Phagocytosis of Gelatin-Latex Particles by a Murine Macrophage Line is Dependent on Fibronectin and Heparin

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ABSTRACT It has been suggested that fibronectin plays a role in clearing particles from the circulation by promoting binding to phagocytes of the reticuloendothelial system. By use of a well-defined system to investigate the possible opsonic role of fibronectin, we have studied the uptake of gelatin-coated latex particles by a murine macrophage cell line ($P388D_1$).

Fibronectin promotes binding of gelatin-coated beads to these cells in both suspension and monolayer cultures. In both cases there is a requirement for heparin as a cofactor. Other glycosaminoglycans (chondroitin sulfates A and C, dermatan sulfate, and keratan sulfate) were inactive, whereas heparan sulfate was somewhat active.

Proof that beads were actually endocytosed was obtained by electron microscopy, which showed beads internalized in membrane-bounded vesicles, and by immunofluorescence analyses, using antibodies to fibronectin to stain external beads. Two rapid assays for the opsonic activity of fibronectin were developed based on differential centrifugation of cell-associated beads and on the immunofluorescence procedure. Binding and endocytosis were time- and temperature-dependent and varied with the amount of gelatin on the beads and with the concentrations of fibronectin and heparin added, and could be inhibited by $F(ab')_2$ antifibronectin. These studies provide a sound basis for a detailed analysis of the interaction of fibronectin with the cell surface and of its involvement in endocytosis.

Phagocytosis is the process by which particles (bacteria, damaged cells, fibrin aggregates, etc.) are bound and endocytosed by phagocytic cells. Recognition of these particles by the cells is due to the presence of humoral factors (opsonins) such as immunoglobulins or complement, which coat the particles and promote their phagocytosis (44). Recently, an α-2-globulin from plasma has been shown to facilitate clearance of certain particles from the circulation (31, 32, 42). It also promotes the binding of particles to liver slices (12, 16, 31, 40) and the uptake of gelatin-coated particles by rat peritoneal macrophages (13, 18).

This α-2-globulin has recently been purified from rat and human sera and found to be immunologically and functionally identical with plasma fibronectin (2, 33), a dimeric glycoprotein with subunits of 230,000 daltons (3). Fibronectin is known to be involved in adhesion of cells to solid substrata and has specific binding sites for gelatin, glycosaminoglycans (i.e., heparin), fibrinogen, and fibrin, and can be crosslinked to fibrin or collagen by Factor XIII (reviewed in references 17, 21, 35, 47, 49). Fibronectin has also been shown to bind to bacteria[1] (27, 36), but has not been shown to promote phagocytosis of bacteria.

To define the conditions necessary for endocytosis of fibronectin-coated particles, we have studied an established mouse macrophagelike cell line ($P388D_1$) by using a centrifugation assay, as well as electron and immunofluorescence microscopy. We find that endocytosis of gelatin-conjugated latex beads by these cells is dependent on both fibronectin and heparin. The assays described that use this cell line will be useful for determinations of the opsonic activity of samples containing fibronectin and for further analysis of the mechanism of uptake of these particles.

MATERIALS AND METHODS

Cell Culture and Preparation

A macrophagelike cell line ($P388D_1$) (10, 26), obtained from Dr. H. Eisen, Massachusetts Institute of Technology, was grown in Dulbecco's modified Eagle's

[1] L. Van De Water et al. Unpublished observations.

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medium with 10% heat-inactivated fetal calf serum on tissue-culture dishes. Adherent cells were harvested with 0.02% EDTA, washed three times in $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (PBS), and resuspended in PBS plus divalent cations. (Nonadherent cells were also tested and found to be less active in uptake of particles.) Another macrophage line, RAW 309 Cr.1 (39), was obtained from Dr. J. Unkeless, Rockefeller University, and grown in the same medium on plastic petri dishes (Falcon Labware Div., Becton & Dickinson, Oxford, Calif.), to which they were loosely adherent. Cells were harvested by pipetting (without EDTA) and washed in PBS. Mouse peritoneal macrophages were collected from BALB/c mice 4 d after intraperitoneal injection of 1 ml of thioglycollate (46). Contaminating erythrocytes were removed by lysis in 0.8% ammonium chloride (7), and cells were washed three times in PBS, resuspended in PBS plus divalent cations, and used directly for phagocytosis (see below). NIL8 cells were grown as previously described (20).

Metabolic Labeling of Cells and Conditioned Media

Cells were labeled for 18–20 h in growth medium containing one-tenth the usual concentration of unlabeled methionine and [^{35}S]methionine (20 μCi/ml; 442 Ci/mmol; New England Nuclear, Boston, Mass.). Conditioned medium was centrifuged at low speed to pellet any cells; adherent cells were scraped from the dishes in 0.2% sodium dodecyl sulfate (SDS) in 0.1 M Tris pH 8.8 with 2 mM phenyl methyl sulfonyl fluoride (PMSF), 2 mM EDTA, 1 mM N-ethyl maleimide (NEM), 1 mM iodoacetic acid (IAA), and heated to 90°C for 3 min. Lysates and conditioned media were centrifuged at 10,000 × *g* for 10 min.

