STUDIES ON THE FIBRONECTIN RECEPTORS OF HUMAN PERIPHERAL BLOOD LEUKOCYTES

Morphologic and Functional Characterization

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It is generally believed that plasma membrane receptors for C3b and for the Fc piece of IgG serve very different functions in phagocytosis. While sheep erythrocytes (E)[1] bearing IgG (EA) are phagocytosed by human polymorphonuclear leukocytes (PMN) and monocytes, E bearing C3b (EC3b) are bound to the plasma membrane but are not ingested (1, 2). The addition of even very small amounts of IgG to EC3b leads to their efficient phagocytosis by both PMN and monocytes (3). Thus, particle-bound IgG is generally considered a signal for phagocytosis, whereas particle-bound C3b has been thought to mediate adherence to phagocytic cells but to be insufficient to initiate the phagocytic event. However, it has been shown that mouse peritoneal macrophages will ingest EC3b after exposure to lymphokines (4, 5). Thus, lymphokine activation of the phagocytic cell, which presumably affects the nature of the interactions of the plasma membrane with the cytoskeleton (6), affects the ability of cellular C3 receptors to mediate phagocytosis.

Recently, we have demonstrated that fibronectin (Fn), a 440,000 D plasma glycoprotein, is able to activate freshly isolated human peripheral blood monocytes to ingest EC3b (7). In these experiments, Fn-exposed monocytes also ingested more EA than did unactivated monocytes; this increased phagocytosis did not require interaction of Fn with the erythrocyte and thus reflected a change in the phagocytic potential of the monocyte itself. This work, as well as that of others (8–10), suggests that monocytes bear a plasma membrane receptor for

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DGVB, low ionic strength isosmotic Veronal-buffered saline with dextrose, gelatin, Ca^{++}, and Mg^{++}; E, sheep erythrocytes; EA, sheep erythrocytes bearing IgG; EC3b, sheep erythrocytes bearing C3b; EDTA-GVB, Veronal-buffered saline containing gelatin and 10 mM EDTA; fmet-leu-phe, N-formyl-methionyl-leucyl-phenylalanine; Fn, fibronectin; GVB^{++}, Veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$; ms, microsphere; PBS, phosphate-buffered saline; PMN, human polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VBS, Veronal-buffered saline.

Fn. In the present study, we have explored the interaction of Fn with human leukocyte membranes by studying the binding of Fn-coated fluorescent particles to the cells, using flow cytometry. In addition, we have examined the binding of radiolabeled Fn to monocytes and PMN and have explored the functional consequences of interaction with Fn for phagocytosis. We have shown that PMN, like monocytes, bind Fn. In marked contrast to monocytes, however, phagocytosis by freshly isolated PMN is not affected by exposure to Fn. On the other hand, if the PMN are first activated by exposure to C5a or N-formyl-methionyl-leucyl-phenylalanine (fmet-leu-phe), then Fn has a marked effect on PMN phagocytosis. Most dramatically, these chemotaxin-activated neutrophils, which do not phagocytose EC3b in the absence of Fn, do, like monocytes, become actively phagocytic for these same particles in its presence.

Materials and Methods

Buffers. Isotonic Veronal-buffered saline (VBS), containing 0.1% gelatin, 0.15 mM $CaCl_2$, 1 mM $MgCl_2$ (GVB^{++}); low ionic strength isosmotic VBS with dextrose, gelatin, Ca^{++}, and Mg^{++} (DGVB), and VBS containing gelatin and 10 mM EDTA (EDTA-GVB), were prepared as described previously (11). Phosphate-buffered saline (PBS) and RPMI 1640 containing glutamine, penicillin, and streptomycin were obtained from the Media Unit, National Institutes of Health.

Purification of Fn. Human plasma Fn was purified by a modification (7) of the method of Vuento and Vaheri (12). Briefly, the 5% polyethylene glycol 3350 precipitate from EDTA, benzamidine, and phenylmethylsulfonyl fluoride (PMSF)-treated plasma was re-suspended in a buffer of 150 mM NaCl, 50 mM KH_2PO_4/K_2HPO_4, and 10 mM EDTA, pH 7.4. This plasma fraction was then absorbed by passage over Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) and the Fn was purified by elution from gelatin-Sepharose with 1 M arginine. Further purification was obtained by adsorption to, and elution from, arginine-Sepharose. All buffers used for chromatography and elution contained 5 mM benzamidine, 1 mM PMSF, and/or 25 μM para nitrophenyl para amidino benzoate to inhibit residual serine proteases. The purified Fn showed a single line on immunoelectro-phoresis vs. anti-whole human serum, and a single major band at 440,000 D on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Upon the reduction of disulfide bonds, SDS-PAGE revealed a closely spaced doublet, as has been reported previously for human plasma Fn (13). Antibodies raised against this Fn preparation in rabbits and goats gave a monospecific response on immunoelectrophoresis and Ouchter-lony double diffusion against whole human plasma.

