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In Vitro Parasite-Monocyte Interactions in Human Leishmaniasis: Possible Role of Fibronectin in Parasite Attachment

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Leishmania* spp. must attach to mononuclear phagocyte surfaces before entering this host cell. We investigated the potential role of fibronectin in facilitating parasite attachment. Human plasma fibronectin bound to axenically cultured promastigotes, and promastigotes and amastigotes preferentially bound to fibronectin-coated cover slips. Promastigotes grown in the absence of fibronectin were strikingly deficient in their ability to attach to human monocytes compared with promastigotes grown in the presence of fibronectin. Rabbit anti-human plasma fibronectin antiserum decreased promastigote and amastigote attachment to monocytes. Immunoglobulin G F(ab')$_2$ and Fab fragments also reduced the ability of amastigotes to bind to monocytes. Antiserum pretreatment of amastigotes followed by washing resulted in reduced parasite binding, whereas antibody pretreatment of monocytes did not. Addition of exogenous fibronectin did not enhance parasite attachment to monocytes. These findings suggest that *Leishmania* spp. can bind fibronectin and may utilize this glycoprotein to facilitate attachment to the mononuclear phagocytes that they infect.

Leishmania spp. are obligate protozoan parasites that are transmitted by vector sand flies and in their mammalian hosts replicate exclusively within mononuclear phagocytes. After inoculation by sand fly bite, promastigotes (the flagellated extracellular form of the parasite) enter host cells and transform into amastigotes (the aflagellate intracellular form that persists in the host). Amastigotes replicate in the mononuclear phagocytes and are presumably released into the interstitium. They must then rapidly gain entry into adjacent host cells. In view of the importance of this infection process to parasite survival and disease pathogenesis, the attachment and uptake of promastigotes (and to a lesser extent amastigotes) by mononuclear phagocytes in vitro have received considerable investigative attention (7). Some of these studies have led to the conclusion that parasite-host cell membrane interactions involved in infection may involve specific ligand-receptor systems (15, 35). For example, mannose on the parasite surface may engage the mannose-fucose receptors on host mononuclear phagocytes and thereby mediate parasite-host cell association (7; R. D. Pearson, personal communication). Deposition of complement components on the parasite surface also appears to facilitate their interaction with complement receptors on macrophages (21). Specific antileishmanial antibody may also enhance parasite association with monocytes via the Fc receptors of host cells (Pearson, personal communication). From these studies it is unclear, however, whether these specific interactions serve primarily to mediate parasite attachment or whether they also are important in phagocytosis, the apparent mechanism by which *Leishmania* spp. enter these host cells (7, 34).

Mononuclear phagocytes possess plasma membrane receptors for fibronectin (5), a large glycoprotein that is a major constituent of the intercellular matrix and is also synthesized by these host cells (2). Because fibronectin has been shown to bind to certain bacteria, yeasts, viruses, and spirochetes and may aid in the association of these pathogens with host cells (16, 20, 24, 28, 29), we reasoned that *Leishmania*-mononuclear phagocyte interactions might also involve host-derived fibronectin, perhaps adsorbed onto the parasite surface. The studies we report here provide initial support for this hypothesis and specifically suggest that fibronectin may be important in the attachment phase of intracellular infection.

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MATERIALS AND METHODS

Parasites. *Leishmania mexicana amazonensis* (Josefa strain) was kindly provided to us by Miercio Pereira; it was originally isolated from a Brazilian case of cutaneous leishmaniasis by C. A. Cuba-Cuba. *Leishmania tropica major* (National Institutes of Health S strain; also called *L. major*) was isolated by one of us (D.J.W.) from a patient who acquired a cutaneous infection in West Africa (22). Parasites were maintained by serial passage of amastigotes in footpads of BALB/c AnNCrlBr mice (Charles River Breeding Laboratories, Inc., Kingston, N.Y.) or cultivated axenically as promastigotes in fetal bovine serum (FBS)-supplemented medium by previously described methods (10). For experiments involving promastigotes, parasites were used at or near the stationary phase of growth from passages 2 to 5 after in vitro transformation from tissue-derived amastigotes. Amastigotes were isolated from infected tissue of nonulcerated footpad lesions by previously published methods (3, 13). On the basis of our electron microscopic observations, amastigotes isolated in this manner are apparently free of surrounding host membrane (35).

