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ARTICLE

Destiny and Intracellular Survival of *Leishmania amazonensis* in Control and Dexamethasone-treated Glial Cultures: Protozoa-specific Glycoconjugate Tagging and TUNEL Staining

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SUMMARY *Leishmania amazonensis*, an obligatory intracellular parasite, survives internalization by macrophages, but no information is available on the involvement of microglia. We have investigated microglia–protozoa interactions in mixed glial cultures infected with promastigote forms of *L. amazonensis* after lipopolysaccharide (LPS) or dexamethasone (DM) treatment. After 2 hr of exposure to parasites in control cultures, there was a small number of infected microglia (1%). Preincubation with LPS or DM led to 14% or 60% of microglial cells with attached parasites, respectively. DM treatment resulted in 39% of microglial cells with internalized parasites (controls or LPS-treated cells had ≤1%). Scanning electron micrographs showed numerous filopodia in DM-treated cells, whereas these projections were rarely observed in LPS-treated or control cells. DM treatment also affected the intramicroglial survival of *Leishmania*. In control cultures, internalized parasites, tagged with an anti-lipophosphoglycan (anti-LPG) antibody, showed fragmented DNA [terminal deoxyribonucleotide transferase-mediated dUTP-X nick end labeling (TUNEL+)] after 4 hr of interaction, but changes seemed slightly delayed in DM-treated cultures. After 12 hr, there were no LPG+/TUNEL+ profiles in controls, whereas rare LPG+ profiles still persisted in DM-treated cells. Our results suggest that microglia are highly effective in the elimination of *Leishmania* and that the process can be effectively studied by LPG/TUNEL double labeling. **(J Histochem Cytochem 52:1047–1055, 2004)**

KEY WORDS
microglia–protozoa interactions
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lipopolysaccharide

MICROGLIA are the main immune effectors of the central nervous system (for reviews, see Kreutzberg 1996; Streit 2002). They can be activated in response to a wide range of injuries that trigger brain inflammatory responses, including head injury and ischemia, neurodegenerative and autoimmune diseases, viral and bacterial infections, prion diseases, and brain tumors (Sasaki et al. 1993; for reviews, see Dubois-Dalcq et al. 1995; McGeer and McGeer 1995; Kreutzberg 1996). Microglia may also play a role in host defense against some protozoa, such as *Plasmodium* (Medana et al. 1997,2000), or against others that affect the immunocompromised host, such as *Toxoplasma gondii* (Rozenfeld et al. 2003; for review, see Suzuki 2002). In addition to these relatively well-known parasites that invade the neural tissue, others, such as *Leishmania*, have become objects of concern by virtue of reports on the presence of anti-*Leishmania* antibodies and/or *Leishmania* amastigotes in the cerebrospinal fluid or of meningeal affection in naturally infected humans and animals (Garcia-Alonso et al. 1996; Nieto et al. 1996; Prasad and Sen 1996; Vinuelas et al. 2001) plus transmission from infected patients to experimental animals under conditions that simulate needle sharing (Morillas-Marquez et al. 2002). More-

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over, recent work has shown parasitized macrophages in the cerebral parenchyma in experimental tegumentary leishmaniasis in mice (Abreu-Silva et al. 2003).

Leishmania are obligatory intracellular pathogenic parasites that must gain entrance into mononuclear phagocytes to successfully complete their cell cycle and parasitize various species of mammalian hosts (for reviews, see Cunningham 2002; Sacks and Sher 2002; Vannier-Santos et al. 2002). However, it is not known how the "macrophages" of the central nervous system (the microglia) interact with this obligatory intracellular parasite in their physiological environment, i.e., in the presence of other major components of the neural tissue, such as astrocytes. Thus, we have chosen to study the interactions of *Leishmania amazonensis* with microglia in mixed glial cultures from neonatal tissue and compare these interactions with known features of *Leishmania*–macrophage interactions. We have also used treatment of microglia in mixed glial cultures with immune modulators [lipopolysaccharide (LPS) or dexamethasone (DM)] before interaction with *L. amazonensis* and have followed the destiny of the parasites by their specific lipophosphoglycan (LPG) membrane labeling plus terminal deoxyribonucleotide transferase-mediated dUTP-X nick end labeling (TUNEL) staining.

