CHARACTERIZATION OF THE MACROPHAGE RECEPTOR FOR COMPLEMENT AND DEMONSTRATION OF ITS FUNCTIONAL INDEPENDENCE FROM THE RECEPTOR FOR THE Fc PORTION OF IMMUNOGLOBULIN G*

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The plasma membranes of mononuclear phagocytes contain externally disposed receptors for the Fc portion of immunoglobulin G(IgG) and for the third component of complement (C3). Although neither of these receptors has been isolated, some data are available concerning their physical and functional properties. The Fc receptors of mouse peritoneal macrophages are resistant to digestion by trypsin (1), do not require divalent cations for binding IgG-coated particles (1,2), and mediate both binding and ingestion of these particles (3,4). In contrast, the receptors on the macrophage plasma membrane for the third component of complement are destroyed by tryptic proteolysis (1), require Mg^{++} ions for binding C3-coated particles (1), and, except under special circumstances (5,6), mediate binding, but not ingestion, of C3-coated particles to the cell's plasma membrane (3,5,6). These findings suggest that the Fc receptors and complement receptors of mouse peritoneal macrophages are chemically and physically distinct entities.

In this paper, we identify C3b as the form of C3 responsible for binding complement-coated erythrocytes to macrophage complement receptors and demonstrate the functional independence of these receptors from receptors for the Fc portion of IgG and from the membrane factors which mediate binding and ingestion of latex particles.

Materials and Methods

Macrophages. The methods for harvesting and maintaining mouse peritoneal macrophages were those of Cohn and Benson (7), as previously modified (8). Cells were cultivated for 24 h in medium 199 (Microbiological Associates, Bethesda, Md.) with 20% heat-decomplemented (56°C, 30 min) fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. In some experiments, mice were injected intraperitoneally with 1 ml of Brewer thioglycollate medium 4 days before their peritoneal macrophages were harvested. Macrophages from these animals are designated activated macrophages.

Lymphocytes. Spleens were obtained from mice and teased apart with forceps. The spleen cells were washed twice in medium 199 and resuspended in medium 199 at a concentration of 2×10^6

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cells/ml. More than 95% of the cells were lymphocytes as judged by morphology and exclusion of latex beads. Lymphocytes were not separated from the few contaminating cells.

Erythrocytes. Sheep erythrocytes (E)[1] in Alsever's solution (Animal Blood Center, Syracuse, N.Y.) were washed three times in PD [solution "a" of Dulbecco's PBS (9)] and suspended in medium 199 or Veronal-buffered glucose containing Ca^{++}, Mg^{++}, and gelatin (VBG) (10).

Complement Source. Serum was prepared from C5-deficient AKR mice and stored immediately in 0.2-ml aliquots at −70°C. It served as the source of C1423. Latex beads, pneumococci, and antipneumococcal antiserum were obtained and used as previously described (8).

Rabbit antisheep E IgM (lot no. 30704), at a concentration of 350 μg/ml, was obtained from Cordis Laboratories, Miami, Fla. Its hemagglutination titer for E, determined as previously described (8), was 1:320.

Preparation of Antibody-Coated E. 1 ml of 5% (vol/vol) E in medium 199 was incubated with 13 μg of rabbit antisheep E IgM for 15 min at 37°C. The suspension was centrifuged for 5 min at 750 g, and the pelleted E were suspended in medium 199 at a concentration of 0.5%. This preparation (0.5% E coated with anti-E IgM) is designated E(IgM).

Preparation of Complement-Coated E(IgM). Freshly thawed AKR mouse serum was diluted 1:10 in VBG and mixed with an equal volume of a suspension of 5% (vol/vol)E(IgM) in VBG. The mixture was incubated for 10 min at 37°C and centrifuged for 5 min at 750 *g*. The pelleted E were suspended in medium 199 at a concentration of 0.5%. This preparation [0.5% complement-coated E(IgM)] is designated E(IgM)C3b.

One volume of 5% E(IgM)C3b in PD (Ca^{++}- and Mg^{++}-free) was mixed with an equal volume of freshly thawed AKR mouse serum 50% in PD containing 0.01 M EDTA. The suspension was incubated for 30 min at 37°C. Under these conditions, C3b inactivator in mouse serum cleaves erythrocyte-bound C3b to C3c and C3d (11), but activation of the complement cascade cannot occur because of the absence of divalent cations. The mixture was centrifuged for 5 min at 750 *g*, and the pelleted E were suspended in medium 199 at a concentration of 0.5%. This preparation [E(IgM)C3b treated with EDTA-treated AKR mouse serum] is designated E(IgM)C3d. E(IgM)C3d were also prepared by incubating E(IgM)C3b with heat-decomplemented (56°C, 30 min) mouse serum for 30 min at 37°C.