Antibodies and Complement Components. C1, C4, and C2 were purchased from Cordis Laboratories Inc., Miami, FL. Rabbit IgG and IgM anti-Forssman (14) and human C3 were prepared as previously described (15). Human C5a was purified by immunoabsorp-tion of zymosan-activated, pooled, citrated plasma followed by ion exchange chromatog-raphy and gel filtration (L. Renfer, K. Yancey, C. Hammer, and M. Frank, manuscript in preparation). In brief, 1 M epsilon-aminocaproic acid, 10 mM $MgCl_2$, and citrated plasma were incubated with boiled zymosan. The supernatant of an acid precipitation of the mixture was adjusted to pH 5.0 with 10 N NaOH, exposed to a solid-phase immunoad-sorbent bearing antibody directed against C5a. C5a was eluted from the immunoadsorbent and then chromatographed sequentially on CM 52 ion exchange resin (Whatman Ltd., Maidstone, Kent, England) and Sephadex G-75 (Pharmacia Fine Chemicals). C5a was identified in column fractions by Lowry protein determinations, bioassay, and immunoblot techniques using specific antibody staining. The C5a prepared in this manner migrated as a 15,000 D band on SDS-PAGE. When this band was transferred to nitrocellulose sheets after electrophoresis (16), reactivity with specific anti-C5 antibody was confirmed. Fmet-leu-phe was purchased from Sigma Chemical Co., St. Louis, MO.

Production of Monoclonal Anti-Fn Antibodies. Monoclonal anti-Fn antibodies were pro-

duced by the method of Köhler and Milstein (20). Briefly, BALB/c mice were injected with 100 μg of purified Fn subcutaneously in complete Freund's adjuvant. 2–4 wk after initial immunization, selected mice were injected intravenously with 50 μg of Fn. 3 d later, the spleens were removed and individual spleen cells were fused with the SP2/0 nonsecretor myeloma cell line. Anti-Fn antibody production by fusions was detected using an enzyme-linked immunosorbent assay in which purified Fn was adsorbed to flat-bottomed microtiter wells (Immulon I; Dynatech Laboratories, Inc., Alexandria, VA). Cell culture supernatants were added in a dilution of 1:10 in PBS containing 0.05% Tween 20 (Mallinkrodt Chemical Co., St. Louis, MO) and incubated for 2 h at 37°C. Then, antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA). Monoclonal antibodies were used in experiments as unfractionated culture supernatants. All the anti-Fn monoclonals used were IgG1 antibodies.

Leukocyte Purification. Whole human blood was collected from normal volunteers in full units drawn with citrate phosphate dextrose anticoagulant by the Washington D.C. Regional Blood Services. Platelet-rich plasma was removed within 3 h of collection by centrifugation at 2,600 g for 3.5 min. The buffy coat (40 ml of the top layer of packed cells) was collected and then used immediately for isolation of leukocytes. Peripheral blood mononuclear cells were separated from the buffy coat on Ficoll-Hypaque gradients. Monocytes were isolated using a J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) equipped with a JE-6 elutriator rotor with two Sanderson separation chambers, following a procedure modified from Lionetti et al. (17). The purity of the monocytes was ~95% as determined by both morphology and nonspecific esterase staining (Technicon Instruments Corp., Tarrytown, NY). Peripheral blood lymphocytes were similarly isolated from the Ficoll-Hypaque gradients by pooling non–monocyte-containing fractions from the elutriation of mononuclear cells. Less than 5% of these cells stained with nonspecific esterase or exhibited phagocytosis of optimally sensitized EA. Alternatively, in some experiments, lymphocytes and monocytes were separated on preformed gradients of polyvinylpyrrolidone-modified colloidal silica (Percoll; Pharmacia Fine Chemicals) as described (18). Monocytes isolated in this manner were indistinguishable from elutriated monocytes with respect to the percentages that bound Fn-coated fluorescent microspheres (ms), the number of ms bound per cell, and the ability to phagocytose EC3b in the presence of Fn. Neutrophils were isolated from the Ficoll-Hypaque pellets by dextran sedimentation (19) without hypotonic lysis since there was no visable erythrocyte contamination.

Opsonized Sheep E. E were collected, washed, and stored using standard methods (13). For the preparation of C3b-opsonized particles, E were sensitized with IgM anti-Forssman antibody. C1, C4, C2, and C3 were then added sequentially as previously described (10). The concentration of C4 was limited to prevent interaction of EAC14 with C3b receptors. For experiments with monocytes, EAC142 were incubated with 5 U of C3 to yield 100–200 C3b molecules per cell. After sensitization with C3, EAC1423b were incubated for 1 h in EDTA-GVB to remove C1 and to allow C2 to decay, and then washed twice in GVB^{++} (EC3b). For the preparation of EA, 1 × 10^9 E/ml were incubated for 30 min at 37°C with a 1:2,000 dilution of IgG anti-Forssman antibody. The cells were then washed twice in GVB^{++} and resuspended in DGVB. Preliminary experiments revealed that PMN rosetted with EA and EC3b less well than with monocytes. Therefore, for use in PMN experiments, the IgG anti-Forssman was diluted only 1:250–1:500 during preparation of EA, and EC3b were prepared that bore 10,000 C3b per cell.

Rosetting and Phagocytosis Assay. For the assay of leukocytes in suspension, 50 μl of cells at 2 × 10^6/ml in RPMI 1640 supplemented with 5 mM MgCl$_2$ were incubated for 30 min in a 5% CO$_2$/95% air incubator at 37°C with Fn or, as a control, Fn passed over gelatin-Sepharose immediately before the experiment. In all experiments, this gelatin-Sepharose–passed Fn was used as the "no Fn added" control. Without exception, gelatin-Sepharose–passed Fn and buffer have given identical results in assays of monocyte and PMN phagocytosis of EA and of EC3b. In some experiments, leukocytes were incubated with varying concentrations of C5a or fmet-leu-phe for 10 min at 37°C and then washed once

in RPMI 1640 before incubation with Fn. Except where noted, after incubation with Fn, the leukocytes were mixed with 50 μl of EC3b or EA at 1.5 × 10⁸ E/ml without washing. The cells and indicator particles were sedimented by centrifugation for 5 min at 50 g, incubated for a further 30 min in a 5% CO_2/95% air incubator at 37°C without mixing, and then gently resuspended and examined under light microscopy. To quantitate phagocytosis, extracellular E were lysed by mixing the cells with a buffer of 1 part PBS and 4 parts H_2O for a few seconds. In all experiments, at least 200 leukocytes were counted to quantitate E rosetting or ingestion. Three or more E bound to a leukocyte was considered a rosette. Total intracellular E were counted as a function of the number of white blood cells observed.

