OmpL1 is an extracellular matrix- and plasminogen- interacting protein of *Leptospira* spp.

Luis G. V. Fernandes¹,²; Monica L. Vieira¹,²; Karin Kirchgatter³; Ivy J. Alves¹; Zenaide M. de Morais⁴; Silvio A. Vasconcellos⁴; Eliete C. Romero⁵ and Ana L. T. O. Nascimento¹*

¹Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil; ²Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, São Paulo, Brazil; ³Núcleo de Estudos em Malária, Superintendência de Controle de Endemias (SUCEN)/Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, São Paulo, Brazil; ⁴Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, Brazil; ⁵Divisão de Biologia Médica, Instituto Adolfo Lutz, Sao Paulo, Brazil.

*To whom correspondence should be addressed. Telephone: (5511) 37220019; fax: (5511) 37261505; e-mail: tabet@butantan.gov.br.

Running title: OmpL1 binds ECM and PLG

2012
Leptospirosis is a zoonosis of multisystem involvement caused by pathogenic strains of the genus *Leptospira*. OmpL1 is an outer membrane protein of *Leptospira* spp. that is expressed during infection. In this work we investigated novel features of this protein. We describe that OmpL1 is a novel leptospiral extracellular matrix (ECM)-binding protein and a plasminogen (PLG) receptor. The recombinant protein was expressed in *E. coli* BL21 (DE3) Star pLysS, as inclusion bodies, refolded and purified in metal chelating chromatography. The protein presented a typical β-strands secondary structure as evaluated by circular dichroism spectroscopy. The recombinant protein reacted with antibodies in serum samples of convalescent leptospirosis patients with high specificity when compared to serum samples from individuals with unrelated diseases. These data strengthen the usefulness of OmpL1 as a diagnostic marker of leptospirosis. Characterization of recombinant OmpL1 immunogenicity in inoculated BALB/c mice showed that the protein has the capacity to elicit humoral and cellular immune responses, as denoted by high antibody titers and proliferation of lymphocytes. We demonstrate that OmpL1 has the ability to mediate the attachment to laminin and plasma fibronectin, with a $K_D$ value of $2099.93 \pm 871.03$ nM and $1239.23 \pm 506.85$ nM, respectively. OmpL1 is also a PLG- receptor, with a $K_D$ of $368.63 \pm 121.23$ nM, capable of generating enzymatically active plasmin. This is the first report that shows and characterizes OmpL1 as an ECM- interacting and a PLG-binding protein of *Leptospira* spp. that being expressed during infection may play a role in the bacterial pathogenesis.
INTRODUCTION

Leptospirosis is been considered an important re-emerging infectious disease. It is a zoonosis caused by pathogenic *Leptospira* spp. that is transmitted from reservoir hosts to humans through water and soil contaminated with their urine (18). Symptoms of the disease include fever, vomiting, headache, diarrhea, abdominal and generalized muscle pain. Progression to multi-organ system complications, known as Weil’s syndrome, occurs in 5-15% of cases, with mortality rates of 5-40% (18, 31, 35, 56).

At present, available vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. They confer protective responses mostly through the induction of antibodies against leptospiral lipopolysaccharide (1, 14). These vaccines, however, do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation (1). Due to the large number of leptospiral serovars (8), conserved and protective antigens are being pursued.

Surface exposed proteins are potential targets for inducing immune responses during infection and may also mediate the initial adhesion process to host cells. Indeed, several of these proteins have been reported as leptospiral adhesins (2, 4, 12, 29, 45, 50). Furthermore, well conserved outer membrane proteins (OMPs) are promising vaccine targets, because they would have the advantage in inducing cross-protective immunity (32). After adherence, pathogens have to overcome host tissue barriers to reach blood circulation and organs. We have reported that leptospires bind PLG at their surface and that proteolytic activity is achieved due to plasmin generation (78).
OmpL1 is a 31 kDa leptospiral transmembrane OMP, containing 320 amino acid residues, characterized by Haake and colleagues (24). Several studies have pointed out the importance of OmpL1 as a serological marker for the diagnosis of human (17, 21, 48, 57, 68, 79) and canine leptospirosis (30, 49).

This work describes the expression and characterization of recombinant OmpL1, the immune response induced in BALB/c mice and the reactivity of this protein with leptospirosis positive serum samples. We also describe the ability of OmpL1 to mediate the attachment to various extracellular matrix (ECM) and serum components. We report that OmpL1 is a novel surface adhesin that binds to laminin and plasma fibronectin, interacts with PLG, is expressed during infection (6) and may participate in the leptospiroid pathogenesis.
MATERIALS AND METHODS

ECM and biological components. Macromolecules, including the control protein fetuin, were purchased from Sigma Aldrich. (St. Louis, Mo., USA). Laminin-1 and collagen Type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was derived from human foreskin fibroblasts, plasma fibronectin, human complement serum and fibrinogen were isolated from human plasma and collagen Type I was isolated from rat tail. Plasminogen native, purified from human plasma, and human factor H were purchased from EMD Chemicals, Inc. (San Diego, CA, USA). C4bp was from Complement Technology, INC. (Tyler, TX, USA).

Bacteria strains and serum samples. The pathogenic high passage non-virulent *Leptospira* strains used were *L. interrogans* serovar Canicola strain Hound Utrech IV, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona strain Pomona, *L. borgpetersenii* serovar Castellonis strain Castellon 3, *L. borgpetersenii* serovar Whitcombi strain Whitcomb, *L. kirshneri* serovar Grippotyphosa strain Moskva V, *L. kirshneri* serovar Cynoptery strain 3522C, *L. santarosai* serovar Shermani strain 1342 K, *L. noguchii* serovar Panama strain CZ 214 and the non-pathogenic *L. biflexa* serovar Patoc strain Patoc. The leptospires were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco®, BD, Franklin Lakes, NJ ,USA) with 10% rabbit serum, enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%), calcium chloride (wt/vol:0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol:0.03%) and meat extract (wt/vol: 0.02%) (70). Low passage, virulent *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 and *L. interrogans* serovar
Kennewicki strain Pomona Fromm were also used. Recently weaned male Golden Syrian hamsters were intraperitonially infected with 500 μL of approximately $1 \times 10^4$ virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm. The animals were bled from the retro-orbital plexus after the appearance of symptoms, such as loss of weight and mobility (approximately 5 days post-infection). *Leptospira* cultures were maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Serum samples of confirmed leptospirosis hamsters were obtained by experimental infection with virulent leptospires. Confirmed leptospirosis human serum samples were from Instituto Adolfo Lutz collection, São Paulo, Brazil. Serum samples from patients with unrelated infectious diseases were obtained from serum collection of Laboratorio Imunopatologia, SUCEN, SP, Brazil. *E. coli* DH5α was used as the cloning host strain and *E. coli* BL21 (DE3) Star pLysS (67) was used as the host strain for the expression of the recombinant protein.

**In silico sequence analysis.** All sequences available in GeneBank were employed to study conservation and identity of OmpL1 using CLUSTAL 2.1 multiple sequence alignment at [http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/) (34).

