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Research Article

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In Vitro Parasite-Monocyte Interactions in Human Leishmaniasis

EVIDENCE FOR AN ACTIVE ROLE OF THE PARASITE IN ATTACHMENT

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ABSTRACT *Leishmania* are obligate intracellular parasites of mononuclear phagocytes in the mammalian host. To more clearly define features of the early events in host-parasite interaction, human monocytes were co-cultured with *Leishmania tropica* amastigotes in vitro. Infection of monocytes was time dependent and inhibited at 4°C and in the presence of cytochalasin B. Pretreatment of amastigotes with cytochalasins prevented their attachment to normal monocytes. Untreated amastigotes attached normally but could not enter cytochalasin-pretreated monocytes. This suggests that amastigotes actively participate in attachment but require host cell participation for interiorization.

INTRODUCTION

The precise mechanisms whereby pathogenic protozoa enter host cells are unknown. An understanding of these mechanisms might be exploited in designing novel chemotherapeutic strategies. Electron microscopic investigations of host cell-parasite interactions in vitro have suggested that *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Leishmania* species enter mononuclear phagocytes by endocytosis (1–3). Furthermore, cytochalasin (antiphagocytic agents) prevented parasite interiorization into host cells in vitro (2, 4, 5). Whether the parasites play a completely passive role in attachment to and entry of these cells is uncertain, however, since these cytochalasin studies failed to distinguish between effects of the drug on the parasite and the host cell. On the other hand, cytochalasin B treatment of *Plasmodium knowlesi* merozoites permitted attachment to but prevented their entry into erythrocytes (6), suggesting that *Plasmodia* might actively participate in infection of these nonphagocytic host cells.

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The interaction of *Leishmania* with mononuclear phagocytes is of considerable interest since once infection is established in a susceptible mammalian host, these parasites grow exclusively in monocytes and macrophages. Prior in vitro studies of these interactions (e.g., 3, 5, 7) examined attachment and entry of the motile flagellated extracellular forms of the parasite (promastigotes) that exist only briefly in the host before they transform into the aflagellate obligate intracellular forms, the amastigotes. Once infection is established, only amastigotes persist. These grow within macrophages, and upon release, invade adjacent cells. Little information is available regarding the early interactions between the amastigote and host mononuclear phagocytes (8), however. Accordingly, studies have been initiated to examine these interactions. In this study, the effects of cytochalasins (fungal metabolites that interfere with microfilament function) on in vitro parasite attachment to and infection of human mononuclear cells is reported. Evidence is presented that attachment involves active participation of the parasite because cytochalasin pretreatment inhibited their ability to attach to monocytes. On the other hand, infection of monocytes appears to require endocytosis, because cytochalasin pretreatment of host cells inhibited infection without inhibiting attachment.

METHODS

Materials. Cytochalasins B, dihydro B, E, and A were obtained from Sigma Chemical Co., St. Louis, MO, and fresh stock solutions were prepared by dissolving 100 mg of material/ml dimethylsulfoxide (DMSO, Fisher Scientific Co., Fairlawn, NJ). Gluteraldehyde (Polyscience, Inc., Warrington, PA) was used at 2% in 0.15 M phosphate-buffered saline (pH 7.4). Hypaque (Winthrop Laboratories, New York) and Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) were used in preparation of mononuclear cell suspensions by previously described techniques (9). Latex beads (polystyrene, 1.101-μm Diam) were obtained from Dow Chemical Co., Indianapolis, IN. Anti-sheep erythrocyte IgG was obtained from Cordis Laboratories Inc., Miami, FL, and used to sen-

sitize sheep erythrocytes by previously described methods (10). Supplemented medium RPMI 1640 (Gibco Laboratories, Grand Island, NY) contained penicillin 100 U/ml, streptomycin 100 μg/ml, and 10% heat-inactivated fetal bovine serum (HIFBS Gibco). For nonspecific esterase staining (11) we used diagnostic kit 90, Sigma Chemical Co.

