20°C. These stock solutions were diluted in veronal buffer immediately before each experiment.

Preparation of Dialysate.—Human serum was dialyzed against an equal volume of physiological saline at 0°C for 4 hr (22). The dialysate was stored at −65°C.

Quantitation of Phagocytosis

Spectrophotometric Measurement of Reduced Nitro Blue Tetrazolium (NBT).—The method of Baehner and Nathan (23) was modified for the study of bacterial phagocytosis. A 0.2% solution of NBT, Grade III (Sigma Chemical Co., St. Louis, Mo.) in physiological saline was filtered through Whatman No. 1 paper, and stored at 4°C in an opaque bottle. The standard test system consisted of 0.4 ml NBT solution, 0.2 ml sensitized pneumococci, and 0.08 ml fresh human serum in 12 ml siliconized conical centrifuge tubes to which 2.5×10^6 phagocytes were added after a 10 min preincubation at 37°C. Reaction volumes were equalized when necessary with veronal buffer. After a 20 min incubation at 37°C in a reciprocating water bath, the reaction was terminated by the addition of 0.1 N HCl in physiological saline. The tubes were then centrifuged at 1400 *g* for 15 min at 4°C, the supernatant was discarded, and the purple cell button was extracted with 2 ml pyridine, reagent grade (Fisher Scientific Co., Fair Lawn, N. J.) in a boiling water bath for 10 min. The tubes were centrifuged at 500 *g* for 5 min and the extraction repeated with 2 ml pyridine. The extracts were combined and their optical density measured in a Zeiss spectrophotometer at 515 mμ. All assays were run in duplicate.

Microscopic Enumeration of Ingested Bacteria.—2.5 million phagocytes in 0.1 ml volume were added to 0.2 ml sensitized pneumococci suspension, 0.08 fresh human serum, and 0.4 ml physiological saline in 10 × 71 mm siliconized glass tubes. These were incubated on a revolving wheel at 37°C for 20 min, then coverslip smears of the mixture were stained with Wright's stain and coded. The number of cocci in 100 consecutive polymorphonuclear and monocytic phagocytes was counted, and the average number of cocci per phagocyte (the phagocytic index) was determined. In this system 98–100% of phagocytes ingested at least one bacterium in the presence of normal serum.

Uptake of ^{125}I-Pneumococci by a Phagocytic Monolayer.—The method of Michell and coworkers (24) was used to study the uptake of labeled pneumococci by phagocytes attached to the surface of a Petri dish. Pneumococci were labeled with ^{125}I (25), sensitized with specific rabbit γG and suspended as described above. The reaction mixture was that of the NBT assay except that 0.2 ml of the labeled bacteria were suspended in veronal buffer to 0.52 ml, making the reaction volume 1 ml.

Bactericidal Capacity.—The method used was based on that of Maaløe as modified by Quie et al. (26). Immediately before use, a 5 hr broth culture of pneumococci was suspended in a 17 mm diameter tube to an OD of 0.100 at 620 mμ in a Coleman Junior spectrophotometer and diluted 1:10 in phosphate buffered saline, pH 7.4, containing 10% Todd-Hewitt broth and 10% serum albumin. To 12 × 75 mm sterile capped plastic tubes (Falcon Plastics Co., Los Angeles, Calif.) were added 0.05 ml of type-specific rabbit antiserum heated to 56°C for 30 min, 0.08 ml of fresh serum or buffer, 0.07 ml serum albumin, 2.5×10^6 phagocytes suspended in 0.5 ml sterile Krebs-Henseleit buffer, and finally, 0.3 ml of the pneumococcal suspension. This was calculated to give 1 or 2 bacteria per phagocyte in a total reaction volume of 1 ml. The contents of the tube were immediately mixed, and 0.01 ml of the mixture was transferred by calibrated platinum loop or micropipette to a 100 × 17 mm capped plastic tube (Falcon) containing 6 ml of sterile water. After vigorous shaking of the tube for 30 sec, a 0.5 ml aliquot was removed to make a pour plate with 12 ml of Todd-Hewitt agar. The original reaction tube was then placed on a revolving wheel at 37°C, and additional samples were obtained for colony counting at intervals up to 120 min. Colonies were counted the following day and expressed as number of surviving bacteria per ml of incubation mixture.