Some E(IgM)C3b were incubated with EDTA-treated AKR mouse serum in the presence of 10 mg/ml of Suramin (IBA Pharmaceuticals, New York, N.Y.), which prevents the cleavage of C3b by C3b inactivator. VBG, E(IgM), E(IgM)C3b, and E(IgM)C3d were prepared fresh on the day of an experiment.

Assay of Erythrocyte Rosetting of Lymphocytes. 0.5 ml of a lymphocyte suspension containing 2×10^6 cells/ml was mixed with 0.5 ml of a 0.5% suspension of either E(IgM)C3b or E(IgM)C3d in a test tube, centrifuged for 5 min at 200 *g*, and incubated for 30 min at 37°C. The cells were resuspended by gentle tapping of the tube and rosetting scored by phase-contrast microscopy.

Phase-Contrast and Electron Microscopy. Cover slip cultures of macrophage monolayers were fixed with glutaraldehyde and examined by phase-contrast microscopy as previously described (8). Cells were processed for electron microscopy as described (12,13) and examined in a Siemens Elmiskop 1A (Siemens Corp., Medical/Industrial Groups, Iselin, N.J.).

Miscellaneous. Protein determinations were performed by the method of Lowry et al. (14), using bovine serum albumin as a standard. VBG was prepared as described by Rapp and Borsos (10).

Results

Interaction of Erythrocyte Preparations with Macrophages. Macrophage monolayers on glass cover slips were incubated for 30 min at 37°C with a suspension of E, E(IgM), or E(IgM)C3b. Unattached erythrocytes were removed by washing, and the preparations were fixed and examined by phase-contrast

[1] Abbreviations used in this paper: E, sheep erythrocytes; E(IgM), E coated with anti-E IgM; E(IgM)C3b, E(IgM) coated with the first four complement components; E(IgM)C3d, E(IgM)C3b treated by one of the methods described in Materials and Methods; PBS, phosphate-buffered saline with Ca^{++} and Mg^{++} ions; PD, PBS without Ca^{++} and Mg^{++} ions; VBG, Veronal-buffered glucose with Ca^{++}, Mg^{++}, and 0.1% gelatin.

and electron microscopy. The results are presented in Table I. Neither E nor E(IgM) were bound to or ingested by macrophages. E(IgM)C3b avidly attached to macrophages, in many instances completely covering the cell (Fig. 1). Very few E(IgM)C3b were ingested by the macrophages. That E(IgM)C3b were only rarely ingested was confirmed by electron microscopy.

Effect of the Form of Erythrocyte-bound C3 upon the Interaction of Erythrocytes with Macrophages. Early in the course of the above experiments, we noted that incubating E(IgM) with AKR mouse serum for periods longer than 10–15 min resulted in a complement-coated E(IgM) preparation which bound poorly to the macrophage surface, but which bound well to mouse spleen lymphocytes. Fig. 2 shows the results of studies in which E(IgM) were incubated

TABLE I
*Interaction of Macrophages with Sheep Erythrocytes**

Erythrocyte preparation	Attachment of erythrocytes to macrophages			Phagocytosis of erythrocytes by macrophages		
	%	No. per macrophage	Index‡	%	No. per macrophage	Index§
E	0	0	0	0	0	0
E(IgM)	0	0	0	0	0	0
E(IgM)C	81	15	1,215	4	2	8

* Each value represents the average of three experiments.

‡ Attachment Index is the percentage of macrophages with erythrocytes attached multiplied by the average number of erythrocytes attached per macrophage times 100.

§ Phagocytic Index is the percentage of macrophages with erythrocytes ingested multiplied by the average number of erythrocytes ingested per macrophage times 100.

