Review Article

Plasminogen Binding Proteins and Plasmin Generation on the Surface of *Leptospira* spp.: The Contribution to the Bacteria-Host Interactions

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Leptospirosis is considered a neglected infectious disease of human and veterinary concern. Although extensive investigations on host-pathogen interactions have been pursued by several research groups, mechanisms of infection, invasion and persistence of pathogenic *Leptospira* spp. remain to be elucidated. We have reported the ability of leptospires to bind human plasminogen (PLG) and to generate enzimatically active plasmin (PLA) on the bacteria surface. PLA-coated *Leptospira* can degrade immobilized ECM molecules, an activity with implications in host tissue penetration. Moreover, we have identified and characterized several proteins that may act as PLG-binding receptors, each of them competent to generate active plasmin. The PLA activity associated to the outer surface of *Leptospira* could hamper the host immune attack by conferring the bacteria some benefit during infection. The PLA-coated leptospires obstruct complement C3b and IgG depositions on the bacterial surface, most probably through degradation. The decrease of leptospiral opsonization might be an important aspect of the immune evasion strategy. We believe that the presence of PLA on the leptospiral surface may (i) facilitate host tissue penetration, (ii) help the bacteria to evade the immune system and, as a consequence, (iii) permit *Leptospira* to reach secondary sites of infection.

1. Introduction

The spirochete *Leptospira interrogans* is a highly invasive pathogen and the causal agent of leptospirosis, one of the most widespread zoonosis of human and veterinary concern. The disease represents a great economic burden because it has an effect on the public health system and the livestock [1–4]. The disease occurs mainly in urban regions lacking adequate sanitary conditions, associated with activities that involve direct contact with contaminated water, soil, or animals [3, 5, 6]. The leptospires chronically infect mammal species, which harbor the bacteria in their renal tubules, shedding them through the urine into the environment, thus constituting a source of reinfection to other animals. Humans are accidental and terminal hosts in the transmission process of leptospirosis [1, 7]. The leptospires enter the body via abrasions on skin or actively through mucosa, spreading to any tissue, but specially colonizing kidneys and liver [2].

The understanding of molecular aspects of the pathogenesis, the virulence, and invasion processes by which the leptospires infect the hosts and initiate tissue colonization remains to be elucidated, despite the availability of genomic sequencing of five strains of *Leptospira* that have identified more than 200 putative membrane proteins that might be involved in pathogenesis [8–12]. Yet, only few virulence

factors related to the pathogenesis of the disease have been reported [13–16].

The ability of the leptospires to adhere to extracellular matrix (ECM) proteins is assumed to be crucial during the first steps of pathogenesis [17]. Indeed, this ability has been recently and firstly described by our group [18] and a number of adhesins, ECM-binding proteins, have been identified [15, 18–26]. After leptospiral adherence, the next fundamental step must be to overcome the barriers imposed by epithelial tissues and extracellular matrixes, to reach the bloodstream and secondary sites of infection within the hosts. Proteolytic activity has been demonstrated to be important during the penetration of several pathogenic microorganisms [27]. Because the presence of ECM degrading enzymes is limited to few bacterial pathogens, one of the strategies employed to surmount this deficiency is the interaction with protease-mediated systems of the hosts [27].

Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has PLG as a main component. PLG is a circulating single-chain zymogen that is converted to plasmin by cleavage of the peptide bond between Arg-560 and Val-561, mediated by PLG activators-like uPA (urokinase-type PLG activator) and tPA (tissue-type PLG activator). Once activated to plasmin the bacteria are endowed with membrane-associated proteolytic activity, a characteristic that have been demonstrated to contribute to the degradation of ECM components, tissue penetration, and invasion. It has been reported that several pathogens, including the spirochetes *Borrelia* spp. and *Treponema* spp., bind PLG on the surface and converts it to plasmin by host activators [28–30]. The binding of PLG and plasmin generation promotes ECM molecules degradation and it is essential for dissemination of the bacteria through the host tissues, thus suggesting its importance in invasiveness process [27, 31–35].

In this paper, we highlight and compile the recent studies performed by our group describing and characterizing the leptospiral binding to PLG [36], the identification of possible PLG receptors [37–40], and the aspect of the leptospiral immune escape strategy associated to plasmin proteolytic activity [41]. The disclosure of this proteolytic system on the surface of *Leptospira* spp. and the implications associated to bacterial infectivity are discussed.

2. Materials and Methods

2.1. Bacteria Isolates and Culture Conditions. Virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 is routinely cultured at Faculdade de Medicina Veterinária da Universidade de Sao Paulo by iterative passages in Golden Syrian hamsters for maintenance of virulence [1]. The organs-derived leptospires were cultured at 28°C in semisolid modified Elinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% rabbit sera. Nonvirulent culture-attenuated *L. interrogans* serovar Copenhageni strain M20 are equally routinely cultured by maintenance in liquid-modified EMJH medium supplemented with 10% rabbit sera [42]. For the experiments

of PLG binding, to exclude the PLG interference from the rabbit serum supplementing the culture medium, serum-free leptospires were obtained by three passages in liquid modified EMJH medium supplemented with 10% *Leptospira* enrichment EMJH (BD, Difco), cultured at 28°C.

2.2. Labeling of Leptospires with Plasmin. The leptospires were treated with PLG and uPA based in the protocol described by Coleman et al. [43], with modifications. A total of 7.0×10^9 leptospires were centrifuged at $6,000 \times$g for 10 min at 25°C, resuspended in 1.4 mL of EMJH culture medium supplemented with 10% *Leptospira* enrichment EMJH, divided into 7 aliquots of 0.2 mL each (1.0×10^9 leptospires) in 2 mL microcentrifuge tubes, and recentrifuged. The 7 tubes containing the leptospires received different treatments: (a) 30% plasma and 3 U uPA (urokinase, Sigma) in 100 μL low-salt PBS (lsPBS—with 50 mM NaCl), (b) 5 μg PLG (native PLG purified from human plasma was from Calbiochem) and 3 U uPA in 100 μL lsPBS, (c) 2 μg PLG and 3 U uPA in 100 μL lsPBS, (d) 0.5 μg PLG and 3 U uPA in 100 μL lsPBS, (e) 5 μg PLG in 100 μL lsPBS, (f) 3 U uPA in 100 μL lsPBS, and (g) 100 μL lsPBS. All the preparations were incubated for 1 h at 37°C, under gentle shaking, prior to the addition of the uPA, and followed by 1 h incubation at 37°C. Leptospires were then centrifuged and washed three times with 0.7 mL lsPBS.

2.3. Measurement of Enzymatic Activity of Plasmin-Coated Leptospires. The treated leptospires (1.0×10^9 per sample) were resuspended in 300 μL lsPBS and divided into 3 aliquots. Each aliquot received 100 μL of 0.5 mg/mL of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in lsPBS, to a final substrate concentration of 0.25 mg/mL. The suspensions were incubated for 1.5 h at 37°C, under gentle shaking, and then centrifuged at $6,000 \times$g for 10 min at 25°C. The supernatants (150 μL) were transferred to 96-well microplates and the cleavage of the specific plasmin substrate was quantified with a microplate reader set at a wavelength of 405 nm.

2.4. 6-Aminocaproic Acid (ACA) Binding-Inhibition Assay. Low-passage virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (1.0×10^9 leptospires/sample) was treated with PLG and uPA, as described above, except for the addition of increase concentrations of ACA (Sigma) ranging from 0 to 1,000 mM, added together with the PLG. PLG quantity was set at 5 μg and uPA at 3 U. Aliquots treated with only PLG, uPA, or ACA, and just lsPBS treated, were used as controls. The enzymatic plasmin activity was measured as described before.

2.5. Liquid-Phase Immunofluorescence Assay (L-IFA). Live bacteria suspensions (2.5×10^9) were harvested at $12,800 \times$ g for 15 min, washed twice with lsPBS, resuspended in 200 μL lsPBS containing 8 μg of human PLG, and incubated for 45 min at 37°C. After the incubation, 6 μg/mL of propidium iodide (Sigma) were added to stain the nuclei, and the suspensions were incubated for more 45 min at 37°C. After

this time, the leptospires were gently washed three times with lsPBS and incubated for 45 min at 37°C with mouse-produced antiserum against human PLG (Sigma) at a 1 : 50 dilution. The leptospires were washed three times and incubated with goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma) at a dilution of 1 : 50 for 45 min at 37°C. After this incubation, the leptospires were washed twice and resuspended in lsPBS-antifading solution (ProLong Gold; Molecular Probes). The immunofluorescence-labeled leptospires were examined by the use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany). As control for cell integrity, we used antibodies against recombinant *Leptospira* proteins LipL32 or GroEL, produced in mice, following all the mentioned procedures for PLG.

