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Role of 67 kDa Cell Surface Laminin Binding Protein of *Leishmania donovani* in Pathogenesis[1]

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The role that interaction with laminin may play in *Leishmania donovani* infection was investigated. Binding of ^{125}I-radiolabeled laminin, in a liquid-phase assay, by the parasite was rapid, saturable, specific, reversible, and of high affinity. Using a Western blotting procedure, a 67 kDa laminin-binding protein (LBP) was identified from the membrane of both the promastigote and amastigote forms of *L. donovani*. Subsequently, the protein was purified by affinity chromatography. Immunofluorescence with a polyclonal antibody against LBP as well as flow cytometric analysis demonstrated its presence at the parasite surface. After stimulation with phorbol-12-myristate-13-acetate (PMA), U937 cells exhibited the ability to adhere to laminin and LBP specifically inhibited this adhesion. The reduced parasite adhesion after tunicamycin treatment suggested the importance of sugar residues in cell adhesion. Although co-administration of either laminin or LBP or anti LBP antibody reduced parasite virulence, resulting in a lower level of infection in the BALB/c mouse model, an *in vitro* macrophage culture–enhanced level of infection was observed in the case of laminin-coated parasites. The results collectively suggest a role for LBP in the interaction of the parasite with extracellular matrix elements, which may constitute a basis for the homing of the parasite to its physiological address.

Key words: adhesion, extracellular matrix, infection, laminin-binding protein, *Leishmania donovani*.

Visceral leishmaniasis is a widespread parasitic disease throughout much of the third world. The causative parasite, *Leishmania donovani*, exists in two forms: a promastigote, which lives in the gut of the sandfly vector, and an amastigote, which resides in macrophages of the mammalian host (*1*). The infective promastigotes are injected into the bloodstream when the sandfly takes a blood meal. The injected promastigotes then migrate through the bloodstream into various definite organs like the liver and spleen, where they successively adhere, penetrate, change into amastigotes and replicate, primarily in the resident macrophages. In this process, the host macrophage is lysed, and when thus released in the interstitial tissue, the parasites move in search of fresh target cells, where they seek refuge, multiply and lyse the host cell, and so once again the cycle is repeated (*2*). Thus a chain reaction is triggered leading to colonization of an organ and subsequently its dysfunctioning, which results in death if untreated.

Organs like the liver and spleen are not just a conglomeration of cells, they also contain acellular materials like collagen, laminin, fibronectin, vitronectin, *etc.*, which constitute the important set up of the extracellular matrix (ECM) and basement membrane (BM). Although we know a great deal about the host–parasite interaction in leishmaniasis (*3*), we have no knowledge regarding the molecular recognition events which enable these parasites to home to the target organs, bypassing the intricate meshwork of ECM and BM. In all likelihood, *L. donovani* promastigotes express on their surface molecules capable of recognizing the ECM macromolecules, which help them to glide through the interstitial tissue during their transit from the blood to target cells (*4*). Interaction with ECM proteins has been correlated with the invasive ability of different pathogens (*5*). The recognition of laminin, in particular, is implicated in the attachment of a variety of extracellular and intracellular pathogens to host tissues or cells (*6–10*). However, the molecular mechanisms involved in these adhesive interactions remain undefined.

Understanding of the mechanisms mediating the adherence of *L. donovani* to the ECM or host cells could lead to the development of antiparasitic agents whose mechanisms of action would involve competition with the endogenous ligands for binding to the pathogen receptors or adhesins. These inhibitors may prevent adhesion to host tissues and thereby prevent invasive infections. We previously detected a laminin-binding protein (LBP) on the surface of both the promastigote and amastigote forms of *L. donovani* (*4*). In

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Abbreviations: LBP, laminin-binding protein; PMA, phorbol-12-myristate-13-acetate; ECM, extracellular matrix; BM, basement membrane; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenyl methyl sulfonyl fluoride; LDU, Leishman-Donovan unit.

the present paper, we report the localization of the binding protein on the surface of *L. donovani* by immunofluorescence and flow cytometry. We have also demonstrated that the specific binding of the *L. donovani* surface protein to laminin is correlated with cell adhesion and the parasite's adhesiveness *in vitro* as well as to the enhancement of its pathogenesis, using the BALB/c mouse model of visceral leishmaniasis.

MATERIALS AND METHODS

Parasites—L. donovani AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with visceral leishmaniasis (*11*). Parasites were maintained in BALB/c mice by intravenous passage every 6 weeks. For experiments involving promastigotes, parasites were used at or near the stationary phase of growth from passages 2 to 5 after *in vitro* transformation from liver and spleen-derived amastigotes. Promastigotes were cultured at 22°C in medium 199 with Hanks salts (GIBCO Laboratories, Grand Island, NY, USA) containing HEPES (12 mM), L-glutamine (20 mM), 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin. Promastigotes were surface labeled with ^{125}I using lactoperoxidase-glucose oxidase as described previously (*12*).

Radioiodination of Laminin—Laminin was labeled with ^{125}I (sodium salt; specific activity 14.5 mCi/μg; Amersham, Arlington Heights, IL, USA) by the chloramine-T method (*13*). The specific activity of the labeled laminin was 3–5 × 10^6 cpm/μg. The protein concentration was determined by the method of Lowry *et al.* (*14*).