RESULTS

Spectrophotometric Quantitation of Phagocytosis.—Quantitation of phagocytosis was achieved by the spectrophotometric measurement of nitro blue tetrazolium (NBT) dye reduced to a blue color in proportion to the number of pneumococci ingested. As shown in Fig. 1, the optical density of the dye reduced was proportional to the number of bacteria in the system until the ratio of bacteria to phagocytes reached approximately 75:1. Increasing this ratio with additional bacteria did not increase the amount of NBT reduced.

When the bacteria:phagocyte ratio of approximately 75:1 was maintained and the volume of fresh human serum in the reaction mixture was varied, an arithmetic plot of the dye reduction was sigmoidal (Fig. 2). Optimal dye reduction was achieved with approximately 0.08 ml serum.

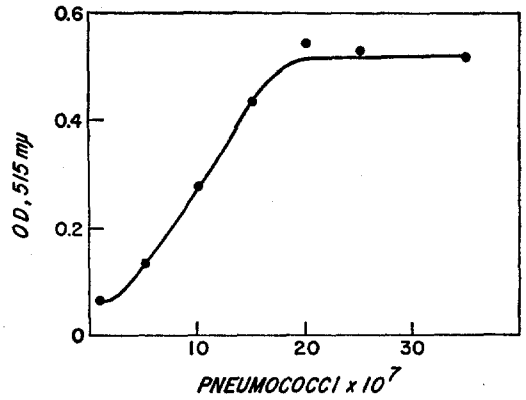

FIG. 1. The optical density of NBT dye reduced during the phagocytosis of pneumococci plotted as a function of the number of bacteria available for ingestion. Type-specific antibody and 0.08 ml fresh serum were present in the system.

The effect of the duration of incubation of the reaction mixture on the dye reduction and on the number of ingested bacteria per phagocyte (phagocytic index) is compared in Fig. 3. Both reactions proceeded rapidly for approximately 15 min, and the plot of the dye reduction was parallel to but slightly slower than that of the phagocytic index. After 15 min, the rate of both reactions decreased, and by 25 min no further increase in phagocytosis was detected by either method. The effect of the length of incubation on the uptake of ^{125}I-pneumococci by fixed phagocytes was also studied. The increase in phagocytosis with time noted in this system was the same as that described for NBT reduction and phagocytic index. Uptake of labeled bacteria was rapid for 15 min and complete by 25 min. When viable pneumococci were incubated with antibody, fresh serum, and phagocytes, and the rate of bacterial killing was determined, it was found that ingestion, measured by the phagocytic index, and dye reduction were complete before most of the bacterial killing occurred (Fig. 3).

Quantitation of Phagocytosis in the Presence of Complement-Deficient Sera.— Various sera specifically deficient in one of the complement components were tested for their ability to enhance bacterial phagocytosis in order to ascertain

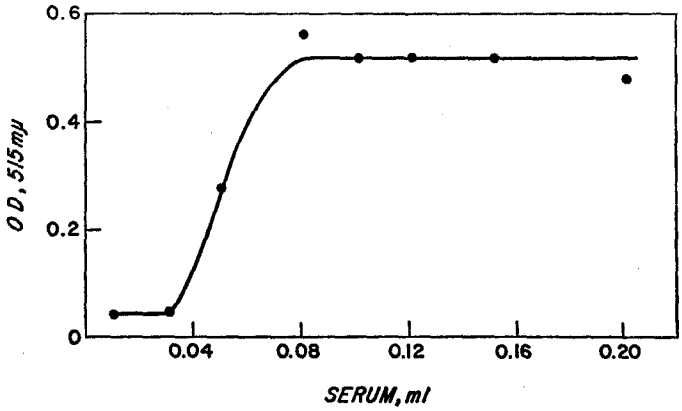

FIG. 2. The optical density of NBT dye reduced during the phagocytosis of pneumococci plotted as a function of the volume of fresh normal serum in the system. The bacteria were sprevioulv prepared with type-specific antibody.

