Brief Definitive Report

FIBRONECTIN AND SERUM AMYLOID P COMPONENT STIMULATE C3b- AND C3bi-MEDIATED PHAGOCYTOSIS IN CULTURED HUMAN MONOCYTES*

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Receptors for C3 exist in two states, an active state in which the receptors promote phagocytosis and an inactive state in which they do not. For example, cultured human monocytes (MO) bear separate receptors that bind C3b- and C3bi-coated erythrocytes, but neither receptor promotes phagocytosis of such C3-coated erythrocytes (1). Brief stimulation of the MO with the tumor promoter phorbol myristate acetate (PMA), however, renders both C3 receptors competent to generate a phagocytic response. Similarly, C3 receptors of resident murine peritoneal macrophages do not promote phagocytosis but are activated when these cells are incubated with a specific lymphokine (2). We have been unable to identify a comparable human lymphokine, and therefore sought other molecules that might represent physiological regulators of C3 receptor activity in human mononuclear phagocytes.

Recenty, Pommier et al. (3) reported that soluble fibronectin (FN) activates C3b receptors of freshly isolated human monocytes. Other investigators, however, failed to observe this effect (4, 5). While soluble FN may cause modest activation of C3 receptors on freshly explanted blood monocytes (3), we report here that substrate-bound FN as well as substrate-bound serum amyloid P component (SAP) cause a marked activation of the C3b and the C3bi receptors of MO and that this activation of C3 receptor activity occurs by a novel mechanism: interaction of substrate-bound FN or SAP with the basal portion of the MO plasma membrane activates C3 receptors on the apical portion of the MO plasma membrane.

Materials and Methods

Reagents. FN and anti-FN were generous gifts of Dr. M. Furie, The Rockefeller University. FN was purified as described (6), and anti-FN antibodies were raised in a rabbit injected with FN further purified on sodium dodecyl sulfate (SDS) polyacrylamide gels. Purified SAP (7) was a gift of R. Glaviano, Rush Medical College, Chicago. Antibodies against SAP were from Atlantic Antibodies, Scarborough, ME. FN and SAP ran as pure proteins on heavily overloaded SDS polyacrylamide gels. The SAP preparation contained no C-reactive protein detectable by a sensitive radial immunodiffusion procedure.

Human serum deficient in complement component C7 (R7) was produced by adsorption on an anti-C7 affinity column as described (8). Before use, R7 was adsorbed with gelatin-

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coated erythrocytes (see below) to remove anti-erythrocyte antibodies and FN.

FN and SAP were adsorbed with immobilized antibodies as follows. *Staphylococcus aureus* (BRL, 1 μg) were incubated for 40 min at 20°C with 20 μg of anti-FN or anti-SAP antibodies, washed, then incubated with 5 μg of FN or SAP for 30 min at 20°C, and removed by centrifugation.

Cells. Human monocytes were purified on Percoll gradients and cultured in Teflon beakers (1). Human neutrophils were purified on Ficoll-Hypaque gradients (9). Sheep erythrocytes (E) were coated with IgM (EIgM), IgG (EIgG), C3b (EC3b), or C3bi (EC3bi) as described (1). E were coated with gelatin (Difco Laboratories Inc., Detroit, MI) using tannic acid (4). The resulting EGel were incubated with 2 mg/ml human serum albumin (HSA) (Worthington Biochemical Corp., Freehold, NJ) for 1 h at 20°C before use. EGel were coated with IgM anti-E antibodies (1) to yield EGelIgM. Complement- and gelatin-coated E were ordinarily suspended at 10^8/ml in 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 $MgCl_2$ ($DGVB^{++}$). For incubations with soluble FN, we used a buffer ($DHVB^{++}$) that contained HSA (1 mg/ml) instead of gelatin.

EIgM and EGelIgM were coated with complement proteins (principally C3b and C3bi) by incubating 2×10^8 E/ml for 15 min at 37°C with R7 diluted to 1/7 of normal serum concentration (based on protein content) in $DGVB^{++}$. The resulting EIgMC or EGelIgMC were washed twice in $DHVB^{++}$ and used immediately.

Substrates. Gelatin (1.5%) spontaneously adsorbed to plastic in a 24-h incubation at 5°C. Proteins were covalently cross-linked to poly-L-lysine (PLL)-coated culture surfaces using glutaraldehyde (10). PLL-coated plastic surfaces were coated with a monolayer of E or EIgM, and the E were subjected to hypotonic lysis to yield ghost surfaces as described (1).

Phagocytosis. $0.5–1.0 \times 10^6$ MO/ml in phosphate-buffered saline (PBS) containing 3 mM glucose, 1 mg/ml HSA, and 0.3 U/ml Aprotinin (Sigma Chemical Co., St. Louis, MO) were added to protein-coated or ghost-coated surfaces for 45 min at 37°C. The monolayer was washed and ligand-coated E were added. After 45 min at 37°C, the uptake of E was scored (1). The number of attached or ingested E per 100 phagocytes is termed the attachment index or phagocytic index, respectively.