Cell cultures. Mononuclear cell suspensions were obtained from normal volunteers without prior exposure to *Leishmania* and were washed twice in Hanks' buffer and resuspended in supplemented medium to a final concentration of 10^6 cells/ml. In some experiments, monocyte-derived macrophage monolayers were prepared on sterile acid-washed glass slides (3 × 1 in.) using a silicone rubber matrix (Micro-Slide Culture Chamber, Bellco Glass, Inc., Vineland, NJ). 2.5×10^5 mononuclear cells, suspended in 0.2 ml of supplemented medium containing 10% heat-inactivated fetal bovine serum, were added to each 5.5-mm Diam well. After overnight incubation of cultures, (37°C in 5% CO_2-95% air), nonadherent cells were removed by washing twice with Hanks' buffer, and adherent cells were further incubated in supplemented medium for 3 d. In related experiments, monocytes were allowed to adhere for 4 h (37°C) to round glass cover slips (3448; Clay-Adams, Oxnard, CA). After removing nonadherent cells by washing, cover slips were placed in Dvorak-Stotler chambers (12). Parasite-macrophage interactions were observed in these chambers with an inverted microscope equipped with Planapo 63X objective (model ICM 405; Carl Zeiss, Inc., Oberkochen, West Germany) under conditions of controlled temperature (37±2°C) maintained with a heat screen and monitored with a thermistor probe (model 47; Yellow Springs Instrument Co., Inc., Yellow Springs, OH) placed on the chamber. The chamber was perfused with supplemented medium at a flow rate of 1 ml/h (model 341 A syringe pump; Orion Research, Inc., Cambridge, MA).

Amastigotes of *L. tropica* (National Institutes of Health S strain [13]) were obtained by described methods (14) from footpad tissue of Balb/c mice infected 1 mo previously. Amastigotes obtained in this manner were proven viable by their ability to replicate in vitro in human monocyte-derived macrophages (14) and to transform into promastigotes and replicate in axenic culture (15). By transmission electron microscopy, these amastigotes were observed to be free of the surrounding host membrane that comprises the parasitophorous vacuole in the intact infected macrophage (Aikawa, M., and D. Wyler. Unpublished observations). Host cells and parasites were incubated separately at 37°C for 1 h in either medium alone or medium containing cytochalasin. Cells were washed at least twice in 50 vol of medium, resuspended, and combined at a ratio of 1:1 in a volume of 200 μl in 1.5-ml conical polypropylene microtest tubes (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY). After further incubation at 37°C in 5% CO_2-95% air, humid atmosphere, cells were harvested by cytocentrifugation (Cytospin, Shandon-Southern Instruments, Inc., Sewickley, PA), fixed in methanol, and stained with Wright-Giemsa (Diff-Quik, Harleco, Hartman-Leddon, Philadelphia, PA). Infectivity of cells with characteristic monocyte morphology (16), mean number of amastigotes per infected monocyte, and attachment rate (% uninfected monocytes with ≥1 amastigotes in close apposition) were determined for triplicate cultures by counting 100 monocytes in random oil immersion fields. These scores were not significantly different whether 100 or 1,000 cells were counted ($P > 0.5$).

Amastigotes of *L. tropica* have been previously shown capable of infecting and growing in vitro within human monocyte-derived macrophage cultures (14). However, cytochalasin treatment of these host cells were found in preliminary studies to result in substantial loss of cells from the monolayer. Therefore, monocyte suspension cultures were used in these studies.

In control experiments, amastigotes incubated for 1 h in 100 μg/ml cytochalasin B or medium alone were washed once and allowed to incubate in medium (10^6 parasites/ml) for 2 h. 1 million monocytes/ml were suspended in supernatants of these cultures and their ability to ingest latex beads (10^7/ml) or IgG-sensitized sheep erythrocytes (EA, 10^6/ml) in 2 h at 37°C was assessed. In related studies, latex and EA ingestion was assessed in the presence of treated amastigotes added to monocyte suspensions in a ratio of 1:1.

