

Fibronectin-mediated Binding and Phagocytosis of Polystyrene Latex Beads by Baby Hamster Kidney Cells

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ABSTRACT The binding and phagocytosis of fibronectin (pFN)-coated latex beads by baby hamster kidney (BHK) cells was studied as a function of fibronectin concentration and bead diameter. Cells were incubated with radioactive pFN-coated beads, and total bead binding (cell surface or ingested) was measured as total radioactivity associated with the cells. Of the bound beads, those that also were phagocytosed were distinguished by their insensitivity to release from the cells by trypsin treatment. In continuous incubations, binding of pFN-coated beads to cells occurred at 4°C or 37°C, but phagocytosis was observed only at 37°C. In addition, degradation of ³H-pFN from ingested beads occurred at 37°C, as shown by the release of trichloroacetic acid-soluble radioactivity into the incubation medium. When the fibronectin density on the beads was varied, binding at 4°C and ingestion at 37°C were found to have the same dose-response dependencies, which indicated that pFN densities that permitted bead binding were sufficient for phagocytosis to occur. The fibronectin density for maximal binding of ingestion was ~250 ng pFN/cm². When various sized beads (0.085–1.091 μm), coated with similar densities of pFN, were incubated with cells at 4°C, no variation in binding as a function of bead size was observed. Under these conditions, the absolute amount of pFN ranged from <100 molecules on the 0.085-μm beads to >15,000 molecules on the 1.091-μm beads. Based upon these results it can be concluded that the critical parameter controlling fibronectin-mediated binding of latex beads by BHK cells is the spacing of the pFN molecules on the beads. Correspondingly, it can be suggested that the spacing between pFN receptors on the cell surface that is optimal for multivalent interactions to occur is ~18 nM. When phagocytosis of various sized beads was compared, it was found that the largest beads were phagocytosed slightly better (two fold) than the smallest beads. This occurred both in continuous incubations of cells with beads and when the beads were prebound to the cells. Finally, the kinetic constants for the binding of 0.085 μM pFN-coated beads to the cells were analyzed. There appeared to be ~62,000 binding sites and the *K_b* was 4.03 × 10⁻⁹ M. Assuming a bivalent interaction, it was calculated that BHK cells have ~120,000 pFN receptors/cell and the binding affinity between pFN and its receptor is ~6 × 10⁻⁵ M.

Fibronectins are a group of high molecular weight glycoproteins found in soluble form in body fluids and in insoluble form in extracellular matrix material (17, 26). Considerable evidence has been presented indicating that fibronectin can promote cell-substratum adhesion of a variety of different cell types (17, 26) to such diverse substrata as fibrin (9), denatured collagen (13), and inert material surfaces (3). In addition, fibronectin has been proposed to play a major role as a nonimmune opsonic factor required for reticuloendothelial function (20). In support of this idea, it has been shown that fibronectin enhanced the phagocytosis of gelatin-coated particles by macrophages (11, 24), and that phagocytosis of

immune-coated particles by monocytes is enhanced when the particles also have fibronectin on their surfaces (1).

Recently, we reported that fibroblasts were able to phagocytose fibronectin-coated latex beads (4), an observation that was subsequently confirmed by others (25). This finding was of particular interest because it raised the possibility that fibroblast spreading on material surfaces such as tissue culture substrata was actually an attempt by the cells to phagocytose beads of infinite diameter, as was suggested previously for macrophages (18).

In order to understand more about fibroblast-bead interactions, a variety of experiments have been carried out to

determine the effects of fibronectin concentration and bead size on the binding and phagocytosis of beads by baby hamster kidney (BHK) cells. The results of these studies are reported herein.

MATERIALS AND METHODS

Cells: BHK cells adapted for growth in suspension culture were a gift from Dr. Adrian Chappel (Center for Disease Control, Atlanta, GA). The cells were grown in suspension as described previously (9). Exponentially growing cells were harvested by centrifugation at 800 g (Sorvall HL-4 rotor; DuPont Instruments-Sorvall Biomedical Div., Dupont Co., Wilmington, DE) for 4 min at room temperature. Cells were washed in Dulbecco's PBS (150 mM NaCl, 3 mM KCl, 6 mM Na₂HPO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, pH 7.2) containing 2% bovine serum albumin (BSA, crystallized; Miles Laboratories Inc., Research Products Div., Elkhart, IN).

Fibronectin: Human plasma fibronectin (pFN) was prepared by ammonium sulfate precipitation and ion-exchange chromatography as described previously (9). The specific activity of the plasma fibronectin preparations used was ~200 U/mg. 1 U of activity has been defined as the amount of fibronectin required to promote complete cell spreading in a standardized assay (9). Radiolabeled ³H-pFN was prepared similarly as described previously (4). To a 5-ml solution of pFN (2.5 mg/ml dialyzed overnight against 0.05 M Na borate, pH 9.0) was added 0.076 ml of [³H]formaldehyde (specific activity 100 μCi/μM; New England Nuclear, Boston, MA). After 40 min at 4°C the reaction was completed by 3 × 0.05 ml additions (at 5-min intervals) of 0.33 M Na borohydride, and the preparation was dialyzed exhaustively against 0.1 M Na phosphate, pH 6.8. The specific activity of radiolabeled fibronectin resulting from this procedure was 9,000–16,000 cpm/μg. In all radioactive measurements, samples to be tested were mixed with 10 ml of Budget Solve (RPI Corp., Mt. Prospect, IL) and counted in a Nuclear-Chicago Mark II scintillation spectrophotometer (Nuclear-Chicago, Des Plaines, IL).