In some experiments, promastigotes were cultured in medium containing 20% FBS depleted of fibronectin (kindly supplied by Magnus Höök, University of Alabama, Birmingham). For these experiments, FBS was initially filtered through columns of washed Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.). One sample of filtered material was stored (−10°C) after filter sterilization (0.22-μm pore; Millipore Corp., Bedford, Mass.) and used as a standard FBS control in the relevant experiments. Another sample was depleted of fibronectin by sequential affinity

chromatography with columns of gelatin conjugated by cyanogen bromide to Sepharose 4B (11), followed by Sepharose 4B-conjugated rabbit anti-bovine plasma fibronectin (prepared by a modification of methods described in reference 36). Depletion of fibronectin from FBS was confirmed by an enzyme-linked immunosorbent assay (26) with rabbit anti-human plasma fibronectin and by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of FBS after reduction with 5% 2-mercaptoethanol (17). Fibronectin was prepared by previously published methods (11).

Amastigotes were enumerated in suspension by previously described methods (3). Promastigotes were quantitated in a hemacytometer chamber after immobilization with 70% ethanol.

Binding of fibronectin to promastigotes. To determine whether fibronectin would bind to *Leishmania* spp., we obtained iodinated (^{125}I) human plasma fibronectin from a commercial source (fibronectin prepared as previously described [11] and iodinated by the chloramine-T method; specific activity, 2.1 μCi/μg; New England Nuclear Corp., Boston, Mass.). Approximately 2 μg of ^{125}I-fibronectin (10^7 cpm) was added in a volume of 100 μl to approximately 3 $\times$ 10^8 extensively washed *L. mexicana amazonensis* promastigotes suspended in 10 ml of Hanks balanced salt solution (HBSS) and incubated at room temperature for 1 h. Promastigotes were then extensively washed with HBSS until supernatants contained less than 100 cpm/100 μl above background as determined in a gamma counter (model 5500; Beckman Instruments, Inc., Fullerton, Calif.). The promastigote pellet was then treated for 1 h at 4°C with a mixture containing 1% Triton X-100 (Bio-Rad Laboratories, Richmond, Calif.), 10 μM leupeptin (Sigma Chemical Co., St. Louis, Mo.), 1.0 μM phenylmethylsulfonyl fluoride (Sigma), 1 typsin inhibitory unit aprotinin (Sigma), and 0.5% epsilon-aminocaproic acid (Sigma). Next, 2-mercaptoethanol (5%; Bio-Rad) was added to the mixture along with 0.5 ml of sample buffer (17), and the mixture was heated to ~100°C for 10 min. The material (100 μl) was subjected to electrophoresis in a 5% sodium dodecyl sulfate-polyacrylamide slab gel (17). Molecular weight standards also loaded on the gel were myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin (Bio-Rad). The gel was stained with Coomassie blue, and autoradiographs were prepared by exposing the dried gel to X-Omat RP film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. Densitometry of radiograms was carried out with a Zeiheh scanning densitometer (Biomed Instruments, Fullerton, Calif.) with a tungsten light source.

Attachment of parasites to fibronectin-coated cover slips. Round glass cover slips (15-mm diameter) were washed in solutions of nitric acid and EDTA and extensively rinsed in distilled water. Air-dried cover slips were placed in wells of 24-well culture trays (24 Cluster; Costar, Cambridge, Mass.) and incubated at 37°C for 18 h in a protein solution (100 μg/ml) of human plasma fibronectin (Collaborative Research, Inc., Waltham, Mass.), bovine serum albumin (Sigma), ovalbumin (Sigma), gelatin (Sigma), or FBS. Cover slips were washed, and parasite suspensions in HBSS were added. After incubation for 2 h with parasites, cover slips were gently washed with HBSS, fixed with 95% ethanol, stained with Wright-Giemsa stain (Diff-Quick; Dade Diagnostics, Inc., Aguada, Puerto Rico), and mounted on microscope slides. Parasites were counted under oil immersion in 50 fields in a predetermined pattern that covered all quadrants of the cover slips.