Purification of Fibronectin and Preparation of Opsonins

Plasma fibronectin (pFN) was purified from human plasma on gelatin-Sepharose columns by a modification of the method of Engvall and Ruoslahti (14). Buffers for the washing, elution and dialysis of protein were degassed and flushed with nitrogen. Human plasma was applied to a gelatin-Sepharose column (8 mg gelatin/ml Sepharose) at a ratio of 1 ml of plasma/ml of gel bed. The column was washed with 5–10 vols of PBS, eluted with 8 M urea in CAPS buffer (10 mM cyclohexylaminopropane sulfonic acid, 150 mM NaCl, 1 mM $CaCl_2$, pH 11), dialyzed, and stored at −30°C in CAPS buffer without urea. In some cases, protein was eluted with 1 M NaBr in 0.05 M Tris-HCl, pH 5.3 (11), or with 1 M arginine in 0.05 M Tris-HCl, pH 7.5 (48). Protein concentrations were determined by the absorbance at 280 nm (1.3 OD unit per mg/ml) or by the method of Lowry et al. (29), using BSA as the standard. Normal human serum was depleted of plasma fibronectin by chromatography on gelatin-Sepharose (1 ml of serum/ml gel bed), and flow-through fractions were pooled. Control experiments showed that this procedure depletes plasma of >99% of its fibronectin. Depleted plasma is negative for fibronectin as assayed by Ouchterlony double diffusion, antibody staining of gels (5), and rerunning on a second gelatin-Sepharose column. Samples of normal human serum or pFN-depleted human serum were heat-inactivated at 56° for 30 min.

Antisera

Rabbit antisera to hamster cellular fibronectin have been described and characterized (8, 30, 43). The antisera were raised to hamster cellular fibronectin purified by successive runs of SDS-gel electrophoresis. The preparation of affinity-purified, rhodamine-conjugated antifibronectin has been described (22). $F(ab')_2$ fragments of rabbit antifibronectin were prepared as follows. The immunoglobulin fraction (from 10 ml of serum) in 0.1 M sodium phosphate, pH 7.0, was passed over a 5-ml protein A-Sepharose column (Pharmacia Inc., Piscataway, N.J.); the column was washed, and IgG was eluted with 0.1 M glycine-HCl, pH 3.0. The eluted protein was digested for 20 h at 37°C with pepsin (Sigma Chemical Co., St. Louis, Mo.; 2 mg/100 mg Ig) in 0.1 M sodium acetate, pH 4.5. The reaction was stopped by raising the pH to 7.5, and the $F(ab')_2$ fragments (in 0.1 M sodium phosphate, pH 7.0) were purified by application to a protein A-Sepharose column (5 ml); the flow-through contained purified $F(ab')_2$ fragments, as confirmed by SDS–polyacrylamide gel analysis.

Rabbit antisera to human plasma fibronectin were raised to plasma fibronectin purified on gelatin-Sepharose and subsequently by preparative SDS-gel electrophoresis of reduced samples. The sera give single immunoprecipitation lines in Ouchterlony double diffusion against human plasma or plasma fibronectin and no precipitation line with pFN-depleted human serum. Direct rhodamine-conjugated, affinity-purified Ig against human fibronectin was prepared as described (22).

Immunoprecipitation

Samples of cell lysates or conditioned media were mixed with an equal volume of 1% Nonidet P40 (NP40), 1% sodium deoxycholate (DOC), 0.1 M Tris HCl, pH 8.8, 2 mM NEM, 1 mM IAA, 2 mM PMSF, 2 mM EDTA, or with PBS, respectively, and either preimmune or anti-hamster cellular fibronectin rabbit serum was added. Samples were incubated for 1 h at 37°C with the first rabbit serum; goat anti-rabbit IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) was then added for an additional hour. Samples were then incubated overnight at 4°C. Samples were washed three times in 0.1% SDS, 0.5% NP40, 0.5% DOC in 0.1 M Tris, pH 8.8, 2 mM PMSF, 2 mM EDTA, 1 mM NEM, 1 mM IAA, and washed pellets were resuspended in electrophoresis sample buffer and heated to 90°C for 3 min.

Electrophoresis and Fluorography

Samples were prepared and analyzed on 5% slab gels, using the buffers described by Laemmli (28). Gels were fixed and stained by the method of Fairbanks et al. (15). SDS gels containing labeled proteins ([^{35}S]methionine) were impregnated with 2,5-diphenyloxazole (PPO) as described by Bonner and Laskey (4), dried, and placed in contact with Kodak X-Omat R film.

Preparation of Gelatin-conjugated Latex

Gelatin was conjugated to latex by a modification of the procedure of Molnar et al. (33). One part gelatin (10 mg/ml) in distilled water, two parts latex particles (Dow Diagnostics, Indianapolis, Ind.; 10% vol/vol, 0.455 μm), and one part 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide-metho-p-toluene sulfonate in 0.2 M acetate buffer, pH 6, were mixed and incubated for 3 h at room temperature and then incubated overnight at 4°C. The conjugated latex was washed and stored in PBS with 2 mM azide. In one set of experiments (Fig. 1 *c*), the gelatin concentration during conjugation was varied to generate beads with 2.9, 1.2, 0.7, and 0.4 fg/bead.

The conjugated beads were iodinated by the method of Hunter and Greenwood (19). A mixture of 100 μl of 25% (vol/vol) gelatin-latex beads in 0.5 M sodium phosphate buffer (pH 7.2), 250 μCi Na^{125}Iodine, and 10 μl chloramine T (5 mg/ml stock) was incubated at room temperature for 60 s. The reaction was stopped by the addition of 0.5 ml of sodium metabisulfite (25 mg/ml in 0.05 M sodium phosphate buffer); the particles were then washed 5–10 times and stored in PBS with 2 mM sodium azide. Before use, beads were sonicated to ensure a suspension of single beads. The concentration of beads was determined by comparing their OD_{550} to a standard set of diluted beads of known concentration (as supplied by Dow).

Centrifugation Assay for Phagocytosis

Iodinated gelatin-latex (50 μl) was added to a mixture of heparin (50 μl of 100 U/ml stock) and serum (100 μl) with or without pFN, and the mixture was preincubated at 37°C, usually for two min, in a 12 × 75-mm polystyrene tube. Washed cells (250 μl; 2×10^7 cell/ml) were then added and the incubation was continued at 37°C. At intervals, aliquots (100 μl) were transferred to tubes containing 2 ml of ice-cold PBS with divalent cations and 1 mM NEM. Samples were then washed two times by rapid centrifugation in a clinical centrifuge (700 *g* for 10 s) and counted for ^{125}Iodine in a gamma counter (Beckman Gamma 300; Beckman Instruments, Fullerton, Calif.).