2.6. SDS-PAGE and Affinity Blotting. Total leptospiral protein extracts for SDS-PAGE were prepared from 10 mL of $\sim 10^9$ bacteria in EMJH serum-free cultures. The cells were harvested by centrifugation, washed three times with 5 mM $MgCl_2$ in lsPBS, and resuspended in 100 μL PBS. The proteins were loaded into 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in semidry equipment. The membranes were blocked for 2 h at 37°C with 5% BSA, washed three times (10 min for each wash) with PBS-0.5% Tween-20 solution (PBS-T), and incubated overnight with 3 μg/mL PLG or 3 μg/mL PLG + 100 mM ACA at 4°C, followed by a 2 h incubation at room temperature. Then, the membranes were washed three times and incubated with mouse anti-human PLG (1 : 750, Sigma) for 3 h at room temperature, following by more than three washings and 1 h incubation at room temperature with anti-mouse IgG-peroxidase conjugated (1 : 5,000, Sigma). The membranes were washed and the protein's reactivity was revealed by ECL reagent (GE Healthcare) with subsequent exposition to X-Ray films (Kodak).

2.7. Assay for the Degradation of Immobilized ECM Macromolecules. 96-well plates were coated overnight at 37°C with 0.5 μg/well of cellular fibronectin (Sigma) or laminin (Sigma), washed four times with 200 μL per washing with PBS-T, and blocked with 2% BSA in PBS-T for 2 h at 37°C, followed by two washings. The spirochetes (1.0×10^8 leptospires/per sample) were treated as described above with 10 μg PLG and 3 U uPA or lsPBS (untreated). Bacteria were washed, resuspended in 100 μL lsPBS, and transferred to the plate previously coated wells. The plates were centrifuged at 180 $\times$g for 15 min to ensure the contact of the leptospires with the immobilized ECM components. The plates were incubated at 37°C for 20 h and washed 5 times to remove the bacteria. The degradation of the ECM components were detected by reduction in absorbance followed by incubation with antifibronectin or antilaminin IgG antibodies (1 : 5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma), anti-IgG peroxidase conjugated antibodies (1 : 5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma), and 100 μL/well of 1 mg/mL *o*-phenylenediamine—OPD—in citrate phosphate buffer, pH 5.0 plus 1 μL/mL H_2O_2. The reaction was stopped

with 50 μL/well 4N H_2SO_4, and the absorbance was measured at 492 nm. Percentage degradation was calculated by de formula $(A - B)/A(100)$, where A = the mean PBS samples absorbance (positive control group) and B = the mean experimental group absorbance (untreated or plasmin).

2.8. Bioinformatics Characterization of the Proteins. Predicted coding sequences (CDSs) were analyzed as their cellular localization predictions by PSORT program, http://psort.hgc.jp/ [44, 45]. The SMART http://smart.embl-heidelberg.de/ [46, 47], PFAM http://www.sanger.ac.uk/resources/software/ [48], and LipoP, http://www.cbs.dtu.dk/services/LipoP/ [49] web servers were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs.

2.9. Cloning, Expression, and Purification of Recombinant Proteins. Amplification of the CDSs was performed by PCR from *L. interrogans* serovar Copenhageni genomic DNA using complementary primer pairs. The gene sequences were amplified without the signal peptide tag and predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). The final constructs were verified by DNA sequencing on an ABI Prism 3730_L sequencer (Seq-Wright, Houston, TX, USA) with appropriate vector-specific T7 (F: TAATACGACTCAC-TATAGGG) and pAE (R: CAGCAGCCAACTCAGTTCCT) primers. Cloning, expression, and purification of the recombinant proteins have been previously described as summarized in Table 1.

2.10. Plasminogen Binding to Recombinant Proteins. The binding of the recombinant proteins to PLG was evaluated by a modified ELISA, based in the protocol described by Brissette et al. [50], as follows: 96-well plates (Costar High Binding, Corning) were coated overnight in PBS at 4°C with 100 μL of 10 μg/mL of the recombinant proteins or bovine serum albumin (BSA, Sigma) as negative control. Plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) nonfat dry milk. The blocking solution was discarded and 100 μL of 10 μg/mL human PLG (Calbiochem) in PBS was incubated for 2 h at 37°C. Wells were washed four times with PBS-T and incubated for 1 h at 37°C with mouse anti-human PLG (1 : 4,000 in PBS, Sigma). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG, Sigma), diluted 1 : 5,000 in PBS. After three washings, 100 μL/well of 1 mg/mL OPD plus 1 μL/mL H_2O_2 in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 μL/well of H_2SO_4 (4 N). Readings were taken at 492 nm.

2.11. Enzymatic Activity Assay of Plasmin Bound to Recombinant Proteins. ELISA plates were coated overnight with 10 μg/mL recombinant proteins or BSA in PBS at 4°C. Plates were then washed once with PBS-T and blocked with PBS with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The blocking solution was discarded and 100 μL/well of 10 μg/mL human PLG (Calbiochem) was added, followed by

TABLE 1: Leptospiral selected proteins for characterization of the binding to PLG. Gene locus, protein name, NCBI reference sequence, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Gene locu[1]	Recombinant protein given name	NCBI reference sequence number[2]	Description/Function	Conservation (identity)[3]	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC11352	LipL32[a,h]	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100%); LBH (98%)	F: 5′ CACCGGTGCTTTCGGTGGTCTG 3′ / R: 5′ ATTACTTAGTCGCGTCAGAAGC 3′	30.2
LIC10314	Lsa63[b,h]	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98%); LBH (87%); LBP(39%)	F: 5′ GGATCCTTATTTTCTCAGGAAAG 3′ (BamH I) / R: 5′ GGTACCCTAAGGTTTAATTTTTTT 3′ (Kpn I)	63.0
LIC10509	rLIC10509[c,h]	YP_000493	Putative lipoprotein	Lai (98%)	F: 5′ CCGGGGATCCAAAAAGAGAGCAAAGAAAG 3′ (BamH I) / R: 5′ GGTACCCTACTCGAGACAGCCAGGACCTTC 3′ (Kpn I)	22.0
LIC12892	Lp29[d,h]	YP_002808	Putative lipoprotein		F: 5′ CTCGAGGCAGTACATTACAATCTTGCT 3′ (Xho I) / R: 5′ CCATGGCTCTTAGGAGCCTGGAAA 3′ (Nco I)	29.6
LIC10793	Lp49[d,h]	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5′ CTCGAGAGCGGAGACTTTTCTTTACTT 3′ (Xho I) / R: 5′ CCATGGTTAAAAACCATCTCTACGATAAAC 3′ (Nco I)	49.1
LIC12895	Lsa27[e,h]	YP_002811	Putative lipoprotein	Lai (79%)	F: 5′ GGATCCCTGAAATATACGAA 3′ (EcoRI) / R: 5′ GAATTCTTACTGTTCTCCTTC 3′ (BamHI)	27.0
LIC13131	MPL21[a,f,h]	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5′ CACCAGTCTCAAAGTTACGCTTCAG 3′ / R: 5′ TTCTCACCATCCAGCTCGG 3′	21.9
LIC10765	MPL17[a,f,h]	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%); LBP (41%)	F: 5′ CACCGAAAGTCCCGTAAGGTTCAAA 3′ / R: 5′ TGCAGGAGTTCCCACATTTTA 3′	15.4
LIC10091	LipL40[a,h]	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5′ CCATGGGACTCGAGACGCCTCCTCCTAAAGATCC 3′ / R: 5′ CTCCATGGTCATTTCAAAACTTCTACGGGGC 3′	39.0
LIC10054	MPL36[a,h]	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%); LBP (50%)	F: 5′ CACCACGTCTTGTCGCTCGGGTAGAG 3′ / R: 5′ CCAAGTATTCTATTTATACGTCCGAG 3′	35.1
LIC10494	rLIC10494[g,h]	YP_000478	Putative lipoprotein	Lai (99%)	F: 5′ CACCACTGCTAGGGCTGCAGAAA 3′ / R: 5′ ACTTTGAGAGCTTCGTCTCGT 3′	25.8
LIC12730	rLIC12730[g,h]	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%); LBP(37%)	F: 5′ CACCAGTTCTGACGGACTTCCCAA 3′ / R: 5′ TCTTGCGAATGAGTTGATCC 3′	77.4

Table 1: Continued.