Interaction of parasites and human monocytes. The ability of parasites to attach to or infect peripheral blood monocytes under different conditions was assessed in experiments using methods we have previously described (34). Briefly, mononuclear cells were obtained from heparinized whole blood of normal volunteers by Ficoll-Hypaque density centrifugation (6). Washed mononuclear cells were suspended at 2.5 $\times$ 10^6/ml in HBSS and incubated (37°C; in 5% CO_2–95% air) in polypropylene microtest Eppendorf tubes (1.5 ml; Brinkmann Instruments, Inc., Westbury, N.Y.) with freshly harvested amastigotes or with promastigotes at a ratio of 1:1 or 2:1 (parasites to monocytes) in a total volume of 200 μl. After incubation, cultures were subjected to cytocentrifugation, and fixed slides were stained with Wright-Giemsa stain. The percentage of infected monocytes and the percentage of uninfected monocytes with $\geq$1 amastigote attached were determined by counting at least 200 cells in several remote oil immersion fields (OIF). Monocytes were identified morphologically (4). We previously validated the morphological criteria of monocytes in our related studies (34). Good accordance between morphologically identified cells, latex-ingesting mononuclear cells, and nonspecific esterase-staining cells was observed.

In some experiments, phagocytosis of parasites was prevented by pretreating mononuclear cell suspensions with cytochalasin E (10 μg/ml; Sigma) as previously described (34). These cells were extensively washed before coincubation with parasites.

Preparation and use of antifibronectin antiserum and antibodies. According to the supplier's specifications (Collaborative Research), rabbit anti-human plasma fibronectin antiserum used in some of our experiments formed a precipitin arc at a 1:4 (but not 1:8) dilution when tested in an Ouchterlony double-immunodiffusion assay against 3.0 and 1.5 μg of human plasma fibronectin per well; it had a titer of 1:4,500 by radioimmunoassay. Immunoglobulin G (IgG) and IgG Fab and F(ab')$_2$ fragments were prepared from rabbit antiserum by published methods (19, 31). Controls included serum and IgG F(ab')$_2$ fragments prepared by identical methods from unimmunized rabbits or from rabbits before immunization with human plasma fibronectin. Polyclonal antibodies to human fibronectin react extensively with fibronectin of many vertebrate and invertebrate species (1).

Statistical analysis. Results of replicate assays were compared by Student's *t* test.

RESULTS

Binding of fibronectin to *Leishmania* spp. Promastigotes (*L. mexicana amazonensis*) grown in the presence or absence of fibronectin attained similar densities in culture at 6 days (stationary phase) whether cultures were initiated with amastigotes or promastigotes. The only difference in growth pattern under the different conditions was that in the absence of fibronectin parasite replication early during incubation was apparently slower than in the presence of fibronectin. Cultures (2 ml) were seeded with 5 $\times$ 10^5 parasites (day 0). By day 4, (3.0 $\pm$ 0.2) $\times$ 10^6 promastigotes were present in cultures containing fibronectin-depleted serum; (5.4 $\pm$ 0.1) $\times$ 10^6 promastigotes were present in cultures with complete serum ($P < 0.01$). By day 6, cultures depleted of fibronectin contained (6.8 $\pm$ 1.1) $\times$ 10^6 parasites, and cultures containing complete serum contained (7.0 $\pm$ 1.2) $\times$ 10^6 parasites ($P > 0.2$). For all subsequent experiments, promastigotes were used on day 6 of culture.

Promastigotes bound iodinated human plasma fibronectin, as revealed by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis autoradiography (Fig. 1). Interestingly, promastigotes grown in the absence of fibronectin bound roughly twice the amount of iodinated fibronectin (as determined by densitometry of radiograms), suggesting that a greater number of fibronectin-binding sites were available when parasites were not previously exposed to fibronectin. More detailed efforts to characterize and quantitate the binding of fibronectin to these parasites are in progress.

