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Full Length Research Paper

Anti-*Leishmania donovani* antibodies enhance promastigotes internalization into host macrophage

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***Leishmania* spp. promastigotes preferentially infect host macrophages, where parasite internalization is facilitated by several host and parasite surface molecules. This study aimed to demonstrate the role of humoral immunity in *Leishmania* parasite internalization into host macrophages. First, informed consent sera were obtained from 67 parasitologically confirmed visceral leishmaniasis patients reporting to our field treatment centre, Eastern Sudan. Then following titre determination, sera that had a titre of >102,400 were selected for parasite coating. An *in vitro* parasite internalization system was developed to enhance the *Leishmanial* macrophage interactions. The mean parasite number per monocytes was 626 ± 91 for antibody-coated *Leishmania donovani*, compared to 412 ± 70 uncoated isolates (p= 0.01). On the other hand, the percentage of infected cells was significantly higher for all antibody-coated isolates (100%) compared to uncoated ones (40%). This evidence of high infectivity probably points to the fact that anti-*Leishmania* antibodies facilitated the parasite uptake by host macrophages and monocytes-derived macrophages (MDM). Moreover, the rate of parasite uptake by MDM was significantly higher compared to monocytes (p= 0.00). This could be explained by the fact that the functional capabilities of fully differentiated macrophages differ from monocytes. In conclusion, host humoral immunity probably plays a pivotal role in *Leishmania* parasites internalization into host macrophages.**

Key words: *Leishmania donovani,* macrophages, monocyte-derived macrophages, humoral immunity.

INTRODUCTION

Leishmania promastigotes and amastigotes preferentially infect macrophages, where several host cell surface molecules have been proposed to mediate internalization of *Leishmania* into macrophages. The internalization of the promastigote form has been shown to be mediated by the mannose-fucose receptor, the fibronectin receptor, and the complement receptors type 1 and 3 (CR1 and CR3) on the surface of host macrophages (Wilson and Pearson, 1988). Promastigotes may interact directly with these molecules, or interaction may occur after parasite opsonization by soluble host molecules, as has been demonstrated in studies with the third component of the complement system (Allen and Aderem, 1996). It is generally thought that random attachment of the flagellated promastigotes to mononuclear phagocytes initiates their uptake via circumferential pseudopods. Intracellularly, the promastigotes become located in phagolysosomes in which they transform to and survive

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Abbreviations: DAT, Direct agglutination test; **SSG,** sodium stibogluconate; **MDMs,** monocyte-derived macrophages; **PBMCs,** peripheral blood monocytes; **NNN,** Novy-Macneal-Nicolle; **PBS,** phosphate buffered saline; **RPMI,** Rosuell Park Memorial Institute; **FCS,** foetal calf serum; **EDTA,** ethylenediaminetetraacetic acid; **PKDL,** post-kala-azar dermal leishmaniasis.

as ‘aflagellated’ amastigotes that hide their shortened flagellum within the flagella pocket. Unrestricted replication of these amastigotes in susceptible hosts is assumed to cause the eventual burst of the host cell, thereby releasing the infectious parasites (Rittig and Bogdan, 2000).

Considerable efforts have been made to define the ligands on the parasite surface and the receptors on the macrophage, which mediate binding and subsequent uptake by phagocytosis. On the promastigote surface, two abundantly expressed molecules, lipophosphoglycan (LPG) and the metalloproteinase gp63, have been shown to bind either directly or after opsonization with complement to theCR3 of macrophages; the mannose and the fibronectin receptors have also been proposed to mediate binding of promastigotes (Mosser and Rosenthal, 1994). On the other hand, the following macrophage receptors have been invoked for the recognition of defined but yet unknown ligands on the amastigote surface: (i) for *Leishmania major*, a lectin-like receptor recognizing LPG (Kelleher et al., 1995), the CR3 and the receptor for the Fc domain of IgG via complement components or immunoglobulins, respectively (Guy and Belosevic, 1993); (ii) for *Leishmania mexicana amazonensis*, heparan sulfate and a receptor for fibronectin (Wyler et al., 1985; Love et al., 1993) (iii) in *Leishmania donovani*, the mannose receptor and CR3 (Blackwell et al., 1985). In addition, amastigotes exhibit promiscuous binding to several different mammalian cell types while the binding of promastigotes is largely restricted to mononuclear phagocytes (Mosser and Rosenthal, 1994).

