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Research brief

Leishmania amazonensis: Multiple receptor–ligand interactions are involved in amastigote infection of human dendritic cells

Maira Cegatti Bosetto, Selma Giorgio *

Departamento de Parasitologia, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, Cep 13083-970, Campinas, São Paulo, Brazil

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Abstract

In their mammalian hosts, *Leishmania* are obligate intracellular parasites that reside in macrophages and dendritic cells (DCs). In the present study, we have investigated in vitro the mechanisms of entry into human DCs of *Leishmania amazonensis* amastigotes isolated from lesions in nude mice (Am nude). The DC infection rate with Am nude was approximately 36%, while opsonization of Am nude with normal human serum and infected human serum increased the DC infection rates to 60% and 62%, respectively. Heat inactivation and depletion of antibodies in sera brought the DC infection rate down to 40%. The DC infection rate was inhibited after pre-treatment of Am nude with heparin. We were unable to implicate mannose–fucose receptors in the uptake of Am nude by DCs. Our data suggest that the ability of *L. amazonensis* amastigotes to infect human DCs involves the participation of at least three multiple receptor–ligand interactions, antibodies/FcR, complement components/CR and proteoglycans/heparin-binding protein.

Index Descriptors and Abbreviations: *Leishmania amazonensis*; Human dendritic cells; Amastigotes nude; Receptors; Mannan; Heparin; CR, complement receptor; FcR, Fc receptor; MFR, mannose–fucose receptor; DC, dendritic cell; IHS, infected human serum; NHS, normal human serum

1. Introduction

Leishmania are protozoan parasites with two developmental forms, promastigotes that develop in the phlebotomine insect vectors, and amastigotes that replicate in host mammalian cells, mainly macrophages and dendritic cells (DCs) (Handman, 1999). Numerous studies have identified that the uptake of *Leishmania* promastigotes by macrophages is mainly mediated by complement receptors (CRs), while are the receptors for Fc domain of immunoglobulins (FcRs) and for mannose–fucose (MFRs) are also of importance (Blackwell et al., 1985; Chakraborty et al., 2001; Mosser and Edelson, 1985; Wilson and Pearson, 1986). Less is known of the molecules that mediate entry of the amastigote form into host cells, even though amastigotes are responsible for sustaining the infection within the host (Kima et al., 2000). The suggested macrophage receptors are FcR, CR and a cellular proteoglycan containing heparan sulfate (Guy and Belosevic, 1993; Kima et al., 2000; Love et al., 1993; Peters et al., 1995). Thus, the entry of *Leishmania* into macrophages involves deposition of complement components and antibodies, and the presence of mannose and heparin-binding protein on parasite surface (Blackwell et al., 1985; Butcher et al., 1992; Guy and Belosevic, 1993; Kima et al., 2000; Love et al., 1993; Mosser and Edelson, 1985; Peters et al., 1995; Wilson and Pearson, 1986).

Dendritic cells efficiently ingest *Leishmania* and become involved in the induction and shaping of T-cell dependent immune responses (Brandonisio et al., 2004; Prina et al., 2004). Recently, considerable efforts have been made to define the opsonic requirements for *Leishmania* ingestion by DCs. Although Blank et al. (1993) suggested that the uptake of *Leishmania major* amastigotes by murine

* Corresponding author. Fax: +55 19 35213124.
E-mail address: sgiorgio@unicamp.br (S. Giorgio).

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Langerhans cells was mediated by CR, more recent studies have shown that CR-mediated uptake is not relevant for infection, and that *L. major* is predominantly phagocytosed by murine DCs via FcR (Woelbing et al., 2006). Colmenares and co-workers (2004) demonstrated that mannan-binding C-type lectin is a receptor in human DCs for promastigote and amastigote forms of *L. infantum* and *L. pifanoi*, but not for *L. major* promastigotes (Colmenares et al., 2002, 2004; Woelbing et al., 2006). In addition, opsonization of *L. pifanoi* amastigotes with serum from patients with leishmaniasis has exhibited a low capacity to bind to DCs (Colmenares et al., 2004). These apparently conflicting results reflect species-specific interactions between *Leishmania* and DCs.