Binding of Protein-coated Microspheres to Leukocytes.   Fluorescent microspheres (ms) (Cov-aspheres-MX; Covalent Technology Corp., Ann Arbor, MI), with an average diameter of 1.0 μm, were covalently bound to Fn (Fn-ms), gelatin (gelatin-ms), or bovine serum albumin (BSA-ms), according to manufacturer's instructions. Briefly, 10 μl of an ms suspension were mixed with 10 μg of protein in 100 μl of PBS, sonicated, and incubated at room temperature for 1 h. The coated spheres were then washed twice in RPMI 1640 plus 1% BSA, resuspended to 0.5 ml, and resonicated. Each preparation was checked by microscopy to assure a suspension of unaggregated particles. In some experiments, gelatin-ms were incubated with Fn at a final concentration of 200 μg/ml for 30 min at room temperature. The beads were then washed and sonicated once more before incubation with monocytes. To evaluate cell binding, the ms were mixed with an equal volume of monocytes or PMN at 1 × 10⁶ cells/ml, centrifuged at 50 g for 5 min, and incubated for 30 min at 37°C in a buffer containing 0.05% azide to inhibit endocytosis of the ms. Finally, leukocytes were separated from free beads by centrifugation through 1 ml of 6% BSA in RPMI 1640 for 1 min at 12,000 g. The cell pellet was resuspended and a drop was placed on a microscope slide. Initially, the cells were examined by fluorescence and phase microscopy. Cells binding at least 3 ms were considered positive, and the percent of leukocytes positive for ms bearing each protein were determined by counting 100–200 cells. Subsequently, the cells were examined by flow cytometry to analyze ms binding in greater detail, and the total number of ms bound to each cell was determined from the cell-associated fluorescence.

To study the inhibition of Fn-ms binding to cells by fluid phase Fn or by monoclonal antibodies, ms were incubated initially with only 1 μg of Fn. After washing, Fn-ms were then incubated with 10 μg/ml of BSA to block unreacted sites on the ms. To examine the inhibition of Fn-ms binding by fluid phase Fn, monocytes and PMN were first incubated for 30 min at 37°C with varying concentrations of Fn in RPMI 1640/azide and then with Fn-ms exactly as described above. To examine the effect of monoclonal anti-Fn antibodies, 50 μl of Fn-ms were incubated for 30 min at 37°C with 75 μl of culture supernatant containing a monoclonal anti-Fn and then incubated as described above with 2.25 × 10⁵ leukocytes in a total volume of 200 μl for 30 min at 37°C. After the separation of the free from the cell-bound Fn-ms, the cells were analyzed for ms binding by flow cytometry. In some experiments, monocytes were preincubated with a human IgG1 myeloma known to interact with the monocyte Fc receptor, and the PMN were incubated with monoclonal antibody 3G8 (New England Nuclear, Boston, MA), which recognizes the PMN Fc receptor. After 30 min, Fn-ms were added without washing. Although these preincubations totally inhibited EA rosetting with monocytes and PMN they did not influence the effect of monoclonal antibodies on Fn-ms binding.

Binding of ¹²⁵I-Fn to Monocytes and PMN.   Fn was radiolabeled with ¹²⁵I by the iodobead method according to manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Final specific activity of the radiolabeled Fn ranged from 3 to 5 × 10⁵ cpm/μg. Immediately prior to binding experiments, ¹²⁵I-Fn was centrifuged for 10 min at 12,000 g in a microfuge (Beckman Instruments, Inc., Irvine, CA) or for 10 min at 180,000 g in an airfuge (Beckman Instruments, Inc.) to remove aggregates. Preliminary experiments indicated that the specific uptake of radiolabeled Fn was not significantly different for the two preparations, or in comparison to uncentrifuged ¹²⁵I-Fn. Aliquots of radiolabeled Fn were incubated in duplicate with 2 × 10⁶ purified monocytes or 4 × 10⁶ PMN in RPMI 1640

containing 1% BSA and 0.05% azide in a total volume of 0.5 ml. Nonspecific binding was assessed by including a 100-fold excess of unlabeled Fn in the incubation mixture. Free and cell-bound radiolabel were separated by centrifugation of the mixture through Versilube F-50 (General Electric Co., Waterford, NY). Unbound and cell-associated radioactivity were determined by counting aliquots of the aqueous supernatant and the entire cell pellet, respectively. Initial kinetic experiments indicated that binding equilibrium was reached after a 1-h incubation. Therefore, saturation binding experiments were performed by incubating ^{125}I-Fn with leukocytes as described above at 37°C for 1 h. Nonspecific binding represented 1.0% of input at all concentrations of the ^{125}I-Fn used for both monocytes and PMN. Total binding was 6–7 times higher than background for monocytes and for PMN at the lowest inputs of Fn. When cells bearing ^{125}I-Fn were washed and reincubated in buffer at 37°C, >70% of the specifically bound radiolabel was released.

Expression of Data.   Statistical analyses were performed using Student's *t* test on population means. Except where indicated, data are expressed as mean ± standard deviation. Figures show representative experiments except as indicated.