Immunofluorescence Assay for Phagocytosis

Two immunofluorescence procedures have been used to determine whether opsonized particles were internalized by cells. In the first, a mixture of gelatin-latex particles (10 μl), heparin (10 μl; 100 U/ml), and serum with or without pFN (20 μl) was layered over a cover slip of cells which had been washed three times in PBS. The cells were incubated for 30 min at 37°C, washed in PBS, and fixed in 3.7% formaldehyde in PBS for 30 min at room temperature. Cells were then stained for 30 min at 37°C with rhodamine-labeled, affinity-purified antifibronectin, washed in PBS, and then stained for 30 min at 37°C with rhodamine-conjugated $F(ab')_2$ fragment of goat anti-rabbit $F(ab')_2$ fragments (Cappel). The cover slips were then washed in PBS, mounted in Gelvatol (38), and viewed and photographed in a Zeiss Photo microscope III with epifluorescence optics.

In the second procedure, cells were stained before fixation. Cover slips incubated with opsonized beads were washed in PBS and immediately stained for 30 min at 37°C with rhodamine-labeled, affinity-purified antifibronectin in PBS plus 1 mM azide. The cells were then washed in PBS, fixed in 3.7% formaldehyde, washed again in PBS, and mounted in Gelvatol. This second staining procedure resulted in a very low background staining, but the cells had a more rounded morphology. Parallel cover slips stained by either procedure contained the same percentage of cells with internalized beads.