In this study, we established an *in vitro* parasite internalization system to determine if the antibodies would enhance the *Leishmania*/ macrophage interactions. The design is based on coating the promastigotes with anti-*Leishmania donovani* antibodies obtained from visceral leishmaniasis patients and exposed to monocytes and monocyte-derived macrophage (MDM).

MATERIALS AND METHODS

Statement of ethics

This study was approved by the Ethics Committee of the Institute of Endemic Diseases, University of Khartoum. All participants were given written consent.

Patients and samples

Sixty-seven parasitologically-confirmed visceral leishmaniasis (VL) patients who reported to the Leishmaniasis Centre at Kassab village, Eastern Sudan were enrolled. Aspirates were collected from lymph nodes and/or bone marrow for smear examination and culture. Five milliliters of peripheral blood were also collected from each selected patient into plain containers and serum was separated and kept at -20°C for further testing. Sera that had reciprocal tires of >102,400 using the direct agglutination test (DAT) with the diagnostic cutoff level of 6400, as was previously described by Harith et al. (1986), were selected for coating.

Parasites isolation and cultivation

Four *L. donovani* isolates were used; 2 strains were obtained from clinically antimony resistant VL patients who received multiple courses of sodium stibogluconate (SSG) (Pentostam GlaxoWellcome) and the other two isolates from clinically sensitive patients. For comparison, an *L. major* isolate was donated by Dr. Abdalla H. Sharief, Tropical Medicine Research Institute, Sudan. Lymph node/bone marrow aspirates were aseptically inoculated into sterile culture tubes containing Novy-Macneal-Nicolle (NNN) medium. Cultures were kept at 27°C and parasite growth was checked daily; the medium was changed every 3 days until cultures were positive.

Preparation of gelatin-coated

Primarily, gelatin-coated flasks were prepared by adding 10 ml of 2% (w/v) gelatin to 75cm^2 tissue culture flasks. Vessels were incubated at 37°C for 2 h and then the gelatin solution was removed by aspiration. Flasks were dried at 56°C overnight and kept at room temperature.

Separation of donor plasma

Fifty milliliters heparinized blood of healthy adult volunteer was taken, centrifuged at 1650 rpm for 15 min, then the plasma was collected and inactivated (for complement components) at 56°C for 30 min and centrifuged at 2500 rpm for 10 min. Subsequently, the supernatant was transferred to flasks pre-coated with gelatin. After incubation at 37°C for 1 h, the solution was removed and the flasks were washed 3 times with phosphate buffered saline (PBS).

Separation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy adult volunteer using density gradient centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). After plasma separation, an equal amount of Ficol was added to the blood, and then centrifuged at 1400 rpm for 40 min. PBMCs were washed twice, re-suspended in 10 ml RPMI-1640 (Sigma Chemical Co., St Louis, MO) and transferred to gelatin coated flasks. This was further incubated for 1 h at 37°C with 5% CO_2. Non-adherent cells were removed by repeated vigorous washings (3 times) with warm RPMI medium. Adherent monocytes were detached by incubation with 5 mM ethylenediaminetetraacetic acid (EDTA) solution (1:1 mixture of 10 mM EDTA in PBS with RPMI medium) for 10 min at 37°C. Monocytes were washed twice, re-suspended in RPMI-1640 and plated on chamber slides at 0.25 × 10^6 cells/well at 37°C with 5% CO_2.

Culture of human MDMs

Freshly isolated human monocytes were grown in chamber slides (8 wells) in RPMI 1640 medium containing 10% FCS, 1% (vol/vol) penicillin/streptomycin, and 20% (vol/vol) autologous plasma (added 24 hrs post monocytes isolation). The cells were incubated at 37C in 5% CO2/95% air atmosphere. The medium was replaced every 3 days and the cells were used for experiments at day 7 of plating.

Table 1. Mean numbers of internalized *Leishmania* parasites into macrophages.

