Fibronectin-Enhanced Attachment of Gelatin-Coated Erythrocytes to Isolated Hepatic Kupffer Cells

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The phagocytic process is a combination of a sequence of events which includes a recognition attachment phase and a subsequent internalization phase. The present study was designed to investigate the effect of plasma fibronectin on the attachment and ingestion of gelatinized sheep erythrocytes to isolated rat Kupffer cells in a monolayer assay. Kupffer cells were isolated by sequential collagenase-pronase digestion followed by metrizamide density gradient centrifugation and subsequent adherence to plastic. Classification as Kupffer cells was confirmed by the presence of functional Fc receptors, a positive peroxidase reaction, and phagocytic activity. Purified plasma fibronectin as well as rat serum containing fibronectin promoted attachment of gelatinized fixed sheep erythrocytes to Kupffer cells in a dose-dependent manner, whereas fibronectin-deficient serum did not. Heparin did not enhance the fibronectin-mediated attachment or ingestion of gelatinized sheep erythrocytes at lower particle doses, whereas at higher particle doses heparin augmented the response. These results indicate that fibronectin can enhance the binding and ingestion of foreign gelatin-coated particulates by Kupffer cells.

Key words: Kupffer cells, fibronectin

INTRODUCTION

Kupffer cells represent a major population of reticuloendothelial (RE) cells whose primary function is phagocytosis [18]. The recognition process can be enhanced by the presence of circulating opsonic factors, of which immunoglobulins and complement have been extensively studied [23]. Plasma fibronectin (FN), a large
molecular weight glycoprotein, can enhance the phagocytic uptake of gelatinized sheep red blood cells (SRBC) [4,14] and gelatinized latex particles [11,24] by rat and mouse peritoneal macrophages. The functional ability of fibronectin to mediate phagocytic uptake has, in part, been primarily assayed with the liver slice bioassay [3,20]. In the liver slice assay, the Kupffer cells are believed to be responsible for the removal of the gelatinized RE test lipid emulsion [18,19,21]. While EM studies have emphasized the Kupffer cells as the primary site of uptake in the liver slice assay [8], it is difficult to evaluate the relative contribution of interiorization versus adherence in the uptake measured by isotopic assay. In addition, the distribution of phagocytic cells in the liver is nonhomogeneous [26], and, thus, variability can be encountered between tissue slices with the assay even though it is normalized on a tissue weight basis. More recently, peritoneal macrophages have been used to assess FN-mediated phagocytosis of gelatin-coated particles [4,11,14]. However, these cells are not the cell type primarily responsible for the removal of blood-borne particulates. As a result, several assumptions are made in order to extrapolate in vitro observations to the in vivo RES clearance activity. The present study was designed to investigate the influence of plasma fibronectin on the interaction of sheep erythrocytes with isolated hepatic Kupffer cells. We tested the hypothesis that plasma fibronectin would directly promote the attachment and ingestion of these particles to Kupffer cells.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 250–350 g, maintained on food and water ad libitum, were used as both blood and liver donors.

Kupffer Cell Isolation and Monolayer Preparation

Kupffer cells were isolated using a modification of previously described methods [13,16,29,30]. Under nembutal anesthesia, a midline incision was made and the portal vein cannulated. The liver was pump-perfused at a flow rate of 15–20 ml/min with calcium- and magnesium-free Hanks’ balanced salt solution (HBSS). At the onset of perfusion, the inferior vena cava was clamped. When the liver became swollen, the inferior vena cava was cut and the liver excised. The liver was perfused with approximately 100 ml of 0.03% collagenase Type II solution (Sigma, St. Louis, MO) dissolved in HBSS. It was then rinsed briefly in Geys balanced salt solution (GBSS), placed on a petri plate and a small volume of 0.2% protease Type XIV (Sigma) dissolved in GBSS was added. With light manual manipulation using a spatula, the liver began to dissociate, at which time additional protease solution was added to yield a final volume of 100 ml. To the protease-supplemented digested liver suspension, 0.5 mg of DNAse I (Sigma) was added. This mixture was incubated for 1 hr at 37°C in a shaking water bath and gassed with 95% O2 - 5% CO2. The suspension was filtered on a nylon gauze, centrifuged for 5 min at 350 g, and washed twice in GBSS. The final pellet was resuspended in 5 ml of GBSS and 7 ml of 30% metrizamide suspension and centrifuged for 15 min at 1400 g. The top layer containing a Kupffer cell-rich fraction was collected, washed twice in GBSS, and centrifuged for 5 min at 350 g.