**Cloning, expression and purification of recombinant OmpL1.** Amplification of the OmpL1 gene was performed by PCR from *L. interrogans* serovar Copenhageni strain M20 genomic DNA using the primer pairs F: 5´GGATCCAAAACATATGCAATTGTAGG 3´ and R: 5´GGTACCTTAGAGTTCGTGTTTATAACC 3´. The PCR reaction was performed with an initial denaturation step of 94°C for 5 min, 35 cycles (94°C for 50 seconds; 60°C for 50 seconds and 72°C for 2 min) and a final step of 72°C for 7 min. Reaction mix contained 1X PCR buffer, 0.2mM dNTP,
2mM MgCl$_2$, 0.2mM of each primer (F and R); 2.5U Taq polymerase and 100ng of genomic DNA for a final volume of 25 µL. Gene sequence was amplified without the signal peptide tag, which was determined by software SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). PCR fragment was cloned into pGEM-T easy vector (Promega, Prodimal, MG, Brazil) and subcloned into the E. coli expression vector pAE (58) at BamHI and KpnI restriction sites. The plasmid pAE-OmpL1, containing the correct DNA sequence, was used to transform E. coli BL21 (DE3) Star pLysS. Protein expression was obtained by inoculating 10 mL of a culture grown overnight in 800 mL of Luria-Bertani medium containing 50µg/mL ampicillin and 34µg/mL chloranfenicol. The culture was grown with continuous shaking at 37 ºC to an optical density 0.6 at 600 nm and then induced for 3 h under constant agitation at 37 ºC in the presence of 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The cells were harvested by centrifugation, the bacterial pellet was resuspended in sonication buffer (20mM Tris-HCl, pH 8.0, 200mM NaCl, 200 mg/mL lysozyme, 2mM PMSF and 1% Triton X-100) and lysed on ice with the aid of a sonicator tip (Ultrasonic Processor, GE Healthcare Bio-Sciences Corp, Piscataway, NJ,USA). The insoluble fraction was resuspended in a buffer containing 20mM Tris-HCl, pH 8.0, 500 mM NaCl, 8M urea and 1mM β-mercaptoethanol. The protein was purified through Ni$^{2+}$-charged beads of chelating fast-flow chromatographic column (GE Healthcare), extensively washed with buffer containing 20mM Tris-HCl, pH 8.0, 500mM NaCl and increasing imidazole concentrations (5-60mM). Bound protein was eluted with buffer containing 1M imidazole. The efficiency of the purification was evaluated by 12% SDS-PAGE. Fractions containing the recombinant protein were extensively dialyzed against PBS (phosphate-buffered saline pH 7.4) containing 0.1% glycine for 24 h.
Circular Dichroism spectroscopy. Purified recombinant protein was dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm-path-length cell at 0.5 nm intervals. The spectra were presented as an average of five scans recorded from 185 to 260 nm. The residual molar ellipticity is expressed in degree X centimeter per decimole. Spectra data was submitted to K2D2 software, http://ogic.ca/projects/k2d2, using the method that calculated the secondary structure content from the ellipticity experimental data (53).

Microscopic agglutination test (MAT). The microscopic agglutination test was performed according to Faine et al. (19). In brief, 22 serovars of Leptospira spp. were employed as antigens: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolffi. All the strains were maintained in EMJH liquid medium at 29 °C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50 % of agglutination. MAT was considered negative when the titer was below 100.

Antiserum production against recombinant protein and isotype determination. The recombinant protein was mixed with 10 % (vol/vol) of Alhydrogel (2 % Al(OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Ten micrograms of OmpL1 were subcutaneously administered in five female BALB/c mice (4-6 weeks old). Two subsequent booster injections were
given at two-week intervals with the same protein preparation. Negative control mice were injected
with PBS. Two weeks after each immunization, the mice were bled from the retro-orbital plexus and
the resulting sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination
of antibody titers. Prior to experiments, anti-recombinant protein sera were adsorbed to a suspension
of \textit{E. coli} to avoid the reactivity with anti-\textit{E. coli} antibodies (23). For isotype determination, total
IgG (immunoglobulin G), IgG1 and IgG2a (Southern Biotech, Birmingham, Alabama, USA) were
measured by incubation of the pooled mice sera with recombinant protein followed by incubation
with horseradish peroxidase (HRP)-conjugated anti-mouse total IgG or goat anti-mouse IgG1 or
IgG2a (1:2,000) followed by incubation with HRP-conjugated anti-goat IgG (1:10,000). The OD492
nm values exhibited by different dilution of mice sera were compared to a curve generated by
coating the plates with different concentrations of mice total IgG, IgG1 or IgG2a. IgM and IgA titers
were also determined. In brief, plates were coated with recombinant protein (250ng/well), anti-
OmpL1 sera and anti-PBS sera (negative control) were previously diluted (1:200-1:409600),
transferred to the OmpL1-coated wells and incubated for 1h at 37 °C. Plates were washed and goat
HRP-conjugated anti-mouse IgA (1:5,000) (Sigma) was added and incubated as described above.
For IgM determination, plates were incubated with goat anti-mouse IgM (1:5000) (Sigma) followed
by incubation with rabbit HRP-anti-goat IgG (1:50000) (Sigma). The titers were considered as the
maximum dilution that presented OD492nm values above 0.1.

\textbf{Ethics Statement}. All animal studies were approved by the Ethics Committee of the Instituto
Butantan, São Paulo, SP, Brazil under protocol nº 767/10. The Committee in Animal Research in
Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation.
Confirmed- leptospirosis human serum samples were from Instituto Adolfo Lutz collection, São
Paulo, Brazil. Serum samples from patients with unrelated infectious diseases were obtained from 197 serum collection of Laboratorio Imunoepidemiologia, SUCEN, SP, Brazil; Laboratorio Protozoologia, IMT/USP, Brazil, Laboratorio Virologia, IMT/USP, Brazil and Nucleo Estudos em Malária, SUCEN/IMT/USP, Brazil. Human sera from collections of the above Institutions were donated to be used for research purposes.

**Immunoblotting assay.** The purified recombinant protein was loaded into 12% SDS-PAGE and fractioned proteins were transferred into nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi-dry equipment. Membranes were blocked with 10% non-fat dry milk, 1% BSA in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-OmpL1 (1:1,600) mouse polyclonal serum or monoclonal anti-his tag antibodies (1:1,000) (GE Healthcare) for 2 h at room temperature. After washing, the membranes were incubated with HRP -conjugated anti-mouse IgG (1:5,000; Sigma) in PBS for 1 h. The protein reactivity was revealed by ECL reagent kit chemiluminescence substrate (GE Healthcare); the luminescence generated by the reaction was detected with the aid of Carestream Molecular Imaging (Equilab, Whitestone, NY, USA) connected to Gel Logic 2200PRO.

