Fibronectin (Fn) is a 440,000-dalton glycoprotein found in plasma at a concentration of ~300 µg/ml (1). The structure of the molecule has been carefully examined, and domains that bind gelatin, heparin, and Staphylococcus aureus have been described (2). Fibronectin also mediates the binding of gelatin-coated particles to Kupffer cells in vivo and in vitro (3), and it has been alleged to be an important component in the optimal function of the reticuloendothelial system (RES) (4). However, the role of Fn in normal host phagocytic function remains unclear. While some investigators have gathered evidence that Fn is an opsonin (5, 6), others have been unable to show that Fn-coated particles will be phagoeytosed (7, 8). In the experiments reported here, we have discovered that Fn binding to particles is not required for Fn to affect their ingestion by human peripheral blood monocytes. Fn enables monocytes to phagocytose erythrocytes bearing C3b molecules and increases the phagocytosis of IgG-sensitized erythrocytes without binding to the opsonized particles. Thus, Fn may be important in host defense in the development and maintenance of an optimally phagocytic state in the mononuclear phagocytes of the RES.

**Materials and Methods**

**Buffers.** Isotonic Veronal-buffered saline (VBS), VBS containing 0.1% gelatin, 0.15 mM Ca ++, and 1 mM Mg ++ (GVB++), low ionic strength isosmotic Veronal-buffered saline with dextrose, gelatin, Ca ++, and Mg ++ (DGVB), and VBS containing gelatin and 10 mM ethylenediaminetetraacetate (EDTA-GVB) were all prepared as previously described (9). RPMI 1640 was obtained from the Media Unit, National Institutes of Health.

**Purification of Fn.** Human plasma Fn was purified by a modification of the method of Vuento and Vaheri (10). Briefly, 1 U of plasma was diluted while stirred with a buffered

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**Abbreviations used in this paper:** DGBV, Veronal-buffered saline with dextrose gelatin Ca ++ and Mg ++; E, sheep erythrocytes; EA IgM and EA IgG, sheep erythrocytes sensitized with IgM or IgG anti-Forsmann antibody; EDTA, ethylenediaminetetraacetate; EDTA-GVB, Veronal-buffered saline containing gelatin and 10 mM ethylenediaminetetraacetate; Fn, fibronectin; GVB++, Veronal-buffered saline containing 0.1% gelatin, 0.15 mM Ca ++, and 1 mM Mg ++; HSA, human serum albumin; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PMSF, phenylmethyl sulfonyl fluoride; RES, reticuloendothelial system; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VBS, isotonic Veronal-buffered saline.
inhibitor solution containing 1 M KH₂PO₄, 0.2 M Na₂EDTA, and 0.2 M benzamidine HCl, pH 7.4 (20 parts plasma to 1 part inhibitor). Phenylmethyl sulfonyl fluoride (PMSF) was then added to a final concentration of 1 mM. The inhibitor-treated plasma was then made 5% wt/vol in polyethylene glycol 3350 (PEG) (J.T. Baker Chemical Co., Phillipsburg, NJ). The precipitate that formed after 1 h at 4°C was resuspended in 150 mM NaCl, 50 mM KH₂PO₄/K₂HPO₄, and 10 mM EDTA, pH 7.4, and snap frozen by adding approximate 20-μl drops directly to liquid N₂. The pellets thus formed were stored at −70°C until further use. Approximate 15-ml aliquots of this 5% PEG precipitate were thawed and passed over a 5-ml column of Sepharose 4B. The protein-containing fractions were pooled and passed over gelatin-Sepharose prepared as described (10). The column was then thoroughly washed and eluted as described. The protein-containing fractions were pooled, dialyzed vs. 0.05 M Tris-HCl to remove arginine, centrifuged for 20 min at 12,000 g, and passed over arginine-Sepharose. The retained material was eluted with a buffer of 0.1 M NaCl, 0.05 M Tris, pH 7.5. All buffers used for the chromatography on Sepharose, gelatin-Sepharose, and arginine-Sepharose contained 5 mM benzamidine and/or 1 mM PMSF to inhibit the possible activity of contaminating proteases on Fn. The purified Fn showed a single line on immunoelectrophoresis vs. anti-whole human serum and a single major band at 440,000 daltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). On reduction of disulfide bonds, a closely spaced doublet was seen at 220,000 daltons as has been previously reported for human plasma Fn (1, 2). With increased sample size, the doublet was obliterated, but these heavily loaded gels showed no lower molecular weight bands even when developed with a sensitive silver stain (Bio-Rad Laboratories, Richmond, CA) (Fig. 1). Antibody prepared against this antigen in a rabbit gave a monospecific response on immunoelectrophoresis and Ouchterlony double diffusion against whole human plasma.