Leishmania amazonensis, a species transmitted mainly in the Brazilian Amazon is associated with benign cutaneous lesions and diffuse cutaneous disease (Grimaldi and Tesh, 1993). A single report has demonstrated that *L. amazonensis* amastigotes, purified from nude mouse lesions, opsonized with immune serum enhances the infection rate of murine bone marrow-derived dendritic cell cultures; the effect of other ligand–receptor systems on the infectivity of amastigotes was not evaluated (Prina et al., 2004). In the present study, we examined the ligands required for in vitro infection of human monocyte-derived dendritic cells by *L. amazonensis* amastigotes isolated from nude mouse lesions (Am nude).

2. Materials and methods

2.1. Parasites and generation of dendritic cells

Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were isolated from footpad lesions from C3H nude/Uni mice (Cemib—Centro Multidisciplinar para Investigação Biológica, Unicamp, Campinas SP, Brazil) as described previously (Barbieri et al., 1993), and used immediately after isolation. Promastigotes were cultured as described by Arrais-Silva et al. (2005). The generation of dendritic cells from peripheral blood monocytes from healthy donors was as previously described (Nair et al., 1998). DCs were cultured in 24 well plates with coverslips for 7 days in Iscove's medium (Sigma, St. Louis, MO) supplemented with interleukin 4 (250 ng/ml) and granulocyte macrophage colony-stimulating factor (50 ng/ml) (Sigma, St. Louis, MO). The DCs were routinely $CD14^{low}$, $CD83^{low}$, $CD80^{+}$, $CD1a^{+}$, HLA-DR^{+}, $CD86^{+}$, and $CD40^{+}$ as determined by flow cytometry.

2.2. Parasite opsonization conditions

Serum samples were obtained from healthy Brazilian individuals with no history of exposure to *Leishmania* (normal human serum—NHS) and individuals with visceral leishmaniasis (infected human serum—IHS), generous gifts from Dr. C. L. Barbiéri (Universidade Federal de São Paulo, SP, Brazil) and Dr. E. Deberaldini (Superintendência de Controle de Endemias, SP, Brazil). These sera were used in the experiments at dilution determined by titration obtained in immunofluorescence reactions, i.e., 1:10 for NHS and 1:80,000 or 1:160,000 for IHS. For complement inactivation, sera were treated at 56 °C for 1 h (Mosser and Edelson, 1985; Navin et al., 1989). For antibody depletion, sera were adsorbed against *L. amazonensis* promastigotes (10^7 parasites/ml) for 2 h at 4 °C (Kolb-Maurer et al., 2001). To confirm antibody removal, an immunofluorescence reaction was performed. For complement inactivation and antibody depletion, sera were first inactivated at 56 °C for 1 h, and then incubated with promastigotes for 2 h at 4 °C. The opsonization of the amastigotes was achieved by incubation at 4 °C for 1 h, and two washes in PBS (Prina et al., 2004). The parasites were counted using a hemocytometer, then added to dendritic cell cultures.

2.3. Heparin and mannan treatments

Amastigotes (10^6/ml) were pre-treated with soluble heparin (100 μg/ml) (Cristália, Campinas, SP, Brazil) for 20 min on ice, washed twice in PBS (Love et al., 1993) and added to DC cultures. In other experiments DC cultures (5×10^5 cells/ml) were pre-incubated for 30 min at 37 °C with soluble mannan of *Saccharomyces cerevisiae* (Sigma, St. Louis, MO) (5, 100, or 750 μg/ml) (Cantos et al., 1993; Colmenares et al., 2002), before parasite infection.