Statistical analysis compared mean and standard error of the mean by Student's *t* test (17).

RESULTS

Observations in live preparations. The interaction of amastigotes with adherent monocyte-derived macrophages were examined by phase-contrast and diffusion interference contrast (DIC; Nomarski Optics) microscopy in Dvorak-Stotler chambers. Amastigotes were observed to be nonmotile, in contrast to the highly motile flagellated promastigotes. When amastigotes made contact with the plasma membrane of the macrophages (by settling or under the influence of Brownian movement), they characteristically appeared to remain attached and frequently could be observed to move slowly over the macrophage surface (Fig. 1). Occasionally, they would detach within minutes of this interaction. Increased perfusion pressure applied to the chamber by increasing flow rate to 5.9 ml/h failed to detach amastigotes from the macrophage surface. This confirmed the impression that actual attachment rather than merely image juxtaposition had taken place. Following attachment, pseudopods were noted to form slowly around the amastigotes. Amastigotes finally came to reside within a parasitophorous vacuole (Fig. 2), as previously confirmed by transmission electron microscopy (8, 14). Quantitation of these interactions was subsequently achieved by examining fixed preparations.

Observations in fixed preparations. Mononuclear cell suspensions contained 49±4% (mean±SEM; three experiments) cells with morphologic features of monocytes, 43±4% that stained for nonspecific esterase, and 39±4% which ingested latex beads in 1 h at 37°C with gentle rocking. Attachment of and infection of cells with characteristic monocytes morphology was achieved by examining stained cytocentrifuge preparations (Fig. 1). This method was validated by the demonstration that attachment and infectivity scores were similar, whether obtained from cytocentrifuge preparations in which monocytes were identified by their ability to stain for nonspecific esterase or by their characteristic morphology following staining with Wright-Giemsa (Table I). Furthermore, it was possible