We have found that, in contrast to macrophages (Kane and Mosser 2000; for reviews, see Cunningham 2002; Vannier-Santos et al. 2002), microglia eliminate intracellular parasites very rapidly and that this process seems to be slowed by DM treatment. Our results indicate that microglia represent a highly effective barrier to the invasion of the brain by *L. amazonensis* and suggest that an extensive investigation of the mechanisms involved may provide additional clues to microglial physiological functions vis-a-vis intracellular parasites.

Materials and Methods

Primary Glial Cell Cultures

Forebrains isolated from newborn Swiss mice were dissected out, minced, and incubated at 37C with 0.002% trypsin (Difco; Detroit, MI) in Ca^{2+}/Mg^{2+}-free 0.85% PBS for 5 min. The brain cells were dissociated by successive cycles of mild incubation with trypsin, with periodic interruption of the enzymatic activity by the addition of 10% fetal calf serum (FCS) (Sigma Chemical; St. Louis, MO). After trypsinization, the cell suspension was freed from large aggregates and centrifuged for 7 min at 650 × g, and the cell pellet was resuspended in DMEM (Sigma) supplemented with 10% FCS, 2% chick embryo extract, 1 mM glutamine (Sigma), 1000 U/ml penicillin (Sigma), and 50 μg/ml streptomycin (Sigma) (complete medium). The cells were counted in a hemocytometer and the cell density adjusted to 2×10^6 cells/ml. The cell suspension was plated in culture dishes or cover slips and incubated with a humidified 95% air/5% CO_2 atmosphere at 37C. After 24 hr of incubation, the cells were washed three times with DMEM and maintained for 4 additional days with a change of the medium at 3 days of incubation.

Macrophages

Murine macrophages were obtained from adult Swiss mice by harvesting of the peritoneal cavity, followed by plating of the cell suspension in 24-well culture dishes and incubation in DMEM in a humidified 95% air/5% CO_2 atmosphere at 37C for 1 hr. Afterward, nonadherent cells were removed by washing and the adherent cells were cultured in complete medium. Macrophages were preincubated with 0.1 μg/ml DM or 1 μg/ml LPS at 37C for 24 hr and used for comparisons of adhesion to and/or internalization of *L. amazonensis* by microglial cells in mixed glial cultures after 2 hr of interaction of either mammalian cell with parasites (Table 1).

Parasite

L. amazonensis (MCAN/BR/94 DCB-16) were isolated from dogs with cutaneous lesions as amastigote forms and afterward maintained in Schneider medium (Sigma) supplemented with 10% FCS for 6–7 d. Within this period, the culture was followed at daily intervals to verify when the parasites transformed into promastigote forms and reached the stationary growth phase. The promastigote forms (Figure 1B) were used to infect the cultured cells at this phase.

Cell Identification

For identification of the microglia, the cultures were treated with either the BSI-B4 isolectin of *Griffonia simplicifolia* (Sigma) or with the F_{4-80} monoclonal antibody (Caltag Laboratories; San Francisco, CA). In the first case, the cells were incubated with 25 μg/ml BSI-B4 coupled to FITC at 37C for 1 hr or by binding of biotinylated BSI-B4 isolectin followed by reaction with horseradish peroxidase (HRP)-avidin (Sigma; EXTRA-2) and the peroxidase activity was revealed

Table 1 *L. amazonensis* adhesion to or internalization by microglial cells or macrophages after 2-hr interactions[a]

	Microglial Cells				Macrophages			
Treatment	Percent Total with Adhered Parasites	Adhered Parasites/Cell	Percent Total with Internalized Parasites	Internalized Parasites/Cell	Percent Total with Adhered Parasites	Adhered Parasites/Cell	Percent Total with Internalized Parasites	Internalized Parasites/Cell
Control	1.0 ± 0.1	1.0 ± 0.5	1.0 ± 0.1	2.0 ± 1.4	55.0 ± 0.4	3.2 ± 0.6	80.0 ± 5.5	3.1 ± 0.3
LPS	14.0 ± 0.4	1.4 ± 0.8	0	0	19.0 ± 0.4	2.5 ± 0.6	90.0 ± 3.5	3.2 ± 0.2
DM	60.0 ± 0.4	1.7 ± 2.6	39.0 ± 0.7	2.1 ± 1.8	50.0 ± 2.6	2.5 ± 0.3	75.0 ± 1.1	3.0 ± 0.3

[a]Values shown are means ± SEM of five separate experiments.

