RECEPTORS FOR C3b AND C3bi PROMOTE PHAGOCYTOSIS BUT NOT THE RELEASE OF TOXIC OXYGEN FROM HUMAN PHAGOCYTES*

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Monocytes, macrophages, and polymorphonuclear leukocytes (PMN)[1] bear receptors for C3 and for the Fc domain of IgG that allow these phagocytes to bind particles coated with the corresponding ligands (1). The consequences of ligand-receptor interaction, however, are different for IgG and C3. While Fc receptors constitutively promote phagocytosis of IgG-coated particles, the ability of C3 receptors to promote phagocytosis is regulated. Murine peritoneal macrophages do not ingest C3-coated erythrocytes, but macrophages "activated" by a T-cell derived lymphokine readily ingest them (2). Similarly, human PMN, monocytes, and cultured monocytes (which resemble macrophages) do not phagocytose particles coated with C3b or C3bi, but cultured monocytes adherent to immobilized fibronectin (FN) or serum amyloid P component do phagocytose both C3b and C3bi-coated particles (3). Complement receptors thus appear to exist in one of two states: Receptors in the "inactive" state bind ligands but fail to promote ingestion, while receptors in the "active" state bind ligands and promote ingestion.

Ligation of Fc receptors signals not only phagocytosis but also the synthesis and release of O_2^- and H_2O_2, powerful oxidants that either alone or in concert with leukocyte enzymes efficiently kill many potential pathogens (4). We have examined the ability of C3 receptors to promote the release of toxic oxygen from human phagocytes. Here we report that neither the C3b nor the C3bi receptor causes the release of H_2O_2 from monocytes, cultured monocytes, or PMN. Further, the complement receptors of cultured monocytes cannot be rendered capable of triggering H_2O_2 release even under conditions in which they readily promote phagocytosis.

[1] *Abbreviations used in this paper:* DGVB^{++}, 2.5 mM veronal buffer, pH 7.5, 75 mM NaCl, 2.5% dextrose, 0.05% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$; DNP, dinitrobenzene; E, sheep erythrocytes; EC3b or GC3b, EIgM or GIgM coated with complement components C1, C4, C2, and C3; EC3bi or GC3bi, EC3b or GC3b treated with I; EGelC, E coated with gelatin by the tannic acid procedure then coated with rabbit anti-E IgM then incubated with R7; EGelCFN, EGelC incubated with FN; EIgG or GIgG, E or G coated with rabbit anti-EIgG; EIgM or GIgM, E or G coated with rabbit anti-E IgM; EIgMC, EIgM treated with R7; FN, plasma fibronectin; G, sheep erythrocyte ghost; HSA, human serum albumin; PBS, phosphate-buffered saline; PLL, poly-L-lysine; PMA, phorbol-12-myristate-13-acetate; PMN, polymorphonuclear leukocytes; R7, human serum immunochemically depleted of C7; SDS, sodium dodecyl sulfate.

Materials and Methods

Reagents. FN, purified as described (5), was a generous gift of Dr. M. B. Furie (The Rockefeller University). Human serum deficient in complement component C7 (R7) was produced by adsorption of normal human serum on an anti-C7 affinity column (6). Before use, R7 was adsorbed with gelatin-coated erythrocytes (see below) to remove FN and anti-erythrocyte antibodies (3).

Cells. Human monocytes were purified on Percoll gradients and cultured in Teflon beakers as previously described (7). Human neutrophils were purified on Ficoll-hypaque gradients (8). Sheep erythrocytes (E) coated with IgM (EIgM), IgG (EIgG), C3b (EC3b), or C3bi (EC3bi) were prepared as described (7). E were coated with gelatin (9) and IgM (3) to yield EGelIgM. EIgM and EGelIgM were coated with complement proteins (principally C3b and C3bi) by incubating them for 15 min at 37°C with R7 to yield EIgMC or EGelIgMC (3).

Substrates. IgG-bearing surfaces were prepared by sequentially treating tissue culture plastic with poly-L-lysine (PLL), dinitrophenol (DNP), and anti-DNP IgG exactly as described by Michl et al. (10). IgM-coated surfaces were prepared in a similar fashion by substituting monoclonal murine IgM anti-DNP (a gift of Dr. V. Nussenzweig) for the IgG anti-DNP.

Plastic surfaces were coated with a monolayer of ligand-coated E as described (7). Briefly, 16-mm plastic culture wells were treated with PLL, then 2×10^7 E were applied to the surface for 10 min at 500 g in the absence of soluble protein. Exposed PLL was quenched with human serum albumin (HSA) (2 mg/ml, 30 min, 20°C), and the E were subjected to hypotonic lysis to yield ghost (G) surfaces.