Binding Assay—Liquid phase binding assays were performed as described previously (*4*). Briefly, *L. donovani* promastigotes after several washings were suspended in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) to reduce non-specific binding and then incubated with increasing concentrations of laminin at 4°C for 30 min. Radiolabeled laminin was separated from bound ^{125}I-laminin by layering the parasites on an oil gradient consisting of one part bisphthalate and one and a half parts dibutyl phthalate (Kodak Eastman Rochester, NY, USA). Non-specific binding was determined in the presence of a 100-fold excess of unlabeled ligand and was <15% of the total binding.

To assess the influence of glycosylation of laminin on parasite binding, ^{125}I-laminin (1.2 × 10^6 cpm/μg) was treated at 20°C for 16 h with 20 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 5.0), and then tested for parasite binding. Similarly, the influence of sialylation of laminin on parasite binding was assessed after 1 μg of ^{125}I-laminin had been incubated with sialidase (2 U) in 10 mM sodium acetate buffer (pH 5.0) for 12 h. To assess the effect of temperature on laminin binding, parasite suspensions (10^8 cells in 10 μl PBS) were treated in a water bath at either 20°C for 60 min or 80°C for 10 min before use in the binding assay. The involvement of surface proteins in laminin binding was assessed by treating parasite suspensions with trypsin (50 μg/ml), proteinase K (5 μg/ml) and pronase E (5 μg/ml) at 37°C for 1 h.

Isolation of LBP—Outer membrane proteins were isolated by biotinylation and streptavidin-agarose extraction. *L. donovani* promastigotes (2 × 10^8) were incubated at 22°C for 10 min with 100 μg of sulfo-NHS biotin (Pierce, Rockford, IL, USA). The cells were then washed and lysed in 1 ml lysis buffer [5 mM Tris-HCl, pH 7.5, containing 0.5% Triton X-100, 25 mM KCl, 5 mM $MgCl_2$, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 50 μg/ml soybean trypsin inhibitor, and 10 μg/ml phenyl methyl sulfonyl fluoride (PMSF)]. The cells were then centrifuged at 12,000 ×*g* for 30 min at 4°C, the supernatant was absorbed onto a streptavidin-agarose column (1 ml; Pierce), and then membrane proteins were eluted with 25 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 30 mM β-octylglucoside.

Membrane proteins were first passed through a DEAE-cellulose column (1 cm × 10 cm), previously equilibrated with buffer I (50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.5 mM PMSF, and 25 U/ml aprotinin). Bound proteins were eluted with 100 ml of a linear gradient of 0–400 mM NaCl in buffer I. The eluate was then passed through a Con A–Sepharose column previously equilibrated with buffer II (10 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, and 0.1% Nonidet P40) and eluted with buffer II containing 1 M α-methyl-D-mannopyranoside. The purified LBP was obtained by mixing the eluate with an equal volume of laminin-Sepharose [prepared by coupling laminin (25 μg) with 100 μl of cyanogen bromide–activated Sepharose CL-4B] and incubated for 16 h at 4°C. The bound protein was eluted with 2 M glycine, dialyzed against 10 mM Tris-HCl, pH 7.4, and stored at −70°C.

Anti-LBP Antibody—A polyclonal antibody to the LBP was raised by intraperitoneal injection of 20 μg LBP emulsified in complete Freund's adjuvant into a male New Zealand rabbit. Three booster doses were administered at intervals of 2 weeks by injecting LBP emulsified in incomplete Freund's adjuvant. At 10 days after the fourth injection blood was collected from the rabbits ear and the anti-LBP antibodies separated according to Hall *et al.* (*15*).

Immunoprecipitation—Metabolically (^{35}S-methionine) labeled *L. donovani* promastigotes (2 × 10^6) were lysed in 50 mM Tris-HCl, pH 7.5, containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A (lysis buffer) under ice cold conditions according to Nandan and Reiner (*16*). The lysate was centrifuged, and anti-LBP IgG (1: 250 dilution) was added to the clear supernatant, which was then kept at 4°C for 18 h. Protein A–agarose was added and the mixture was incubated with shaking for another 2 h at 4°C. After washing with lysis buffer, agarose beads were boiled in SDS-sample buffer for 2 min to release the immune complexes. The released proteins were then electrophoresed under reducing conditions in a 7.5% SDS-PAGE gel. In control experiments, instead of the anti-LBP antibody, preimmune sera were used. After electrophoresis the gels were dried and exposed to X-Omat RP film (Eastman Kodak Rochester, NY, USA) at −70°C for autoradiography.

Electrophoresis and Immunoblotting—The promastigote membrane proteins were resolved by 7.5% SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes (0.45 μm; Scheleicher and Schuell, Keene, NH, USA). The residual binding sites were blocked by incubation with 5% non-fat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine for 30 min at room temperature with gentle shaking. The membranes were washed for 5 min each with 20 mM Tris-HCl, pH 7.4–50 mM NaCl

(TBS) containing 0.1% Nonidet P40 (TBSN), and then incubated for 1 h at 37°C with laminin (50 μg/ml) in TBS supplemented with 1% BSA (TBS-BSA). After washing with TBSN, the membranes were treated with anti-laminin antibodies in TBS-BSA at 37°C for 30 min, followed by another round of washing and incubation with alkaline phosphatase–conjugated goat anti-rabbit IgG $F(ab')_2$ (Sigma Chemical St. Louis, MO, USA) at 1: 500 dilution in TBS-BSA. The protein bands were developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-indolyl phosphate in 50 mM Tris-HCl, pH 9.5, containing 150 mM NaCl and 5 mM $MgCl_2$ (*17*).