Monocyte Preparation. Peripheral blood mononuclear cells were separated from the buffy coat from 500 ml of blood of normal volunteers on Ficoll-Hyphaque gradients. Monocytes were isolated using a J2-21 centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-6 elutriator rotor with two Sanderson's separation chambers following a procedure modified from Lionetti et al. (11). The purity of the monocytes was >95% as determined by both morphology and nonspecific esterase staining (Technicon Instruments Corp., Tarrytown, NY).

Antibody and Complement Components. C1, C4, and C2 were purchased from Cordis Laboratories Inc., Miami, FL. Rabbit IgG and IgM anti-Forssman (12) and human C3 were prepared as previously described (13). C3b was prepared by trypsin incubation of purified C3 followed by Biogel A 0.5-m chromatography as previously described (14).

Optimized Sheep Erythrocytes. Sheep erythrocytes (E) were collected, washed, and stored using standard methods (12). For preparation of C3b-opsonized particles, E were sensitized with IgM anti-Forssman antibody (EAIgM). C1, C4, C2, and C3 were then added sequentially as previously described (15). The concentration of C4 was limited to prevent interaction of EAIgM C14 with C3b receptors. EAIgM C14 were incubated with 5 U of C3 to yield 100-200 C3 molecules per cell. Following sensitization with C3, EAIgM C1423b were incubated for 1 h in EDTA-GVB to remove C1 and to allow C2 to decay, and then washed twice in GVB++. For preparation of EAIgM, 1 × 10⁹ 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++ (EAIgM).

Rosetting and Phagocytosis Assay. For assay of leukocytes in suspension, 50 μl of cells at 2 × 10⁸/ml in RPMI 1640, supplemented with 5 mM MgCl₂, were incubated for 30 min in a 5% CO₂/95% air incubator at 37°C with Fn or as a control, Fn passed over gelatin-Sepharose immediately before the experiment. Except where noted, the cells were then mixed with 50 μl of EAIgM C143b, or EAIgM 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% CO₂/95% air incubator at 37°C without mixing, and then gently resuspended and examined under light microscopy. In assays of monocyte monolayers, monocytes were suspended at 2 × 10⁶ cells/ml in RPMI 1640 without added protein; 250 μl was added to each chamber of an eight-chamber tissue culture plate (Lab-Tek Div. Miles Laboratories Inc., Naperville, IL); and the cells were allowed to adhere to glass during a 45-min incubation in a 5% CO₂, 37°C incubator. The cells were then washed in RPMI 1640. Various concentrations of Fn, buffer, or
Fig. 1. SDS-PAGE of purified fibronectin (Fn). 5 μg of the purified Fn preparation was electrophoresed in 4-10% SDS-PAGE gels both in the presence (left lane) or the absence (middle lane) of 50 mM dithiothreitol. The right lane contains Bio-Rad high molecular weight standards (myosin, Mr 200,000; β-galactosidase, Mr 130,000; phosphorylase B, Mr 93,000; bovine serum albumin; Mr 68,000; and ovalbumin, Mr 43,000). After electrophoresis, the gel was stained with the Bio-Rad silver stain.