Results

Effect of Fn on EC3b and EA Phagocytosis.   As previously reported (7), fluid-phase Fn induced phagocytosis of EC3b by monocytes. However, exposure of PMN to fluid-phase Fn did not lead to EC3b phagocytosis (Fig. 1). Fn also increased monocyte phagocytosis of EA in a dose-dependent fashion and increased monocyte rosettes with both EC3b and EA as described (7, 8). In striking contrast, Fn affected none of these functions of neutrophils.

Binding of Fn-ms to Peripheral Blood Leukocytes.   To investigate the difference between monocytes and PMN in their phagocytic response to Fn, we first tested the hypothesis that neutrophils did not possess a membrane receptor for Fn. Therefore, we examined the binding of Fn-ms to freshly isolated human peripheral blood leukocytes. In initial experiments, monocytes and PMN were incubated with increasing numbers of Fn-ms, gelatin-ms, or BSA-ms for 30 min at 37°C. At all inputs of ms, <1% of the cells bound gelatin-ms or BSA-ms as assessed by fluorescence microscopy. In contrast, the majority of monocytes bound Fn-ms. These visual data were confirmed by flow cytometry. Whereas very little cell fluorescence was found when cells were incubated with gelatin-ms
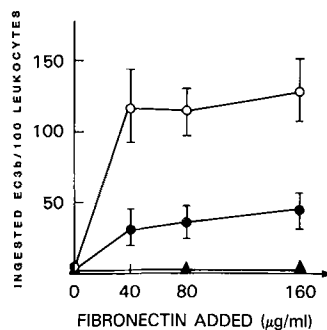

FIGURE 1.   The effect of Fn on phagocytosis by PMN and monocytes. The ingestion of EC3b by PMN and monocytes is plotted as a function of fluid-phase Fn concentration for monolayer monocytes (○), fluid-phase monocytes (●), and PMN (▲). None of the cells ingested EC3b in the absence of Fn. Whereas monocytes responded to Fn with EC3b phagocytosis, PMN did not. Data are represented as the mean ± standard deviation of five separate experiments.
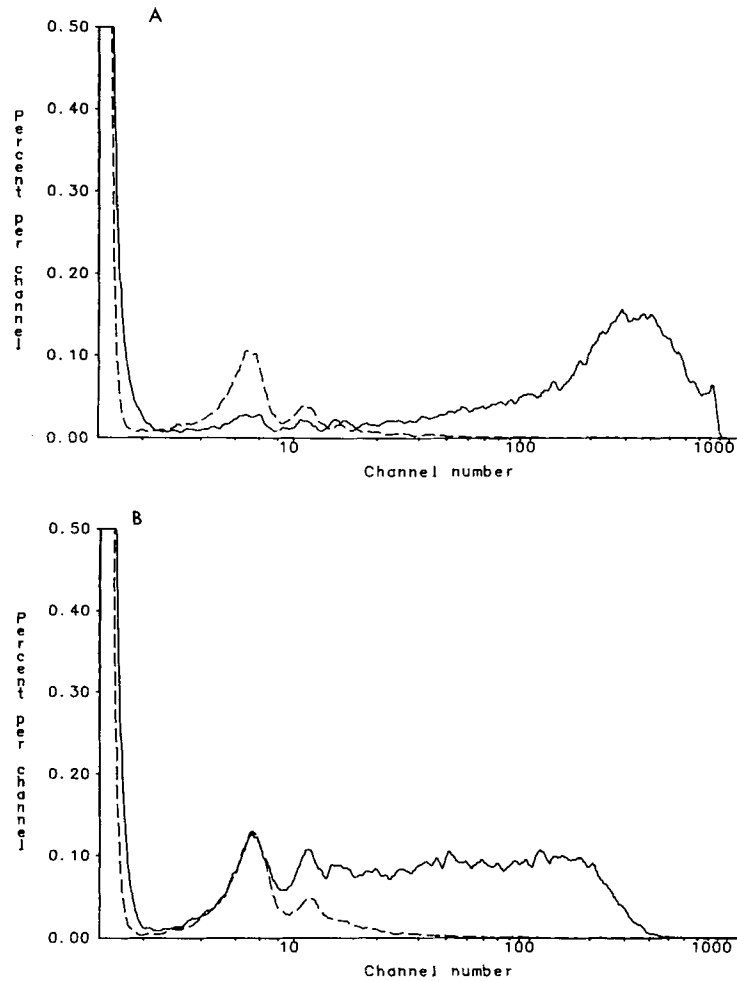
FIGURE 2.   Binding of gelatin-ms and Fn-ms by peripheral blood leukocytes. In *A*, a representative flow cytometric analysis of ms binding to monocytes is shown. *B* illustrates a representative analysis of binding by PMN. In both panels, the dashed line illustrates binding of gelatin-ms and the solid line shows the binding of gelatin-ms that were first incubated with 200 µg/ml of Fn. In both panels, binding of Fn-ms greatly exceeds binding of gelatin-ms. The average number of Fn-ms bound per monocyte exceeded the average number bound to individual PMN, although somewhat more PMN than monocytes bound Fn-ms.

