Brief Definitive Report

LIGATED COMPLEMENT RECEPTORS DO NOT ACTIVATE THE ARACHIDONIC ACID CASCADE IN RESIDENT PERITONEAL MACROPHAGES

By ALAN A. AderEM, SAMUEL D. WRIGHT, SAMUEL C. SILVERSTEIN, and ZANVIL A. COHN

From The Rockefeller University, New York 10021.

Murine resident peritoneal macrophages are a rich source of arachidonic acid (20:4) metabolites (1, 2). When these cells phagocytose zymosan, or particles coated with complexes of IgG or IgE, 20:4 is released from the macrophage membrane phospholipid by the reaction of one or more phospholipases. The released 20:4 is then quantitatively oxygenated via either the cyclooxygenase or lipoxygenase pathways (2, 3). Interaction of the surface-bound IgG with Fc receptors is sufficient to trigger both phagocytosis and the release of 20:4 (4). Here, we examine two other phagocytosis-promoting receptors, and report that the C3b and C3bi receptors do not initiate the release of 20:4. Since these receptors do promote phagocytosis, we infer that the intracellular signal leading to phospholipase activation is distinct from that leading to engulfment.

Materials and Methods

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of specific pathogen-free female ICR mice (Trudeau Institute, Saranac Lake NY) weighing 25–30 g, as previously described (5). Peritoneal cells (~9 × 10^6 cells/ml) in minimal essential medium (MEM) (Gibco, Grand Island NY) containing 10% fetal calf serum (FCS) were added to 35-mm-diameter plastic culture dishes (1 ml/dish) or to 12-mm glass coverslips (0.1 ml/slip). After 2 h at 37°C in a 5% CO_2, 95% air atmosphere, cultures were washed three times in calcium- and magnesium-free phosphate buffered saline (depleted phosphate buffer, PD), to remove nonadherent cells. Fresh MEM plus 10% FCS (1 ml/dish), containing 0.5 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]20:4) (specific activity, 70 Ci/nmol; New England Nuclear, Boston MA) was added, and the cells were incubated overnight (16 h).

Preparation of Ligand-coated Particles. Glass beads (8 μm, Duke Scientific Corp., Palo Alto, CA) were derivitized with dinitrophenyl (DNP), as described previously (6) for glass coverslips, and were suspended in phosphate-buffered saline (PBS) at 50 mg/ml. IgG-coated beads (BIgG) or IgM-coated beads (BIgM) were obtained by incubating 10 mg of DNP-coated beads in 250 μl 10% FCS for 30 min at 20°C with 25 μg/ml affinity-purified rabbit anti-DNP IgG (BIgG) or 3 μl of murine monoclonal anti-DNP IgM ascites fluid (BIgM) (7). The beads were then washed three times and suspended at 50 mg/ml in PBS. Complement-coated beads (BIgMC) were prepared by incubating BIgM at 25 mg/ml with 20% normal human serum for 15 min at 37°C followed by three washes in PBS.

Sheep erythrocytes (SE) were coated with IgM (EIgM), IgG (EIgG), or human C3b

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(EC3b), or C3bi, as previously described (8). In some experiments, murine C3 was deposited on EIgM by incubating 5×10^8 cells/ml with 20% mouse serum at 37°C for 15 min. These cells probably bear both C3b and C3bi and are designated EIgMC.

Assays of Phagocytosis and Attachment. Phagocytosis and attachment of ligand-coated SE was measured as described (8). The results were scored with a phase-contrast microscope by counting the number of SE ingested or attached per 100 phagocytes. These numbers are termed phagocytic index and attachment index, respectively. In some experiments, ligand-coated glass beads were added with the ligand-coated SE. Under such conditions, attachment of SE to macrophages could not be scored by phase-contrast microscopy. In such cases, the following procedure was used. After a 45 min incubation of macrophages with both beads and SE, monolayers were washed, cooled to 0°C, and incubated for 30 min with fluoresceinated anti-SE. The monolayers were washed again, and attachment of SE was scored using fluorescence microscopy. The presence of beads that did not bear ligand (BIgM) caused a 15% depression in the attachment index of all ligand-coated SE.

Assay of Total [^{3}H]20:4 Release. Macrophages prelabelled with [^{3}H]20:4 were washed four times in PD and were overlaid with 1 ml of serum-free MEM. Zymosan (160 μg/ml) and BIgM, BIgMC, and BIgG (all at 5 mg/ml) were added as indicated. The cells were incubated at 37°C in a 95% air, 5% CO_2 atmosphere, and at the times indicated, aliquots of medium were removed and counted in Hydrofluor (National Diagnostics, Inc., Somerville NJ). The cells were washed in PD and scraped twice into 1 ml of Triton X-100. Portions of the cell lysates were assayed for radiolabel content, and protein was determined by the method of Lowry et al. (9) with bovine serum albumin as a standard.