Polystyrene Beads: Polystyrene latex beads (Dow Diagnostics, Indianapolis, IN) with diameters of 0.085, 0.305, 0.76, and 1.091 μm were obtained from the manufacturer or from Sigma Chemical Co. (St. Louis, MO). According to the manufacturer, the concentration (particles/ml) of the various bead solutions used were: 0.085 μm: 2.91 × 10¹⁴; 0.305 μm: 6.38 × 10¹²; 0.76 μm: 4.14 × 10¹¹; and 1.091 μm: 1.40 × 10¹¹.

Preparation of Fibronectin-coated Beads: Polystyrene latex beads were incubated with either pFN or ³H-pFN for 10 min at room temperature. The incubation volume was generally <0.5 ml, and the bead number and pFN concentrations were varied to give the desired density of fibronectin on the beads as indicated in the figure legends. At the end of the incubations, the samples were diluted 10-fold with phosphate saline (PS) (0.1 M NaCl, 0.005 M Na phosphate, pH 7.4) containing 2% BSA and incubated an additional 10 min at room temperature after which the beads were collected by centrifugation (0.085 μm beads, 20 min at 100,000 g, Beckman SW 27 rotor; Beckman Instruments, Inc., Fullerton, CA, all other beads, 15 min at 27,000 g, Sorvall SS-34 rotor). The sedimented beads were resuspended in Dulbecco's PBS containing 0.2% BSA and sonicated for 5 s at 60 W (Heat Systems-Ultrasonics, Inc., Plainview, NY) immediately before addition to cells. The sonication step was necessary because the latex beads aggregated when fibronectin was first added. After sonication, however, the beads tended to remain unaggregated, and in previous microscopic studies the beads that bound to the cells were found to be individual and not in aggregates (4).

Binding of Fibronectin-coated Beads to Cells: In all experiments, BHK cells (2 × 10⁶) were suspended in 0.5 ml of Dulbecco's PBS containing 0.2% BSA and brought to 4°C or 37°C. pFN-coated beads (0.5 ml) were added to the cell suspensions and incubated for the time periods indicated in the figure legends. The sample were then diluted with a fivefold excess of ice-cold PS and the cells were collected by centrifugation for 2 min at 800 g. The cells were washed by gently resuspending the pellets in 1 ml ice-cold PS, overlaying the suspensions onto 2 ml of PS containing 5% BSA, and centrifuging as before. Unbound beads remained in the supernatant, whereas cells and cells with bound beads sedimented (4).

RESULTS

Adsorption of Plasma Fibronectin onto Polystyrene Latex Beads

To measure phagocytosis of pFN-coated beads as a function

¹ *Abbreviations used in this paper:* BHK, baby hamster kidney; BSA, bovine serum albumin; pFN, plasma fibronectin; PS, phosphate saline, TCA, trichloroacetic acid.

of fibronectin density on the beads, it was necessary to establish appropriate pFN binding conditions. Using the techniques described previously (4), the adsorption of pFN to the beads was determined as a function of pFN concentration. As shown in Fig. 1, radiolabeled pFN adsorbed to the beads with typical saturation kinetics. The binding constants were analyzed by double reciprocal plots (Fig. 1, inset). In seven different experiments using 0.76-μm beads and three different ³H-pFN preparations saturation binding was 723 ± 168 ng pFN/cm² with a dissociation constant of 7.1 ± 2.2 × 10⁻⁷ M (based on a molecular weight for pFN of 440,000). These constants appeared to be independent of bead diameter. For example, with the 0.085-μm beads, saturation binding was 618 ng pFN/cm² with a dissociation constant of 3 × 10⁻⁷ M. It should be noted that these saturation binding values are similar to those previously reported for polystyrene tissue-culture dishes (7, 12).

Quantitation of Ingestion of pFN-Coated Beads

That ingestion of pFN-coated beads by BHK cells can occur was established previously by antibody binding and electron microscopic observations (4). Experiments were carried out with pFN-coated beads to determine whether the portion of bound beads that were ingested could be distinguished based upon their sensitivity to trypsin treatment as has been found by others (10). This possibility seemed likely since previous studies had shown that treatment of cells with trypsin destroyed their ability to bind fibronectin-coated beads (4). Cells were incubated with pFN-coated beads at 4°C or 37°C. Under these conditions, beads were found randomly distributed on the cells (Fig. 2, A and C, respectively). When cells with beads bound at 4°C were washed and incubated with trypsin, all of the bound beads were removed (Fig. 2B), which is consistent with the expectation that no ingestion would occur at 4°C. On the other hand, trypsin treatment removed only some of the beads that had bound to cells in the 37°C incubations. The remainder appeared to have been ingested and were located intracellularly in a perinuclear distribution (Fig. 2D). Similar results were obtained in quantitative experiments

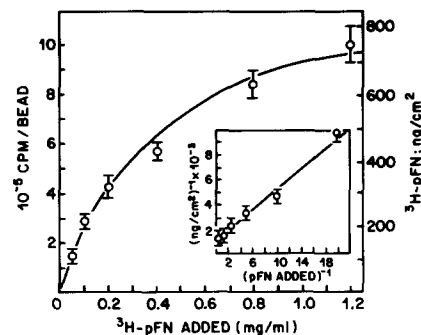


FIGURE 1 Binding of ³H-pFN to 0.76-μm latex beads. To 1.65 × 10⁹ 0.76-μm latex beads were added increasing concentrations of ³H-pFN (specific activity 16,000 cpm/μg) in PS giving a total volume of 0.3 ml. The bead/³H-pFN mixture was incubated and processed as described in Materials and Methods. After the beads were dispersed by sonication, samples from each bead preparation were analyzed for radioactivity. Data points are the mean and SD of three samples. These data were used to form a double reciprocal plot (inset). Fibronectin density on the beads at saturation was 723 ± 168 ng pFN/cm², calculated from the double reciprocal plot as (Y-intercept)⁻¹. The K_d was 7.1 ± 2.2 × 10⁻⁷ M, calculated according to the equation, K_d = (slope) ÷ (Y-intercept).

