THE ROLE OF MEMBRANE RECEPTORS FOR C3b and C3d IN PHAGOCYTOSIS*

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A variety of evidence, both in vivo and in vitro, has shown that complement (C), and in particular C3, is involved in the opsonization of particles (1–3). The importance of C3 in defense mechanisms is highlighted by the finding of patients with hereditary deficiency in C3, whose serum cannot sustain opsonization of bacteria (4). These patients have repeated infections, although they have normal levels of immunoglobulin (Ig) and form antibodies to both thymus-dependent and thymus-independent antigens (5).

The mechanism whereby C3 participates in opsonization is a matter of controversy. Many investigators (6–10) find that C3-coated bacteria and erythrocytes adhere to phagocytes, forming "rosettes," but are not ingested. In contrast, others (11) claim that the deposition of C3 on particles enhances their rate of ingestion without enhancing the binding of the particles to the cells. Furthermore, their results suggest that C3b is the opsonically active moiety of C3, while the C3d fragment is not relevant to opsonization (12). Cooperation between IgG and C3 in opsonization has also been reported (6–10, 13, 14).

In this paper we re-examine the role of particle-bound IgG and C3 in phagocytosis of sheep erythrocytes (E)[1] by monolayers of purified human monocytes and polymorphonuclear leukocytes (PMN). We conclude that (*a*) two fragments of the C3 molecule, that is, C3b and C3d, can function as opsonins if the phagocytes have the appropriate membrane receptors. Monocytes, that bind both C3b and C3d (10, 15) respond to both as opsonins. Since PMN lack the C3d receptor (16, 17), only C3b is opsonic for these cells. (*b*) C3 and IgG have separate but synergistic roles in phagocytosis. IgG, through its Fc fragment, directly stimulates particle ingestion, but is relatively inefficient at inducing particle binding. C3, on the other hand, mediates the binding of the particle to the phagocyte without directly stimulating ingestion. However, the opsonic effect of C3 is manifested by a marked synergy with IgG in the induction of phagocytosis. Depending on the experimental conditions, the presence of particle-bound C3 can reduce by 100-fold the amount of IgG required to produce particle interiorization. Opsonization with C3 can thus be a necessary condition for phagocytosis to occur, although by itself, C3 does not induce ingestion. (*c*) The opsonic effect of C3 can be reproduced by a variety of nonimmunologic agents which enhance binding of the particle to the phagocyte without directly stimulating ingestion.

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[1] *Abbreviations used in this paper:* E, sheep erythrocytes; FCS, fetal calf serum; PMN, polymorphonuclear leukocytes.

The contact-inducing agents used include centrifugation of particle and phagocyte, high molecular weight dextran, protamine, and treatment of E with neuraminidase. These results strongly suggest that the role of C3 in opsonization is mainly or exclusively one of establishing contact between particle and phagocyte.

Materials and Methods

Medium. RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with added bicarbonate and 40 ml of 0.25 M Hepes, pH 7.4, per liter. Dextran (110 and 10) were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Protamine chloride and heparin were obtained from Sigma Chemical Co., St. Louis, Mo.

Phagocytes. Monocytes were isolated from normal human peripheral blood by a modification of the technique described by Koller et al. (18). 30 ml of heparinized venous blood was diluted with an equal volume of medium and divided in two equal portions. Each portion was layered over 15 ml of Lymphoprep (Nyegaard & Co., Oslo, Norway) in 50-ml plastic conical tubes. These were centrifuged at 400 *g* for 30 min and the interfaces, containing mononuclear cells and platelets, harvested with a Pasteur pipette. Care was taken to avoid including much of the volume beneath the interface. A total of approximately 10 ml was removed from each tube. The two portions were combined, and diluted with medium to 45 ml in 50-ml tubes. 3 ml of Lymphoprep was then added beneath the cell suspension using a Pasteur pipette, to provide a "cushion" for the cells during centrifugation and to avoid pelleting of the cells, which caused loss of monocytes. The preparation was then centrifuged at 100 *g* for 8 min at room temperature, which was sufficient to bring down the cells while most platelets remained in suspension. The volume above the interface (about 42 ml) was removed, and the cells diluted up to 45 ml with medium. A second Lymphoprep cushion was added and the low speed centrifugation process repeated. After removal of the upper 42 ml, the cells were diluted to 50 ml with medium and spun down without a cushion (300 *g* for 15 min). The pelleted cells were resuspended in 4 ml of medium containing 15% autologous serum (fresh, or frozen at −10°C). 100-μl aliquots of the mononuclear cell suspension were layered on clean 18 × 18 mm cover glasses, and these were incubated for 30 min at 37°C in an incubator with 5% CO_2 atmosphere. Nonadherent cells and serum were removed by dipping the cover slip 20 times in three successive beakers of fresh medium. The phagocytes were then incubated for 30–60 min at 37°C in fresh medium and washed once more before being used in the phagocytic assays. Such preparations were more than 99% mononuclear, and 97% or more of the cells were capable of ingesting opsonized E.

Phagocytosis by PMN was also assayed with the cells adherent to glass cover slips. PMN were isolated from normal venous blood by a variation of a technique proposed by Newsome (19). 10 ml of venous blood was drawn without heparin into a plastic syringe and immediately transferred into a 12 ml plastic tube cooled in ice. This tube was centrifuged at 1,500 *g* for 15 min at 0°C and the supernatant plasma, along with the upper 2 or 3 mm of the cell pellet, were harvested. The cells were resuspended in the plasma and 200 μl of the suspension layered on 18 × 18 mm cover glasses. These were incubated in Petri dishes in a moist, 37°C incubator with 5% CO_2 atmosphere for 25 min. At the end of this time, the Petri dishes were removed from the incubator and warm medium added until the red "clots" floated off the cover glasses. The cover glasses were then rinsed gently with medium, until all erythrocytes had been removed. The cover slips were washed gently in three successive beakers of fresh medium and incubated for 30 min at 37°C in 100 × 15 mm Petri dishes filled with medium containing 15% fetal calf serum (FCS; GIBCO). They were rinsed in medium once more before use in phagocytic assays. As Newsome reported, polymorphs are damaged if exposed to thin liquid films; therefore, during all manipulations, cover slips were handled in a horizontal fashion with as much medium above as could be carried. Preparations were generally 90% or more polymorphonuclear. PMN and mononuclear cells were differentiated on morphologic grounds by examination of stained preparations.

Erythrocytes. E in Alsever's solution were obtained from the New York City Department of Health.