Gene locu[1]	Recombinant protein given name[2]	NCBI reference sequence number[2]	Description/Function	Conservation (identity)[3]	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC12922	rLIC12922[h]	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%); LBP (48%)	F: 5′ CACCGAATCACTCAACAGAGTCATTGC 3′ R: 5′ ATCAATCTAAATGAAACGTCTCTTC 3′	40.0
LIC12238	rLIC12238[h,i]	YP_002173	Hypothetical protein	Lai (99%); LBH (77%); LBP (39%)	F: 5′ CTCGAGTGTTTAAACCTACCGGAG 3′ (Xho I) R: 5′ AAGCTTCTACTTCATCGCTTTTTCTATATC 3′ (Hind III)	17.6
LIC10258	Lsa66[i]	YP_000249	Hypothetical protein with ompA domain	Lai (99%); LBH (79%)	F: 5′ GGATCCGAAGCCTTCTCACCCAATTG 3′ (BamH I) R: 5′ CCATGGTAAAGTGAAAGATAAAATCGATTC 3′ (Nco I)	65.7
LIC12880	Lp30[i]	YP_002796	Putative lipoprotein	Lai (99%); LBH (76%)	F: 5′ CTCGAGGAAGTTGTCCGAGTCTAT 3′ (Xho I) R: 5′ CCATGGTTATTGATTGTTTAATTCAG 3′ (Nco I)	30.7
LIC11469	Lsa20[i]	YP_001430	Hypothetical protein	Lai (100%); LBH (82%); LBP (29%)	F: 5′ CTCGAGCCAATTTCTTTCGATCCAAATC 3′ (Xho I) R: 5′ AAGCTTTCAATCCTCACTGCAGCCC 3′ (Hind III)	20.0
LIC11030	rLIC11030[g,j]	YP_001000	Putative lipoprotein with unknown domain (DUF1565)	Lai (100%)	F: 5′ CTCGAGTGTACAAACGACAAAAAGAAGGT 3′ (Xho I) R: 5′ AAGCTTTTAGTTGCAAGGATTTGGA 3′ (Hind III)	37.0
LIC11834	rLIC11834[k] (Lsa33)	YP_001783	Putative lipoprotein	Lai (99%); LBH (87%); LBP (31%)	F: 5′ CTCGAGGATCTACAAGGTGGGGTTTTTAC3′ (Xho I) R:5′ CCATGGTTACTGAGGTTTTACTTGGTCC3′ (Nco I)	33.1
LIC12253	rLIC12253[k] (Lsa25)	YP_002188	Conserved hypothetical protein	Lai (100%); LBH (77%); LBP (39%)	F: 5′ CTCGAGGAGGAGAAACCGGACGATAC 3′ (Xho I) R: 5′ CCATGGTTAGGGAAGACTTCTAACACATC 3′ (Nco I)	24.0

[1] http://aeg.lbi.i.c.unicamp.br/world/lic/.
[2] http://www.ncbi.nlm.nih.gov/protein/.
[3] Protein BLAST—http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.
[a] Previously published by Gamberini et al. [78];
[b] Previously published by Vieira et al. [37];
[c] Previously published by Gómez et al. [79];
[d] Previously published by Neves et al. [80];
[e] Previously published by Longhi et al. [19];
[f] Previously published by Oliveira et al. [81];
[g] Previously published by Barbosa et al. [18];
[h] Previously published by Vieira et al. [37];
[i] Previously published by Oliveira et al. [38];
[j] Previously published by Mendes et al. [39];
[k] Previously published by Domingos et al. [40];
Lai: *L. interrogans* serovar Lai [8]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [11]; LBP: *L. biflexa* serovar Patoc [12].

incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma) were added. Subsequently, 100 μL/well of the plasmin-specific substrate D-valyl-leucyl-lysine 4-p-nitroanilide dihydrochloride (Sigma) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate was measured by taking readings at 405 nm.

2.12. Dose-Response Curves and Dissociation Constant of the Proteins-PLG Interactions.

ELISA plates were coated overnight in PBS at 4°C with 100 μL of 10 μg/mL PLG. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0-1 μM) were added (100 μL/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at appropriate dilutions, followed by horseradish peroxidase-conjugated anti-mouse IgG (1 : 10,000 in PBS, Sigma). The binding was evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [51] and Lin et al. [52], based on the equation: $A = A_{max} [\text{protein}]/(K_D + [\text{protein}])$, where A is the absorbance at a given protein concentration, A_{max} 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).

2.13. Two-Dimensional Gel Electrophoresis (2-DE Gels).

Virulent low passage leptospires culture was harvested by centrifugation at 12,800 ×g at 4°C for 10 min. Pellet was washed (×5) by resuspension in PBS containing 5 mM MgCl$_2$. The pellet was resuspended in DeStreak rehydration solution(GE Healthcare, USA) and lysed by vigorous vortexing. The cellular debris was separated by centrifugation at 20,800 ×g for 10 min, and the supernatant was collected. Total protein content was determined according to the Bradford method (Pierce Biotechnology, USA). Samples containing 200 μg of protein were adjusted to 125 μL with DeStreak rehydration solution(GE Healthcare, USA), along with 0.8% (v/v) IPG buffer, with a pH range of 3–10 (GE Healthcare).

First-dimension isoelectric focusing was performed using the IPGphor-System (GE Healthcare, USA), and the second dimension was conducted on the Ettan DALT*six* system (GE Healthcare). The IPG gel strips (7 cm) with a linear separation of immobilized pH ranging from 3 to 10 were rehydrated directly with the solubilized samples. The focusing protocol was 30 V for 180 Vh, 150 V for 300 Vh, 350 V for 350 Vh, 500 V for 500 Vh, 1000 V for 1,000 Vh, 3,000 V for 3,000 Vh, and 5,000 V for 40,000 Vh, with a 50 μA/strip maximum-setting at 20°C. The strips were equilibrated twice (reduced and alkylated) for 15 min in 15 mL equilibration solution (0.05 M Tris-HCl, pH 8.8, 6.0 M urea, 30% [v/v] glycerol, and 2% [w/v] SDS), first with the addition of 1% DTT, and finally with 2.5% iodoacetamide. After equilibration, the strips were attached to the 12%

SDS-PAGE. The gels were stained by Coomassie Blue R350 (PhastGel BlueR-GE Healthcare). The gels were analyzed with Image Master-2D Platinum version 6.0 software (GE Healthcare, USA).

2.14. Mass Spectrometry and Protein Identification.

The samples were analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry, using α-cyano-4-hydroxycinnamic acid as the matrix on an Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, USA). The spots preparation, mass spectrometry, and database search for protein identification were performed according to the protocol already described in Vieira et al. [53]. Prediction for protein localization was performed by LipoP program [49].

2.15. Human Sera and Microscopic Agglutination Test.

Confirmed-leptospirosis serum samples were obtained from Instituto Adolfo Lutz collection, Sao Paulo, Brazil, as previously described [41]. In brief, 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 the titer that was the highest sample dilution with 50% of agglutination. MAT (microscopic agglutination test) was considered negative when the titer was below 100. In addition to the MAT-negative and MAT-positive paired samples of the same individuals with laboratory and clinic leptospirosis confirmations, we also employed sera from normal healthy donors (normal human sera—NHS), without a known history of leptospirosis, with confirmed negative MAT.

2.16. Human IgG and C3b Deposition on Leptospires.

L. interrogans serovar Pomona strain LPF (2.5×10^7 cells/well) were diluted in 50 μL lsPBS, coated onto microplates, and allowed to stand at 37°C for 1 h, followed by overnight at 4°C. The plates were washed twice with 200 μL lsPBS and blocked with 5% nonfat dry milk and 2.5% BSA for 2 h at 37°C. After addition of 50 μL of pooled sera/well (NHS, MAT-negative or MAT-positive) as a source of IgG, the plates were allowed to incubate for 30 min on ice, and then washed three times. The coated leptospires were treated with 50 μL/well of 5 μg/mL uPA (urokinase, Sigma) and 40 μg/mL PLG (Calbiochem) for 2 h at 37°C, or only with uPA, only with PLG, or no additions, as negative controls. After three washings, the plates were incubated with anti-human IgG Fc region specific antibodies (Calbiochem) at a dilution of 1 : 5,000 for 1 h at 37°C, and then with secondary antibodies conjugated with peroxidase at a dilution of 1 : 10,000 for 1 h at 37°C. The IgG depositions on leptospires were evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. When dilutions of NHS were used, they were performed with lsPBS.