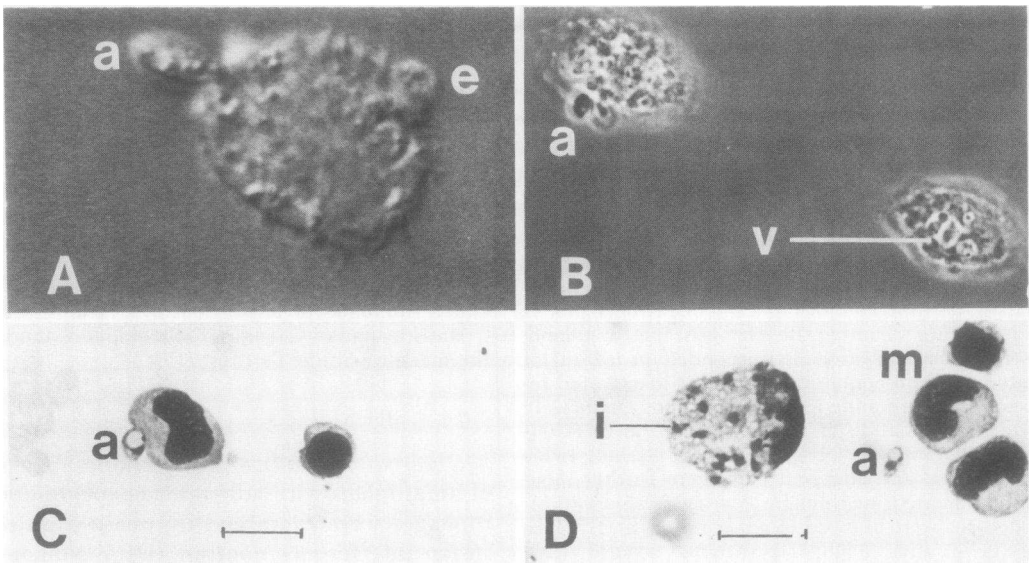

FIGURE 1 Attachment to and infection of human monocytes by *L. tropica* amastigotes in vitro. A: Amastigote attached to adherent monocyte (a) and beginning to enter (e). (Nomarski DIC microscopy using Dvorak-Stotler chamber; ×2,000) B: Amastigote attached to monocytes (a) and within parasitophorous vacuole (v) (phase contrast; ×800) C: Amastigote (a) attached to monocyte (Wright-Giemsa-stained cytocentrifuge preparation; ×800). D: Monocyte heavily infected in vitro with *L. tropica* amastigotes (i) extracellular amastigote (a) and uninfected monocytes (m) are also present (Wright-Giemsa-stained cytocentrifuge preparation; ×800).

to exclude that cytocentrifugation artefactually influenced apparent attachment and infectivity. Aliquots of cultures were either subjected to cytocentrifugation or fixed with gluteraldehyde in suspension. Attachment and infectivity scores obtained by phase-contrast and DIC microscopy of fixed suspensions were similar to those obtained from parallel cytocentrifuge preparations (Table I). Finally, it was determined that retrieval of monocytes from culture tubes occurred with high efficiency, since >90% could be recovered after 6 h incubation and ≥75% after 24 h incubation.

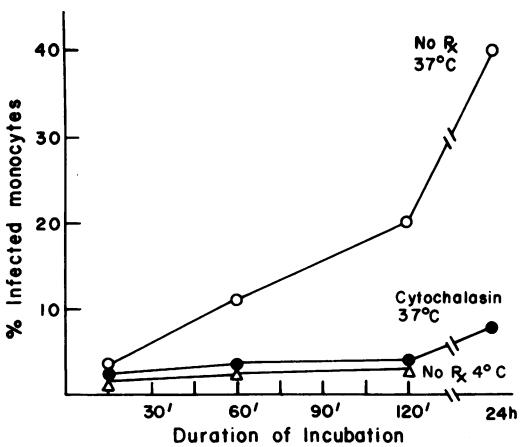

FIGURE 2 Effects of temperature and cytochalasin on the in vitro infection of human monocytes by *L. tropica* amastigotes. Infectivity in cultures incubated at 4°C without addition of drug (△) or in cultures incubated at 37°C in the presence of cytochalasin B (10 μg/ml) (●) is compared with infectivity in medium alone, incubated for various periods of time at 37°C. The mean±SEM of triplicate cultures is shown. SEM ≤ 15% of mean at all points.

Infection of monocytes was time dependent and inhibited at 4°C and in the presence of cytochalasin B (Fig. 2). This inhibitory effect of cytochalasin was dose dependent (Table II) and not due to the presence of the DMSO diluent (0.001–0.1%) in cultures (data not shown). These observations were consistent with the notion that the parasite gains entry into monocytes by endocytosis, but did not distinguish whether the cytochalasin affected host cells, parasites, or both. Therefore, amastigotes and mononuclear cells were separately pretreated with cytochalasin or medium alone (1 h at 37°C), washed twice with 50 vol of HBSS, and then coincubated. Pretreatment of either monocytes or amastigotes strikingly reduced infectivity (Fig. 3). These inhibitory effects were largely reversible over the subsequent 22 h of incubation (Fig. 3). Infection of treated monocytes by normal amastigotes was decreased by 75% ($P < 0.001$), and infection of normal monocytes by treated amastigotes was reduced by 80% ($P < 0.02$). These effects were cytochalasin dose dependent and resulted from treatment with a variety

TABLE I

Attachment (A) to and Infection (I) of Human Monocytes by L. tropica Amastigotes, Assessed by Three Different Methods

	Cytocentrifuge preparations, stain							
	Wright-Giemsa		Esterase		Fixed suspensions		*P* value	
	A*	I‡	A	I	A	I	A	I
Experiment 1	47±2	30±3	48±1	28±2	ND§	ND	>0.1	>0.1
Experiment 2	18±3	44±3	ND	ND	18±0.7	40±0.7	>0.1	>0.1

* Percent uninfected monocytes with ≥1 parasites attached.
‡ Percent of infected monocytes.
§ ND, study not performed.

of cytochalasins (Table II). Cytochalasin E was the most potent inhibitor. The degree of reduction in infectivity resulting from cytochalasin B treatment of amastigotes, monocytes, or both was similar ($P > 0.1$).