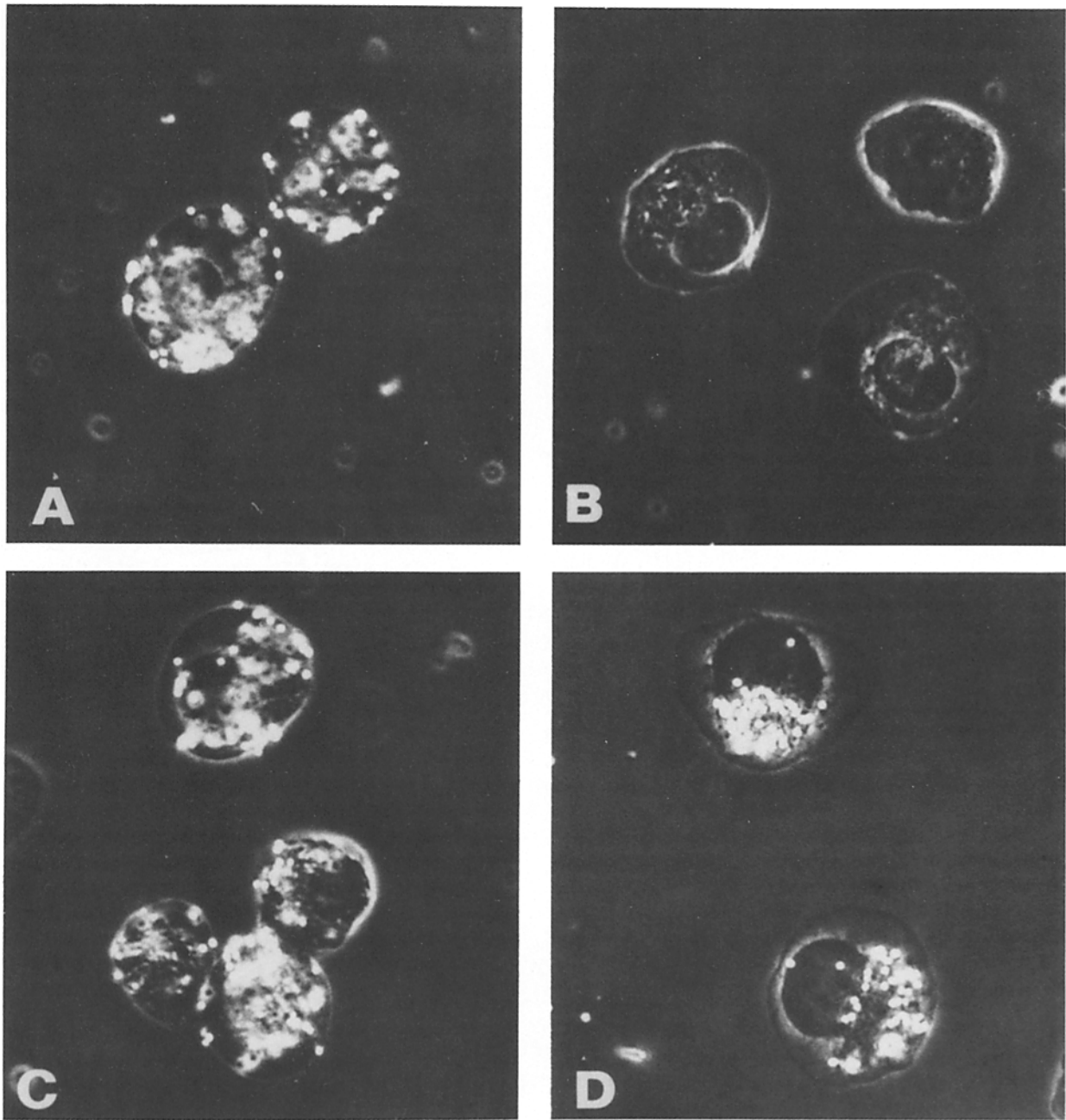


FIGURE 2 Bound vs. phagocytosed pFN-coated beads. BHK cells were incubated at 4°C (A and B) or 37°C (C and D) for 30 min with 2×10^9 beads ($0.76 \mu\text{m}$, pFN density $\sim 500 \text{ ng/cm}^2$). At the end of the incubations, the cells were collected and washed as described in Materials and Methods. Half of the samples were immediately fixed with 3% formaldehyde (A and C). The other half were incubated with 1 ml of 0.25% Gibco trypsin for 5 min at 37°C (B and D), after which the cells were centrifuged through the BSA gradient and fixed as above. Samples were photographed using a Zeiss Photomicroscope III equipped with dark field phase contrast. $\times 1,800$.

using ^3H -pFN as shown in Table I. Trypsin treatment removed 95% of the radioactivity associated with cells that had bound pFN-coated beads at 4°C, but only 54% of the radioactivity associated with cells that had bound beads at 37°C. This result suggested that $\sim 45\%$ of the bound beads had been ingested during the 30-min incubation at 37°C. In the studies to be described using the radioactive technique, the cell-associated radioactivity was taken as a measure of total bead binding (i.e., cell surface or ingested), and trypsin-resistant radioactivity was taken as a measure of the bound beads that

had been ingested.

When experiments similar to those described in Fig. 2 were carried out with albumin-coated beads, no binding or ingestion of beads by the cells was observed, as reported previously (4). When the experiments were carried out with uncoated beads in protein-free incubations, extensive binding of the beads to the cells occurred, but $<5\%$ of the bound beads appeared to be ingested. These results show that nonspecific binding of beads to the cells is not sufficient for ingestion to occur.