The protocol of C3b deposition on leptospires was similar to the IgG deposition described before, with exception of the incubation with NHS, placed at 37°C for 50 min, and use of anti-human C3b primary antibodies (1 : 5,000)
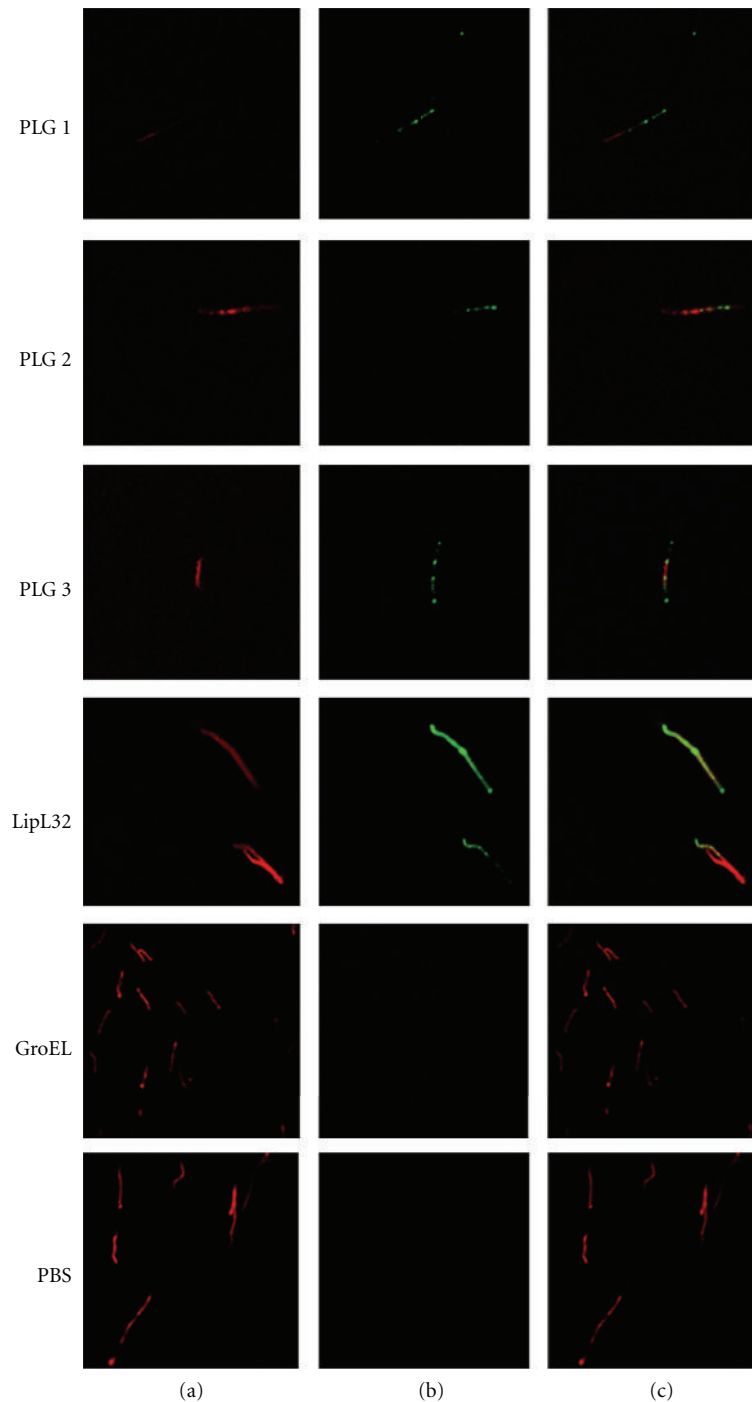
FIGURE 1: Recognition of PLG binding to *Leptospira* by L-IFA. Live virulent *L. interrogans* serovar Copenhageni isolates were treated with PLG, and the recognition was assessed through polyclonal anti-PLG antibodies under a confocal immunofluorescence microscope (PLG 1 to 3). PLG-treated leptospires stained for the proteins LipL32 (outer surface protein marker) or GroEL (a protoplasmic-cylinder marker) and untreated leptospires (PBS), are shown as controls (a) DNA propidium iodide-stained, (b) FITC-stained and (c) A+B composite images.

(Calbiochem), followed by secondary antibodies conjugated with peroxidase (1 : 5,000).

2.17. Human C3b Degradation by Leptospires. L. interrogans serovar Pomona strain LPF (10^8 leptospires/sample) were treated in 200 μL lsPBS with the addition of 10 μg PLG and 3 U uPA (plasmin), 10 μg PLG, or no additions (untreated). The cells were incubated for 1 h at 37°C with the PLG, and for one more hour after the addition of uPA. The cells were washed three times with lsPBS, and then resuspended in 100 μL lsPBS containing 15 μg/mL human purified C3b (Calbiochem), being incubated for 20 h at 37°C. The leptospires were removed by centrifugation, 20 μL of the supernatants
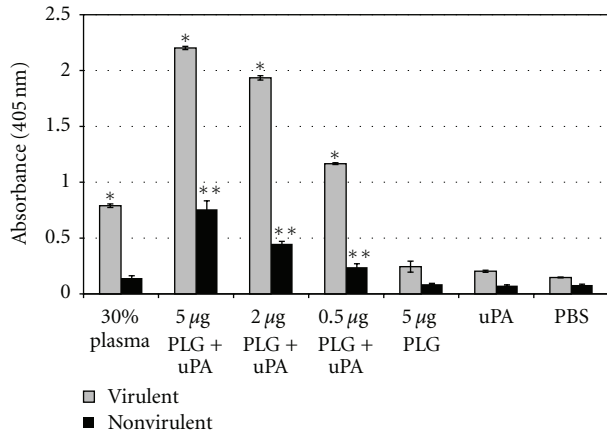
Figure 2: Cleavage of the plasmin-specific chromogenic substrate by plasmin-bound leptospires. Live low-passage virulent and high-passage nonvirulent *L. interrogans* serovar Copenhageni cells received the following treatments: PBS only (PBS), uPA alone (uPA), 5 μg PLG alone (PLG), PLG in crescent quantities (0.5, 2 and 5 μg) together with uPA, and 30% human plasma together with uPA (30% plasma). Bars represent mean absorbance as a measure of relative substrate degradation ± the standard deviation of three replicates for each experimental group and are representative of three independent experiments. *Virulent leptospires experiments: statistically significant ($P < 0.0001$) in comparison to the PBS control; **nonvirulent leptospires experiments: statistically significant ($P < 0.01$) in comparison to the PBS control. The 30% plasma, as PLG source, and all the PLG + uPA samples of the virulent leptospires were statistically significant ($P < 0.001$) in comparison to the same nonvirulent bacteria tested samples.
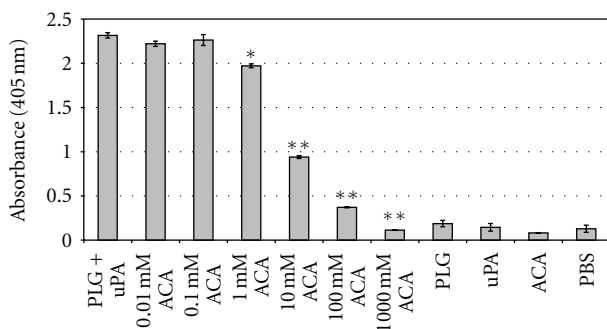

Figure 3: Inhibition of PLG binding to *L. interrogans* by ACA. Low-passage virulent *L. interrogans* serovar Copenhageni cells were treated with the following: PLG together with uPA (PLG + uPA), PLG together with uPA with the addition of crescent concentrations of ACA (0.01 to 1,000 mM ACA), PLG alone (PLG), uPA alone (uPA), ACA alone (ACA), and no additions (PBS). The cleavage of the plasmin-specific substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloridein by the treated spirochetes was measured by absorbance readings at 405 nm. Bars represent mean absorbance ± the standard deviation of three replicates for each experimental group and are representative of three independent experiments. Statistically significant substrate degradation inhibition results in comparison to the positive control (PLG + uPA) are shown: $P < 0.01$ (*) and $P < 0.0001$ (**).

were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes in semi-dry equipment. The membranes were blocked by incubating overnight at 4°C with 5% nonfat dry milk and 1% BSA. The C3b detection was made by incubations with anti-human C3b antibodies and secondary antibodies conjugated with peroxidase, followed by ECL (GE Healthcare) development.

2.18. Serum Susceptibility Testing for Treated Leptospires. Complement-mediated killing of leptospires was evaluated after incubation with nonimmune NHS, as described by Meri et al. [54], with some modifications. Approximately 5×10^8 *L. interrogans* serovar Pomona strain LPF per sample were treated with 40 μg PLG and 4 U uPA in 200 μL lsPBS for 3 h at 37°C. As a control, one aliquot of *L. interrogans* serovar Pomona strain LPF was incubated only with PBS. Then, the samples were divided into aliquots containing 1.0×10^8 cells, which were added by 80 μL NHS and 80 μL EMJH medium supplemented with 10% *Leptospira* enrichment. The bacteria suspensions were incubated at 37°C for one hour, followed by a incubation of 5 min on ice for stopping complement activation. After, 20 μL of each sample (four replicates) were transferred to microplates filled with 180 μL of EMJH medium/well. The plates were sealed with a sterile adhesive film and incubated at 30°C for 4 days. Bacterial growth was determined by counting leptospires in a Petroff-Hausser chamber under dark-field microscopy.

2.19. Statistics. For the data that were tested for statistical significance, the student's two-tailed test was applied, considering the minimum of $P < 0.05$.

3. Results and Discussion

3.1. Binding of Human Plasminogen by L. interrogans Cells. Live-immunofluorescence microscopy (L-IFA) was employed in order to evaluate the ability of *L. interrogans* cells to bind human PLG. L-IFA revealed that leptospires bind PLG in their outer surface along the entire cell, as seen when the bacteria were probed with antibodies anti-PLG (Figure 1). PLG bound to leptospires does not seem to damage the bacterial membrane because no fluorescence of GroEL, a cytoplasmatic heat-shock protein [55], was achieved when serum anti-GroEL was employed (Figure 1). The leptospiral recombinant proteins LipL32, an abundant outer membrane protein of *Leptospira* [56], used as positive control, showed an intense surface fluorescence. We also observed that the spirochete regular movement was conserved, suggesting that the flagella remained undamaged. Taken together, these data suggest that the PLG-binding does not interfere with the membrane structure or dynamics [36].