or BSA-ms (<2% of cells bound more than one ms), a broad peak of fluorescence was obtained when monocytes were incubated with Fn-ms (Fig. 2*A*). As the ratio of ms to monocytes was increased, the percentage of monocytes binding to at least three Fn-ms reached a plateau at ~60% (Fig. 3). Examination of the monocytes of 18 different unrelated healthy blood donors with an optimal ratio of Fn-ms to monocytes revealed that 58 ± 5% SD of elutriator-purified monocytes specifically bound Fn-ms. When gelatin-ms were allowed to bind Fn before incubation with monocytes, binding of the ms also occurred (48 ± 7% monocytes positive, $n = 4$). This demonstrated that Fn-ms would bind to monocytes whether the Fn was incubated directly with ms or was allowed to bind to gelatin that had
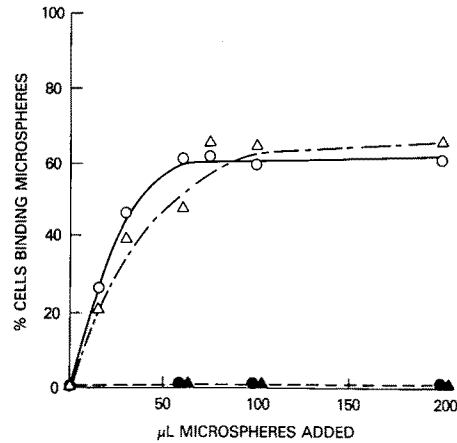
FIGURE 3. Dose response of Fn-ms binding to PMN and monocytes. The dose response of the addition of increasing numbers of Fn-ms (O, △) and gelatin-ms (●, ▲) to monocytes (O, ●) and PMN (△, ▲) is plotted as the percent of cells binding ms vs. the number of ms added. Gelatin-ms did not bind significantly to PMN or monocytes. Fn-ms binding to both cell types reached a plateau at similar inputs of ms. Since not all PMN or monocytes could bind Fn-ms, these data imply that there are subpopulations of PMN and monocytes which bind Fn.
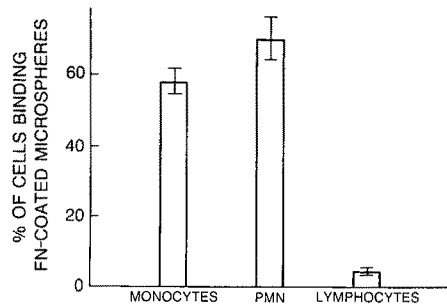

FIGURE 4. Binding of Fn-ms by PMN, monocytes, and lymphocytes. The number of cells binding Fn-ms was determined for PMN, monocytes, and lymphocytes of 15–18 different normal individuals. The data are represented as mean percent of cells binding ± SD.

been linked to the ms. Moreover, the elutriation technique for purifying monocytes was not selecting a subset of cells, since purification of monocytes on preformed Percoll gradients rather than by elutriation did not change the percentage of cells binding Fn-ms.

Although Fn had no effect on phagocytosis by freshly purified PMN, these cells also specifically bound Fn-ms (Fig. 2B). Like monocytes, PMN did not bind significant numbers of gelatin-ms or BSA-ms, and incubation of gelatin-ms with Fn led to ms binding similar to that when Fn-ms were incubated with PMN. The plateau for Fn-ms binding to PMN occurred at a bead/leukocyte ratio similar to that for monocytes (Fig. 3). In fact, a higher percentage of PMN (70 ± 8%, n = 15) than monocytes bound Fn-ms ($P < 0.001$) (Fig. 4). This was true when the monocytes and PMN of individual donors were compared, as well as for the population means. However, monocytes bound more Fn-ms per cell than PMN. The mean fluorescent channel for monocytes binding Fn-ms was 300, corre-
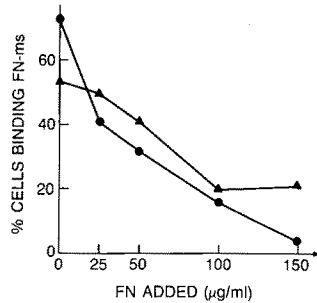
FIGURE 5.   Inhibition of Fn-ms binding by fluid-phase Fn. PMN (●) and monocytes (▲) were incubated with increasing concentrations of Fn and then assessed for binding of Fn-ms as described in Materials and Methods. Fluid-phase Fn inhibited the binding of Fn-ms to both cell types in a dose-related fashion.
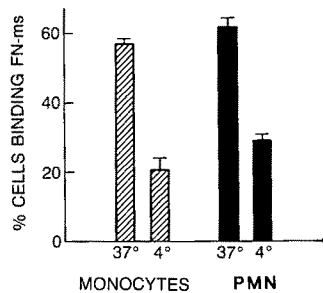

FIGURE 6.   Temperature dependence of Fn-ms binding. The ability of monocytes and PMN to bind Fn-ms was evaluated at 4°C and 37°C. Both cell types showed increased binding of Fn at elevated temperatures.

sponding to ~55 ms per cell; for PMN, it was 35, corresponding to seven ms per cell. Lymphocytes did not bind more Fn-ms than gelatin-ms or BSA-ms (Fig. 4).

To confirm the specificity of binding of Fn-ms, PMN and monocytes were incubated with fluid-phase Fn before mixing with Fn-ms bearing 1/10 the usual amount of Fn. A dose-dependent inhibition of Fn-ms binding by fluid-phase Fn was demonstrated for both PMN and monocytes (Fig. 5).