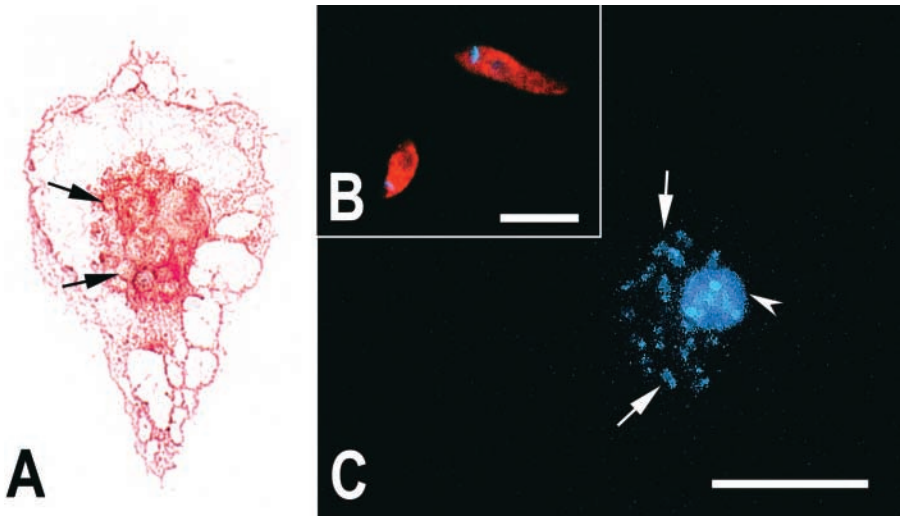

Figure 1 Phenotypic identification of infected microglia and *Leishmania* by glycoconjugates. (**A**) Light-field micrograph of BS1-B4 isolectin binding of infected microglia. Arrows point to parasitophorous vacuoles. (**B**) Dark-field micrograph of 45D3 (anti-LPG) antibody binding (Cy3 label) of *L. amazonensis*. Blue dots show DAPI counterstaining of parasite nuclei. (**C**) Lack of staining of the same microglial cell or internalized parasites with Cy3-tagged secondary antibody only. The microglial cell nucleus (arrowhead) and internalized parasite nuclei plus kinetoplasts (arrows) are stained with DAPI. Bars: **B** = 5 μm; **C** = 20 μm.

with H_2O_2 using 3-amino-9-ethyl carbazol (AEC; Sigma) as coupler (Figure 1A). For double staining of microglial cells, the cultures were also treated with the $F_{4–80}$ primary antibody. Briefly, the samples were washed three times in PBS and incubated in blocking solution (10% normal rabbit serum diluted in PBS plus 1% BSA) plus 0.1% saponin for 1 hr at 37C, incubated with $F_{4–80}$ diluted 1:10 at 37C for 1 hr, washed three times in PBS, and incubated with a Cy3-tagged rabbit anti-rat IgG secondary antibody diluted 1:300. Controls were treated by omission of the primary antibody. The nuclei of the cells were stained with 4′,6-diamino-phenylindole (DAPI; 0.1 μg/ml) (Sigma). The samples on cover slips were mounted on slides with diazabicyclo[2.2.2] octane (DABCO) (Sigma) and analyzed with a Zeiss epifluorescence photomicroscope (Zeiss do Brasil, Sao Paulo, Brazil).