3.2. Activation and Enzymatic Activity of Plasminogen-Bound Leptospira. The binding of PLG to leptospiral surface was further analyzed by plasmin enzymatic activity. Leptospires were incubated with purified PLG, followed by plasmin activation after addition of uPA, conferring the bacteria a surface-associated plasmin activity (Figure 2), as measured
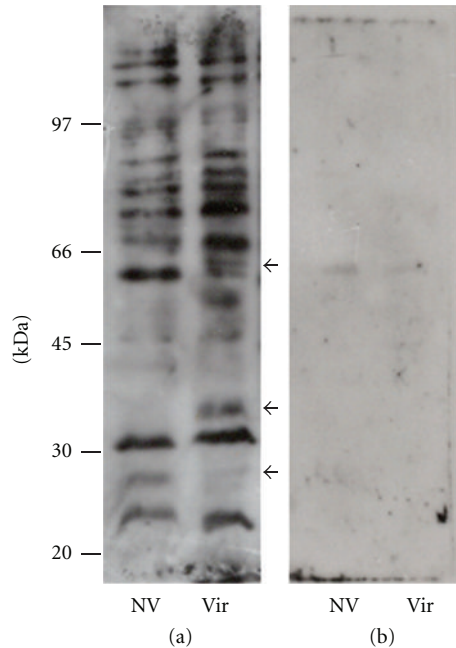
Figure 4: Binding of human PLG to *L. interrogans* proteins by affinity blotting. Total protein extracts were resolved by 10% SDS-PAGE and electroblotted into nitrocellulose membranes. The membranes were incubated with PLG (a) or PLG + ACA (b), and the binding was detected by anti-PLG antibodies and peroxidase-conjugated antibodies. The reactivity was revealed by ECL (GE Healthcare) and exposure to X-Ray films. NV: high passage, nonvirulent *L. interrogans* serovar Copenhageni strain M 20; Vir: low-passage, virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The arrows indicate reactivity protein regions seen in the virulent but absent in the nonvirulent bacterial extract. Positions of protein molecular mass markers are shown on the left.

by the degradation of plasmin's chromogenic specific substrate [36].

The labeling with PLG alone did not trigger proteolytic activity suggesting that *L. interrogans* serovar Copenhageni is dependent on the host PLG activation system, probably not possessing an endogenous mechanism, as already reported for other pathogens [57–64]. In contrast, the ability of PLG activation is shown for a number of pathogenic microorganisms [57–64].

Both the virulent and nonvirulent strains of *L. interrogans* serovar Copenhageni tested were capable of capturing PLG, although the virulent strain seems to be more efficient in the PLG-binding than the nonvirulent bacteria (∼2.5 fold) (Figure 2) [36]. PLG is present in plasma at a concentration of approximately 20.8 ± 1.9 mg/100 mL [65]. Our data shows that virulent *L. interrogans*, but not the attenuated strain, have the ability to sequester PLG from human plasma in the conditions assayed. At any rate, once in the host, the infectious leptospires can acquire PLG when reach the blood circulation.

3.3. Inhibition by 6-Aminocaproic Acid (ACA) of Proteolytic Activity Acquired by L. Interrogans.
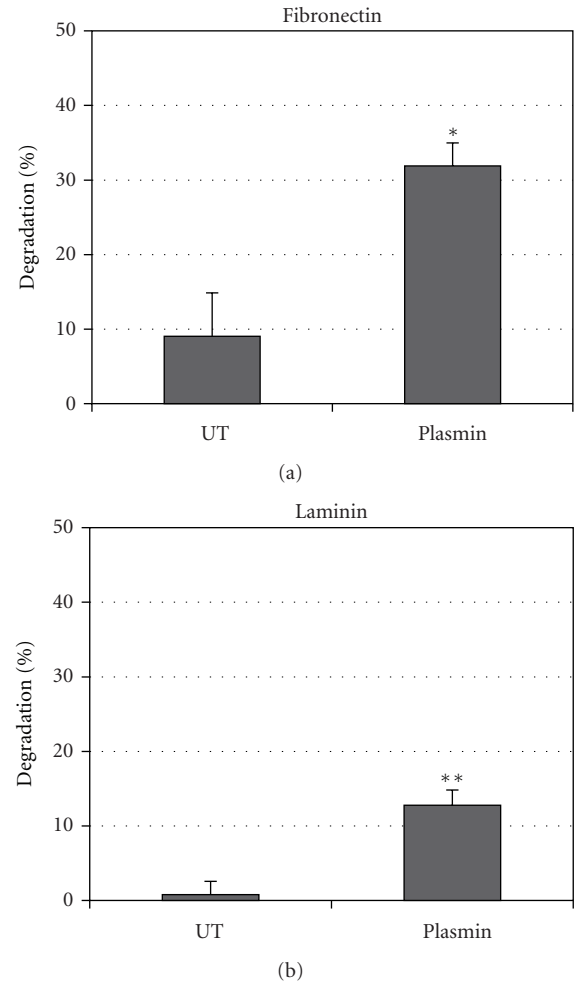
It is well known that the

Figure 5: Fibronectin and laminin degradation by plasmin-coated leptospires. Spirochetes were incubated in lsPBS with no additions (UT) and with addition of PLG and uPA (plasmin). ELISA plate wells coated with $0.5 \mu g$/well fibronectin (a) or laminin (b) were incubated for 20 h with the spirochetes from each experimental group to allow substrate digestion. Plates were then incubated with antibodies antilaminin or antifibronectin. A reduction in absorbance was interpreted as substrate digestion, relative to controls lacking bacteria ($0.5 \mu g$/well purified laminin or cellular fibronectin, corresponding to 0% degradation). Percent degradation (a reduction in absorbance value relative to control lacking bacteria) was calculated as described in Section 2. Bars represent mean percent substrate degradation relative to the positive control (0% degradation) $\pm$ the standard deviation of three replicates. The experiment was independently performed three times with similar results. $P < 0.001$ (*) and $P < 0.01$ (**).

lysine-binding sites of PLG kringle domains frequently mediate interactions with lysine residues of the cellular receptors [27]. ACA, a lysine analogue, decreased the proteolytic activity of virulent *L. interrogans* serovar Copenhageni incubated with PLG, in a dose-dependent manner (Figure 3). The total inhibition (100%) of plasmin activity achieved with the higher ACA concentrations employed shows that the PLG interaction with these spirochetes occurs trough the lysine-binding sites of the kringle domains [36].
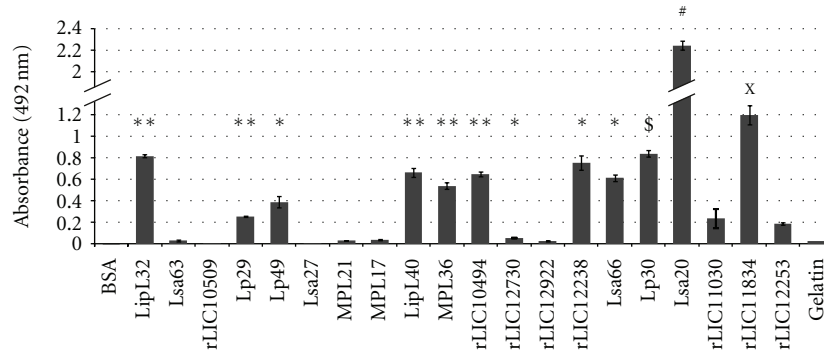
FIGURE 6: Binding of leptospiral recombinant proteins to human plasminogen. The recombinant proteins LipL32, Lsa63, rLIC10509, Lp29, Lp49, Lsa27, MPL21, MPL17, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922, rLIC12238, Lsa66, Lp30, Lsa20, rLIC11030, rLIC11834, and rLIC12253 were coated onto 96-well ELISA plates (10 μg/mL) and allowed to interact with purified human PLG (10 μg/mL). BSA and gelatin were used as a negative control for nonspecific binding. Binding was detected and quantified by specific antibodies. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of four replicates for each protein and are representative of three independent experiments. Statistically significant binding in comparison to negative control BSA are shown by $^{*}(P < 0.001)$; $^{**}(P < 0.0001)$; $^{\$}(P < 0.02)$; $^{\#}(P < 0.0005)$. The binding of rLIC11834 and rLIC12253 was compared to its binding to gelatin $^{X}(P < 0.005)$.
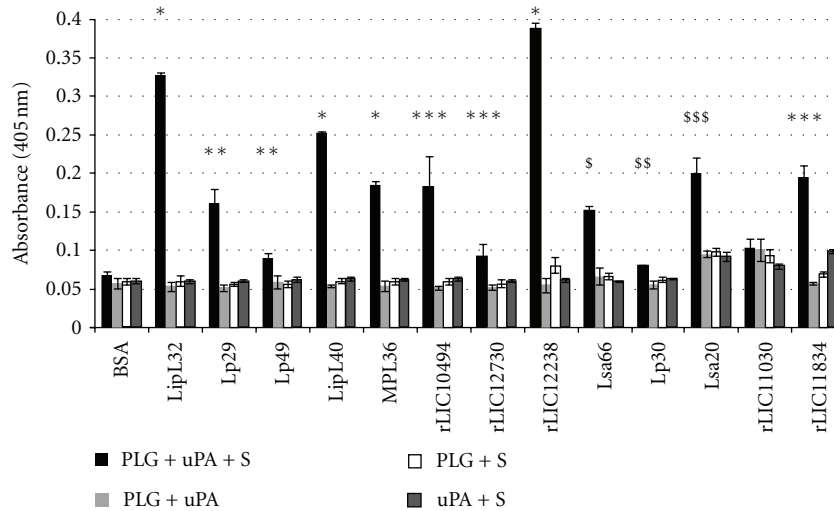