TABLE I
Quantitation of pFN-coated Bead Binding and Phagocytosis*

Incubation temperature	Cell-associated radioactivity		% Trypsin releasable
	Total	After trypsin	
4°C	11,130 ± 307	572 ± 47	95
37°C	10,422 ± 122	4,783 ± 76	54

* BHK cells were incubated for 30 min at 4°C or 37°C with 0.76- μ m beads (1.65×10^9) coated with ^3H -pFN (pFN density $\sim 500 \text{ ng/cm}^2$, 163,200 cpm/incubation). At the end of the incubations, the cells were collected and washed as described in Materials and Methods, and resuspended in 1.0 ml of 0.25% trypsin (Gibco) at 37°C. After 5 min, samples of the suspensions were used to determine total cell-associated radioactivity. The remaining portions of the suspensions were centrifuged through the BSA gradient, resuspended in 1.0 ml of PS, and radioactivity measured to determine trypsin-resistant (i.e. ingested) radioactivity. Data presented are the mean and SD from duplicate experiments.

Time Course of pFN-Coated Bead Interaction with BHK Cells

The time course of pFN-coated bead interaction with BHK cells was measured as shown in Fig. 3. In 4°C incubations, the binding of pFN-coated beads to the cells increased with time up to 60 min. Little ingestion of the beads was observed, and there was no release of trichloroacetic acid (TCA)-soluble radioactivity into the incubation medium. It should be noted that in these experiments pFN-coated beads were present in excess. That is, <15% of the input beads were bound by the cells. At 37°C, binding of the beads increased with incubation time up to 30 min and then leveled off or declined. In addition, a substantial portion of the bound beads were ingested by 15 min. Although no further increase in ingested radioactivity was observed after 15 min, TCA-soluble radioactivity was detected in the medium after 30 min and by 60 min had reached a level equal to the amount of radioactivity within the cells. The release of TCA-soluble radioactivity suggested that continuous ingestion of beads was occurring after 15 min, but that a portion of the ingested fibronectin was degraded and released from the cells. Such an interpretation is consistent with the previous finding that ingested beads were delivered to lysosomes (4).

Relationship of pFN-Coated Bead Binding and Ingestion to pFN Density

It was of interest to determine the relationship between fibronectin density on the beads, bead binding, and bead ingestion. For instance, it might have required a different density of fibronectin for ingestion of beads to occur than for binding of beads to occur. To make this determination, beads coated with varying densities of fibronectin were incubated with cells at 4°C or 37°C. Since there were different absolute amounts of radioactivity per bead, the data are presented in terms of the number of beads per cell bound at 4°C or ingested at 37°C. The results (Fig. 4) demonstrated a close correspondence in the dose response curves. That is, fibronectin densities necessary to permit bead binding were sufficient to permit ingestion. In addition, the binding and ingestion curves reached a maximum level at a fibronectin density of $\sim 250 \text{ ng/cm}^2$.

Relationship of pFN-Coated Bead Binding and Ingestion to Bead Diameter

The above experiments could be interpreted in two different ways. On one hand, the distribution of pFN receptors on the

cell surfaces might require a particular spacing of pFN molecules on the beads in order for efficient binding to occur. Alternatively, a certain number of cell-fibronectin interactions might be required regardless of how the fibronectin molecules are spaced. Stated differently, the cells might be responding either to the number of fibronectin molecules per unit area on the beads or to the absolute number of fibronectin molecules on the beads.

In order to distinguish between the above possibilities, binding experiments at 4°C were carried out with beads of varying diameters. In these studies there were similar densities of fibronectin per bead and similar amounts of total bead surface area were added to the incubations so that the parameter varied was the absolute number of fibronectin molecules

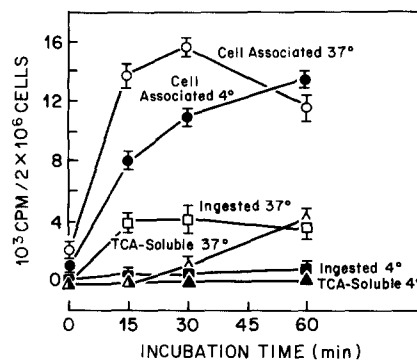


FIGURE 3 Time course of binding and phagocytosis of pFN-coated beads. BHK cells were incubated at 4°C or 37°C with 1.65×10^9 ^3H -pFN beads (0.76 μ m, ^3H -pFN density 300 ng/cm^2). At the times indicated, the total cell-associated and -ingested radioactivity were determined as in Table I. The supernatants from the cell collection step were further centrifuged at 27,000 g (Sorvall SS-34) for 10 min at 4°C to sediment unbound beads; 100% TCA was added to this supernatant to attain a final TCA concentration of 10%. These samples were incubated for 20 min at 4°C, centrifuged at 1,800 g for 5 min to sediment TCA-precipitable material, and samples of the supernatants were assayed for TCA-soluble radioactivity. Data points are the mean and SD of duplicate experiments.

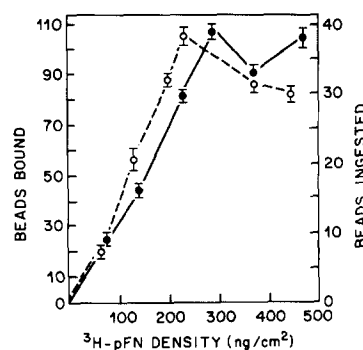


FIGURE 4 Binding and phagocytosis of pFN-coated beads as a function of pFN density on the beads. BHK cells were incubated for 30 min at 4°C or 37°C with 1.65×10^9 beads (0.76 μ m) coated with ^3H -pFN at the densities indicated. The cells were collected and washed as described in Materials and Methods. Samples from the 4°C incubations were assayed for cell associated radioactivity. Cells from the 37°C incubation were subjected to the trypsin procedure as in Table I, and aliquots of these samples were analyzed to determine ingested radioactivity. Beads bound per cell at 4°C (●) or ingested per cell at 37°C (○) were calculated according to counts per minute (bound or ingested) \div (cells per incubation) (counts per minute/bead). Data presented are the mean and SD of duplicate experiments.