Results

MO that have spread on HSA-coated, gelatin-coated, or uncoated plastic (data not shown) readily ingest EIgG but ingest very small numbers of EC3b or EC3bi (Table I and reference 1). On substrates covalently derivitized with FN, however, MO avidly ingest both EC3b and EC3bi (Table I, part I). A similar but less dramatic stimulation of C3-mediated phagocytosis is seen in MO on surfaces coated with SAP. The degree of C3-mediated phagocytosis induced by FN is similar to that observed in MO stimulated with PMA. As with PMA, the effect of substrate-bound FN or SAP is not a general enhancement of phagocytic capacity since the uptake of EIgG is not enhanced in MO spread on FN- or SAP-coated surfaces (Table I). Because the FN and SAP are covalently bound to the substrate, it is unlikely that they are acting as opsonins; rather, these proteins stimulate the activity of receptors on the opposite surface of the MO.

Two lines of evidence indicate that stimulation of C3-mediated phagocytosis is caused by SAP and FN rather than by a contaminant in both preparations. First, FN binds to gelatin through a specialized domain (6), whereas SAP does not. When FN was added to a gelatin-coated surface, C3-mediated phagocytosis was stimulated in adherent MO; when SAP was added, no stimulation was observed (Table I, part II). Second, anti-FN antibodies immobilized on *S. aureus* cell walls removed phagocytosis-promoting activity from solutions of FN but not from SAP. Similarly, adsorption with anti-SAP antibodies removed activity from

TABLE I

FN and SAP Promote Phagocytosis of Erythrocytes Coated with C3b or C3bi

	Surface	Test particle		
		EC3b (%)	EC3bi (%)	EIgG (%)
I. MO*	HSA	84 (40)	211 (55)	1,554 (90)
	HSA + soluble PMA‡	812 (80)	1,444 (93)	1,375 (85)
	FN	993 (100)	1,660 (100)	1,785 (87)
	SAP	397 (72)	845 (96)	1,375 (91)
II. MO§	Gel	109 (38)	389 (84)	2,036 (100)
	Gel-FN	1,009 (97)	2,042 (100)	2,550 (100)
	Gel-SAP	82 (33)	310 (73)	2,467 (100)
III. Neutrophils‖	Gel	21 (17)	7 (7)	124 (65)
	Gel-FN	7 (6)	8 (5)	107 (54)
	Gel + soluble PMA‡	381 (75)	381 (81)	141 (66)

* Plastic surfaces were covalently derivitized with HSA, FN, or SAP. MO cultured for 7 d in Teflon beakers were allowed to spread on the coated surface for 45 min at 37°C; then the phagocytic index was determined using the indicated ligand-coated E. The percent of phagocytes ingesting at least one E is given in parenthesis. A similar number of phagocytes attached to all the surfaces used. Results of a representative experiment (of five) are shown.

‡ 30 ng/ml PMA was included with ligand-coated E during phagocytosis.

§ Surfaces coated with gelatin were incubated for 30 min with 100 μg/ml of FN or SAP, washed, then used immediately in an experiment as described above.

‖ Neutrophils were used in place of MO in the assay of phagocytosis. Results are averaged from two experiments.

solutions of SAP but not from FN (data not shown).

The capacity to respond to FN-coated surfaces increases as MO are maintained in culture (Fig. 1). Freshly explanted monocytes adherent to FN-coated surfaces showed minimal phagocytosis of EC3b (Fig. 1) or EC3bi (data not shown), but after 3 d in culture, MO C3 receptor activity was markedly stimulated when phagocytes were plated on FN-coated surfaces. Thus the capacity of MO to activate their C3 receptors in response to FN-coated substrates requires maturation and occurs at a similar stage of MO development to that described previously for the activation of C3 receptors by PMA (1).

C3b receptors of human neutrophils mediate binding but not ingestion of C3-coated E (11). Neutrophils lack FN receptors (4), and therefore attachment of neutrophils to FN-coated substrates does not activate their C3 receptors (Table I, part III). PMA, however, activates both the C3b and the C3bi receptors of human neutrophils (Table I, part III).

To determine if soluble, as opposed to surface-bound FN stimulates MO C3 receptor activity, we developed a method for incubating MO with soluble FN in the absence of surface-bound FN. A special procedure was necessary because FN spontaneously attaches to plastic culture surfaces and enhances C3 receptor activity of MO plated on these surfaces even after the soluble FN is washed away (data not shown). We plated MO on plastic substrates coated with a monolayer of red cell ghosts. Soluble FN does not significantly stimulate C3-mediated phagocytosis in MO spread on such ghost surfaces (Table II), indicating that soluble FN cannot stimulate phagocytosis in adherent MO.