The pellet was resuspended in GBSS, cells were counted, and viability determined by trypan blue exclusion. The average yield of nonparenchymal cells per liver was $1.5 \times 10^7$. The nonparenchymal cells were plated at $2 \times 10^5$/ml and cultured for
18 hr in Dulbecco's Modified Eagle Medium (DMEM), 10% calf serum, 50 units penicillin, and 50 mg streptomycin/ml. Following incubation, the cells were washed twice in DMEM. Fresh DMEM-calf serum (10%) was added and the cells were cultured an additional 6 hr before use in the monolayer assay.

Isolation of Plasma Fibronectin

Blood was collected using a 10-cc plastic syringe containing 0.8 ml of citrate phosphate dextrose (CPD) anticoagulant with 3 mM benzamidine HC1 from ether-anesthetized rats by inferior vena cava puncture. The blood was centrifuged at 5,000 g for 20 min at 5°C. Following this, the citrated plasma was collected. The addition of 15 mg BaCl2/ml of plasma removed the vitamin K-dependent proteins. This suspension was stirred gently for 30 min at 4°C and centrifuged at 12,000 g for 10 min at 4°C. Excess barium was removed by the addition of 9.5 mg/ml (NH4)2 SO4, and the BaSO4 was removed by centrifugation [3]. The plasma was then applied to a gelatin-Sepharose column and the fibronectin was purified by an affinity procedure previously described [5].

Preparation of Gelatinized Sheep Red Blood Cells (SRBC)

Formalin-fixed sheep red blood cells were used as the target particle. They were gelatinized on the day of study as previously described [4]. The volume of target SRBC preparation was adjusted to a 1% hematocrit with DMEM (2 × 10^9 SRBC/ml). A 10 μl aliquot of the SRBC was added to the washed monolayer of Kupffer cells in a volume of 1 ml DMEM. Attachment of this test particle was measured following a 30-min incubation at 37°C.

Preparation of Gelatin-Coated Tanned Sheep Erythrocytes

The erythrocyte preparation used was a modification of Czop and Austen's procedure [7]. Sheep erythrocytes were collected from whole heparinized blood. Two milliliters of packed SRBC were washed twice in PBS (pH 7.4) and radiolabeled with 0.5 μCi 51Cr in a total volume of 20 ml. This was allowed to incubate for 60 min at room temperature. Cells were washed with PBS, pH 7.4, and 100 μl of a 12.5 mg/ml tannic acid solution was added to a total volume of 50 ml PBS (pH 7.4). Following a 30-min incubation at 37°C, the cells were washed three times in PBS (pH 6.4). The cells were then resuspended in 25 ml of PBS, pH 6.4, and an additional 25 ml of a 2 mg/ml gelatin solution in PBS (pH 6.4) was added. This suspension was allowed to shake for 30 min at room temperature and subsequently washed three times in PBS, pH 7.4. The sheep red blood cells were adjusted to a 10% hematocrit by adding appropriate amounts of DMEM.

Rosetting Assay

The degree of binding of opsonized or unopsonized gelatinized sheep erythrocytes to Kupffer cells was determined microscopically. In this procedure, we randomly counted four groups of 100 Kupffer cells, and the number of target SRBC bound per 100 Kupffer cells was recorded.

Kupffer Cell Monolayer Isotopic Phagocytic Assay

Twenty-four-hour cultured Kupffer cell monolayers were washed in DMEM to remove nonviable or nonadherent cells. DMEM (no calf serum added) supplemented with fibronectin or IgG was added to each well (Costar 24-well plate). The monolay-
ers were challenged with $^{51}$Cr gelatinized sheep erythrocytes at a dose of $4 \times 10^7$ SRBC (40:1 particle to cell ratio) or $8 \times 10^7$ SRBC (80:1 particle to cell ratio). The challenged monolayers were incubated for 1 hr and subsequently washed three times with ice cold PBS without Ca$^{2+}$ or Mg$^{2+}$. The monolayers were then solubilized in 0.5 M NaOH and percent uptake of SRBC was determined. In studies in which tannic acid-treated red cells were used, the percent ingested was determined by subjecting the monolayers to brief hypotonic lysis prior to solubilization.