TABLE II
Binding of pFN-coated Beads of Different Diameters to Cells at 4°C

Beads added				Beads bound		
Diameter	No. added	Total bead surface area added	pFN Density	cpm associated per 2 × 10 ⁶ cells [‡]	Number beads bound per cell [§]	% Cell surface area occupied by beads [¶]
μm		μm ²	ng/cm ²			
0.085	1.31 × 10 ¹¹	2.97 × 10 ⁹	356	5,399 ± 1,342	3,552	3.8
0.305	1.05 × 10 ¹⁰	3.07 × 10 ⁹	322	4,514 ± 370	255	3.5
0.76	1.66 × 10 ⁹	3.01 × 10 ⁹	334	4,395 ± 39	39	3.3
1.091	8.05 × 10 ⁸	3.01 × 10 ⁹	351	4,145 ± 97	17	2.9

* BHK cells were incubated at 4°C for 15 min with ³H-pFN beads as indicated. At the end of the incubations, the cells were collected and washed as described in Materials and Methods. The cell pellets were resuspended in 1.0 ml of PS, and samples of the suspensions were used to determine cell-associated radioactivity.

‡ Average ± SD from duplicate experiments.

§ Number of beads bound/cell = (cpm associated) ÷ (cells per incubation) (cpm per bead).

¶ Percent of cell surface occupied was calculated based upon the cross-sectional area of the beads, and the average diameter of BHK cells was measured to be ~13 μm.

TABLE III
Binding of pFN-coated Beads of Different Diameters to Cells at 37°C*

Beads added to cells		Beads bound by the cells				
Diameter	pFN Density	cpm associated per 2 × 10 ⁶ cells [‡]	cpm ingested per 2 × 10 ⁶ cells [‡]	% Bound beads ingested [§]	No. of beads ingested per cell [¶]	Bead surface area ingested per cell [¶]
μm	ng/cm ²					μm ²
0.085	256	4,414 ± 209	1,033 ± 172	23.4	945.5	21.5
0.305	303	8,010 ± 200	1,936 ± 235	24.2	116	33.9
0.760	329	8,860 ± 34	2,368 ± 21	26.7	21	38.1
1.091	330	8,218 ± 955	2,509 ± 116	30.5	10.5	39.3

* BHK cells were incubated at 37°C for 15 min with ³H-pFN beads as indicated. The number of beads added and total bead surface area added are shown in Table II. At the end of the incubations, the cells were collected and assayed for bound or ingested radioactivity as described in Materials and Methods and Table I.

‡ Average ± SD from duplicate experiments.

§ % Ingested = cpm ingested ÷ cpm associated.

¶ Number ingested/cell = (cpm ingested) ÷ (cells per incubated) (cpm per bead).

¶ Area ingested/cell = (number ingested/cell) (surface area/bead).

per bead (Table II). The beads used ranged from 0.085 μm to 1.091 μm, and the absolute number of fibronectin molecules per bead ranged from <100 to >15,000 at the fibronectin density tested (~300 ng/cm²).

When the 4°C binding data were analyzed, it was found that many more small beads bound to cells than large ones (Table II), but the area occupied by the bound beads was calculated to be ~3% of the cell surface regardless of size. This result indicated that the extent of bead binding at 4°C was similar even though there was more than a two orders-of-magnitude difference in the absolute number of fibronectin molecules per bead.

Experiments also were carried out with different sized beads at 37°C. The time course of bead binding, ingestion, and release of TCA-soluble material was similar as observed in Fig. 3, regardless of bead diameter. As shown in Table III, the number of small beads ingested per cell was much greater than the number of large beads. When, however, these data were analyzed in terms of total bead surface area ingested by the cells, it was found that the smaller beads were not ingested as well as the larger ones. In part, this appeared to occur because the percentage of bound beads that were ingested was lower with the small beads.

The results of the experiments presented in Tables II and III also suggested that the ratio of total bead binding (cell-

associated cpm) at 37°C compared with 4°C was less with the smaller beads than with the larger ones. Differences in the pFN densities on the beads, however, might have accounted for this result. To test this point, experiments were carried out at 4°C and 37°C using the same bead preparations. The results shown in Fig. 5 indicate that small beads bound less well to cells at 37°C than at 4°C, whereas larger beads bound better to cells at 37°C than at 4°C.

Ingestion of pFN-Coated Beads Bound at 4°C

It appeared in the above experiments that bead interactions with cells at 37°C, unlike the situation of 4°C, depended upon parameters other than fibronectin density. It was of interest, therefore, to study phagocytosis of beads of varying sizes under conditions in which the beads were previously bound to the cells at 4°C. To accomplish this, pFN-coated beads were incubated with cells at 4°C, and then the cells were washed and warmed up to 37°C as shown in Fig. 6. The total cell-associated radioactivity rapidly decreased once the cells were placed at 37°C. This appeared to result from the release of some of the bound beads back into the incubation medium. Also, there was release into the medium of TCA-precipitable radioactivity, most of which required cells. That is, the release was not a result of nonspecific dissociation of radiolabeled

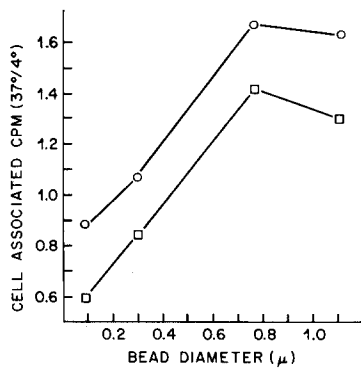


FIGURE 5 Association of pFN-coated beads with cells at 4°C or 37°C as a function of bead diameter. BHK cells were incubated at 4°C or 37°C with various sized ³H-pFN beads as follows: 0.085 μm (301 ng pFN/cm², 1.45 × 10¹¹ beads), 0.305 μm (367 ng pFN/cm², 9.58 × 10⁹ beads), 0.76 μm (315 ng pFN/cm², 1.65 × 10⁹ beads), 1.091 μm (210 ng pFN/cm², 1.40 × 10⁹ beads). After the specified incubation times (○, 15 min; □, 30 min), the cells were collected and assayed for cell-associated radioactivity as described in Materials and Methods. Experiments were carried out in duplicate, and data presented are mean values of cell associated CPM at 37°C divided by cell associated CPM at 4°C.

