Phosphatidylserine Exposure and Surface Sugars in Two Leishmania (Viannia) braziliensis Strains Involved in Cutaneous and Mucocutaneous Leishmaniasis

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Background. Phosphatidylserine (PS) and surface carbohydrates (SC) are known as virulence factors that may contribute to the different clinical symptoms ranging from self-healing cutaneous leishmaniasis lesions to fatal visceral disease. Leishmania (Viannia) braziliensis causes localized cutaneous leishmaniasis (LCL) and mucocutaneous leishmaniasis (MCL).

Methods. We analyzed PS exposure and SC expression associated with 2 primary L. braziliensis isolates from patients with LCL or MCL. The role of PS exposure was also addressed during promastigotes phagocytosis by macrophages.

Results. We observed higher PS exposure on the surface of late stationary growth phase promastigotes from patients with LCL, compared with those from patients with MCL, and both strains were alive during PS display. Reduction in the infectivity index was observed during macrophage interaction with late stationary growth phase promastigotes in which PS was blocked by annexin V. The major surface carbohydrates detected on LCL and MCL promastigotes were α-Man, α-Glc, and α-Gal. However, α-β-GalNAc, although observed on the surface of the LCL strain during the late stationary growth phase was highly expressed on the surface of early stationary growth phase promastigotes.

Conclusions. Our results suggest that PS and SC can modulate interactions between Leishmania organisms and host cells and may be important for the outcome of the clinical course of diseases caused by L. braziliensis.

Keywords. Leishmania braziliensis; leishmaniasis; phosphatidylserine; carbohydrates; lectin.

Leishmania (Viannia) braziliensis is the main etiological agent of American cutaneous leishmaniasis, which has 2 clinical forms: localized cutaneous leishmaniasis (LCL) and mucocutaneous leishmaniasis (MCL). LCL is characterized by an induced cellular response through CD4+ and CD8+ T cells [1], leading to ulcerated cutaneous lesions in which few macrophages and parasites are observed [2]. However, nasopharyngeal mucosa destruction due to MCL is associated with an exacerbated cellular immune response, predominantly by CD4+ T cells. The infiltrated lesions have elevated amounts of plasmatic cells and lymphocytes, but parasites and histiocytes are infrequently observed [1].

Different macrophage receptors are implicated in the ability of Leishmania organisms to recognize multiple parasite ligands [3]. Lipophosphoglycan (LPG), surface carbohydrates, and phosphatidylserine (PS)
have been implicated as some of the ligands recognized by receptors present on macrophages, inducing the decrease in inflammatory cytokine production, leading to infection establishment and maintenance [3–7]. These ligands, usually known as virulence factors, contribute to produce different clinical symptoms, varying from self-healing cutaneous lesions to potentially fatal visceral disease [8]. The finding that PS exposure on the amastigote surface plays an important role in parasite survival [9] has led to the investigation of the role of this molecule in some species of Leishmania during its interaction with the host cell [6, 10]. Furthermore, recent evidence suggests that the presence of apoptotic promastigotes of Leishmania amazonensis enable disease progression in vertebrate hosts [11]. However, to date, display of PS on the surface of L. braziliensis promastigotes has not been described. Conversely, parasite surface carbohydrates play an important role in the survival of Leishmania organisms during macropage infection and also during parasite development in the digestive tract of the vector [12, 13]. A previous study showed that, in Leishmania species, the glyocalyx is composed of LPG and glycoinositolphospholipids, which have several functions either in the mammalian host or in the insect vector [14, 15]. Thus, the present study was undertaken to characterize early stationary (E-STAT) growth phase and late stationary (L-STAT) growth phase L. braziliensis promastigotes obtained from patients with LCL and MCL, with respect to surface PS and carbohydrate expression patterns and the role of PS in the interaction between promastigotes and murine macrophages.