For identification of the parasites, the cultures infected as described above or the promastigotes attached onto poly-L-lysine-coated microscope slides were fixed in 4% paraformaldehyde (Sigma) at room temperature for 5 min. Afterward, the samples were washed three times in PBS and incubated in blocking solution (10% normal rabbit serum diluted in PBS plus 1% BSA) plus 0.1% saponin at 37C for 1 hr. Later, the cover slips were incubated with 45D3 monoclonal (IgG1 anti-LPG) antibody (kindly donated by Dr David Sacks, National Institutes of Health, Bethesda, MD) diluted 1:50 at 37C for 1 hr. After incubation with the primary antibody (Lang et al. 1991), the samples were washed three times in PBS and incubated with a Cy3-tagged rabbit anti-mouse IgG secondary antibody (Caltag) diluted 1:200 (Figure 1B). Controls were treated by incubating the samples in the absence of the primary antibody (Figure 1C). The nuclei and kinetoplasts of the parasites were stained with DAPI (Sigma) (Figures 1B and 1C; see Figure 5) at a concentration of 0.1 μg/ml. The samples on cover slips were mounted on slides with DABCO (Sigma) and analyzed with a Zeiss epifluorescence photomicroscope.

Surface Morphology of Infected Microglial Cells

Five-day-old cultures were pretreated for 24 h with 0.1 μg/ml DM or complete medium, washed three times with PBS, and then incubated with promastigote forms at 4C for 1 hr (parasite/microglia ratio = 10:1). DM-treated and untreated infected cells were rinsed, fixed for 1 hr in 2.5% glutaraldehyde (Sigma), postfixed with 1% osmium tetroxide (Sigma) for 15 min, and dehydrated in acetone. Afterward, they were dried in CO_2 in a critical point dryer (Balzers CPD030; Balzers Union, Lichtenstein) and coated with gold (Balzers MED 010). Samples were examined in a scanning electron microscope (Zeiss DSM 940).

Kinetic studies of Microglia–*Leishmania* Interaction

Mixed glial cultures were preincubated in DMEM/FCS with 0.1 μg/ml DM or 1 μg/ml LPS or maintained without treatment (control) at 37C for 24 hr. After that, the cultures were washed several times with serum-free DMEM and the total number of cells was estimated by counting several fields. Promastigote forms of *L. amazonensis*, previously centrifuged and suspended in serum-free DMEM, were added to the cultures to achieve a ratio of ~10:1 parasites/glial cells, and the cell/parasite contact was maintained for 2 hr. After this period, cultures were rinsed with PBS to remove extracellular parasites, DMEM with 2% BSA was added, and the infection was followed at 37C for up to 72 hr, with fixation of infected cells at 0, 2, 4, and 10 hr after PBS rinsing (2, 4, 6, and 12 hr of interaction). Infected cells were fixed with Bouin's solution and stained with Giemsa. The percentage of microglial cells containing parasites attached or internalized was then determined by examining, in bright-field optics, randomly selected cells in at least 300 fields at 1000× magnification with a Zeiss photomicroscope (see Figures 3 and 4). To avoid missing internalized parasites that might have undergone shape or size changes, every microglial cell was analyzed in a through-focus mode.

DNA Degradation of Internalized Parasites

To approach the question of microglial killing of internalized parasites, a triple-labeling procedure was used. First, microglial cells in fixed cultures were identified by binding of biotinylated BSI-B4 isolectin followed by HRP-avidin

(Sigma), and the peroxidase activity was revealed with H_2O_2 and AEC. After that, components of the parasites were detected with the 45D3 monoclonal antibody as described above. Finally, the infected cells were incubated with the TUNEL kit as described by the manufacturer (Boehringer, Mannheim, Germany).

For the TUNEL labeling, the samples on cover slips, previously fixed in 4% paraformaldehyde at room temperature, were permeabilized with 0.1% saponin (Sigma) on ice for 30 min. After washing, the samples were incubated with the TUNEL reaction mixture containing Terminal deoxyribonucleotide Transferase (enzyme solution) and fluorescein-dUTP (labeling solution) at 37C for 1 hr. The positive controls were treated by incubating the infected cells in the presence of DNase I (Sigma) and applying the TUNEL procedure. The cover slips were mounted on slides with DABCO and analyzed under bright-field and epifluorescence conditions.