2.4. Infection of DCs with L. amazonensis

DCs were infected with *L. amazonensis* Am nude at a parasite-DC ratio of 3:1 at 37 °C in 5% CO_2/95% humidified air. After 24 h, the cultures were washed with PBS to remove extra-cellular parasites, fixed with methanol and stained with Giemsa. The percentage of infected DCs and the number of intracellular parasites were determined by light microscopy examination of at least 600 cells on triplicate coverslips.

2.5. Statistics

All experiments were repeated at least twice in triplicate wells, and the results are expressed as the mean ± standard deviation. Statistical analyses were performed using the two-tailed Student's *t* test and Microcal Origin 6.0 (Microcal Software, Northampton, MA).

3. Results and discussion

In our experiments we examined the opsonic requirements for entry of *L. amazonensis* amastigotes isolated from nude mouse lesions in human DCs. These amastigotes did not have antibodies bound in their surface (Prina et al., 2004) and in our study, the antibody isotypes IgG1, IgG2a, IgG2b, and IgG3 were not detectable on the parasite surface by immunofluorescence (data not shown).

Previously, *L. amazonensis* was found to be able to infect dendritic cells of murine bone marrow (Prina et al., 2004). Our data have extended this finding to human monocyte-derived DCs. Twenty-four hours after adding Am nude, the percentage of infected cells was 36 ± 1.7% (Fig. 1). The number of intracellular amastigotes in DCs was 3.8 ± 0.2% amastigotes per cell, and infection could be maintained for at least 72 h. The effect of human sera on the entry of Am nude into human DCs was then assessed. As shown in Fig. 1, the percentage of infected DCs increases significantly when Am nude are opsonized with IHS (62.3 ± 2.5% of infected DCs). Interestingly, the entry of Am nude opsonized with NHS in DCs was also significantly enhanced as compared with unopsonized Am nude (60 ± 2.9% versus 36 ± 1.7%) (Fig. 1). The opsonization of Am nude with IHS or NHS did not change the number of intracellular parasites in DC cultures, when compared to DC cultures infected with unopsonized Am nude (data not shown). Next, we examined which of the two major factors present in sera might be responsible for enhancement of the *L. amazonensis* Am nude uptake by human DCs. As shown in Fig. 1, the heat treatment of IHS and NHS did not reduce the percentage of infected DCs to the level observed in DCs infected with unopsonized parasites. To establish the importance of serum antibodies to promote DCs infection by Am nude, IHS and NHS were depleted of antibodies, i.e., pre-adsorbed against promastigotes and then used for Am nude opsonization. The drastic reduction in antibodies (for IHS: IF 1:80,000 and IF 1:10 before and after adsorption, respectively; for NHS: IF 1:80 and 0 before and after adsorption, respectively) did not alter the ability of the sera to opsonize Am nude for DC infection (Fig. 1). However, the opsonization of Am nude with IHS or NHS, heat inactivated and depleted of antibodies, reduced the infection rate to the levels observed in DC cultures infected with unopsonized Am nude (Fig. 1).

We also assessed the involvement of other two ligands involved in *Leishmania* infection, namely mannan and heparan sulfate (Blackwell et al., 1985; Love et al., 1993; Wilson and Pearson, 1986). Yeast mannan from *S. cerevisiae*, a molecule that displays mannose residues, and is known to bind to the MFRs (Wilson and Pearson, 1986). We found no inhibition in the uptake of Am nude when DCs were treated with mannan, irrespective of the concentration used (Fig. 2). Human DCs cultures pre-treated with 750 μg/ml of mannan and infected with *L. amazonensis* promastigotes (10:1 parasites/cell ratio) were used as a positive control and showed 34% inhibition of parasite uptake.