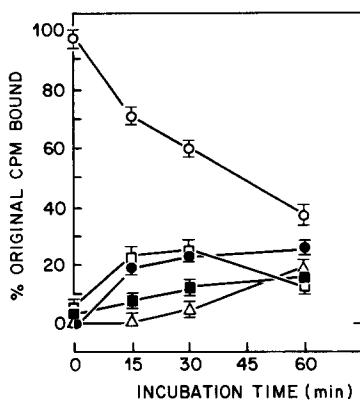


FIGURE 6 Time course of phagocytosis of pFN-coated beads previously bound to BHK cells. BHK cells were incubated for 30 min at 4°C with 1.65 × 10⁹ ³H-pFN beads (0.76 μm, ³H-pFN density 300 ng/cm²). The cells were collected and washed as described in Materials and Methods and further incubated in 1.0 ml of DPBS containing 0.2% BSA at 37°C for the times indicated. At each time, cell-associated (○), ingested (□), TCA-precipitable (●), TCA-nonprecipitable (Δ), and radioactivity associated with released beads (■) were determined (Fig. 3). Greater than 95% of the radioactivity originally bound to the cells at 4°C was recovered in the various fractions at each time point analyzed. Data points are expressed as the percent of radioactivity originally bound to the cells at 4°C (11,345 ± 52 cpm/2 × 10⁶ cells) and are the mean and SD of duplicate experiments.

fibronectin from the beads. In addition, bead ingestion and the appearance of TCA-soluble radioactivity in the medium were observed to occur at a similar time as in experiments carried out with cells in the continuous presence of beads (Fig. 3). The extent of ingestion as a function of bead size is shown in Fig. 7. The percentage of the beads previously bound at 4°C that subsequently were ingested at 37°C increased with increasing bead diameter, and the magnitude of the increase was similar to that shown in Table III.

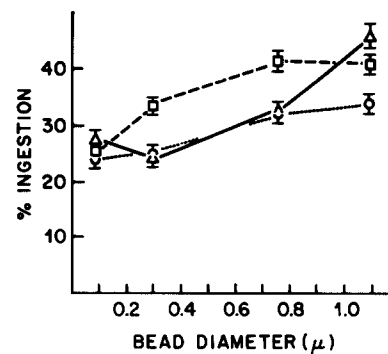


FIGURE 7 Phagocytosis of pFN-coated beads as a function of bead diameter. ³H-pFN beads of various sizes (described in Table II) were prebound to BHK cells as in Fig. 6. Cells with beads bound at 4°C were further incubated at 37°C for the time periods indicated (○, 15 min; □, 30 min; Δ, 60 min) after which ingestion was analyzed as in Table I. The extent of bead ingestion is expressed as a percentage of the beads originally bound to the cells, and the data points are the mean and SD of duplicate experiments.

Distribution of Bound pFN-Coated 0.085-μm Beads

The apparent lessening in the ability of cells to ingest beads of increasingly smaller sizes might have been a result of a difference in the surface distribution in which the small beads bound to the cells. Also, surface redistribution of the small beads, e.g., capping, might have occurred rather than ingestion. To examine these possibilities, the distribution of 0.085-μm beads bound to the cells and their susceptibility to release by trypsin were assessed microscopically after 4°C and 37°C incubations. This was accomplished using indirect immunofluorescence since the beads were too small to be directly visualized by phase contrast microscopy. The distribution of beads on the cell surfaces was similar whether binding occurred at 4°C (Fig. 8A) or 37°C (Fig. 8C), and the beads bound at 4°C were completely removed by trypsin treatment (Fig. 8E). At 37°C, however, some of the beads, presumably those that were ingested, were not released by trypsin treatment (Fig. 8G). These results are similar to the findings with the larger beads (Fig. 2).

Analysis of Binding Constants of 0.085-μm pFN-Coated Beads

Finally, it was of interest to determine the number of binding sites on the cells for the 0.085-μm beads. Based upon the cross-sectional area of these beads it could be calculated that a maximum of 94,000 beads could bind to a spherical cell, 13 μm in diameter. Binding of pFN-coated 0.085-μm beads to cells as a function of bead concentration demonstrated saturation kinetics, as shown in Fig. 9. A double reciprocal plot of the data (inset) indicated that there was a maximum of 62,000 bead-binding sites per cell, and the dissociation constant was 4.03 × 10⁻⁹ M.