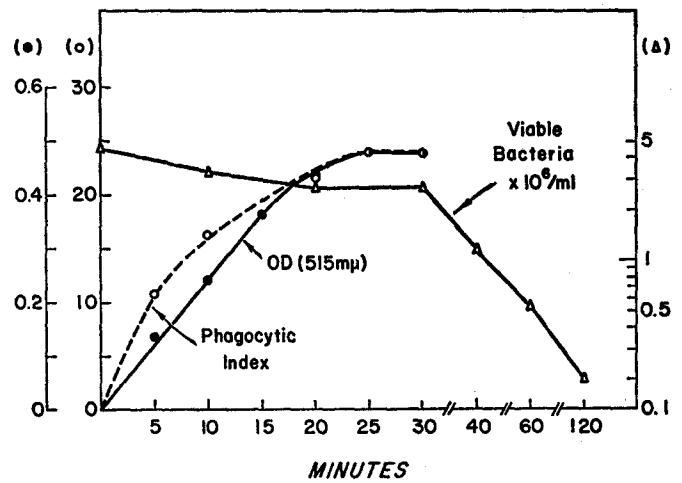

FIG. 3. The optical density of reduced NBT dye, the number of pneumococci per phagocyte (phagocytic index), and the number of viable bacteria per ml of reaction mixture plotted as a function of the length of time during which phagocytosis was allowed to occur.

which components are necessary for this process. When normal serum was replaced in the assay system by an equal volume (0.08 ml) of human serum deficient in C2 or C3, no enhancement of phagocytosis was observed (Table I). The results obtained with varying amounts of sera deficient in C2 or C3

are compared in Fig. 4. The C2-deficient serum which contained less than 5% of the normal C2 concentration (10) was more effective in promoting phagocytosis than equal volumes of C3-deficient serum which contained approximately 8% of the normal C3 concentration. When serum was heated at 56°C for 30 min, or treated with hydrazine, EDTA, or cobra venom, it no longer enhanced phagocytosis (Table I).

When serum from mice with an inherited deficiency of C5 or serum from rabbits genetically deficient in C6 were compared to the normal serum of their species, there were no significant differences in their ability to promote phagocytosis (Figs. 5 and 6).

TABLE I

*Enhancement of Phagocytosis of Antibody-Sensitized Pneumococci by Complement-Deficient Sera**

C-Deficient serum		Serum C2 concentration	OD	C-Deficient serum		Serum C3 concentration	OD
		mg/100 ml‡				*mg/100 ml*‡	
No serum			0.042	C3 Deficiency:			
Heated			0.055	Hereditary	1	81	0.183
Hydrazine-treated			0.045		2	62	0.128
EDTA-treated			0.027		3	54	0.140
Cobra venom-treated			0.017	Acquired	1	14	0.074
C2 deficiency:					2	12	0.055
Hereditary					3	<5	0.030
Homozygous	1	<0.05	0.071				
	2	<0.05	0.068				
Heterozygous	1	0.3	0.271				
	2	0.3	0.317				

* Measured by the optical density (OD) at 515 mμ of NBT dye reduced during phagocytosis. Serum volume was 0.08 ml. The mean OD for 72 determinations of normal serum was 0.386, and the range was 0.304 to 0.520.

‡ Normal value for C2, 1 ± 0.2 mg/100 ml, for C3, 150 ± 50 mg/100 ml.

Enhancement of Phagocytosis by Purified Complement Components.—It was apparent from these results that C1, C4, C2 and C3 were needed to enhance bacterial phagocytosis, whereas C5 and C6 and, presumably, the later-reacting components had no demonstrable effect on this process.

Pneumococci were prepared for phagocytosis by the addition of purified complement components. Equal volumes of the pneumococci-antibody suspensions (0.3 ml) and C1 in veronal buffer were incubated together at 30°C for 30 min, centrifuged at 8000 *g* at 0°C for 15 min, and washed once in cold veronal buffer. The washed complex was resuspended in 0.3 ml of C4 in veronal buffer, incubated at 37°C for 30 min, and centrifuged again at 8000 *g* for 15 min. The pneumococci-antibody-$\overline{C1}$,4 complex was resuspended in 0.2 ml oxidized C2 and incubated at 30°C for 8 min; 0.3 mg C3 was added and incubation was continued at 30°C for 10 min, after which NBT and phagocytes were added.