Effect of Temperature and Protease Inhibitors on Fn-ms Binding to PMN and Monocytes.   Fn-ms bound to both PMN and monocytes, yet, only monocytes were stimulated by Fn to induce phagocytosis of EC3b. Hence, we considered the possibility that the plasma membrane molecules that bound Fn differed for the two cell types. To investigate this possibility, several experiments were performed. As previously detailed, the binding of Fn-ms to both PMN and monocytes was inhibited by fluid-phase Fn, demonstrating that the membrane receptor on both cells recognize the Fn molecule in solution. We next compared the temperature dependence of the binding of Fn-ms to the two cell types. For both PMN and monocytes, Fn-ms binding was greatly decreased but not abolished at 4°C (Fig. 6). Because PMN enzymes can cleave Fn (21, 22), the possibility that cleavage of Fn was required for Fn-ms binding to PMN was considered. However, PMN and monocytes both bound equal numbers of Fn-ms in the presence and absence of 2 mM PMSF, an inhibitor known to prevent cleavage of Fn by PMN
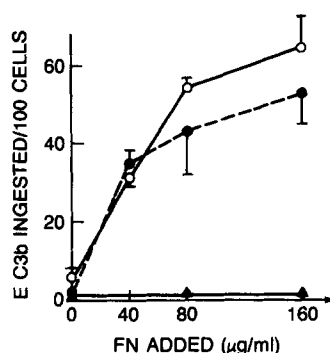
FIGURE 7.   Effect of C5a and fmet-leu-phe on the ability of Fn to induce EC3b phagocytosis by PMN. The ingestion of EC3b by PMN exposed to C5a (O), fmet-leu-phe (●), or buffer (▲) is plotted as a function of Fn concentration. In the absence of Fn, C5a- and fmet-leu-phe-stimulated PMN did not phagocytose EC3b. In the absence of C5a or fmet-leu-phe exposure, Fn did not induce phagocytosis of EC3b. When PMN were first exposed to either chemotactic agent, they showed an Fn dose-related ingestion of EC3b.

TABLE I

Inhibition of Fn-ms Binding by Monoclonal Anti-Fn IgG *

Monoclonal Antibodies	Percent inhibition[‡]	
	Monocytes (n = 5)[§]	PMN (n = 3)[§]
None	0	0
3G4-2	57.6 ± 2.7[∥]	80.1 ± 1.1
3C11	53.0 ± 3.4	82.1 ± 2.7
5C9	8.6 ± 2.1	17.9 ± 9.8
6D4	16.9 ± 7.6	11.7 ± 13.9

* Fn-ms were prepared with 1 μg of Fn, as described in Materials and Methods, before incubation with monoclonal antibodies.
‡ Percent inhibition = 100 × [1 − (cells binding Fn-ms incubated with monoclonal antibodies)/(cells binding Fn-ms in absence of monoclonal antibodies)].
§ n, number of different occasions on which each experiment was performed, singly or in duplicate.
∥ Mean percent inhibition ± SEM.

proteases (22).

Effect of Monoclonal Anti-Fn Antibodies on the Binding of Fn-ms to PMN and Monocytes.   We next used monoclonal anti-Fn antibodies to examine whether the PMN and monocyte Fn receptors recognized similar sites on the Fn molecule. Two monoclonal antibodies that inhibited Fn-ms binding to monocytes and two that did not were tested for their ability to inhibit ms binding to PMN. There was a complete correspondence between the inhibition of binding by the monoclonals on PMN and on monocytes, implying that the membrane Fn binding site on the cells recognized similar domains on the Fn molecule (Table I).

Effect of C5a and fmet-leu-phe on Fn-induced EC3b Phagocytosis by PMN.   The data comparing Fn-ms binding to PMN and monocytes suggested that the surface molecules that recognized Fn on the two types of phagocytic cells were similar. To determine if PMN could be induced to respond to Fn by increasing phagocytic activity, neutrophils were incubated with C5a or fmet-leu-phe for 10 min at 37°C

before exposure to Fn. PMN thus treated bound more EA and EC3b than did unactivated cells, as has been previously reported (23). However, PMN incubated with C5a or fmet-leu-phe remained unable to ingest EC3b in the absence of Fn. In marked contrast, when these activated neutrophils were incubated with Fn, they ingested EC3b efficiently and in a Fn dose-dependent manner (Fig. 7). Similar data were obtained with EA: although buffer-incubated cells did ingest some EA in the absence of Fn, Fn did not increase phagocytosis by these cells. In contrast, Fn increased EA ingestion by PMN incubated with C5a and fmet-leu-phe from 47 to 94 E per 100 PMN and from 42 to 82 E per 100 PMN, respectively. The dose-response curves for both the C5a and the fmet-leu-phe requirement for PMN phagocytosis of EC3b showed that increased concentrations of the chemotaxins led to increased effect (Fig. 8), as has been demonstrated for neutrophil granule secretion, without the high dose unresponsiveness that has been demonstrated in assays of PMN chemotaxis (24). Exposure of PMN to fmet-leu-phe or C5a was required before exposure to Fn to achieve efficient
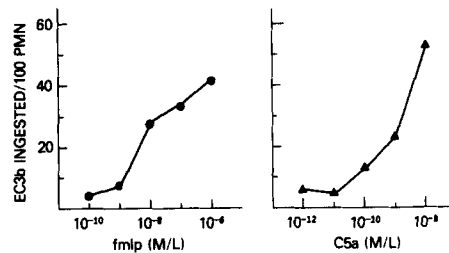

FIGURE 8.   Dose response of fmet-leu-phe (fmlp) and C5a effect on PMN. The effect of preincubation with fmet-leu-phe on subsequent EC3b ingestion by PMN incubated with 80 μg/ml of Fn is shown in the left panel. A similar dose-response curve for C5a is shown in the right panel.
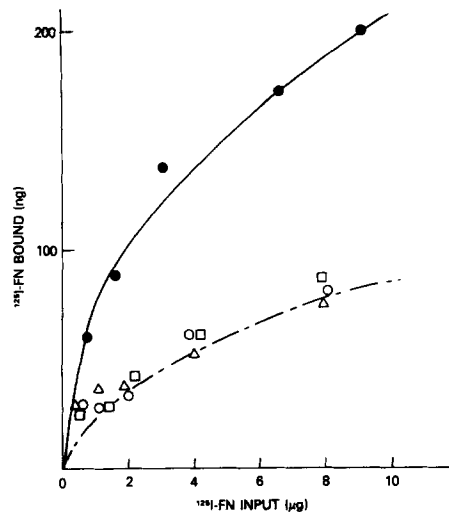