DISCUSSION

The studies reported in this paper were carried out in order to determine the effects of fibronectin concentration and particle size on the phagocytosis of fibronectin-coated beads by fibroblasts. Initial experiments established conditions for

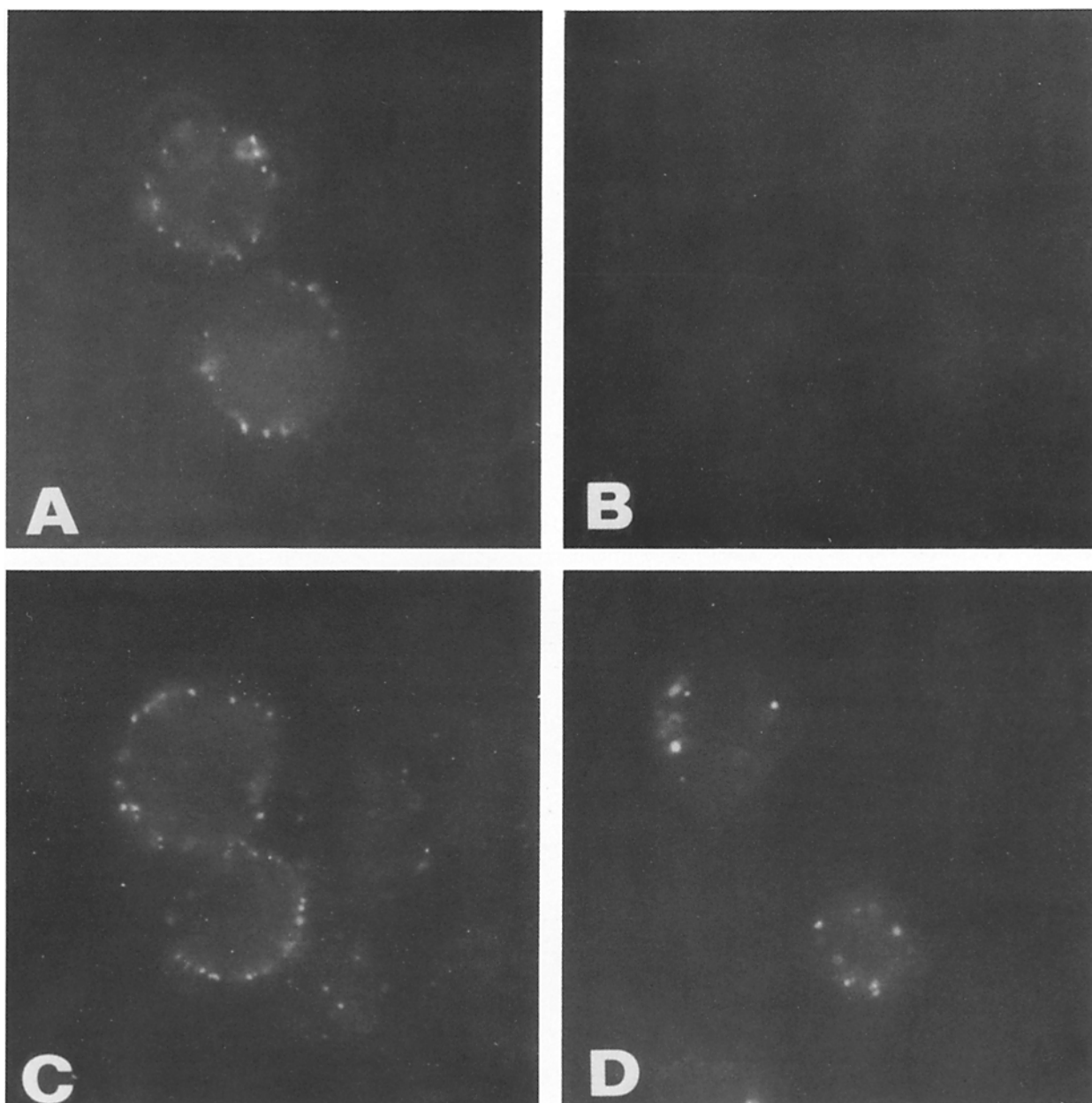


FIGURE 8 Detection of binding and phagocytosis of 0.085- μm pFN-coated beads by indirect immunofluorescence. BHK cells were incubated with beads (0.085 μm , pFN density 300 ng/cm²) for 60 min at 4°C (A and B) or 37°C (C and D). The cells were collected and washed as described in Materials and Methods. Half of the samples (A and C) were immediately fixed with 3% formaldehyde, permeabilized with Triton X-100, and processed for indirect immunofluorescence using anti-pFN as described previously (6). The other samples (B and D) were trypsinized as in Fig. 2 and processed for indirect immunofluorescence as above. $\times 1,500$.

preparing beads coated with various concentrations of fibronectin. In addition, a method was demonstrated for distinguishing between total bound beads (cell surface or ingested) and bound beads that were ingested.

Fibronectin-coated beads were bound and ingested by the cells. Based upon the appearance of TCA-soluble radioactivity in the medium, it appeared that degradation of the radiolabeled fibronectin bound to the beads was occurring. When binding at 4°C was compared to ingestion at 37°C, there appeared to be a similar dependence on the density of fibronectin adsorbed to the beads. This is significant because there might have been a different amount of fibronectin required for binding than for ingestion.

The binding of fibronectin-coated beads to the cells at 4°C appeared to be independent of bead size over the range of 0.085 μm to 1.091 μm when the beads were coated with similar densities of pFN. Since the absolute number of fibronectin molecules varied from <100 on the 0.085- μm beads to >15,000 on the 1.091- μm beads, it can be concluded that fibronectin density per se is more important than the absolute number of fibronectin molecules in determining binding behavior at 4°C.