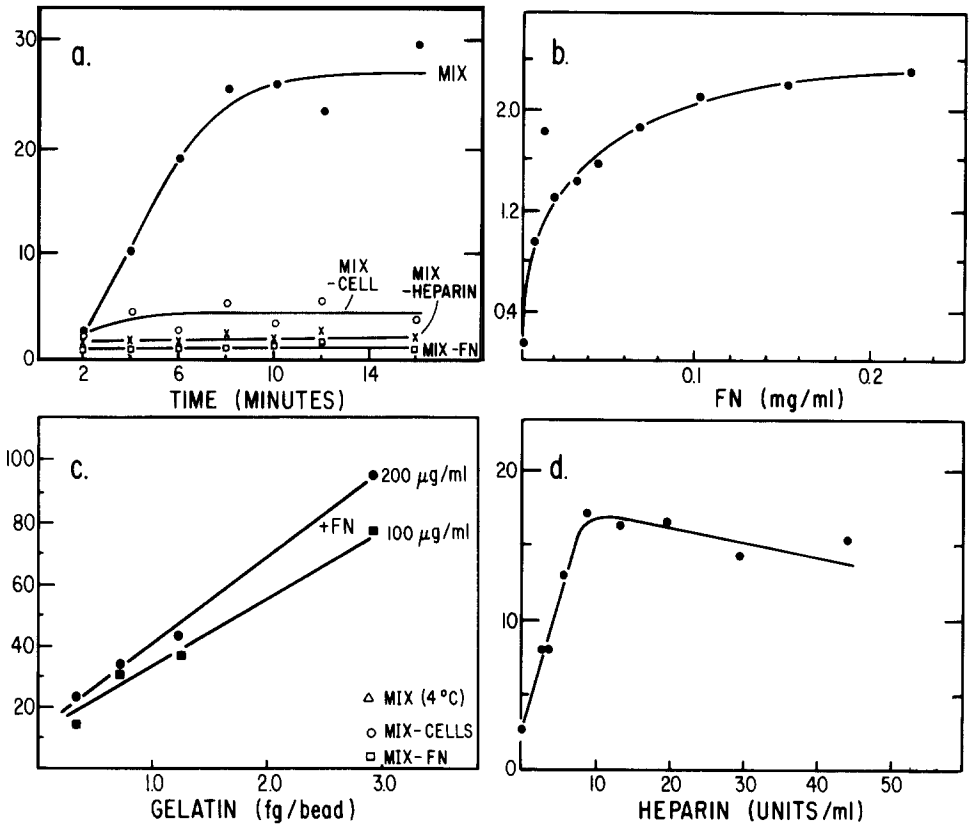

FIGURE 1 Binding of ^{125}I-gelatin-latex by P388D$_1$ cells. Cells were incubated with beads under various conditions and cell-bound beads were separated by centrifugation through several washes. Each panel depicts a separate experiment with different preparations of ^{125}I-gelatin beads; the ordinate differs between experiments (see below). (*a*) Dependence of binding on cells, fibronectin (FN), and heparin. The complete mix consisted of fibronectin-depleted serum plus purified fibronectin (200 μg/ml), heparin (10 μg/ml, 59 μg/ml), and ^{125}I-gelatin-latex beads (3.3×10^9 beads/ml, 6.10^{-4} cpm/bead). Samples were preincubated for 2 min at 37°C, and P388D$_1$ cells (10^7 cells/ml) were added and the incubations continued at 37°C for the times indicated when equal aliquots (0.1 ml) were taken. Control incubations lacked cells, fibronectin, or heparin. Ordinate: cpm $\times 10^{-3}$ associated with cell pellet. (*b*) Dependence on concentration of fibronectin. Incubations contained heat-inactivated fibronectin-depleted serum, ^{125}I-gelatin-latex beads (3×10^8 beads/ml; 3×10^{-4} cpm/bead), heparin (10 U/ml; 59 μg/ml), and various concentrations of fibronectin together with 2.5×10^6 P388D$_1$ cells in a total volume of 225 μl. Incubation was carried out for 20 min at 37°C. Ordinate: cpm $\times 10^{-3}$ associated with cell pellet. The fibronectin was eluted from gelatin-Sepharose with urea. Similar stimulation was observed with fibronectin eluted with NaBr. (*c*) Dependence on density of gelatin on beads. Beads (8.8×10^8 bds), previously prepared with differing amounts of conjugated ^{125}I-gelatin, were mixed with fibronectin-depleted serum, heparin (10 U/ml; 59 μg/ml), fibronectin (200 μg/ml (●) or 100 μg/ml (■)), and incubated for 3 min at 37°C. P388D$_1$ cells (2.5×10^6 cells) were added for 15 min at 37°C. Control samples lacked fibronectin (□) or contained fibronectin at 200 μg/ml, but were incubated at 0-4°C (△), or without cells (○) at 37°C. Ordinate: number of beads ($\times 10^{-6}$) associated with cells, corrected for different amounts of ^{125}I-gelatin on beads. (*d*) Dependence on concentration of heparin. Incubations contained heat-inactivated fibronectin-depleted serum plus fibronectin (200 μg/ml), various amounts of heparin and ^{125}I-gelatin latex beads (3.5×10^8 beads). After 2 min preincubation at 37°C, 1×10^6 P388D$_1$ cells were added and the incubation was continued for 18 min. Ordinate: cpm ($\times 10^{-3}$) associated with cell pellet.

With either protocol, all beads that were not associated with cells were stained and were readily visible in both phase and fluorescence. Beads associated with cells were of two classes: fluorescent (i.e., not internalized) and unlabeled (i.e., internalized). Accurate quantitation of the percentage of cells showing phagocytosis required that the plane of focus be changed to be certain that all beads were visualized. Controls using preimmune serum or antifibronectin preabsorbed with fibronectin-Sepharose (22) gave no staining in either protocol. In the case of beads incubated in the absence of fibronectin, the very few beads that do associate with cells naturally do not stain. In other experiments, these have been visualized by inserting an extra staining step using fibronectin to label the gelatin.

Electron Microscopy

Mixtures of cells and beads were prepared as for the centrifugation assay, incubated at 37°C for different times, and immediately fixed in 2% glutaraldehyde, 0.2% tannic acid in 0.1 M cacodylate buffer, pH 7.2, washed, postfixed in 1% OsO_4, followed by 1% uranyl acetate, dehydrated through graded ethanols, embedded in Epon/Araldite, and sectioned. Sections were stained with uranyl acetate and lead citrate and viewed in a Philips EM 201 microscope.

RESULTS

Fibronectin Promotes the Binding of the Latex Particles by Phagocytic Cells

Initially, to compare our data with those of others, we used an assay in which slices of rat liver were incubated with the beads plus various serum preparations and heparin (33). In agreement with others (16, 33, 40), we observed that fibronectin and heparin were required for the binding of gelatin-latex to liver slices (data not shown). Whereas the data are consistent with the phagocytosis of these particles by Kupffer cells as suggested by others (3), this assay is unable to discriminate between ingestion and membrane attachment of these particles or between binding to hepatocytes and binding to other cells.

Because of the difficulties in adequately defining ingestion in a complex system such as the liver, we investigated endo-

cytosis of gelatin-conjugated latex particles by cultured cells. In screening different populations of phagocytic cells for efficient uptake of gelatin-latex, we observed a high level of binding by the P388D_1 cell line. These cells can be easily grown in continuous culture, are a homogeneous cell type, and can be easily labeled with radioactive precursors (26). Experiments were first carried out using these cells in a suspension assay with iodinated gelatin-latex, various sera, and heparin. We observed binding of gelatin-latex particles to the cells which was dependent upon the presence of pFN (Fig. 1). The activity of human sera depleted of FN could be restored by the addition of affinity-purified pFN in a dose-dependent fashion (Fig. 1 *b*). Similar results were obtained with human plasma depleted of FN with and without heat inactivation (not shown). The increase in cell-associated gelatin-latex particles reached a maximum within a 20-min incubation at 37°C. The maximum level of cell-associated counts in the presence of pFN ranged from 10 to 30% of the total particles and generally represented a tenfold stimulation of binding over background. If incubations were carried out at 37°C but without cells present, little or no activity was observed above background (Fig. 1 *a* and *c*). Thus, the observed pelleting of particles was dependent on the presence of cells and did not merely represent aggregation of the beads by pFN. The binding of beads by cells was also dependent on the amount of gelatin conjugated per bead (Fig. 1 *c*). This panel also shows that the association of beads with cells is inhibited by low temperatures.

Previous reports (33, 40) had indicated a dependence on heparin of the liver slice assay, so we investigated its role in our assay system. If heparin was omitted from the incubation mixture, the level of binding in the presence of pFN approximated that of samples without pFN (Fig. 1 *a*). Fig. 1 *d* shows the dependence of binding on heparin concentration. 10 U/ml (59 μg/ml) were used in all subsequent experiments. Experiments (not shown) were also carried out using chondroitin sulfate, dermatan sulfate, keratan sulfate, or hyaluronic acid at the same concentration as heparin. None of these compounds substituted for heparin. Heparan sulfate (59 μg/ml), on the other hand, did stimulate phagocytosis, but less well than did the commercial heparin used in the experiments described.

In conclusion, association of gelatin-coated latex with macrophages is dependent on temperature and on the concentrations of fibronectin, gelatin, and heparin.

Evidence that Gelatin-Latex Particles Are Ingested: Electron Microscopy

The centrifugation experiments suggested that particles were bound by cells. To determine whether the test particles were actually ingested or simply associated with the cell surface, we used electron and light microscopy.