The supernatant of an absorption of the Fn preparation with gelatin-Sepharose (done immediately before the experiment) were added to the cells, and incubation was allowed to proceed for a further 30 min. After incubation, indicator particles were added to the cells, and incubation was allowed to proceed for a further 30 min. After incubation, indicator particles were added to the wells, the plates centrifuged at 50 g for 5 min, and an additional incubation was carried out as described for fluid phase cells. After this incubation, chambers were washed two times, fixed with 0.5% glutaraldehyde, and stained with Giemsa stain. To quantitate phagocytosis, assays were carried out exactly as described, except that an erythrocyte lysis step (hypotonic lysis, in 1 part phosphate-buffered saline [PBS] and 4 parts H₂O, with vigorous mixing for several seconds) was added before fixing, staining, and counting. In all experiments, at least 200 leukocytes were counted to quantitate erythrocyte rosetting and/or ingestion. Three or more erythrocytes bound to a leukocyte was considered a rosette. Total intracellular erythrocytes were counted as a function of the number of monocytes observed.

Preincubation of Monocytes or Indicator Particles with Fn. In some experiments, monocytes or EAaM C43b were incubated with Fn and washed before mixing for assays of particle ingestion. For these studies, fluid phase monocytes were incubated for 30 min at 37°C with varying concentrations of Fn in RPMI/Mg⁷⁺ buffer or with buffer alone and then washed in warm buffer. This wash removed >95% of the Fn as determined by inclusion of 125I-Fn in the
incubation mixture. Thereafter, monocytes and EA_{igM} C43b, which had been incubated with buffer, were mixed, and ingestion was quantitated as described. Parallel sets of experiments were performed in which EA_{igM} C43b were preincubated with Fn and then mixed with monocytes which had been incubated in buffer.

Determination of Fc Receptor Number. The binding of 125I-labeled monomeric myeloma IgG1 was used to determine the number of IgG receptors present on monocytes before and after incubation with Fn. For these experiments, monocytes were incubated with RPMI 1640 supplemented with 5 mM MgCl2 or this buffer containing 20 μg/ml Fn for 30 min at 37°C before performance of the binding studies, which were then carried out exactly as previously described (16).

Results

Effect of Fn on Monocyte Rosetting and Ingestion of EA_{igG}. The ability of fluid phase Fn to affect monocyte rosetting and ingestion of EA_{igG} was examined. In preliminary experiments, IgG anti-Forssman was titrated so that in the absence of Fn, rosetting was less than maximum. Incubation of monocytes with Fn in concentrations of 40-160 μg/ml caused a modest increase in the number of monocyte rosettes. Fn caused a dose-related increase in rosetting for both fluid phase and monolayer monocytes from 13% to a maximum of 55%. Monocytes did not form rosettes with E or EA_{igM} with or without Fn.

Fibronectin caused a marked increase in the number of EA_{igG} ingested by monocytes (Fig. 2A). As with rosetting, Fn caused a dose-related increase in ingestion for both fluid phase and monolayer monocytes. For fluid phase monocytes the number of EA_{igG} ingested increased from 113 ± 15.9 to 250 ± 44.4 with 160 μg Fn/ml (P <
For monolayer monocytes, a plateau was reached at a lower concentration of Fn than for fluid phase monocytes with a maximum of 296 ± 24.5 EAgG ingested per 100 monocytes at 40 µg/ml Fn. When the percentage of monocytes ingesting EAgG was quantitated with and without Fn, only a small increase, from 48% to 61%, was observed (Fig. 2, inset). Thus, most of the increased ingestion of the antibody-sensitized erythrocytes resulted from more avid phagocytosis by a portion of the monocytes in the presence of Fn. As was the case in every experiment, gelatin-Sepharose absorption of the Fn abolished its effect on monocyte phagocytosis of EAgG. When fluid phase monocytes were incubated under identical conditions with equivalent amounts of C3b, human serum albumin (HSA), and Fn, Fn caused a twofold increase in total ingestion of EAgG; neither HSA nor C3b affected the ability of monocytes to bind or ingest EAgG.