A heparin-binding activity on *L. amazonensis* amastigotes surface has been implicated with the attachment to macrophages (Love et al., 1993). To determine whether Am nude heparin-binding activity is involved in the infection of human DCs, assays were performed in the presence of heparin. A significant 50% reduction in the percentage of infected DCs was observed when Am nude were pre-treated with 100 μg/ml of heparin (Fig. 2). Our results are consistent with the findings of Prina et al. (2004) who were able to detect a significant rise of the percentage of murine DCs when Am nude were pre-incubated with *Leishmania*-specific immune serum. The authors suggested that the opsonization with antibodies is involved in the uptake of parasites by DCs. In addition, we have found that opsonization of Am nude with NHS also enhanced the percentage of infected DCs (Fig. 1). In contrast to these results, Woelbing et al. (2006) have reported the inability of normal mouse serum-opsonized *L. major* amastigotes isolated from SCID mice to enhance the infection of murine DCs. Explanations for this discrepancy could be differences in DCs and origins of the serum (murine versus human), *Leishmania* species (*L. major* versus *L. amazonensis*) and opsonization conditions (10 min at 37 °C in Woelbing's protocol, compared to 1 h at 4 °C in our protocol). Indeed, natural antibodies recognizing

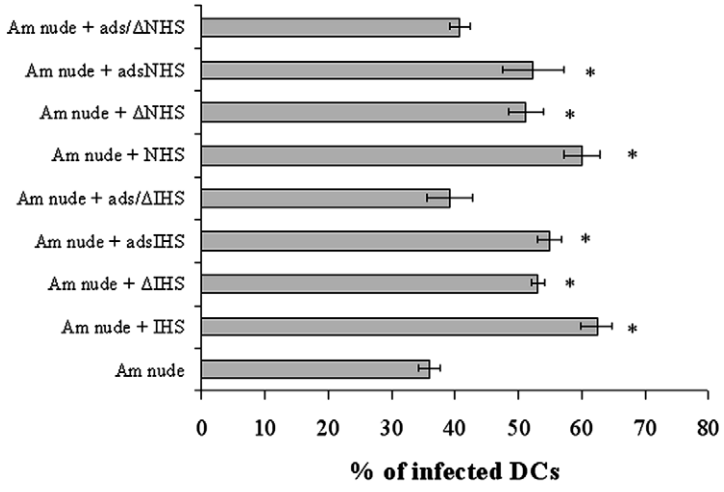

Fig. 1. Infection of DCs with *L. amazonensis* Am nude. Am nude were unopsonized or previously opsonized with infected human serum (IHS), heat inactivated IHS (ΔIHS), a serum (NHS), heat inactivated NHS (ΔNHS), adsorbed NHS (adsNHS) or heat inactivated and adsorbed NHS (ads/ΔNHS). The NHS and IHS were used at dilution of 1:10 and 1: 80,000, respectively. Data are averages of three representative experiments, bars indicate SD. ∗, statistically significant difference relative to unopsonized Am nude ($P < 0.01$).

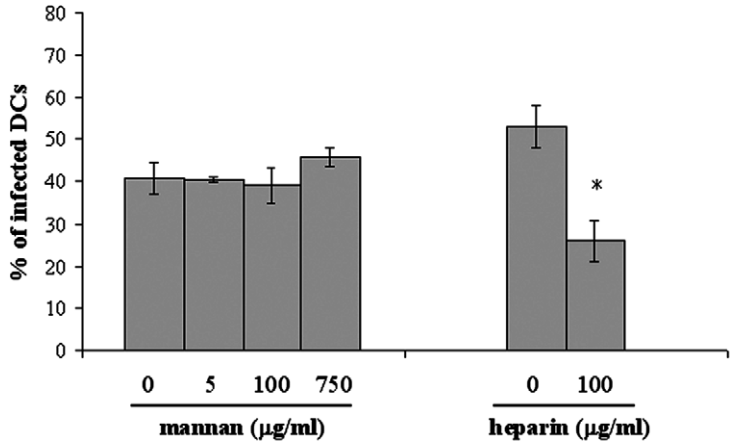

Fig. 2. Infection of DCs with *L. amazonensis* Am nude. DC cultures were untreated (0) or pre-treated with soluble mannan (5, 100, or 750 μg/ml) for 30 min before parasite addition. In other experiments, Am nude were untreated (0) or pre-treated with soluble heparin (100 μg/ml) for 20 min on ice before parasite addition. One out of two independent experiments with similar results and performed in triplicate is shown, bar indicate SD. ∗, statistically significant difference relative to untreated ($P < 0.01$).