Samples were prepared for electron microscopy by mixing opsonin and latex beads with cells in suspension, washing, fixing the pellets, and sectioning. When human serum was used as an opsonin and incubated at 37°C with P388D_1 cells, beads were found inside the cells (Fig. 2 *a*). If the serum was depleted of pFN, very few beads were associated with the cells (Fig. 2 *b*). The decrease in endocytosis by depletion of pFN could be reversed by reconstituting the sample with purified pFN (Fig. 2 *c*). Samples prepared with pFN present (Fig. 2 *a* and *c*) had beads both inside and outside the cells. The internalized beads were enclosed in membrane-bounded vesicles (Fig. 2 *d*) and these vesicles often contained more than one bead. If samples were incubated at 4°C in the presence of pFN, beads were not internalized (not shown).

To quantify binding and endocytosis, samples were prepared with or without pFN, incubated at either 0° or 37°C, and the percentage of cells with beads inside and the numbers of beads inside cells were determined. In the absence of pFN, very few beads were found inside the cells. However, in the presence of pFN at 37°C an increase was observed in the percentage of cells with beads internalized (Fig. 3 *a*). A corresponding increase in the number of beads inside the cells (Fig. 3 *b*) was also observed with pFN at 37°C. If a mixture of pFN, beads, heparin, and P388D_1 cells was incubated at 0–4°C instead of 37°C, very few beads were observed inside the cells (Fig. 3 *a* and *b*).

Evidence that the Gelatin-Latex Particles Are Ingested: Immunofluorescence Microscopy

Because the electron microscopic assay was time consuming, we developed an immunofluorescence assay that allowed ready quantitation of binding and uptake of beads. P388D_1 cells grown on cover slips were incubated with gelatin-latex, heparin, and different opsonins. After incubation, the cover slips were washed and stained for fibronectin by indirect immunofluorescence (see Materials and Methods). The cells were not permeabilized, allowing visualization of beads outside (fluorescent and phase-dense) contrasted with those inside (phase-dense only). Examples ares shown in Fig. 4. When human serum was used as an opsonin, beads were found inside the cells (Fig. 4 *a*). If serum fractions depleted of FN were used, very few beads were found in the field (Fig. 4 *b*). If serum was reconstituted with fibronectin, phagocytosis was again clearly observed (Fig. 4 *c*). In all samples containing fibronectin, beads were found in aggregates both inside and bound to the outside of the cells.

To obtain a more detailed picture of the conditions necessary for phagocytosis, we determined the percentage of total cells with associated beads (inside and outside) as a function of various conditions (Fig. 5). Binding and internalization were dependent on both fibronectin and heparin, and both were inhibited by low temperature. Addition of $F(ab')_2$ fragments of antifibronectin antibody inhibited both binding and internalization, indicating that fibronectin is involved in both processes.

Do Macrophages Produce or Bind Fibronectin?

If P388D_1 cells are to serve as a useful assay for exogenous fibronectin, it is important to determine whether they produce fibronectin. As shown in Fig. 6, they do not. Whereas fibronectin can readily be detected in the culture medium of two other phagocytic murine cell types (RAW and peritoneal exudate cells [PEC]) by immunoprecipitation, none could be detected in either culture medium or cell lysates of P388D_1 cells. P388D_1 cells were also negative for fibronectin by indirect immunofluorescence (data not shown). Therefore, assays for fibronectin effects on endocytosis in this system are not complicated by endogenous production of fibronectin. The same is not true for all phagocytic cells.

DISCUSSION

Our results provide convincing evidence for fibronectin-mediated stimulation of binding and endocytosis of particles by a pure population of phagocytic cells. We have developed two rapid assays for binding and endocytosis of gelatin-coated latex by the mouse macrophagelike cell line, P388D_1, first isolated