Effect of Fn on Monocyte Rosetting and Ingestion of EAgM C43b. The effect of Fn on rosette formation and ingestion by monocytes with EAgM C43b was examined in experiments analogous to those performed with EAgG. As in the case of IgG, a C3 input was chosen in the preparation of EAgM C43b that was less than that which would produce optimal rosetting with monocytes. Fn caused a dose-related increase in the percentage of monocytes forming rosettes from 15% to 32% with fluid phase monocytes, and from 18% to 40% with monolayer monocytes. No phagocytosis of these E bearing C3b as the only opsonin was seen in the absence of Fn for either fluid phase or monolayer monocytes. The inability of monocytes to ingest C3b-coated erythrocytes in these experiments is in agreement with previous observations (17). However, incubation of monocytes with Fn led to phagocytosis of EAgM C43b by both fluid phase and monolayer cells. The extent of ingestion was dependent on the Fn input (Fig. 2B). As had been observed with EAgG ingestion, C3b-mediated ingestion by Fn-treated monocytes was quantitatively greater for monolayer monocytes, and the Fn dose response reached a plateau at lower concentrations of Fn. For both EAgG and EAgM C43b, addition of 0.1% gelatin to the buffer did not affect the Fn enhancement of phagocytosis.

To examine whether Fn was interacting with the monocyte or the opsonized particle to cause ingestion, Fn was preincubated with EAgM C43b or with monocytes alone and removed by washing as described in Materials and Methods. Complement-sensitized erythrocytes that had been preincubated with varying doses of Fn were mixed with monocytes that had been incubated in buffer. In other tubes, monocytes that had been preincubated with Fn were mixed with EAgM C43b that had been incubated in buffer. As a positive control, some monocytes were incubated with Fn, and indicator particles were added without washing, exactly as in the usual protocol. The results are shown in Fig. 3. Phagocytosis of EAgM C43b occurred when monocytes were preincubated with Fn or when Fn was present throughout the assay. Significantly, no phagocytosis occurred when only EAgM C43b had been incubated with Fn. Thus, a direct interaction of Fn with monocytes, but not with opsonized particles, was required for ingestion of EAgM C43b.

Several experiments were performed to ensure that the biologic activity of the Fn preparation on monocytes was not caused by a trace contaminant in the preparation. The ability of gelatin-Sepharose to remove the phagocytosis-promoting activity from Fn preparations was examined and compared to protein-A Sepharose (because IgG may be a contaminant of Fn preparations [18]) (Table I). Absorption with 1 ml of
Fig. 3. Preincubation of monocytes and target cells with fibronectin (Fn). Fluid phase monocytes (▲) or EA<sub>lgG</sub> C43b (△) were incubated with Fn for 30 min at 37°C, washed, and then mixed with buffer-incubated EA<sub>lgM</sub> C43b or monocytes. As a positive control monocytes were incubated with Fn and then with EA<sub>lgM</sub> C43b without washing (●). Phagocytosis is plotted as a function of Fn concentration. Only when monocytes were exposed to Fn does erythropagocytosis occur.

Table I

| Absorption       | Erythrocytes ingested/100 monocytes |
|------------------|-------------------------------------|
|                  | EA<sub>lgG</sub> | EA<sub>lgM</sub> C43b |
| None             | 203           | 69                   |
| Sepharose 4B-CL  | 210           | ND<sup>†</sup>       |
| Protein A-Sepharose | 215   | 72                   |
| Gelatin Sepharose | 106           | 0                    |
| No Fn added      | 114           | 0                    |

* 200-μg aliquots of Fn, purified as described, were incubated with 1 ml of Sepharose 4B-CL, with protein A Sepharose, or with gelatin Sepharose. The absorbed supernatant was then assayed for phagocytosis-promoting activity using EA<sub>lgG</sub> or EA<sub>lgM</sub> C43b. Gelatin-Sepharose absorbed all phagocytosis promoting activity, but protein A Sepharose or Sepharose alone did not affect the biologic activity of the Fn preparation.