TABLE II

Inhibition of Amastigote Infection of Monocytes by Cytochalasin Treatment

Treatment (incubation period)	% inhibition* of infection by different concentrations of cytochalasin (μg/ml)			
	0.1	1.0	10	100
A Cytochalasin B (2 h)				
Addition to culture	41	77	93	99
Monocytes treated	2	30	71	70
Amastigotes treated	14	28	86	96
Both treated	ND‡	29	83	89
B Dihydro-cytochalasin B (2 h)				
Monocytes treated	26	49	59	63
Amastigotes treated	0	22	37	66
Both treated	15	26	33	59
C Cytochalasin E				
Monocytes treated				
(2 h)	ND	91	95	Tox.§
(24 h)	ND	73	83	Tox.
Amastigotes treated				
(2 h)	ND	66	73	100
(24 h)	ND	83	87	96
D Cytochalasin A (2 h)				
Monocytes treated	ND	83	98	Tox.
Amastigotes treated	ND	0	58	94

* % inhibition = $\left[\frac{\text{\% infection with treated cells}}{\text{\% infection with untreated cells}} - 1\right] \times 100$ and was calculated from the mean of triplicate cultures. SEM ≤ 15% of mean in all cases.
‡ ND, study not performed.
§ Tox., cytochalasin caused visibly toxic effects on treated cells.

Cytochalasin treatment of amastigotes also resulted in their inability to infect human monocyte-derived macrophage monolayers (76% reduction in infectivity; $P = 0.02$). Doses of cytochalasin used to pretreat monocytes also inhibited uptake of IgG-sensitized sheep erythrocytes (Table III).

The effects of cytochalasin on parasite attachment to monocytes were also assessed (Fig. 4). Pretreatment of monocytes with cytochalasin did not reduce the percent of monocytes with ≥1 amastigote attached, whereas pretreatment of parasites resulted in significant ($P < 0.01$) inhibition of attachment scores (Fig. 3). The inability of cytochalasin-treated amastigotes to attach to mononuclear phagocytes was confirmed by observations in live preparations of adherent monocytes in Dvorak-Stottlar chambers, as well as by the failure of treated amastigotes to attach to monocyte-derived macrophage monolayers (40% reduction in attachment; $P = 0.02$). Further analysis indicated that

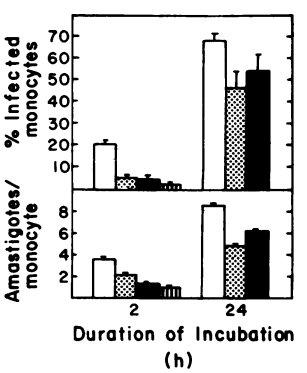

FIGURE 3 Effects of cytochalasin (10 μg/ml) pretreatment on infectivity. Infectivity was compared for cultures containing untreated cells (□), treated monocytes (■), treated amastigotes (■), and treated monocytes and treated amastigotes (▥) after 2 and 24 h coincubation at 37°C. Mean of triplicate determinations and SEM are shown. DMSO diluent (0.001 − 0.1%) pretreatment of cells did not affect scores ($P > 0.5$).

TABLE III
Comparison of Cytochalasin (Cyto) Inhibition of Amastigotes and EA Uptake by Human Monocytes

Treatment of monocytes	Amastigotes		EA	
	% infection	% attachment	% uptake	% attachment
None	34±3.4°	15±2.2	36±3.5	19.7±2.6
Cyto E, 1 μg/ml	2±0.8	27±3.0	0.3±0.2	26.8±3.0
Cyto E, 10 μg/ml	0	28.3±3	0	31.2±5.7
Cyto E, 100 μg/ml	0	32.7±3.3	0	30.5±3.2

° Mean±SEM; triplicate determinations.
EA, erythrocyte antibodies.

monocytes rarely had >1 amastigotes attached to their surface at the time cells were harvested after a 2-h incubation (Table IV). Upon further incubation, amastigotes apparently entered normal monocytes, with a concomitant reduction in attachment scores. In contrast, amastigotes remained on the surface of cytochalasin-treated monocytes which they were unable to enter (Table IV).