Immunoglobulin and Complement. Rabbit IgM anti-E was obtained from Cordis Laboratories, Miami, Fla. Each lot was tested for possible contamination with IgG anti-E by assaying ingestion of preparations of EIgM and EIgMC3b by normal human monocytes. As the text will show, these

cells can recognize and ingest E coated with 100 molecules of IgG, particularly if C3 is also present on the E. Any lot which induced more than 5% of human monocytes to ingest EIgM or EIgMC3b was discarded.

Rabbit IgG anti-E was obtained from a rabbit antiserum to E (gift of Dr. Michel Rabinovitch, New York University School of Medicine). IgG was isolated by chromatography in DEAE-cellulose, and passaged twice through a column of Sephadex G-200. It was frozen at the concentration of 1 mg/ml until used. $F(ab')_2$ of IgG anti-E was prepared as described previously (20).

Human C1, C2, C3, and C4 were obtained from Cordis Laboratories. In experiments with ^{125}I-labeled C3, purified human C3 was kindly provided by Dr. Brian Tack, and labeled as described below. The labeled C3 showed a single peak by sodium dodecyl sulfate-acrylamide gel electrophoresis. No conversion of C3 was detected by immunoelectrophoresis.

Immune Adherence. Immune adherence tests were performed as described by Lachmann and Müller-Eberhard (21).

Iodination of Proteins. IgG and C3 were labeled by the chloramine-T method as described previously (22).

Treatment of E with Neuraminidase. This was performed as described previously (23), in Hanks' balanced salt solution, pH 6.5 (Neuraminidase, type VI from *Clostridium perfringens*; Sigma Chemical Co.)

Preparation of EIgMC3b and EIgMC3d. To prepare EIgMC3b, E were suspended at 10^9/ml and sensitized with an equal volume of an appropriate dilution of IgM anti-E. After washing, the cells were sensitized with C3b via sequential addition of purified C1, C4, C2, and C3. Usually 5,000 CH_{50} units of C1 and 500 CH_{50} units of C4, C2, and C3 were used for each 10^9 E. The sensitized cells were washed in medium containing 0.01 M NA_3H EDTA to remove C1, and incubated at 37°C for 2 h to decay C2.

Deposition of C3b on the E was shown by agglutination with specific antisera to C3, and by immune adherence with human erythrocytes. In experiments with labeled C3, the same amounts of IgM, C1, C4, and C2 were used, but the amount of C3 bound to the cells was calculated directly from the specific activity of the C3 preparations.

We found that binding of the E to phagocytes was C3 dependent. Therefore the cells will be referred to as EIgMC3b although they also contained C4b.

EIgMC3d was prepared as described previously (24) using normal human serum, either heat inactivated or EDTA treated, and absorbed extensively with E, as a source of C3b inactivator. In some experiments purified C3b inactivator was purchased from Cordis Laboratories. E prepared in this fashion were still agglutinable by anti-C3 but did not show immune adherence. When labeled C3 was used to prepare EIgMC3b, conversion of C3b to C3d was also assayed by release of label from the cell surface. After incubation with purified C3b inactivator, 50–60% of the label was specifically released from the red cells.

Preparation of EIgG, EIgMC3b·IgG, and EIgMC3d·IgG. E, EIgMC3b, or EIgMC3d were suspended at 10^9/ml and mixed with equal volumes of medium containing various dilutions of IgG anti-E. The medium also contained 0.1% gelatin in order to minimize nonspecific protein losses. The cells were incubated at room temperature for 30 min with constant mixing and washed twice before using. The IgG anti-E preparation contained 40% specific antibody as determined by specific absorption with E of a ^{125}I-labeled aliquot of this preparation. In phagocytosis experiments, unlabeled antibody was used to sensitize E to avoid possible alteration in the Fc fragment which might occur during the labeling procedure. The number of antibody molecules bound per E was calculated assuming that the binding activity of the labeled and unlabeled preparations was equivalent. As reported previously (6) no alterations in the binding capacity of IgG anti-E are induced by this labeling procedure. It is assumed that the actual numbers of antibody molecules on individual E follow a distribution with the average equal to the expected value. Calculations, based on a Poisson distribution, showed that if the average number of IgG molecules per E was 100 or more, less than one cell in 10^8 would exceed the average by a factor of two or more. In the text, the calculated number of IgG molecules bound per E will be indicated as a subscript. Thus, $EIgG_{400}$ are E sensitized with an average of 400 IgG molecules per E.

Rosette Formation. Rosette formation with human monocytes or PMN was performed by overlaying monolayers of about 5×10^4 phagocytes, on 18 × 18 mm cover glasses, with 0.1 ml of E suspensions at a 2×10^8/ml concentration. After incubation for 30 min at 37°C, free E were removed by gently dipping cover slips in beakers of fresh medium. Rosettes were fixed with 0.1 ml

of 2% glutaraldehyde in Dulbecco's phosphate-buffered saline (GIBCO) and examined as wet preparations.

Phagocytes with three or more E bound were considered rosettes. Rosette formation with human peripheral blood lymphocytes has been described (24).

Assays for Phagocytosis. Phagocytosis was examined in two basic protocols. In overlay experiments, 18 × 18 mm cover slips containing about 5×10^4 phagocytes were overlaid with 0.2 ml of medium containing 3×10^7 E (1.5×10^8 cells/ml) and incubated for 30–45 min at 37°C. Under these conditions the E quickly settle on top of the phagocyte monolayer and provide a uniform saturating particle concentration. After the incubation, ingestion was quantitated as described below.

In suspension experiments, the conditions were designed to maximize effects of particle motion and/or particle concentration. In these experiments, 12 × 100 mm test tubes were filled with 4.0 ml of E suspensions at various concentrations. 9 × 21 mm cover slips containing about 2×10^4 phagocytes were added and the test tubes were sealed. Continuous agitation was provided by inclining the test tubes at about 20° on an oscillating platform moving at about 60 strokes per minute. The entire apparatus was placed in a 37°C incubator for 30 min. Quantitation of ingestion was then done as in the overlay situation.

Quantitation of Ingestion. Quantitation of ingestion was accomplished by lysis of external E using techniques that do not destroy leukocytes or ingested E. In experiments with monocytes, the following procedure was used: monolayers were first rinsed in media to remove free E and agglutinating reagents (when used). The cover slips were then immersed in 0.037 M NaCl for 15 s, drained, and immediately dried with a hot air blower. Polymorphs required a more delicate technique. Monolayers were removed from the incubator and cooled on ice. The cover slips were then soaked in an E-lysing solution, as described previously (24) at 4°C for 2–3 min with gentle agitation. The monolayers were then immersed in cold medium containing 10% FCS, drained, and then dried using an air blower without heat.

