

A Heparin-binding Activity on *Leishmania* Amastigotes Which Mediates Adhesion to Cellular Proteoglycans

Dona C. Love, Jeffrey D. Esko,* and David M. Mosser

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140; and *Department of Biochemistry, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract. The intracellular amastigote form of leishmania is responsible for the cell-to-cell spread of leishmania infection in the mammalian host. In this report, we identify a high-affinity, heparin-binding activity on the surface of the amastigote form of leishmania. Amastigotes of *Leishmania amazonensis* bound approximately 120,000 molecules of heparin per cell, with a K_d of 8.8×10^{-8} M. This heparin-binding activity mediates the adhesion of amastigotes to mammalian cells via heparan sulfate proteoglycans, which are expressed on the surface of mammalian cells. Amastigotes bound efficiently to a variety of adherent cells which express cell-surface proteoglycans. Unlike wild-type CHO cells, which bound amastigotes avidly, CHO cells with genetic deficiencies in heparan sulfate proteoglycan biosynthesis or cells treated with heparitinase failed to bind amastigotes even at high

parasite-input dosages. Cells which express normal levels of undersulfated heparan bound amastigotes nearly as efficiently as did wild-type cells. The adhesion of amastigotes to wild-type nonmyeloid cells was almost completely inhibited by the addition of micromolar amounts of soluble heparin or heparan sulfate but not by the addition of other sulfated polysaccharides. Binding of amastigotes to macrophages, however, was inhibited by only 60% after pretreatment of amastigotes with heparin, suggesting that macrophages have an additional mechanism for recognizing amastigotes. These results suggest that leishmania amastigotes express a high-affinity, heparin-binding activity on their surface which can interact with heparan sulfate proteoglycans on mammalian cells. This interaction may represent an important first step in the invasion of host cells by amastigotes.

LEISHMANIA are dimorphic intracellular protozoan parasites. The flagellated promastigote form is delivered to the mammalian host from an infected sandfly. This developmental form invades mammalian cells and quickly transforms to the intracellular amastigote form. It is the amastigote form of the parasite which replicates in mammalian cells (20) and spreads the infection to neighboring cells.

A great deal of information concerning the interaction of the promastigote form with mammalian macrophages has been compiled (28). Promastigotes bind to specific receptors on macrophages and enter cells by receptor-mediated endocytosis (22). Several groups have demonstrated that the macrophage is the primary, if not exclusive, cell infected by promastigotes (8). The binding of promastigotes to macrophages involves a variety of apparently redundant mechanisms. Both complement and lectin-like receptors have been shown to participate in this interaction (3, 21). The coexpression of complement and lectin-like receptors on macrophages may define the cell type specificity of promastigote invasion. In contrast to promastigotes, little is known about how the amastigote form interacts with mammalian cells.

Neither the amastigote surface structures involved in parasite adhesion nor the receptors on mammalian cells for amastigotes have been identified. The cell specificity of amastigote binding also remains largely undefined. Unlike the promastigote form, whose infectivity appears to be restricted to macrophages (17), amastigotes have been shown to infect fibroblasts in vitro (7, 10). Furthermore, in leishmania lesions, intact amastigotes can be found within both macrophages and nonmyeloid cells (32). Despite the fact that amastigotes are the developmental form found in the infected host and the form responsible for spreading the disease within the host, little is known about the molecular structures used during parasite adhesion and invasion.

Most animal cells express proteoglycans. Proteoglycans consist of a protein core which contains one or more covalently attached glycosaminoglycan (GAG)¹ chains. GAGs are linear polysaccharides that consist of repeating disaccharide units bearing a net negative charge (15). The most com-

1. *Abbreviations used in this paper:* GAG, glycosaminoglycan; MPM, murine peritoneal macrophages; PB, phagocytosis buffer; PD, cation-free Dulbecco's PBS.

mon GAGs found in cellular proteoglycans are chondroitin sulfate, dermatan sulfate, and heparan sulfate. Heparin is very similar to heparan sulfate, except it is more highly sulfated, contains a higher proportion of iduronic acid, and is only produced by connective tissue mast cells. Proteoglycans can be anchored to the plasma membrane and expressed on the surface of cells (11). They can also be secreted from cells and deposited in the extracellular matrix, or they can be found intracellularly in secretory granules. Proteoglycans have many physiological functions, some of which are the mediation of cell/cell and cell/extracellular matrix interactions, the maintenance of tissue integrity, and the sequestration of growth factors near the cell surface (16). Recent reports have indicated that GAGs provide a mechanism of adherence for several human pathogens. *Chlamydia trachomatis* express heparan sulfate-like GAGs which mediate cellular attachment to eukaryotic host cells (31). Herpes simplex virus (29) and cytomegalovirus (25) bind to heparan sulfate proteoglycans on mammalian cells, and this interaction represents the first step in viral infection. Recently, heparan sulfate proteoglycans, localized on the basolateral domain of hepatocytes, have been identified as a receptor for the malaria circumsporozoite protein (6, 27). A heparin-binding protein has also been identified on *Trypanosoma cruzi* (26). This molecule is believed to aid in migration of the organism through the extracellular matrix by attaching to extracellular matrix components.

In this report, we identify a high-affinity, heparin-binding activity on the surface of leishmania amastigotes. We propose that this activity mediates attachment of amastigotes to mammalian cells by binding to cellular proteoglycans containing heparan sulfate.

Materials and Methods

Parasites

The U.S. National Institutes of Health S strain of *Leishmania major* (2) was isolated from a West African patient with cutaneous leishmaniasis and was originally provided by Dr. David Wyler (Tufts University Medical Center, Boston, MA). The Josefa isolate of *Leishmania mexicana amazonensis* (23) was isolated from a Brazilian patient with persistent cutaneous leishmaniasis and was provided by Dr. Janet Keithly (New York State Public Health Center, Albany, NY). Promastigotes were cultured in Schneider's complete medium containing Schneider's *Drosophila* medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 20% FCS, penicillin G/streptomycin (100 U/ml and 100 µg/ml, respectively), and 2 mM glutamine. Stationary-phase promastigotes were radiolabeled overnight with [³H]uracil as described (21). Amastigotes were isolated from the footpad of BALB/c mice which were infected 6 to 8 wk earlier as described (20). Briefly, the excised foot was forced through a wire mesh using the plunger from a 10-ml syringe while washing with 10 ml Schneider's complete medium. The mixture was then subjected to repeated passages through 21-, 23-, and 25-gauge needles to release the amastigotes from host cells. Cellular debris was removed from the mixture by centrifugation at 50 g for 5 min. Amastigotes were incubated in Schneider's medium on ice for 60 min to allow any associated cellular debris to detach. Amastigotes were then sedimented by centrifugation at 600 g for 10 min. For cell-binding assays, amastigotes were resuspended in an isotonic phagocytosis buffer (PB) which consists of equal parts of medium 199 and DME buffered with 12.5 mM Hepes (Mediatech, Herndon, VA) (21). For radiolabeling, amastigotes were added to the macrophage-like cell line J-774A.1, and the infected cells were incubated with [³H]uracil as described (20).