Results

In phase-contrast optics or preparations stained by the Giemsa method, most cells (~76%) in our 5-day cultures presented morphological features consistent with those of protoplasmic astrocytes (Moura Neto et al. 1985), occurring either as single cells or as small clusters of confluent cells (data not shown). Cells of varied morphologies (~23%) were also found either on top of the astrocyte clusters or adherent to the plastic and were tentatively identified as microglia. This morphological identification was confirmed by phenotypic cell markers both before (data not shown) and after (Figure 1A) infection. Approximately 1% of the cells remained unidentified.

Surface Morphology of Infected Microglial Cells

To demonstrate the in vitro influence of immune function mediators on the morphology of microglial cells, we used the macrophage function modulators DM and LPS during the interaction of microglia with promastigote forms of *L. amazonensis*. After pretreatment of the host cell for 24 hr, followed by washing and interaction with the parasite at 4C for 1 hr, scanning electron microscopy analysis showed numerous filopodia (Figure 2A) in DM-treated cells, whereas these projections were rarely observed in LPS-treated

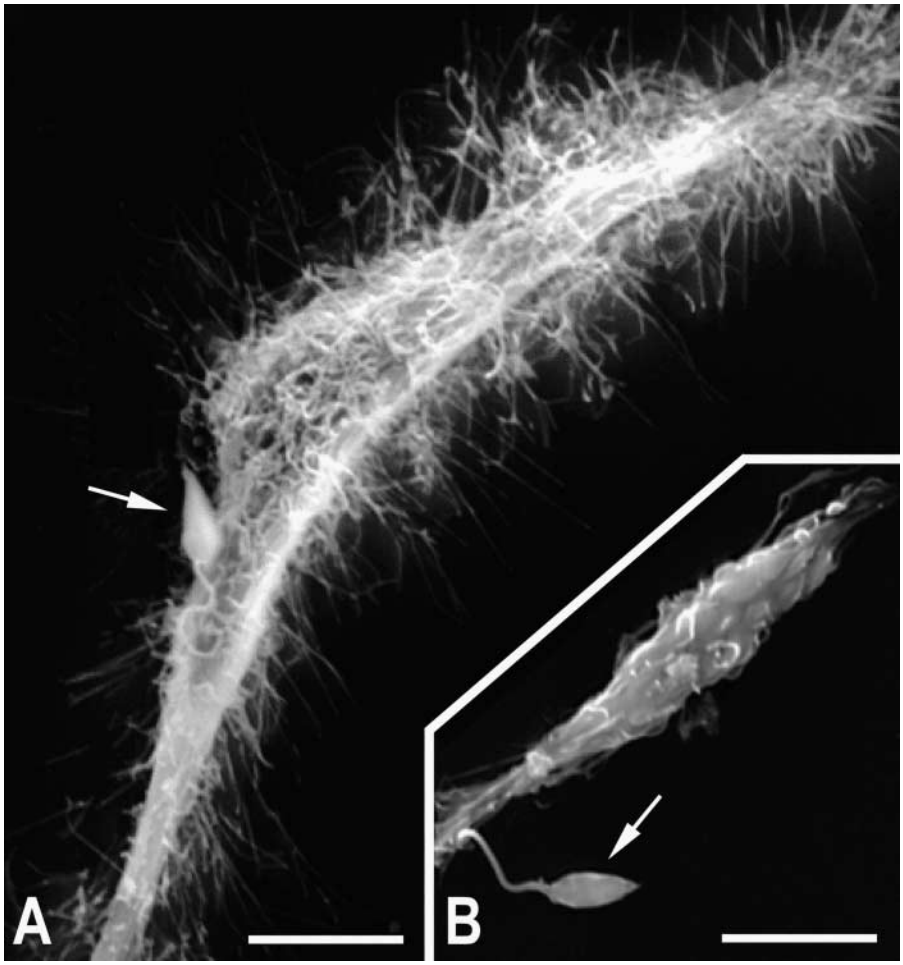

Figure 2 Ultrastructural analyses showing adhesion of promastigotes of *L. amazonensis* to DM-treated microglia (**A**) or to untreated controls (**B**) after interactions at 4C for 1 hr. DM-treated cells showed numerous philopodia, whereas in control cultures these projections were rarely observed, even in the presence of adhered parasites (arrows). Bars: **A** = 10 μm; **B** = 15 μm.