DAY 1				DAY 7			
L. donovani		***L. major***		***L. donovani***		***L. major***	
Coated	**Uncoated**	**Coated**	**Uncoated**	**Coated**	**Uncoated**	**Coated**	**Uncoated**
626 ± 91*	412± 70*	598± 30*	228± 49*	1170± 122*	418±85*	692 ±59*	234± 60*
620	380	600	230	1220	420	670	220

Continuous data presented as (Mean ± SD, Median). *p-value < 0.05.

Table 2. Percent of infected cells by antibodies-coated and uncoated *L. donovani* and *L. major* at days 1 and 7.

Strain	Day 1 (%)	Day 7 (%)
Antibodies-coated *L. donovani*	100*	100*
Uncoated *L. donovani*	40*	54*
Antibodies-coated *L. major*	50*	60*
Uncoated *L. major*	20*	25*

*p-value < 0.05.

Parasites coating and infection of monocytes and MDMs

Sera of high antibody titers (> 102,400) were inactivated at 56°C for 30 min, and a count of 2.5×10^6 parasites of six days old promastigotes (at stationary phase) were added into tubes containing 100 µL patients' inactivated sera and incubated at 37°C for 2 h. Monocyte and MDMs that were exposed to antibody-coated promastigotes at cell: parasite ratio of 1: 5 were incubated at 37°C and 5% CO_2 for 48 h. The infection was done at day 1 (for monocytes) and day 7 (for MDMs) after monocytes isolation. Un-coated (no sera added) *L. donovani* and *L. major* were used to infect human monocytes in the same ratio as a control.

Determination of parasite/cells interactions

Supernatants were decanted from each well and chambers were removed, then the slides were fixed with methanol, stained with 3% Giemsa stain for 10 min, and finally examined under the microscope using a ×100 objective. The percentage of macrophages that contained amastigotes and the number of amastigotes per infected cells were determined in replicate cultures by counting 100 cells per well. Data analysis was performed using Epi Info and statistical significance was set at $p < 0.05$.

RESULTS

More than 90% of sera (94%; 63/67) had titers ≥ the diagnostic cutoff level of 6400, about 50% (32/67) of those had high antibodies titers (>102400), and they were included in parasite coating while a minority (1.4%; 1/67) had borderline titers of 3200 and only 4.4% (3/67) were direct antiglobulin test (DAT) non-reactive. The monocytes from a single donor phagocytosed promastigotes and supported intracellular multiplication for both *L. donovani* and *L. major* parasites. On day 1, the mean parasite number per monocyte/MDM was 626 ± 91 and 598 ± 30 for antibody-coated *L. donovani* and *L. major*, respectively, compared to 412 ± 70 and 228 ± 49 for un-coated isolates respectively (p= 0.01 for *L. donovani;* p= *0.00* for *L. major*) (Table 1). On the other hand the percentage of infected cells was significantly higher (p=00) for all antibody-coated isolates compared to uncoated ones (Table 2). The highest infection rate was demonstrated in cells infected with antibody-coated *L. donovani* (100%), unlike *L. major* isolates where only 50% of the cells were infected. Only 40% and 20% of cells were infected by un-coated *L. donovani* and *L. major* promastigotes respectively (Table 2). There was a significant difference between infectivity of *L. donovani* and *L. major;* the infection rate of *L. donovani* was two-fold higher than *L. major* in all experiments (p=0.00).

The ability of monocytes-derived macrophage to support growth of *Leishmania* parasite was determined using cells at day 7 following cells isolation. The cells formed a confluent monolayer, mainly due cell spreading as observed by phase microscopy. There was insignificant increase in the mean parasite number and infectivity rate of both antibody-coated leishmania strains in all experiments done compared to day 1. At day 7 post parasites coating, a significant increase in the mean number per infected macrophages was noticed *(p = 0.00)* with 100 and 60% infectivity rates. However, these rates were diminished to 60% and 25% for uncoated *L. donovani* and *L. major*, respectively. The mean antibody-coated parasite number per infected macrophage was 1170 ± 122 for *L. donovani* and 692 ± 59 for *L. major*, while uncoated isolates showed a mean of 418 ± 85 and 234 ± 60 for *L. donovani* and *L. major*, respectively (Table 1).