Cells

Several mutant CHO cells with specific deficiencies in proteoglycan biosyn-

thesis were used. Mutants pgsA-745 and pgsB-618 lack xylosyltransferase and galactosyltransferase I, respectively, enzymes necessary for the initiation of GAG biosynthesis (13, 14). Mutant pgsD-677 does not express heparan sulfate, but overexpresses chondroitin sulfate by a factor of three, causing the total amount of sulfated glycosaminoglycan to be comparable to that of wild-type cells (18). The mutant cell line pgsE-606 has reduced *N*-sulfotransferase activity which results in undersulfation of heparan sulfate without altering the levels of GAG synthesis (1). CHO-K1 and mutant CHO cells were maintained in Ham's F12 medium (GIBCO-BRL) supplemented with 10% FCS, penicillin G/streptomycin (100 U/ml and 100 µg/ml, respectively), and 2 mM L-glutamine (GIBCO-BRL). HEp-2 human epidermoid carcinoma cells, NIH-3T3 cells, and L-929 cells (American Type Culture Collection, Rockville, MD) were maintained in DME supplemented with 10% FCS, penicillin G/streptomycin (100 U/ml and 100 µg/ml, respectively), and 2 mM L-glutamine (D-10). Human foreskin fibroblast cells were obtained from 2-d-old males and were provided by Dr. Earl Hendersen (Temple University School of Medicine, Philadelphia, PA). Human foreskin fibroblast cells were maintained in D-10. The macrophage-like cell line J-774A.1 (ATCC) was maintained in DME supplemented with 7% FCS, penicillin G/streptomycin (100 U/ml and 100 µg/ml, respectively), and 2 mM L-glutamine (D-7). Murine peritoneal macrophages (MPM) were washed from the peritoneal cavity of BALB/c mice with cold cation-free Dulbecco's phosphate-buffered saline (PD) as previously described (21). Cells were washed with DME and incubated overnight on glass coverslips in D-7. Mononuclear cells were isolated from human peripheral blood using Lymphoprep (Nycomed Phara, Oslo, Norway) according to the manufacturer's instructions (24). Cells were resuspended in RPMI-1640 (Mediatech) and adhered to 13-mm glass coverslips in 24-well plates for 30 min at 37°C. After washing, the monocytes were used immediately. Before use in binding assays, all monolayers were washed free of serum. All binding assays were performed in PB.

Amastigote-binding Assay

Cells were incubated on 13-mm glass coverslips in 24-well plates overnight in medium containing FCS. Monolayers were washed three times with PB before use, and then amastigotes were added to the monolayers in PB at the indicated concentrations. The amastigotes were allowed to bind to the monolayers for 45 min at 35°C. All binding studies were carried out in a total volume of 400-µl per well. After the incubation, the cells were washed at least three times with PB to remove unbound amastigotes from the monolayer. The monolayers were then fixed with 2.5% glutaraldehyde in PD, stained with Giemsa dye, and bound organisms on triplicate coverslips were quantitated by light microscopy. One hundred cells were counted per coverslip. For the radiobinding assay, monolayers were solubilized with 0.5% Triton X-100 for 30 min, transferred to scintillation vials, and the amount of radiolabel present was quantitated by a TRI-CARB 1900 CA liquid scintillation analyzer (Packard, Sterling, VA).

To inhibit the binding of amastigotes to cells, either the amastigotes were pretreated with competitors for 20 min on ice and washed two times before their addition to the monolayers, or inhibitors and amastigotes were simultaneously added to the monolayers and left in throughout the 45-min assay. The competitors used were hyaluronic acid (Sigma Immunochemicals, St. Louis, MO), heparin, chondroitin sulfate (Calbiochem-Behring Corp., San Diego, CA) and heparan sulfate isolated from bovine aorta as described (12). Enzymatic treatment of cells was performed according to Neyts et al. (25). Briefly, heparitinase (Seikagaku America, Rockville, MD) was resuspended in medium 199 + 0.1% BSA. The monolayers were treated for 2 h at 37°C and washed three times with medium 199 + 0.1% BSA. The monolayers were then used in binding assays as described above.

Heparin-radiobinding Assay

Freshly isolated, washed amastigotes were incubated on ice for 1 h in PB before the binding assay. The washed amastigotes were resuspended in fresh PB containing 0.5% BSA. Increasing amounts of radiolabeled heparin were added to a total of 2×10^7 amastigotes. In some experiments, unlabeled competitors were also included. The amastigotes were incubated with radiolabeled heparin for 30 min at 4°C. After this incubation, amastigotes were washed four times in PB containing 1% BSA. In some experiments free ¹²⁵I-labeled heparin was separated from bound ¹²⁵I-labeled heparin by layering the parasites on an oil gradient consisting of one part bis phthalate and one and a half parts dibutyl phthalate (Eastman Kodak Co., Rochester, NY). The radioactivity bound to amastigotes was determined using a Gamma trac 1191 automatic gamma counting system (Tracer Analytic, Elk

Grove Village, IL). In all cases, the amount of specific parasite-bound radioactivity was compared to the amount of nonspecific binding defined as the binding which occurred in a 100-fold excess of unlabeled heparin. Heparin was radiolabeled according to Cardin et al. (5). Briefly, 2.5 mg of heparin was reacted with 1 mg of Bolton-Hunter reagent (*N*-succinimidyl-3-(4-hydroxyphenyl)propionate) (Pierce, Rockford, IL) in 0.5 ml of 0.1 M borate buffer, pH 9.0, for 20 min on ice. The unreacted, insoluble Bolton-Hunter reagent was removed by centrifugation for 30 s at 12,000 g. The sample was then reacted with 1 mg of fresh Bolton-Hunter reagent for 20 min. The unreacted Bolton-Hunter reagent was separated from the derivatized heparin by passing the sample over a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ). Fractions (0.5 ml) were collected and analyzed for heparin content according to MacIntosh (19). Fractions containing heparin were then added to Iodo-vials containing two Iodobeads (Pierce). 1 μ Ci of carrier-free Na¹²⁵I (Amersham Corp., Arlington Heights, IL) was added to the vial. The reaction was allowed to proceed for 15 min at room temperature and was terminated by removing the sample from the vial. Free Na¹²⁵I was removed by passage over a PD-10 column which had been equilibrated with PD. The radiolabeled heparin was eluted with PD, and the fractions containing radiolabeled heparin were determined using a γ counter. The concentration of heparin recovered from the procedure was determined as described above.