Control experiments. Although the precise mechanism of cytochalasin-mediated inhibition of attachment was not defined by those studies it was possible to exclude a lethal effect of the drug on the parasite, and a secondary effect of parasite treatment on macrophage function. Amastigotes were incubated for 1 h at 37°C in medium alone or medium containing cytochalasin B (1 and 10 μg/ml) or cytochalasin E (1 and 10 μg/ml). For viability studies, parasites were washed once and resuspended to 2.5×10^5/ml in supplemented medium 199 (15), permitted to transform to promastigotes and grown at 20°C for 5 d. Promastigote density was then assessed. Parasites underwent ~32-fold replication and there was no significant difference ($P > 0.1$) in parasite density in cultures of treated and untreated amastigotes. In the presence of treated amastigotes or their culture supernatants the ingestion of latex beads and IgG-sensitized sheep erythrocytes by normal monocytes during a 2-h incubation at 37°C was the same ($P > 0.2$) as in the presence of untreated amastigotes or their culture supernatants.

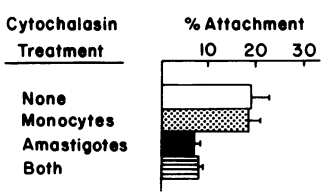

FIGURE 4 Effects of cytochalasin (10 μg/ml) pretreatment on attachment. Attachment of amastigotes to uninfected monocytes after preincubation of cells in medium alone (□), preincubation of monocytes in cytochalasin (■), preincubation of amastigotes in cytochalasin (■), or preincubation of both host cells and parasites in cytochalasin (▥) are compared for the same experiments summarized in Fig. 3. Mean±SEM of triplicate determinations are shown.

DISCUSSION

The ability of *Leishmania* amastigotes to infect and grow within mononuclear phagocytes is an important aspect of pathogenesis in leishmaniasis. Infection involves two processes: attachment, and interiorization. The data presented here suggest that the attachment phase of infection depends upon the active participation of this nonmotile stage of the parasite, whereas interiorization probably occurs by endocytosis. Because inhibition of endocytosis by cytochalasin treatment of monocytes did not seem to interfere with parasite attachment (Fig. 4; Table IV) this observation can be exploited to further investigate the attachment process in greater detail.

In these studies, attachment and infection scores were obtained primarily by inspection of fixed specimens from co-cultures of amastigotes and mononuclear cells. A potential pitfall in this method was the possibility that some of the attached parasites might have been superimposed on the monocyte image and have thus been erroneously scored as interiorized. Because this possibility cannot be entirely excluded, the scores obtained should not be construed to represent precise quantitation, but rather possess only semiquantitative value. Nonetheless, this visual method for analyzing the effects of cytochalasin on attachment and infection can be justified. The conclusion that amastigotes enter by phagocytosis is supported by observations with live preparations under partially controlled environmental conditions (Fig. 1), by the apparent inhibition of infection with cytochalasin treatment of monocytes (Fig. 3), or incubation at 4°C (Fig. 2), and by transmission electron microscopic studies that revealed that monocyte pseudopods pro-

TABLE IV

Attachment of L. tropica to Normal and Cytochalasin E-treated Monocytes during 2 and 4 h of Incubation

Incubation time	Monocytes treated	% attachment: Number of amastigotes per monocyte: 1	2	≥3	Total	% infection
h						
	−	22±2.2*	5±0.8	2±0.1	29	28.3±2.8
2	+	46±5	11±1.5	1±0.4	58	0
	−	13±0.7	1±0.3	0	14	43.7±6.1
4	+	49±3.4	9±0.7	1±0.4	59	0