In related experiments designed to assess fibronectin-parasite interactions, we found that parasites preferentially bound to glass cover slips coated with plasma fibronectin (Table 1). Interestingly, amastigotes bound more readily to cover slips coated with all proteins than did promastigotes. By direct microscopic observation under Nomarski optics (model ICM 405 microscope; Carl Zeiss, Inc., Obekochen, West Germany) of parasites in Dvorak-Stotler chambers (9), we could attribute these quantitative differences to the intrinsic motility of promastigotes but not amastigotes. Promastigotes could be observed in some cases attaching to and then migrating away from cover slip surfaces. In contrast, amastigotes simply settled to the cover slip surface. Binding of *Leishmania* spp. to fibronectin-coated cover slips (65.6 ± 11 per 10 OIF) was reduced in the presence of rabbit antifibronectin antiserum (22.7 ± 6 per 10 OIF), whereas binding to bovine serum albumin-coated cover slips (20.2 ± 2 per 10 OIF) was not reduced in the presence of antifibronectin antiserum (26.9 ± 3 per 10 OIF).

Interaction of promastigotes with monocytes. The relative ability of promastigotes grown in the presence or absence of fibronectin to associate with human monocytes was assessed in vitro. As summarized in Table 2, data from two representative experiments clearly indicate that promastigotes grown in the absence of fibronectin are relatively deficient in their ability to infect monocytes compared with those grown in the presence of fibronectin. The difference in infectivity rates (mean number of promastigotes per 100 total monocytes) was statistically significant ($P < 0.02$). The possibility that these differences were due at least in part to the decreased binding of promastigotes to monocytes was suggested by the observation that the rate of attachment of fibronectin-free promastigotes to uninfected monocytes in these cultures was also substantially reduced (Table 2).

To quantitate parasite-host cell attachment independent of uptake, we exploited our previously published method of incubating parasites with cytochalasin-treated mononuclear cells (34). Parasites attach to but do not enter treated host cells. This treatment totally inhibited infection in all our experiments. Parasites grown in the absence of fibronectin were strikingly deficient in their attachment to cytochalasin-treated monocytes compared with those grown in the presence of fibronectin (Fig. 2). The frequency distribution of attachment (i.e., number of parasites that attached per monocyte to which any parasite attached) was the same ($P >$ 0.2) for both groups, however. In related experiments, promastigotes were grown in medium containing standard FBS, fibronectin-depleted FBS, or depleted FBS enriched with human plasma fibronectin (50 μg/ml; Collaborative Research). Attachment to cytochalasin-treated monocytes of promastigotes grown under these conditions was then assessed. Promastigotes grown in the absence of fibronectin attached to 25.7 ± 1.8% of the monocytes. The addition of fibronectin (10 μg/ml) to culture medium before promastigote cultivation restored parasite attachment rates (42 ± 2.5%, three experiments) to levels characteristic of promastigotes grown in standard (nondepleted) FBS (45 ± 2.5%, three experiments) ($P > 0.2$). This indicated that the

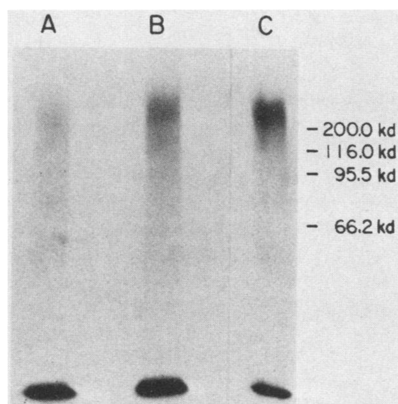

FIG. 1. Autoradiographs of sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gels of Triton X-100-extracted *L. mexicana amazonensis* promastigotes after their incubation with ^{125}I-labeled human plasma fibronectin. Lanes: A, promastigotes grown in medium containing 20% FBS (standard); B, promastigotes grown in medium containing the same lot of 20% FBS that was depleted of fibronectin; C, ^{125}I-plasma fibronectin (human) used in this experiment. Lane C was exposed to radiographic film for 6 h; lanes A and B were exposed for 7 days. Molecular weight standards are shown (kd, kilodalton).