Dot Blot Assay

Approximately 1×10^9 *L. amazonensis* amastigotes were solubilized in 1 ml of lysis buffer containing 0.5% Triton X-100, 25 mM KCl, 5 mM MgCl₂, and 5 mM Tris, pH 7.5. The following protease inhibitors were added to the lysis buffer: leupeptin (0.5 μ g/ml); aprotinin (1 μ g/ml); soy bean trypsin inhibitor (50 μ g/ml); PMSF (1.0 mM); and 1,10-phenanthroline (2 mM). Amastigotes were solubilized on ice for 30 min. Insoluble protein was removed by centrifugation at 12,000 g for 10 min. Soluble protein was removed and either used immediately or stored for up to 1 wk at -20°C. Soluble amastigote protein was blotted onto nitrocellulose (Schleicher & Schuell, Keene, NH) as described (30), using a vacuum manifold dot blot apparatus. The nitrocellulose was previously washed with PD, 100 μ l per well. After addition of lysate, each well was washed with 300 μ l of PD. After incubation for 1 h at room temperature, the blot was blocked for 15 min with 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.3, 1 mg/ml BSA, and 0.05% Tween 20. Next, the blot was placed in a sealable bag containing 5 ml of the same buffer and incubated with radiolabeled heparin for 30 min at room temperature. Blots were washed three times with blocking buffer, 30 min per wash. The blots were air dried and exposed to a Fuji imaging plate for 1 h and visualized using a Fuji Bio-imager (Stamford, CT).

Results

The Binding of Amastigotes to Mammalian Cells

Increasing concentrations of radiolabeled *L. amazonensis* amastigotes were added to murine peritoneal macrophages for 45 min at 35°C in the absence of exogenous serum. Under these conditions, amastigotes bound avidly to macrophages and withstood vigorous washing. Amastigotes bound to macrophages in a dose-dependent manner (Fig. 1). The efficiency of amastigote binding in vitro was defined as the number of parasites added per cell required to achieve one organism bound per cell. This binding efficiency, calculated at multiple parasite-input doses, was 7.0 ± 1.25 (SEM, $n = 16$). In this in vitro model, this efficiency is between two- and fivefold greater than that of the binding of the promastigote form of leishmania to macrophages (21). Leishmania amastigotes were also added to parallel monolayers of the murine fibroblastoid cell line L-929. These cells also bound amastigotes in a dose-dependent manner (Fig. 1), but the relative efficiency of this binding was approximately threefold lower than the efficiency with which macrophages bound amastigotes. Thus, the efficiency of amastigote binding to L-929 cells was comparable to the efficiency of promastigote binding to macrophages.

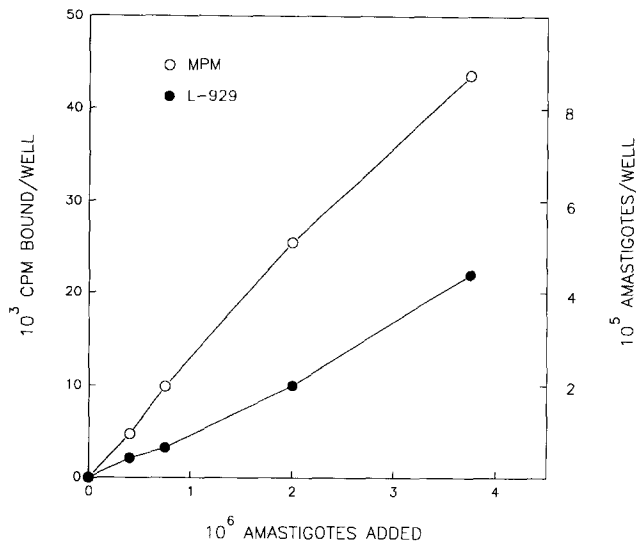


Figure 1. A comparison of the binding of *L. amazonensis* amastigotes to macrophage and nonmacrophage cells. Increasing numbers of radiolabeled *L. amazonensis* amastigotes were added to monolayers of either mouse peritoneal macrophages (○) or L-929 cells (●). Binding proceeded for 45 min at 35°C. Monolayers were washed three times, solubilized in Triton X-100, and the amount of radioactivity associated with the monolayers was determined using a β -counter. Data represent the average of two determinations. The experiment shown is representative of three experiments. Amastigotes were radiolabeled to a specific activity of 0.051 cpm per organism. In this experiment the maximum input of 3.75×10^6 amastigotes resulted in approximately 43,600 cpm or 8.5×10^5 organisms bound per macrophage monolayer, for a binding efficiency of 4.4. Background binding to blank wells never exceeded 350 cpm and was not subtracted.

Given the substantial binding of leishmania amastigotes to L-929 cells, freshly isolated amastigotes were added to a variety of different adherent mammalian cells, and their binding was quantitated. All cells tested, including the CHO-K1 cell line, human foreskin fibroblasts, the human epithelioid cell line HEP-2, and the two murine fibroblastoid cell lines NIH-3T3 and L-929 (not shown), as well as murine peritoneal macrophages, bound leishmania amastigotes (Fig. 2). A single parasite-input dose was chosen to illustrate relative binding efficiencies of each of the cell types in parallel. Freshly isolated monocytes bound leishmania amastigotes least efficiently, whereas HEP-2 cells bound amastigotes almost as avidly as did macrophages (Fig. 2). At a relatively high parasite-input dose multiple amastigotes bound to virtually every HEP-2 cell in the monolayer (Fig. 3).

Inhibition of Amastigote Binding by Soluble Heparin

A characterization of amastigote adhesion to mammalian cells was undertaken as a first step toward identifying the molecular basis of this interaction. The binding of amastigotes to fibroblastoid and macrophage cells was unaffected by treating the cells with reagents known to inhibit other receptor-mediated adhesion events, including EDTA, cytochalasin, mannan, β -glucan, or mAbs to the macrophage complement receptor Mac-1 (data not shown). The adhesion of amastigotes to fibroblastoid and epithelioid cells, however, was dramatically inhibited by the addition of soluble

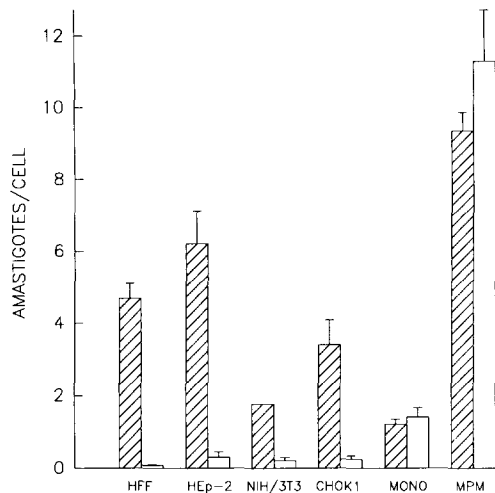


Figure 2. The binding of *L. amazonensis* amastigotes to a variety of adherent cells. A single dose of 2.5×10^6 *L. amazonensis* amastigotes was added to cell monolayers of human foreskin fibroblasts (HFF), human epithelial cells (HEp-2), murine fibroblastoid cells (NIH/3T3), CHO cells (CHOK1), freshly isolated human peripheral blood monocytes (MONO), and murine peritoneal macrophages (MPM). Amastigotes were added to the monolayers in the absence (▨) or presence of 100 µg/ml heparin (□). Binding proceeded for 45 min at 35°C. Monolayers were then washed three times with PB, fixed, stained, and quantitated by light microscopy. The indicated number of amastigotes bound to NIH-3T3 cells in the absence of heparin represents the average of two determinations. All other bars represent the mean of triplicate determinations \pm the standard deviation. This experiment is representative of three separate experiments.