When the pneumococci were prepared with C1 alone; C1 and C3; C1, C4, and C2; or C1, C4, and C3 prior to the addition of NBT and phago-

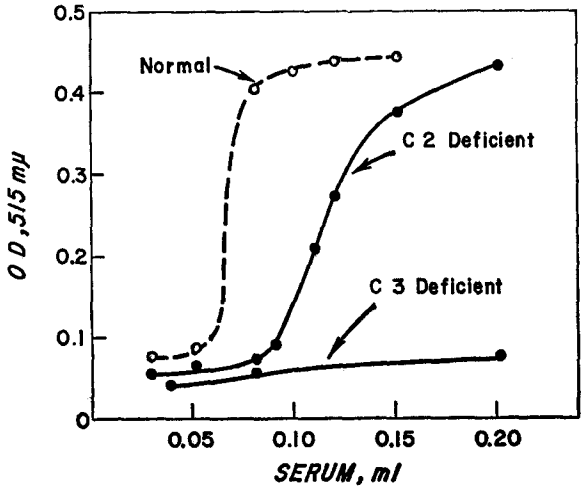

FIG. 4. A comparison of the optical density of NBT dye reduced during the phagocytosis of sensitized pneumococci in the presence of normal, C2-deficient, and C3-deficient fresh human serum, plotted as a function of the volume of serum in the reaction mixture.

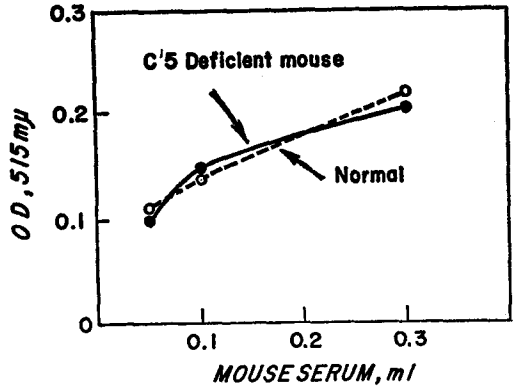

FIG. 5. A comparison of the optical density of NBT dye reduced during the phagocytosis of pneumococci in the presence of normal or C5-deficient mouse serum, plotted as a function of the volume of serum in the reaction mixture.

cytes, no increase in phagocytosis was observed (Table II). The sequential addition of C1, C4, C2, and C3 did result in significant enhancement of phagocytosis (Table II); however, repeated experiments with these purified complement components in concentrations in excess of those in normal serum

failed to achieve an OD that was more than 50% to 60% of the average OD of 72 determinations with fresh serum (0.386, range 0.304–0.520). Therefore, it seemed possible that other factors which are not complement components might be required for optimal sensitization.

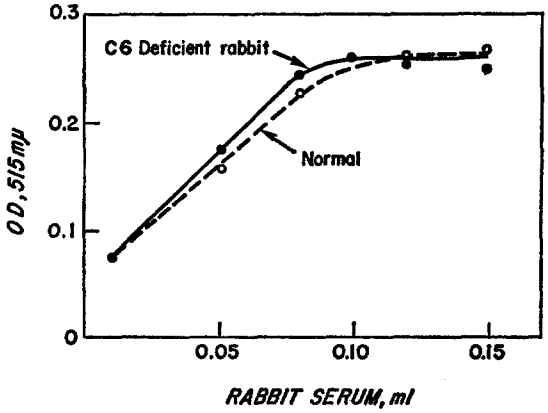

FIG. 6. A comparison of the optical density of NBT dye reduced during the phagocytosis of pneumococci in the presence of normal or C6-deficient rabbit serum, plotted as a function of the volume of serum in the reaction mixture.

TABLE II

Enhancement of Phagocytosis by the Addition of Purified Complement Components to Sensitized Pneumococci

Components added	OD*
None	0.058
C1	0.054
C1,3	0.074
C1,4,2	0.058
C1,4,3	0.082
C1,4,2,3	0.217

* Optical density at 515 mμ of NBT dye reduced during phagocytosis.