Assays. H_2O_2 was measured by the scopoletin procedure (11). Briefly, 0.75 ml of phosphate-buffered saline (PBS, reference 12) containing 20–50 nmol scopoletin, 2 mM NaN_3, 3 mM glucose, and 1 U/ml horseradish peroxidase was added to 16-mm culture wells. An additional 0.75 ml of PBS containing 3 mM glucose and $2–5 \times 10^5$ phagocytes was added, the wells were agitated, and the cells were allowed to settle for 15 min at 4°C. The cultures were then warmed to 37°C for 60 min, and the decrease in fluorescence of the scopoletin in the supernatant fluid was determined. H_2O_2 release from phagocytes was calculated by comparison with parallel wells containing no phagocytes. Where indicated, phorbol myristate acetate (PMA) was added with the scopoletin solution to a final concentration of 100 ng/ml.

Phagocytosis was measured as described (7). Results are reported as phagocytic index, the number of E ingested per 100 phagocytes.

Results

We measured the H_2O_2 evolved as phagocytes spread on ligand-coated surfaces. Such surfaces were prepared in two ways. In the first, EIgM, EIgG, EC3b, or EC3bi were deposited as a monolayer on PLL-coated plastic culture wells, and the wells were dipped in hypotonic buffer to lyse the E (7). Monocytes or PMN were then plated on the resulting ligand-coated ghost (G) surfaces (GIgM, GIgG, GC3b, or GC3bi). Monocytes and PMN do not release H_2O_2 as they spread on control surfaces (HSA or GIgM, Table I). The release of H_2O_2 is not inhibited by the monolayers of ghosts since PMA-stimulated evolution of H_2O_2 is essentially identical to that seen with phagocytes spreading on surfaces without ghosts (Table I). In keeping with previous observations (13), PMN and monocytes readily evolve large amounts of H_2O_2 when they engage IgG-bearing ghost monolayers. However, little or no H_2O_2 release is caused by GC3b- or GC3bi-surfaces (Table I). Previous experiments have shown that under the conditions employed, the phagocyte's complement receptors are specifically engaged by ligands on such ghost monolayers: receptor activity is lost from the apical portion

TABLE I

Ligand-induced Release of H_2O_2*

Surface	Monocytes		Neutrophils	
	No PMA	+ PMA	No PMA	+ PMA
	$nmol/10^5$ cells/h			
GIgM	0.12	3.25	0.09	4.06
GIgG	1.52	3.18	1.68	4.90
GC3b	0.09	3.21	0.08	4.25
GC3bi	0.39	3.32	0.11	4.30
GIgG + C	1.75	3.25	ND	ND
HSA‡	0.18	3.10	0.11	4.44

* Sheep erythrocytes (E) coated with IgM, IgG, C3b, or C3bi were attached as a monolayer to plastic tissue culture wells as described in Materials and Methods. Where indicated, human serum (1:5, 10 min, 37°C) was added as a source of complement (C). After osmotic lysis of the E, 5×10^5 freshly isolated monocytes or neutrophils were added, and the preparation was then warmed to 37°C for 60 min. H_2O_2 production was assayed as in the presence or absence of 100 ng/ml PMA. Data represent the averages of duplicate wells from a representative experiment (of 6).
‡ Plastic surfaces incubated with HSA (5 mg/ml, 30 min, 20°C) were used in place of ghost surfaces.
ND, not done.

of the phagocyte after adherence to ghost-surfaces bearing the corresponding ligand (7). This results from diffusion of receptors to the basal surface of the macrophage where they are "trapped" by interaction with ligand (14). Thus, the failure of the cells to secrete H_2O_2 in response to C3b or C3bi is not due to inefficient interaction between ligand and receptor, rather, ligated C3b and C3bi receptors are unable to stimulate secretion of H_2O_2.

In a second series of experiments, ligand-bearing surfaces were constructed by derivatizing PLL-coated plastic with the hapten, DNP, and then adding anti-DNP antibodies to yield culture surfaces coated with antigen-antibody complexes. Cultured monocytes release large amounts of H_2O_2 as they spread on these DNP-anti-DNP IgG-coated surfaces (Table II). This release is triggered by the Fc domain of IgG since surfaces coated with IgM anti-DNP or with $F(ab)_2$ fragments of IgG anti-DNP cause no release of H_2O_2 (Table II). Complement-bearing surfaces were generated by the action of whole serum on surfaces treated with DNP and IgM anti-DNP. As shown in Table II, such C3-bearing surfaces do not promote release of H_2O_2 from cultured monocytes. However, these surfaces do bear sufficient C3b and C3bi to ligate all of the phagocyte's C3 receptors since both C3b and C3bi receptors are lost from the apical surface of cultured monocytes adherent to such C3-bearing surfaces (data not shown). Thus, the C3 receptors of these phagocytes were engaged by ligands, but H_2O_2 was not released.