heparin (Fig. 2). 1 µg/ml of heparin inhibited the binding of *L. amazonensis* amastigotes to CHO-K1 cells by >90% (Fig. 4). This inhibition was specific, since 500 µg/ml of chondroitin sulfate was required to inhibit amastigote adhesion by 50%. Heparin inhibited virtually all of the binding of amastigotes to all of the nonmyeloid cells tested. In contrast to nonmyeloid cells, the addition of heparin to macrophages did not

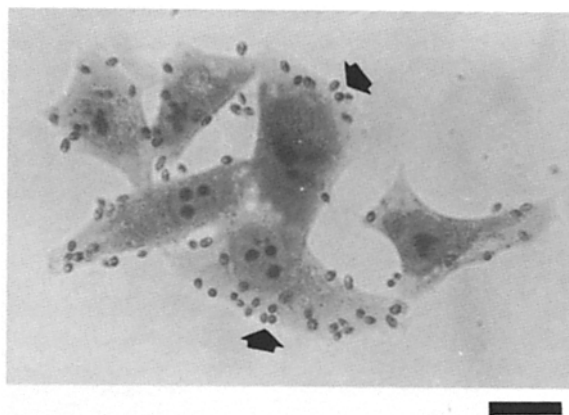


Figure 3. The binding of *L. amazonensis* amastigotes to HEp-2 cells. A single dose of 3.75×10^6 *L. amazonensis* amastigotes was added to a monolayer of HEp-2 cells. Binding proceeded for 45 min at 35°C. Monolayers were washed three times with PB, fixed, stained with Giemsa dye, and examined by light microscopy. Bar, 25 µm.

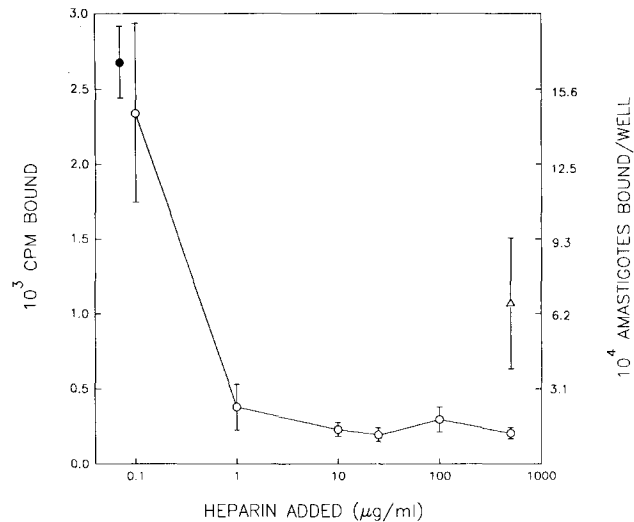


Figure 4. Inhibition of *L. amazonensis* amastigote binding by soluble heparin. The binding of radiolabeled *L. amazonensis* amastigotes to monolayers of CHO-K1 cells was measured in the absence (●) or presence of soluble GAGs (○, △). Heparin was added in increasing concentrations (○). Chondroitin sulfate was added at a single dose of 500 µg/ml (△). Data represent the mean of triplicate samples \pm the standard deviation. The experiment is a representative of four separate experiments.

inhibit amastigote adhesion, regardless of the concentration of heparin used (Fig. 2). When amastigotes were pretreated with heparin and washed before their addition to macrophage monolayers, however, a 60% inhibition of the binding of amastigotes to macrophages was observed (Fig. 5).

The Binding of Amastigotes to Cell Surface Proteoglycans

Several cell-surface proteoglycans contain heparan sulfate GAGs. To determine whether the cell-surface heparan sulfate proteoglycans were involved in amastigote adhesion, a number of CHO cells with defined deficiencies in GAG biosynthesis were analyzed. Increasing concentrations of leishmania amastigotes were added to wild-type CHO cells and CHO cell lines which lacked specific GAGs (18). Organisms bound efficiently to wild-type CHO cells in a dose-dependent manner (Fig. 6). In contrast to their binding to wild-type cells, amastigotes failed to bind to several CHO cell lines with specific deficiencies in GAG biosynthesis. Two cell lines (pgsA-745 and pgsB-618) which express very little, if any, GAGs failed to bind amastigotes (Fig. 6). A third cell line (pgsD-677), which expresses no heparan sulfate but overexpresses chondroitin sulfate so that the total amount of sulfated proteoglycan present is equal to that of wild-type cells, also failed to bind amastigotes. A fourth cell line (pgsE-606) produces heparan sulfate that contains about one-third less sulfate. These cells bound amastigotes, albeit with slightly less efficiency than did the wild-type cells at the highest parasite-input dose tested. Thus, the binding of leishmania amastigotes to CHO cells correlates with cellular expression of proteoglycans containing heparan sulfate.

Two additional approaches were taken to confirm that cellular proteoglycans are the ligands for adhesion of leishmania amastigotes to mammalian cells. First, adhesion

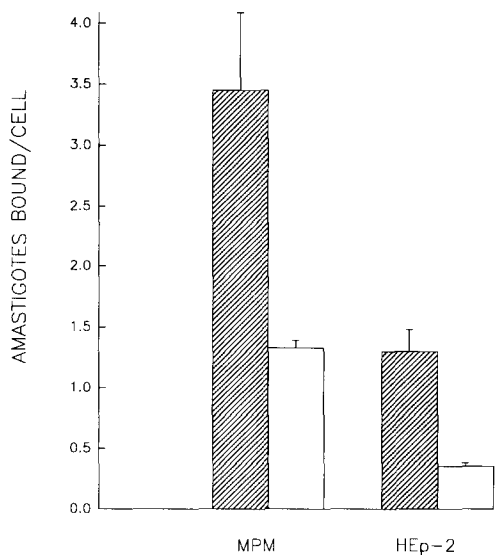


Figure 5. Inhibition of *L. amazonensis* amastigote binding by pretreatment with soluble heparin. Amastigotes were pretreated with heparin (10 $\mu\text{g/ml}$), before their addition to monolayers of either MPM or HEp-2 cells. After incubation in heparin for 20 min on ice, the amastigotes were washed twice with PB and added to the monolayers at a dose of 1.25×10^6 amastigotes per well. Incubation proceeded for 45 min at 35°C. Monolayers were washed, fixed, stained, and quantitated by light microscopy. Data represent the mean of triplicate samples \pm the standard deviation from a single experiment representative of three. The average inhibition of amastigote binding to MPM from three separate experiments was $59.4\% \pm 4\%$.