"Activation" of Complement Receptors With Lymphokines. To obtain cells that were capable of ingesting C3-coated particles, macrophage monolayers were incubated at 37°C for16 h in supernatants containing a lymphokine secreted by appropriately triggered T lymphocytes (TCF) (generously provided by Dr. Frank Griffin, University of Alabama in Birmingham) (10).

Results

Macrophages were challenged with glass beads coated with a variety of ligands. Since IgM-coated glass surfaces do not promote the release of H_2O_2 from phagocytes (11), IgM-coated glass beads were chosen as a negative control. BIgM bound very well to macrophages, but they neither triggered release of 20:4 nor inhibited the release of 20:4 stimulated by zymosan (Fig. 1 *A*). In keeping with previous findings (4), IgG-coated beads did trigger the release of ~12% of cellular 20:4. Complement-coated beads caused no release of cellular 20:4 (Fig. 1 *A*).

The inability of complement-coated beads (BIgMC) to stimulate release of 20:4 does not result from insufficient amounts of the ligand, C3. This was demonstrated by measuring the number of available C3 receptors on the cell surface following interaction with ligand-coated beads. While BIgM caused no reduction in either C3b or C3bi receptors, BIgMC caused a marked reduction (~85%) of both C3b and C3bi receptors (Table I). This reduction likely results from diffusion of receptors to the glass-adherent surface where they are occupied and trapped by interaction with the ligand. Thus, our observations suggest that the failure of macrophages to secrete 20:4 in response to BIgMC is not due to inefficient interaction between ligand and receptor, rather, ligated C3b and C3bi receptors are unable to stimulate secretion of 20:4.

The resident peritoneal macrophages employed in the above experiment have "inactive" complement receptors, i.e. receptors that do not promote phagocytosis of C3b- or C3bi-coated particles (12, 13, and Table II). Therefore, it may not

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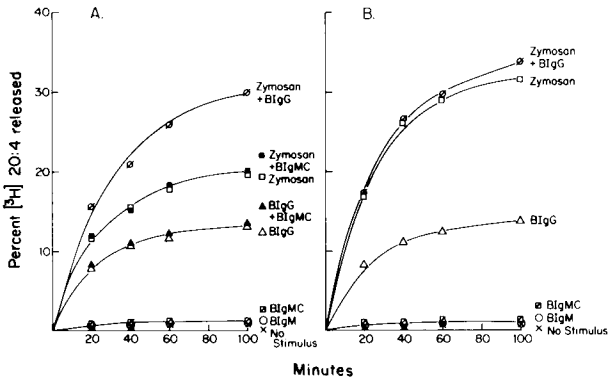

FIGURE 1. Time course of 20:4 release in response to various stimuli. Macrophages were isolated and labeled with [^{3}H]20:4 for 16 h in MEM plus 10% FCS (*A*), or in culture supernatants containing TCF (*B*). The cells were then washed four times with PD, overlaid with 1 ml MEM, and challenged with 160 μg zymosan (□) or with 5 mg of ligand-coated beads (BIgM [○], BIgMC [▨], BIgG [△]). Control cells receiving no stimulus (X) were similarly treated and incubated at 37°C in a 95% air, 5% CO_2 atmosphere. At the specified times, duplicate aliquots of the medium were removed and counted. The cells were scraped twice in 0.05% Triton-X 100, and the radiolabel and protein content of the cell lysates were determined. Values are expressed as percent of total cellular 20:4 released into the medium, and are the mean of triplicate cultures.

TABLE I

Complement Receptors Bind to C3 on Complement-coated Glass Beads

Ligand-coated bead	Attachment index							
	Resident macrophage				TCF-treated macrophage			
	EC3b	EC3bi	EIgMC	EIgG	EC3b	EC3bi	EIgMC	EIgG
BIgM	1,130	1,009	1,665	650	958	900	1,183	977
BIgMC	194	164	343	644	172	124	175	1,050
BIgG	703	820	1,640	350	1,022	800	ND*	222

Monolayers of macrophages were cultured overnight in MEM plus 10% FCS, or TCF. The monolayers were then washed in PBS and incubated for 45 min at 37°C with a mixture of the indicated ligand-coated erythrocytes and ligand-coated beads as described in Materials and Methods. The monolayers were again washed in PBS and incubated at 0°C for 30 min with fluoresceinated antibody directed against sheep erythrocytes. Attachment of erythrocytes to macrophages was scored using a fluorescent microscope.
* Not done.

