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Participation of D-galactose-specific receptors of liver macrophages in recognition of fibronectin-opsonized particles*,†

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ABSTRACT

The interaction of immobilized human or rat plasma fibronectin with isolated rat liver macrophages was studied in a model system using colloidal gold of 17-nm diameter (Au-17) as test particles. Plasma fibronectin (pFn)-coated gold particles were rapidly bound and endocytosed *via* the coated pit–coated vesicle pathway as demonstrated by photometry, and light and electron microscopy. The isolated macrophages bind 2.5 ± 2 particles/10 μm of plasma membrane (incubation at 4°), equalling a binding capacity of ~3.5 × 10^4 pFn–Au-17 particles per cell. Binding and uptake (at 37°) was specifically inhibited by D-galactose-related carbohydrates, but not by D-mannose, *N*-acetyl-D-glucosamine, nor by excess soluble pFn. Uptake was also inhibited by lactosylated bovine serum albumin at a concentration of 10^{-6} M but not by bovine serum albumin. India ink uptake by the liver macrophages in the presence of fibronectin was also inhibited by D-galactose-related monosaccharides. The presence of terminal, nonreducing D-galactosyl groups on pFn could be demonstrated by agglutination experiments with the D-galactose-specific plant lectin, *Ricinus communis* agglutinin (RCA), which could also be used for isolation of pFn from rat plasma.

The 29-kDa molecular mass D-galactose-specific receptor, known to be expressed on the liver macrophage membrane and recently shown to be a membrane-bound form of C-reactive protein, was found to bind the pFn-coated gold particles in dot blotting experiments. It was concluded that the D-galactose-specific macrophage receptor binds to terminal D-galactose-related units of immobilized pFn and participates in recognition of fibronectin-opsonized particles.

INTRODUCTION

Plasma fibronectin has been shown to promote the clearance of gelatin-coated particles *in vitro*[1–3] and *in vivo*[4–6]. Therefore, plasma fibronectin has been termed "opsonic α_2-glycoprotein" before its structural identity with fibronectin became apparent. However, experiments using fibronectin-coated bacteria or agarose beads gave rise to doubts as to whether fibronectin can really act as an opsonin for isolated peritoneal macrophages[7–10]. On the other hand, monocytes have been clearly shown to express receptor activity for attached but not soluble fibronectin in the presence of divalent cations[11,12]. This receptor activity was shown[13,14] to be attributable to an integrin of the

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VLA family (VLA-5) and to another integrin recently identified (or to both)[15]. Both receptors recognize a RGD-containing peptide sequence on the carboxy-terminal, cell-adhesion domain of fibronectin. We were recently able to identify a membrane-bound form of the acute-phase reactant, C-reactive protein (CRP) as the D-galactose-specific particle receptor of rat liver macrophages[16]. Since CRP has been shown to bind to immobilized pFn[17] and since *in vivo* phagocyte-mediated clearance of particles occurs mainly in the liver[18], we examined the binding and uptake of fibronectin-coated particles by isolated rat liver macrophages (Kupffer cells). As test particles, fibronectin-coated colloidal gold was used. Owing to their red color and their electron density, the fate of these particles can be followed by light and electron microscopy, and by photometric measurement[19,20]. Furthermore, it has been reported that the concentration of plasma fibronectin is reduced following the injection of colloidal particles in animals [21,22], thereby suggesting the consumption of fibronectin during *in vivo* clearance of such inert particles. The results of our experiments demonstrate that the lectin-like binding site of CRP recognizes a D-galactosyl group of attached pFn and that this recognition participates in the uptake of fibronectin-opsonized particles.

EXPERIMENTAL

Materials. — Fibronectin from human plasma (hFn) was purchased from Sigma Chemical Co. (Deisenhofen Corp., FRG), Calbiochem Corp. (Frankfurt/Main, FRG), and Behringwerke (Marburg, FRG). Rat fibronectin (rFn) was from Calbiochem Corp. and Paesel (Frankfurt/Main, FRG). Purity of all fibronectin preparations was checked by sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS–PAGE) prior to experiments. Antihuman pFn antiserum was purchased from Behringwerke. *Ricinus communis* agglutinin A (RCA_I) was from Sigma. RCA-Sepharose from Medac (Hamburg, FRG), gelatin-Sepharose from Pharmacia (Freiburg, FRG), collagenase from Boehringer (Mannheim, FRG), and Eagle's medium from Gibco (Karlsruhe, FRG). Bovine serum albumin (BSA, defatted) and human serum albumin (HSA) were from Sigma. All other reagents were from Merck (Darmstadt, FRG). Lactosylation of BSA was performed as described earlier[23].