assays were performed on HEp-2 cells which were enzymatically treated with heparitinase to remove proteoglycans. Treatment of cells with heparitinase decreased amastigote adhesion in a dose-dependent manner (Fig. 7 A). Treatment of HEp-2 cells with 5 mIU heparitinase decreased the adhesion of amastigotes by $>70\%$ (Fig. 7 A). Second, adhesion assays were performed in the presence of exogenous heparan sulfate isolated from bovine aorta. The addition of 100 $\mu\text{g/ml}$ heparan sulfate diminished the binding of amastigotes to HEp-2 cells by $>90\%$ (Fig. 7 B) and to murine peritoneal macrophages by $\sim 40\%$ (Fig. 7 B).

The Binding of ^{125}I -labeled Heparin to *Leishmania* Amastigotes

The inhibition of amastigote binding to cells by soluble heparin or heparan sulfate, and the correlation of amastigote binding with cellular expression of heparan sulfate suggested the presence of a heparin-binding activity on amastigotes. To detect this activity, increasing concentrations of iodinated heparin were added to a constant number of *L. amazonensis* amastigotes. Heparin bound to leishmania amastigotes in a dose-dependent, specific, and saturable manner (Fig. 8). Scatchard analysis of these data indicates $\sim 120,000$ high-affinity receptors, with a $K_d = 8.8 \times 10^{-8}$ M. The analysis also suggested a lower-affinity interaction with a $K_d = 5 \times 10^{-7}$ M. The binding of radiolabeled heparin to freshly isolated amastigotes was almost completely inhibited by excess nonradioactive heparin, but binding was not inhibited by excess chondroitin sulfate or hyaluronic acid (Table I). Radio-

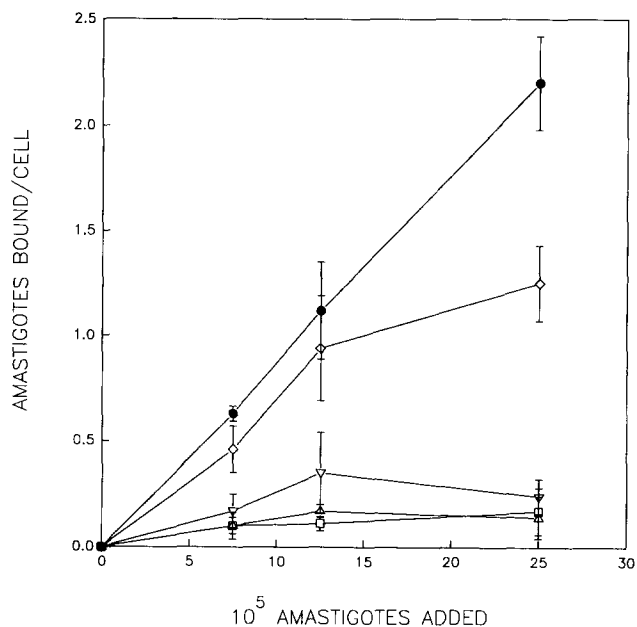


Figure 6. The binding of *L. amazonensis* amastigotes to cell-surface proteoglycans. Increasing numbers of *L. amazonensis* amastigotes were added to monolayers of wild-type CHO-K1 cells (●) or cells with specific mutations in GAG biosynthesis (○, △, ▽, ◇). pgsA-745 and pgsB-618 cells (△, ▽, respectively) express essentially no glycosaminoglycans. pgsD-677 cells (□) specifically lack heparan sulfate but overexpress chondroitin sulfate. pgsE-606 cells (◇) express undersulfated heparan sulfate chains. Incubation proceeded for 45 min at 35°C. Monolayers were washed extensively, fixed, stained, and quantitated by light microscopy. Data represent the mean of triplicate samples \pm standard deviation. This experiment is representative of three separate experiments.

labeled heparin also bound to a second species of leishmania amastigotes, *L. major* (Fig. 9 A). In contrast to the amastigote form, promastigotes bound heparin relatively poorly (Fig. 9). The binding of heparin to either *L. major* or *L. amazonensis* promastigotes was not detected at the low heparin-input concentrations at which amastigotes bound heparin avidly. Even at high heparin doses *L. major* promastigotes failed to bind heparin; however, at the highest doses tested (8–30 $\mu\text{g/ml}$ heparin) *L. amazonensis* promastigotes bound low levels of heparin (Fig. 9 B). The dose-dependent, -specific, and -saturable binding of radiolabeled heparin to leishmania amastigotes is consistent with the presence of specific heparin-binding sites on leishmania amastigotes.

Dot Blot Analysis

Amastigotes were solubilized in Triton X-100, and the lysates were analyzed for heparin-binding activity. The untreated lysate bound radiolabeled heparin (Fig. 10), indicating that it contained an intact heparin-binding activity. Like the activity on the intact amastigotes, radiolabeled heparin binding was competed with cold heparin, but not with cold chondroitin sulfate. The heparin-binding activity of amastigote lysates was also of high apparent affinity, because binding occurred not only in physiological saline but also in higher salt concentrations. Binding in 1 M salt was decreased by $\sim 50\%$ relative to binding under isotonic conditions (Fig. 10). Heparin binding occurred in SDS, as has been reported

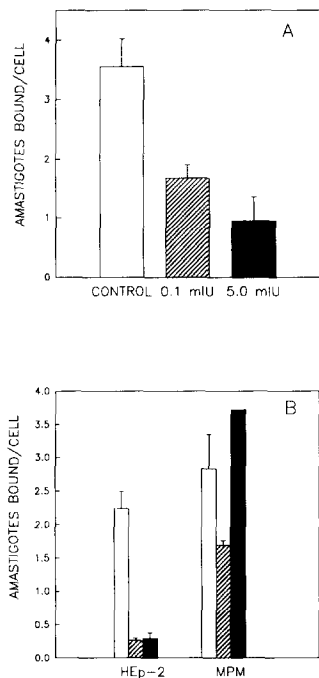


Figure 7. Inhibition of the binding of amastigotes to mammalian cells. (A) Monolayers were pretreated with heparitinase (0.1 mIU, ▨; 5.0 mIU, ■) for 2 h at 37°C and washed three times. A single dose of 2.5×10^6 amastigotes was added to each monolayer. Binding proceeded for 45 min at 35°C. Monolayers were washed, fixed, and stained. Adhesion was quantitated by light microscopy. (B) Soluble glycosaminoglycans were used to inhibit amastigote binding. A single dose of 2.5×10^6 amastigotes was added to monolayers of either HEP-2 cells or MPM. Binding proceeded either in the absence (□) or presence of heparan sulfate (▨) or heparin (■) at a concentration of 100 $\mu\text{g/ml}$. Amastigote adhesion was quantitated as described above. Data represent the mean of triplicate samples \pm standard deviation.

for other heparin-binding proteins (5). Treatment of the lysate with the reducing agent 2-mercaptoethanol or with trypsin completely abolished heparin binding, suggesting that the binding activity was most likely mediated by a protein on the surface of amastigotes.