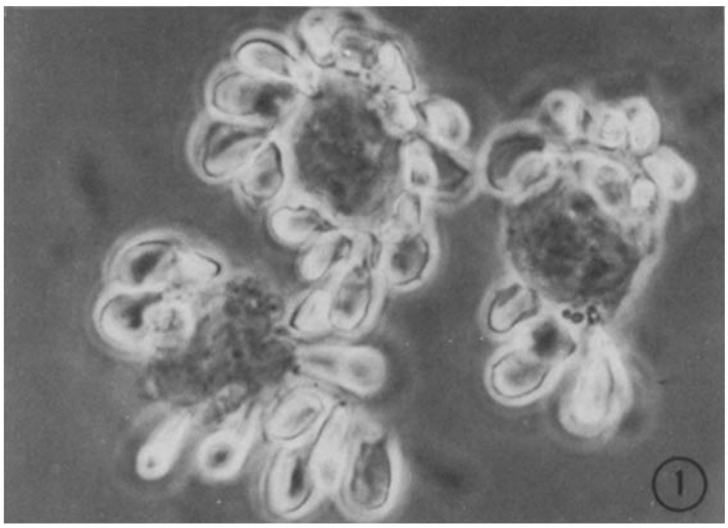

FIG. 1. Interaction of macrophages with E(IgM)C3b. Phase-contrast microscopy. Macrophages were incubated with E(IgM)C3b. Several E(IgM)C3b have bound to the complement receptors of the macrophage plasma membrane. No erythrocytes have been ingested. × 1,000.

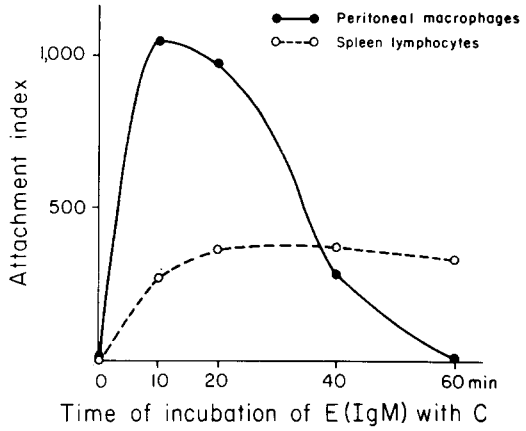

FIG. 2. Effect of the time of incubation of E(IgM) with a complement source (C5-deficient mouse serum) upon the binding of complement-coated erythrocytes to mouse macrophages and to mouse lymphocytes. Attachment index is the percentage of macrophages or lymphocytes binding erythrocytes multiplied by the average number of erythrocytes bound per cell times 100.

with AKR mouse serum at 37°C for periods from 10–60 min, and the resulting complement-coated erythrocytes were then incubated with either macrophages or lymphocytes. IgM-coated erythrocytes incubated with AKR mouse serum for 20 min or longer bound maximally to mouse spleen lymphocytes. IgM-coated erythrocytes incubated with AKR mouse serum for up to 20 min bound avidly to mouse peritoneal macrophages. However, incubation of these E(IgM)C with AKR mouse serum for more than 20 min inhibited their capacity to adhere to mouse macrophages. These findings suggested that, during the longer incubation periods, serum C3b inactivator cleaves C3b to C3c and C3d and that the macrophage complement receptor recognizes only C3b and not C3d. This hypothesis was supported by the results of experiments presented in Table II. Incubation of E(IgM)C3b with either heat-inactivated AKR mouse serum or with fresh AKR mouse serum containing 0.01 M EDTA abolished erythrocyte binding to macrophages. This effect could be partially prevented by including in the incubation mixture Suramin, a drug which inhibits the action of C3b inactivator (15,16).

All complement-coated E(IgM) preparations remained agglutinable by an antimouse C3 antibody even after prolonged incubation in mouse serum and after treatment with heat-inactivated or EDTA-treated mouse serum. Thus, the treatments with mouse serum did not remove C3 from the erythrocyte surface but converted C3b to a form of C3 not recognized by the macrophage complement receptor. Identical results were obtained when activated macrophages were used. These cells bound and ingested E(IgM)C3b (5,6,17), but failed to interact at all with E(IgM)C3d (data not shown). We conclude therefore that the complement receptor of the plasma membrane of the mouse peritoneal macrophage recognizes particle-bound C3b but does not recognize particle-bound C3d.

Effect of Ingestion of Latex Particles or Opsonized Pneumococci upon the Fate of E attached to the Macrophage Plasma Membrane via the C3b Receptor. Al-

TABLE II
*Interaction of Macrophages with Sheep Erythrocytes Coated with C3b or C3d**

Pretreatment of E(IgM)C3b‡ for 30 min at 37°C	Presumed form of C3 on E(IgM)	Attachment of erythrocytes to macrophages		
		%	No. per macrophage	Index§
Veronal-buffered glucose	C3b	97	13.4	1,300
Heat-decomplemented AKR mouse serum	C3d	28	2.1	59
AKR mouse serum + 0.01 M EDTA	C3d	33	2.3	76
AKR mouse serum + 0.01 M EDTA + Suramin, 10 mg/ml	C3b and C3d	87	5.2	452

* Each value represents the average of three experiments.
‡ E(IgM)C3b prepared by incubation of E(IgM) with fresh AKR mouse serum for 10 min at 37°C, as described in Materials and Methods.
§ As in Table I.