Enhancement of Phagocytosis of Pneumococci Prepared with Antibody and C1,4,2,3 by a Dialysate of Human Serum.—It was observed in 1957 that peptides containing an aromatic amino acid inhibited immune hemolysis by guinea pig complement (27, 28). In 1965 Basch showed that this inhibition occurred between the erythrocyte-antibody (EA) $C\bar{1},4,\bar{2}$ step and the intermediate EA C1 $\sim$ 7, and that these peptides inhibited immune adherence in a rank order similar to that for their inhibition of immune hemolysis (29). Thus, the evidence suggested that the activity of cell bound C3 was inhibited

by the peptides. Cooper and Becker confirmed this in 1967 when they showed that complexed guinea pig C3 hydrolyzed peptides containing aromatic amino acids (30). Cooper also demonstrated that this peptidase activity of C3 was enhanced by a dialysate of whole guinea pig serum (22). If human C3 has similar peptidase activity and if this activity is essential for phagocytosis, aromatic amino acid-containing peptides might inhibit phagocytosis.

When glycyl-L-tyrosine in concentrations greater than 5×10^{-4} M was present in the phagocytic system, inhibition of phagocytosis occurred (Fig. 7). Inhibition was also obtained with 4×10^{-3} M and 9×10^{-4} M glycyl-L-leucyl-L-tyrosine, but not with 8×10^{-3} M glycyl-L-leucine, glycyl-L-valine, or glycyl-L-

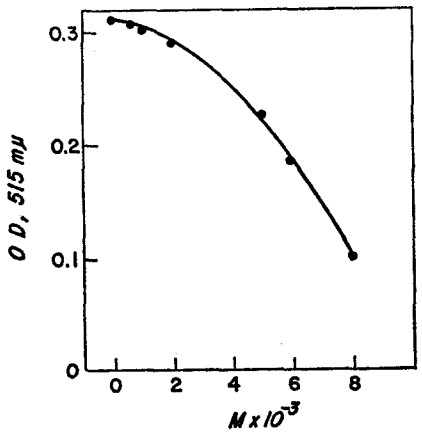

FIG. 7. The optical density of NBT dye reduced during phagocytosis of pneumococci prepared with antibody and complement, plotted as a function of the concentration (molarity) of glycyl-L-tyrosine in the reaction volume.

serine (Table III). NBT dye reduction after the ingestion of latex particles (0.81 μ diameter, Difco Laboratories, Detroit, Mich.), which do not require serum for ingestion, was not inhibited by 8×10^{-3} M glycyl-L-tyrosine. These results suggested that the peptidase activity of C3 was essential for phagocytosis of pneumococci.

When 0.19 mg of purified C3 was added to 0.08 ml C3-deficient serum in the presence of 0.2 ml of the dialysate of human serum, a further 20% enhancement of phagocytosis was achieved over that obtained with 0.19 mg C3 alone (Table IV). Heating the dialysate at 56°C for 30 min partially eliminated this effect, and heating at 56°C for 60 min completely eliminated it. When dialysate as well as C1, C4, C2, and C3 were used to prepare the pneumococci, phagocytosis was further improved by approximately 13% (Table IV).

Enhancement by Pseudoglobulin of Phagocytosis of Pneumococci Prepared

with Antibody, C1,4,2,3, and Dialysate.—It was apparent from the foregoing experiments that the peptidase activity of C3 is required for the enhancement of phagocytosis by complement. However, sensitization of the pneumococci with the first four components in excess and the dialyzable cofactor of

TABLE III

Activity of Various Peptides in Inhibiting the Phagocytosis of Pneumococci in the Presence of Serum

Peptide added	Concentration of peptide (M)	OD*
None		0.322
Glycyl-L-leucine	8×10^{-3}	0.313
Glycyl-L-serine	8×10^{-3}	0.310
Glycyl-L-valine	8×10^{-3}	0.319
Glycyl-L-leucyl-L-tyrosine	4×10^{-3}	0.151
Glycyl-L-leucyl-L-tyrosine	9×10^{-4}	0.209
Glycyl-L-tyrosine	8×10^{-3}	0.125

* Optical density at 515 mμ of NBT dye reduced during phagocytosis.

TABLE IV

Enhancement of Phagocytosis by a Dialyzable Serum Factor

Serum and serum factors added to sensitized pneumococci	OD*
Normal serum	0.325
C3-deficient serum	0.072
C3-deficient serum + dialysate	0.096
C3-deficient serum + C3	0.290
C3-deficient serum + C3 + dialysate	0.340
C3-deficient serum + C3 + dialysate heated 56°C, 30 min	0.327
C3-deficient serum + C3 + dialysate heated 56°C, 60 min	0.280
C1,4,2,3	0.217
C1,4,2,3 + dialysate	0.237

* Optical density at 515 mμ of NBT dye reduced during phagocytosis.

