Communication between Receptors for Different Ligands on a Single Cell: Ligation of Fibronectin Receptors Induces a Reversible Alteration in the Function of Complement Receptors on Cultured Human Monocytes

SAMUEL D. WRIGHT, MARK R. LICHT, LYDIA S. CRAIGMYLE, and SAMUEL C. SILVERSTEIN
The Rockefeller University, Laboratory of Cellular Physiology and Immunology, New York 10021

ABSTRACT   Receptors for the third component of complement (C3) on cultured human monocytes (MO) bind ligand-coated particles but do not initiate phagocytosis. The function of these receptors, however, is altered dramatically after MO attach to surfaces coated with fibronectin (FN) or after MO are exposed to phorbol esters. FN and phorbol esters "activate" C3 receptors such that they promote vigorous phagocytosis. Here we show that activation of C3 receptors requires the continuous presence of FN or phorbol esters and is rapidly reversible when these stimuli are removed. Activation does not change the number or distribution of C3 receptors on the surface of MO. We conclude that the function of C3 receptors is regulated by reversible reactions that are initiated by ligation of a different class of receptors on the surface of the same cell.

A central problem in modern biology is the mechanism by which signals arising at the cell surface influence cellular behavior. Specific membrane receptors for hormones, neurotransmitters, growth factors, connective tissue components, chemoattractants, antibodies, and complement have been identified on a wide variety of cells. Ligation of each of these receptors generates transmembrane signals that produce specific cellular responses that are uniquely associated with the receptor stimulated (e.g., muscle cell contraction in response to ligation of acetylcholine receptors by acetylcholine; directed cell movement in response to chemoattractants; T-cell growth in response to interleukin 2). Some of these responses occur immediately (milliseconds to minutes), while others require activation of dormant genes and take many hours. In this manuscript we are concerned with receptors that promote immediate responses.

In some specialized cells, ligation of one type of surface receptor causes a change in the number of receptors for other ligands. For example, in fat cells, insulin signals the movement of glucose transporters from internal membranes to the plasma membrane (1). The resultant high rate of glucose transport is thus caused by an increased number of glucose transporters. Of potentially greater importance are membrane receptors whose ligation promotes *qualitative* changes in other receptor systems on the same cell. In theory, such receptors have the capacity to switch other receptor systems on or off, thereby determining whether or not the cell is responsive to a specific environmental stimulus. The first indication of such an interaction between different classes of receptors was the

report of Griffin and Griffin (2). They showed that a secretory product of mouse thymocytes regulates the endocytic capacity of complement receptors on murine macrophages. This lymphokine rapidly and reversibly converts these receptors from an inactive state (i.e., one in which they bind complement-coated particles but do not promote their ingestion) to an active one (i.e., one in which they mediate both binding and ingestion of complement-coated particles). The availability of purified human complement components and the ease with which human leukocytes can be purified and cultured prompted us to use human materials to explore this system further.

Human monocytes and monocyte-derived macrophages express two distinct surface receptors for the third component of complement, C3. One receptor, CR1, binds the first cleavage product of C3 termed C3b; a second receptor, CR3, binds a further cleavage product of C3b termed C3bi. CR1 and CR3 have been isolated and characterized. CR1 is a M_r 205,000 glycoprotein (3), and CR3 is composed of two externally disposed polypeptides; an α chain of M_r 185,000 and a β chain of M_r 105,000 (4) in an $\alpha_1\beta_1$ configuration (5). In freshly explanted blood monocytes and macrophages derived from monocytes by in vitro cultivation (MO),[1] CR1 and CR3

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[1] *Abbreviations used in this paper:* FN, fibronectin; HSA, human serum albumin; MO, monocytes cultivated in vitro for 5–10 d; PBS, phosphate-buffered saline; PDB, phorbol dibutyrate; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

bind particles coated with their respective ligands, but neither receptor mediates ingestion of these particles. Treatment of MO with phorbol esters, or plating of these MO on fibronectin (FN)-coated surfaces activates the phagocytosis promoting properties of their complement receptors (6, 7). Of special interest is the finding that ligation of FN receptors on the basal surface of the MO activates complement receptors on the apical portion of its plasma membrane (7). Thus C3 receptors throughout the surface of the MO can be activated by signals initiated within a small segment of the plasma membrane.