Immunofluorescence—Immunofluorescence was used to visualize the localization of LBP on *L. donovani* promastigotes. Thoroughly washed promastigotes (2×10^7) were incubated with 100 μl of rabbit anti-LBP antibodies at a dilution 1: 50 in PBS containing 0.5% BSA. After 1 h at 22°C, the cells were washed in PBS and then incubated for 30 min at 37°C in 100 μl of fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA, USA) at 1:50 dilution in PBS containing 0.5% BSA. After several washings, the cells were placed on glass slides, air dried, mounted in glycerol: PBS (9: 1, by volume), and then observed under a Leitz Ortholux Microscope equipped for epifluorescence.

Flow Cytometric Analysis—Thoroughly washed promastigotes (1×10^7) were first treated with PBS containing 2% goat serum for 1 h at 22°C. After washing, the cells were subjected to treatment with anti-LBP antibodies and FITC-conjugated goat anti-rabbit IgG as described under Immunofluorescence. After several washings, the cells were suspended in PBS containing 1% paraformaldehyde and then analyzed with a FACSCalibur cytofluorometer using the CellQuest software (BD Biosciences, San Jose, CA, USA). The area of positivity was determined using preimmunized serum.

Cell Adhesion and Spreading—Cell adhesion was assayed according to Bauvois *et al.* (*18*) by coating 24-well micro titer plates at 37°C overnight with 100 μl of laminin (100 μg/ml) in PBS. After washing, non-specific sites were blocked by incubation with 100 μl of 1% BSA in PBS for 1 h at 37°C. U937 cells (2×10^5 in 100 μl) were added to substrate coated wells and then incubated for 90 min at 37°C in 5% CO_2, 95% air. U937 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 1 mM pyruvate, and 50 μg/ml gentamycin. Cells were also added to wells that had been coated with BSA alone. After the incubation, the wells were gently washed three times with PBS to remove unattached cells. Attached cells were trypsinized and then counted with a hemocytometer. The number of bound cells/total number of cells × 100 was determined as % cell adhesion. For attachment inhibition studies, wells were coated with laminin as above. Prior to the addition of U937 cells, various concentrations of affinity-purified binding protein or anti-laminin antiserum in PBS containing 0.05% BSA were added to the wells. The attachment assay was then carried out as above.

Solid Phase Adhesion Assay—Micro titer wells were coated with 50 μl of laminin (100 μg/ml) and then blocked with BSA. To the wells, ^{125}I-labeled parasites (5×10^5 parasites/ml) were added and allowed to incubate for 60 min at 22°C. The wells were then washed extensively with PBS containing 0.1% Tween 20 and the radioactivity was measured. All readings were corrected for background values, which represented radioactivity recovered in wells coated with BSA alone.

Tunicamycin Treatment—L. donovani promastigotes were treated for 24 h with tunicamycin (solubilized in DMSO) at a final concentration of 5 μg/ml.

In Vitro Assaying of L. donovani Growth in Macrophages—Promastigotes were used to infect cultures of adherent macrophages on glass cover slips (18 mm^2; 5×10^5 macrophages/cover slip) in 0.5 ml of RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, at a ratio of 10 parasites/macrophage. In the incubation medium different doses of laminin, LBP or anti-LBP antibodies were also included. Infection was allowed to proceed for 4 h, unphagocytized parasites were removed by washing with the medium and the cells were placed in the medium for 72 h. The cells were fixed in methanol and then stained with Giemsa for determination of the intracellular parasite number. At least 300 cells were counted on each cover slip and the numbers of parasites per 100 infected cells were determined.

Effects of Various Agents on In Vivo L. donovani Infection—To investigate the influence of laminin in BALB/c mouse infection, 6-wk-old mice (~20 g body weight) were divided in four groups of six animals each. Three groups were intravenously administered 2×10^6 viable promastigotes/mouse in the presence of either laminin (50 μg), LBP (10 μg), or anti-LBP antibodies (50 μg). The fourth group served as an infected control, which received only parasites. After the indicated periods of infection, the animals were sacrificed, and multiple spleen impression smears were prepared and stained with Giemsa. The spleen parasite burden expressed as Leishman-Donovan units (LDU) was calculated as the number of amastigotes per 1000 nucleated cells × spleen weight in grams (*19*).

RESULTS

Characterization of Laminin Binding by L. donovani—Both the promastigote and amastigote forms of *L. donovani* bound ^{125}I-laminin, although to different extents, in a time-dependent manner (data not shown). The binding of ^{125}I-laminin by both forms was rapid and saturable, maximum binding being attained at 60 min. Prolonged incubation for up to 3 h did not result in additional binding of ^{125}I-laminin. Quantification of bound laminin as a function of increasing concentration of radiolabeled protein showed that the binding of both forms was saturable (Fig. 1), and indicated that the parasites contain a limited number of laminin receptors. Scatchard plot analysis (Fig. 1, A and B, inset) of the binding data yielded a binding constant K_d of 3.56 nM for promastigotes and one of 3.98 nM for amastigotes. Assuming that the molecular mass of laminin is about 850 kDa, there are about 9,000 binding sites on promastigotes and 800 sites on amastigotes with the aforementioned binding constants.

The inhibitory effects of various proteins on the binding of ^{125}I-laminin by promastigotes are shown in Table I. All assays were performed in the presence of 0.1% BSA to minimize any possible contribution of non-specific protein-protein interactions. Of the proteins tested, only fibronectin and ovalbumin did induce some inhibition ($p < 0.02$) of binding. Since the only common chemical property of fibro-

nectin, ovalbumin and laminin is that they are glycoproteins, this partial inhibition of laminin binding by fibronectin and ovalbumin may reflect the importance of protein glycosylation in binding. However, sialic acid may not be involved in laminin binding as the preincubation of both fetuin and asialofetuin resulted in no significant inhibition. Unlabeled laminin caused the greatest and most significant inhibition ($p < 0.001$) of laminin binding, thereby confirming the specificity of promastigote binding.

