In Vitro Parasite-Monocyte Interactions in Human Leishmaniasis: Effect of Enzyme Treatments on Attachment

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Essential to the pathogenesis of leishmaniasis is the ability of *Leishmania* spp. to attach to mononuclear phagocyte surfaces before entering this host cell which they parasitize. We have investigated the attachment phase of infection in vitro by quantitating the percent of human peripheral blood monocytes pretreated with cytochalasin (to prevent parasite entry) to which tissue-derived *L. tropica* amastigotes will attach during coincubation at 37°C in serum-free medium. We determined that pretreatment of parasites with trypsin, chymotrypsin, Pronase, and neuraminidase reduced attachment. In contrast, parasites treated with β -galactosidase had an enhanced ability to attach to host cells. Treatment of monocytes with chymotrypsin and Pronase, but not with trypsin or neuraminidase, reduced attachment of untreated amastigotes. We propose that in vitro amastigote attachment under serum-free conditions depends on the interaction of protein determinants on the surface of both parasite and host cell.

An essential feature in the pathogenesis of diseases due to obligate intracellular parasites is the ability of the pathogen to attach to, enter, and replicate within appropriate host cells. It has been proposed that when parasites are highly selective in the cell types which they infect, infection might depend on specific recognition systems between host cell and parasite. One concept which has gained widespread interest is that specific receptors and ligands are expressed on host cell and parasite surfaces and that the interaction of these molecules is essential for infection (10). These molecular determinants might include ones which mediate initial attachment between host cell and parasite and others which are involved in triggering the cellular events involved in parasite entry. In the search for such molecules, investigators have studied host cell-parasite interactions in vitro. In some cases, such as studies of *Plasmodium falciparum* merozoite interactions with erythrocytes (14), the interpretation of observations regarding putative receptors has been complicated by the difficulty in functionally separating the attachment and entry phases in vitro.

Leishmania spp. are obligate intracellular parasites of mononuclear phagocytes in mammalian hosts and are an important cause of morbidity and mortality in several Third World nations (18). Disease pathogenesis depends on the amplification of the parasite population in a susceptible host and is achieved by repeated cycles of intracellular growth of amastigotes (nonflagellated intracellular form of the parasites), their

release from host cells, and subsequent attachment to and entry into other susceptible mononuclear phagocytes. In contrast to the several in vitro studies (1, 5, 12, and others) of the interactions of host cells and promastigotes (the infective flagellated extracellular form transmitted by sandflies), very little attention has been given to the early interactions between amastigotes and host cells (7).

We previously described a simple in vitro system for studying the early interactions between tissue-derived amastigotes and human monocytes (17). Employing this assay, we obtained evidence to suggest that these nonmotile parasites attached to monocytes by an active process but entered the cells passively by phagocytosis. When monocytes were treated with cytochalasins to prevent phagocytosis, amastigotes attached normally to their surfaces but did not enter. This observation has afforded us the opportunity to functionally isolate the attachment from the entry phase and thereby investigate the possibility that attachment may involve specific receptor-ligand interactions. We now report that selected enzyme treatment of parasites and host cells alter attachment, suggesting that attachment may depend on the interaction of specific molecular determinants expressed on the amastigotes and the monocyte surfaces.

MATERIALS AND METHODS

Animals and parasites. BALB/c mice (female, 18 to 20 g [Charles River Breeding Laboratories, Wilming-

FIG. 1. *L. tropica* amastigote attaching to the surface of a cytochalasin-treated monocyte. The amastigote apparently is devoid of surrounding host membrane, including at the attachment sites. The subpellicular microtubular system of the parasite is noteworthy ($\times 30,000$; prepared by M. Aikawa, Case Western Reserve Institute of Pathology, Cleveland, Ohio).

ton, Mass.]) were used to propagate parasites by footpad infection with 5×10^5 amastigotes of *Leishmania tropica* (NIH S strain [13]) suspended in sterile saline. Amastigotes were harvested from infected tissue 4 to 5 weeks later by our previously described methods (2). Briefly, footpad tissue was aseptically excised from mice euthanized by CO_2 narcosis and cervical dislocation. Cells were dissociated in Hanks balanced salt solution (HBSS) by passage through stainless steel mesh and disrupted to release intracellular parasites by passage through no. 27-gauge hypodermic needles. Intact cells, cell debris, and amastigote aggregates were pelleted ($40 \times g$ for 5 min at 10°C), and monodispersed free amastigotes were retrieved in the supernatant. Amastigotes were washed twice or thrice in HBSS ($150 \times g$ for 10 min) and resuspended to a concentration of $2 \times 10^6/\text{ml}$ in serum-free RPMI 1640 medium (pH 7.2 to 7.4). Electron microscopy revealed that these amastigotes were not surrounded by host-derived vacuolar membrane (Fig. 1).