C3 peptidase activity did not promote phagocytosis as well as did a small volume of whole serum.

Another serum component necessary for the optimal phagocytosis of pneumococci was obtained by separation of serum into pseudoglobulin and euglobulin fractions after overnight dialysis against 10 volumes of 0.02 M Na acetate buffer, pH 5.5. Fractions were dialyzed against veronal buffer before use, and 0.05 ml of a fraction was added to the assay mixture prior to the addition of purified C3. The pseudoglobulin fraction markedly enhanced the phagocytosis

of pneumococci prepared with antibody, C1,4,2,3 and the dialysate of human serum (Table V). Pseudoglobulin obtained from serum of an individual with hereditary angioneurotic edema (HANE pseudoglobulin), which contained no hemolytically demonstrable C4 or C2 activity (31), was equally effective. Heating pseudoglobulin to 56°C for 30 min partially destroyed its ability to enhance phagocytosis (Table V).

Pseudoglobulin was eluted from a DEAE cellulose column with 0.02 M Na phosphate buffer, pH 7.3 (conductance at 25°C of 2875 μ mho/cm). The DEAE eluate was concentrated 10-fold by evaporation through dialysis tubing and separated by electrophoresis on Pevikon (32) with barbital buffer, pH 8.6, μ 0.1. Fractions were eluted at 1 cm intervals from the Pevikon and dialyzed against veronal buffer; 0.1 ml of a fraction was added to a reaction tube before the addition of C3.

TABLE V

Enhancement of Phagocytosis by a 5-6S, Beta Pseudoglobulin

Complement components and co-factors added to sensitized pneumococci	OD*
Pseudoglobulin	0.065
C1,4,2,3-dialysate	0.232
C1,4,2,3-dialysate-pseudoglobulin	0.330
C1,4,2,3-dialysate-HANE pseudoglobulin	0.348
C1,4,2,3-dialysate-heated pseudoglobulin (56°C, 30 min)	0.287
C1,4,2,3-dialysate-DEAE eluate	0.338
C1,4,2,3-dialysate-β pseudoglobulin	0.308
C1,4,2,3-dialysate-5-6S globulin	0.334

* Optical density at 515 mμ of NBT dye reduced during phagocytosis.

As shown in Table V phagocytosis-enhancing activity was found in the region of the β globulins, 1–3 cm toward the anode from the origin.

In order to estimate the molecular size of the β pseudoglobulin, serum was eluted from Sephadex G-200 with physiological saline. The fraction eluted between the 4S and 7S peaks (5–6S) contained significant phagocytosis-enhancing activity (Table V).

DISCUSSION

Study of the role of serum factors in the enhancement of bacterial phagocytosis has been facilitated by a spectrophotometric assay, utilizing the reduction of nitro blue tetrazolium (NBT) dye. NBT dye enters polymorphonuclear and monocytic phagocytes *pari passu* with ingested material and is reduced intracellularly (33), apparently as the result of release of reductases from lysosomal granules which rupture after ingestion (34). Thus, the results of this assay are not influenced by particles merely adherent to the surface of phago-

cytes. The rate of dye reduction follows closely the rate of ingestion of pneumococci as measured by the phagocytic index and by the uptake of ^{131}I-pneumococci by a monolayer of phagocytes (24).

By using sera deficient in specific complement components and by the sequential fixation of purified human C1, C4, C2, and C3, it has been shown that these four components, but not the later-acting components C5 through C9, are necessary for the optimal phagocytosis of pneumococci. This finding is in agreement with the work of Stiffel and coworkers (35) and Glynn and Medhurst (36), who showed that in vivo phagocytosis of *Salmonella typhimurium* and *Escherichia coli*, respectively, was comparable in normal and C5-deficient mice. Similarly, Rother and Rother (37) and Biro and Garcia (38) showed that C6-deficient rabbits cleared the bloodstream of *Salmonella typhi* and aggregated gamma globulin as well as normal rabbits.

Several previous investigations have suggested that serum factors other than antibody and complement might enhance bacterial phagocytosis (39). The unavailability of purified complement components at the time of these investigations prevented the precise separation of complement activity from that of other heat-labile factors. Although the exposure of sensitized pneumococci to C1,4,2,3 increased phagocytosis, an excess of these components did not promote phagocytosis as well as whole serum.