TABLE II

C3 Receptors of TCF-stimulated Macrophages Promote Phagocytosis

Ligand-coated erythrocyte	Phagocytic index	
	Resident macrophage	TCF-treated macrophage
EIgMC	18 (10%)	140 (64%)
EIgG	480 (86%)	404 (83%)
EC3b	23 (13%)	72 (34%)
EC3bi	25 (14%)	91 (46%)

Monolayers of macrophages were cultured overnight in MEM or TCF as described in Materials and Methods. The macrophages were washed and the phagocytic capacity was determined using the indicated ligand-coated erythrocytes. Results are expressed as phagocytic index, and the percent of phagocytes ingesting at least one erythrocyte is given in parentheses. Data are averaged from two separate experiments.

be surprising that these receptors are incapable of promoting the release of 20:4. In order to measure the effect of active phagocytosis-promoting C3 receptors on the release of 20:4, we cultured the macrophages for 18 h in a source of an activating lymphokine (TCF). Griffin et al. (10, 13) have shown that this lymphokine renders C3 receptors competent to promote phagocytosis, and Table II shows that complement-mediated phagocytosis is stimulated 7-fold by the conditions we employed. Nevertheless, BIgMC triggered no release of 20:4 from lymphokine-stimulated macrophages (Fig. 1 *B*). Thus, C3 receptors signal phagocytosis without signalling the release of 20:4 from peritoneal macrophages.

Though C3 receptors of resident peritoneal macrophages may not themselves promote phagocytosis, Ehlenberger and Nussenzweig (12) have shown that C3 receptors can act synergistically with Fc receptors to promote phagocytosis. Therefore, we examined the ability of C3 receptors to augment the release of 20:4 stimulated by BIgG and zymosan. In unstimulated macrophages, saturating doses of BIgG and zymosan individually promote the release of 10–20% of cellular 20:4 (Fig. 1 *A*). A combined stimulus of zymosan plus BIgG provokes the release of an amount of 20:4 equal to the sum of the release triggered by either zymosan or BIgG alone (Fig. 1 *A*). This observation demonstrates that the engagement of either the Fc receptor, or those receptors that bind zymosan, does not exhaust the capacity of the macrophage to release 20:4, and that these receptors act in an additive manner. Engagement of C3 receptors by BIgMC, on the other hand, causes no augmentation of the release of 20:4 initiated by either BIgG or zymosan (Fig. 1 *A*). Thus, C3 receptors appear incapable of synergy with Fc receptors, or with those receptors that bind zymosan.

Even though treatment of macrophages with lymphokine does not enable the C3 receptors to promote the release of 20:4, it does induce the cells to secrete double the amount of 20:4 in response to zymosan (Fig. 1, *A* and *B*). In contrast, the Fc receptor–mediated release of 20:4 is unaffected by this treatment (Fig. 1, *A* and *B*).

Discussion

Macrophages express receptors for the principle opsonic molecules in mammals, IgG and C3 (14). Both the Fc and C3 receptors are transmembrane glycoproteins which bind appropriately opsonized particles, and which are capable of initiating changes in cellular behavior (14). Ligation of Fc receptors by IgG-coated particles results not only in phagocytosis of the particle, but also in the concomitant release of H_2O_2 and 20:4 (2, 14). C3 receptors differ from Fc receptors in several respects. While the Fc receptor is composed of a single polypeptide chain of ~55 kD (14), the C3 receptors are much larger. The C3b receptor is a 190 kD protein (15), and the C3bi receptor is composed of two polypeptide chains of 190 and 105 kD (16). Consonant with the structural difference between Fc and C3 receptors is their different behavior. C3 receptors can exist in two states, an inactive state, in which the C3 receptors bind C3-coated particles but do not promote their ingestion, and an active state, in which particle binding is followed by phagocytosis (13). Resting macrophages bear inactive receptors that can be converted by the action of a lymphokine to the activated state (13). Second, while C3 receptors do promote phagocytosis, they

do not trigger the release of H_2O_2 (11, 17), and we report here that they do not cause the secretion of 20:4 metabolites.

The observation that C3 receptors do not promote the release of 20:4 and H_2O_2 suggests that complement-mediated phagocytosis may provide a means of clearing opsonized particles without initiating or perpetuating an inflammatory response.