To estimate the viability of amastigotes, the parasites were suspended at an initial density of $5 \times 10^5/\text{ml}$ in medium 199 supplemented with 25 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [Sigma Chemical Co., St. Louis, Mo.]), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20% heat-inactivated fetal bovine serum (9). A 1-ml amount of the suspension was dispensed into each

polypropylene culture tube (no. 2053 [Falcon, Cockeysville, Md.]) and maintained in air atmosphere at room temperature (20 to 22°C). After 5 days, the concentration of parasites which had transformed to promastigotes and divided in axenic cultures was determined.

Amastigote and promastigote concentrations were determined by thoroughly mixing equal volumes of parasite suspension and suspensions of chicken erythrocytes of known concentration. The ratio of parasites to nucleated erythrocytes was then determined from Wright-Giemsa-stained smears, and parasite concentration was calculated from this ratio.

Mononuclear cells. Peripheral blood from normal volunteers was drawn into heparinized plastic syringes and subjected to density gradient fractionation on Ficoll-Hypaque cushions by methods previously described (4). Mononuclear cells were retrieved and washed twice in HBSS and then suspended at a density of 2.5×10^6 mononuclear cells per ml in serum-free RPMI 1640 medium. This population was comprised of $49 \pm 4\%$ (mean $\pm$ standard error of the mean) cells with morphological characteristics of monocytes (3) as determined from Wright-Giemsa-stained cytocentrifuge preparations; $43 \pm 4\%$ cells which stained for nonspecific esterase activity (Diagnostic kit no. 90 [Sigma]; methods according to the suggestions of the manufacturer); and $39 \pm 4\%$ cells

which ingested latex beads (polystyrene; 1.101- μ m diameter [Dow Chemical Co., Indianapolis, Ind.]) during a 1-h incubation at 37°C in a 5% CO₂-95% air atmosphere.

To inhibit parasite uptake, mononuclear cell suspensions were incubated for 30 min at 37°C in 5% CO₂-95% air mixture in the presence of cytochalasin E (10 μ g/ml [Sigma]; initially dissolved to 1 mg/ml in dimethylsulfoxide before subsequent dilution in medium). Cells were washed twice with HBSS (centrifuged at 200 $\times$ g for 10 min) and finally resuspended to their original concentration in serum-free medium.

Enzyme treatment and mononuclear cell-parasite co-cultures. Amastigotes and cytochalasin-treated mononuclear cells were incubated for 1 to 2 h at 37°C (5% CO₂-95% air atmosphere) in either serum-free medium alone or in medium containing one of the following enzymes: trypsin (type III [Sigma]; 1, 10, and 100 U/ml); chymotrypsin (type II [Sigma]; 1, 10, and 100 U/ml); Pronase (type XIV, from *Streptomyces griseus* [Sigma]; 1, 2, 5, and 20 U/ml); neuraminidase (from *Vibrio cholera* [GIBCO Laboratories, Grand Island, N.Y.]; 1 and 10 U/ml); and β -galactosidase (grade VIII, from *Escherichia coli* [Sigma]; 1, 10, and 100 U/ml). Cells were then washed twice in 50 volumes of HBSS and resuspended to their original concentration in serum-free medium.

A total of 100 μ l each of amastigote and mononuclear cell suspension (parasite-to-monocyte ratio = 2:1) were combined in each polypropylene microtest Eppendorf tube (1.5 ml [Brinkmann Instruments, Inc., Westbury, N.Y.]), gently mixed, and incubated for 2 h (unless otherwise indicated) at 37°C in 5% CO₂-95% air atmosphere. Just before harvesting cells by cyto-centrifugation, bovine serum albumin (100 μ g/ml) was added to cell suspensions. Cultures were carried out in triplicate, and one cyto-centrifuge preparation from each culture was prepared followed by methanol fixation, staining with Wright-Giemsa, and examining by light microscopy ($\times$ 1000). Uninfected cells with morphological features of monocytes were scored for the percent which had $\geq$ 1 amastigote closely associated to its surface; a total of 200 cells were counted in different portions of the slide. The percent of infected cytochalasin-treated monocytes was $\leq$ 1% in all experiments.

Since control attachment rates varied (18 to 52%) for different experiments, effects of enzyme treatment are expressed relative to attachment rates of untreated cells:

$$\% \text{ change in attachment} = \frac{\% \text{ attachment (control)} - \% \text{ attachment (treated)}}{\% \text{ attachment (control)}} \times 100$$

The mean of triplicate attachment rate determinations for control and treated cells were used in these calculations.