FIGURE 9.   Binding of ^{125}I-Fn by monocytes and PMN. The binding of ^{125}I-Fn by monocytes (●) and by PMN incubated with buffer (O), 10^{-8} M fmet-leu-phe (△), or 10^{-8} M C5a (□) is plotted as a function of ^{125}I-Fn input. Monocytes bound more ^{125}I-Fn than PMN. C5a and fmet-leu-phe incubation did not change the amount of ^{125}I-Fn binding by PMN.

phagocytosis of EC3b. When the order of exposure was reversed and the neutrophils were first exposed to 160 μg/ml Fn, a maximum of only nine EC3b per 100 PMN were ingested by cells subsequently incubated with fmet-leu-phe and only 16 EC3b per 100 PMN were ingested by cells subsequently incubated with C5a.

Since PMN bound fewer Fn-ms than monocytes, we tested whether C5a and fmet-leu-phe acted on PMN to increase the number of neutrophil Fn receptors. Using Fn-ms suboptimally coated with Fn, neither the number of cells binding Fn-ms nor the number of ms bound per cell changed after C5a or fmet-leu-phe treatment. This lack of effect of the PMN activators on Fn receptor number was confirmed by studies of the uptake of radiolabeled Fn, which showed that the curves for ^{125}I-Fn binding to buffer, C5a-, and fmet-leu-phe-treated PMN were similar (Fig. 9). At each input of radiolabel, less Fn was bound by either activated or buffer-treated PMN than by monocytes. This finding is in agreement with the differences noted between the two cell types in the binding of Fn-ms.

Discussion

In this study we have investigated both the morphological characteristics and the functional consequences of the binding of Fn by human peripheral blood phagocytic cells. We have been able to study the interaction of Fn with PMN and monocytes in two independent ways. One approach involved direct study of the binding of either Fn-ms or ^{125}I-Fn to the cells. The other required that Fn affect the cell membrane in a way to cause the phagocytic cell to recognize C3b bound to E, normally only a signal for leukocyte adherence, as a signal for phagocytosis. This latter functional assay apparently required Fn effects on the cell beyond simple membrane binding since freshly isolated PMN bound both Fn-ms and fluid-phase Fn, but were not stimulated by Fn to ingest EC3b. The disparity between human peripheral blood monocytes and PMN in this latter phagocytic assay was the starting point for the investigations reported here.

There is evidence from a number of systems that monocytes and macrophages bind Fn at the plasma membrane (7–10). It has been suggested (25) that fluid-phase Fn will not bind to cells and that a conformational change in Fn, induced by binding to other molecules, is required for cell binding. However, our data clearly demonstrate that fluid-phase Fn will bind to monocytes. Free Fn inhibited the binding of Fn-ms to monocytes, ^{125}I-Fn in solution bound specifically to monocytes, and Fn coupled directly to ms bound to monocytes, as efficiently as Fn that adhered to gelatin-ms. Since Fn that was aggregated on a fluorescent microsphere or a gelatin-coated latex bead would have higher avidity for the receptor than monomeric Fn, the major effect of ligand binding by Fn for subsequent cell binding may be to aggregate Fn molecules rather than to expose a cryptic cell-binding domain by inducing a conformational change in the molecule. This hypothesis is supported by the identification and isolation of a cell (fibroblast)-binding fragment of Fn that is exposed in the native molecule and that retains its ability to bind to cells when cleaved from all other domains of the molecule necessary for ligand binding (26).

The existence of a plasma membrane receptor for Fn on PMN has been less certain. Although some investigators have been able to demonstrate adherence

of Fn-coated particles to PMN (27, 28), in other systems, binding of PMN to Fn has not been observed (8). No investigators have been able to demonstrate that PMN will ingest particles opsonized solely by Fn. In this study, we have demonstrated that PMN indeed can bind Fn at the cell membrane. However, binding is quantitatively less than for monocytes, as judged by both Fn-ms and ^{125}I-Fn binding. This may explain the discrepancies among other investigations since, in systems that require high avidity interaction, PMN binding to Fn might not be observed. As with monocytes, PMN binding of Fn-ms was inhibitable by sufficient concentrations of fluid-phase Fn, and PMN bound ^{125}I-Fn directly, implying that a ligand-induced conformational change in the Fn molecule was not required for cell binding.

The heterogeneity among peripheral blood PMN and monocytes with respect to Fn-ms binding suggests that this property may mark a subset of each of these cell types. Although it is possible that cells bearing Fn receptors were lost during the assay of phagocytes in the fluid phase, it is interesting that the percentage of monocytes that bound Fn-ms was equal to the percentage that were stimulated by Fn to ingest opsonized E (cf., Fig. 2 in reference 7). Since this was true of monocytes that were allowed to adhere to glass before assay, as well as of fluid-phase cells, it is likely that Fn-ms binding marks a monocyte subpopulation that will be stimulated by Fn to phagocytose. Because of the low number of E ingested by PMN, it was impossible to be certain that Fn-ms binding correlated with E ingestion for these cells. However, the percentage of PMN engaged in Fn-induced phagocytosis never exceeded the number that bound Fn-ms.