Table II shows the effects of treatment of laminin as well as promastigotes with various agents before determination of binding to *L. donovani* promastigotes. The decreased binding after periodate treatment supports the importance of carbohydrates in laminin binding. Consistent with this, the recombinant B1 chain of human laminin, which contains only high mannose oligosaccharides, markedly inhibited the binding (Table I). The inability of sialidase pretreatment to decrease the binding further suggests that sialic acid may not be involved in laminin binding. The binding of laminin was greatly inhibited by heating *L. donovani* promastigotes and also by treating cells with proteolytic enzymes (Table II), suggesting the involvement of a protein receptor in the binding of laminin.

Identification and Purification of Laminin Binding Protein—Indirect immunoblotting of the outer membrane proteins of both promastigotes and amastigotes gave a 67 kDa protein band, with laminin as the primary probe, followed by treatment with anti-laminin antibodies and alkaline phosphatase-conjugated secondary antibodies (Fig. 2, lanes 1 and 2). The control nitrocellulose strip (lane 3), which was not subjected to laminin treatment, did not show any band, suggesting the specificity of the reaction. Also, autoradiography of the promastigote membrane proteins after binding to ^{125}I-laminin revealed a 67 kDa band (lane 4), confirming that radioiodination of the glycoprotein did not cause steric or conformational changes affecting binding. Anti-LBP antiserum and protein A–Sepharose beads were used to selectively precipitate out LBP from metabolically (^{35}S-methionine) labeled *L. donovani* promastigotes. The same 67 kDa band was observed when the immunocomplexes were dissociated, subjected to SDS PAGE and autoradiographed (lane 5). To isolate the laminin-binding component, *L. donovani* promastigote membrane proteins were subjected to a three-step purification procedure involving DEAE-Cellulose, Con A–Sepharose, and a laminin-Sepharose affinity chromatography. Silver staining of the electrophoretically separated proteins showed that the material bound at the final laminin-Sepharose step and subsequently was eluted with 2 M glycine as a single band corresponding to a molecular mass of 67 kDa (Fig. 3).

TABLE I. **Inhibition of ^{125}I-laminin binding to *L. donovani* promastigotes by various proteins.**

Inhibitor	cpm bound[a]	Laminin bound (ng)[b]
None	22,465 ± 3,050	7.5 ± 1.0
Gamma globulin	21,395 ± 2,925	7.2 ± 1.0
Collagen type I	21,433 ± 2,135	7.2 ± 0.7
Collagen type IV	22,165 ± 2,745	7.4 ± 0.9
Fetuin	21,267 ± 1,575	7.1 ± 0.5
Asialofetuin	20,968 ± 2,725	7.0 ± 0.9
Ovalbumin	12,348 ± 1,220	4.1 ± 0.4*
Fibronectin	14,596 ± 1,830	4.8 ± 0.6*
Laminin	3,724 ± 1,005	1.2 ± 0.3**
Laminin B1	4,268 ± 1,193	1.4 ± 0.4**

[a]Unlabeled competitors were used at a final concentration of 1 mg/ml. [b]The amount of ^{125}I-labeled laminin per 10^6 cells. Data represent means of three determinations ± standard deviation. Values include the significance (*$p < 0.02$; **$p < 0.001$) of the difference between inhibition in the presence and absence of inhibitors as determined by analysis of variance.

TABLE II. **Effect of modification of the receptor or ligand on binding of ^{125}I-laminin.**

A. Effect of treatment of *L. donovani* on binding of ^{125}I-laminin.

Treatment	% inhibition[a]
None	0 ± 2
Heat (100°C for 5 min)	83 ± 5**
Trypsin	97 ± 4**
Proteinase K	65 ± 5**
Pronase E	85 ± 6**
α-Mannosidase	58 ± 3**

B. Modification of the ligand by various treatments.

Compound	% inhibition[a]
Control (native laminin)	0 ± 3
Periodate-treated laminin	72 ± 3**
Sialidase-treated laminin	17 ± 6*

[a]Means of three determinations ± standard deviation. Values include the significance (*$p < 0.02$; **$p < 0.001$) of the difference between inhibition in the presence and absence of inhibitors as determined by analysis of variance.