Identification of Kupffer Cells

The isolated adherent nonparenchymal liver cells were identified as Kupffer cells by three other criteria in addition to their adherence properties. These criteria were the presence of Fc receptors, the presence of peroxidase enzyme, and the phagocytic activity of the cells.

Fc Receptors

Sheep red blood cells were opsonized with purified rabbit antisheep erythrocyte IgG [1,2] (Cordis Corp., Miami, FL) for 30 min and incubated with the Kupffer cells for an additional 30 min followed by determination of binding by microscopy.

Peroxidase

As previously documented, among the liver sinusoidal cells, only Kupffer cells give a positive staining reaction for peroxidase [25, 27]. In the present study the percentage of cells which were peroxidase positive was determined [10]. Cultured cells were fixed in 1.25% glutaraldehyde, washed in 0.05 M Tris buffer (pH 7.6), and replaced with diphenyltetraamine tetrahydrochloride-Tris-H$_2$O$_2$ solution. This was incubated for 20 min, washed with distilled water and examined microscopically.

Kupffer Cell Phagocytosis-Electron Microscopy

Kupffer cells were prepared for electron microscopy by fixing 24-hr cultures for 15 min in 2% glutaraldehyde in half strength DMEM containing 0.1 mM cacodylate, 0.05 M sucrose, and 5% calf serum. The fixative-culture media was removed by aspiration, and fresh 2% glutaraldehyde buffer was added and incubated at room temperature for 1 hr. The fixed cells were washed three times for 15 min in 0.1 M cacodylate buffer. The monolayers were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at room temperature. The cells were washed once in distilled water for 15 min followed by 10 min dehydration steps in 30, 50, 70, 90, and 100% ethanol. Embedding of the fixed cells was carried out by two incubations in epon-ethanol (1:3) for 1 hr each. This was followed by a 1 hr incubation in epon-ethanol (1:1). Then the monolayers were embedded overnight in epon-ethanol (3:1). On the following day undiluted epon was added and the monolayers were incubated for 1 hr. Fresh epon was then applied to the monolayers and polymerized in a 70°C oven overnight. Cultures were cut and reembedded in beem holders where 60-nm sections were cut on a Sorvall MT 5000 ultramicrotome with a Diatome Ltd. diamond knife. Sections were placed on a 200-mesh copper grid and stained with 2% uranyl acetate in 100% methanol for 15 min and then stained with Reynold's lead citrate stain for 25 min. Micrographs were taken on a JEOL 100 B electron microscope. A durst enlarger was used with a Kodak dry printer for a ×2.5 enlargement of the original magnification.
Data Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by utilizing the two-tailed unpaired Student's t test employing a confidence level of 95%.

RESULTS

After the nonparenchymal cells were incubated for 24 hours the adherent population was found to be predominantly Kupffer cells. The majority (85%) of these adherent cells showed a weak peroxidase reaction at the cell periphery. This reaction was more defined when the Kupffer cell population was stained prior to the adherence step. Peripheral blood monocytes served as a positive control for peroxidase activity. Kupffer cells in culture were additionally identified by their ability to bind and ingest antibody-coated sheep erythrocytes, which was consistent with data of others [2,17]. We observed that 90-95% of the adherent cells expressed a functional Fc receptor.

Kupffer cells were further characterized by their ability to ingest large particles. An electron micrograph of a 24 hr cultured Kupffer cell demonstrated the phagocytic capacity of these cells. As shown in Figure 1, the Kupffer cell has ingested five formalin-fixed gelatinized sheep erythrocytes which have an approximate diameter of 4 µm.