TABLE 1. *L. mexicana amazonensis* binding to fibronectin-coated glass cover slips

Protein on cover slips	No. of parasites per 10 OIF[a]	
	Amastigotes	Promastigotes
Fibronectin	93.7 ± 22.5	25.5 ± 5.5
Bovine serum albumin	43.0 ± 14.0	6.3 ± 1.3
Gelatin	36.0 ± 13.6	3.7 ± 1.3
FBS	21.0 ± 4.0	3.7 ± 0.7

[a] Promastigotes grown in medium with 20% heat-inactivated FBS (standard) and amastigotes harvested from footpad tissue of infected mice were centrifuged onto treated glass cover slips and incubated for 2 h. Washed, fixed, and stained cover slips were examined microscopically. The presence of parasites was scored by examining 50 OIF in a predetermined pattern that covered all quadrants of the round cover slip. The mean ± the standard error of the mean of parasites identified per 10 OIF in three separate cover slips is indicated for a representative experiment.

TABLE 2. *L. mexicana amazonensis* promastigotes grown in the absence of fibronectin are defective in their infection of monocytes in vitro[a]

Expt	Culture conditions	% Infected monocytes	No. of parasites per infected monocyte	% Uninfected monocytes with ≥1 parasite attached
1	FBS	16.7 ± 3.5	1.6 ± 0.4	35.3 ± 7.2
	Fibronectin depleted	7.3 ± 1.7	1.2 ± 0.1	5.0 ± 0.6
2	FBS	20.3 ± 2.4	2.1 ± 0.2	63.3 ± 1.5
	Fibronectin depleted	11.3 ± 2.7	1.7 ± 0.2	25.3 ± 1.2

[a] Washed promastigotes that were grown in standard or depleted FBS were incubated for 2 h at 37°C (5% CO_2–95% air atmosphere) with human mononuclear cells at a ratio of 2:1 suspended in serum-free RPMI 1640 medium.

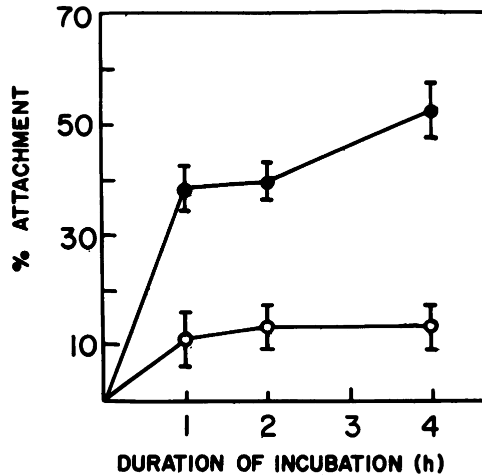

FIG. 2. Attachment of promastigotes grown in the presence (●) or absence (○) of fibronectin to cytochalasin-treated monocytes. Percent attachment (percentage of monocytes with ≥1 parasite attached) was assessed microscopically from cytocentrifuged samples of cultures at different times during incubation at 37°C. Shown are mean ± standard error of the mean for triplicate determinations. Representative experiment of three performed.

specific absence of fibronectin from FBS was responsible for decreased attachment of promastigotes grown in depleted medium.

In related studies, we investigated whether the addition of rabbit anti-human plasma fibronectin antiserum interfered with the ability of promastigotes (axenically cultured in standard FBS) to attach to cytochalasin-treated monocytes. In a representative experiment, we observed that whereas 47 ± 1.4% of monocytes had ≥1 promastigote attached to their surface after a 2-h coincubation period in the presence of normal rabbit serum, 27 ± 7.2% monocytes had parasites attached if antiserum was present (1:4 final dilution; $P < 0.02$).

Interaction of amastigotes with monocytes. We attempted to determine whether the potential role of fibronectin in the interaction of promastigotes with host cells might also be characteristic of amastigotes. In this endeavor we utilized *L. mexicana amazonensis* amastigotes freshly obtained from infected mouse tissue. Although methods for axenic cultivation of *Leishmania* spp. in forms morphologically similar to tissue amastigotes have been published (14, 18), stringent criteria to confirm their biological and biochemical identity with tissue amastigotes have not been applied. We therefore did not attempt to compare the interaction of such putative amastigotes grown in the presence or absence of fibronectin with host cells. Instead, we examined the effects of antifibronectin antibody on tissue-derived amastigote-monocyte interaction.