The dried monolayers were stained with Mac Neal's tetrachrome and examined at 1,000 × magnification under oil. Random fields were examined and cells scored for ingestion. All counts were done "blind," that is, the observers did not know the nature of the preparation being counted. At least 100 cells were examined per slide, but if the observed frequency of ingestion was low, 200–500 cells per slide were examined. All experiments were done at least in duplicate, and the results are the average of the individual preparations. Results are expressed in two indices of phagocytosis. One index is the percentage of cells which show ingestion at all, i.e., which have ingested one or more E. The second index is the percentage that have ingested three or more E. With these two indices, we give information both on the frequency of phagocytosis among the cell population and the frequency of phagocytes which show a "large" amount of ingestion. In terms of these indices, variations between duplicates were ±10% of the reported value.

Results

Role of C3b and C3d Receptors in Phagocytosis. Phagocytosis by human monocytes and human PMN were both examined under two different protocols, the "overlay" and "suspension" techniques described in the Materials and Methods section. As shown below, both protocols demonstrate a marked synergy between C3 and Fc receptors in the phagocytic process.

In the overlay technique, a large excess of particles is allowed to settle down on the phagocyte monolayer, providing a saturating particle density and absence of shear forces between particle and phagocyte. Fig. 1*a* compares the ingestion by PMN of E, sensitized either with IgG alone (EIgG) or with IgG and C3b (EIgMC3b·IgG) in an experiment performed in the "overlay" fashion. Several points are evident: (*a*) without IgG neither E nor EIgMC3b are ingested; (*b*) without C3b, neutrophils require large amounts of IgG bound to the particle to stimulate phagocytosis. 6,000 molecules of IgG per E induces virtually no ingestion, and even with 60,000 molecules of IgG per E, only 70% of PMN ingested; (*c*) C3 and IgG act synergistically in opsonization; with C3b also bound to the E, ingestion occurs with less than 1/10 the amount required with IgG

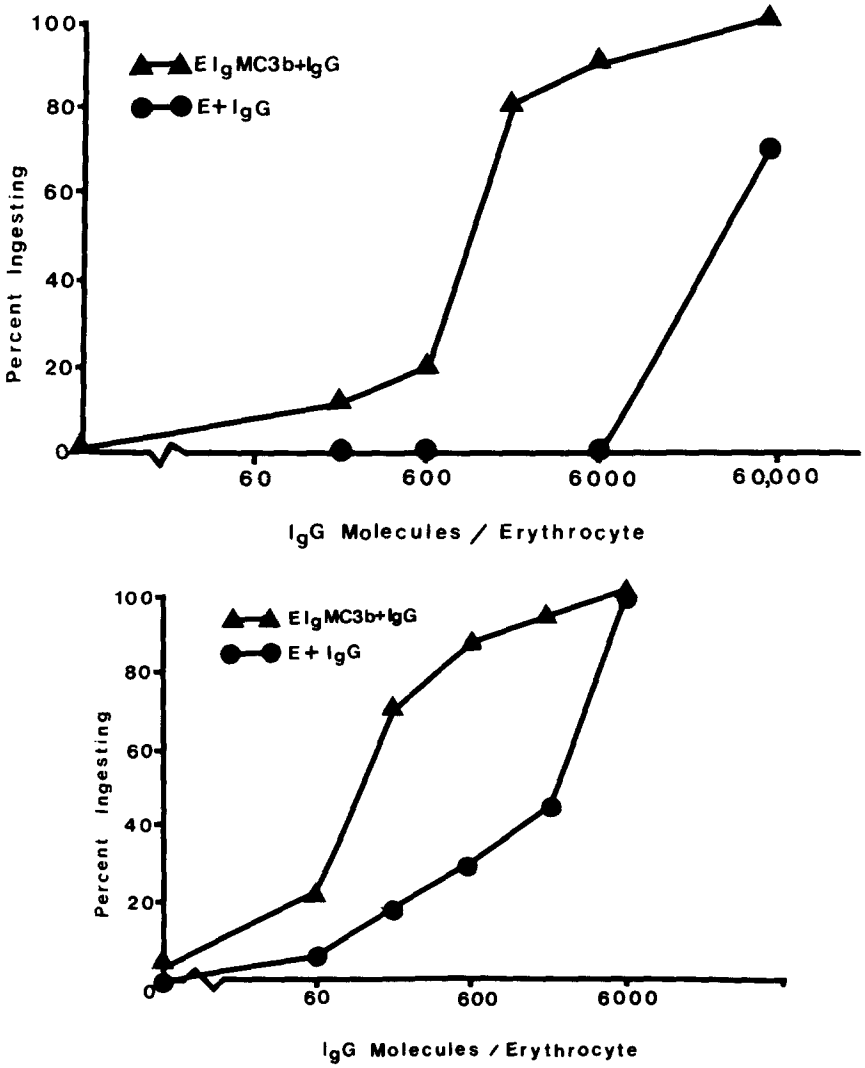

FIG. 1. (*a*, upper) Ingestion of EIgG and EIgMC3b·IgG by human PMN. E were sensitized with IgG alone (E + IgG) or sensitized with C3b and IgG (EIgMC3b + IgG). Assays for phagocytosis were performed with the overlay technique. The figure shows the percentage of phagocytes ingesting E vs. the average number of IgG molecules bound per E. (*b*, lower) Ingestion of EIgG and EIgMC3b·IgG by human monocytes. These experiments were also carried out with the overlay technique.

alone. In other experiments using labeled C3, it was demonstrated that E coated with 1,000 molecules of C3b and 2,000 molecules of IgG were ingested more avidly by PMN than E coated with 60,000 molecules of IgG without C3b.

The opsonic effect of C3 was dependent on its presence on the E in sufficient quantities to induce rosette formation. Greater amounts showed little, if any, further enhancement. With PMN, cleavage of C3b to C3d abolished rosette formation, and at the same time the opsonic effect was lost.