Incubation of gelatinized erythrocytes with Kupffer cells for 30 min in the absence of added fibronectin resulted in minimal binding and minimal ingestion of these particles (1 SRBC/Kupffer cell). Addition of 5 µg of purified plasma fibronectin resulted in a significant increase in the number of particles bound (3 SRBC/Kupffer cell). It was observed, using nongelatinized sheep erythrocytes, that the ability of plasma fibronectin to mediate attachment of these erythrocytes to Kupffer cells was markedly limited (Cardarelli et al., unpublished data). Also, attachment of SRBC to Kupffer cells in the absence of purified plasma fibronectin or whole serum was the same whether gelatinized or nongelatinized sheep erythrocytes were used. Kupffer cells, which were pretreated with fibronectin for 30 min, washed, and challenged with SRBC, showed no significant increase in target particle binding above nonpretreated cells, suggesting that fibronectin-gelatin interaction was necessary for attachment as opposed to a direct effect of fibronectin on the cell in the presence or absence of the particle.

Figure 2 shows the uptake observed in the presence or absence of fibronectin as a function of incubation time. After 30 min, significant (P < 0.01) increase in rosette formation or particle attachment was seen in the presence of fibronectin. However, in the absence of fibronectin a significant (P < 0.01) increase did not occur until 60 min. The extent of rosette formation in the presence of fibronectin was also greater at all time points. The number of attached SRBC was highest at 1 hr, and the percentage of Kupffer cells that internalized the particles was also highest at this time (~40%). Thus, a 30-min incubation period was selected for subsequent rosetting experiments since this time point showed definite attachment with minimal particle internalization. This incubation period allowed specific evaluation of particle attachment as influenced by fibronectin.

Increasing the concentration of fibronectin from 3 µg/ml to 20 µg/ml at a constant 30-min incubation interval led to a concomitant increase both in particle
Fig. 1.
Fig. 2. Time course of fibronectin-mediated particle attachment to Kupffer cells. Kupffer cells were incubated with $2 \times 10^6$ gelatinized SRBC in the presence or absence of $3 \mu g/ml$ fibronectin for various times, washed, and evaluated for rosette formation. The number of SRBC bound per 100 Kupffer cells is expressed (RBC/100 K cells). Numbers in parentheses indicate the percentage of Kupffer cells that bound more than eight SRBC (too numerous to count). A significant difference (*) was observed when rosette formation at various time points was compared to that obtained for 15 min for each group ($P < 0.01$).

Fig. 1. Electron micrograph of a cultured Kupffer cell. Monolayers were incubated with gelatinized formalin-fixed sheep erythrocytes ($4 \times 10^7$ SRBC) in the presence of plasma fibronectin (50 $\mu g/ml$). Kupffer cells were washed, fixed in 2% glutaraldehyde, postfixed in 1% $O_3O_4$, and embedded in epon overnight. $\times 10,269$. 
attachment and the percentage of cells that had more than eight SRBC bound (too numerous to count) (Fig. 3). Therefore, at higher concentrations of fibronectin the number of attached SRBC represents an underestimated value. Specificity as it relates to fibronectin was suggested since addition of varying concentrations of albumin did not augment particle attachment to the Kupffer cells.

Figure 4 demonstrates the ability of rat serum containing fibronectin at various concentrations to promote attachment of gelatinized SRBC to Kupffer cells. Addition of 1% normal rat serum showed a threefold increase in attachment. Only a modest
increase in attachment was observed when the percentage of normal rat serum added to the culture wells exceeded 5%. At a concentration of normal rat serum greater than 5%, the percentage of cells, which were too numerous to count, increased significantly, and at 20% normal serum approximately 90% of the Kupffer cells showed attachment of SRBC, which were too numerous to visually enumerate. Whole plasma made deficient of fibronectin by passage over a gelatin-Sepharose column lost its ability to enhance SRBC attachment (Table 1). With fibronectin-deficient plasma (1%), we observed approximately 1.5 SRBC attached per Kupffer cell in contrast to four SRBC/Kupffer cell in fibronectin-rich plasma (1%). Reconstitution of this fibro-
TABLE 1. The Effect of Removal of Fibronectin From Plasma on its Ability To Promote the Attachment of Gelatinized SRBC to Kupffer Cells