A striking finding in this study is that, as isolated from the blood, only monocytes are induced by Fn to ingest EC3b, although both PMN and monocytes possess membrane receptors for the Fn molecule. Since Fn is known to be able to bind to a wide variety of ligands through different domains of the molecule, we tested whether the difference in functional consequences of Fn binding could be explained by differences in the portion of the molecule recognized by the cells. However, several lines of investigation suggested that the Fn receptors on PMN and monocytes were quite similar and recognized very similar domains on Fn. Binding of Fn-ms to both cell types was inhibited by fluid-phase Fn at similar concentrations, implying that there was no major difference in the affinity of the Fn-receptor interaction between PMN and monocytes. The two cell types showed similar decreases in binding Fn-ms at 0°C, and neither required Fn cleavage by cellular proteases before binding the Fn-ms. Most importantly, monoclonal anti-Fn antibodies had similar effects on Fn-ms binding by monocytes and PMN. Anti-Fn monoclonals that did not block binding to monocytes did not block binding to PMN; similarly, monoclonals that blocked ms binding to one cell blocked binding to the other as well. Thus, it was unlikely that the difference in the functional effect of Fn binding to PMN and monocytes could be explained by the presence of different membrane receptor molecules on the two cell types.

The apparent similarities between the PMN and the monocyte membrane receptors for Fn led to an investigation of whether PMN could be induced to demonstrate a functional change upon exposure to Fn. We tested whether activation of PMN by a separate signal is required for Fn to induce PMN ingestion of EC3b. Indeed, exposure of PMN to C5a and fmet-leu-phe did cause

PMN to phagocytose EC3b upon subsequent incubation with Fn. Thus two stimuli were required for ingestion of EC3b by PMN. This contrasts strikingly with monocyte ingestion of EC3b, for which Fn alone was sufficient to induce phagocytosis. The two signals to the PMN did not have independent effects since, if the order in which they were incubated with PMN was reversed, no EC3b phagocytosis was induced. Exposure to C5a or fmet-leu-phe was required to make PMN responsive to Fn. Since PMN have fewer Fn receptors than monocytes, it was possible that C5a and fmet-leu-phe caused PMN to become responsive to Fn by increasing the number of Fn receptors. However, chemotaxin exposure did not induce a change in Fn receptor number, as judged by both Fn-ms and ^{125}I-Fn binding. Therefore, freshly isolated PMN possess a sufficient quantity of Fn receptors to mediate the Fn effect on phagocytosis of EC3b. Rather, C5a and fmet-leu-phe must affect the nature of the transmembrane signal generated by the occupancy of the Fn receptor.

The mechanisms by which these changes occur in the phagocytic state of PMN and monocytes are unknown. The number of monocyte IgG Fc membrane receptors is not influenced by Fn (7), even though the ingestion of EA is dramatically increased. The number of PMN Fn receptors is not changed by C5a or fmet-leu-phe, even though the cell passes from a Fn-unresponsive to a Fn-responsive state. Thus, the effects on phagocytosis demonstrated here are unlikely to reflect changes in the number of expressed receptors and more likely arise from changes in the interaction of the receptors with other membrane proteins and the cell cytoskeleton. It is possible that, after C5a or fmet-leu-phe exposure, the mobility of the Fn receptors in the membrane is changed (29), that new associations with cytoskeletal elements are induced (30), or that a new physical association of the Fn receptor with the C3b receptor occurs (31). The potential physiologic importance of this requirement for activation of PMN before Fn-induced phagocytosis of EC3b will occur is clear. The phagocytic process is accompanied by the release of proteolytic enzymes and mediators of inflammation potentially harmful to the host. The requirement for activation by chemotactic factors before the full phagocytic potential of PMN is achieved would effectively limit phagocytosis by granulocytes to the inflammatory foci into which they have migrated.

Summary

We have investigated the interactions between plasma fibronectin (Fn) and human peripheral blood phagocytic cells. As shown by studies of the binding of Fn-coated fluorescent microspheres (Fn-ms), both polymorphonuclear leukocytes (PMN) and monocytes had specific binding sites for Fn at the plasma membrane. However, as purified from blood, only monocytes were stimulated by Fn to become more actively phagocytic. This increase in phagocytosis was reflected by an Fn-induced increase in the ingestion of IgG-coated erythrocytes and, more dramatically by an Fn-dependent initiation of phagocytosis of C3b-coated erythrocytes. Despite this difference between PMN and monocytes in the functional consequences of Fn binding, the cell surface molecules responsible for Fn binding on the two cell types shared many characteristics. On both cells, binding of Fn-ms was inhibited by sufficient concentrations of fluid-phase Fn; both PMN and

monocytes bound fewer Fn-ms at 4°C than at 37°C; both achieved maximal binding at similar Fn-ms/cell ratios; and phenylmethylsulfonyl fluoride did not inhibit Fn-ms binding to either cell type. Most dramatically, monoclonal anti-Fn antibodies that inhibited binding of Fn-ms to one cell type inhibited binding to both; conversely, monoclonal anti-Fn antibodies that did not inhibit Fn-ms binding to either cell type did not inhibit binding to the other.

Fn will stimulate PMN to a more actively phagocytic state, like that induced in monocytes, if the PMN are first exposed to C5a or N-formyl-methionyl-leucyl-phenylalanine. This effect occurs without apparent change in the number of Fn receptors. We conclude that the PMN and monocyte receptors for Fn are very similar, but that their milieu is very different in the two cells as purified from peripheral blood. Whereas Fn induces increased phagocytosis in monocytes, PMN must be activated before the Fn can be effective.

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