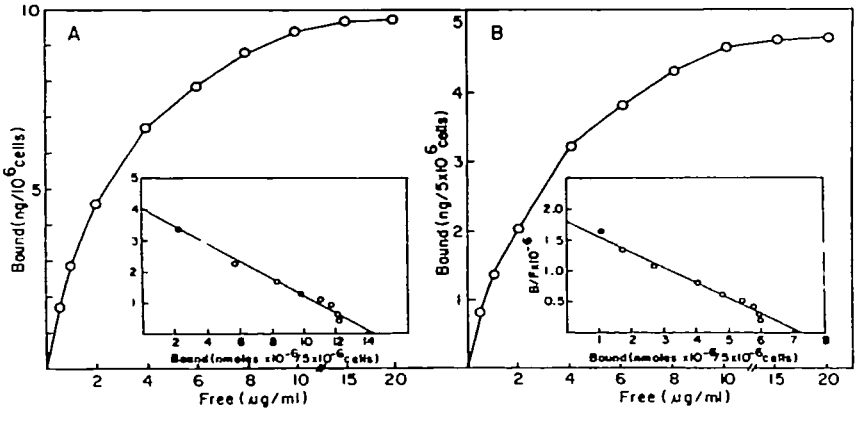

Fig. 1. **Radiolabeled laminin binding to *L. donovani* parasites.** Increasing amounts of radiolabeled laminin (1.2×10^6 cpm/μg) were added to a constant number of promastigotes (1×10^6) (A) and amastigotes (5×10^6) (B). Parasites were incubated for 30 min at 4°C in a volume of 0.2 ml and washed three times. The amount of ^{125}I-labeled laminin bound to the parasites was determined. Non-specific binding (*i.e.* binding in the presence of a 100-fold molar excess of unlabeled laminin) was subtracted from the total. A Scatchard plot (inset) was constructed from the specific binding data, yielding binding constants (K_d) of 3.56×10^{-9} M for promastigotes (A, inset) and 3.98×10^{-9} M for amastigotes (B, inset), and a stoichiometry of ~9,000 and ~800 receptors per cell for promastigotes and amastigotes, respectively. Data represent duplicate determinations in three separate experiments.

Visualization of the Binding of Laminin to L. donovani—In order to examine cell surface localization of the laminin receptor, immunofluorescence and flow cytometric studies were performed with mono specific antibodies directed against affinity-purified LBP. Treatment of *L. donovani* promastigotes first with anti-LBP antibodies followed by probing with FITC conjugated goat anti-rabbit antibodies revealed strong and homogeneous fluorescence on their surface (Fig. 4A). On the other hand, parasites treated with irrelevant immune serum in place of anti-LBP antibodies did not show any fluorescence. Similar results were obtained on flow cytometric analysis, where preimmunized serum was used as a control (Fig. 4B).

Effect of LBP on Cell Adhesion—The presence of LBP on

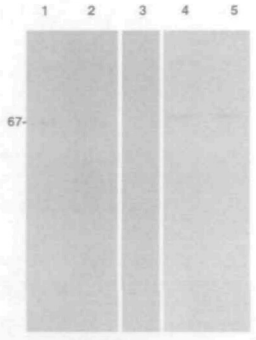

Fig. 2. **Presence of 67 kDa LBP on both the promastigote and amastigote forms of *L. donovani*.** *L. donovani* promastigote (lane 1) and amastigote (lane 2) membrane proteins, isolated by biotinylation and streptavidin-agarose extraction, were resolved under denaturing conditions in a 7.5% SDS-PAGE gel and then transferred to a nitrocellulose membrane. The transferred proteins were subjected to indirect immunoblot analysis with laminin as the primary probe, followed by treatment with rabbit anti-laminin IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG. Lane 3 was incubated with BSA instead of laminin. Promastigote membrane proteins transferred to a nitrocellulose membrane were incubated with radiolabeled laminin and then subjected to autoradiography (lane 4). In lane 5, promastigotes were metabolically labeled with [^{35}S]methionine and lysed, and LBP was immunoprecipitated with anti-LBP antibodies and autoradiographed. The molecular mass is indicated to the left of the panel.

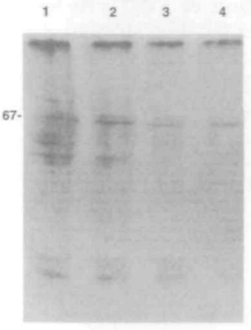

Fig. 3. **Purification profile of LBP from *L. donovani* promastigotes on SDS-PAGE.** *L. donovani* membrane proteins isolated by biotinylation and streptavidin-agarose extraction were passed through various chromatographic columns. Membrane proteins (lane 1) as well as the eluates from DEAE-cellulose (lane 2), Con A–Sepharose (lane 3) and laminin-Sepharose (lane 4) columns were analysed by 7.5% SDS-PAGE under reducing conditions and then silver stained. The molecular mass is indicated to the left of the panel.