though C3b receptors of nonactivated mouse peritoneal macrophages mediate binding but not ingestion of C3b-coated erythrocytes, C3b receptors of thioglycollate-activated macrophages mediate ingestion of E(IgM)C3b (5,6,17). Thus, under some circumstances, the C3 receptor mediates ingestion of C3-coated erythrocytes. These findings prompted us to test whether a phagocytic stimulus initiated by another particle via a different membrane receptor would trigger the ingestion of erythrocytes bound to C3b receptors of nonactivated macrophages. For this purpose, we used latex particles, which are phagocytized via an as yet uncharacterized membrane receptor, and opsonized encapsulated pneumococci, which are ingested via the Fc receptor.

E(IgM)C3b were attached to macrophages; the monolayers were washed to remove unattached erythrocytes and then incubated for 30 min at 37°C with 1–2 $\times$ 10^8 latex particles or opsonized pneumococci. These preparations were then washed, fixed, and examined by phase-contrast microscopy. More than 80% of macrophages had E(IgM)C3b bound to their plasma membranes and had ingested 10–20 latex particles (Table III, line 2) or opsonized pneumococci (Table III, line 3) each. Ingestion of attached E(IgM)C3b was not prompted by phagocytosis of either latex particles or opsonized pneumococci (Fig. 3). That the erythrocytes remained bound to the macrophage surface was confirmed by electron microscopy. Addition of anti-C3 IgG to E(IgM)C3b attached to macrophages promotes ingestion of these erythrocytes[2]; thus, binding via C3b receptors does not constitute an impediment to the ingestion of the bound erythroytes. These results provide additional evidence that the various phagocytic receptors function independently of each other and indicate that a signal initiating ingestion via one type of receptor is not transmitted to other potentially phagocytic receptor sites.

[2] Griffin, F.M., J.A. Griffin, J.E. Leider, and S.C. Silverstein. Manuscript in preparation.

TABLE III

*Fate of Macrophage-Bound E(IgM)C3b During Ingestion of Latex and Pneumococci by Macrophages**

Particle added	Attachment of E(IgM)C3b to macrophages			Phagocytosis of E(IgM)C3b by macrophages			No. of latex particles or opsonized pneumococci ingested per macrophage
	%	No. per macrophage	Index‡	%	No. per macrophage	Index§	
None	81	15	1,215	4	2	8	—
Latex	80	14	1,120	4	2	8	10–20
Opsonized pneumococci	77	15	1,155	2	2	4	10–20

* Each value represents the average of three experiments.
‡ As in Table I.
§ As in Table I.

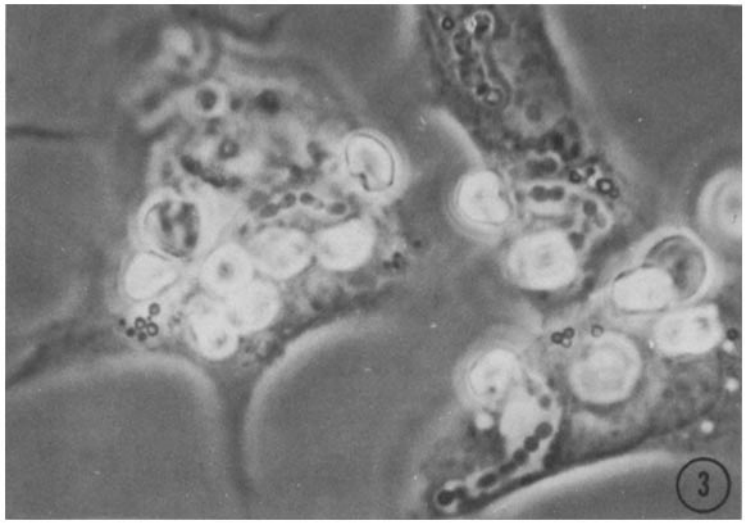

FIG. 3 Failure of test particle ingestion to promote phagocytosis of membrane-bound E(IgM)C3b. Phase-contrast microscopy. E(IgM)C3b were attached to macrophages as in Fig. 1. The macrophages were then incubated with opsonized pneumococci. Pneumococci have been ingested, while erythrocytes remain bound to the complement receptors of the macrophage plasma membrane. Identical results were obtained when latex particles were used as test particles. × 1,000.