The above findings are of considerable importance in understanding the idea of a "threshold" level of fibronectin required on the substratum for cell spreading to occur. That a threshold exists was inferred from the observation that

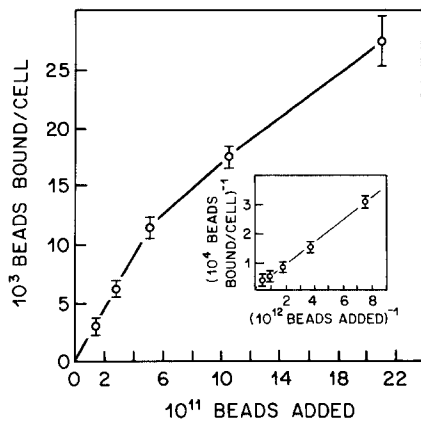


FIGURE 9 Binding of 0.085- μ m pFN-coated beads as a function of beads number. BHK cells were incubated for 15 min at 4°C with 0.085- μ m bead (pFN density 292 ng/cm²) as indicated. The cells were collected and washed and assayed for bound radioactivity as described in Materials and Methods. Data point are the mean and SD of duplicate experiments. Bead binding at saturation was 61,728 beads/cell, calculated from the double reciprocal plot (inset) as (Y-intercept)⁻¹. The K_d was 4.03×10^{-9} M, calculated according to the equation $K_d = (\text{slope}) \div (\text{Y-intercept})$.

maximal cell-spreading activity required a certain density of fibronectin on the substratum (12). On plain polystyrene dishes, for instance, the threshold level required for complete cell spreading was ~ 100 ng of fibronectin/cm² (7). Based upon the present experiments, it can be suggested that the key feature of the threshold is the spacing of fibronectin molecules on the substratum. The density of fibronectin required for maximal binding and phagocytosis was ~ 250 ng/cm², which is 1 fibronectin molecule per 300 nm² (based on a molecular weight of 440,000). Assuming that binding of fibronectin-coated beads to cells required more than one cell receptor-fibronectin interaction (4), then it can be suggested that the threshold density corresponds to the spacing between adjacent fibronectin receptors on the cells necessary for multiple interactions to occur (i.e., ~ 18 nm).

A lower estimate for the number of fibronectin receptors was 60,000/cell, based upon the binding studies with 0.085 μ m. This assumed, however, only one interaction between fibronectin and its receptor for each bead. If there were two interactions/bead, then the actual number of receptors would be 120,000/cell, which is close to the value of 100,000 specific pFN-binding sites/cell measured in binding studies with soluble pFN (10). The dissociation constant for the 0.085- μ m beads of 4.03×10^{-9} M indicates that multivalent forms of pFN have a high affinity for the cells. In the case of multivalent interactions, the binding constant is multiplicative (i.e., $k_{\text{apparent}} = k_a k_b \dots$) (16). If binding of the 0.085- μ m beads involves a two-site interaction, then the binding constant between each individual pFN molecule and its receptor would be on the order of 6×10^{-5} M, which is consistent with the low affinity of soluble pFN for the cells that has been discussed elsewhere (10). A caveat to the theoretical treatment above is the presupposition that the 0.085- μ m beads are interacting with the cells as individual beads and not as microaggregates. By analogy to the other beads, it seems likely that this is the case (see preparations of beads under *Materials and Methods*). Nevertheless, without carrying out electron microscopic studies this point cannot be confirmed.

The ratio of binding of beads at 37°C to binding at 4°C

appeared to depend upon bead size. The smaller the beads, the lower the 37°C/4°C ratio. The explanation for this finding is unclear, but may be related to the finding that the binding of soluble pFN to cells at 37°C was much less than the binding that occurred at 4°C (10). It is possible that the affinity between fibronectin and its cell surface receptor is different at 4°C and 37°C. For instance, the affinity between low density lipoprotein and its cell surface receptor is five times less at 37°C than at 4°C (2). If the affinity between fibronectin and its receptor is much less at 37°C than at 4°C, then binding of beads to cells at 37°C compared to 4°C may be dependent on bead size, because the absolute number of fibronectin-receptor interactions that could occur simultaneously would increase as bead size increases. This multiple binding effect could compensate for the lower affinity of individual fibronectin-receptor interactions at 37°C.

A difference depending upon bead size also was observed for ingestion. That is, the percentage of bound large beads that was ingested was higher than the percentage of bound small beads that was ingested. Considering the difference in the absolute number of pFN molecules on the smallest and largest beads (100-fold), the twofold difference in ingestion was minimal. Nevertheless, that there should be any difference was unexpected. Generally, phagocytosis has been suggested to proceed by a zipper mechanism (21). Since fibronectin adsorbs onto polystyrene surfaces in a random and uniform manner (5), the spacing of fibronectin molecules around the beads should be similar at similar fibronectin densities regardless of bead size. Therefore, the ability of cells to zipper around the beads should have been independent of bead size. The possibility that the small beads were bound or reorganized on the cell surface differently from the larger beads was ruled out by immunofluorescence experiments, but high resolution analyses at the electron microscopic level have yet to be carried out. It is also possible that large beads and small beads were internalized by different mechanisms, e.g., phagocytosis and pinocytosis, or that efficient phagocytosis of the small beads required that they precluster on the cell surface (c.f. reference 14).

A variable that cannot be ruled out as a parameter determining differences in how well small and large beads are ingested by the cells is the radius of curvature of the beads. For instance, given an approximate spacing between pFN molecules on the bead surfaces of 18 nm (see above), if the conformation of adsorbed pFN molecules is similar regardless of bead diameter, then the angle between adjacent pFN molecules would be $\sim 24^\circ$ on 0.085- μ m beads but only 1.9° on 1.091- μ m beads. These differences in orientation between the pFN molecules may affect how the cell surface receptors can interact with them during ingestion.

Finally, an observation of potential interest was the release of TCA-precipitable radioactivity into the medium when pFN-coated beads were previously bound to the cells at 4°C and then switched to 37°C. This phenomenon was cell dependent and may indicate an effect of cell surface protease activity. Under the same conditions, the release of beads from the cell surface may have been normal dissociation or may have occurred because some of the fibronectin was removed from the bead surfaces.

Previously, the phagocytic capabilities of fibroblasts have not been examined in detail, and many interesting features of this process have been described by the present studies. This subject seems to be of considerable importance since fibro-

blasts may phagocytose collagen during the remodeling of connective tissue in vivo (15, 22). In this regard, it is interesting that fibronectin binds much better to denatured than to native type I collagen (19), which might provide a signal for phagocytosis of the denatured material under wound-healing conditions (8, 23).

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