C3 can potentiate the release of H_2O_2 caused by IgG. If C3 is deposited on an IgG-bearing surface (Tables I and II), or if IgG anti-E is added to a C3b- or C3bi-bearing erythrocyte ghost-surface (data not shown), the resulting substrates cause phagocytes to release ~15% more H_2O_2 than do substrates bearing IgG alone. Thus, the absence of H_2O_2 secretion from phagocytes spread on C3-

TABLE II

*Ligand-induced Release of H_2O_2 from Cultured Monocytes**

Surface	No PMA	+ PMA
	nmol/10^5 cells/h	
IgG	1.90	3.56
F(ab)$_2$	0.10	3.10
IgM	0.01	3.30
IgM + C	0.02	3.10
IgG + C	2.17	3.38

* DNP-surfaces were coated with rabbit IgG-anti-DNP, F(ab)$_2$ fragments of this IgG-anti-DNP (15), or monoclonal IgM-anti-DNP as described in Materials and Methods. Some IgM-bearing wells were subsequently incubated with a 1:5 dilution of fresh human serum (10 min, 37°C) in order to deposit C3 on the surface. Monocytes were cultured in Teflon beakers for 5 d, then 2 × 10^5 phagocytes were added to each well and the release of H_2O_2 was measured. Data represent the average of duplicate wells from a representative experiment (of 6).

TABLE III

Phagocytosis and Generation of H_2O_2 Induced by Ligand-coated Erythrocytes

Target	H_2O_2 release*	Phagocytic index‡	Molecules H_2O_2§ per E
	nmol/10^5 cells/h	*E/10^2 cells/h*	*× 10^8*
EIgG	1.03	346	18.0
EGeIC	0.14	36.7	2.3
EGeICFN	0.01	150	0.42

* Monocytes were cultured in Teflon beakers for 72 h, then assayed for the release of H_2O_2 during spreading on ghost monolayers prepared from the indicated ligand-coated E. 72-h cultures were used because the resulting phagocytes activate their complement receptors in response to FN (3) and exhibit a high capacity to release H_2O_2 (16).
‡ Phagocytic index was measured using parallel preparations of spread phagocytes and ligand-coated E.
§ Calculated from the data in the first two columns assuming that H_2O_2 release by a phagocyte settling on a monolayer of ghosts will approximate that from a phagocyte overlain by a monolayer of intact erythrocytes. Data are averaged from four separate experiments.

coated surfaces does not result from inhibition of H_2O_2 secretion by ligated C3 receptors.

The phagocytes employed above have "inactive" complement receptors, i.e., receptors that do not promote phagocytosis of C3b- or C3bi-coated particles. Therefore, it may not be surprising that these receptors are incapable of promoting the release of H_2O_2. In order to measure the effect of "active" C3 receptors on the release of H_2O_2, we constructed particles that bear both C3 and FN. Previous work has shown that such particle-bound FN activates both C3b and C3bi receptors of cultured human monocytes (3). We performed parallel experiments in which we measured both the phagocytosis of ligand-coated E and the H_2O_2 released when the phagocytes were allowed to spread on monolayers prepared with these E (Table III). Cultured monocytes readily phagocytose E coated with both FN and C3. However, these phagocytes do not secrete meas-

urable H_2O_2 as they spread on monolayers of like erythrocyte ghosts. Using the assumption that the H_2O_2 release from phagocytes settling on a monolayer of ligand-coated ghosts approximates that from phagocytes overlaid with intact erythrocytes, we calculated the amount of H_2O_2 released per E ingested. Cultured monocytes release 18×10^8 molecules of H_2O_2 per EIgG ingested. However, $<0.5 \times 10^8$ molecules of H_2O_2 are released for each C3- and FN-coated E ingested. Thus, C3 receptors signal phagocytosis without signaling the release of H_2O_2 from cultured monocytes.

Discussion

The results reported here show that neither the C3b nor the C3bi receptors of human PMN, monocytes, or macrophages are capable of promoting the release of H_2O_2. These results are in keeping with the observations of Newman and Johnston (17) who showed that the C3b receptor of human PMN cannot mediate degranulation or release of O_2^- and of Hed and Stendahl (18) who showed that in cytochalasin B–treated PMN, binding of IgG-coated zymosan promoted vigorous release of H_2O_2 while the binding of C3-coated zymosan did not. While it has been reported that C3b and C3bi induce a chemiluminescent response in human PMN (19), in our hands the chemiluminescent response of both monocytes and PMN to surfaces bearing C3b and C3bi is 1–2% of that evoked by 100 ng/ml PMA (Wright and Silverstein, unpublished observations). This chemiluminescence may reflect the small amount of C3-mediated H_2O_2 release shown in Table I or generation of products of other pathways of O_2 utilization. C3-mediated release of toxic oxygen appears, therefore, to be of a magnitude far lower than that associated with microbicidal activity (20).