MATERIALS AND METHODS

Parasites
Promastigotes of L. braziliensis were isolated by inoculation of lesion tissue fragments from patients with LCL (MHOM/BR/M17593) and MCL (MHOM/BR/M17323) in axenic Novy-MacNeal-Nicolle medium. The infectivity of the parasites was maintained by regular passage in hamsters (Mesocricetus auratus). Promastigotes used in the different assays were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS) at 27°C. To determine the growth phases, L. braziliensis promastigotes at a concentration of 1 x 10^6 cells/mL were cultured as described above, and promastigotes were quantified daily for up to 11 days in triplicate cultures. For all experiments, promastigotes were obtained at the E-STAT growth phase (7 days of growth) and L-STAT growth phase (10 days of growth).

Lectin Agglutination Assay
Lectin agglutination assays were performed using 2-fold serial dilutions of several lectins in 25 μL of PBS. The same volume of the E-STAT and L-STAT growth phase promastigote suspensions (2 x 10^7 cells/mL) was incubated with the different lectins for 1 hour at room temperature. Agglutination was determined by microscopic observation, as described elsewhere [16]. Parasites incubated without lectins were used as controls. The experiments were repeated at least 3 times. The lectins used for agglutination were as follows: concanavalin A (ConA), Ulex europaeus I, wheat germ agglutinin, peanut agglutinin from Arachis hypogaea, Ricinus communis I (RCA-I), soybean agglutinin (SBA), and Dolichos biflorus agglutinin (DBA), all from Vector Laboratories (Peterborough, United Kingdom).

Flow Cytometry Analysis of PS Display on L. braziliensis
E-STAT and L-STAT growth phase promastigotes were collected by centrifugation at 2500 g for 10 minutes, washed in PBS, and resuspended in binding buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2). Parasites were then incubated with 10 μg/mL of annexin V–fluorescein isothiocyanate (FITC) for 1 hour at room temperature. Cells were washed and resuspended in PBS, and propidium iodide (0.4 μg/mL) was added 15 minutes before analysis. The negative control was obtained by incubating parasites with annexin V–FITC in the absence of Ca++. Results were obtained by flow cytometry, using a FACScan cytometer with CellQuest (Becton Dickinson) software for acquisition and analysis. Results were analyzed by FlowJo 7.

Mitochondrial Membrane Potential of L. braziliensis Promastigotes Displaying PS
For measurement of mitochondrial membrane potential, JC-1 dye (Molecular Probes) was used. This lipophilic cationic dye selectively enters mitochondria and forms complexes known as J-aggregates, emitting an intense red fluorescence and a change of color from red to green as the membrane potential decreases. LCL and MCL promastigote strains, in both E-STAT and L-STAT growth phases, were incubated with JC-1 (1 μM) for 15 minutes at 27°C. Subsequently, parasites were incubated with Alexa Fluor 350 annexin V conjugate in binding buffer, and cells were analyzed with a Zeiss LSM 510 Laser Scanning Confocal Microscope.

Host Cells
Macrophages were harvested from the peritoneal cavity of BALB/c mice with Dulbecco’s modified Eagle’s medium (DMEM; pH 7.2) and incubated at 37°C in a humidified atmosphere containing 5% CO2. After 1 hour of incubation, nonadherent cells were washed away with PBS (pH 7.2), and macrophages were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO2 for 24 hours. All experiments were performed in agreement with the current Brazilian animal protection laws (014/2005/CEPAN/IEC/SVS/MS).
Blocking of Promastigote PS Display During Macrophage-Parasite Interaction

L-STAT growth phase promastigotes of LCL and MCL strains of *L. braziliensis* were washed with annexin V binding buffer and resuspended in the same buffer at a concentration of 10^6 cells/mL. Cells were then incubated with or without annexin V at 26°C for 30 minutes. Host cells were allowed to interact with both strains of the parasite at a ratio of 10 promastigotes to 1 macrophage in DMEM without FBS. After 2 hours, cultures were washed to remove nonadherent parasites, and DMEM and FBS were added. Subsequently, after 1 and 24 hours, the cultures were rinsed with PBS, fixed with Bouin’s fixative, stained with Giemsa stain, and mounted with Entellan (Merck). For each coverslip, at least 200 macrophages were randomly counted using a 100× objective in an Olympus BX41 microscope. Results were expressed as an infectivity index, calculated as follows [17]: [percentage of macrophages with ingested parasites] × [mean number of intracellular parasites per macrophage].