Little is known of the biochemical mechanisms by which CR1 and CR3 are converted from an inactive to an active state. In both human and murine phagocytes, activation of these receptors occurs rapidly (minutes), does not require protein synthesis, and is prevented by agents that depolymerize microtubules (2, 6). Here we report that activation of the endocytic capacity of C3 receptors of human MO by phorbol esters and by FN requires the continuous presence of these compounds, that it is reversed completely upon their removal, and that receptor activation is not accompanied by changes in the number or distribution of C3 receptors on the MO surface.

MATERIALS AND METHODS

Reagents: Phorbol myristate acetate (PMA) and phorbol dibutyrate (PDB) were obtained from Sigma Chemical Co. (St. Louis, MO). FN, purified from human plasma (8), was a generous gift of Dr. M. B. Furie (The Rockefeller University). Monoclonal antibody OKM10, directed against the ligand-binding domain of CR3 (4), was a gift of Dr. G. Goldstein (Ortho Pharmaceuticals). Fab fragments of OKM10 were prepared by digestion with papain (9). Fab fragments of the monoclonal antibody 57F, directed against CR1 (10) were a generous gift Drs. K. Iida and V. Nussenzweig (New York University). Fab fragments were iodinated by the "Iodogen" procedure (11) to specific activities of 10^6–10^7 cpm/µg.

Cells: Human blood monocytes were purified on Percoll gradients and cultured in Teflon beakers as described (6). The MO mature during culture, and after 5–10 d they closely resemble macrophages. Such macrophage-like cells are referred to here as MO.

Monolayers of MO were prepared as follows. Cells were harvested from Teflon beakers, washed, and suspended at 0.5–1.0 × 10^6/ml in phosphate-buffered saline (PBS [12]) containing 3 mM glucose, 0.5 mg/ml human serum albumin (HSA [Worthington Biochemical Corp., Freehold, NJ]), and 0.3 U/ml Aprotinin (Sigma Chemical Co.). Plastic culture surfaces were coated with HSA (1 mg/ml) or FN (0.1 mg/ml) by a 60 min incubation at 20°C. The surfaces were washed, MO were added to a density of ~1,500/mm², and cells were allowed to spread for 45 min at 37°C. Where indicated, PMA (30 ng/ml) or PDB (500 ng/ml) were included during plating.

Sheep erythrocytes were coated with IgG, C3b or C3bi as previously described (6).

Phagocytosis: Monolayers of spread MO were washed and ligand-coated erythrocytes were added at a density of 3 × 10^5/mm². After 45 min at 37°C, uningested erythrocytes were lysed by brief exposure to distilled water and the phagocytosis of erythrocytes was scored by phase-contrast microscopy (6). The number of ingested erythrocytes per 100 phagocytes is termed the phagocytic index.

Binding of Radiolabeled Antibodies to MO: Monolayers of MO in 16-mm culture wells were cooled on ice and radiolabeled antibody was added to duplicate wells in the presence or absence of a 100 fold excess of unlabeled antibody. After 60 min on ice, unbound antibody was washed away by dipping the wells in successive beakers of cold PBS containing 1% fetal bovine serum. The cells were scraped up with cotton swabs and cell-associated radioactivity was counted in a gamma counter. Specific binding was calculated by subtracting the amount of radiolabel bound to wells containing 100-fold excess unlabeled antibody. Preliminary experiments (not shown) established that 60 min was sufficient to establish binding equilibrium and that binding was fully reversible with a single off rate. Radiolabeled Fab fragments of the immunoglobulins were employed to avoid binding through Fc receptors on MO.

Immunofluorescence: Monolayers of MO were washed and incu-

bated with Fab fragments of OKM10 or 57F, each diluted to 10 µg/ml in PBS. After 40 min at 20°C, the monolayers were washed in four beakers of PBS, and fluoresceinated Fab fragments of sheep anti-murine IgG (diluted 1:100, [Cappell Laboratories, Cochranville, PA]) was added. After an additional 40 min at 20°C, the live MO were washed and viewed on a Zeiss photomicroscope III equipped for fluorescence microscopy. Control slides were prepared by omitting the primary antibody. We employed monovalent reagents and avoided fixatives to prevent artifactual clustering of receptors.