Discussion

The complement receptors of guinea pig polymorphonuclear leukocytes (18), human monocytes (19), human polymorphonuclear leukocytes (20), and activated mouse peritoneal macrophages (5,6,17) mediate particle ingestion. It has been reported previously (3,5) that the complement receptor of the mouse peritoneal macrophage mediates binding of E(IgM)C to the cell surface but prompts little, if any, ingestion of E(IgM)C. The data reported in Table I also

show that the complement receptor of mouse macrophages does not mediate ingestion of complement-coated erythrocytes. Thus, the nonactivated mouse peritoneal macrophage differs from many phagocytes in that its complement receptor mediates only binding of particles to the cell's surface.

C3b inactivator (KAF, conglutinogen activating factor) is a heat stable enzyme present in normal serum which cleaves particle-bound C3b into a C3c fragment which is released into the fluid phase and a C3d fragment which remains bound to the particle (reviewed in 11). Many leukocytes bear receptors for C3, and the interaction of antigen-antibody complexes with one or another form of C3 has physiological importance in determining the fate of these complexes. Polymorphonuclear leukocytes have receptors for only C3b (18,21). B lymphocytes, on the other hand, have receptors for both C3b and C3d-bearing erythrocytes (21,22). Little information is available concerning the forms of C3 mediating particle attachment to macrophages. Reynolds et al. (23) reported that rabbit and human alveolar macrophages have receptors for C3b but not for C3d.

We have shown that the complement receptor of the mouse peritoneal macrophage does not recognize all forms of erythrocyte-bound C3. When E(IgM) were incubated with C5-deficient mouse serum for 10 min, the resulting complement-coated E(IgM) bound avidly to both mouse macrophages and mouse lymphocytes. When the time of incubation of E(IgM) with serum was prolonged, however, binding of these particles to macrophages was markedly reduced, while their binding to lymphocytes was unaltered. The component of mouse serum which abolishes the binding of complement-coated E(IgM) to macrophages is heat stable and does not require divalent cations for its action, properties which are identical to those of C3b inactivator of rabbit, guinea pig, and human serum. Suramin, a drug which inhibits the action of C3b inactivator (15,16), partially prevents the serum-mediated inhibition of binding of complement-coated E(IgM) to macrophages. None of these treatments of complement-coated E(IgM) removes C3 from the erythrocyte surface, for the erythrocytes can be agglutinated by an anti-C3 antiserum. All of these results strongly suggest that the mouse macrophage complement receptors recognize particle-bound C3b but do not recognize particle-bound C3d, and in that regard are similar to the complement receptors of human and rabbit alveolar macrophages (23).

In a previous study (8), we bound mouse erythrocytes to mouse peritoneal macrophages using $F(ab')_2$ immunoglobulin links. When these macrophages with erythrocytes bound to their plasma membranes were fed either latex particles or opsonized pneumococci, the latex paritcles and opsonized pneumococci were ingested, but the erythrocytes remained bound to the macrophage plasma membrane. We concluded from these studies that the stimulus to ingest one particle does not trigger the generalized ingestion of other particles attached to the cell's plasma membrane and that the phagocytic stimulus is confined to that segment of the cell's plasma membrane immediately adjacent to the particle being ingested.

Erythrocytes bound to macrophages by the C3b receptor provide better membrane markers than those bound by $F(ab')_2$ immunoglobulin fragments for several reasons. First, the binding is more stable; attached E(IgM)C3b are not so easily washed off the macrophage surface as the immunologically attached

erythrocytes. Second, the complement receptor is a single functional entity, whereas the sites on the macrophage plasma membrane which bind the $F(ab')_2$ reagent used previously have not been characterized and are probably quite heterogeneous. Finally, the complement receptor is a membrane structure the function of which closely resembles that of the Fc receptor; in thioglycollate-activated mouse peritoneal macrophages, both the complement and Fc receptors mediate phagocytosis. Thus, our demonstration that ingestion mediated by either the Fc receptor (opsonized pneumococci) or a nonspecific phagocytic receptor (latex particles) fails to initiate phagocytosis of particles bound to the C3b receptor provides additional evidence that the various phagocytic receptors function independently of each other and indicates that a signal initiating ingestion via one type of receptor is not transmitted to all receptors which have the potential to mediate phagocytosis

Summary

The complement receptor of the macrophage plasma membrane recognizes particle-bound C3b but does not recognize particle-bound C3d. C3b-coated sheep erythrocytes were bound to macrophages via their C3b receptors, and the preparations were then incubated with either latex particles or opsonized pneumococci (test particles). Macrophages ingested the test particles, but erythrocytes were not ingested; they remained bound to C3b receptors of the macrophage plasma membrane. Thus, a signal initiating ingestion via one type of receptor is not transmitted to all receptors which have the potential to mediate phagocytosis.

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