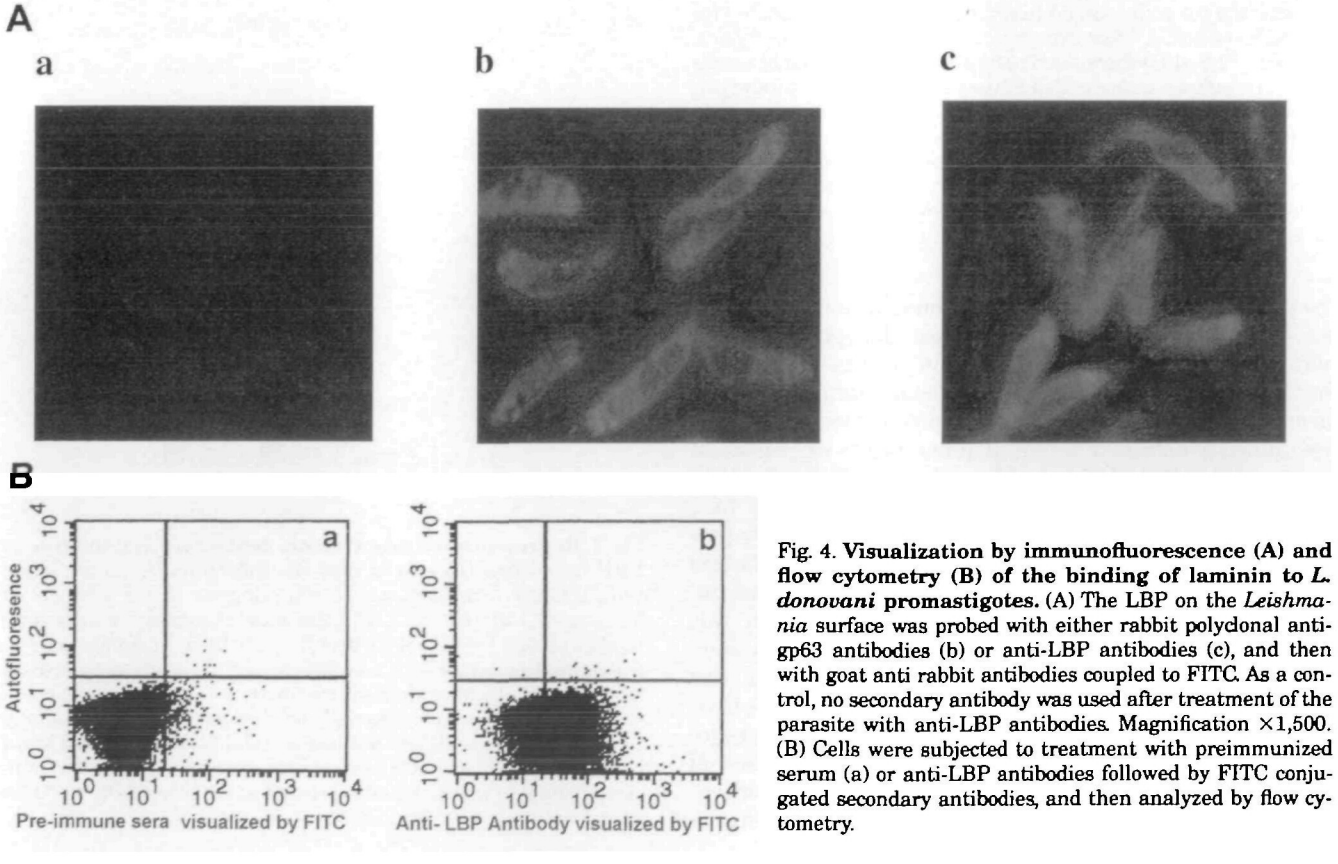

Fig. 4. **Visualization by immunofluorescence (A) and flow cytometry (B) of the binding of laminin to *L. donovani* promastigotes.** (A) The LBP on the *Leishmania* surface was probed with either rabbit polyclonal anti-gp63 antibodies (b) or anti-LBP antibodies (c), and then with goat anti rabbit antibodies coupled to FITC. As a control, no secondary antibody was used after treatment of the parasite with anti-LBP antibodies. Magnification ×1,500. (B) Cells were subjected to treatment with preimmunized serum (a) or anti-LBP antibodies followed by FITC conjugated secondary antibodies, and then analyzed by flow cytometry.

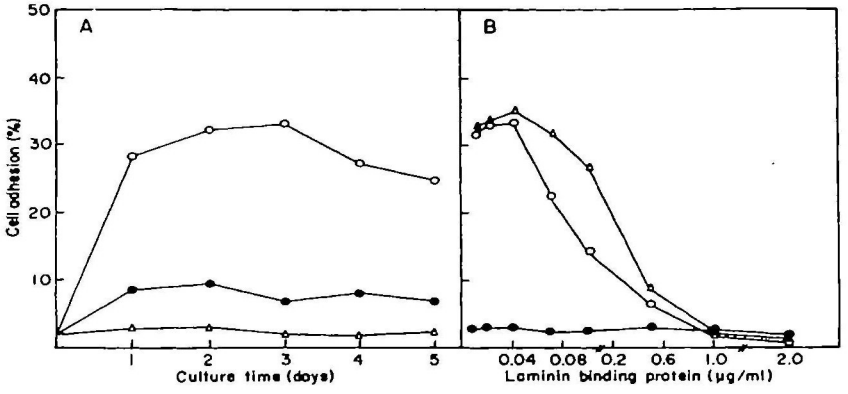

Fig. 5. **(A) Time course of U937 cell adhesion to laminin-coated surfaces after stimulation with PMA.** 24-well micro titer plates were coated with laminin (10 μg/well) or BSA (50 μg/ml) as described under "MATERIALS AND METHODS." U937 cells stimulated with 1 ng/ml PMA at 37°C for various times up to 5 days were added to the protein-coated wells and then the plates were incubated at 37°C for 90 min. Attached cells were quantitated as described under "MATERIALS AND METHODS." △, adhesion of unstimulated cells to laminin; ○, adhesion of PMA stimulated cells to laminin; ●, adhesion of stimulated cells to BSA. **(B) Inhibition of U937 cell adhesion to laminin by LBP.** The adhesion of unstimulated (●), day 1 PMA-stimulated (△), and day 3 PMA-stimulated (○) U937 cells to laminin (10 μg/well) was examined in the presence and absence of different concentrations of affinity purified binding protein as described under "MATERIALS AND METHODS." Data represent the means of three separate experiments.