Fewer monocytes become infected when cocultured for 2 h with amastigotes in the presence of than in the absence of antifibronectin antiserum (Table 3). Furthermore, attachment of amastigotes to uninfected monocytes observed in the presence of antifibronectin antiserum was also reduced (Table 3). The low titer of the antiserum we employed was reflected by our failure in most experiments to detect a reduction in infection when it was used at a 1:8 dilution; decreased amastigote attachment to uninfected monocytes compared with that under control conditions was observed at this dilution, however (Table 3). These findings contrasted with our in vitro observations on the failure of rabbit

TABLE 3. Infection of monocytes by amastigotes treated with antifibronectin antiserum[a]

Antifibronectin antiserum added (dilution)	% Infected monocytes	Amastigotes per infected monocyte	Uninfected monocytes with ≥1 amastigote attached
0	34.0 ± 2.6	2.4 ± 0.7	23.7 ± 5.2
1:4	15.7 ± 1.9[b]	1.8 ± 0.2[c]	8.0 ± 3.4[d]
1:8	36.0 ± 1.5	2.3 ± 0.07	12.3 ± 4.1

[a] Tissue-derived *L. mexicana amazonensis* amastigotes and human monocytes were incubated at 37°C for 2 h in the presence of normal rabbit serum or rabbit anti-human plasma fibronectin antiserum.
[b] $P < 0.005$; comparison with results in the absence of antiserum.
[c] $P > 0.2$; comparison with results in the absence of antiserum.
[d] $P < 0.05$; comparison with results in the absence of antiserum.

anti-leishmanial amastigote (or promastigote) antiserum (prepared by the method of Chang [8]; titer, 1:128 by indirect immunofluorescence assay) to interfere with amastigote-monocyte interactions.

In a series of experiments, we assessed the effects of rabbit antifibronectin antiserum, IgG antibody, and IgG antibody fragments on amastigote attachment to cytochalasin-treated monocytes (Table 4). In the presence of antifibronectin antiserum, amastigote attachment was substantially reduced, whether compared with attachment rates in the presence or absence of normal rabbit serum tested at the same dilutions as antiserum. Furthermore, $F(ab')_2$ fragments of antifibronectin rabbit IgG antibody also reduced attachment rates compared with those observed in the presence of preimmune $F(ab')_2$ fragments tested at the same concentration (100 μg/ml). Fab fragments of the antibody also reduced attachment. On the other hand, preincubation (37°C; 1 h) of

TABLE 4. Effects of antifibronectin antiserum and antifibronectin IgG fragments on attachment of amastigotes to cytochalasin-treated monocytes[a]

Expt	Antiserum or IgG fragments	Dilution or concn (μg/ml)	% Attachment	% Reduction in attachment
1	Serum-free medium	0	49.7 ± 7.7	
	Antifibronectin antiserum	1:8	29.0 ± 5.1	42
		1:16	30.9 ± 4.5	38
		1:32	46.3 ± 4.8	7
2	NRS[b]	1:4	26.0 ± 6.0	
		1:8	27.3 ± 1.5	
		1:16	34.0 ± 6.4	
	Antifibronectin antiserum	1:4	11.0 ± 3.6	58
		1:8	10.0 ± 1.0	63
		1:16	32.0 ± 3.3	6
3	Preimmune $F(ab')_2$	100	40.0 ± 3.0	
	Antifibronectin $F(ab')_2$	100	25.0 ± 1.5	36
4	NRS	1:10	54.7 ± 2.4	
	Antifibronectin Fab	1	40.7 ± 4.3	25
		10	36.3 ± 8.1	34
		100	35.0 ± 3.2	46

[a] Parasites and monocytes were coincubated at 37°C for 2 h with or without antiserum or IgG fragments.
[b] NRS, Normal (nonimmunized) rabbit serum.

antifibronectin antiserum with 1 μg of human plasma fibronectin per ml before addition of the mixture to amastigote-monocyte cultures abrogated the attachment-blocking effects of the antiserum (data not shown). This strongly suggests that the attachment-blocking effect of the antiserum depends on its antifibronectin specificity.