Leishmania have been found in sera of people with no history of exposure to the parasite, and they can enhance parasite phagocytosis by human monocytes and human peripheral blood neutrophils (Laufs et al., 2002; Navin et al., 1989). In addition to these studies with human blood leukocytes, *L. donovani* and *L. amazonensis* promastigotes and amastigotes opsonized with human non-immune serum containing IgG and IgM that recognize parasite epitopes, bind to human erythrocytes (Dominguez and Torano, 1999; Avila et al., 1989). The opsonization of Am nude with NHS or IHS depleted of antibodies did not reduce the percentage of infected DCs (Fig. 1), suggesting that complement components are involved in the infection of *L. amazonensis* amastigotes to human DCs. However, the opsonization of Am nude with heat inactivated NHS or IHS did not reduce the percentage of infected DCs (Fig. 1). The results also showed that opsonization of Am nude with IHS or NHS which had been heat inactivated *and* depleted of antibodies, significantly reduced the infection rate of DCs (Fig. 1). These data suggest that the presence of one of two serum components (i.e., complement or antibodies that recognize the parasite) is necessary and sufficient to enhance the entry of Am nude in human DCs. It is possible, however, that the presence of unknown heat-labile molecules other than the complement components would fix to the amastigote surface ligands and would be involved in parasite uptake by human DCs. On the other hand, it has been proposed that the attachment of *L. pifanoi*, *L. amazonensis* and *L. major* amastigotes to macrophage occur through both FcR and CR3 receptors (Guy and Belosevic, 1993; Kima et al., 2000; Prina et al., 2004). Thus, in the tissue microenvironment, complement components and antibodies facilitate the infection of human DCs by *L. amazonensis*, and in the absence of one of these opsonins, the other would compensate.

We also recognized the possibility that other mechanisms might contribute to serum-independent infection of DCs. In fact, a 36% infection rate was observed in DC cultures infected with unopsonized Am nude (Fig. 1). Recently, C-type lectin receptors have also been implicated in the uptake of *L. pifanoi* and *L. infantum* amastigotes by human DCs, but not in the uptake of *L. major* (Colmenares et al., 2004; Woelbing et al., 2006). Our results did not, however, support a role of mannan-binding C-type lectins in the phagocytosis of *L. amazonensis* Am nude by human DCs (Fig. 2).

Interestingly, we found that DC infection was inhibited after pre-treatment of Am nude with heparin (Fig. 2). This would suggest that DCs such as macrophages have an additional mechanism for recognition of amastigotes. Love et al. (1993) reported heparin-binding activity on the surface of *L. amazonensis*. The authors suggested that *L. amazonensis* amastigotes interact with non-myeloid cells and macrophages, which express in their surface proteoglycans containing heparan sulfate (Love et al., 1993). We can speculate that binding of *L. amazonensis* amastigotes to heparin is an important mechanism in the infection of human DCs.

In summary, our data suggest that the ability of *L. amazonensis* amastigotes to infect human DCs involves the participation of multiple receptor–ligand interactions, at least the three shown in this report, namely antibodies/FcR, complement components/CR, and proteoglycans/heparin-binding protein. This mechanism of *L. amazonensis* invasion into DCs seems to be similar to that described for the infection of macrophages (Guy and Belosevic, 1993; Love et al., 1993) and represent advantages for the parasite. One benefit to the parasite is that, in the absence of one or two opsonins, other receptor–ligand interactions could be used. Another functional advantage is that parasites can enter different leukocyte populations, including macrophages and DCs, allowing the establishment and the persistence of infection.

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