<table>
<thead>
<tr>
<th>Media conditions</th>
<th>SRBC/100 Kupffer cells&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>DMEM (N = 4)</td>
<td>143 ± 18</td>
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<tr>
<td>Whole plasma (1%) (N = 4)</td>
<td>406 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fibronectin-deficient plasma (1%) (N = 4)</td>
<td>149 ± 10</td>
</tr>
<tr>
<td>Purified fibronectin (3 µg/ml) (N = 4)</td>
<td>308 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fibronectin-deficient plasma (1%) + fibronectin (3 µg/ml) (N = 4)</td>
<td>341 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SEM are expressed.
<sup>b</sup>Significantly (P < 0.001) different from DMEM control samples.

TABLE 2. Fibronectin-Promoted Ingestion of Gelatinized Tannic Acid-Treated Sheep Erythrocytes<sup>a</sup> by Kupffer Cells

<table>
<thead>
<tr>
<th>Fibronectin concentration</th>
<th>No heparin (% Uptake)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Heparin (10 U/ml) (% Uptake)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Ingested</td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>20.48 ± 1.36</td>
<td>5.49 ± 0.73</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>30.43 ± 1.37</td>
<td>6.64 ± 0.73</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>42.51 ± 1.07</td>
<td>13.62 ± 0.36</td>
</tr>
<tr>
<td>40 µg/ml</td>
<td>46.52 ± 1.40</td>
<td>16.11 ± 0.87</td>
</tr>
<tr>
<td>Antisheep erythrocyte IgG (1:10,000)</td>
<td>20.73 ± 0.65</td>
<td>7.32 ± 0.17</td>
</tr>
<tr>
<td>Antisheep erythrocyte IgG (1:5,000)</td>
<td>30.39 ± 2.23</td>
<td>12.76 ± 1.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>Particle dose 4 × 10<sup>7</sup> sheep erythrocytes (particle to cell ratio = 40:1).
<sup>b</sup>Mean ± SEM of 12 samples are expressed.
<sup>c</sup>Significantly (P < 0.01) different from nonheparinized cells for a given concentration of fibronectin.

deficient plasma with affinity purified plasma fibronectin restored this biological activity (~ 3.4 SRBC/Kupffer cell) to nearly that of whole plasma.

Utilizing a 24 hr cultured Kupffer cell monolayer, radioactive uptake of formalin-fixed gelatinized 51Cr labeled sheep erythrocytes in the presence of varying concentrations of fibronectin was assessed by isotopic assay (Fig. 5). Increasing the concentration of fibronectin to 50 µg/ml resulted in a 40% increase (P < 0.001) in the percentage of erythrocytes taken up. The results obtained in this study provide further evidence that fibronectin enhances both particle attachment and, by visual observations, ingestion by Kupffer cells. When gelatinized tannic acid-treated sheep
Fig. 5. Fibronectin-mediated uptake of particles by isolated Kupffer cells. Kupffer cells were treated with fibronectin in the presence or absence of 10 U/ml heparin. The percent uptake of gelatinized sheep red blood cells was assessed following a 60-min incubation at 37°C. Particle dose, $4 \times 10^7$, sheep erythrocyte (particle to cell ratio = 40:1).

erthrocytes were used, which by hypotonic lysis allowed us to assess ingestion, fibronectin promoted particle ingestion in a dose-dependent manner (Tables 2 and 3). Addition of IgG-coated sheep erythrocytes, which served as our positive control, also demonstrated increase in both total and ingested erythrocyte uptake by the Kupffer cells.