Cell preparation. — Liver macrophages were prepared from male Wistar rats (150–180 g body weight) by perfusion with collagenase and differential centrifugation of the resulting cell suspension, essentially as described earlier[23]. The final cell preparation consisted of 2×10^6 macrophages/mL of Eagle's medium and contained 5–10% hepatocytes and 5–15% endothelial cells. Viability was better than 90% as tested by Trypan Blue exclusion.

Fibronectin-coated colloidal gold particles (pFn–Au–17). — Colloidal gold particles of 17-nm diameter were prepared by reduction of an aqueous chloroauric acid solution with sodium citrate exactly as described earlier[18]. Plasma fibronectin [1 mg/mL in Tris·HCl buffered saline solution (TBS); 0.05M Tris·HCl and 0.15M NaCl, pH 7.4] was adsorbed onto colloidal gold by mixing 10 μg of protein per mL of gold sol and addition of 0.05% poly(ethylene glycol) (mol. wt. 20 000). Successful coating was monitored by

mixing an aliquot of the sol with NaCl[19]. The protein–gold complex was washed twice by centrifugation (30 min at 32 000*g*), resuspended in TBS, and used as a concentrated stock solution (containing 2–4 × 10^{12} particles/mL).

Binding and uptake experiments. — Macrophages (4 × 10^5 in 200 μL of Eagle's medium plus mM $CaCl_2$) were incubated with or without saccharides (80mM final concentration) for 5 min prior to addition of ligand. pFn–Au-17 was added at a final concentration of ~10^{12} particles/mL and cells were incubated for the times indicated. Binding experiments were performed at 4° for all incubation steps, and uptake experiments at 37°. The reaction was stopped by addition of an equal volume of ice-cold cacodylate-buffered glutaraldehyde (0.2%) and the cells were washed immediately. At this low concentration and followed immediately by a washing step, glutaraldehyde does not affect the amount of bound particles, as has been shown previously[25]. The washed cells were used for light microscopical examination, or processed for electron microscopy or for photometric measurement of ligand uptake.

India ink (from an old bottle of Guenther Wagner India ink) was dialyzed against 0.9M NaCl and used in a final dilution of 1:50 and mixed with rat fibronectin in a final concentration of 160 μg/mL.

Electron microscopy. — Washed cells were further fixed, dehydrated, and embedded in resin as described earlier[25].

Morphometry of electron micrographs. — Electron micrographs of the surface of any macrophage sectioned through its nucleus were used at a final magnification of 18 000 ×. The length of plasma membrane was measured with a HP 85 computer, equipped with a digitizing board (Hewlett–Packard, Cupertino, CA, U.S.A.) and an area–distance program (HP menu) as software. The number of gold particles adhering to the plasma membrane were counted.

Photometric measurement of ligand uptake. — Uptake experiments were performed as described above, the fixed cells were washed three times in Eagle's medium plus $CaCl_2$ to completely remove free ligands, and then homogenized by sonification. Gold particles were spun down and freed from cell debris by washing in phosphate-buffered solution (PBS; Ca^{2+} and Mg^{2+} free). The extinction of resuspended particles was measured at 510 nm wavelength by use of an ELISA photometer.

Agglutination experiments. — pFn–gold complexes were incubated with RCA (200 μg/mL) with or without saccharides (100mM) or pFn (500 μg/mL), or with anti-hFn-antiserum (1:100). The particles were incubated overnight at room temperature and agglutination was determined by light microscopy.