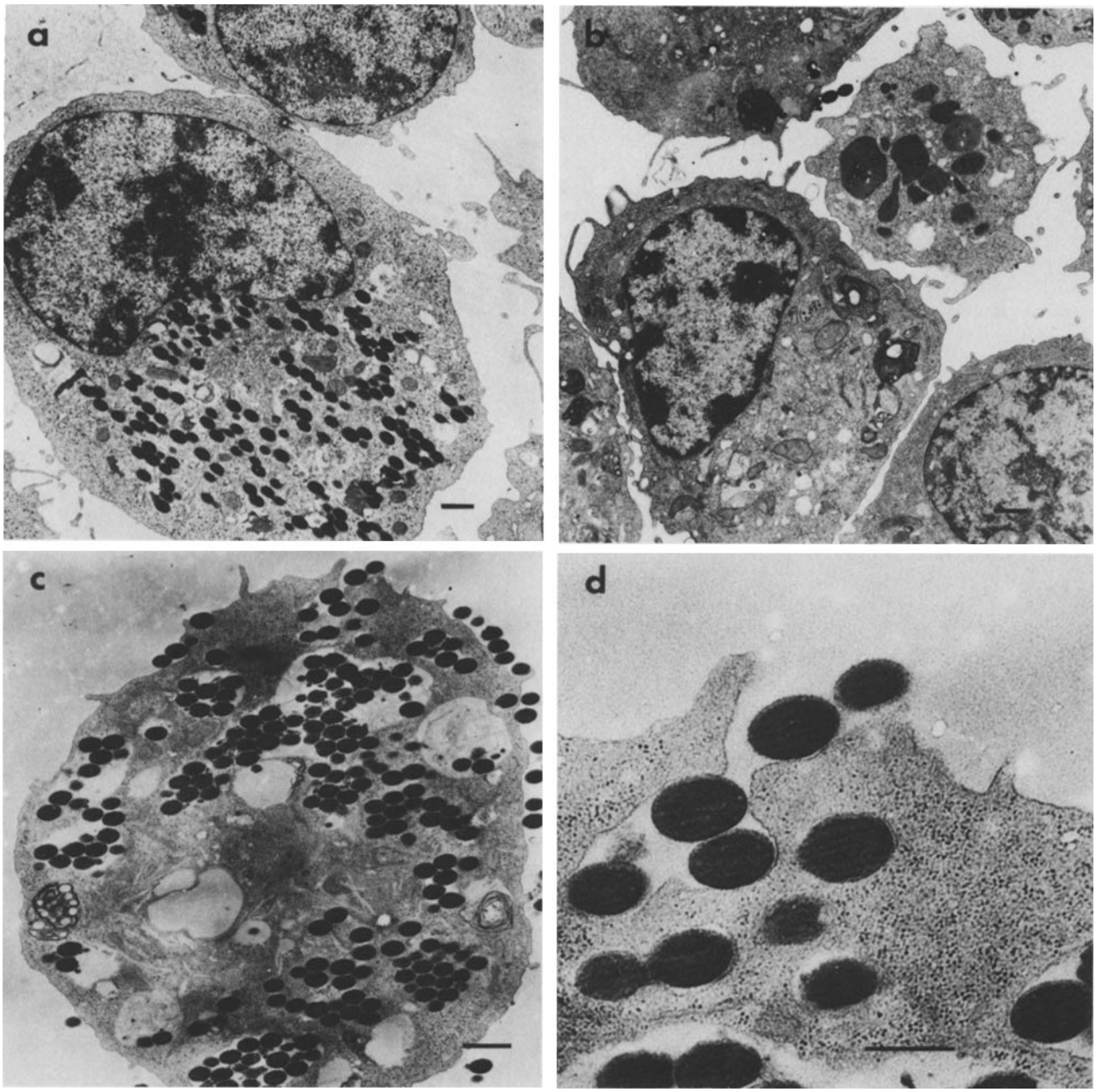

FIGURE 2 Endocytosis of fibronectin-coated gelatin-latex beads. Gelatin-latex beads (5×10^9 beads) were mixed with heparin, P388D$_1$ cells (2.5×10^6 cells), and different serum samples, and incubated for 60 min at 37°C (*a*, *b*, and *c*). The serum samples were human serum (*a*), heat-inactivated, fibronectin-depleted human serum (*b*), or fibronectin added to (44 μg/ml) heat-inactivated fibronectin-depleted human serum (*c*). *d* is a higher magnification of portion of *c*. *a*, *b*, and *c*: bar, 1 μm; × 5,000. *d*: bar, 0.5 μm; × 26,000.

by Dawe and Potter (10) and characterized in detail by Koren et al. (26). This cell line is easily grown in suspension or monolayer culture, can be metabolically labeled, will readily phagocytose particles coated with IgG or complement, and secretes neutral hydrolases characteristic of macrophages (see reference 34 for review).

The dependence of binding and endocytosis on fibronectin was shown by a strict dose dependence (Fig. 1*b*) and by inhibition with F(ab′)$_2$ antifibronectin (Fig. 5). Fibronectin active in our assays can be prepared by elution from gelatin-Sepharose with either urea or sodium bromide and with or without low levels of reducing agent (0.01% 2-mercaptoethanol, data not shown). This latter result is in contrast with reports that inclusion of mercaptoethanol during purification is a requirement for protein active in the liver slice assay (2). Binding and internalization were also dependent on heparin, consistent with the results of others using other systems (3, 13, 16, 18).

The reason for the heparin dependence is obscure. Several studies have shown that fibronectin, gelatin, and heparin can participate in the formation of ternary aggregates (6, 23, 24, 45). However, aggregation is not a sufficient explanation for our results because (*a*) in the presence of F(ab′)$_2$ fragments of antifibronectin, aggregates of beads still formed but internali-

zation was inhibited (Fig. 5); and (*b*) aggregation of beads in the absence of fibronectin sometimes occurs and is not associated with phagocytosis (e.g., when beads are not sonicated before use).

We cannot, however, rule out the possibility that aggregation is a necessary concomitant of phagocytosis in this system, and some observations are consistent with this idea. As the amount of gelatin on the beads is varied (Fig. 1 *c*), lower levels of gelatin lead to reduced aggregation and also produce lower

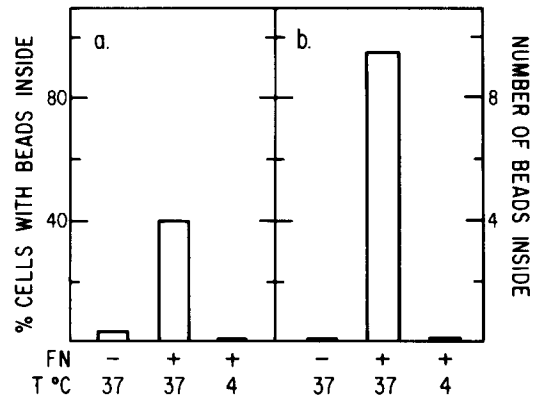

FIGURE 3 Quantitation of cell-associated beads by electron microscopy. Counts were made from prints such as those in Fig. 2 *a* (human serum), 2 *b* (heat-inactivated, fibronectin-depleted human serum), or human serum at 4°C (not shown in Fig. 2). Results are presented as the percent of total cells with internalized beads (*a*) and as the number of beads internalized divided by the total number of cells counted (*b*).