Discussion

Amastigotes are the developmental form of leishmania which exists within mammalian cells. They multiply intracellularly and spread the disease to neighboring cells. Very little is known about how amastigotes interact with mammalian cells. Neither the receptors on mammalian cells involved in mediating amastigote adhesion nor the ligand(s) on amastigotes with which mammalian cells interact is known. In this report, we present several lines of evidence that are consistent with a heparin-binding activity being an adhesion factor for leishmania amastigotes. Adhesion of amastigotes to cells was inhibited by the addition of exogenous GAGs. The addition of micromolar amounts of heparin abolished the binding of amastigotes to several mammalian cells, including CHO cells, human foreskin fibroblasts, and HEP-2 cells. This inhibition was specific to heparin, since other GAGs, such as chondroitin sulfate or hyaluronic acid, did not inhibit amastigote adhesion to these cells unless added at a 500-fold higher molar concentration. The addition of intact heparan sulfate proteoglycans also inhibited adhesion of amastigotes to mammalian cells. Pretreatment of amastigotes with heparin prior to their addition to HEP-2 cells or to macrophages resulted in decreased amastigote binding, indicating that the heparin-binding activity resides on the amastigote and not on the mammalian cell. Addition-

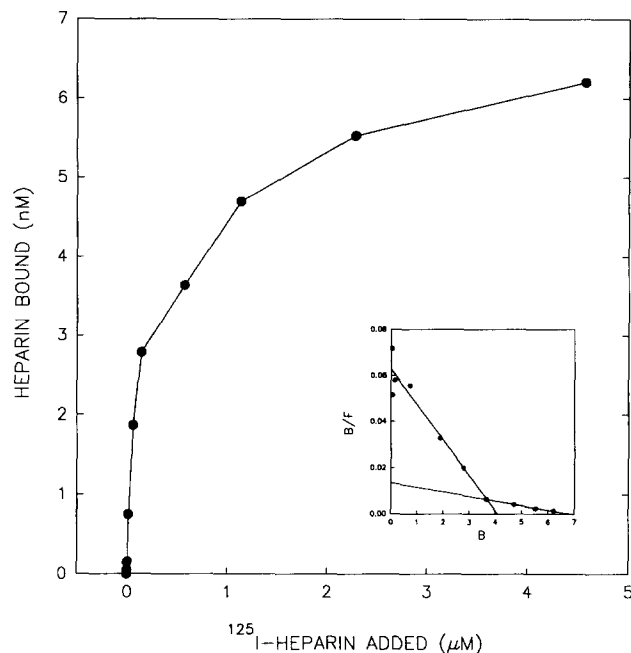


Figure 8. Radiolabeled heparin binding to *L. amazonensis* amastigotes. Increasing amounts of radiolabeled heparin (sp act = 3.1×10^5 cpm/ μg) were added to a constant number (2×10^7) of *L. amazonensis* amastigotes. Organisms were incubated for 30 min at 4°C and washed three times. The amount of ^{125}I -labeled heparin bound to the parasites was determined. A Scatchard plot of the binding data (inset) shows two lines. From the slope and x intercept of the first line, a binding constant (K_d) of 8.8×10^{-8} M and a stoichiometry of 120,000 receptors per organism were determined. From the second line, a K_d of 5.0×10^{-7} and a stoichiometry of 210,000 receptors per organism were determined. Data represent the average of two determinations. This experiment is representative of three separate experiments.

ally, the removal of cellular heparan sulfate by pretreatment of mammalian cells with heparitinase resulted in decreased adhesion of amastigotes to these cells, indicating that cell-surface heparan sulfate proteoglycans are the ligands for amastigote adhesion. Finally, amastigotes fail to adhere to three CHO cell lines with single, defined mutations in heparan sulfate biosynthesis. These mutant CHO cell lines express low or absent levels of heparan sulfate. However, amastigotes bound efficiently to the parent CHO cell line and to a cell line with undersulfated heparan. Even heparan sulfate-deficient cells which express the same amount of sul-

Table 1. Inhibition of Radiolabeled Heparin Binding to *L. amazonensis* Amastigotes by Soluble Glycosaminoglycans

Competitor*	cpm bound [‡]	Heparin bound [‡] (ng)
None	18,385 \pm 2,770	94 \pm 14
Heparin	2,494 \pm 1,212	13 \pm 6
Chondroitin sulfate	19,010 \pm 4,507	97 \pm 23
Hyaluronic acid	15,921 \pm 1,046	81 \pm 5

* Unlabeled competitors were used at a final concentration of 1.6 mg/ml.
[‡] The amount of radiolabeled heparin bound per 2×10^7 amastigotes. Values represent the mean \pm SD of triplicate determinations.

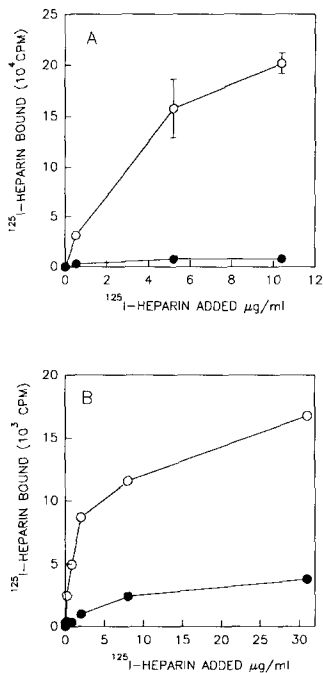


Figure 9. Binding of radiolabeled heparin to *L. major* (A) and *L. amazonensis* (B) amastigotes and promastigotes. (A) Radiolabeled heparin (sp act = 1.96×10^5 cpm/μg) was added to freshly isolated *L. major* amastigotes (○) or stationary-phase promastigotes (●). Parasites were washed three times with PB supplemented with 1% BSA, and the amount of ¹²⁵I-labeled heparin bound to the parasites was determined. Data represent the mean of three determinations ± standard deviation. (B) Increasing amounts of radiolabeled heparin (sp act = 4.0×10^6 cpm/μg) were added to freshly isolated *L. amazonensis* amastigotes (○) or stationary-phase promastigotes (●). Free ¹²⁵I-labeled heparin was separated from bound ¹²⁵I-labeled heparin by pelleting

the parasites through an oil gradient (see Materials and Methods). Data represent the average of two determinations. This experiment is representative of three separate experiments.

fated proteoglycan, due to the overexpression of chondroitin sulfate, failed to bind amastigotes.