Similar results were obtained with human monocytes (Fig. 1*b*). IgG was from 10 to 30 times more effective in inducing phagocytosis of E sensitized simultane-

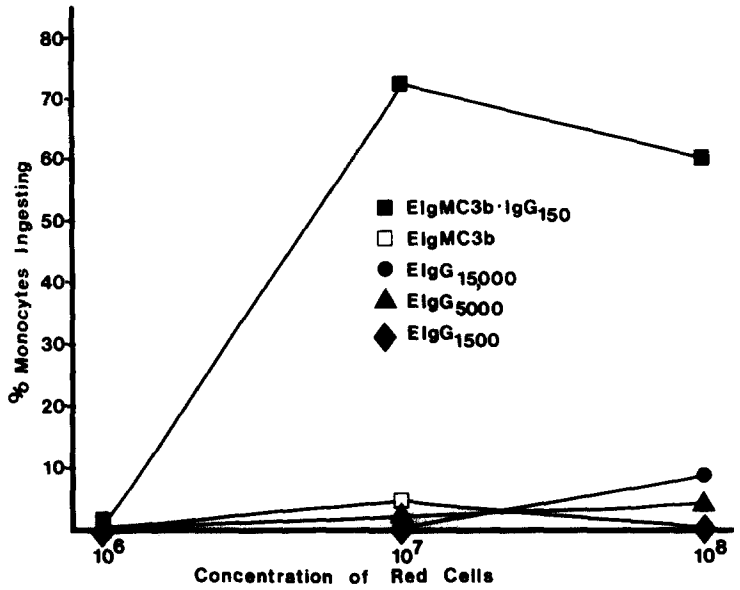

FIG. 2. Phagocytosis of EIgG and EIgMC3b·IgG in "suspension." Human monocytes were exposed to dilute suspensions of E opsonized with IgG, C3b, or IgG and C3b. The figure shows the percentage of monocytes ingesting E vs. the E concentration. Note that E with 15,000 molecules of IgG were not ingested in suspension, and that under these conditions opsonization with C3 was a necessary, but not sufficient condition for phagocytosis. The subscripts represent the average number of molecules of IgG bound per E.

ously with C3b. However, one consistent difference between PMN and monocytes was observed. While PMN did not ingest E sensitized with 6,000 molecules of IgG per E, monocytes phagocytosed E coated with less than 600 antibody molecules. In various experiments, as few as 100 molecules of IgG, in the presence of C3b, stimulated ingestion in 15–45% of monocytes. Even without C3, some ingestion of E sensitized with 100 molecules of IgG was observed. In contrast, up to 10,000 molecules of C3b, without IgG, did not induce ingestion.

In "suspension" experiments, monolayers of phagocytes were exposed to dilute suspensions of E which were kept in constant motion. Under these conditions, the effects of particle concentration and shearing forces between particle and phagocytes may be observed, and the opsonic effect of C3b is even more remarkable. For example, in suspension, EIgG sensitized with as many as 15,000 molecules of IgG did not form rosettes and were not phagocytosed by monocytes, even at relatively high particle concentrations (10^8/ml). In contrast, E sensitized with C3b and 150 molecules of IgG were taken up very effectively at a concentration of 10^7/ml (Fig. 2).

Next, we studied the opsonic effect of C3d. As stated earlier, cleavage of membrane-bound C3b to C3d by C3b inactivator simultaneously removes the ability of the E to bind to PMN and the synergistic interaction between C3b with IgG in phagocytosis. However, the situation is quite distinct in the case of monocytes, which have membrane receptors for C3d. As shown in Fig. 3, the uptake by monocytes of particles sensitized with IgG is strikingly enhanced in the presence of C3d. While EIgMC3d were not ingested by monocytes,

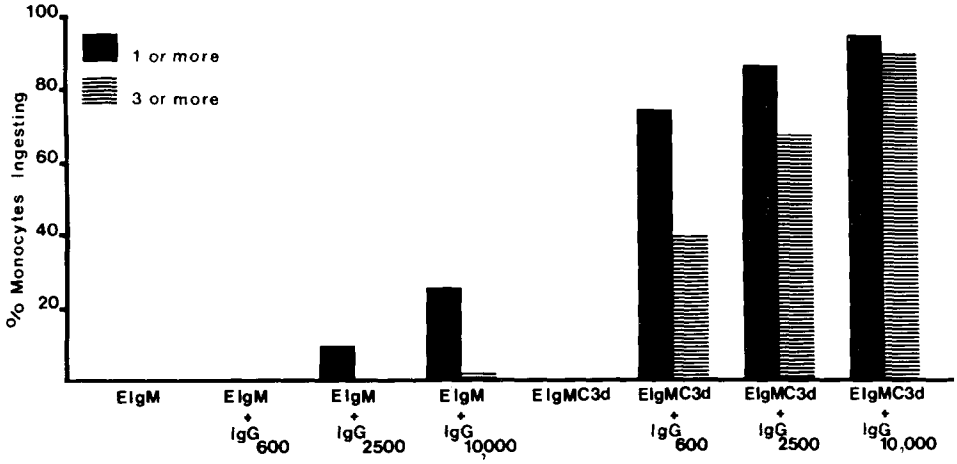

FIG. 3. Opsonic effect of C3d. These experiments were carried out in suspension with glass-adherent human monocytes. Radiolabeled C3 was used to prepare EIgMC3d. EIgM or EIgMC3d bearing 500 molecules of C3d were sensitized with different amounts of IgG anti-E. Erythrocytes were suspended at 10^8/ml. Two indices of phagocytosis are presented; that is, the percentage of monocytes which ingested one or more E, and the percentage which ingested a large number of E (three or more). Note that ingestion of E opsonized with 500 molecules of C3d and 600 molecules of IgG is far greater than ingestion of E opsonized with 10,000 molecules of IgG.

EIgMC3d·IgG coated with 500 molecules of C3d and 600 of IgG were ingested more effectively than E opsonized with 10,000 molecules of IgG.

Role of Contact-Inducing Agents in Phagocytosis. Since in all cases the opsonic effect of C3 was correlated with the induction of binding between particle and phagocyte, we asked whether enhancement of contact alone could explain the opsonic activity of C3.

In order to answer the question, we examined the effect on phagocytosis of a number of nonimmunologic agents which also enhance binding of the E to the phagocyte. These experiments were carried out in the "overlay" situation. The first contact-inducing agent used was high molecular weight dextran, a polymer that is known to agglutinate cells nonspecifically. The effect of dextran as an "opsonin" with human PMN is shown in Fig. 4. It is clear that dextran, by itself, does not induce ingestion of E or EIgMC3b. However, the presence of dextran with IgG-coated E causes a very marked increase in phagocytosis. In terms of the number of particles ingested per phagocyte, addition of dextran to the medium caused an increase of over 5,000% in the ingestion of $EIgG_{10,000}$. This synergistic interaction with IgG disappears when the particle also has C3b bound to its surface. Ingestion of EIgMC3b·IgG is not enhanced at all by the presence of dextran. The same effects were observed in overlay experiments with human monocytes (data not shown). Dextran enhanced ingestion most markedly in concentrations of 2–6%, the same concentrations in which agglutination of E by dextran was maximal. Dextran preparations of lower molecular weight (dextran 10), which do not effectively enhance agglutination, were much less effective at increasing ingestion.