FIGURE 7: Activation of recombinant protein-PLG to enzymatically active plasmin. Cleavage of specific plasmin substrate by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG + uPA + specific plasmin substrate (PLG + uPA + S) or controls lacking one of the three components (PLG + uPA; PLG + S; uPA + S). BSA was employed as a negative control. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. Statistically significant differences are shown by $^{*}(P < 0.00001)$, $^{**}(P < 0.005)$, $^{***}(P < 0.05)$, $^{\$}(P < 0.001)$, $^{\$\$}(P < 0.02)$, and $^{\$\$\$}(P < 0.003)$.

3.4. Analysis of Plasminogen-Binding L. Interrogans Proteins on Cell Lysates.

Assessment of leptospiral proteins binding to PLG was first visualized by affinity blotting using total whole-cell lysates (Figure 4(a)). Our results are indicative that multiple leptospiral proteins are capable of binding PLG, although these affinity blotting experiments do not simulate the protein-PLG interaction *in vivo*. As expected, the data also demonstrate that there are similarities between the PLG-binding proteins of the virulent and nonvirulent strains, but also revealed the presence of some reactive bands on the low-passage virulent strain that are absent in the nonvirulent one, indicating that virulent strains have more PLG binding proteins. Thus, based on the data from enzymatic activity

and affinity blotting presented here we cannot discriminate whether the increased bound activity of the virulent strain is due to only differential expression of a different set of membrane receptors, an increased expression of the same receptors, or both. Comparable to the results obtained with the intact leptospires, the affinity blotting was inhibited by the presence of the lysine analog, ACA, supporting the central role of lysine residues of the proteins in the PLG interactions (Figure 4(b)) [36].

3.5. Plasmin-Coated Leptospira Degrades Immobilized ECM Molecules.

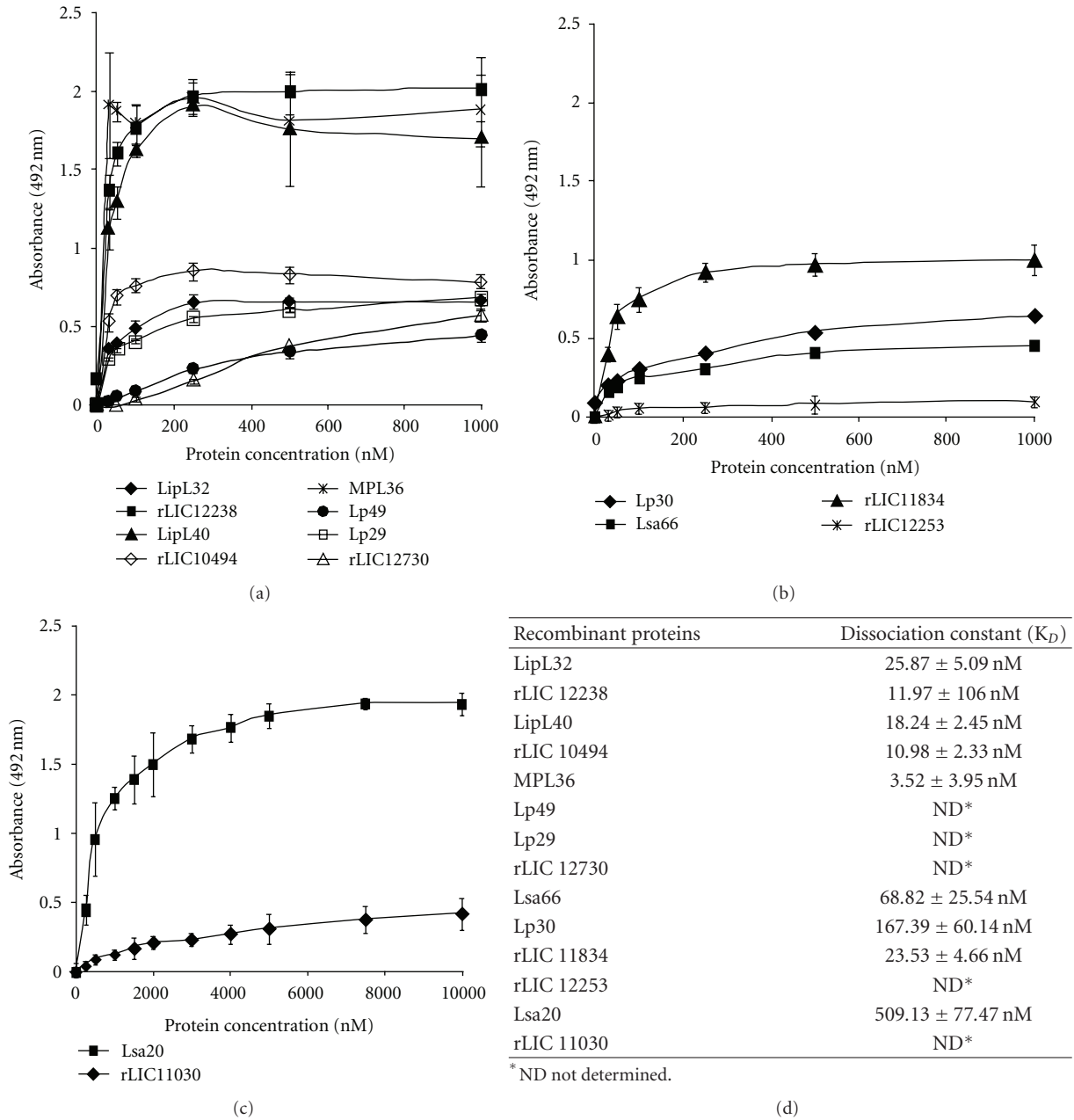Plasmin is a broad-spectrum serine protease that

(a)

(b)

(c)

Recombinant proteins	Dissociation constant (K_D)
LipL32	25.87 ± 5.09 nM
rLIC 12238	11.97 ± 106 nM
LipL40	18.24 ± 2.45 nM
rLIC 10494	10.98 ± 2.33 nM
MPL36	3.52 ± 3.95 nM
Lp49	ND*
Lp29	ND*
rLIC 12730	ND*
Lsa66	68.82 ± 25.54 nM
Lp30	167.39 ± 60.14 nM
rLIC 11834	23.53 ± 4.66 nM
rLIC 12253	ND*
Lsa20	509.13 ± 77.47 nM
rLIC 11030	ND*

*ND not determined.

(d)

FIGURE 8: Characterization of recombinant proteins binding to PLG. In (a), (b), and (c) PLG ($10\,\mu$g/mL) was immobilized in 96-well ELISA plates, and each recombinant protein at 0 to 1,000 nM was added for interaction. The binding was detected using antiserum raised in mice against each protein at appropriate dilutions (1 : 4,000 for LipL32; 1 : 5,000 for rLIC12238, LipL40, and MPL36; 1 : 1,000 for Lp29, Lp49, Lsa20, and rLIC11030; 1 : 500 for rLIC12730, Lsa66, and Lp30; 1 : 750 for rLIC11834 and rLIC12253), followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values $\pm$ the standard deviation of six replicates for each experimental group. The results are representative of two independent experiments. In (d) The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that reached equilibrium up to a concentration of 1,000 nM.

is capable to degrade directly laminin and fibronectin. To evaluate whether the plasmin associated to the leptospires surface can directly break these ECM components, purified soluble human fibronectin and laminin were adhered into 96-well plates, and the reaction was evaluated by exposing the ECM proteins with low-passage virulent *L. interrogans*, pretreated with PLG/uPA. As shown in Figure 5, the plasmin-coated leptospires were able to significantly cleave down the

immobilized ECM macromolecules, when compared to the ones exposed to untreated bacteria [36].