* Mean±SEM; triplicate determinations.

gress to engulf amastigotes, and that amastigotes do not penetrate host plasma membrane (Aikawa, M., and D. J. Wyler. Manuscript in preparation.). If a substantial number of parasites were merely attached and visually superimposed on the monocyte image, and erroneously scored as interiorized, inhibition of phagocytosis with cytochalasin (Table III) should not have reduced apparent infectivity to the extent observed (Table II). This conclusion is valid since cytochalasin treatment of monocytes did not inhibit parasite attachment (Table IV). Furthermore, in studies with untreated monocytes infectivity scores increased with time, in association with decreased attachment scores, only if monocytes were capable of phagocytosis. If phagocytosis was inhibited by cytochalasin, attachment scores did not change significantly (Table IV). Taken together, these observations validate the semiquantitative methods used in establishing the conclusion that parasites only enter monocytes capable of phagocytosis.

Attachment appears to be largely parasite-dependent, since cytochalasin treatment prevented their ability to attach to monocytes (Fig. 4). It is unlikely that these observations represented secondary effects on the monocytes of cytochalasin eluting from the parasite. The ability of monocytes to ingest latex and IgG-sensitized erythrocytes was unaffected in the presence of cytochalasin-treated amastigote or their culture supernatants. Moreover, treatment of monocytes with cytochalasin did not prevent parasite attachment (Table IV). On the other hand, the precise mechanisms whereby cytochalasins inhibit attachment are not revealed by this study. Since cytochalasins interfere with microfilament function, it is tempting to speculate that parasite attachment might require cytoskeletal reorganization. For example, attachment might involve capping of low-affinity ligands expressed on the amastigote plasma membrane. The feasibility of such a process is suggested by observations that anti-leishmanial antibodies could induce in vitro capping of antigenic determinants on amastigotes (18). Electron micrographic studies of *L. tropica* amastigotes have failed to disclose organized microfilaments (Aikawa, M., and D. J. Wyler. Unpublished observations.). However, this technique may be too crude to detect the presence of actin microfilaments, and in no way excludes the potential importance of actin in the attachment process. The effects of cytochalasins on actin microfilament structure and function may also be subtle. It has been shown, for example, that cytochalasin can interfere with cross-linking of the actin network without altering grosser parameters such as specific viscosity of actin solutions (19). On the other hand, it seems unlikely that inhibition of attachment resulted from effects of cytochalasin unrelated to actin, such as inhibition of glucose transport (20). Dihydrocytochalasin B and cytochalasin E apparently do not inhibit glucose transport in mammalian cells (21), and yet inhibited parasite attachment (Table II).

The effects of cytochalasin treatment of leishmanial amastigotes on attachment to mononuclear phagocytes may be unique. In the presence of cytochalasins, attachment of *T. gondii* trophozoites and *T. cruzi* epimastigotes and trypomastigotes to macrophages was not decreased (2, 4). Cytochalasin B treated *P. knowlesi* merozoites attached normally to erythrocytes (6). A previous in vitro study of *Leishmania* promastigotes suggested that in the presence of cytochalasin, attachment via the flagellum was reduced (7). However, the overall quantitative effects of the drug on attachment were not reported, and the study did not distinguish between direct effects on the parasite and host cells. In preliminary studies it was observed that cytochalasin E (10 μg/ml) pretreatment of promastigotes reduced by 63% the ability of the parasites to attach to human monocytes (Wyler, D. J. Unpublished data.).

These observations suggest a similar participation of promastigotes and amastigote in attachment. However, since cytochalasin treatment of promastigotes drastically reduced their motility (Wyler, D. J. Unpublished observations), observations with this stage of the parasite may be difficult to interpret. Whether the active participation of *Leishmania* amastigotes in attachment represents a truly novel adaptation in intracellular parasitism must await further investigation of other intracellular parasites.

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