The effect of dextran 110 in enhancing contact between EIgG and phagocytes

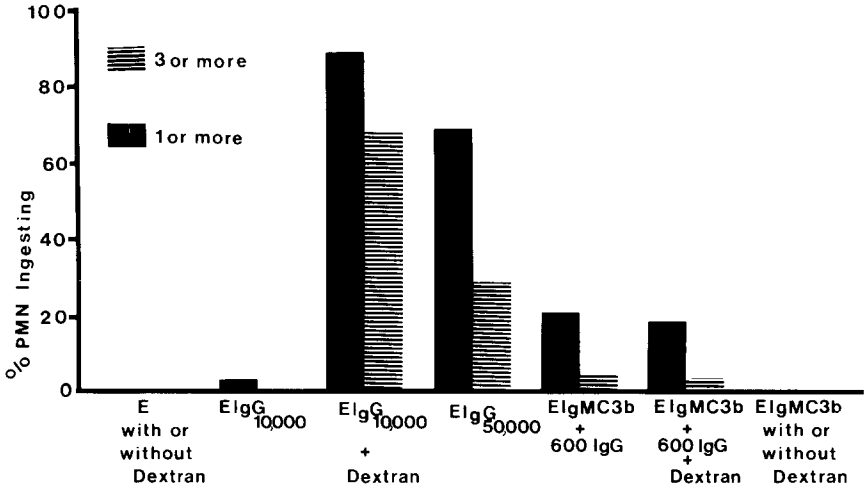

FIG. 4. Dextran 110 as an opsonin for human PMN. Experiments were carried out in overlay fashion. E were suspended at 3×10^8/ml. 0.1-ml aliquots were mixed with 0.1 ml of medium alone or medium containing 8% wt/vol dextran 110 and immediately added to cover slips containing PMN. After incubation for 30–45 min phagocytosis was assayed. Note that dextran, while not opsonic to E, greatly increases ingestion of IgG-coated cells. Furthermore, this effect is not seen when C3b is present.

could be directly demonstrated. Human monocytes were incubated with $EIgG_{400}$ in the presence or absence of dextran 110 under overlay conditions. Without dextran, no rosettes were formed (about 4,000 molecules of IgG per E were required to form Fc-mediated rosettes under these conditions). In the presence of 4% dextran 110, 50% of monocytes formed rosettes with $EIgG_{400}$ but not with E alone.

The same effect that dextran provided could also be obtained by simply centrifuging the E and phagocytes together. In these experiments, monolayers of phagocytes on 12-mm round cover slips were placed in the bottom of 18-mm wells in tissue culture chambers. 10^9 E were added in 1 ml of medium and the chambers sealed. The entire plate was centrifuged at 37°C for 20 min and then incubated for 20 min more at 37°C without centrifugation. Controls were incubated for 50 min at 37°C (the additional 10 min were to compensate for faster E settling under centrifugation). The preparations were processed as previously described. As shown in Fig. 5, centrifugation does not induce phagocytosis of unsensitized E or EIgMC3b, but enhances the sensitivity to IgG opsonization by nearly 10-fold. Also, as expected, centrifugation did not enhance ingestion of EIgMC3b·IgG. Centrifugation also showed the same effect as dextran in the formation of rosettes between EIgG and phagocytes: only 1/10 as many IgG molecules on the E were required for rosette formation when centrifugation was used, as compared to the overlay situation.

Enhancement of phagocytosis was also observed by treating E with neuraminidase, a procedure that is known to decrease the surface charge of cells through removal of sialic acid (25). This treatment, which is known to enhance the agglutinability of E (26) and the formation of rosettes between human T cells and E (23) also increased ingestion of EIgG without being directly opsonic. The

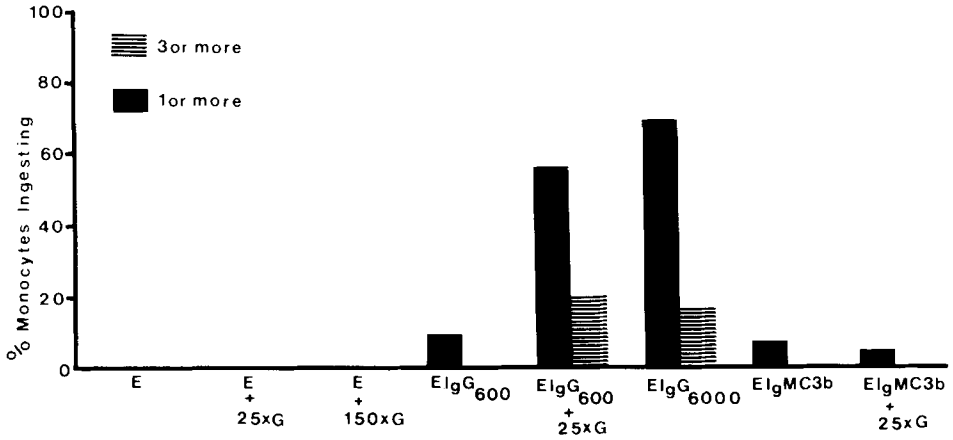

FIG. 5. Centrifugation as an opsonin. In these experiments, monocytes were exposed to saturating concentrations of E at various *g* forces, as described in the text. Note that centrifugation, like C3 opsonization and dextran, does not induce interiorization of E, but greatly enhances the effect of IgG. Centrifugation has no effect on ingestion of cells opsonized with C3b.