Affinity chromatography and SDS–PAGE. — Autologous rat plasma fibronectin was purified from pooled rat plasma by affinity chromatography on gelatin-Sepharose according to Engvall and Ruoslahti[26]. Aliquot samples of the same plasma pool were also examined by affinity chromatography on an RCA-Sepharose column. This column was eluted with 200mM D-galactose in TBS after extensive washing with TBS.

For absorption experiments RCA was adsorbed onto colloidal gold as described above. Sedimented particles were incubated with pFn (100mM) in TBS (10^{11} particles/50 μg of pFn) with or without D-galactose. After incubation for 60 min at room temper-

ature, the particles were spun down and the supernatant was applied to SDS–PAGE. All preparations were reduced with 5% mercaptoethanol prior to SDS–PAGE (7.5% SDS) according to Laemmli[27], and gel slabs were stained with silver[28].

Dot blots. — Macrophage-receptor protein and serum amyloid P protein were purified as described previously[16]. Dots of antiserum, or lectin, or purified receptor were applied onto strips of nitrocellulose sheets. After being dried and blocked with 0.3% Tween in TBS for 60 min at room temperature, the strips were incubated with pFn–Au-17 in Tris-buffered saline plus 0.005% Tween and 2mM $CaCl_2$ for 2 h and stained by silver enhancement exactly as described earlier[29].

RESULTS

Quantitation of binding and uptake in electron microscopy. — Fibronectin-coated gold particles were prepared by use of highly purified plasma fibronectin from both rat or human plasma. The minimal amount of fibronectin needed to stabilize the colloid and to result in a monodisperse particle solution was determined, as described previously[19,25], by addition of sodium chloride and electron microscopy. The stable complex was achieved by addition of 8 μg of plasma fibronectin to 1 mL of Au-17 sol, giving a complex of 10–12 molecules of fibronectin per single Au-17 particle.

Freshly prepared fibronectin–gold colloid conjugates (pFn–Au-17) were coincubated with isolated liver macrophages, either for 10 min at 4° to demonstrate binding or for 15 min at 37° to allow for uptake. The cells were then fixed and processed for electron microscopy. As shown in Fig. 1, both binding (Fig. 1a) and uptake (Fig. 1d) of the fibronectin particles are prominent events. Initial binding (Fig. 1b) as well as endocytosis (Fig. 1e) is blocked in the presence of 80mM *N*-acetyl-D-galactosamine, whereas the presence of *N*-acetyl-D-glucosamine at the same concentration is not inhibitory (Figs. 1c and f). Uptake of pFn–Au-17 proceeded *via* the coated-pit–vesicle pathway as expected for receptor-mediated endocytosis (Fig. 1g).

By morphometry of electron micrographs of macrophages from two binding experiments, a medium binding capacity of 25 ± 3 particles/10 μm of plasma membrane was determined (Table I). Assuming the sections to be 60 nm in thickness and a medium diameter of 15 μm for the isolated liver macrophages (determined in light micrographs), an overall minimum binding capacity of $\sim 3.5 \times 10^4$ pFn–Au-17 particles per macrophage could be calculated. Morphometry of cells, incubated in the presence of mono-

Fig. 1. Electron micrographs of the binding and uptake of pFn–Au-17 by isolated rat liver macrophages: (a–c) Binding of human pFn. (d–g) Uptake of rat pFn. The different pFns show no significant differences in binding and uptake. Incubation for 10 min at 4° led to binding of pFn–Au-17 clusters to the plasma membrane (a). The presence of 80mM *N*-acetyl-D-galactosamine in the incubation mixture completely blocked this binding (b), whereas the same concentration of *N*-acetyl-D-glucosamine did not interfere (c). Incubation for 15 min at 37° led to uptake and intracellular accumulation of pFn–Au-17 (d), which was also blocked completely by the presence of *N*-acetyl-D-galactosamine (e), but not by *N*-acetyl-D-glucosamine (f). Uptake of the particles proceeded *via* the coated pit–vesicle pathway (arrowheads in g). The bar in F indicates magnification (30 600 ×) for (a–f), magnification in (g) is 50 400 ×.


a
b
c
d
e

TABLE I

Morphometric quantitation of pFn–Au-17 binding to the plasma membranes of isolated liver macrophages after a coincubation of 10 min at 4°[a]