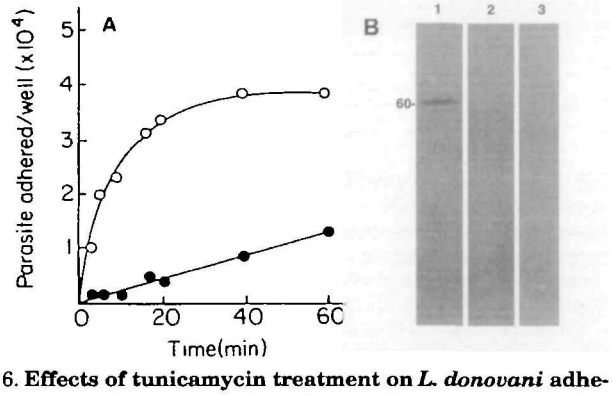

Fig. 6. **Effects of tunicamycin treatment on *L. donovani* adhesion.** (A) Laminin-coated surfaces (2 μg/well) were overlaid with a suspension of ^{125}I-labeled parasites, 5×10^5 cells, either treated (●) or untreated (○) with 5 μg/ml tunicamycin, and then incubated for the indicated times. After extensive washing of the unbound parasites with PBS, the adherence of parasites was determined by counting with a gamma counter. (B) LBP was purified from tunicamycin-treated cells, and subjected to 7.5% SDS PAGE and then autodradiography (lane 1). The protein was transferred to a nitrocellulose strip and then subjected to indirect immunoblotting with laminin as a primary probe (lane 2), followed by treatment with anti-laminin antibodies and alkaline phosphatase-conjugated second antibodies. BSA was used instead of laminin in lane 3.

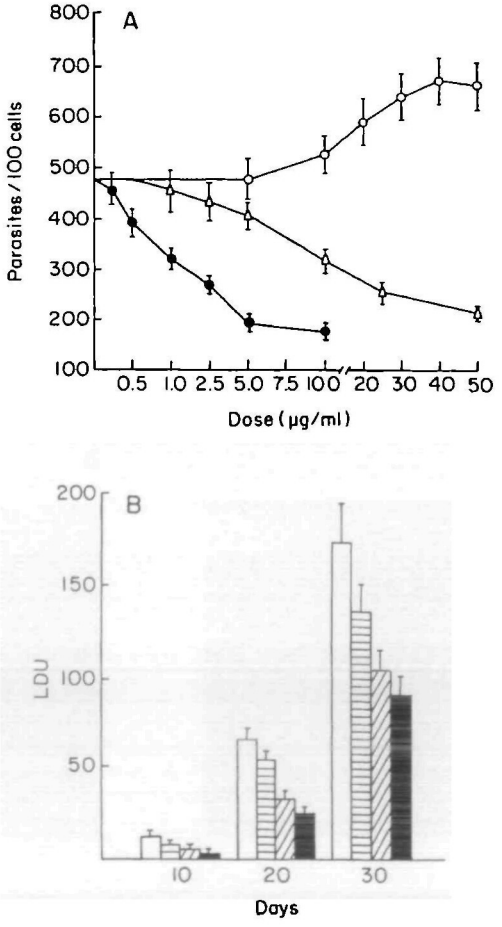

Fig. 7. **Representative experiments demonstrating the role of LBP in *in vitro* (A) and *in vivo* (B) infection.** (A) Macrophages were infected with *L. donovani* promastigotes in the presence of laminin (○), LBP (●), anti-LBP antibodies (△), excess parasites were washed off and the cells were kept for 72 h, then the number of parasites inside macrophages was determined. Data are presented as means ± SD. (B) Mice were infected with 2×10^6 promastigotes in the presence of laminin (diagonally hatched bars), LBP (horizontally hatched bars), or anti-LBP antibodies (solid bars), or nothing (open bars). The spleen parasite burden was determined after the indicated periods of infection and expressed as the mean LDU ± SD for six animals.

the parasite surface as well as the abundance of laminin in the extracellular matrix suggest that this protein might play a role in cell adhesion. Adhesion studies were, therefore, carried out using cells as well as laminin-coated substrata. U937 cells, a human macrophage monocyte cell line, are known to exhibit differential adherence to various ECM proteins after stimulation by phorbol esters. Cells (5×10^5/ml) were cultured in the presence of 1 ng/ml PMA for various times (up to 5 days). PMA stimulation significantly promoted U937 cell adhesion to laminin (almost 30% adhesion after 24 h compared to <2% adhesion in the case of unstimulated cells) (Fig. 5A). This increased adhesion was found to be inhibited by the affinity-purified LBP in a dose-dependent manner (Fig. 5B). Almost total inhibition was observed with a dose of 1 μg/ml. This inhibition seems to be specific because in the case of PMA-stimulated adherence to fibronectin, used as a positive control, no inhibition of U937 cell adherence to fibronectin was noted with concentrations of up to 2 μg/ml of LBP. In adhesion studies involv-

ing laminin-containing substrata, radiolabeled parasites were incubated in micro titer wells coated with laminin or BSA. The adhesion of parasites to laminin-coated wells was time-dependent and reached a maximum after 3-h incubation, whereas very few parasites had attached to BSA after 3 h incubation (Fig. 6A). Significant reduction of the adhesion to laminin-coated wells was observed in the case of tunicamycin-treated parasites, suggesting the importance of sugar residues in this interaction. Moreover, silver staining of LBP isolated from tunicamycin-treated *L. donovani* showed a reduced molecular wt. (60 kDa), and the isolated protein showed little response when subjected to indirect immunoblotting with laminin and anti-laminin antibodies (Fig. 6B).