The leishmania heparin-binding activity is expressed on intact organisms, and it was also detected in amastigote lysates. Intact amastigotes of *L. amazonensis* bound an average of approximately 120,000 molecules of heparin, with an apparent affinity of 8.8×10^{-8} M. Amastigotes of a second species of leishmania, *L. major*, also expressed a heparin-binding activity. We were unable to detect this high-affinity activity on the promastigote form of either species. In our hands, promastigotes bound heparin poorly compared to amastigotes. Others have previously identified a lower-affinity, heparin-binding activity on promastigotes of *Leishmania donovani* (4). We have not examined that species, but we speculate that the activity on promastigotes is distinct from the high-affinity activity described here on amastigotes. As we do detect some binding of heparin to promastigotes at high heparin input concentrations, this low-affinity binding of heparin to promastigotes may correspond to the lower-affinity activity detected on amastigotes. The K_d value for this second activity (5×10^{-7} M) approximates that reported for *L. donovani* promastigotes (4). Importantly, the low-affinity activity on promastigotes does not appear to be essential for parasite binding to cells. Promastigotes bound very poorly to epithelioid and fibroblastoid cell lines which express proteoglycans and which bound amastigotes avidly (data not shown). Furthermore, in contrast to amastigotes, the addition of excess soluble heparin has been reported to actually enhance the binding of promastigotes to macrophages (4).

We hypothesize that the binding of amastigotes to heparan sulfate proteoglycans is an important first step in the invasion of mammalian cells. We demonstrate that leishmania amastigotes bind to several different types of mammalian cells in

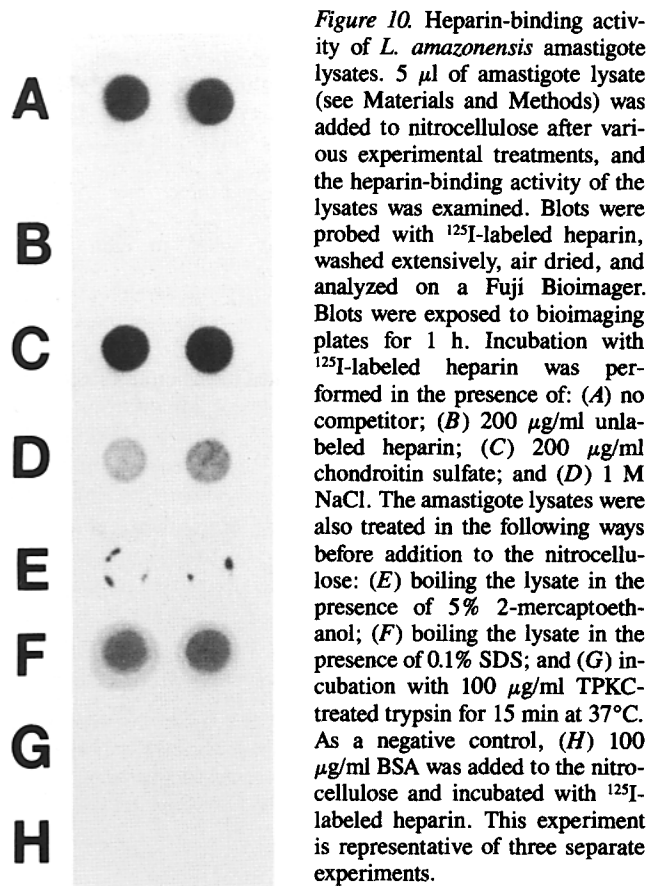


Figure 10. Heparin-binding activity of *L. amazonensis* amastigote lysates. 5 μl of amastigote lysate (see Materials and Methods) was added to nitrocellulose after various experimental treatments, and the heparin-binding activity of the lysates was examined. Blots were probed with ¹²⁵I-labeled heparin, washed extensively, air dried, and analyzed on a Fuji Bioimager. Blots were exposed to bioimaging plates for 1 h. Incubation with ¹²⁵I-labeled heparin was performed in the presence of: (A) no competitor; (B) 200 μg/ml unlabeled heparin; (C) 200 μg/ml chondroitin sulfate; and (D) 1 M NaCl. The amastigote lysates were also treated in the following ways before addition to the nitrocellulose: (E) boiling the lysate in the presence of 5% 2-mercaptoethanol; (F) boiling the lysate in the presence of 0.1% SDS; and (G) incubation with 100 μg/ml TPKC-treated trypsin for 15 min at 37°C. As a negative control, (H) 100 μg/ml BSA was added to the nitrocellulose and incubated with ¹²⁵I-labeled heparin. This experiment is representative of three separate experiments.

vitro. These cells include phagocytic macrophages as well as the nonphagocytic fibroblastoid and epithelioid cells. Although the macrophage has long been considered a focus for the intracellular replication of leishmania, these epithelioid and fibroblastoid cells may be an important reservoir for this organism. Indeed, several in vivo studies have demonstrated the presence of intact parasites within nonmacrophage cells (32). We have begun to examine the efficiency with which leishmania amastigotes are taken up by these nonmacrophage cells in vitro and the extent to which amastigotes survive within these cells.

The efficient and almost complete inhibition of the binding of amastigotes to nonmyeloid cells by soluble heparin suggests that the cell surface proteoglycans are the principal, if not sole, mechanism used by amastigotes to adhere to fibroblastoid and epithelioid cells. The recognition of amastigotes by macrophages, however, may be more complex than the relatively simple mechanism used by nonmyeloid cells. Soluble heparin inhibits amastigote adhesion to macrophages by only 60%, and this inhibition is only observed when the amastigotes are pretreated with heparin prior to their addition to macrophages. Cellular heparan sulfate inhibits amastigote adhesion to macrophages by only 40%, and amastigote adhesion was not affected by treatment of macrophages with heparitinase (data not shown). We speculate that the inability to completely inhibit amastigote binding to macrophages reflects the participation of additional cellular mechanisms by which macrophages recognize leishmania amastigotes. These additional mechanisms may cooperate with the proteoglycan interaction and promote more efficient

parasite internalization. An analogous mechanism has been reported to mediate the binding of cytomegalovirus to mammalian cells (9). Cytomegalovirus adheres to cell-surface proteoglycans (25), but efficient invasion of cytomegalovirus into mammalian cells is dependent on additional cellular receptors. In fact, we observed that macrophages internalize amastigotes much more efficiently than do the nonmyeloid cell lines that we have examined (A. Brittingham, D. C. Love, and D. M. Mosser, manuscript in preparation). This finding would be consistent with the hypothesis that efficient amastigote internalization requires the cooperation of more than one type of receptor.