RESULTS

Stimulation of Complement-mediated Phagocytosis by Phorbol Esters Is Reversible

MO plated on plastic surfaces are unable to ingest C3b- or C3bi-coated erythrocytes, but if the cells are incubated with PMA or other tumor-promoting phorbol esters, phagocytosis of these ligand-coated erythrocytes is vigorous (6; and Fig. 1). In this study, we employed a hydrophilic phorbol ester, PDB. Unlike PMA, its more hydrophobic congener, PDB can be washed away from cells: one half of cell-associated PDB is released from monocytes in ~10 min at 20°C (13). Fig. 1 shows that PDB fully activates C3 receptor-mediated phagocytosis whether added before or together with the C3-coated erythrocytes. The precise speed with which PDB activates C3 receptors cannot be measured because the assay of phagocytosis requires 45 min to reach plateau, but since MO preincubated with PDB ingest as many C3-coated erythrocytes as MO given PDB at the same time as the C3-coated erythrocyte, PDB must act early in the 45-min assay of phagocytosis (Fig. 1). The activation of phagocytic capacity by PDB appears specific for C3 receptors since parallel experiments show no alteration in the phagocytosis of IgG-coated erythrocytes
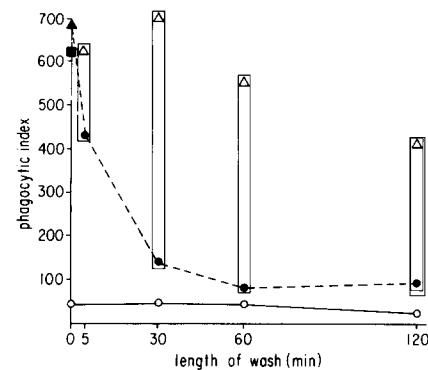

FIGURE 1 The activation of C3 receptors mediated by phorbol esters is reversible. Human MO (6-d cultures) were allowed to spread for 45 min at 37°C in the presence or absence of 500 ng/ml PDB. Cells were washed in 2 l of PBS containing 1 mg/ml HSA at 20°C for the indicated periods of time, and their capacity to ingest C3-coated erythrocytes was then measured with a 45 minute assay at 37°C in the presence or absence of freshly added PDB. Cells not exposed to PDB (O) phagocytose few C3bi-coated erythrocytes. MO continuously exposed to PDB during spreading and phagocytosis (■) or MO exposed to PDB only during the 45-min assay of phagocytosis (▲) show comparable high levels of ingestion. MO stimulated with PDB during plating show a progressive loss of phagocytic capacity as PDB is washed away (●). Washed MO remain sensitive to PDB since fresh PDB added during the assay of phagocytosis restores the phagocytic capacity of these washed cells to control levels (△). Shown here is a representative experiment of five separate experiments. Only the phagocytosis of C3bi-coated erythrocytes is shown, but qualitatively similar results were obtained using C3b-coated erythrocytes.

which is promoted by receptors for the Fc domain of IgG (6; and data not shown).

To examine the reversibility of the activation of C3 receptors caused by PDB, we plated MO in plastic culture dishes at 37°C in the presence of PDB. The resulting monolayers were washed at 0°C and incubated at 20°C for various intervals in PBS that contains 1 mg/ml HSA. At the indicated times, the MO were placed into fresh medium with or without PDB, and were incubated with C3-coated erythrocytes for 45 min at 37°C. Fig. 1 shows that the capacity of PDB-treated MO to ingest C3-coated erythrocytes falls progressively when the PDB is washed away. After a 30-min wash, phagocytosis of C3bi-coated erythrocytes falls to 15% of the level in MO exposed continuously to PDB. The washed MO remain sensitive to restimulation by PDB since addition of fresh PDB fully restores their capacity to ingest C3b- (data not shown) and C3bi-coated erythrocytes (Fig. 1). Thus, the phagocytosis-promoting capacity of C3 receptors can be activated, deactivated, then reactivated in the course of 2 h.