FN and SAP bind to bacteria (12, 13). Thus, MO may encounter FN or SAP either on the substratum or on C3-coated bacteria. To determine if the phagocytosis of C3-coated particles is enhanced when FN is simultaneously bound to the same particle, we used E coated with gelatin and IgM. EGelIgM showed little

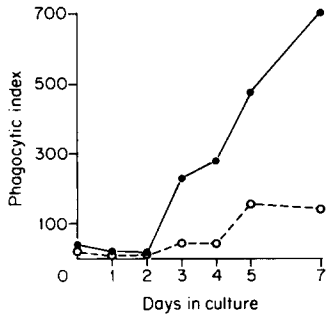

FIGURE 1. Response of C3b receptor activity to surface-bound FN in monocytes cultured in Teflon beakers. After various periods in culture, phagocytes were harvested, washed, then allowed to spread on plastic surfaces coated with gelatin (open circles) or gelatin and FN (closed circles). The phagocytosis of EC3b was then measured.

affinity for MO (attachment index, 119). FN binds to EGelIgM (4), and the resulting EGel-FN-IgM are avidly bound (attachment index, 1,494) but not ingested (Table II) by MO. However, when EGelIgM are coated with both FN and C3, they are efficiently ingested (Table II). Similar effects of FN on phagocytosis of EGelIgMC were obtained with MO cultured for 3–10 d (data not shown). In 13 determinations, the phagocytic index of EGelIgMC by MO adherent to ghost surfaces was enhanced an average of 5.3-fold by the addition of FN. Since FN does not bind to ghost surfaces, the added FN acts by binding to the EGelIgMC. Thus, C3 receptors of MO are activated by FN bound to a C3-bearing particle.

Discussion

FN and, to a lesser extent, SAP augment the capacity of C3b and C3bi receptors of human MO to promote phagocytosis of EC3b and EC3bi (Tables I and II). Since C3-coated particles are readily ingested if they also bear FN (Table II), FN activates C3 receptors on the segment of membrane to which it binds. More importantly, the interaction of substrate-bound FN or SAP with the basal portion of the MO plasma membrane activates C3 receptors on the apical portion of the MO plasma membrane (Table I). Thus, binding of MO membranes to FN and SAP generates a signal that is propagated to the entire MO plasma membrane.

Pommier et al. (3) recently reported that the ingestion of C3b-coated E by human monocytes is stimulated by soluble FN. While our observations are consistent with this finding, the amount of phagocytosis stimulated by soluble FN is small in comparison with that stimulated by substrate-bound FN (Tables I and II). This may explain why other authors have failed to observe FN-mediated enhancement of C3 receptor activity in freshly isolated monocytes (4, 5).

The capacity of substrate-bound FN to stimulate C3 receptor activity of cultured monocytes may explain the divergent reports on this subject (1, 14, 15). Since MO synthesize FN (16), it is likely that MO cultured on glass or collagen substrates coated these substrates with FN and may have thus stimulated the activity of their own C3 receptors.

Our observations suggest new roles for FN and SAP. FN binds to a number of bacterial species (12), and to particulate activators of the alternative comple-

TABLE II

*Phagocytosis of Ligand-coated Erythrocytes by MO Spread on GIgM Surfaces**

Test particle	Phagocytic index	
	No FN (%)	With FN (%)
EC3b	56 (21)	47 (18)
EC3bi	109 (44)	125 (55)
EIgMC	184 (47)	210 (56)
EGelIgM	15 (6)	18 (11)
EGelIgMC	147 (49)	696 (90)

* MO were allowed to spread for 45 min on surfaces coated with GIgM as described (1). The phagocytic index for each type of E was then determined in the presence or absence of 200 μg/ml FN. The percent of phagocytes ingesting at least one E is given in parentheses. Data are averaged from two experiments done with MO cultured for 8 d. Identical results were obtained with MO plated on surfaces coated with ghosts lacking IgM.

ment pathway (17). While FN does not promote phagocytosis of these particles, it may synergize with C3 to generate a phagocytic response. SAP binds in a Ca^{2+}-dependent fashion to agarose, bacteria, bound complement, and FN (13). Thus, SAP may also potentiate the phagocytosis of C3-coated pathogens.

The C3 receptors of granulocytes and freshly explanted blood monocytes are unresponsive to surface-bound FN (Table I, Fig. 1). Thus the greatest effect of FN on phagocytosis may be to enhance the activity of the C3 receptors of mature macrophages. This enhancement may occur either through binding of FN to C3-coated particles or to underlying connective tissue. The observation that FN does not activate the C3 receptors of neutrophils while PMA does (Table I, part III) suggests the existence of additional factors that regulate the C3 receptor activity of these cells.

Summary

Fibronectin (FN) and serum amyloid P component (SAP) markedly enhance phagocytosis mediated by the C3b and C3bi receptors of cultured human monocytes but not of granulocytes. (The C3b and C3bi receptors of granulocytes can be activated by treatment of these phagocytes with PMA.) Activation of monocyte C3 receptors by FN is developmentally regulated: Freshly explanted monocytes respond to FN with a small increase in C3 receptor-mediated phagocytosis while monocytes matured in culture exhibit a much greater response. The mechanism of action of FN on C3 receptors of cultured monocytes is unique in two respects. First, while substrate-bound FN or SAP activate monocyte C3 receptors, soluble FN does not. Second, stimulation of the basal surface of monocyte plasma membranes by substrate-bound FN activates C3b and C3bi receptors on the apical surface of the plasma membrane, i.e., at sites remote from the segments of membrane in contact with the FN or SAP.

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