RESULTS

Table 1 summarizes the effects of the treatment of amastigotes with selected enzymes on their ability to attach to cytochalasin-treated monocytes during a 2-h coincubation. Trypsin most profoundly, but also chymotrypsin, Pron-

TABLE 1. Effects of treating *L. tropica* amastigotes with selected enzymes on their ability to attach to cytochalasin-treated human monocytes in vitro during a 2-h incubation at 37°C

Enzyme	Concn (U/ml) ^a	% Change in attachment ^b		No. of experiments
		Mean	Range	
Trypsin	10	-32	-19 to -45	3
	100	-62	-50 to -80	5
	1,000	-59	-43 to -75	3
Chymotrypsin	100	-36	-20 to -47	5
Pronase	10	-41	-30 to -55	4
Neuraminidase	100	-40	-36 to -43	3
β -galactosidase	100	+43	+21 to +65	3

^a For enzymes other than trypsin, only the concentration resulting in the greatest change in attachment is shown.

^b See text for definition of percent change. 100% (control) attachment ranged from 22 to 35% of monocytes.

ase, and neuraminidase treatment, reduced the rate of attachment. In contrast, β -galactosidase treatment enhanced parasite attachment.

If trypsin (100 U/ml) was first incubated with soybean trypsin inhibitor (SBTI; 100 U/ml [Sigma]) for 1 h at 37°C before treating amastigotes, no significant effect of the enzyme on attachment scores was observed (control attachment, 26.7 $\pm$ 3%; trypsin-SBTI treatment, 22.3 $\pm$ 2.3%; $P > 0.1$). Incubating SBTI alone with amastigotes did not alter their attachment. Similarly, chymostatin (100 U/ml [Sigma]) incubated with chymotrypsin (100 U/ml) abrogated the enzyme effect on attachment but itself had no effect on amastigotes (data not shown). Heating Pronase (in a 100°C water bath for 10 min) abrogated its effect on amastigotes. These findings suggested that enzyme activity rather than a contaminant in the preparation was responsible for the observed effects of these proteases. Amastigotes preincubated in medium or enzymes (at maximum concentrations tested) transformed into promastigotes and proliferated

to a similar extent ($P > 0.05$) when cultured axenically (data not shown).

Increasing the duration of trypsin treatment from 1 to 2 h did not significantly alter the proportional decrease in attachment (Table 2). Amastigotes incubated for increasing periods in medium alone progressively lost their ability to attach to monocytes (Table 2). Furthermore, a comparison of the kinetics of attachment by control and trypsinized amastigotes (Fig. 2) sug-

TABLE 2. Effects of duration of trypsin treatment of amastigotes on their attachment to cytochalasin-treated human monocytes in vitro^a

Treatment of amastigotes	% Attachment ^b during 2 h the following duration (h) of trypsin treatment		
	1	2	3
Medium alone	41 ± 0.7 ^c	28.3 ± 3	16.7 ± 1.6
Trypsin (100 U/ml)	21 ± 3.0	14.0 ± 1	11.5 ± 0.4
% Change in attachment	-49	-51	-31

^a Representative experiment. These findings were confirmed in two additional experiments performed on separate occasions.

^b Percent uninfected monocytes with ≥1% amastigote closely attached.

^c Mean ± standard error of the mean; triplicate cultures.

gested that enzyme treatment had its major effect on the attachment which occurred during the first hour of incubation of cells. Between 1 and 2 h of incubation, the incremental increase in attachment scores was similar (7% cells for controls; 9% cells for trypsinized amastigotes). After 2 h, there was no additional attachment of either control or trypsinized amastigotes.

To determine whether there might also be specific determinants on monocytes required for parasite attachment, we incubated cytochalasin-treated monocytes with medium alone or enzymes before coculturing them with untreated amastigotes. Chymotrypsin and Pronase treatment of the host cells reduced amastigote attachment (Table 3). In contrast, the effects of trypsin and neuraminidase treatment on attachment were not significantly different ($P > 0.1$) from untreated controls.

To exclude the possibility that cytochalasin treatment of monocytes artifactually contributed to our results, we repeated some of these studies in experiments with monocytes which had not been treated with the antiphagocytic agent. We could thereby confirm (data not shown) that treatment of amastigotes with trypsin, chymotrypsin, Pronase, and neuraminidase (tested at optimum concentrations) all significantly reduced attachment and to a quantitatively similar degree as that observed with attachment to cytochalasin-treated monocytes (Table 1).

DISCUSSION

The observations that treatment of tissue-derived amastigotes with certain proteases and neuraminidase decreases attachment of the parasite to monocytes (Table 1) suggest that attachment depends on protein (or glycoprotein) deter-

minants present on the surface of the parasite. It is unlikely that these determinants are on the host-derived vacuolar membrane surrounding the parasites intracellularly, since our electron micrographic studies revealed that the freed amastigotes are apparently not surrounded by host membrane (Fig. 1). Furthermore, to the extent that it is presently possible to assess amastigote viability (by in vitro transformation and growth in axenic cultures), we could also exclude a toxic effect of the enzymes on the parasites. Finally, since treatment of monocytes with chymotrypsin and Pronase (but not with trypsin or neuraminidase) reduced attachment (Table 3), we can exclude the possibility that our observations were due to secondary effects on host cells of enzymes carried over with the treated parasites. On the other hand, experiments with enzyme treatment of monocytes suggests that a protein receptor(s) for parasite surface determinants may be present on the monocyte plasma membrane surface.