In related experiments, we determined that preincubation with antifibronectin antiserum (followed by washing) of amastigotes but not of cytochalasin-treated monocytes resulted in decreased attachment (Table 5). That is, when antiserum-pretreated amastigotes were incubated with monocytes, attachment rates were significantly less ($P < 0.005$). In contrast, cytochalasin-treated monocytes first preincubated with antiserum and then washed permitted untreated amastigote attachment to proceed at normal rates when host and parasite cells were coincubated ($P > 0.2$). These observations are consistent with the conclusion that fibronectin is present on the amastigote surface and that antiserum interferes with attachment by interacting with the parasite rather than with the host-cell surface.

Findings with *L. mexicana amazonensis* amastigotes were confirmed in experiments with *L. major*, which indicated that the observations were not species specific (data not shown).

TABLE 5. Effects of pretreatment of amastigotes but not monocytes with antifibronectin antiserum on parasite attachment[a]

Cell treated	Treatment	% Attachment	% Reduction in attachment
Monocytes	NRS	59.7 ± 0.9	
	Antifibronectin	55.0 ± 3.2	8
Amastigotes	NRS	43.7 ± 1.7	
	Antifibronectin	27.0 ± 3.5	38

[a] Amastigotes and cytochalasin-treated monocytes were separately incubated with normal rabbit serum (NRS) or with rabbit antifibronectin antiserum at a 1:4 dilution for 1 h at 37°C. Cells were extensively washed and then combined either with monocytes or with amastigotes that had not been pretreated with sera.

DISCUSSION

The results of these studies suggest that fibronectin can bind to *Leishmania* promastigotes in vitro (Fig. 1). The presence of fibronectin on the parasites appears to facilitate their association with mononuclear phagocytes (Table 2) and specifically their ability to attach to the host-cell surface (Fig. 2). Indeed, antifibronectin antibody can diminish parasite attachment to monocytes (Table 4), apparently by interacting with fibronectin on the parasite surface (Table 5). These findings provide strong evidence in support of the hypothesis that *Leishmania* attachment to mononuclear phagocytes is mediated in part by the interaction of parasite-bound fibronectin with host-cell fibronectin receptors. On the other hand, since fibronectin-depleted parasites were only partially reduced in their ability to infect host cells (Table 2), it is likely that fibronectin is not the only ligand involved in parasite-host cell interaction. Thus, our findings do not conflict with those of other investigators who have suggested potential roles for mannose and C3b in parasite-host cell association (7, 21; Pearson, personal communication). Indeed, it seems likely that infection may depend upon complex interactions of several ligand-receptor systems on parasite and host-cell surfaces.

The evidence we presented indicating that fibronectin can bind to *Leishmania* spp. (Fig. 1; Table 1) does not indicate whether this binding is to specific plasma membrane receptors on the parasites. In the case of *Staphylococcus aureus*, not only has specificity of binding been demonstrated, but a candidate receptor on these bacteria has been identified (27). It has been proposed that fibronectin bound to *S. aureus* (and perhaps to certain other bacteria as well) may facilitate their association with host cells bearing fibronectin receptors. On the other hand, recent evidence indicates that fibronectin alone does not serve as an opsonin promoting uptake of bacteria by phagocytes (30). Results of the investigations of the interaction of fibronectin with another pathogenic protozoan, *Trypanosoma cruzi*, were reported during the course of our studies with *Leishmania* spp. (23). The investigators reported that fibronectin bound to glutaraldehyde-fixed trypomastigotes in a characteristically saturable and specifically inhibitable manner; they did not report results with viable parasites. Since antifibronectin antiserum interfered with parasite-host cell interactions in vitro, trypanosome-bound fibronectin may also be important in intracellular infection by this parasite. In preliminary studies, we also have observed that iodinated human plasma fibronectin binds to viable (but not to glutaraldehyde-fixed) promastigotes in a saturable manner. However, neither saturability nor specific inhibition of binding proves that the binding is to a specific fibronectin receptor; the candidate receptor should ideally be identified. On the other hand, the consequences of fibronectin binding to a pathogen with respect to its subsequent interactions with host cells can be expected to be similar whether binding is specific or nonspecific. Thus, our observations that promastigotes grown in fibronectin-depleted medium are deficient in their association with monocytes (Table 2) provide compelling evidence for the role of this molecule in facilitating parasite-host cell interactions.