Stimulation of Complement-mediated Phagocytosis by FN Is Reversible

The complement receptors of MO become capable of promoting phagocytosis after MO adhere to FN-coated surfaces (7; and Fig. 2). The reversibility of this phenomenon was tested by removing MO from FN-coated surfaces and replating them on fresh HSA- or FN-coated surfaces. Since the adhesion of monocytes to FN-coated surfaces requires divalent cations (14), MO were released from FN-coated substrates by incubation at 0°C in the presence of 1 mM EDTA. MO removed from FN lose the ability to phagocytose C3-coated erythrocytes when they are replated on control substrates. However, their C3 receptors can be restimulated by plating these MO on fresh FN-coated surfaces (Fig. 2). Thus the activation of complement receptors by FN requires its continuous presence and is fully reversible.

Stimulation of C3-mediated Phagocytosis Is Not Accompanied by a Change in the Number or Distribution of Cell-surface Receptors for C3

Macrophages contain internal pools of membrane that exchange rapidly with the plasma membrane (15), and recent studies show that chemoattractants cause human polymorphonuclear leukocytes (PMN) to exteriorize membrane proteins leading to a two- to fivefold increase in C3b (16) and formyl-methionyl-leucyl-phenylalanine (17) receptors. Thus, it was possible that the enhanced phagocytosis of C3-coated particles observed in MO stimulated with PMA or FN was caused by an increase in cell surface receptors for C3. To test this hypothesis, we determined the number of surface receptors in parallel preparations of FN- or PMA-stimulated and untreated MO using radiolabeled monoclonal anti-receptor antibodies. Fig. 3 shows results obtained with OKM10, an antibody that binds and blocks the ligand-binding site of CR3. Neither the number of CR3 nor the affinity of OKM10 for CR3 is changed when MO are activated by spreading on FN (Fig. 3) or by treatment with PMA (data not shown). Similarly, using the monoclonal antibody, 57F, we detected no change in the number or affinity of CR1 on MO treated with FN or PMA (data not shown). These experiments demonstrate that PMA and FN enhance the phagocytosis-promoting capacity
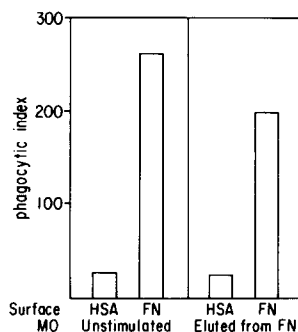

FIGURE 2 Activation of C3 receptors caused by spreading on FN is reversible. Human MO (5-d cultures) were allowed to spread for 45 min at 37°C on plastic surfaces coated with HSA or FN and their capacity to ingest C3b-coated erythrocytes was measured (left panel). A parallel preparation of MO spread on FN was eluted from the FN-coated surface with EDTA, washed, and replated on fresh HSA- or FN-coated surfaces, and the phagocytic index was measured (right panel). In this experiment, MO optimally stimulated with PMA showed a phagocytic index of 280. Only the phagocytosis of C3b-coated erythrocytes is shown, but qualitatively similar results were obtained using EC3bi-coated erythrocytes. Shown is a representative experiment of five separate experiments. Duplicate samples varied by <10%.
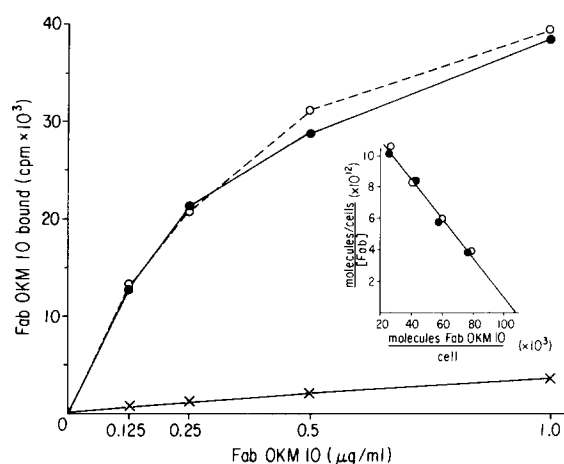