Statistical Analysis

All experiments were performed in triplicate. The mean and SD of at least 3 experiments were determined. Statistical analyses of the differences between mean values obtained for the experimental groups were performed by 1-way analysis of variance, followed by the Tukey test. A *P* value of < .05 was considered statistically significant.

RESULTS

*L. braziliensis* Growth Phase Identification

*L. braziliensis* growth phase was determined during 11 days of culture. Under our culture conditions, promastigotes were in the logarithmic phase of growth until the fourth day of culture, whereas the E-STAT and L-STAT growth phases were found to occur on the seventh and tenth days of culture, respectively (Figure 1), with similar results for both LCL and MCL strains. L-STAT growth phase promastigotes were used for flow cytometry and infectivity index analysis, whereas promastigotes in both E-STAT and L-STAT growth phases were used for the remaining assays.

Agglutination Assay

The *L. braziliensis* (LCL and MCL) agglutination profile with a panel of 7 lectins is shown in Tables 1 and 2. MCL parasites were agglutinated by only 2 of the 7 lectins tested: ConA and RCA-I agglutinated L-STAT growth phase promastigotes at a lower concentration than that for the E-STAT growth phase cells (Table 1). ConA and RCA-I were 15 and 16 times, respectively, more specific for E-STAT. LCL parasites were agglutinated by 3 of 7 lectins assayed (Table 2). ConA agglutinated *L. braziliensis* equally in both growth phases. E-STAT growth phase LCL promastigotes were agglutinated by SBA at a concentration 4 times lower than that required by L-STAT growth phase promastigotes. RCA-I showed a striking specificity for LCL promastigotes, which, unlike MCL promastigotes, were highly agglutinated by the lectin. However, RCA-I was unable to discriminate between E-STAT and L-STAT growth phase LCL promastigotes. All other tested lectins were unable to agglutinate the parasites at the highest concentration assayed (500 µg/mL).

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**Table 1. Lectin Agglutination of Leishmania (Viannia) braziliensis Mucocutaneous Leishmaniasis (MCL) Strain Promastigotes**

<table>
<thead>
<tr>
<th>Lectin Agglutinators</th>
<th>Specificity</th>
<th>Minimum Agglutination Concentrationa (µg/mL)</th>
<th>E-STAT Promastigotes</th>
<th>L-STAT Promastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>α-Man, α-Glc</td>
<td>15.6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Dolichos biflorus agglutinin</td>
<td>GlcNAc</td>
<td>Noneb</td>
<td>Noneb</td>
<td></td>
</tr>
<tr>
<td>Peanut agglutinin</td>
<td>β-Gal(1-3)GalNAc</td>
<td>Noneb</td>
<td>Noneb</td>
<td></td>
</tr>
<tr>
<td>Soybean agglutinin</td>
<td>α-β-GalNAc</td>
<td>Noneb</td>
<td>Noneb</td>
<td></td>
</tr>
<tr>
<td>Ulex europaeus agglutinin I</td>
<td>α-L-Fucose</td>
<td>Noneb</td>
<td>Noneb</td>
<td></td>
</tr>
<tr>
<td>Wheat germ agglutinin</td>
<td>GlcNac2</td>
<td>Noneb</td>
<td>Noneb</td>
<td></td>
</tr>
<tr>
<td>Ricinus communis agglutinin I</td>
<td>α-Gal</td>
<td>32.2</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: E-STAT, early stationary growth phase; L-STAT, late stationary phase promastigotes.

a Minimum concentration required to agglutinate *L. braziliensis* promastigotes (n = 3).
b No agglutination at the maximum lectin concentration tested (500 µg/mL).
The infectivity index was greater for LCL than for growth phase (ie, L-STAT) with the highest PS exposure to interact with murine peritoneal macrophages during the annexin V analysis. To test the role of PS in the L-STAT growth phase by annexin V Flow Cytometry Analysis of PS Exposure on promastigotes, we performed in vitro assays to determine the capacity of Localized Cutaneous Leishmaniasis (LCL) Strain Promastigotes to interact with murine peritoneal macrophages. Blocking of PS with annexin V significantly inhibited (by approximately 42%) the interaction of LCL L-STAT growth phase promastigotes with macrophages (Figure 3A). Interestingly, at 24 hours of macrophage interaction, annexin V binding to the promastigotes strongly inhibited (by 70%–90%) the infectivity index of both MCL and LCL strains in the L-STAT growth phase (Figure 3B).