**OmpL1 genomic DNA and protein conservation among leptospiral strains.** Leptospiral genomic DNAs were extract as previously described (50) and sample integrity was verified by amplification of 16S ribosomal DNA. Cloning primers of OmpL1 were used for DNA amplification of *Leptospira* spp., as describes above. Fragments were visualized in 1% agarose gel. For protein conservation, cultures of *Leptospira* spp. were harvested by centrifugation, washed three times with PBS low-salt containing 5mM MgCl₂ and resuspended in PBS. A sample of each strain was applied onto a 12%
SDS-PAGE and after separation, proteins were transferred into nitrocellulose membrane on a semidry equipment (GE Healthcare). The membrane was blocked overnight at 4 °C with 10% nonfat dry milk, 1% BSA in PBS-T. After three washings with PBS-T, the membrane was incubated for 3 h at room temperature with the antiserum against OmpL1 (1:500) produced in mice, followed by washings and incubation with HRP-conjugated goat anti-mouse IgG (1:5,000) for 1 h at room temperature. The membrane was then washed and the reactivity was revealed as described above;

**ELISA for detection of human and hamsters antibodies.** Human and hamster IgG antibodies against OmpL1 were detected by ELISA as previously described (51). In brief, negative and positive MAT serum samples from 32 confirmed-leptospirosis patients and 44 experimentally infected hamsters, were diluted (1:100) and evaluated for total IgG using HRP-conjugated anti-human IgG antibodies, 1:5,000 (Sigma, USA), and total IgG using HRP-conjugated anti-hamster IgG antibody, 1:5,000. Cut-off values were set at three standard deviations above the mean OD492 nm of sera from 10 healthy individuals from the city of São Paulo, Brazil, or 5 non-infected hamsters. Reaction specificity was evaluated with serum samples from patients diagnosed with unrelated infection diseases, as follows: dengue (n =13), malaria (n =12), HIV infection (n=15) and Chagas’ disease (n=20). The sera were used at 1:100 dilutions and the values for specificity were determined by the method of Galen and Gambino (22)

**Lymphoproliferation assay.** At the end of the immunization protocols, BALB/c mice were sacrificed their spleens were aseptically removed and suspended in RPMI (Roswell Park Memorial Institute Medium-RPMI-1640 medium containing 2mM L-glutamine, 100 IU/mL of Penicillin, 100 µg/mL of Streptomycin and 50 µg/mL of Gentamycin). Spleen were macerated and after
erythrocytes lysis splenocytes were resuspended in 1 mL of RPMI containing 10% fetal bovine serum and counted after staining with 0.4% trypan blue for viability. Splenocytes (5x10⁶ cells/well) were plated in triplicate in a 96-well flat bottom cell culture plates (Costar, Corning). Spleen cells were stimulated with 5 µg/mL of ConA (Sigma), employed as positive control, 5µg/mL of OmpL1 or medium alone, used as negative control. Cells were cultured for 48 h at 37 °C and 5% CO₂ in a humidified atmosphere and proliferative rates were determined as a function of DNA synthesis, measured by the incorporation of bromodeoxyuridine (BrdU) by BrdU ELISA colorimetric kit (Roche Diagnostic, Indianapolis, IN, USA). Cells were labeled for 2 h with 20 µL of BrdU labeling solution. Subsequently, anti-BrdU conjugated with HRP (1:100) was added and incubated for 90 min. The enzyme substrate solution was added and incubation was carried at room temperature for 15 min. The enzymatic reaction was stopped by adding 1M H₂SO₄ and the optical density was measured at 450 nm in a microplate reader. Stimulation Index (S.I.) was calculated as the ratio between the mean OD of cells cultured with the antigen and the mean OD of cells cultured in medium only, in the immunized group.

**Evaluation of cytokine production.** For analysis of secreted cytokines, spleen cells were isolated and cultured as described above, except that the culture was made in 24 well tissue culture plates, each well containing 5x10⁶ cells. After 48 h, cell-free culture supernatants were collected and stored for short-term at -20 °C. IL-4, IL-10, IFN-gamma and TNF-alpha were measured by ELISA (PreproTech, Colonia Narvarte, DF, Mexico) according to the manufacturer’s instructions. Briefly, capture antibody was added to each well and the plates were incubated overnight at 4 °C, followed by incubation with blocking buffer for 1h. Standard cytokines were diluted to create a concentration curve. Then, 100 µL of each supernatant sample were added and plates were incubated for at least 3 hours.
2h. After that, antibody detection solution was added (1:2,000) followed by 2h of incubation; substrate was added to each well and incubation proceeded at room temperature for reaction development; readings were taken at 405 nm with wavelength correction set at 650 nm in an ELISA plate reader. Results are expressed in pg/mL.

**Binding of OmpL1 to ECM and to serum components.** Protein attachment to individual macromolecules of the extracellular matrix and serum components was analyzed according to a previously published protocol (4). Briefly, ELISA plates (Costar High Binding, Corning) were coated with 1 µg of ECM component or bovine serum albumin (BSA, negative control), gelatin and fetuin (highly glycosylated attachment-negative control protein) in 100 µL of PBS for 3 h at 37 ºC, and then blocked overnight at 4º C. One µg of OmpL1 was added per well in 100 µL of PBS, and protein was allowed to attach to the different substrates for 2 h at 37 ºC. After washing six times with PBS-T, bound proteins were detected by adding an appropriate dilution of mouse antiserum that gives an OD492 nm value of 1 determined by titration in 100 µL of PBS (1:1,600). Incubation proceeded for 1 h at 37 ºC, and after three washings with PBS-T, 100 µL of a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG in PBS was added per well and incubated for 1 h at 37 ºC. The wells were washed three times, and o-phenylenediamine (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 µL/mL H2O2 was added (100 mL per well). The reaction was allowed to proceed for 10 min and interrupted by the addition of 50 µL of 8 M H2SO4. Readings were taken at 492 nm in a microplate reader (Multiskan EX; Thermo Fisher Scientific, Helsinki, Finland). For statistical analyses, the binding of recombinant protein to ECM macromolecules was compared to its binding to all three negative controls using Student’s two-tailed t test, and P value was given related to comparison to gelatin, used as negative control for the following experiments. Bindings were also
confirmed by using HRP-conjugated anti-his tag monoclonal antibodies, previously titrated against
the recombinant protein and used in a dilution that generates and OD492 nm value of approximately
1.

**Dissociation equilibrium constant (K_D) for the OmpL1 binding to ECM and to serum components.** First, ELISA plates were coated with 100μl of 10 μg/mL ECM or serum components and allow adhering for 3h at 37 °C. Plates were then blocked overnight and increasing concentrations of the purified OmpL1 were added (100 μl/well in PBS) and incubated for 2 h at 37 °C. The assessment of bound protein was performed by incubation for 1 h at 37 °C with the antiserum raised against the protein at the dilution of 1:1,600, followed by HRP-conjugated goat anti-mouse IgG (Sigma) (1:5,000 in PBS). The ELISA data were used to calculate the dissociation constant (K_D) according to the method previously described (38) based on the equation: 

\[ A = \frac{A_{max} \text{[protein]}}{K_D + \text{[protein]}} \]

where A is the absorbance at a given protein concentration, Amax is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

**Antibody inhibition assay.** We accessed the effect of anti-OmpL1 antibodies on the binding of the recombinant protein to laminin, plasma fibronectin and plasminogen in a dose-dependent manner. As a control, pre-immune serum was employed. Briefly, plates were coated with 1 μg of each component and blocked with 10% non-fat dry milk. At the same time, 1μg of OmpL1 was incubated with different concentration of anti-serum raised in mice against the recombinant protein or pre-immune serum (from 1:50 to 1:400 dilutions) for 2h at 37° C. After that, the recombinant protein...
was allowed to interact with the coated component, for 2h at 37°C. After washing with PBS-T, HRP-conjugated anti-his tag monoclonal antibodies, previously titrated against the recombinant protein and used in a dilution that generates and OD492 nm value of approximately 1, were added. The wells were washed three times and o-phenylenediamine (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 μL/mL H2O2 was added (100 mL per well). The reaction was allowed to proceed for 10 min and interrupted by the addition of 50 μL of 8 M H2SO4. Readings were taken at 492 nm in a microplate reader. For statistical analyses, the percentage of binding of recombinant protein was compared to its binding in the treatment in which no anti-OmpL1 antiserum was added (100% of binding) by Student’s two-tailed t test.