† ND, not done.

gelatin-Sepharose entirely removed the phagocytosis-promoting activity of 200 μg of the Fn preparation for EA<sub>lgG</sub>, but the activity was unaffected by passage over similar amounts of protein-A Sepharose or Sepharose 4B-Cl alone. In fact, supernatants from gelatin-Sepharose-absorbed Fn were routinely used as a negative control in all experiments on Fn-enhanced phagocytosis of EA<sub>lgG</sub> and EA<sub>lgM</sub> C43b; rosetting and ingestion in these tubes never exceeded binding and phagocytosis in tubes containing buffer alone.

Because of a report that the phagocytosis-enhancing effect of Fn preparations for alternative pathway activators resides in a 180,000-dalton co-purifying protein (19–21), our Fn preparation was chromatographed on a calibrated Biogel A 1.5-M column (Fig. 4). If the phagocytosis-promoting activity were smaller than the Fn protein, fractions lighter than the main protein peak should have contained biologic activity. However, the biologic activity of the preparation exactly co-chromatographed with the 440,000-dalton protein peak.

Because many investigators use 4 M urea to elute Fn from gelatin Sepharose (1),
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Fig. 4. Biogel A 1.5m chromatography of purified fibronectin (Fn) and of phagocytosis-promoting activity. Fn, purified as in Materials and Methods, was chromatographed on a calibrated Biogel A 1.5m column. Fractions were assayed for protein concentration by absorbance at 280 nm (○) and for phagocytosis-promoting activity by ingestion of EA IgG after incubation of 1 × 10⁶ monocytes with 50 μl of the various column fractions (▲). The phagocytosis-enhancing activity coincides with the Fn protein peak.

Fig. 5. Binding of IgG to monocytes. ¹²⁵I-IgG binding was assessed to monocytes which had been incubated in buffer (○) or with 20 μg/ml Fn (▲). The binding curves for the Fn- and buffer-incubated cells overlie one another. Scatchard transformations of the binding curves show that the control cells showed an average of 28,700 IgG-binding sites per cell with $K_a = 4.03 \times 10^6$ M⁻¹, while Fn-incubated cells showed 31,900 IgG binding sites per cell with $K_a = 3.57 \times 10^6$ M⁻¹. These values for Fn- and buffer-incubated cells are not statistically significantly different.

this procedure was used to purify Fn, and the eluted material was examined for biologic activity. Although slightly less effective on a weight basis than Fn purified as described above, this urea-eluted material also promoted ingestion of EA IgG and EA IgM C43b.
Enumeration of Fc receptors on Fn-Treated Monocytes. To examine the possibility that Fn augmented EAI~ phagocytosis by increasing the expression of Fc receptors on monocytes, Fc receptors were enumerated in a well-characterized quantitative assay for the binding of monomeric monoclonal IgG1 (Fig. 5). Monocytes that had been incubated with 20 μg/ml Fn for 30 min at 37°C showed no difference in IgG receptor number (30 × 10^5/cell) or affinity (3.8 × 10^8 M⁻¹) compared to monocytes incubated with buffer.

Discussion

In 1969 Saba and DiLuzio (22) suggested that depletion of a plasma protein could be responsible for RES dysfunction in experimental RES blockade. This opsonie protein could mediate the binding of gelatin-coated particles to Kupffer cells in vivo and in vitro (23). Subsequent work from several laboratories showed that this protein, α2 opsonic surface-binding glycoprotein, was identical to cold insoluble globulin, or Fn (4). Recent work by Bevilacqua et al. (8) demonstrated a receptor for Fn on human monocytes. Taken together, these data suggested that Fn might play an important role in monocyte and macrophage phagocytosis. Our initial experiments using E showed that Fn could markedly affect monocyte binding and phagocytosis but only when the erythrocytes were already opsonized. This suggested that Fn acted directly on the monocyte without binding to the phagocytic target, unlike conventional opsonins such as IgG or complement. In support of this hypothesis we found that incubation of erythrocyte targets with Fn had no effect on subsequent monocyte phagocytosis.