Extending previous work (27, 28, 29), Cooper and Becker showed that EAC1,4,2,3 hydrolyzed peptides containing aromatic amino acids (30), and Cooper showed that the hemolytic, immune adherence, and peptidase activities of bound guinea pig C3 were enhanced by a dialyzable factor from guinea pig serum (22). A dialyzate of human serum enhanced the phagocytosis of pneumococci prepared with γG antibody and purified human components C1,4,2,3 in the experiments described here. Moreover, phagocytosis promoted by whole serum could be inhibited by the aromatic amino acid-containing peptides, glycyl-tyrosine and glycyl-leucyl-tyrosine. It would appear, therefore, that the peptidase activity of C3 fixed to an immune complex is essential for optimal phagocytosis.

Cooper and Müller-Eberhard found that effective C3 peptidase activity (hydrolysis) by EAC1,4,2,3 required the binding of 300–400 molecules of C4,5 molecules of oxidized C2, and less than 100 molecules of C3 (40). They suggested that the generation of peptidase activity may depend on a critical spatial relationship between C3 and a bound, active C4,2 site. A requirement for $\frac{1}{20}$ as much C2 as C3 for peptidase activity might explain why C3-deficient serum was much less effective in supporting phagocytosis than equal volumes of C2-deficient serum.

Although the dialyzable factor improved phagocytosis achieved by sensitization with purified complement components, the effect was still less than that of whole serum. However, when a 5–6S, beta pseudoglobulin was added to the

assay system before the addition of C3, phagocytosis was comparable to that attained with fresh serum. This pseudoglobulin is similar in physicochemical characteristics to that protein which restored complement-mediated functions to the serum of a young man with recurrent pyogenic infections and rapid in vivo catabolism of C3 (41). Augmentation of phagocytosis by this cofactor might result from stabilization of C3 peptidase activity or inhibition of enzymatic inactivation of C3 by cell bound or fluid phase C4,$\overline{2}$ (42) or by other C3-inactivating factors (43, 44).

The complement requirement of C1,4,2,3 for bacterial phagocytosis is in accord with recent observations on the complement requirements for erythrophagocytosis. Gigli and Nelson have demonstrated that the first four components of guinea pig complement are required for efficient phagocytosis of sensitized erythrocytes (45). Huber et al. (46) have shown that human monocytes possess distinct receptor sites for the adherence and phagocytosis of erythrocytes prepared with antibody and C1,4,2,3 and erythrocytes prepared with γG antibody alone (EA). Prior treatment of the monocytes with trypsin inhibited ingestion of EAC1,4,2,3 but not EA. The role of C3 peptidase activity in erythrophagocytosis has not been determined.

It is interesting to recall that Metchnikoff suggested over 80 years ago that serum influenced phagocytosis by interaction with the phagocytic cell. Since C3 peptidase activity would seem essential for bacterial phagocytosis and the attachment of EAC1,4,2,3 to monocytes (46), it is quite possible that a natural substrate of this enzymatic activity resides on the membrane of the phagocyte.

SUMMARY

The role of serum factors in the phagocytosis of pneumococci was studied employing a spectrophotometric assay which measures reduced nitro blue tetrazolium (NBT) dye. Dye reduction occurs within the phagocyte shortly after bacterial ingestion as measured by the phagocytic index technique and by the uptake of ^{125}I-pneumococci. Bacteria prepared with γG antibody were not phagocytosed unless a small volume of fresh normal serum was added. Using fresh sera deficient in single complement components, it was demonstrated that the first four components are necessary for optimal bacterial phagocytosis. When highly purified complement components were added to the antibody-coated pneumococci, enhancement of phagocytosis was achieved only with the sequential addition of C1, C4, C2, and C3.

Evidence has been presented that human C3 bound to an immune complex exhibits peptidase activity and that this activity is essential for phagocytosis. A heat-labile, dialyzable serum cofactor which enhances C3 peptidase activity enhanced the phagocytosis of pneumococci prepared with purified complement components. A second phagocytosis-promoting cofactor, which is not a com-

plement component, was found to be a heat-labile, 5–6S, beta pseudoglobulin. This protein may stabilize C3 peptidase activity or inhibit enzymatic inactivation of C3.

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