Characterization of the binding of OmpL1 to laminin and to PLG. In order to evaluate sugar moiety contribution to the laminin-OmpL1 interaction, microplates were coated with 1 μg of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three times with the same buffer, and immobilized laminin was treated with different sodium metaperiodate concentrations (5 to 100 mM) for 15 min at 4°C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 μl of PBS-T 10% (w/v) non-fat dry milk for 2 h at 37°C. Binding of OmpL1 (1 μg in PBS per well) to periodate-treated laminin was assessed as described above. The effect of ionic strength on the binding of OmpL1 to PLG was assessed by increasing NaCl from 137 mM, which is the concentration in PBS solution, to 437 mM. To determine the role of lysines in PLG-recombinant protein interactions, the lysine analog 6-aminocaproic acid (ACA) (Sigma-Aldrich) was added together with the recombinant protein at a final concentration of 2 mM or 20 mM to the PLG-coated wells. The detection of bound protein was performed as previously described.
Plasmin enzymatic activity assay. 96-well ELISA plates were coated overnight with 10 μg/mL recombinant protein, or BSA and Lsa63 (74), for negative control, in PBS at 4°C. Plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) non-fat dry milk. The blocking solution was discarded and 100 μL/well of 10 μg/mL human PLG was added followed by incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma-Aldrich) was added. Subsequently, 100 μL/well of plasmin-specific substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (Sigma-Aldrich) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate degradation was measured by taken the readings at 405 nm.

Binding interference of OmpL1 with ECM and PLG. 96-well plates were coated overnight at 4°C with 100 μl of 10 μg/mL laminin, plasma fibronectin or PLG in PBS, washed three times and blocked with 10% (w/v) non-fat dry milk for 2 h at 37 °C. Concomitantly, OmpL1 (1 μg in 100 μL of PBS) was incubated with increasing concentrations (0-1 μg in 100 μL of PBS) of the other components separately, and then transferred to the coated plates, which was then incubated for 2 h at 37 °C. After four washings, recombinant protein binding was quantified by specific antibodies as described above.

Inhibition of live leptospires binding to laminin, plasma fibronectin and PLG by OmpL1. ELISA plates were coated with laminin, plasma fibronectin or PLG (1 μg/well). The plates were washed and blocked with 10% non-fat dry milk in PBS-T for 2 h at 37°C. The blocking solution was discarded, and the wells were incubated for 2 h at 37°C with increasing concentrations of recombinant protein (0 to 1.5μM). After three washings, 100 μL/well of 4x10^7 live L. interrogans...
serovar Copenhageni strain M20 in PBS low salt buffer (containing 50mM NaCl) were added for 90 min at 37°C. The unbound leptospires were washed with PBS low salt and the quantification of bound leptospires was performed indirectly by anti-LipL32 antibodies produced in mice (1:4,000), due to the fact that LipL32 is a major outer membrane leptospiral protein (25) the procedure was followed by HRP-conjugated anti-mouse IgG antibodies, essentially as described in Barbosa et al.(4). The detection was performed by OPD as previously described.

Statistical analysis. All results are expressed as means ± SEM. Student's paired t test was used to determine the significance of differences between means, and P lower than 0.05 was considered as statistically significant.
RESULTS