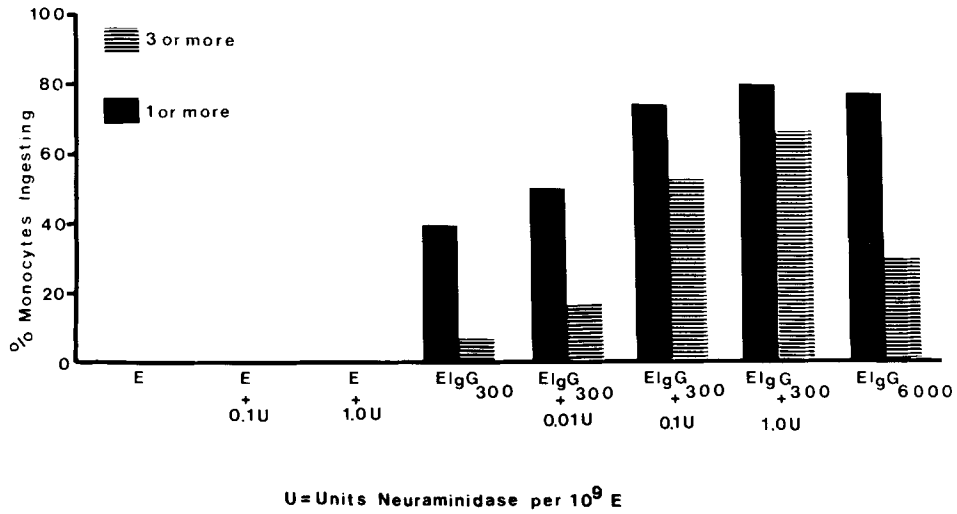

FIG. 6. Neuraminidase as an opsonin. These experiments were carried out in overlay fashion with human monocytes. E were incubated by Hanks' balanced salt solution (pH 6.5) alone or with various amounts of neuraminidase. After washing, they were sensitized with various amounts of IgG. E treated with the enzyme were not ingested. However, enzyme treatment greatly enhanced the effect of IgG anti-E.

results shown in Fig. 6 make clear that while large amounts of neuraminidase (1 U/10^9 E) did not induce ingestion of E, small amounts (0.01 U/10^9 E) enhanced ingestion of EIgG.

Protamine, a polycation with agglutinating properties, also had a similar effect (Fig. 7). That is, it enhanced ingestion of EIgG without inducing phagocytosis of unopsonized E. Heparin, a polyanion without agglutinating properties, did not stimulate ingestion of EIgG. In fact, a slight depression of EIgG inges-

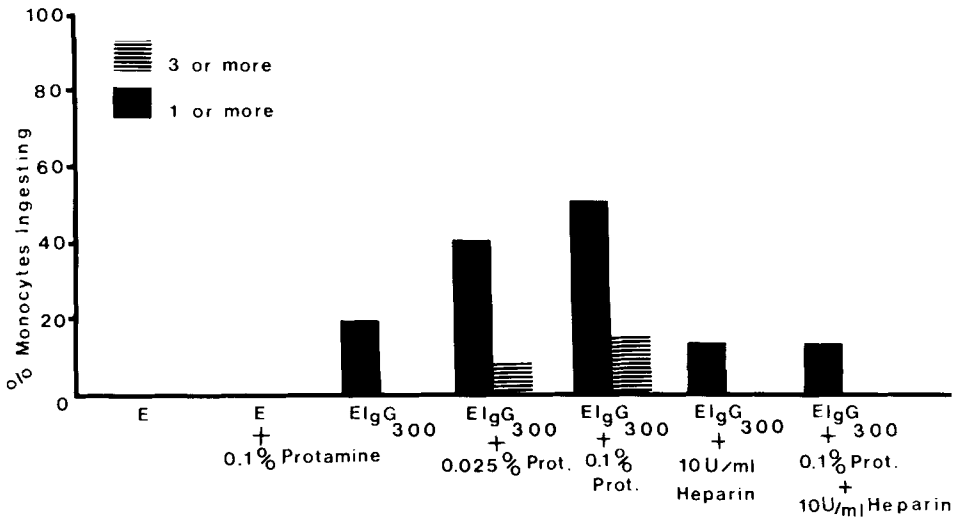

FIG. 7. Effect of a polycation (protamine) and a polyanion (heparin) on phagocytosis by human monocytes. E were suspended at 3×10^8/ml and 0.1-ml aliquots mixed with medium alone or medium containing the polyion. Phagocytosis was then assayed in the overlay fashion. The final concentrations of the polyions are shown in the figure. Again, as in the case of treatments shown in previous figures, protamine enhances phagocytosis of EIgG but not of E alone. Heparin neutralizes the effect of protamine.

tion was noted. However, 10 U/ml of heparin, added to 0.1% protamine, abolished the agglutinating property of protamine for E, and returned phagocytosis of EIgG to control levels.

Discussion

The data show that while C3b receptors on monocytes and PMN, and C3d receptors (on monocytes only) do not induce ingestion directly, they interact synergistically with IgG in the internalization process. This effect was highlighted in experiments with particles in suspension. In this protocol, monolayers of fixed phagocytes are exposed to dilute suspensions of opsonized particles kept in motion by continuous agitation. This many be analogous to the "in vivo" situation, where fixed phagocytes (in liver, spleen, and lymph node, for example) are exposed to circulating, opsonized particles. Under these conditions, opsonization with IgG alone was remarkably inefficient; even with monocytes, 15,000 molecules of IgG per E proved ineffective at inducing ingestion. While E opsonized with C3 (but not IgG) were not ingested, addition of C3 to the particles markedly enhanced ingestion if IgG was present. For example, E opsonized with 500 molecules of C3d and 600 molecules of IgG were ingested in much greater amounts by monocytes than E opsonized with 10,000 molecules of IgG without C3. The same effect was seen with C3b. Opsonization with C3 can thus be a necessary condition for ingestion to occur, even if it is not a sufficient condition.

In experiments with particles in suspension, much of the enhancement of phagocytosis can be explained by the binding of particle to phagocyte mediated by C3 receptors. This binding overcomes the shearing forces present between particle and phagocyte which prevent ingestion. Furthermore, the formation of "rosettes" on the surface of the phagocyte represents an enormous increase in

the particle concentration "seen" by the phagocyte. The local concentration of E in a rosette is about 2×10^{10}/ml. If the concentration of E in suspension is 10^7/ml, the formation of rosettes represents an increase of 2,000-fold in the concentration of particles on the phagocyte surface.

Overcoming shearing forces is probably not the only role of C3 receptors in phagocytosis, since the synergy is also apparent in the overlay experiments, in which fixed phagocytes are saturated with particles not subject to agitation. Even under these circumstances, IgG without C3 is a poor opsonin, particularly for PMN. E opsonized with up to 10,000 molecules of IgG are ignored by PMN under these conditions. Again, while the presence of C3b, without IgG, does not induce ingestion, E coated with 1,000 molecules of C3b, and 2,000 molecules of IgG are ingested more avidly than E coated with 60,000 molecules of IgG.