Given that leptospires are highly invasive pathogens that are thought to penetrate the skin or break in the skin to initiate infection, the ability of digesting ECM macromolecules via plasmin activity might be an important step for leptospiral pathogenesis. To date, this is the first proteolytic activity detected in *Leptospira* that can promote
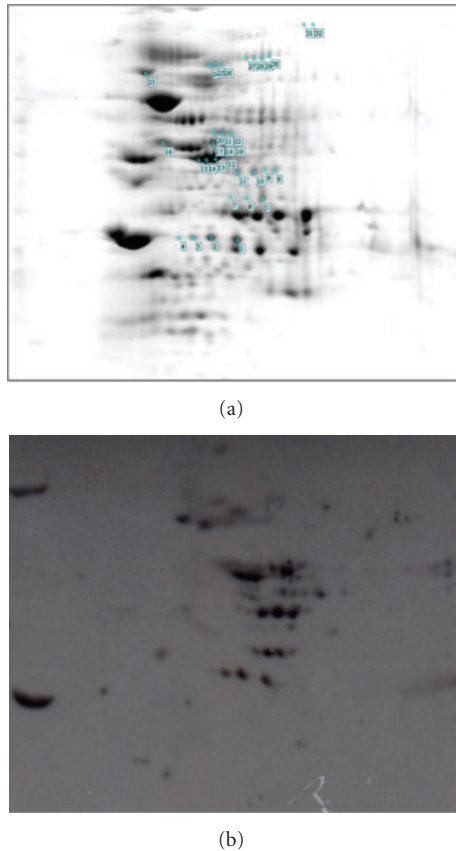
(a)

(b)

Figure 9: Two-dimensional gel analysis of *L. interrogans* PLG binding proteins. Leptospiral total cell lysates were separated by isoelectric point (pH 3–10) and molecular mass on 12% SDS-PAGE. Replicate gels were Coomassie Blue stained (a) or transferred to membranes and submitted to PLG affinity immunoblotting (b). The reactive spots are enumerated on (a). The results are representative of three independent experiments performed in duplicates.

(a)

(b)

Figure 10: Human IgG and C3b binding to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG (a) or C3b (b)were bound by incubating bacteria with 100% NHS. Bacteria were treated with PBS, $40\,\mu$g/mL PLG (PLG), $5\,\mu$g/mL uPA (uPA), or PLG + uPA (Pla). Presence of human IgG or C3b deposited on leptospires was determined by ELISA. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

ECM degradation and it is a plausible mechanism that can contribute to leptospiral invasiveness [1, 66].

3.6. Recombinant Leptospiral Proteins Bind Human Plasminogen.

In order to investigate PLG binding proteins in *Leptospira* we benefitted from the availability of several recombinant proteins of *L. interrogans* available in our laboratory. We evaluated the ability of these proteins to interact with PLG *in vitro*. Table 1 summarizes the features of these proteins and gene conservation within the sequenced genomes [8–12]. The binding of these proteins to PLG was quantified by ELISA and the results are shown in Figure 6. Proteins LipL32, Lp29, Lp49, LipL40, MLP36, rLIC10494, rLIC12730, rLIC12238, Lsa66, Lp30, Lsa20, and Lsa33 (rLIC11834) presented significant binding to PLG while proteins Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12922, rLIC11030, Lsa25 (rLIC12253), BSA, and gelatin did not show significant binding activity (Figure 6) [37–40].

3.7. Activation of Plasminogen-Bound Proteins.

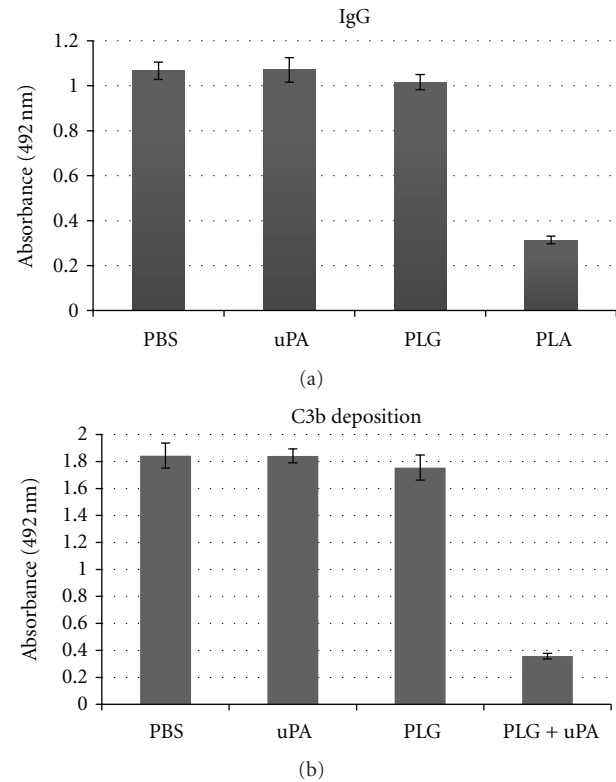To evaluate whether purified PLG-binding proteins were independently capable to generate active plasmin in the presence of uPA PLG activator, we measured the degradation of plasmin specific chromogenic substrate in a modified ELISA. The PLG captured by the proteins could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity (Figure 7). The negative control BSA, that showed no PLG binding, produced no significant proteolytic activity, as well as the controls lacking PLG, uPA, or the chromogenic substrate [37–40].

3.8. Characterization of the Binding of Recombinant Proteins to PLG.

The binding between each recombinant protein and PLG was assessed on a quantitative basis as illustrated in Figures 8(a), 8(b), and 8(c). Dose-dependent and saturable binding was observed when increasing concentrations (0 to 1,000 nM) of the recombinant proteins MPL36, LipL40, LipL32, rLIC10494, rLIC12238, Lsa66, Lp30, Lsa33 (rLIC11834), and Lsa20 was allowed to individually adhere to a fixed amount of PLG (1 μg), indicating the specificity of the binding. For the proteins Lp29, Lp49, rLIC12730, Lsa25 (rLIC12253), and rLIC11030, the saturation level was not

TABLE 2: *L. interrogans* PLG-binding proteins identified by MALDI-TOF mass spectrometry. The proteins are identified as the genomic nomenclature (LIC: *Leptospira interrogans* serovar Copenhageni), and are listed and respective NCBI reference sequence, predicted name/function and predicted cellular localization, in theoric molecular mass and isoelectric point (pI).

Gene locus[1]	NCBI reference sequence number[2]	Gene/protein function	Localization[3]	Theoric molecular mass (kDa)	pI
LIC12816	YP_002733	Hypothetical protein	Membrane	14.7	9.5
LIC10788	YP_000767	flaA-1 (Flagellar filament sheath protein)	Membrane	35.0	7.6
LIC11194	YP_001164	Putative citrate lyase	Cytoplasm	37.2	6.1
LIC12875	YP_002791	tuf (Elongation factor Tu)	Cytoplasm	43.6	5.7
LIC12795	YP_002712	Acetyl-CoA acetyltransferase	Cytoplasm	47.4	6.0
LIC12407	YP_002339	Glutamine synthetase protein	Cytoplasm	55.4	5.9
LIC10524	YP_000508	dnaK (Heat shock protein)	Cytoplasm	69.1	5.1

[1] http://aeg.lbi.ic.unicamp.br/world/lic/.
[2] http://www.ncbi.nlm.nih.gov/protein/.
[3] Lipo P: Juncker et al., [49].

reached, even at the highest concentration tested (1,000 nM). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG are depicted in Figure 8(d); for the ones that reached equilibrium, the highest and the lowest K_D values were for Lsa20 (509.13 ± 77.47 nM) and MPL36 (3.52 ± 3.95 nM), respectively, [37–40].

3.9. Proteomic Approach to Leptospiral PLG Binding Protein Identification. In order expand our knowledge on PLG binding proteins, we decided to employ two-dimensional gel electrophoresis followed by affinity immunoblotting, as an alternative strategy to identify novel leptospiral PLG-binding proteins. Total protein extracts of low passage *L. interrogans* were separated by isoelectric point and molecular mass (Figure 9(a)), transferred to membranes, and incubated with PLG and anti-PLG antibodies for detection of reactive spots (Figure 9(b)). The reactive spots were excised and prepared for MALDI-TOF mass spectrometry and protein identification. Under the conditions assayed, we could identify seven proteins capable of binding PLG, as depicted in Table 2. Two were annotated as membrane and five as cytoplasmatic proteins (Table 2). Along with the membrane proteins, one is hypothetical and the other is a putative flagellar filament sheath protein (FlaA-1); the cytoplasmic proteins are citrate lyase, Tuf (elongation factor), acetyl-CoA acetyltransferase, glutamine synthetase, and the heat shock protein DnaK. While the role of membrane proteins on the PLG sequestering within the host is expected, the meaning of cytoplasmatic proteins remains to be investigated. Intriguingly, none of the PLG-binding recombinant proteins was detected in these experiments. One possible explanation is that the identified proteins, except LIC12816, are expressed in high amounts per cell, while the proteins assayed in our laboratory were below the detection limit, as reported by quantitative proteomics [67]. However, LipL32 that was shown to have the highest expression level per leptospiral cell, was not detected in our proteomics studies. Although the reason for this is unknown, we might speculate that because LipL32 is always presented as isoforms in 2D gels [53, 68, 69], it is possible that these isoforms do not

bind PLG. Several cytoplasmatic proteins of other pathogenic microorganisms have been identified as PLG binding proteins, such as *Bifidobacterium animalis* DnaK [70], *Paracoccidioides brasiliensis*, *Schistosoma bovis* and *Streptcoccus pneumoniae* enolases [71] and *Streptococcus pneumoniae* GAPDH [72]. For some of these proteins, there is the possibility that at some moment they could be exposed in the bacterial outer membrane. Indeed, it has been recently reported that *B. burgdorferi* enolase, which is both a cytoplasmic and membrane-associated protein, is also a PLG-binding protein [73]. It is also possible that the PLG binding to these cytoplasmic proteins are not relevant for the bacteria.