Added inhibitor	*pFn–Au-17/10 μm²*[b]	*BSA–Au-17/10 μm²*[b]
None	25 ± 13 (22)	26 ± 13 (25)
N-acetyl-D-glucosamine (50mM)	18 ± 8 (25)	n.d.
N-acetyl-D-galactosamine (50mM)	1 ± 1 (25)[c]	36 ± 20 (26)[d]

[a] Cells were coincubated with ligands for 10 min at 4°, processed for electron microscopy, and morphometrically quantified as described in the Experimental section. [b] Number in parentheses give the number of cells measured. [c] $p < 0.001$; significantly different. [d] $p = 0.9$; not significantly different.

saccharides otherwise treated identically, demonstrated the inhibitory effect of *N*-acetyl-D-galactosamine, which was of no effect to binding of BSA–Au-17 particles used as nonglycosylated control particles.

Endocytosis of colloidal particles as assessed by light microscopy. — Endocytosis was also monitored by light microscopy. Here, the accumulation of endocytosed gold particles within the macrophage was easily recognized as a dark (red) stain over the cell

Fig. 2. Light microscopy revealing a massive accumulation of freshly prepared fibronectin–gold particles by the isolated liver macrophages as evidenced by the dark (red) stain within all cells (A). In the presence of 80mM *N*-acetyl-D-galactosamine, they were no longer taken up (B). The same effect was observed with India ink particles, which in the presence of pFn accumulated inside the macrophages (C) but not when *N*-acetyl-D-galactosamine was present (D).

cytoplasm (Fig. 2A). Uptake of pFn–Au-17, as well as uptake of India ink in the presence of pFn (Fig. 2C), were both blocked by coincubation in the presence of 2-acetamido-2-deoxy-D-galactose (Figs. 2b and d). India ink uptake in the absence of pFn or plasma was very poor. Three experiments with India ink were evaluated by light microscopy only, the colloidal-carbon particles being rather diffuse in electron contrast microscopy, hetrogeneous in size, and only available in small amounts since its production was stopped. Here, we have used India ink to compare the results of gold colloid with those of another colloid that was widely used in the past for *in vivo* labeling of liver macrophages. It showed that D-galactose-inhibitable uptake of chemically inert particles in the presence of pFn is not restricted to colloidal gold only. Endocytosis of fibronectin-coated gold particles was very similar when three different preparations were used, *i.e.*, commercially available human fibronectin (chemically pure, a single band in SDS-gel electrophoresis), a rat fibronectin preparation, and an autologous fibronectin purified from the plasma of Wistar rats, the same source from which the liver macrophages were obtained.

Photometric quantitation of uptake and inhibition. — Particle uptake by the isolated macrophages was quantified by measuring the absorbance of the colloidal gold[19] bound to or taken up by the cells. As can be seen from the data in Table II, uptake of freshly prepared particles did not differ significantly, whether the incubation mixture contained *N*-acetyl-D-galactosamine, D-mannose, or no added saccharides. *N*-Acetyl-D-galactosamine or lactose were potent, and D-galactose a less effective inhibitor at the concentration used. Soluble fibronectin did not compete with attached fibronectin for uptake by macrophages. Inhibition of particles ingestion was also achieved in the presence of lactosylated bovine serum albumin (LacBSA) at concentrations down to 10^{-6}M; at a concentration of 10^{-7}M inhibition was not significant. Nonglycosylated BSA did not interfere with pFn–Au-17 uptake (Table II).

TABLE II

Photometric quantitation of human pFn–Au-17 uptake by isolated rat liver macrophages

Ligand	*Addition to incubation*	*Concentration*	*Percent of control uptake*[a]
pFn–Au-17	None (control)		100 (9)
pFn–Au-17	Soluble hFn	50 μg/mL	105 ± 16 (3)
pFn–Au-17	*N*-Acetyl-D-galactosamine	80mM	25 ± 5 (9)
pFn–Au-17	*N*-Acetyl-D-glucosamine	80mM	110 ± 15 (8)
pFn–Au-17	D-Mannose	80mM	82 ± 8 (6)
pFn–Au-17	D-Galactose	80mM	43 ± 7 (5)
pFn–Au-17	Lactose	80mM	29 ± 18 (4)
pFn–Au-17	Lactosylated BSA	10μM	22 ± 8 (3)
pFn–Au-17	Lactosylated BSA	1μM	28 ± 10 (3)
BSA–Au-17	None		37 ± 16 (3)
BSA–Au-17	Lactose	80mM	32 ± 14 (3)
BSA–Au-17	Lactosylated BSA	10μM	38 ± 10 (3)