**DISCUSSION**

The interaction between *Leishmania* parasites and phagocytic cells relies on molecules present on the parasite surface that are recognized by macrophage receptors. Studies have shown that the infectivity of *Leishmania* promastigotes is cell cycle dependent and restricted to the stationary growth phase [18]. Other studies also demonstrate that clinical manifestations are directly related to parasite surface molecules [4, 8, 19]. Despite numerous studies of *L. braziliensis* biology, few are related to virulence factors that could define the different clinical forms of the disease. In addition, there are many open questions concerning disease mechanisms in the most severe *Leishmania* infections, such as MCL. Thus, the present study was performed to analyze the relationship between clinical manifestations of 2 distinct strains of *L. braziliensis* and 2 *Leishmania* virulence factors, PS exposure and carbohydrate expression.

Previous observations demonstrated by quantitative methods that *L. braziliensis* promastigotes had higher PS exposure on their surface during the L-STAT growth phase (10 days of culture), compared with the E-STAT growth phase (7 days of culture) and logarithmic growth phase (4 days of culture; data not shown). We observed greater PS exposure during the L-STAT growth phase of the LCL strain, compared with the MCL strain. This higher level of exposure could be related to the clinical manifestation of LCL, which is characterized by a cutaneous lesion that is less aggressive than the MCL lesion. This variability in disease manifestation could be related to the display of PS, which is one of the molecules responsible for host antiinflammatory response [11, 20]. Some studies have shown that PS exposure on *Leishmania major* promastigotes [6] and *L. amazonensis* amastigote [7, 9, 20] and promastigote [11] forms inhibits the inflammatory response of the host cell. Moreover, amastigotes of *L. amazonensis* display PS on their surface when not undergoing apoptotic cell death [9, 21]. In this study, promastigotes of *L. braziliensis* displayed PS without undergoing apoptotic cell death, as shown by the maintenance of mitochondrial transmembrane potential.

**Flow Cytometry Analysis of PS Exposure on *L. braziliensis* Promastigotes**

PS exposure was analyzed in both promastigote strains during the L-STAT growth phase by annexin V–FITC binding, in relation to the parasite cell size. It was possible to observe, in both strains, that PS-exposing promastigotes were smaller than PS-nonexposing promastigotes (Figure 2A and 2B). PS exposure analysis demonstrated that the LCL promastigote strain had a higher percentage of PS-exposing cells than the MCL strain (Figure 2C and 2D).

In addition, it was possible to observe slender, PS-exposing promastigotes in which the mitochondrial transmembrane potential was not lost (Figure 2E–G). This was observed in both strains in the L-STAT growth phase; however, it was not possible to differentiate these strains qualitatively.

**Infectivity Index**

To test the role of PS in *Leishmania*–macrophage interactions, we performed in vitro assays to determine the capacity of annexin V–treated LCL and MCL *Leishmania* promastigotes to interact with murine peritoneal macrophages during the growth phase (ie, L-STAT) with the highest PS exposure (Figure 3). The infectivity index was greater for LCL than for MCL promastigotes after 1 hour of interaction with macrophages. Blocking of PS with annexin V significantly inhibited (by approximately 42%) the interaction of LCL L-STAT growth phase promastigotes with macrophages (Figure 3A). Interestingly, at 24 hours of macrophage interaction, annexin V binding to the promastigotes strongly inhibited (by 70%–90%) the infectivity index of both MCL and LCL strains in the L-STAT growth phase (Figure 3B).