The experiments with dextran, centrifugation, neuraminidase, and protamine strongly suggest that this effect can be explained by the enhanced cell to cell contact mediated by C3-C3 receptor interaction. These contact-inducing agents mimic the effect of C3, in that they markedly (by 1,000% or more) enhance the effect of particle-bound IgG without inducing ingestion directly. Furthermore, their effect vanishes when the particle is already bound to the phagocyte by C receptors. The experiments with centrifugation show that the effect does not seem to depend on alteration of the chemical properties of the surface of either the E or phagocyte, nor on the presence of a physical structure linking the two cells, but rather depends on approximating the two cell surfaces together. The experiments with neuraminidase further exclude any stimulating effect on the phagocyte itself, since in these experiments, the E were pretreated, washed, and then exposed to the monocytes without the neuraminidase being present.

One may ask why there is a need for enhanced contact in the recognition of particle-bound IgG by phagocytes even when the phagocyte is saturated by these particles. The experiments with neuraminidase and polycations suggest an explanation. Both these agents reduce the negative charge present on the cell surface. Most cells and particles have an intrinsic negative charge, and can be expected to repel one another over short distances. Calculations have shown that in physiological saline, the electrostatic field surrounding a particle or cell decays in an exponential fashion, but remains significant for perhaps 20–30 Å (27). Overcoming two such barriers may represent a considerable obstacle to the recognition of an antibody molecule (100 Å), or other surface moieties, by phagocytic cells. While C3 receptors may serve to overcome this electrostatic repulsion, the effect of C3 itself is clearly not mediated by a reduction of particle surface charge, since electrophoretic studies have shown that cells become more negatively charged when C3 is added (28).

Our experiments demonstrate that both C3 and contact-inducing agents can appear to induce ingestion directly when ingestion without them is negligible or nonexistent. Phagocytosis in these cases may actually be triggered by IgG or other moieties on the particle surface which are only effective when contact is established.[2] Claims that binding of C3 to a particle triggers the phagocytic act

[2] The important role of contact in phagocytosis was clearly demonstrated in the work of Wood and associates (29). They showed that many bacteria are intrinsically ingestible, in the absence of antibody, but that recognition and phagocytosis only occurred when the phagocyte trapped the bacteria against a surface.

directly require the demonstration that the particle is not intrinsically ingestible; that is, that interiorization cannot be achieved by simple approximation of particle and phagocyte by physical means.

Some of the observations which can be re-interpreted in the light of the present findings are as follows: (*a*) Gigli and Nelson (30) studied the ingestion of E coated with antibodies and purified guinea pig C components by guinea pig neutrophils. In this classical paper, the role of C3 as an opsonin was established. They also postulated that C3b directly stimulated particle interiorization. However, in their experiments both C3b and IgG[3] were present on the E. It is probable that ingestion was actually triggered by IgG, present in insufficient amounts to mediate effective phagocytosis unless C3b was also present to induce particle binding.

(*b*) Stossel (11) studied the ingestion of various lipid emulsions by human PMN and other phagocytes. Based on a mathematical analysis which treated phagocytosis as an enzyme-substrate reaction, they concluded that particle-bound C3 directly mediates phagocytosis. However, there are several shortcomings to this mathematical analysis (8), and furthermore, the particles used in these studies were intrinsically ingestible in the absence of C3. Therefore, C3 might have enhanced ingestion by simply approximating the particle to the phagocyte. It should be stressed that these experiments were carried out under suspension conditions, where the synergistic effect of C3 is crucial for efficient phagocytosis.

In addition, Stossel et al. (12, 31) as well as Gigli and Nelson (30), showed that after treatment of C3-opsonized particles with C3b inactivator, phagocytosis by PMN was very reduced. This treatment removes from the particle a large fragment of C3b, namely C3c. A smaller peptide, C3d, remains. It was concluded (31) that C3c is the opsonin moiety of C3 and that C3d has no role in phagocytosis. It is clear from our observations that two separate fragments of the C3 molecule are involved in immune phagocytosis C3b and C3d. Since both function as opsonins if the phagocyte has the appropriate receptors, the conclusion of Stossel et al. is valid only in the case of PMN, which lack C3d receptors (16, 17).

(*c*) It has been reported that activated mouse macrophages ingest EIgMC3b, and suggested that this occurs via an alteration in the function of the C receptor (32). However, in a succeeding paper[4] we show that EIgM are ingested by activated macrophages in amounts comparable to EIgMC3b if contact between E and phagocyte is enhanced by dextran or centrifugation. In other words, it is possible that for activated macrophages, IgM provides an appropriate ligand for ingestion.

It has recently been proposed that phagocytosis results from a sequential interaction of ligands and receptors around the surface of the particle, the "zipper" theory (33). If one assumes that all receptors function in phagocytosis in the same way, it is difficult to explain why 10^4 C3b moieties on a particle cannot induce ingestion when as few as 10^2 IgG molecules can. It may be that the

[3] Although in the paper it is mentioned that a hyperimmune rabbit antiserum to E was used, most experiments were performed with purified IgG (I. Gigli, personal communication).

[4] Ehlenberger, A. G., and V. Nussenzweig. Manuscript in preparation.

distribution of C3b moieties and C3 receptors does not permit sequential interaction of ligands and receptors around the entire particle. Another explanation is that local changes in the membrane and/or subjacent cytoplasm are required for phagocytosis and that interaction with Fc receptors can initiate these changes, while binding to C3 receptors cannot. In other words, the zippering mechanism may require triggering ligands, like particle-bound IgG, to stimulate the active features of the process. Contact ligands, like C3, may enhance recognition of triggering moieties and also provide binding ligands as the particle is zippered into the interior of the phagocyte.

Summary

In this paper we re-examine the roles of particle-bound IgG and C3 in phagocytosis of sheep erythrocytes (E) by monolayers of purified human monocytes and polymorphonuclear leukocytes (PMN). We conclude that two fragments of the C3 molecule, that is, C3b and C3d, can function as opsonins if the phagocyte has the appropriate membrane receptors. Monocytes, that bind both C3b and C3d, respond to both as opsonins. PMN, which do not bind C3d, respond only to particles opsonized with C3b.

C3 and IgG have separate roles in phagocytosis. IgG, through its Fc fragment, directly stimulates particle ingestion, but is relatively inefficient at inducing particle binding. On the other hand, C3 primarily mediates the binding of the particle via complement receptors. A marked synergy exists between C3 and IgG in inducing phagocytosis. Thus, opsonization of the particle with C3 can be a necessary condition for particle ingestion, although by itself C3 does not trigger phagocytosis.