Cloning, expression and purification. Oligonucleotides for PCR amplification (see Materials and Methods) were designed based on genome sequences of *L. interrogans* serovar Copenhageni, excluding the signal peptide. The amplified coding sequence was cloned into an *E. coli* pAE vector (58) and the protein was expressed with 6XHis tag at the N-terminal. Recombinant protein was expressed in the bacterial pellet, in its insoluble form, as inclusion bodies (Fig. 1A, lane 4). We have failed to obtain the protein in its soluble form, possible due to its structure that is predicted to have 10 β-sheet transmembrane segments (64). Protein was recovered from inclusion bodies after solubilization with 8M urea. The purification was performed by metal chelating chromatography after refolding for removal of urea and was evaluated by SDS-PAGE, as shown in Fig. 1A, lane 6. The recombinant protein bands were confirmed by western blotting probed with polyclonal antibodies raised in mice against OmpL1 (Fig. 1B, lane 1) and with anti-His tag monoclonal antibodies (Fig. 1B, lane 3). The calculated 32.4 kDa molecular mass of the recombinant protein OmpL1 comprise the vector fusion plus the encoded amino acids sequence. Structural integrity of the purified protein was assessed by circular dichroism (CD) spectroscopy. The method evaluates the secondary structure content of protein and it is an important data to obtain after protein refolding. As depicted in Fig. 1C, CD spectrum shows the minima at 215-220 nm, and the maximum at approximately 196 nm, typical of β-strands secondary structure content in the recombinant protein. The OmpL1 β-strands predominance was also confirmed by analysis of the spectrum data by K2D2 software, which showed 34% of β-strand and 14% of alpha helix (53). The data is in agreement with the structure of the native protein, predicted to have a predominance of β-strand (60).
Genomic DNA and protein conservation among leptospiral strains. Genomic DNA integrity was confirmed by amplification of 16S DNA. A single band corresponding to OmpL1 gene was observed in all pathogenic strains tested, while no band was found in the saprophytic *L. biflexa* genomic DNA (Fig. 2A). Protein expression and conservation among *Leptospira* strains was assessed with total protein extracts from the same pathogenic, high passage, non-virulent strains of *Leptospira*, used for genomic DNA conservation, the saprophytic strain *L. biflexa* serovar Patoc and the virulent, low-passage strains, *L. interrogans* serovars Copenhageni strain FIOCRUZ L1-130 and *L. interrogans* serovar Kennewicki strain Pomona Fromm. Cell extracts were gel fractionated, proteins membrane transferred and western blotting analysis was performed by probing the membranes with polyclonal serum from mice immunized with OmpL1. The serological reactivity showed conservation of OmpL1 epitopes in the main serovars of *L. interrogans* (Fig. 2B). However, with the two serovars of *L. borgpetersenii* (lanes 5 and 6) only weak protein bands were detected (Fig. 2B). No protein band was detected with *L. santarosai* serovar Shermani and with the saprophytic *L. biflexa* strain (lanes 9 and 11). Reactivity was also detected with both *L. interrogans* virulent strains employed (lanes 12 and 13). Multiple sequence alignment was performed with CLUSTAL 2.1 program comparing OmpL1 with the sequences available in GenBank and the resulting phylogram is depicted in Fig. 2C. The result clearly shows the high conservation of OmpL1 among leptospiral strains, the similarity/proximity with the sequence present in pathogenic strains and the low similarity of the sequence present in saprophytic and in pathogenic intermediate strains (44), which are organized in a more distant branch. Contrary to the in silico analysis, we did not detect in our experiments the OmpL1 protein in *L. santarosai* serovar Shermani (Fig. 2B and Fig. 2C). This might be due to some sequence problem with the gene or because the analysis was performed with different isolates.
Evaluation of immunological response elicited in mice by OmpL1. The antibody immune response induced by OmpL1 in mice was analyzed by ELISA in a quantitative basis. As depicted in Fig. 3A, antibodies IgG1 and IgG2a against OmpL1 were found, with a predominance of IgG1 isotype. The major Ig response was IgG, with a titer of 100,400, IgM titer was 6,400 (data not shown) and IgA was not detected. Statistically significant value for lymphocyte proliferation was achieved with animals immunized with the OmpL1, stimulated with the recombinant protein (P<0.05) (Fig. 3B), having an average stimulation index of 2.8. High proliferation level was obtained when cells were treated with ConA, employed as positive control of the experiment (not shown). Addition of OmpL1 to lymphocytes from animals that have not been primed with the recombinant protein produced non-significant levels of proliferation (data not shown). Supernatants of cultured spleen cells from OmpL1 immunized mice were analyzed for the presence of cytokines, IL-10, IL-4, IFN-γ and TNF-α, selected to discriminate cellular-Th1 (IFN-γ and TNF-α) and humoral-Th2 (IL-10 and IL-4) immune responses (59, 66). The cytokine profiles obtained showed a predominance of IL-10 and IFN-γ, both occurring in high levels (Fig. 3C and 3D), similar to the ones of the ConA positive control (not shown). A statistically significant cytokine level was obtained in the OmpL1-primed group when cells were stimulated with OmpL1 (P<0.001 for IL-10 and P<0.0001 for IFN-γ); lower value, although statistically significant, was observed with TNF-α level (P<0.05) when the cells from OmpL1-primed group were induced or not with the recombinant protein (Fig. 3E). In contrast, no IL-4 stimulation was achieved when comparing spleen cells from animal groups under the same experimental conditions (Fig. 3F). Measurements of the same parameters with spleen cells from control animals immunized with PBS either stimulated or not with the recombinant protein produced negligible results (not shown).
Reactivity of OmpL1 with human and hamster leptospirosis serum samples. We have performed ELISA to analyze whether the OmpL1 is recognized by antibodies present in confirmed leptospirosis cases from humans and experimentally infected hamsters. We have employed 32 paired human samples of early (MAT negative) and convalescent (MAT positive) phases of the disease and 44 samples of MAT-confirmed serum from hamsters. Our data show that the recombinant protein was able to recognize specific IgG antibodies in 90% of infected hamster’s tested sera (Fig. 4A). High level of responders (75%) was obtained for IgG in human MAT positive sera while a total of 9% of serum samples presented IgG antibodies against OmpL1 in the early phase of the disease (MAT negative) (Fig. 4B). The cutoff value calculated with healthy human sera was 0.374. Due to the non-specific clinical symptoms of leptospirosis, we analyzed the reactivity of the recombinant protein OmpL1 with serum samples from patients with unrelated infectious diseases that did not have a previous history of leptospirosis, including dengue (n=13), malaria (n=12), Chagas’ disease (n=20), and HIV infection (n=15). The reactivity obtained with OmpL1 and these serum samples was similar to the ones obtained with serum samples from healthy donors (Fig. 4B). The specificity of OmpL1 was calculated to be 100% for all unrelated diseases tested, except for dengue, to which the specificity was calculated to be 92.3%.

Adhesion of OmpL1 to ECM components. As native OmpL1 has surface-exposed domains (24, 55), we decided to evaluate whether this protein could mediate host colonization by adhering to extracellular matrix proteins. Thus, laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, ECM gel, elastin and the control proteins gelatin, BSA and fetuin were immobilized on 96-wells microdilution plates and recombinant protein attachment was assessed by ELISA, as previously described (4). As shown in Fig. 5A, OmpL1 protein exhibited statistically
significant adhesiveness to laminin and plasma fibronectin ($P<0.01$). The adherence of OmpL1 to ECM gel was expected, as laminin is one of the components (see M&M). No statistically significant adhesiveness was observed with OmpL1 protein when wells were coated with collagen Type I and IV, cellular fibronectin, elastin or with control proteins. The bindings were confirmed by performing similar assay but using anti-his tag monoclonal antibodies (Fig. 5B). The interaction between OmpL1 to laminin and to plasma fibronectin was also assessed on a quantitative basis by fixing the ECM components and varying protein concentration, as depicted in Fig. 5C and 5D, respectively. Binding saturation level was achieved with protein concentration of ~5 μM for laminin and plasma fibronectin, with $K_D$ of 2099.93 ± 871.03 nM and 1239.23 ± 506.85 nM, respectively. The role of sugar moiety in laminin was also investigated by incubating this component with increasing concentrations of sodium metaperiotade. A reduction of 20% on the binding could be noticed only with the highest concentration tested, suggesting that the carbohydrate moieties do not have a major role in the binding of OmpL1 to laminin (Fig. 5E). The effect of mice anti-OmpL1 polyclonal serum on the interaction of the recombinant protein and ECM was also evaluated. Low antiserum dilution (1:50) almost abolished the binding of OmpL1 with laminin (90%) while 73% binding reduction was obtained with fibronectin (Fig. 5F). Pre-immune serum at the same dilutions had only a moderate effect on the binding of recombinant protein with ligands, 21 and 17% binding reduction for laminin and plasma fibronectin, respectively, reinforcing the interaction of OmpL1 and the two ECM components (Fig. 5F).

Recombinant leptospiral OmpL1 binds to human PLG. In our previous work we have shown that leptospires bind PLG on their surface (78) and we have also described several proteins that are probably PLG receptors (74). Based on this assumption, we decided to evaluate if OmpL1 can also
adhere to human PLG \textit{in vitro}. In addition, we investigated whether the recombinant protein can also bind factor H, human complement and C4bp, as previously reported for other recombinant proteins (4, 5, 11, 16, 72). Our data show that OmpL1 binds to human PLG ($P < 0.01$), while no or very low reactivity was detected with the other components and the control proteins (Fig. 6A). This binding was also confirmed using anti-his monoclonal antibodies (Fig. 6B). The interaction between OmpL1 and PLG was also assessed on a quantitative basis, keeping PLG and varying the protein concentration, as shown in Fig. 6C. Binding saturation level was reached with 2 µM of OmpL1 with a $K_D$ of $368.63 \pm 121.23$ nM. It is well known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors (33). These domains were shown to participate in the binding of PLG with intact live \textit{L. interrogans} serovar Copenhageni strain L1–130, since the derivative and analogue of lysine, ACA, almost totally inhibited binding (78). Based on these findings, the participation of lysine residues in the binding of the recombinant protein was evaluated by the addition of ACA to the assay. As depicted in Fig. 6D, the increasing NaCl concentration from 137 mM, which is the concentration in PBS solution, to 437 mM had no effect on the OmpL1-PLG binding, suggesting that ionic interactions were not important. In contrast, when 2 mM of ACA was added to the reaction, the binding of the protein to PLG was almost completely abolished ($P<0.01$), strongly suggesting the participation of these domains in OmpL1 interaction with PLG. We further evaluated the interaction of PLG with the recombinant protein by adding several dilution of mice anti-OmpL1 polyclonal serum on the binding reaction. An inhibitory effect was observed on the interaction of OmpL1 with PLG that was dependent on the serum dilution, almost totally eliminated at the lowest serum dilution (86%), contrasting with 17% binding reduction obtained with pre-immune serum (Fig. 6E).
Plasmin generation from PLG-bound. It has been demonstrated that enzymatically active plasmin is generated by PLG bound to the surface of *L. interrogans* when its activator is present (78). To assess whether the PLG bound to OmpL1 generates proteolytic activity, as previously reported with other recombinant proteins (45, 50, 71, 72, 74), a microplate was coated with OmpL1, blocked, and then incubated with PLG. Unbound PLG was washed away, uPA (urokinase)-type PLG activator together with a plasmin-specific chromogenic substrate were added. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the plasmin-specific substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride at 405 nm. The PLG bound to OmpL1 protein could be converted into plasmin, as indirectly demonstrated by specific proteolytic activity (Fig. 6F). Reaction controls lacking PLG, uPA or the chromogenic substrate showed no significant enzymatic activity. The adhesin Lsa63 that is not a PLG-binding protein and therefore does not generate plasmin (74) was also included as a negative control (Fig. 6F).