[a] Number of individual experiments are given in parentheses

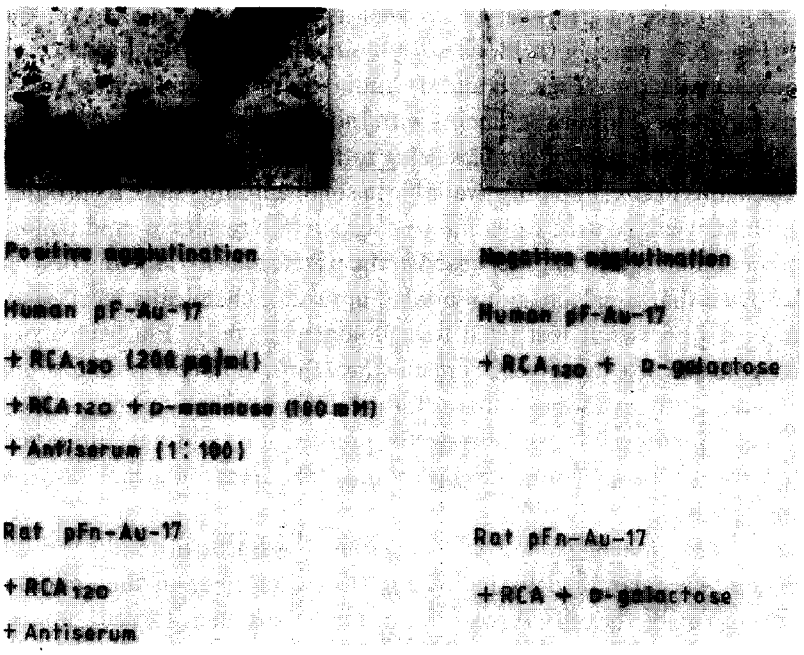

Fig. 3. D-Galactose-specific, lectin-mediated agglutination of pFn–Au-17 particles. Freshly prepared particles were mixed with indicated additives and incubated overnight. Agglutination was monitored by light microscopy, yielding the results shown in the micrographs (magnification 600 ×).

Lectin binding to pFn. — The inhibition experiments suggested that a terminal, nonreducing D-galactosyl group on plasma fibronectin glycans plays a role in its opsonic activity. Therefore, we performed a series of experiments to prove the presence of such a terminal sugar group on plasma fibronectin. Agglutination experiments using RCA (a D-galactose-specific plant lectin) led to strong agglutination of pFn–Au-17 particles (Fig. 3).

The lectin-mediated agglutination was completely inhibited in the presence of 0.1 M D-galactose and not by D-mannose. An antifibronectin antiserum also agglutinated the particles as a control for the presence of fibronectin on the colloidal gold. The data showed that terminal, nonreducing D-galactosyl groups are present on the opsonized particles that are readily recognized by the plant lectin.

Further tests were performed to demonstrate that the recognized D-galactosyl groups are indeed present on the fibronectin and not on some contaminating factor(s). Immobilized RCA, adsorbed onto colloidal gold, was used as the one lectin that also recognizes the D-galactosyl groups of soluble pFn in order to test which component could be adsorbed from the commercially available fibronectin solutions used in our experiments. As shown in Fig. 4, most of the human and all of rat plasma fibronectin are absent after absorption with RCA but not when incubated in the presence of D-galactose. A minor impurity in the pFn preparations was not bound by RCA.