(data not shown) or control (Figure 2B) cultures, even in the presence of adhered parasites.

Quantitative Analysis of *L. amazonensis* Adhesion to and Internalization by Microglial Cells or Peritoneal Macrophages

Rare microglial cells (1% only) displayed parasites adhered to their surface, whereas adhesion to peritoneal macrophages occurred in more than half of these cells (Table 1, control). LPS or DM treatment of cultures showed a differential response for the two cell types. The treatment of microglial cells with LPS was effective in promoting the adhesion of parasites to their cellular surface, with such adhesion increasing from 1% in controls to 14% in the LPS-treated cells. By contrast, after LPS treatment of peritoneal macrophages, the percentage of cells with adhered parasites decreased from 55% in the control to 19% (Table 1). There were no major changes in the number of adhered parasites per microglial cell or per macrophage after treatment with LPS.

After DM treatment, there was a significant increase in the percentage of microglial cells with adhered parasites, increasing from 1% in the untreated cultures to 60% in the DM-treated cultures (Table 1). On the other hand, this increase was not observed in macrophages after the same treatment with DM, so that the percentage of cells with adhered parasites remained nearly identical to half of the cells. There were no major changes in the number of adhered parasites per microglial cell (Figure 3) or per peritoneal macrophage (Table 1) vis-à-vis the treatment with DM.

With respect to the internalization of parasites, similar to what was observed in adhesion assays, a very low number of untreated microglial cells presented intracellular parasites compared with untreated peritoneal macrophages (1% vs 80%; Table 1). No intracellular parasites were observed in microglial cells after treatment with LPS, whereas a very large percentage (90%) of macrophages contained intracellular parasites. The number of intracellular parasites per LPS-treated infected macrophage was similar to that in control cells.

The preincubation of microglial cells with DM favored parasite–cell interactions. Thus, the percentage of cells with intracellular parasites increased from 1% in the control to 39% after DM treatment (Table 1). However, the number of intracellular parasites per cell tended to remain unaltered in this short-term experiment. In the case of macrophages, we observed that 80% of the untreated cells already showed an average of 3.1 parasites per cell, whereas the percentage observed after treatment with DM was 75% with 3.0 parasites per cell (Table 1).

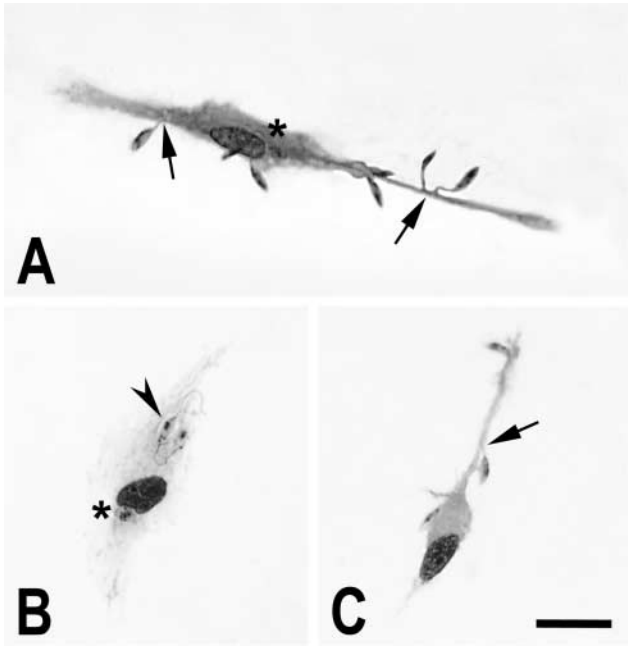

Figure 3 Giemsa-stained microglial cells from DM-treated (**A**,**B**) and untreated (**C**) cultures with adhered (arrows) or internalized (arrowhead) *L. amazonensis*. Note several adhered parasites in (**A**) and one internalized parasite adjacent to the cell nucleus. A profile in (**B**) (arrowhead) is suggestive of a recent internalization (promastigote form maintained), whereas a paranuclear profile (asterisk) seems to have undergone an abortive transformation to amastigote. The cell in (**C**) is representative of untreated cultures by the small number of adhered parasites. Bar = 15 μm.