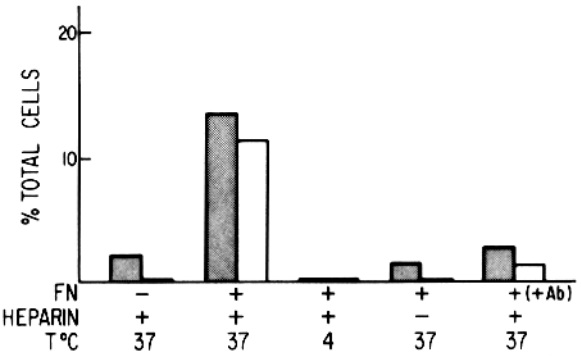

FIGURE 5 Quantitation of cell-associated beads by immunofluorescence microscopy. Procedure as in Fig. 4. Cover slips were stained by the second procedure described in Materials and Methods and scored for the percent of total cells with beads associated (shaded bars) or internalized (open bars). In some samples, heparin was omitted or rabbit anti-hamster cellular fibronectin $F(ab')_2$ fragments (~50 μg/μg FN) was preincubated with pFN (30 min at 37°C) before addition to the bead mixture. Incubation was carried out with cells for 30 min at 37°C in all cases but one (pFN, 4°C).

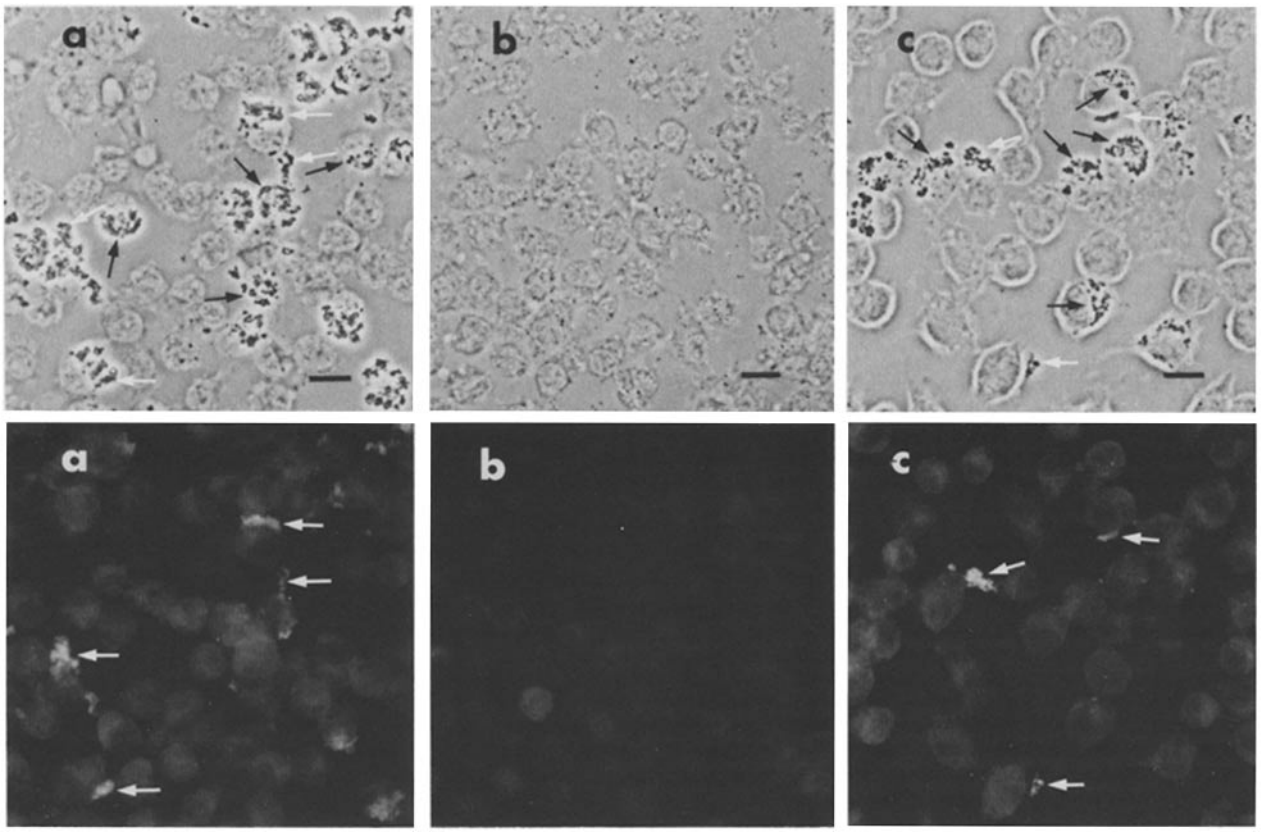

FIGURE 4 Analysis of cell-associated beads by immunofluorescence microscopy. Samples of human serum (*a*), fibronectin-depleted human serum (*b*), or fibronectin-depleted human serum reconstituted with purified plasma fibronectin (*c*) at a final concentration of 0.2 mg/ml were mixed with gelatin-latex. An aliquot (20 μl) of the mixture was placed over $P388D_1$ cells grown on cover slips, incubated for 30 min at 37°C, washed, fixed, and stained by the first procedure outlined in Materials and Methods. Internalized beads (black arrows) are unstained, while external beads (white arrows) are stained by fluorescent antifibronectin. Fixation before staining results in higher background immunofluorescence but flatter cells more suitable for photography. Bar, 10 μm.