We have observed a striking divergence in the cellular responses promoted by receptors for IgG and C3. While Fc receptors and "activated" C3 receptors both promote phagocytosis, only Fc receptors promote the release of H_2O_2. This observation is consistent with the work of Newburger et al. (21) who showed that phagocytosis in a macrophage-like cell line was not associated with O_2^- release, though O_2^- was secreted in response to the soluble stimulus, PMA. These observations indicate that phagocytosis and the release of H_2O_2 can be initiated independently of one another and they suggest that the intracellular signal(s) generated by ligated C3b or C3bi receptors differ from the signal(s) of the Fc receptors.

The basis for this inference is diagrammed in Fig. 1. In the simplest model for the action of Fc and C3 receptors (model A), both receptors generate a common intracellular message (M) that stimulates both phagocytosis and the release of H_2O_2. The observation that C3 receptors stimulate phagocytosis without the release of H_2O_2 makes this model untenable. An alternate model, B, in which peroxide release and phagocytosis are initiated by a common signal but in which H_2O_2 release is blocked by an additional signal generated by C3 receptors, is also untenable since ligation of C3 receptors enhances rather than suppresses the H_2O_2 release stimulated either by Fc receptors or PMA (Tables I and II). The remaining models require that phagocytosis and H_2O_2 release can be independently stimulated and that the intracellular signals generated by Fc receptors and C3 receptors differ. In model C, the Fc receptor and the C3 receptor generate
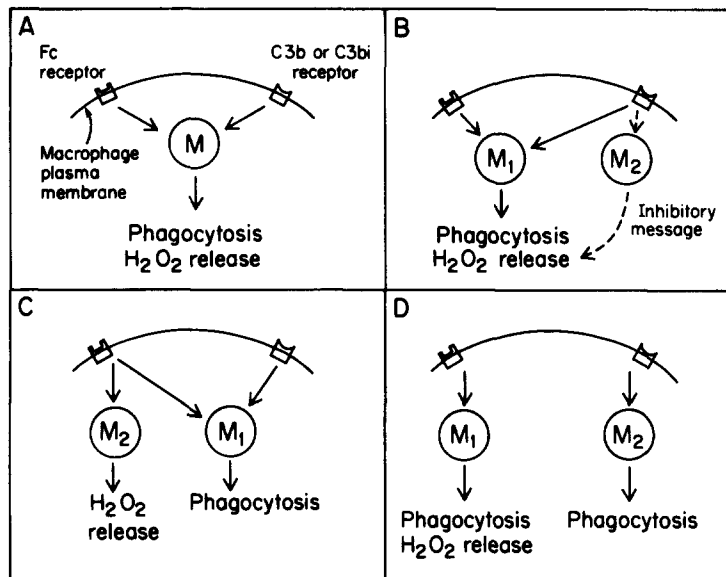
FIGURE 1. Models describing the intracellular messages (M) generated by ligated Fc and C3 receptors. See text for details.

a common intracellular message, M_1, that results in phagocytosis, and the Fc receptor generates an additional message, M_2, that causes H_2O_2 release. In model D, the two types of receptors each generate a distinct intracellular message, M_1 or M_2. Here the message generated by the C3 receptor stimulates only phagocytosis while the message of the Fc receptor stimulates both H_2O_2 release and phagocytosis. (An additional model that employs three separate messages, one each for H_2O_2 secretion, Fc-mediated phagocytosis, and C3-mediated phagocytosis, is formally similar to Model D.) Presently, we cannot distinguish between models C and D.

Summary

We have measured the release of H_2O_2 from granulocytes, monocytes, and macrophages during spreading on ligand-coated culture surfaces. While IgG-coated surfaces stimulate vigorous release of H_2O_2, neither C3b- nor C3bi-coated surfaces promoted appreciable release of H_2O_2 despite full ligation of C3b and C3bi receptors. We also measured release of H_2O_2 from cultured monocytes spreading on surfaces coated with both fibronectin and C3. Under such circumstances, the C3 receptors elicit a strong phagocytic response, but no H_2O_2 release was recorded. We conclude that the C3b and C3bi receptors of monocytes and granulocytes do not signal the generation of toxic oxygen intermediates from these cells.

Note added in proof: Analagous results have been reported recently for murine macrophages by K. Yamamoto and R. B. Johnston, Jr. (submitted for publication).

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