The inability of the C3 receptors to promote the release of 20:4 and H_2O_2 may also provide insight into the mechanisms by which receptor ligation is coupled to metabolic responses. The Fc receptor initiates phagocytosis, the secretion of H_2O_2, and the release of 20:4 (2, 14). Are these three responses triggered by a common intracellular signal, or are separate signals required for each? We have previously shown that C3 receptors initiate phagocytosis without triggering the release of H_2O_2 (11). Thus, it appears that the signals for secretion of H_2O_2 and for phagocytosis are functionally distinct. Similarly, we show here that C3 receptors promote phagocytosis without triggering the release of 20:4, suggesting that the signal for phagocytosis and for 20:4 release are also distinct. Recent evidence from this laboratory suggests that the signals for 20:4 release and for H_2O_2 secretion are also distinct, as they differ in their requirement for extracellular Na^+. This is inferred from ion-replacement studies, which show that the signal(s) between the receptor and the phospholipase is interrupted by the removal of Na^+ from the medium, while Fc receptor–induced H_2O_2 secretion is unaffected (18, and A. Aderem et al., manuscript in preparation).

Since each of the three cellular responses to Fc receptor ligation appears to be triggered by a distinct intracellular signal, we speculate that Fc receptors can generate at least three separate signals. We wish to point out, however, that "the signal" that initiates a complex biological process such as the release of 20:4 need not be synonymous with a single intracellular second messenger such as Ca^{2+}, cyclic nucleotides, or diacylglycerol. The initiation of such a cellular process may require the simultaneous presence of two or more second messengers or, more likely, a defined sequence of events, mediated by two or more second messengers. The pathway by which Fc receptors initiate release of 20:4 is currently under study.

Summary

Receptors for IgG stimulate the release of ~20% of cellular arachidonic acid (20:4) from murine resident peritoneal macrophages. In contrast, C3 receptors do not trigger the secretion of any 20:4 in excess of that released constitutively from the cells. Since the ability of C3 receptors to promote phagocytosis is regulated, we compared resting macrophages, whose C3 receptors do not promote phagocytosis of C3-coated particles, and lymphokine-treated cells, whose receptors do promote ingestion. Despite their ability to promote phagocytosis, the C3 receptor of lymphokine-treated macrophages remain unable to initiate release of 20:4. We speculate that the intracellular signals that initiate phagocytosis are distinct from those that initiate release of 20:4.

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References

1. Scott, W. A., J. M. Zrike, A. L. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152:324.
2. Bonney, R. J., P. Naruns, P. Davies, and J. L. Humes. 1979. Antigen-antibody complexes stimulate the synthesis and release of prostaglandins by mouse peritoneal macrophages. *Prostaglandins.* 18:605.
3. Rouzer, C. A., W. A. Scott, Z. A. Cohn, P. Blackburn, and J. M. Manning. 1980. Mouse peritoneal macrophages release leukotriene C in response to a phagocytic stimulus. *Proc. Natl. Acad. Sci. USA.* 77:4928.
4. Rouzer, C. A., W. A. Scott, J. Kempe, and Z. A. Cohn. 1980. Prostaglandin synthesis by macrophages requires a specific receptor-ligand interaction. *Proc. Natl. Acad. Sci. USA.* 77:4279.
5. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:157.
6. Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607.
7. Wright, S. D., and S. C. Silverstein. 1984. Phagocytosing macrophages exclude proteins from the zones of contact with opsonized targets. *Nature (Lond.).* 309:359.
8. Wright, S. D., and S. C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3b′ receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149.
9. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193:265.
10. Griffin, F. M., Jr., R. A. Luben, and D. W. Golde. 1984. A human lymphokine activates macrophage C3 receptors for phagocytosis: Studies using monoclonal anti-lymphokine antibodies. *J. Leukocyte Biol.* 36:95.
11. Wright, S. D., and S. C. Silverstein. 1983. Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J. Exp. Med.* 158:2016.
12. Ehlenberger, A. G., and V. Nussenzeig. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. *J. Exp. Med.* 145:357.
13. Griffin, J. A., and F. M. Griffin, Jr. 1979. Augmentation of macrophage complement receptor function in vitro. I. Characterization of the cellular interactions required for the generation of a T-lymphocyte product that enhances macrophage complement receptor function. *J. Exp. Med.* 150:653.
14. Unkeless, J. C., and S. D. Wright. 1984. Structure and modulation of Fc and complement receptors. *In* Contemporary Topics in Immunobiology. R. Snyderman, editor. 14:171.
15. Kinoshita, T., and V. Nussenzweig. 1984. Functional properties of factor H and the membrane complement receptor for C3b, C4b (CRI) in mice. *Fed. Proc.* 43:1491*a*. (Abstr.).
16. Beller, D. I., T. A. Springer, and R. D. Schreiber. 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med.* 156:1000.
17. Yamamoto, K., and R. B. Johnston, Jr. 1984. Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. *J. Exp. Med.* 159:405.
18. Aderem, A. A., W. A. Scott, and Z. A. Cohn. 1984. A selective defect in arachidonic acid release from macrophage membranes in high potassium media. *J. Cell Biol.* 99:1235.