The opsonic effect of C3 can be mimicked by a variety of nonimmunologic agents which enhance binding of the particle to the phagocyte without directly stimulating ingestion. The contact-inducing agents used include centrifugation of particle and phagocyte, high molecular weight dextran, protamine, and treatment of E with neuraminidase. These results strongly suggest that the role of C3 in opsonization is mainly or exclusively one of establishing contact between particle and phagocyte.

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References

1. Stossel, T. P. 1975. Phagocytosis, recognition and ingestion. *Semin. Hematol.* 12:83.
2. Ehlenberger, A. G., and V. Nussenzweig. 1976. Immunologically-mediated phagocytosis: role of C3 and Fc receptors. *In* Clinical Evaluation of Immune Functions in Man. G. Siskind and G. S. Litwin, editors. Grune & Stratton, Inc., New York. In press.
3. Rabinovitch, M. 1970. Phagocytic recognition. *In* Mononuclear Phagocytes. R. van Furth, editor. Blackwell Scientific Publications Ltd., Oxford, Great Britain. 299.
4. Alper, C. A., H. R. Colten, F. S. Rosen, A. R. Rabson, G. M. Macnab and J. S. S. Gear. 1972. Homozygous deficiency of C3 in a patient with repeated infections. *Lancet.* 2:1179.

5. Rosen, F. S. 1975. Immunodeficiency. *In* Immunogenetics and Immunodeficiency. B. Benacerraf, editor. University Park Press, Baltimore, Md. 229.
6. Mantovani, B., M. Rabinovitch and V. Nussenzweig. 1972. Phagocytosis of immune complexes by macrophages. *J. Exp. Med.* **135**:780.
7. Mantovani, B. 1975. Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes. *J. Immunol.* **115**:15.
8. Scribner, D. J., and D. Fahrney. 1976. Neutrophil receptors for IgG and complement: their roles in the attachment and ingestion phases of phagocytosis. *J. Immunol.* **116**:892.
9. Ross, G. D., and M. J. Polley. 1974. Human lymphocyte and granulocyte receptors for the fourth component of complement (C4) and the role of granulocyte receptors in phagocytosis. *Fed. Proc.* **33**:759. (Abstr.)
10. Ehlenberger, A. G., and V. Nussenzweig. 1975. Synergy between receptors for Fc and C3 in the induction of phagocytosis by human monocytes and neutrophils. *Fed. Proc.* **34**:854. (Abstr.)
11. Stossel, T. P. 1973. Quantitative studies of phagocytosis: kinetic effects of cations and of heat labile opsonin. *J. Cell Biol.* **58**:346.
12. Stossel, T. P., R. J. Field, J. D. Gitlin, C. A. Alper, and F. S. Rosen. 1975. The opsonic fragment of the third component of human complement (C3). *J. Exp. Med.* **141**:1329.
13. Huber, H., M. J. Polley, W. D. Linscott, H. H. Fudenberg, and H. J. Müller-Eberhard. 1968. Human monocytes: distinct receptors for the third component of complement and for immunoglobulin G. *Science (Wash. D. C.).* **162**:1281.
14. Wellek, B., H. Hahn, and W. Opferkuch. 1975. Quantitative contributions of IgG, IgM and C3 to erythrophagocytosis and rosette formation by peritoneal macrophages, and anti-opsonin activity of dextran sulfate-500. *Eur. J. Immunol.* **5**:378.
15. Reynolds, H. Y., J. P. Atkinson, H. H. Neuball, and M. M. Frank. 1975. Receptors for immunoglobulin and complement on human alveolar macrophages. *J. Immunol.* **114**:1813.
16. Ross, G. D., M. J. Polley, E. M. Rabellino, and H. M. Grey. 1973. Two different complement receptors in human lymphocytes. One specific for C3b and one specific for C3b inactivator cleaved C3b. *J. Exp. Med.* **138**:798.
17. Eden, A., G. W. Miller, and V. Nussenzweig. 1973. Human lymphocytes bear membrane receptors for C3b and C3d. *J. Clin. Invest.* **52**:3239.
18. Koller, C. A., G. W. King. P. E. Hurtubise, A. L. Sagone, and A. F. LoBuglio. 1973. Characterization of glass-adherent human mononuclear cells. *J. Immunol.* **102**:1172.
19. Newsome, J. 1967. Phagocytosis by human neutrophils. *Nature (Lond.).* **214**:1092.
20. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestion products. *Methods Med. Res.* **10**:134.
21. 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.
22. McConahey, P., and F. J. Dixon. 1966. A method for trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.
23. Weiner, M. S., C. Bianco, and V. Nussenzweig. 1973. Enhanced binding of neuraminidase treated sheep erythrocytes to human T lymphocytes. *Blood.* **42**:939.
24. Ehlenberger, A. G., and V. Nussenzweig. 1976. Identification of cells with complement receptors. *In* In Vitro Methods of Cell Mediated Immunity. B. Bloom and J. David, editors. Academic Press, Inc., New York. 2nd edition. In press.
25. Seamen, G. V. F., and G. Uhlenbruck. 1963. The surface structure of erythrocytes from some animal sources. *Arch. Biochem. Biophys.* **100**:493.
26. Uhlenbruck, G., G. V. F. Seamen, and R. A. Coombs. 1967. Factors influencing the

agglutinability of red cells. III. Physicochemical studies on ox red cells of different classes of agglutinability. *Vox Sang.* **12**:240.

27. Eagland, D. 1975. The influence of hydration on the stability of hydrophobic colloidal systems. *In* Water, A comprehensive Treatise. F. Franks, editor. Plenum Press, New York. **5**:1.
28. Okada, H., K. Kajima, T. O. Yoshida, and K. Nishioka. 1972. Electrokinetic behavior of intermediate cells in immune hemolysis. *J. Immunol.* **108**:59.
29. Wood, W. B., Jr., M. R. Smith, and B. Watson. 1946. Studies on the mechanism of recovery in pneumococcal pneumonia. IV. The mechanism of phagocytosis in the absence of antibody. *J. Exp. Med.* **84**:387.
30. Gigli, I., and R. A. Nelson, Jr. 1968. Complement dependent immune phagocytosis. I. Requirement of C'1, C'4, C'2, C'3. *Exp. Cell Res.* **51**:45.
31. 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.)
32. Bianco, C., F. M. Griffin, and S. C. Silverstein. 1975. Studies of the macrophage complement receptor: alteration of receptor function upon macrophage activation. *J. Exp. Med.* **141**:1278.
33. Griffin, F. M., Jr., J. A. Griffin, J. E. Leider, and S. C. Silverstein. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med.* **142**:1263.