The Role of LBP in Parasite Infectivity—The presence of specific surface receptors for laminin is known to increase the invasiveness of some parasites like *T. vaginalis* and *T. foetus*, and the fungus *P. brasiliensis* after binding to their specific surface receptors (*20, 21*). In order to ascertain the role of LBP in parasite infectivity, *L. donovani* was allowed to infect both a macrophage culture and the BALB/c mouse model in the presence of laminin, LBP, and anti-LBP antibodies. On *in vitro* infection, determination of the number of parasites per 100 macrophage cells demonstrated that infection with parasites pretreated with laminin was significantly higher than that with untreated parasites, whereas the presence of externally added LBP and anti-LBP antibodies did not have much effect on infection (Fig. 7A). On the other hand, a lower level of infection was found in all three cases when either laminin, LBP or anti-LBP antibodies were co-administered (Fig. 7B). The increased infection in the presence of laminin under the *in vitro* conditions may be attributed to the presence of the laminin receptor on macrophages, which acted as a bridging molecule facilitating the host-parasite interaction. In the *in vivo* situation, however, the presence of both laminin and anti-LBP antibodies might mask the navigatory signal for infection through interaction with leishmanial LBP resulting in a lower level of infection. LBP, on the other hand, might act as a competitive inhibitor resulting in lower infection.

DISCUSSION

Extracellular matrix (ECM) binding proteins on the surface of *Leishmania* are thought to play a crucial role in the onset of leishmaniasis, as these parasites invade mononuclear phagocytes in various organs after migrating through ECM. In this direction, we previously identified laminin-binding activity on the surface of *L. donovani* (*4*). Binding of laminin by both the promastigote and amastigote forms of *L. donovani* is rapid, saturable, specific, and of high affinity, and the available numbers of sites for laminin per cell are about 9,000 on promastigotes and 800 on amastigotes. Taking into consideration the larger size of promastigotes, the receptor density appears to be comparable in the two forms. Laminin possesses about 40 amide linked oligosaccharides with repeating units of poly-*N*-acetyllactosaminyl side chains attached to the trimannosyl core portion of bi-, tri-, and tetra-antennary complex-type oligosaccharides (*22, 23*). The inhibition of laminin binding by ovalbumin and fibronectin, but not by fetuin or asialofetuin, indicated that glycosylation other than sialylation was important for binding by *L. donovani* promastigotes. This was supported by the unaltered binding to sialidase-treated laminin, and the reduced binding to periodate-treated laminin and the recombinant B1 chain of laminin, which contains only high-mannose type oligosaccharides. Surface proteins are involved in laminin binding by promatigotes since protease treatment and heating of parasite cells reduced the binding. The reduced binding ability exhibited by promastigotes treated at 80°C for 10 min, a temperature that will cause perturbation rather than denaturation of proteins, suggests that a particular protein conformation is crucial for binding. Moreover, since only outer membrane proteins are biotinylated, the detection of the laminin-binding component in the membrane fraction prepared by biotinylation and streptavidin-agarose extraction also supported the surface location of the receptor.

The 67 kDa LBP was purified from the promastigote membrane fraction by a three-step procedure involving DEAE-cellulose, Con A–Sepharose, and laminin-Sepharose affinity chromatography. In addition to the detection of the 67 kDa protein in the biotinylated membrane fraction with an anti-LBP antiserum, the surface localization of the receptor was further highlighted on immunofluorescence microscopy and flow cytometry. *L. donovani* promastigotes showed strong fluorescence when treated with anti-LBP antibodies followed by FITC conjugated goat anti rabbit antibodies. This protein may be similar to the previously described laminin receptor, which is present on many cells (*7, 9, 24, 25*) and is involved in cell adhesion to laminin through the Tyr-Ile-Gly-Ser-Arg site on the B1 chain of laminin (*26*). It has been shown that the recognition of laminin may influence the pathogenesis of several microorganisms, and receptors have been identified in various species of bacteria (*8, 27, 28*), parasites (*19, 29, 30*), and fungi (*6, 9, 31*).

The presence of LBP on the *L. donovani* promastigote plasma membrane suggests that this molecule could mediate the initial contact between the parasite and the ECM network, and could participate in the invasive process as well. U937 cell adhesion-inhibition studies performed in the presence of affinity-purified LBP point to this, although a relatively high concentration (1 μg/ml) was needed for complete inhibition. This was further substantiated by parasite adhesion–inhibition studies in the presence of both LBP and anti-LBP antibodies. Moreover, the presence of laminin bound to the parasite surface appears to facilitate the infectivity in a macrophage culture, whereas both laminin and anti-LBP antibodies decreased the infectivity in the BALB/c mouse model, apparently by interacting with LBP on the cell surface. These findings constitute evidence in support of the hypothesis that *Leishmania* migration through the ECM network prior to macrophage attachment and entry is mediated in part by the interaction of laminin with parasite laminin receptors. On the other hand, since both LBP and anti-LBP antibody co-administration resulted in only a partial reduction of the ability of parasites to infect host cells compared to an infection control, it is likely that laminin is not the only ligand involved in the laminin–ECM interaction. It may be mentioned in this regard that potential roles for heparin in parasite–ECM interactions have also been suggested (*32*). Indeed, it seems likely that infection may depend upon complex interactions of several ligand-receptor systems on parasites and the ECM network. It is possible, however, that *in vivo*, *Leishmania*

attachment to macrophages might be mediated through bidirectional interactions involving laminin. Parasites might adhere to ECM laminin, which in turn, might associate with the host cells through the laminin receptor present on macrophages.

In conclusion, this study provides evidence that LBP, a cell surface protein of *L. donovani*, serves as a ligand that mediates the adhesion and invasion of host cells. In addition, the heavier levels of infection in macrophage cultures with laminin-coated parasites and the reduced infection in BALB/c mice in the presence of laminin, LBP and anti-LBP antibodies suggest that LBP may function as one of the bridging molecules for ECM recognition. Further studies at the molecular and biochemical levels will provide more insights into the structure and function of this protein and its possible role in the homing of the parasites to their physiological address.

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