Inhibition of *L. interrogans* attachment to laminin, PLG or to plasma fibronectin by OmpL1. It has been reported that several recombinant proteins (4, 45, 50, 54) exhibit an inhibitory effect on the binding of leptospires to PLG and ECM macromolecules. We thus carried out similar experiments to evaluate whether OmpL1 has similar effect on leptospiral adherence to laminin, PLG and plasma fibronectin. The results show that the addition of increasing concentration of OmpL1 reduced the leptospiral binding to laminin, PLG and to plasma fibronectin. Binding reduction in the number of leptospires adhering to PLG was statistically significant with 0.1 μM of OmpL1 (*P* < 0.05) while to laminin significance was reached with 1 μM of protein (*P* < 0.05) and to plasma fibronectin with 1.5 μM (*P* < 0.05) (Fig. 7A). These results suggest that OmpL1 compete with the native leptospiral
protein for the component binding sites. The experiment was performed in triplicate and this figure shows one representative data of two independent experiments.

**Binding competition of OmpL1 to ECM and PLG by different ligands.** As OmpL1 showed significant binding to PLG, laminin and plasma fibronectin, we decided to evaluate if these macromolecules compete for the same protein binding sites. Thus, we evaluated the competition among the components in a combination of two each time. The increasing concentration of laminin or PLG had no effect on the binding of OmpL1 to plasma fibronectin (Fig. 7B). Similarly, the addition of laminin or plasma fibronectin caused no changes on the binding of recombinant protein to PLG (Fig. 7C). The binding of laminin to OmpL1 was not affected by the addition of plasma fibronectin (Fig. 7D) contrasting with the effect produced by the addition of PLG, which unexpectedly, caused an increased on the interaction (Fig. 7D).
DISCUSSION

Leptospiral outer membrane proteins are the main focus of our research. Because of their location, these proteins may be involved in mechanisms of leptospiral pathogenesis and protective immunity. We have reported that leptospires have ability to adhere to laminin, cellular fibronectin, plasma fibronectin, collagen I and IV, and to date, several leptospiral adhesins ECM-binding proteins have been identified. These include 36-kDa fibronectin-binding protein, LfhA/Lsa24, LigA and LigB proteins, Len-family proteins, Lsa21, LipL32, Lsa27, Lp95, TlyC, LipL53, Lsa63, OmpL37, Lsa66, Lsa20, Lsa25 and Lsa33. After adherence, leptospires rapidly disseminates to several organs that is thought to be mediated by rapid translocation across host cell monolayers. Besides motility and chemotaxis, *Leptospira* invasion may be mediated by proteolytic activity capable of cleaving host cell membranes. Indeed, we have described that leptospires acquire host-derived plasmin by receptor-bound PLG and can degrade fibronectin and laminin (Vieira et al., manuscript submitted to publication) that could help bacterial dissemination.

OmpL1 is a transmembrane outer-membrane protein of *Leptospira* firstly described by Haake and colleagues. This protein has been identified in pathogenic *Leptospira* spp. by proteomics, exhibited synergistic immunoprotective capacity in combination with LipL41 in the Golden Syrian hamster model of leptospirosis, partial protective immunity, as recombinant protein or as DNA vaccine, and it has been studied by several groups for its human and veterinary diagnostic capability. In this work, we report that OmpL1, encoded by the gene LIC10973, is an adhesin of *Leptospira* that interacts with laminin and plasma.
fibronectin, is a PLG-binding receptor capable of generating plasmin and is specifically recognized by antibodies in serum samples of human confirmed leptospirosis.

The DNA coding sequence of OmpL1 is conserved in all serovars of *L. interrogans* and other pathogenic species tested, but is absent in the saprophytic *L. biflexa*. These results corroborate previous findings that this protein is expressed in high passage pathogenic and low passage, virulent strains of *Leptospira* (24, 26, 27, 80). The LIC10973 gene was cloned and expressed in *E. coli* as a 32.4 kDa full-length recombinant protein, which comprises the native sequence of OmpL1 plus a 6X His tag at N-terminal. The purified protein exhibited a single major band in SDS-PAGE and was recognized by monoclonal anti-His tag antibodies and its homolog antibodies from mice immunized with the recombinant protein. Assessment of secondary structure of the recombinant protein after the purification process has been performed by CD spectroscopy that showed a typical of β-strands secondary structure content in the recombinant protein, which is in agreement with the predicted native structure of OmpL1, characterized as an outer membrane-spanning protein, whose topological model contains ten amphipathic transmembrane β-strands (24, 64).

Several researchers have suggested the importance of OmpL1 as a serological antigen for the diagnosis of human (17, 21, 48, 57, 68, 79) and canine leptospirosis (30, 49). Our data showed high percentage of anti-OmpL1 IgG responders when experimentally infected-hamsters or MAT+ human serum samples were used, which is in agreement with previous published studies. Moreover, the results corroborate previous findings that this protein is expressed during infection (6). Further evaluation of OmpL1 with human serum samples of confirmed unrelated infectious diseases revealed high specificity of this antigen, which reinforces the importance of OmpL1 as an antigen for the development of leptospirosis diagnostic kit.
Lin and colleagues (36) evaluated combined B and T cell epitopes of OmpL1 and of LipL41 conserved regions for their capacity to promote immune responses in BALB/c mice, and reported that the selected epitopes can induce a Th1-response. It has been already shown that OmpL1 is highly immunogenic in animal models, but these antibodies are probably not protective because OmpL1 alone did not protect hamsters against the challenge with virulent leptospires (26). High levels of OmpL1 IgG antibodies detected in the sera from immunized BALB/c mice were mainly from IgG1 subclass indicating Th2-type response. Our data show that the recombinant OmpL1 was also capable of activating cell-mediated immune response indicated by lymphocyte proliferation from OmpL1 immunized mice. Moreover, OmpL1 was capable to induce both cellular and humoral immune response as demonstrated by the levels of IFN-γ and TNF-α (Th1) and IL-10 (possibly Th2) cytokines. These results might be explained in part by the effects exerted by the aluminium salt, employed as adjuvant, which is known to stimulate both Th1 and Th2 immune responses (43). In fact, Vernel-Pauillac and Merien (73) have demonstrated that pathogenic leptospires can elicit Th1 response together with anti-lipopolysaccharide antibodies in animal model. Moreover, the protective immune mechanism of LigA DNA vaccine was evaluated in immunized animals and shown to be conferred by both Th1 and Th2 immune responses (20). It is worth mentioning that the recombinant protein LipL32, a major leptospiral antigen, has failed to confer protection against virulent bacteria in several challenge studies (9, 40). However, protection of this antigen was achieved when hamsters were immunized with recombinant BCG (rBCG) expressing LipL32 (63). Recombinant BCG has the ability to elicit strong cellular as well as humoral immune responses (15). Thus, it is possible that not only humoral but cellular immune responses are important in the immunoprotection against leptospirosis. Understanding the immune response elicited by leptospiral recombinant antigens may