Rat plasma was purified by affinity chromatography on an RCA-agarose column and the elute compared to that from a gelatin-agarose column. Examination by SDS-gel

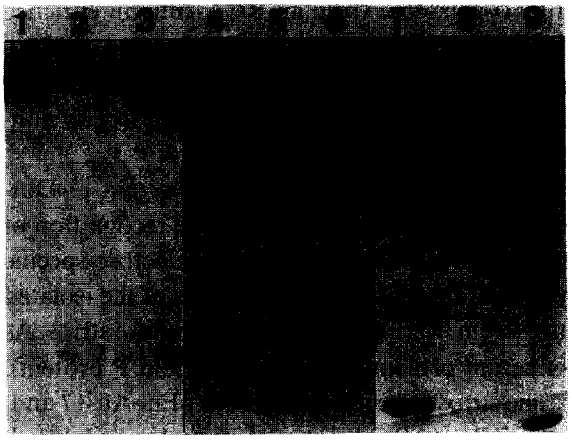

Fig. 4. SDS-slab gels (7% SDS) of a commercially available human (lane 1) and rat (lane 4) plasma fibronectin preparation showing that, after incubation with immobilized RCA, both fibronectins were almost completely removed (lane 2, hFn; and lane 5, rFn) but not when RCA incubation was performed in the presence of 100mM D-galactose (lanes 3 and 6). In both preparations a minor, low-molecular weight impurity was not bound by RCA (arrowheads in lanes 3 and 6). In lanes 8 and 9, rat plasma was split into two aliquot samples. From the first, fibronectin was prepared by the use of gelatin-agarose as shown in lane 8. The other aliquot sample was applied to a RCA-agarose column and eluted with D-galactose. The resulting eluate is shown in lane 9. Four bands were found, one of which represents fibronectin (arrow), the yield being comparable to gelatin-agarose. Lane 7 represents marker proteins, with the following mol. wts.: 200 000, 116 250, 92 500, 66 200, and 45 000.

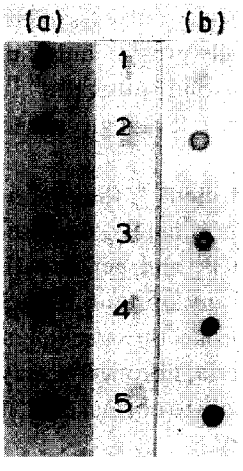

Fig. 5. (a) Dot blotting with human fibronectin-coated gold particles, followed by silver enhancement resulting in positive staining of antihuman-fibronectin antiserum (1), RCA (5), and D-galactose-specific macrophage receptor preparation (4). Both human albumin (2) and serum amyloid P protein (3) stayed negative. (b) Dot blotting with D-galactosylated BSA-coated gold gave essentially the same pattern with the exception of the antifibronectin antiserum (1) that stayed negative here.

chromatography showed that elution by D-galactose from an RCA-column enriched several plasma proteins, including fibronectin, the latter in a proportion comparable to that obtained by chromatography on gelatin-agarose (Fig. 4). Taken together, these results demonstrated that human and rat plasma fibronectin molecules bear non-reducing terminal D-galactosyl groups that are recognized by RCA.

Interaction of isolated receptor protein with immobilized fibronectin. — Dot-blotting experiments were performed as a further direct proof that the recognition of immobilized fibronectin by liver macrophages is mediated by the D-galactose-specific receptor located on these cells, *i.e.*, by membrane-bound CRP[16]. Drops of the isolated receptor protein, and of an anti-hFn-antiserum, were dotted on nitrocellulose strips. As controls, human albumin and another serum protein (rat serum amyloid P protein), closely related to CRP, were included. The strips were incubated with pFn–Au-17 and, after silver enhancement, the antiserum, CRP, and RCA dots were found to be labeled, whereas human albumin and serum amyloid P showed only background labeling (Fig. 5).

DISCUSSION

We used a model system to study the interaction of surface-bound fibronectin with liver macrophages. Incubation of liver macrophages with fibronectin-coated. colloidal gold particles led to their binding, uptake, and massive accumulation within the cells, as shown by light microscopy, photometry, and electron microscopy. Endocytosis of pFn particles was not inhibited by soluble Fn. Thus, fibronectin behaves in this system as a true opsonin; it is not recognized by liver macrophages in its soluble form but only after attachment. This result is best explained by the relatively gross changes in conformation of the fibronectin molecule upon adsorption onto solid substrates[30,31]. This conformational change may be necessary to expose one or more relevant glycans to the membrane-bound receptors.