The phagocytic uptake of gelatin-coated particles by liver slices or peritoneal macrophages has been shown to be enhanced by heparin [4, 11, 20]. However, the possibility that this effect may be primarily on the ingestion phase of phagocytosis as opposed to the attachment phase had not been clarified. In the present study, addition of heparin to Kupffer cell monolayers (particle to cell ratio 10:1) during the 30-min incubation period did not enhance attachment of gelatinized erythrocytes (Fig. 6). Increasing the dose of heparin from 0 to 5 units/ml decreased the number of SRBC
bound both in the presence as well as the absence of fibronectin. Higher heparin doses from 5 to 15 units/ml had a variable effect on the number of SRBC bound. We also assessed the distribution of gelatinized SRBC/Kupffer cell at various heparin concentrations. This was performed by measuring the percentage of Kupffer cells that had 0 SRBC/Kupffer cell, 1–2 SRBC/Kupffer cell, 3–5 SRBC Kupffer cell, 6–8 SRBC/Kupffer cell, and the percentage of Kupffer cells that had greater than 8 SRBC/Kupffer cell. The distribution of bound particles was also not influenced by heparin, although once again fibronectin promoted attachment.
Fibronectin-Kupffer Cell Interaction

TABLE 3. Fibronectin-Promoted Ingestion of Gelatinized Tannic Acid-Treated Sheep Erythrocytes* by Kupffer Cells

<table>
<thead>
<tr>
<th></th>
<th>No heparin (% Uptake)(^b)</th>
<th>Heparin (10 U/ml) (% Uptake)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Ingested</td>
</tr>
<tr>
<td>Fibronectin concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (\mu g/ml)</td>
<td>11.90 ± 0.54</td>
<td>2.42 ± 0.36</td>
</tr>
<tr>
<td>10 (\mu g/ml)</td>
<td>21.16 ± 1.23(^c)</td>
<td>5.65 ± 0.27(^c)</td>
</tr>
<tr>
<td>20 (\mu g/ml)</td>
<td>27.68 ± 1.38(^c)</td>
<td>7.91 ± 0.62(^c)</td>
</tr>
<tr>
<td>40 (\mu g/ml)</td>
<td>28.68 ± 1.33(^c)</td>
<td>8.82 ± 0.67(^c)</td>
</tr>
<tr>
<td>Antisheep erythrocyte IgG (1:10,000)</td>
<td>16.69 ± 0.60(^c)</td>
<td>5.75 ± 0.50(^c)</td>
</tr>
<tr>
<td>Antisheep erythrocyte IgG (1:5,000)</td>
<td>19.89 ± 0.72(^c)</td>
<td>6.96 ± 0.48(^c)</td>
</tr>
</tbody>
</table>

*Particle dose, \(8 \times 10^7\) sheep erythrocyte (particle to cell ratio = 80:1).
\(^b\)Mean ± SEM of 20 samples are expressed.
\(^c\)Significantly (P < 0.001) different from 0 \(\mu g/ml\) fibronectin.
\(^d\)Significantly (P < 0.001) different from nonheparinized cells for a given concentration of fibronectin.

Consistent with observations made in the rosetting assay, addition of 10 units of heparin/ml did not augment fibronectin-mediated particle uptake in the Kupffer cell monolayer isotopic assay (Fig. 5). To determine if heparin promoted particle ingestion, tannic acid-treated sheep erythrocytes were used. At a particle to cell ratio of 80:1, heparin augmented both the number of particles bound and ingested (Table 3). However, at a particle to cell ratio of 40:1, addition of 10 units of heparin per milliliter did not augment either particle attachment or ingestion (Table 2). This effect was observed with both formalin-fixed gelatinized sheep erythrocytes (Fig. 5) and tannic acid-treated gelatinized sheep erythrocytes (Table 2).

**DISCUSSION**

Plasma fibronectin has been implicated in the in vivo [12,19,22] and in vitro [4,11,14,19,20,24] phagocytosis of various test materials. As reviewed [18], a number of investigations have documented that Kupffer cells are primarily responsible for the clearance of fibronectin-opsonized nonbacterial particles from the blood. This concept is supported by the fact that other populations of RE phagocytic cells, such as peritoneal macrophages and peripheral blood monocytes, will bind and ingest particles opsonized with fibronectin. Furthermore, the depletion of circulating fibronectin by the infusion of gelatinized colloids, which are cleared by Kupffer cells [4,19], also reduces subsequent Kupffer cell uptake of circulating gelatinized particles. The present study addressed the hypothesis that plasma fibronectin would promote the adherence as well as ingestion of gelatinized particles by isolated Kupffer cells.