Kinetics of Microglia Infection by *L. amazonensis*

In preparations stained by the Giemsa procedure, we evaluated the kinetics of endocytosis of the promastigote forms of *L. amazonensis* in microglial cells maintained only in medium or preincubated with LPS or DM for 24 hr. The intracellular destiny of the parasites was analyzed after interaction with microglia for 2 hr, after which the cultures were washed and maintained in serum-free DMEM (2% BSA added), and the infection was followed for periods of 2, 4, 6, and 12 hr. In the cultures preincubated with LPS, we observed no internalized parasites or their residues in the host cell at any of the time intervals used (data not shown).

A significant number of internalized parasites was seen in DM-treated cells at 2 hr after infection (Figure 4B) compared with the untreated cells (Figure 4A). At 4 hr, stained profiles that could not be identified as viable parasites were observed in untreated cultures. In these untreated cells, there were structures in vacuoles distributed in different regions of the cytoplasm, but such structures were not clearly identified as parasites (Figure 4C). At the same time interval, in DM-treated

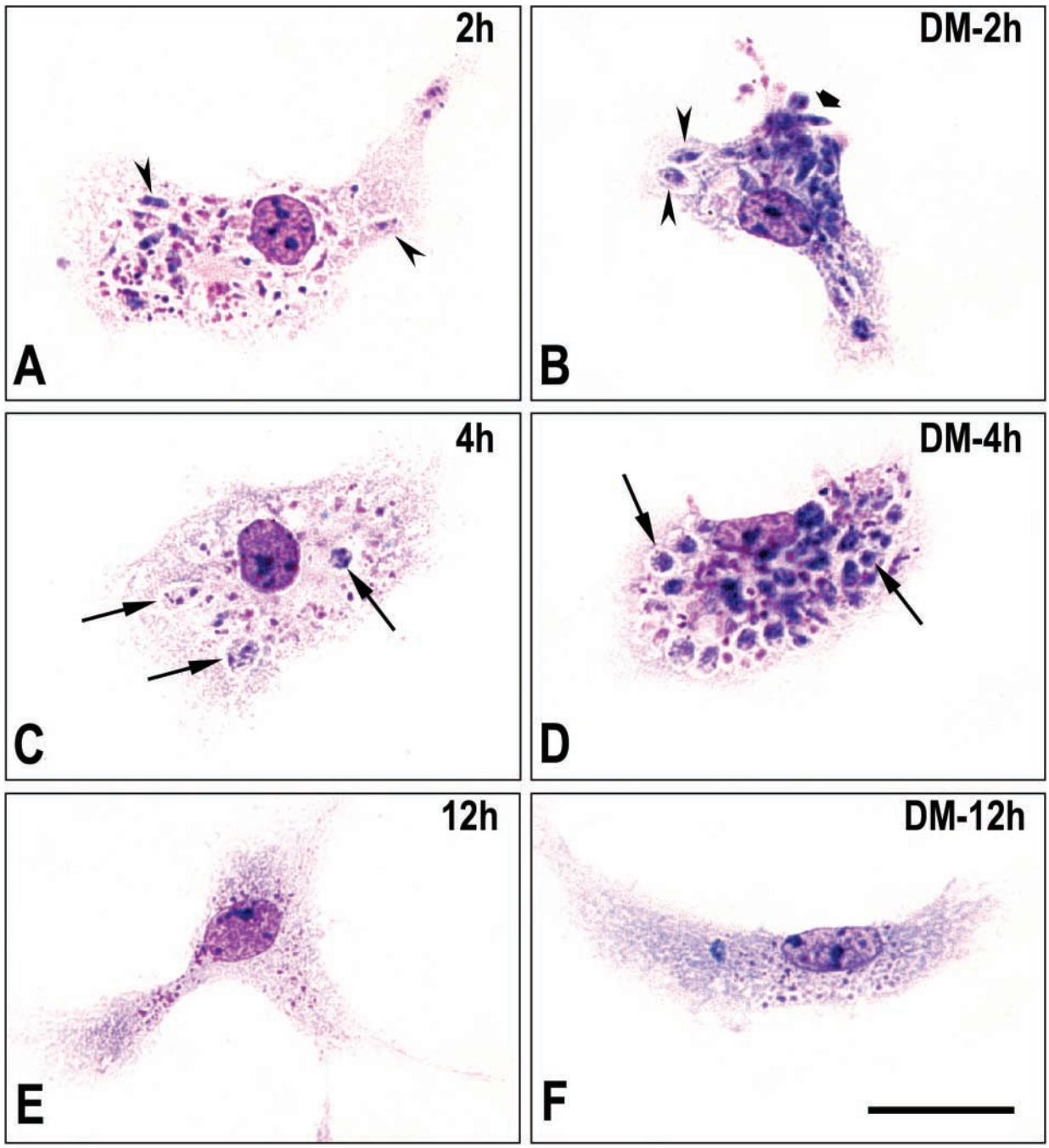

Figure 4 Kinetics of endocytosis of *L. amazonensis* by DM-treated microglia. Untreated control (**A**,**C**,**E**) and DM-treated (**B**,**D**,**F**) microglial cells were rinsed, infected with *L. amazonensis* for 2 hr, and rinsed again. In the control, after 2 hr of infection (**A**), intracellular parasites with altered morphology (arrowheads) are observed. In DM-treated cultures (**B**), there are many adhered (thick arrow) or internalized (arrowheads) parasites with no damaged aspect, together with apparently damaged parasites. After 4 hr of infection, a substantial reduction is found in the number of internalized parasites (arrows) in untreated cultures (**C**), whereas this decrease is delayed in DM-treated cultures (**D**). After 12 hr of infection, no parasites are detected in untreated (**E**) or DM-treated (**F**) cultures. These results are representative of five separate experiments. Bar = 15 μm.