In summary, we have identified a heparin-binding activity on leishmania amastigotes. The expression of this activity correlates with the ability of amastigotes to adhere to mammalian cells which express proteoglycans containing heparan sulfate. We hypothesize that amastigotes use this heparin-binding activity to facilitate their interaction with mammalian cells. Studies are underway to isolate the molecule(s) mediating this activity and to determine its role as a leishmania virulence factor.

This work was supported by National Institutes of Health grants GM33063 and BRS02 S07 RR05417-30.

Received for publication 16 March 1993 and in revised form 10 August 1993.

References

- Bame, K. J., and J. D. Esko. 1989. Undersulfated heparan sulfate in a Chinese hamster ovary cell mutant defective in heparan sulfate N-sulfotransferase. *J. Biol. Chem.* 264:8059-8065.
- Bjorvatn, B., and F. A. Neva. 1979. A model in mice for experimental *Leishmania tropica*. *Am. J. Trop. Med. Hyg.* 28:472-479.
- Blackwell, J. M., R. A. B. Ezekowitz, M. B. Roberts, J. Y. Channon, R. B. Sim, and S. Gordon. 1985. Macrophage complement and lectin-like receptors bind leishmania in the absence of serum. *J. Exp. Med.* 162:324-331.
- Butcher, B. A., L. A. Sklar, L. C. Seamer, and R. H. Glew. 1992. Heparin enhances the interaction of infective *Leishmania donovani* promastigotes with mouse peritoneal macrophages. *J. Immunol.* 148:2879-2886.
- Cardin, A. D., K. R. Witt, and R. L. Jackson. 1984. Visualization of heparin-binding proteins by ligand blotting with ¹²⁵I-heparin. *Anal. Biochem.* 137:368-373.
- Cerami, C., U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M. J. Santos, and V. Nussenzweig. 1992. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. *Cell.* 70:1021-1033.
- Chang, K. P. 1978. *Leishmania* infection of human skin fibroblasts in vitro: absence of phagolysosomal fusion after induced phagocytosis of promastigotes, and their intracellular transformation. *Am. J. Trop. Med. Hyg.* 27:1084-1096.
- Chang, K. P., G. Chaudhuri, and D. Fong. 1990. Molecular determinants of *Leishmania* virulence. *Annu. Rev. Microbiol.* 44:499-529.
- Compton, T., R. R. Nepomuceno, and D. M. Nowlin. 1992. Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. *Virology.* 191:387-395.

- Dedet, P., A. Ryter, E. Vogt, P. Hosli, and L. P. DaSilva. 1983. Uptake and killing of *Leishmania mexicana amazonensis* amastigotes by human skin fibroblasts. *Ann. Trop. Med. Parasitol.* 77:35-44.
- Esko, J. D. 1991. Genetic analysis of proteoglycan structure, function and metabolism. *Cur. Opin. Cell Biol.* 3:805-816.
- Esko, J. D. 1993. Preparation and analysis of glycoconjugates. In *Current Protocols in Molecular Biology*. F. M. Ausubel, R. Brent, R. E. Kingston, D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, editors. Greene Publishing Associates, New York. 1721-1729.
- Esko, J. D., T. E. Stewart, and W. H. Taylor. 1985. Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc. Natl. Acad. Sci. USA.* 82:3197-3201.
- Esko, J. D., J. L. Weinke, W. H. Taylor, G. Ekborg, L. Roden, G. Anantharamaiah, and A. Gawish. 1987. Inhibition of chondroitin and heparan sulfate biosynthesis in Chinese hamster ovary cell mutants defective in galactosyltransferase I. *J. Biol. Chem.* 262:12189-12195.
- Jackson, R. L., S. J. Busch, and A. D. Cardin. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.* 71:481-539.
- Kjellén, L., and U. Lindahl. 1991. Proteoglycans: structures and interactions. *Annu. Rev. Biochem.* 60:443-475.
- Klempner, M. S., M. Cendron, and D. J. Wyler. 1983. Attachment of plasma membrane vesicles of human macrophages to *Leishmania tropica* promastigotes. *J. Infect. Dis.* 148:377-384.
- Lindholt, K., J. L. Weinke, C. S. Kiser, F. N. Lugesma, K. J. Bame, S. Cheifetz, J. Massague, U. Lindahl, and J. D. Esko. 1992. A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc. Natl. Acad. Sci. USA.* 89:2267-2271.
- MacIntosh, F. C. 1941. A colorimetric method for the standardization of heparin preparations. *Biochem. J.* 35:776-782.
- Mosser, D. M. 1990. An assay to quantitate the binding of leishmania amastigotes to macrophages. *J. Immunol. Meth.* 130:235-242.
- Mosser, D. M., and P. J. Edelson. 1985. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of leishmania promastigotes. *J. Immunol.* 135:2785-2789.
- Mosser, D. M., and L. A. Rosenthal. 1993. Leishmania-macrophage interactions: multiple receptors, multiple ligands, and diverse cellular responses. *Semin. Cell Biol.* In press.
- Mosser, D. M., S. K. Burke, E. E. Coutavas, J. F. Wedgwood, and P. J. Edelson. 1986. *Leishmania* species: mechanisms of complement activation by five strains of promastigotes. *Exp. Parasitol.* 62:394-404.
- Mosser, D. M., T. A. Springer, and M. S. Diamond. 1992. Leishmania promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). *J. Cell Biol.* 116:511-520.
- Neyts, J., R. Snoeck, D. Schols, J. Balzarini, J. D. Esko, A. Van Schepdael, and E. De Clercq. 1992. Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. *Virology.* 189:48-58.
- Ortega-Barria, E., and M. E. A. Pereira. 1991. A novel *T. cruzi* heparin binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. *Cell.* 67:411-421.
- Pancake, S. J., G. D. Holt, S. Mellouk, and S. L. Hoffman. 1992. Malaria sporozoites and circumsporozoite proteins bind specifically to sulfated glycoconjugates. *J. Cell Biol.* 117:1351-1357.
- Russell, D. G., and P. Talamas-Rohana. 1989. *Leishmania* and the macrophage: a marriage of inconvenience. *Immunol. Today.* 10:328-333.
- Shieh, M. T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.* 116:1273-1281.
- Smith, J. W., and D. J. Knauer. 1987. Ligand blotting with ¹²⁵I-fluoresceinamine-heparin. *Anal. Biochem.* 160:105-114.
- Zhang, J. P., and R. S. Stephens. 1992. Mechanism of *C. trachomatis* attachment to eukaryotic host cells. *Cell.* 69:861-869.
- Zuckerman, A. 1953. Initial reaction to the subcutaneous inoculation of cultures of *Leishmania tropica* in the hamster. *Acta Med. Orientalia.* 12:238-241.