provide new insights on the mechanisms of anti-
Leptospira immunity and may facilitate the design
for an improved recombinant-based vaccine.

OmpL1 is a surface exposed protein that is expressed during infection. It is therefore possible
that it may play a role in the attachment to the host. Indeed, OmpL1 protein exhibits extracellular
matrix-binding properties. This protein binds laminin and plasma fibronectin contrasting to the
previously reported Lsa24 (4), Lsa27 (39) and Lsa20 (45), that are laminin-binding adhesins, but
comparable to other reported adhesins, namely Len family protein (65), LigA and LigB proteins(12),
Lsa21 (2), LipL32 (28, 29), TlyC (10), OmpL37 (54) and Lsa66 (50) that showed a broader
spectrum binding profile to ECM. The calculated K_D for the binding of OmpL1 to laminin and to
plasma fibronectin (2099.93 + 871.04 and 1239.23 + 506.85 nM, respectively) is, however, higher
when compared to the K_D values obtained with Lsa66 and the same ECM components (55.4 + 15.9
nM and 290.8 + 11.8 nM) (50). Nevertheless, the kinetics of dose-dependence of each ligand with
OmpL1 is specific and saturable binding to immobilized components was observed, fulfilling the
properties of a typical receptor-ligand interaction.

The interaction with the host PLG, a key component of the host fibrinolytic system, has been
shown for several invasive gram-positive and gram-negative bacteria (13, 69). We have reported that
Leptospira species were also capable to bind PLG and generate active plasmin on their surface (78).
Moreover, we have demonstrated that the generation of this proteolytic activity makes virulent L.
interrogans capable of degrading purified extracellular matrix fibronectin [15] and laminin (Vieira et
al., manuscript submitted to publication) a step that may contribute to leptospiral invasiveness (77).
In addition, we have identified proteins that act as leptospiral PLG-receptors (74). OmpL1 also
shows PLG-binding activity that seems to occur via PLG kringle domains with a K_D of 368.63 +- 651
121.23 nM. This K_D value is in the same order of magnitude as the one reported for the adhesin
Lsa20 (45) but higher when compared to the ones reported with several recombinant proteins in our laboratory (74). As previously shown with other protein-PLG-receptors (45, 50, 71, 72, 74), plasmin is actively generated in the presence of urokinase activator by PLG-bound OmpL1. Thus, it is possible that this protein may contribute to leptospiral infectiveness.

The low binding affinity of OmpL1 to ECM and to PLG could be compensated by the high copy number reported for this protein in *L. interrogans* (41) that was shown to increase after 7 days of serum treatment (62). The fact that mice polyclonal anti-OmpL1 serum had a strong reduction effect on the binding of OmpL1 with laminin, plasma fibronectin and PLG and allied to the fact that pre-immune serum had only a moderate effect, strengthen the reactivity between the recombinant protein and these ligands. No interference on the binding of OmpL1 with these ligands was observed except the increased binding effect produced by increased PLG concentration on the binding of recombinant protein to laminin suggesting that PLG is probably binding directly to immobilized laminin. Indeed, the interaction of laminin with PLG has been reported (61). OmpL1 exhibited an inhibitory effect on the binding of intact *L. interrogans* to immobilized laminin, plasma fibronectin or PLG, as was previously observed with the ECM- and PLG-interacting proteins Lsa24(4), Lsa63(75), LigA/LigB(12), OmpL37(54) and Lsa20(45).

In conclusion, we report in this work for the first time that OmpL1 is an ECM and a PLG-binding protein of *Leptospira* spp. The interaction of OmpL1 with PLG in the presence of host activator can generate plasmin, rendering the bacteria with proteolytic activity capable of degrading host cell membranes. Thus, as an ECM- and PLG binding protein, OmpL1 may promote the attachment of the bacteria to mammalian hosts and may help the leptospires to disseminate during the infection process. The high number of human leptospirosis (MAT +) responders associated with the specificity of the IgG antibody response to OmpL among serum samples from patients with...
unrelated febrile diseases such as dengue, malaria, HIV, and Chagas disease, strengthens the potential of OmpL1 as a diagnostic marker for leptospirosis. OmpL1 promotes the proliferation of lymphocytes and activates both Th1 and Th2 cytokines from immunized BALB/c mice. It is conceivable that OmpL1 administered with adequate immune modulator might exhibit protective activity against lethal challenges.

Acknowledgments. This work was supported by FAPESP, CNPq and Fundação Butantan, Brazil; LGVF and MLV have scholarships from FAPESP (Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
REFERENCES


LEGENDS TO FIGURES

Figure 1. Expression, purification and Western blotting analysis of purified recombinant OmpL1. (A) Recombinant OmpL1 expression analysis on SDS-PAGE; M: molecular mass protein marker; 1: non-induced total bacterial extract; 2: total bacterial cell lysates after induction; 3 and 4: soluble and insoluble (pellet) fractions of the induced culture, respectively; 5: flow-through fraction; 6: purified recombinant protein in Comassie blue stained gel. (B) Western blotting analysis of the recombinant protein probed with pooled polyclonal antiserum raised in mice immunized with OmpL1 (1) or PBS (2), or with anti-His tag monoclonal antibodies (3). (C) CD spectrum of OmpL1 protein depicting a predominance of β-sheets in its secondary structure composition. Far-UV CD spectrum is presented as an average of five scans recorded from 185 to 260 nm.

Figure 2: DNA and OmpL1 protein conservation among Leptospira strains. (A) genomic DNA of several species of Leptospira were used as template for PCR amplification of 16S ribosomal DNA (constitutive control) and OmpL1 genes. The negative control (−) denotes a reaction without DNA template. (B) Leptospires whole cell lysates and recombinant OmpL1 (+, used as a positive control) were separated by SDS-PAGE, transferred into membranes and probed with antiserum against the recombinant protein followed by anti-mouse IgG peroxidase conjugated. Reactivity was detected by ECL kit. L. interrogans serovars: Copenhageni (1), Canicola (2), Icterohaemorrhagiae (3) Pomona (4), L. borgpetersenii serovars: Castellonis (5), Whitcombi (6), L. kirschneri serovars Cynoptery (7) and Grippotyphosa (8), L. santarosai serovar Shermani (9), L. noguchii serovar Panama (10), and L. biflexa serovar Patoc (11). For protein conservation virulent L. interrogans serovar Copenhageni
strain Fiocruz L1-130 (12) and serovar Kennewicki, strain Pomona Fromm (13) were also included. (C) Resulted phylogram of all sequence alignments performed using Clustal W2 showing the high sequence conservation among leptospiral strains, the proximity of OmpL1 within the pathogenic strains of Leptospira and the intermediate and saprophytic strains in distant branches.