In several reports where no opsonic activity of pFn was detected[7–10], the experiments were performed with agarose beads[10] which have exposed terminal D-galactose-related glycosyl groups and which are known to interact with rat CRP[32] and, hence, with the D-galactose-specific macrophage receptors in the absence of any opsonin. Other experiments were performed with pFn-coated bacteria[8,9] where fibronectin attachment may not occur in the same way as on colloidal gold. In some bacteria, fibronectin may act exactly in the opposite way, *i.e.*, if the fibronectin attaches as it does to normal cells, it may mediate tissue adherence[9] and even support bacterial mimicry.

The major difference between the aforementioned studies and those presented herein is that the latter were designed to study *endocytosis* of small, chemically inert particles, such as colloidal gold or India ink, known to be rapidly coated by plasma proteins when entering the circulating blood. In contrast to endocytosis, the effective phagocytosis of large particles may require additional signals, such as binding by complement receptors[33–35].

Binding of pFn particles, as well as their uptake, is specifically inhibited by certain monosaccharides as is characteristic for lectin-like recognition. A lectin-like affinity site has been shown to reside in the cell-binding domain of pFn, which is masked in the intact molecule[36]. This binding activity, however, showed a different sugar specificity and was inhibited by *N*-acetylneuraminic acid and *N*-acetyl-D-glucosamine. The latter monosaccharide was always tested in our assays and was without any effect on pFn–Au-17 binding and uptake, which was specifically inhibited in the presence of D-galactose-related carbohydrates. This observation led us to test for the presence of terminal nonreducing D-galactosyl groups on fibronectin and to identify the putative fibronectin receptor on liver macrophages.

There has been a thorough attempt to elucidate the function of the fibronectin glycans[37] by use of nonglycosylated fibronectin from tunicamycin-treated fibroblasts. It was found that this fibronectin exhibited an enhanced proteolytic susceptibility with no other function being impaired. However, opsonic activity was not tested and, in contrast to the numerous other functions, it has not been mapped on the fibronectin molecule so far[38].

The *N*-linked glycans of plasma fibronectin were initially described as to be not fully sialylated, a finding that led to the former name of "galactoprotein"[39] for fibronectin. Recently, most investigations led to the conclusion that plasma fibronectin, in contrast to cell-bound fibronectin, bears fully sialylated *N*-linked glycans, although all investigators[40–43] found a molar ratio of *N*-acetylneuraminic acid to galactose of $< 1:1$, indicating that a minor proportion of terminal nonreducing D-galactosyl groups is present on plasma fibronectins.

Such a minor proportion would be sufficient for effective binding by macrophages since, on one colloidal gold particle, we calculated that 10 to 12 fibronectin molecules are attached in the stable complex; thus, one particle bears more than one recognition site.

The experiments described herein showed evidence for the presence of terminal, nonreducing D-galactosyl groups on fibronectin. Firstly, RCA binds to soluble Fn and, by chromatography on a RCA column, a similar amount of Fn could be isolated from rat plasma by use of a gelatin column; secondly, liver macrophages bind to attached Fn, again this reaction being inhibitable only by D-galactose-related sugars; thirdly, the specific interaction of attached fibronectin with RCA or with the isolated D-galactose-specific macrophage lectin (CRP) could be demonstrated by dot blots.

From many recent reports and the rapidly increasing knowledge on the mechanism of cell adhesion of fibronectin, we could have assumed a role for the signal peptide (for review, see ref. 44) in our experimental setup as well; especially, since a receptor for the signal peptide has been described also on monocytes; this receptor mediates the attachment to fibronectin-coated surfaces[14,15,45]. However, carbohydrate-inhibition experiments clearly showed that the signal-peptide recognition is not solely responsible for the initial event in binding and endocytosis of pFn–Au-17. It may well be that binding of the signal peptide may follow, as a secondary event, the initial D-galactose-specific recognition. There are several convincing, though indirect, evidences that led to the

conclusion that additional binding sites must be involved in fibronectin binding, and the D-galactose-specific recognition *via* surface-bound CRP may represent such an additional signal.

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