DISCUSSION

Immunity against visceral leishmaniasis starts when the parasite is successfully internalized into mononuclear phagocytes and parasite antigen is presented in association with MHC Class II molecules to T-cells. Several host cell surface molecules have been proposed to mediate internalization of *Leishmania* promastigotes into macrophages (Kumar et al., 2002). Although leishmanial infections induce strong humoral response, antibodies appear to play no role in protection. In fact, they are associated with non-healing forms of leishmaniasis in some cases (Tripathi et al., 2007), while Moreno et al. (2010) demonstrate that B lymphocytes have an early role in Leishmania immunity.

In this study, we attempted to demonstrate that humoral immunity probably play a role in subclinical infection and, as well, in clearing of the parasites. First, it is well documented that individuals with reduced immunoglobulins do not heal as effectively as those with normal levels (Musa et al., 2005b). Secondly, data on post-kala-azar dermal leishmaniasis (PKDL) chemotherapy showed that individuals with low immunoglobulins do not heal despite the fact that their leishmanin skin test is reactive (Musa et al., 2005b). Thirdly, it is well documented that some individuals living in VL endemic areas in Sudan develop subclinical infection as evidenced by high anti-leishmanial antibodies that may exists for sometime before they convert in leishmanin skin test. This is unlikely due to a subcellular level of protection through Th1 type of cytokines, since IL-10 reciprocally inhibits IFN-γ secretion, while protection is dependent on an IL12 driven type 1 response and INF- γ production, which results in the induction of parasite killing by macrophages primarily via the production of reactive nitrogen and oxygen intermediates (Ghalib et al., 1995; Sharma and Sing, 2009). Fourthly, HIV/VL co-infected patients are incompetent to clear the leishmania infection.

Co-infected patients tend to have false-negative results with the direct agglutination test (DAT) used to test for *Leishmania* antibodies (Deniau et al., 2003; Herrera et al., 1995), an increased parasite load in blood and bone marrow, lower sensitivity of serological tests and a higher rate of treatment failure (Akuffo et al., 1997; Niamba et al., 2007) Due to the depletion of both the cellular and humoral responses to *Leishmania* in co-infected patients (Moreno et al., 2000), there is an increased risk of disease progression of leishmaniasis after *Leishmania* infection in HIV+ individuals (Ezra et al., 2010), which could probably be due to their failure to produce anti-leishmanial antibodies that lead the initial process of induction of an effective immune response. They are therefore able to internalize parasites but are incapable of activating the macrophages to kill the parasites. Fifthly, it is well documented in animals that interaction between anti-leishmanial antibodies and the macrophages/dendritic cells leads to maturation of these cells (Miles et al., 2005). The findings of the direct agglutination test in the present study showed universal elevation of anti-leishmanial antibodies in sera of confirmed VL. This is according to previous studies from Sudan and abroad (Neogy et al., 1987; Atta et al., 1998; Khalil et al., 2002).

In this study, the increased rate of parasite uptake as well as the numbers of infected cells observed during antibody-coated *L. donovani* interactions is a clear evidence of infectivity and probably points to the fact that anti-leishmania antibodies facilitated the parasite internalization by monocytes and MDMs. The comparative *L. major* strain tested in this study almost showed similar cell infectivity. These conclusions are in consistency with previous findings (Kima et al., 2000; Miles et al., 2005). However, our findings are discordant with animal studies in BALB/c mice where anti-*Leishmania* IgGs did not alter the uptake of amastigotes (Guy and Belosevic, 1993; Woelbing et al., 2006), and therefore our results strengthen the view that FcR-mediated invasion clearly play a pivotal role in the establishment of *Leishmania* infections *in vivo* (Diebold and Bokoch, 2005).

Interestingly, this study demonstrated a reduced rate of phagocytosis observed in uncoated *Leishmania* parasites that could point to a possible alternative mechanism for parasite internalization. In the present study, the monocyte-derived macrophages showed higher percentage of infected cells and increased number of the parasite per cell for both *L. donovani* and *L. major* compared to monocytes. This could be explained by the fact that the functional capabilities of fully differentiated macrophages and blood monocytes differ, and the outcome of infection probably depends on the stage of differentiation of the host cells as documented previously by Bosque et al. (1998). The study showed an increase in parasite uptake and percentage of infected cells with *L. donovani* compared to *L. major* that could probably be due to isolate virulence and not strain-dependent.

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