3.10. Human IgG and C3b Deposition on Leptospires. Plasmin-generation on surface of *L. interrogans* may facilitate the bacteria to degrade opsonizing IgG and C3b molecules, and therefore contribute to immune evasion. To test this hypothesis, microtiter plates coated with virulent *L. interrogans* serovar Pomona were incubated with NHS, as a source of C3b and nonspecific IgG molecules. Bacteria were then treated with PLG and uPA, and the resulting surface-associated opsonins were quantified by specific antibodies. The results show that the incubation of bacteria with PLG and uPA resulted in a decrease in IgG Fc region (Figure 10(a)) and C3b depositions (Figure 10(b)) at the leptospiral surface. No effect on IgG or C3b binding to *L. interrogans* was observed with only PLG or uPA [41].

3.11. IgG Binding from Human Leptospirosis Immune Serum to L. Interrogans. To evaluate whether PLA activity observed against nonspecific human IgG bound to leptospires could also be functional to immune sera from patients diagnosed with leptospirosis, we performed the same experiment using MAT negative (preimmune phase) and MAT positive (convalescent immune phase) pools of human sera. As expected, MAT positive sera resulted in significant higher IgG depositions on bacterial surface when compared to NHS and MAT negative sera (Figure 11). When PLA was generated on leptospires, a noticeable decrease in the amount of IgG
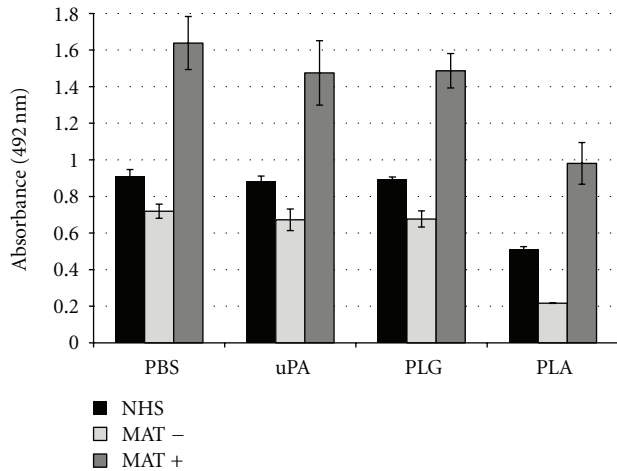
FIGURE 11: IgG binding from human leptospirosis immune serum to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG was bound by incubating bacteria 50% nonimmune human sera (NHS) or confirmed paired leptospirosis patients sera: MAT negative (MAT−) or MAT positive (MAT+). Bacteria were treated with PBS, $40\,\mu g$/mL PLG (PLG), $5\,\mu g$/mL uPA (uPA), or PLG + uPA (Pla). Presence of human IgG deposited on leptospires was determined by ELISA using Fc-specific anti-human IgG antibodies. Bars represent the mean absorbance values at 492 nm ± the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

deposition from both negative and positive MAT sera was detected [41].

Opsonization obstruction by antibodies or complement system appears to be an interesting approach for the bacteria to evade the first line of human immune defense upon infection. In fact, the expression of proteins on the bacterial surface that sequester host complement regulators FH, FHL-1, or C4BP and therefore the inhibition of the complement cascade by the alternative or classical pathways has been employed by several pathogens [74–77]. Moreover, the proteolytic cleavage of antibodies that prevents the activation of complement classical pathway or Fc receptor-mediated phagocytosis, is another well-known strategy employed by pathogenic bacteria [74–77].

3.12. Human C3b Degradation by Leptospires. Meri and colleagues [54] reported the capability of *Leptospira*-bound factor H (FH) to act as a cofactor for the serum protease factor I in cleaving C3b. Thus, we decided to assess whether the decrease in C3b deposition by *Leptospira*-generated PLA was due to C3b cleavage. *L. interrogans* serovar Pomona were coated with PLA by treatment with PLG and uPA, and then incubated with human purified C3b in fluid-phase. The resulting supernatant together with anti-C3b-specific antibodies was analyzed by Western blotting (Figure 12). The data revealed the appearance of two-lower molecular mass bands of estimated 60 and 20 kDa, concurrently with the decrease in the masses of the alpha (105 kDa) and beta (75 kDa) C3b chains that took place only with PLA-coated
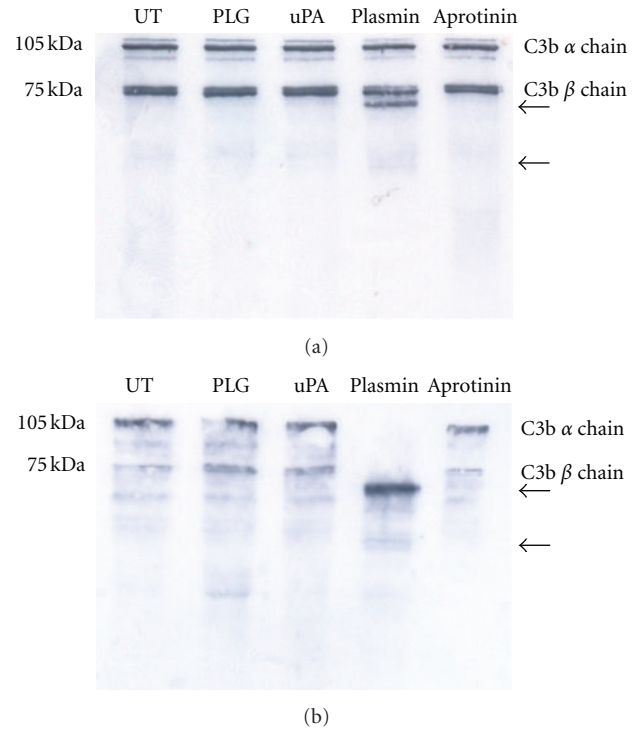

(a)

(b)

FIGURE 12: Detection of human C3b. Leptospires (10^8/sample) were treated with $10\,\mu g$ PLG and 3 U uPA (Plasmin), only with PLG (PLG), or left untreated (UT). The cells were incubated with $15\,\mu g$/mL human purified C3b for 20 h at 37°C, and the supernatants were resolved by SDS-PAGE and transferred to membranes. The C3b was detected by specific antibodies followed by ECL reagent development and exposition to X-Ray films. There are indicated the native human C3b alpha chain (105 KDa) and beta-chain (75 KDa), as well as the degradation products (arrows).

leptospires. Untreated or leptospires only treated with PLG showed no gradation. The results unmistakably show that the human C3b is cleaved by leptospires-coated PLA, indicating that the reduction in deposition is most probably due to degradation [41].

3.13. Serum Susceptibility Testing for Treated Leptospires. It has been described that *L. interrogans* serovar Pomona is partially susceptible to complement killing upon incubation with normal human sera [54]. To evaluate whether the PLG/PLA binding to leptospires confers protection on exposure to NHS, leptospires were treated with PLG+uPA (Pla) or with PBS, as negative control, and incubated for one hour with pooled NHS. The bacteria were harvested and counted in a dark-field microscopy using Petroff-Hausser chamber, at the fourth day of incubation. The number of untreated leptospires was almost four times less when compared to the plasmin-coated leptospires ($0.35 \times 10^8 \pm 0.1 \times 10^8$ versus $1.28 \times 10^8 \pm 0.3 \times 10^8$ leptospires/mL), suggesting that the leptospires endowed with active plasmin are less vulnerable to components present in NHS [41].

4. Conclusions

The interaction of the human PLG system has been suggested to be a feature that significantly contributes to the virulence of many bacterial pathogens by facilitating the initial anchoring to endothelium and penetration [27]. Our group has been studying the interaction of *Leptospira* with PLG/plasmin generation system and the possible implications for pathogenesis. We demonstrated that leptospires interact with PLG and can acquire plasmin activity associated to the surface with no apparent damage to the surface, what occurs through multiple proteins. Although nonvirulent leptospires can interact with PLG, there seems to be a correlation between the efficiency of PLG capturing and virulence, suggesting a role in virulence and infection. Moreover, the generation of enzymatically active PLA on the leptospiral surface decreased C3b and IgG depositions that may constitute a novel mechanism by which leptospires could evade the immune system until reach immunologically safe environments. This latter assertion will also have implication to the bacterial persistence within the host. We believe that the disclosure of this enzymatically system will shed light on the molecular mechanism of leptospiral pathogenesis.

Acknowledgments

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