The process of particle phagocytosis may be divided into phases of adherence and ingestion. In our experiments, adherence of both IgG- and C3b-opsonized E to monocytes was somewhat increased by Fn, which was in agreement with the work of Bevilacqua et al. The more marked effect in our experiments, however, was on the ingestion of opsonized particles. This was particularly true for EAI~ C43b, which were not ingested in the absence of Fn, but which were phagocytosed to the same extent as EAI~ when Fn was present during the incubation. Although this increase in phagocytosis was not found by Bevilacqua et al., the complement-coated particles used in their experiments were less well characterized than in ours since they used mouse serum rather than purified human components as a source of complement. Under the conditions of incubation, it is probable that most of the C3 on their cells was in the form of C3bi or C3d, rather than C3b.

A number of experiments were done to examine whether the biologically active molecule in our Fn preparations was native Fn. Great care was taken during purification of the Fn, particularly during plasma collection, to inhibit enzymatic degradation of native Fn. The use of denaturing buffers containing urea or guanidine was deliberately avoided in order to attempt to preserve the native configuration of plasma Fn. The biological activity of the Fn preparation was not a trace contaminant such as endotoxin or IgG since the activity was absorbed by gelatin Sepharose but not by protein A-Sepharose. Moreover, the biologic activity co-chromatographed with the native protein on a sieving column. These facts led us to conclude that the biologic activity resided in the 440,000-dalton native Fn. However, our data do not exclude the possibility that the tertiary structure of the Fn molecule is changed without cleavage during purification or that a cleavage is made that does not significantly
Several experiments were done to characterize the mechanism by which Fn enhances phagocytosis. These experiments showed that fluid phase Fn influenced monocyte binding and phagocytosis of opsonized particles through interaction with the monocyte and did not require the concomitant presence of the phagocytic target for its effect. This presumably was mediated via a cell membrane receptor for the Fn molecule (8). One possible explanation for the mechanism of the Fn effect on monocytes is that incubation with Fn increases the membrane expression of Fc and C3b receptors. This hypothesis was tested in an experiment in which Fc receptors were enumerated, and their affinity measured, on monocytes with and without Fn preincubation. Fn had no effect on the number or affinity of monocyte receptors for monomeric IgG. Thus, by this assay, membrane expression of phagocytic receptors was not changed by Fn incubation. Although Fn incubation did increase the number of rosettes formed with EAIgG, monocyte Fc receptor number does not correlate well with rosette formation (Fries, L., S. Inada and M. Frank, unpublished data). Griffin et al. (25) have presented evidence that a lymphokine causes ingestion of complement-coated erythrocytes by increasing the mobility of the C3 receptor in the macrophage membrane. It is tempting to speculate that this may also be the mechanism of action of Fn. Increased Fc and C3b receptor mobility in the monocyte membrane after incubation with Fn could explain both the observed increase in rosetting without increase in Fc receptor number and the increased ingestion of opsonized particles. Details of the mechanism by which Fn affects monocyte membranes await further study.

Summary

We have investigated the effect of plasma fibronectin (Fn) on binding and phagocytosis of sheep erythrocytes (E) by human peripheral blood monocytes. Unopsonized E were not phagocytosed in the absence or presence of Fn, but Fn enhanced the phagocytosis of E bearing IgG. Sheep erythrocytes sensitized with IgM and C3b were ingested only when monocytes were exposed to Fn. The Fn enhancement of phagocytosis occurred for both fluid-phase and glass-adherent monocytes. Experiments in which Fn was washed out before mixing monocytes with opsonized E demonstrated that the Fn effect occurred because of interaction with the monocytes and not the opsonized particles. Chromatography of the Fn on Biogel A 1.5m showed that the phagocytosis-enhancing activity exactly co-chromatographed with the Fn protein. Fn did not increase the number of monocyte membrane receptors for the Fc fragment of monomeric IgG. We conclude that Fn enhances monocyte phagocytosis, not by binding to particles as a conventional opsonin, but by stimulating monocytes to ingest already opsonized particles more avidly.

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