FIGURE 3 Activation of C3 receptors caused by spreading of MO on FN is not accompanied by an alteration in the expression of CR3. MO (6-d cultures) were plated for 45 min at 37°C on plastic surfaces coated with HSA (●) or FN (○), and the binding of ^{125}I-Fab OKM10 was determined as described in Materials and Methods. Background binding was determined in the presence of 100 fold excess unlabeled Fab OKM10 (X) and was subtracted from experimental values to yield the specific binding plotted here. Equivalent numbers of MO bound to HSA- and FN-coated surfaces as estimated by measurements of surface-associated protein and by examination with a phase contrast microscope. Scatchard analysis (inset) showed 108,000 sites/cell with K_a 1.0 × 10^8 M^{-1} whether the MO were spread on FN or HSA. Data are averaged from duplicate determinations in two separate experiments.

of CR1 and CR3 through a qualitative alteration in the function of pre-existing receptors, not by a quantitative change in the number of cell-surface receptors. While we have observed no change in the binding affinity of monoclonal anti-receptor antibodies, we cannot rule out the possibility that activation of CR1 and CR3 is accompanied by altered affinity for their respective ligands.

The distribution of receptors on the cell surface may affect their function, and Hafemen et al. (18) have shown that the distribution of CR1 on PMN depends on the substrate to which the cell is adherent. In PMN spread on glass, CR1 appears clustered, while in PMN spread on lipid-coated surfaces they appear diffuse. To examine the possible contribution of receptor clustering to phagocytosis, we examined the

surface distribution of CR1 and CR3 by fluorescence micros-copy. Control MO monolayers or MO activated with PMA or FN were incubated with Fab fragments of OKM10 or 57F. Cells were washed, incubated with fluorescinated Fab anti-mouse IgG, and the living cells were examined under a fluorescence microscope. CR1 and CR3 appear diffusely and homogeneously distributed on the surface of all MO exam-ined, whether activated with PMA, FN, or neither (data not shown). We conclude that activation of complement receptors is not accompanied by an alteration in their distribution on the cell surface.

DISCUSSION

We have shown here that ligation of FN receptors causes greatly enhanced responsiveness to C3b and C3bi without changes in the number or distribution of cell surface receptors for these ligands. Other instances have been described in which ligation of one receptor results in rapid, qualitative changes in another receptor. In a process termed "heterolo-gous desensitization," cells exposed to prostaglandins show a decreased capacity to respond to β-adrenergic agonists despite normal levels of receptor and catalytic subunits of adenylate cyclase (19). The epidermal growth factor receptors of murine fibroblasts exhibit greatly reduced affinity for epidermal growth factor after brief exposure of the cells to phorbol esters (20) or platelet-derived growth factor (21); and the response of a variety of cell types to catecholamines is sharply decreased after ligation of their muscarinic, α-adrenergic, or opiate receptors (22). In all of these examples, ligation of one type of receptor *de*sensitizes or *in*activates the capacity of a second type of receptor to bind its ligand. Our observations differ from these in that we observe sensitization or activation of complement receptors as the result of ligation of FN receptors.

Positive alteration of the activity of one receptor by ligation of a second may represent a common and powerful strategy employed by many different types of cells. For example, during the movements of embryogenesis, the migration of neural crest cells requires the simultaneous presence of both FN and the appropriate neighboring cell types (23). In the nervous system of *Aplysia*, ligation of serotonin receptors on sensory neurons alters the gating of a K^+ channel so that subsequent depolarizations result in enhanced neurotrans-mitter release (24). This interaction between membrane pro-teins in neurons constitutes the physical basis of one form of learning. Viewed in this context, FN-mediated activation of C3 receptors reflects information processing at the unicellular level. The structural basis for such regulatory influences of one receptor by another is thus of general interest.

The effect of ligated FN receptors on the responsiveness of C3 receptors is mimicked by PMA. Since PMA increases the activity of a widely distributed Ca^{++}- and phospholipid-de-pendent protein kinase (25), we are studying the role of phosphorylation in the regulation of receptor activity.

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