As has been observed with peripheral blood monocytes [1], pretreatment of Kupffer cells with soluble fibronectin did not result in subsequent enhancement of
particle uptake ability by these cells. This indicates that Kupffer cells require fibronectin bound to the surface of gelatinized particles so that recognition and/or binding can occur. Additionally, this suggests that the coating of a foreign particle by the dimeric soluble fibronectin may result in a conformational change in the fibronectin molecule and/or the exposure of unique sites on fibronectin that can bind to the surface of the macrophage. Accordingly, Kupffer cells may have a fibronectin receptor. Such a receptor is consistent with the existence of an analogous receptor on blood monocytes [1]. Preliminary studies from our laboratory suggest that a trypsin-sensitive membrane component exists on Kupffer cells that mediates fibronectin-coated particle uptake [6]. This sensitivity is similar to that observed with blood monocyte [1]. The fact that fibronectin did not enhance uptake of nongelatinized fixed sheep erythrocytes points to the specificity of the response since fibronectin has a specific binding site for collagen and gelatin [15,28]. Based upon results of the fibronectin depletion and reconstitution experiments in the present study, enhancement of particle uptake by whole plasma appears mediated by the fibronectin in plasma.

We also investigated the role of heparin on particle attachment to Kupffer cells. It has been previously shown that maximal expression of activity of fibronectin using the liver slice bioassay [19,20] required heparin. Heparin also enhanced ingestion of gelatinized particles by mouse [14] and rat peritoneal macrophages [11,24]. In our rosetting assay, which tested only binding and not ingestion, heparin was not necessary for maximal particle attachment. Perhaps the heparin-enhancing effects observed in other studies [4,11,14,24] were due to several factors. First, heparin may not be a necessary factor for binding of particles to the surface of the Kupffer cell, but may be necessary for internalization of the particles. Second, the ratio of particles to macrophages was much higher in previous studies when compared to our study, which may limit the heparin requirement. Third, it may be possible that Kupffer cells in culture respond to fibronectin, heparin, and gelatinized particles in a manner different from peritoneal macrophages.

Our initial suggestion was that heparin may not be a necessary cofactor for particle attachment but it may be necessary for particle ingestion. Since the Kupffer cell monolayer isotopic assay measures both attachment and ingestion and no heparin-promoting effects were observed, this possibility seems unlikely. Direct measurements of ingestion demonstrated that at a higher particle to cell ratio, heparin augmented ingestion of gelatinized tannic acid-treated sheep erythrocytes, but this was not observed at a lower particle to cell ratio. The ratio of particle to cell was higher in other studies [4,14] when compared to ours. Therefore, the ratio of particle to cell may be a valid difference between studies. These results are consistent with Doran, et al. [9], who showed that heparin enhanced fibronectin-mediated particle uptake by increasing the size of surface bound aggregates. It is possible that when larger particle doses are added to the monolayers in the presence of fibronectin and heparin particle aggregation occurs. This may explain the enhanced particle uptake.

The existence of a recognition system by which blood-borne materials having affinity for fibronectin can be removed from the circulation by hepatic Kupffer cells has important physiological implications [18]. This would promote the clearance of a variety of potentially harmful substances such as fibrin/fibrinogen monomer from intravascular coagulation, cytoskeletal debris, such as actin from trauma, and collagenous tissue debris after burn injury. Indeed, fibronectin has unique domains with affinity for denatured collagen, fibrin, and actin [15,28], which provides the basis for such a structure-function relationship.
Fibronectin-Kupffer Cell Interaction

During tissue injury, it may be crucial that cell debris and other substances be quickly removed from the circulation by the liver to prevent damage to other organs, especially the lung, by microembolization. The fact that trauma, burn, or direct injection of a foreign particle load into the blood will deplete immunoreactive fibronectin dramatically points to a consumptive phenomenon and emphasizes the clinical relevance of this response. The ability to reverse this fibronectin deficiency in patients as reviewed by Saba [18] suggests that this concept has potential clinical importance.

Although evidence from this study and from others [1] suggests the existence of a fibronectin receptor, the mechanism by which it function is unknown. In addition, the functional independence or association (linkage) of a fibronectin receptor with other known receptors for immunoglobulin and complement on the surface of macrophages remains to be clarified.

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