**Figure 3: Immune response evaluation in BALB/c mice immunized with OmpL1.** (A) Total IgG antibodies and subclasses were determined in sera from BALB/c mice immunized with OmpL1. OD values were from different pooled sera dilution and compared against a concentration curve of total IgG, IgG1 and IgG2a. (B) Lymphocyte proliferation response was analyzed from spleens of immunized mice with OmpL1; cells were cultured followed by a pulse with medium alone, ConA (5μg/mL) or OmpL1 (5μg/mL). Lymphocytes stimulated with the medium alone were used as a negative control and those stimulated with ConA were used as positive control. The proliferative response was measured by ELISA BrdU colorimetric assay. The data represent the mean OD values of three determinations ± S.D. of two independent experiments. For statistical analysis, OD values of OmpL1 immunized group treated with the recombinant protein were compared against OmpL1 immunized group treated with medium alone by the two-tailed t test (*P<0.05, ***P<0.001 and ****P<0.0001).
Figure 4: Recognition of OmpL1 by IgG antibodies of hamsters, of individuals diagnosed with leptospirosis and of individuals diagnosed with unrelated diseases. Positive sera (responders) were determined by ELISA with the recombinant protein and (A) serum samples from experimentally infected hamsters; (B) serum samples from leptospirosis patients in both phases of the disease, early (MAT-) and convalescent (MAT+), and serum samples from patients diagnosed with unrelated febrile diseases. The reactivity was evaluated as total IgG antibodies. The cutoff values are defined as the mean plus 3 standard deviations obtained with sera from ten healthy individuals or 5 non-infected hamsters (horizontal bars).

Figure 5: Binding of OmpL1 to ECM components. (A) Wells were coated with 1 μg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, ECM gel, elastin and the control proteins gelatin, BSA and fetuin. 1 μg of the recombinant protein was added per well and the binding was measured by ELISA. Data represent the mean ± the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein to the ECM components was compared to its binding to all negative controls by the two-tailed t test, although the P value given here refers to comparison against gelatin (*P < 0.05 and **P < 0.01). (B) Significant binding activity was confirmed by ELISA employing anti-his tag monoclonal antibodies. Data represent the mean ± the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein was compared to its binding to gelatin by the two-tailed t test (*P < 0.05, **P < 0.01 and ***P < 0.001). OmpL1 dose-dependent binding experiments to laminin (C) and to plasma fibronectin (D): each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control. The equilibrium dissociation constant (K_D) value is depicted for each ligand in C.
and D, and was calculated based on ELISA data for the OmpL1 recombinant protein that reached equilibrium at a given concentration. (E) Immobilized laminin was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark followed by OmpL1 interaction. The mean absorbance values at 492 nm (± the standard deviations of three independent experiments) were compared to those obtained with untreated laminin (0 mM). (F) Effect of pre-immune and mice polyclonal anti-OmpL1 serum dilution upon the binding of OmpL1 with laminin or plasma fibronectin, compared to the binding in the absence of antibodies (*P<0.05; **P<0.01).

**Figure 6. OmpL1 binds to PLG and generates active plasmin.** (A) Wells were coated with 1 μg of PLG, factor H, complement mix, C4bp and the control proteins gelatin, BSA and fetuin. 1 μg of the recombinant protein was added per well and the binding was measured by ELISA. Data represent the mean ± the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein to the serum components was compared to its binding to all negative controls by the two-tailed t test, although the P value given here is relative to comparison against gelatin (**P<0.01). (B) Significant binding activity was confirmed by ELISA employing anti-his monoclonal antibodies. Data represent the mean ± the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein was compared to its binding to gelatin by the two-tailed t test (**P<0.01 and ***P<0.001). (C) OmpL1 dose-dependent binding experiments: each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as negative control. The equilibrium dissociation constant (K_D) is depicted and was calculated based on ELISA data for the OmpL1 recombinant protein that reached equilibrium at a given concentration. (D) Binding of OmpL1 to PLG was carried out in the presence of NaCl concentration from 137 to 437...
mM and in presence or absence (no inhibition) of the lysine analogue 6-aminocaproic acid (ACA).

The bound PLG was detected and quantified by specific antibodies. Bars represent the mean absorbance at 492 nm ± the standard deviation of triplicate and are representative of two independent experiments. For statistical analyses, the attachment of recombinant protein in the presence of ACA was compared to its binding to PLG without ACA (no inhibition) by the two-tailed t test (**P<0.01). (E) Effect of pre-immune and mice polyclonal anti-OmpL1 serum dilution upon the binding of OmpL1 with PLG, compared to the binding in the absence of antibodies (*P<0.05; **P<0.01). (F) Plasmin generation by PLG bound to recombinant protein, indirectly measured by cleavage of specific plasmin substrate, was assayed by modified ELISA; Immobilized recombinant OmpL1 protein received the following treatment: PLG+uPA+specific plasmin substrate (PLG+uPA+S), or controls lacking one of the three components (PLG+uPA; PLG+S; uPA+S). BSA and Lsa63 were employed as negative controls. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, ± the standard deviation of three replicate for each experimental group and are representative of two independent experiments. Statistically significant differences are shown in comparison to BSA (*P<0.05).

Figure 7. Binding inhibition of leptospires to ECM or to PLG by OmpL1 and competition assays of OmpL1-binding to ECM and to PLG. (A) Laminin, plasma fibronectin or PLG (1 μg/well) were adsorbed to microtiter plates followed by incubation with increasing concentrations of OmpL1 (0 to 1.5 μM) for 90 min at 37°C. After washings, live leptospires (100 μL/well of 4 x 10^7) were added and the quantification of bound leptospires was performed indirectly by anti-LipL32 antibodies produced in mice (1: 4,000 dilution), followed by HRP-conjugated anti-mouse IgG antibodies. Each point represents the mean absorbance value at 492 nm ± the standard deviation of
three replicates. Data are representative of 2 independent experiments (*P <0.05). The effect of the
component on each other interaction to OmpL1 was performed by competition assays with the
addition of increasing masses of different components (0 to 1.00 μg in 100μL PBS); (B) The effect
of PLG or laminin on the binding of OmpL1 (10 μg/mL) to immobilized plasma fibronectin (10 μg/mL) was performed with the addition of increasing PLG or laminin masses (0 to 1.00 μg in 100μL PBS); (C) The effect of laminin and plasma fibronectin on the binding of OmpL1 to immobilized PLG and (D) the effect of PLG and plasma fibronectin on the binding of OmpL1 to immobilized laminin. Statistically significant interference was determined by the two-tailed t test (**P<0.01).