Trypsin treatment of amastigotes most profoundly inhibited parasite attachment and perhaps, not surprisingly, the degree of inhibition depended on the concentration of trypsin (Table 1). Unfortunately, our efforts to block attachment by adding supernatants of amastigotes incubated for 1 to 2 h with trypsin (to which SBTI was then added) to cultures of fresh untreated amastigotes and monocytes were unsuccessful (data not shown). We suspect that the enzyme effectively reduced protein fragments to peptides which may have been too small to sterically hinder the putative ligands involved in parasite-monocyte attachment.

In analyzing the kinetics of attachment (Fig. 2), it is apparent that most amastigotes attach in

FIG. 2. Comparison of the kinetics of attachment to cytochalasin-treated monocytes of untreated amastigotes (○) and amastigotes treated with trypsin (100 U/ml) for 1 h (●). The mean ± standard error of the mean of triplicate determinations (cultures) is shown for a representative experiment.

TABLE 3. Effects of treating cytochalasin-treated monocytes with enzymes on the ability of *L. tropica* amastigotes to attach in vitro during a 2-h incubation at 37°C

Enzyme	Concn (U/ml)	% Change in attachment ^a		No. of experiments
		Mean	Range	
Trypsin	100	0	-9 to +15	4
Chymotrypsin	100	-50	-34 to -61	3
Pronase	10	-54	-31 to -81	6
Neuraminidase	10	-13	-11 to -18	4

^a See text for definition of percent change.

the first hour, few additional ones between 1 and 2 h, and no additional ones thereafter. When amastigotes are preincubated in serum-free medium, they also progressively lose the ability to subsequently attach to monocytes in vitro (Table 2). Consequently, it has not been possible to determine whether the trypsin-sensitive determinant on the amastigote might be regenerated.

The notion of receptor-ligand systems for the interaction of plasma membranes of microorganisms with host cells (10) has evolved largely from conceptual models of the interaction of soluble molecules such as peptide hormones and acceptor molecules on mammalian cell surfaces (8). However, the attachment of particulate entities such as amastigotes and monocytes undoubtedly entails highly complex interactions possibly involving a multitude of electrostatic and hydrophobic forces (11). As a result, enzymatic modifications of cell surfaces which decrease attachment rates could result from the removal of specific ligands involved in the attachment process or could alter the cell surface in a nonspecific manner. In the latter case, removal of surface components could prevent certain electrostatic or hydrophobic interactions which normally favor attachment. Thus, although our findings are compatible with the notion that amastigote-monocyte interactions might involve specific receptor-ligand systems, they do not prove their existence. What our findings do clearly indicate is that the search for specific surface molecules, particularly those sensitive to trypsin expressed on the parasite and those sensitive to chymotrypsin and Pronase on the monocyte, should be sought.

The enhancement of attachment after treatment of amastigotes with β -galactosidase (Table 1) raises an interesting possibility. That is, that the removal of galactose from terminal oligosaccharides may expose glucose and mannose residues which could then mediate or enhance attachment via the monosaccharide receptors present on mononuclear phagocytes (15). This possibility derives support from the observations that the addition of exogenous glucose or

mannose or pretreatment of parasites with β -glucosidase or α -mannosidase interfered with the attachment of *Leishmania donovani* promastigotes to hamster peritoneal macrophages in vitro (6). Thus, competitive inhibition with the host cell monosaccharide receptor or removal of these sugars from the parasite surface oligosaccharides apparently inhibited attachment. It is tempting to speculate that β -galactosidase, an enzyme present in mammalian lysosomes (16) and therefore presumably also present in the phagolysosome within which amastigotes grow (2), has an effect in vivo. That is, possibly oligosaccharides on the amastigote surface are modified during intracellular parasite maturation, and these modifications profoundly affect the subsequent early parasite-host cell interactions which follow the amastigote release from infected cells. Our observation that β -galactosidase treatment enhanced attachment suggests that intracellular modification of these oligosaccharides, if this occurred, was incomplete.

In summary, our observations suggest that protein or glycoprotein determinants on amastigotes and determinants on mononuclear phagocytes are involved in the attachment of parasites to host cells. These findings should stimulate efforts to identify the molecules involved to determine whether a specific ligand-receptor system is responsible for parasite-host cell recognition.

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