The ability of host cells and microbes to bind to fibronectin-coated cover slips has been widely exploited for examining their interactions with this molecule (12). On the basis of our studies of *Leishmania* spp. in such a system (Table 1), we conclude that these parasites bind preferentially to fibronectin. Our data suggest that fibronectin-binding sites on the parasite (whether specific or nonspecific) are not saturated as a result of in vitro cultivation in fibronectin-containing medium (promastigotes) or as a result of in vivo growth (amastigotes). Were these sites saturated, preferential binding to fibronectin-coated cover slips might not have been observed. On the other hand, in the interaction of amastigotes with monocytes, it seems more likely that fibronectin on the parasite engages the monocyte fibronectin receptor than that the parasite attaches to host-cell-bound fibronectin. This conclusion is supported by the observation that parasite but not monocyte pretreatment with antifibronectin antiserum interferes with attachment (Table 5). Furthermore, in contrast to macrophages, circulating monocytes lack surface-bound fibronectin (2). It is possible, however, that in vivo, *Leishmania* attachment to macrophages might be mediated through bidirectional interactions involving fibronectin. Parasites might adhere to macrophage-bound fibronectin and associate with the host cells by interaction of fibronectin on parasite surfaces with specific fibronectin receptors.

Observations that antifibronectin antiserum and IgG fragments with antifibronectin specificity interfere with parasite attachment to host cells (Tables 4 and 5) support our hypothesis that fibronectin is important in parasite-host cell interactions. We cannot absolutely exclude the possibility

that antifibronectin antibodies are acting to sterically hinder some other attachment ligand in the vicinity of surface-bound fibronectin. However, the fact that $F(ab')_2$ and Fab antibody fragments can interfere with attachment makes this possibility less likely. Furthermore, rabbit antileishmanial antiserum that lacks antifibronectin specificity but recognizes the immunodominant antigens on the parasite surface (D. J. Wyler and J. P. Sypek, unpublished observations) did not interfere with attachment of amastigotes to monocytes (data not shown).

In this study, we separated the parasite attachment phase from the entry phase of infection by using cytochalasin-treated monocytes. This approach permitted us to conclude that fibronectin was likely to be involved in parasite attachment to mononuclear phagocytes. Whether fibronectin might also play a role in promoting uptake of parasites by phagocytosis remains to be determined, however. The addition of exogenous plasma fibronectin (up to 100 μg/ml) to cultures did not significantly ($P > 0.2$) alter infection rates in our preliminary studies (D. J. Wyler, unpublished observations). This finding contrasts with those obtained in studies of *T. cruzi*-macrophage interactions in which enhanced parasite-host cell association was observed with the addition of exogenous fibronectin (32). One possibility is that through the ligation of macrophage fibronectin receptors, *Leishmania* spp. may promote their uptake via another receptor-ligand system, such as that involving C3b or the Fc portion of immunoglobulin. Evidence for cooperativity between fibronectin and C3 receptors and fibronectin and Fc receptors on macrophages has been reported (25, 33).

In conclusion, our study adds fibronectin to a growing list of ligands potentially involved in *Leishmania*-mononuclear phagocyte interactions. It also serves to add this protozoan parasite to the list of microbes that bind this molecule and may utilize it for association with host cells. Acquisition of host fibronectin by *Leishmania* spp. could occur in the interstitium rich in this glycoprotein or within the mononuclear phagocytes, cells known to produce fibronectin. In view of the multiple ligands potentially involved in *Leishmania*-host cell association, the mechanism whereby this obligate intracellular parasite gains entry into mononuclear phagocytes appears to be complex.

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