**Table 2. Lectin Agglutination of *Leishmania (V.*) braziliensis* Localized Cutaneous Leishmaniasis (LCL) Strain Promastigotes**

<table>
<thead>
<tr>
<th>Lectin Agglutinin</th>
<th>Specificity</th>
<th>Minimum Agglutination Concentrationa (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E-STAT Promastigotes</td>
</tr>
<tr>
<td>GlcNAc Noneb</td>
<td>Noneb</td>
<td>Noneb</td>
</tr>
<tr>
<td>α-Gal(1-3)</td>
<td>Noneb</td>
<td>Noneb</td>
</tr>
<tr>
<td>Soybean agglutinin</td>
<td>Noneb</td>
<td>Noneb</td>
</tr>
<tr>
<td>α-L-Fucose</td>
<td>Noneb</td>
<td>Noneb</td>
</tr>
<tr>
<td>GlcNac</td>
<td>&lt; 4.0</td>
<td>&lt; 4.0</td>
</tr>
</tbody>
</table>

Abbreviations: E-STAT, early stationary growth phase; L-STAT, late stationary phase.

* Minimum concentration required to agglutinate *L. braziliensis* promastigotes (*n* = 3).

* No agglutination at the maximum lectin concentration tested (500 µg/mL).
amastigotes and promastigotes could function as ligands for recognition by macrophages [6, 7, 9, 10]. Results presented here showed that L-STAT growth phase LCL and MCL strains in which PS exposure was blocked by annexin V had a large reduction in infectivity index, compared with controls of both strains. This suggests that a large amount of PS exposure is important for promastigote capture by macrophages. A previous study demonstrated that parasite infectivity was affected by annexin V blocking in vitro [9, 10] and in vivo [7].

Cell surface carbohydrates were evaluated as another *Leishmania* virulence factor. The importance of carbohydrates on the cell surface of *Leishmania* organisms has been shown in previous reports [23–27], and these molecules play an important role in the cell-parasite interaction, in the survival of organisms inside macrophages, and during *Leishmania* development in the digestive tract of the vector [28–30]. Our results showed that the E-STAT growth phase MCL strain was less agglutinated by the mannose- and galactose-specific lectins...
Differentiated by the phases of the LCL strain. However, this strain could be differentiated by the expressions of these carbohydrates between the 2 growth phases of the promastigotes. On the other hand, there was no difference in the expressions of carbohydrates of both strains analyzed could be related to differences in the outcome of the clinical course of human leishmaniasis caused by the different L. braziliensis strains. Further studies are necessary to better determine the specific role of these molecules.

The higher presence of mannose on the surface of L-STAT growth phase parasites, as shown by ConA agglutination, could be related to LPG elongation, which is a compound of repeating units of this carbohydrate. LPG is one of the major molecules on the cell surface of the Leishmania organisms that is required for initial parasite establishment and maintenance inside host cells [35].

The present investigation shows the importance of studying the virulence factors present on the surface of Leishmania organisms to better understand their interaction mechanisms with host cells. Furthermore, this could lead to a better evaluation of Leishmania developmental biology and of different clinical forms of leishmaniasis.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

7. Wanderley JLM, Moreira MEC, Benjamin A, Bonomo AC, Barcinski MA. Mimicry of apoptotic cells by exposing phosphatidylserine

ConA and RCA-I, respectively, than the L-STAT growth phase promastigotes. On the other hand, there was no difference in the expressions of these carbohydrates between the 2 growth phases of the LCL strain. However, this strain could be differentiated by the N-acetyl-galactosamine expression detected by SBA, which was not observed on MCL promastigotes. SBA was also capable of differentiating between E-STAT and L-STAT growth phase parasites, as the minimal amount of SBA necessary to agglutinate L-STAT growth phase parasites was 4 times higher than that for the E-STAT growth phase parasites. Our results are in agreement with other studies that presented similar patterns of agglutination for L. braziliensis, using ConA and RCA-1 [25, 31–34]. However, for the first time, we found that it is possible to distinguish between strains of L. braziliensis isolated from patients with LCL and MCL from the Amazon area, using SBA lectin. The differences observed in the expressions of carbohydrates of both strains analyzed could be related to differences in the outcome of the clinical course of human leishmaniasis caused by the different L. braziliensis strains. Further studies are necessary to better determine the specific role of these molecules.

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