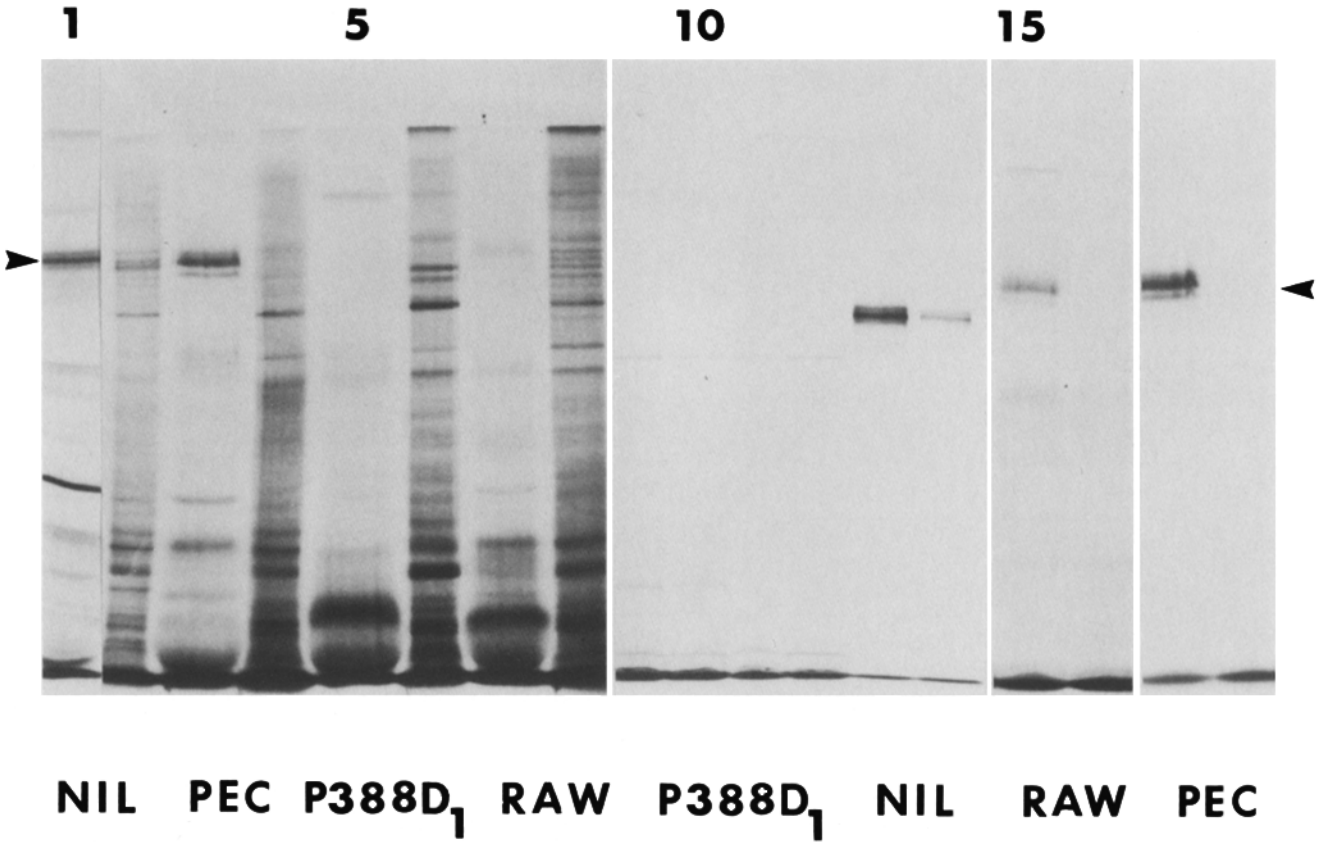

FIGURE 6 SDS gel analysis of conditioned media and cell lysates. Analysis of [^{35}S]methionine-labeled, conditioned media, and cell lysates (*a*) Samples of conditioned media (lanes 1, 3, 5, and 7) and SDS cell lysates (lanes 2, 4, 6, and 8) were prepared from NIL.8 cells (lanes 1 and 2), mouse peritoneal exudate cells (lanes 3 and 4), P388D$_1$ (lanes 5 and 6) and RAW 309 Cr.1 (lanes 7 and 8), and analyzed on 5% SDS-polyacrylamide gels. Samples were normalized to contain equal acid-precipitable radioactivity. Arrow marks position of fibronectin. Immunoprecipitation of culture media or cell lysates. (*b*) Aliquots of conditioned medium (lanes 11–18) and SDS cell lysates (lanes 9 and 10) from P388D$_1$ cells (lanes 9–12), NIL.8 cells (lanes 13 and 14), mouse peritoneal exudate cells (lanes 15 and 16), or RAW 309 Cr.1 cells (lanes 17 and 18) were reacted with preimmune (lanes 10, 12, 14, 16, and 18), or rabbit anticellular fibronectin antiserum (lanes 9, 11, 13, 15, and 17), washed, and analyzed on 5% SDS gels.

levels of cell association of aggregates. Conceivably, the heparin acts as a cofactor by promoting aggregation, and, while the role of heparin in endocytosis remains unclear, it would be premature to suggest that fibronectin acts as an opsonin analogous to IgG or C3b. However, the current results show clearly that, in the cell system studied, addition of fibronectin increases endocytosis from a very low level to a significant level, whether by binding to specific receptors on the cell surface or by promoting aggregation or both.

Less marked stimulation of phagocytosis was observed with several other phagocytic cell types (mouse peritoneal macrophages, RAW 309 Cr.1 cells, and human neutrophils). It may be relevant that we observed that RAW 309 Cr.1 cells and PEC both synthesize fibronectin, whereas P388D$_1$ cells do not (Fig. 6). Pearlstein et al. (37) reported that PEC cells do not have surface fibronectin, whereas Colvin et al. (9) found that they do. Recently, Johansson et al. (25) observed that these cells can synthesize and secrete fibronectin. A report has also appeared on synthesis of FN by human monocytes (1). So it is possible that some phagocytic cells such as P388D$_1$ require exogenous FN whereas other cell types do not, either because they make their own or because they do not use FN at all. The same might also be true for the requirement for heparin: some cells may be able to synthesize polyanionic species such as heparan sulfate which could replace the exogenous heparin.

There has been much interest in the possibility that fibronectin may act as an opsonin in reticuloendothelial clearance of particulates from the circulation (3, 33, 41). The establishment of rapid and straightforward assays using well-defined cells and particles should aid in the further analysis of the proposed opsonic activity of fibronectin.

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