cultures, several parasitophorous vacuoles with a large amount of cellular remains similar to killed parasites were observed in the microglial cytoplasm (Figure 4D), indicating a delay in the elimination of parasites in DM-treated cultures compared with untreated cultures. At 12 hr after infection, only vestiges of presumptive parasites were found in any of the cultures (Figures 4E and 4F).

DNA Degradation of Internalized Parasites

To validate the findings of our study of the kinetics of *L. amazonensis* endocytosis by microglia, we used the TUNEL technique to evaluate the intracellular survival of the parasites. That was done through the identification of fragments of nuclear and kinetoplast DNA generated during the degradation and/or death process by digestion of the parasite by cells identified as microglia via BSI-B4 isolectin staining. We also used the (anti-LPG) 45D3 monoclonal antibody, which recognizes a characteristic LPG on the surface of *Leishmania* promastigotes (Lang et al. 1991) that is absent on amastigotes (Pimenta et al. 1991), for the localization of intracellular parasites or their residues during the infection of microglia. The promastigote forms of *L. amazonensis*, used for infection of the cultures, showed binding of the anti-LPG antibody throughout their surfaces (compare Figure 1B).

The LPG antigen and fragmented DNA of the parasite were detected at the 2-hr interval within both control cells and DM-treated microglia (data not shown). TUNEL+ nuclei and kinetoplasts of the presumptive promastigotes colocalized with LPG+ membrane structures of the parasite. There was a larger number of these LPG-labeled profiles together with nuclei of dead parasites in cells treated with DM compared with control cells (data not shown). At 4 hr, LPG+ profiles and nuclei of the tagged parasites were numerous, with apparent aggregation of LPG+ profiles in the control cells (Figures 5A and 5B) and their punctual distribution close to the nucleus of the DM-treated cells (Figures 5C and 5D). At 6 hr, the amounts of parasite membrane profiles and fragmented DNA were noticeably reduced in relation to the previous times, but the distribution of both membrane remains and DNA fragments was not altered largely in either group (Figures 5E–5H). Similarly, there was a com-

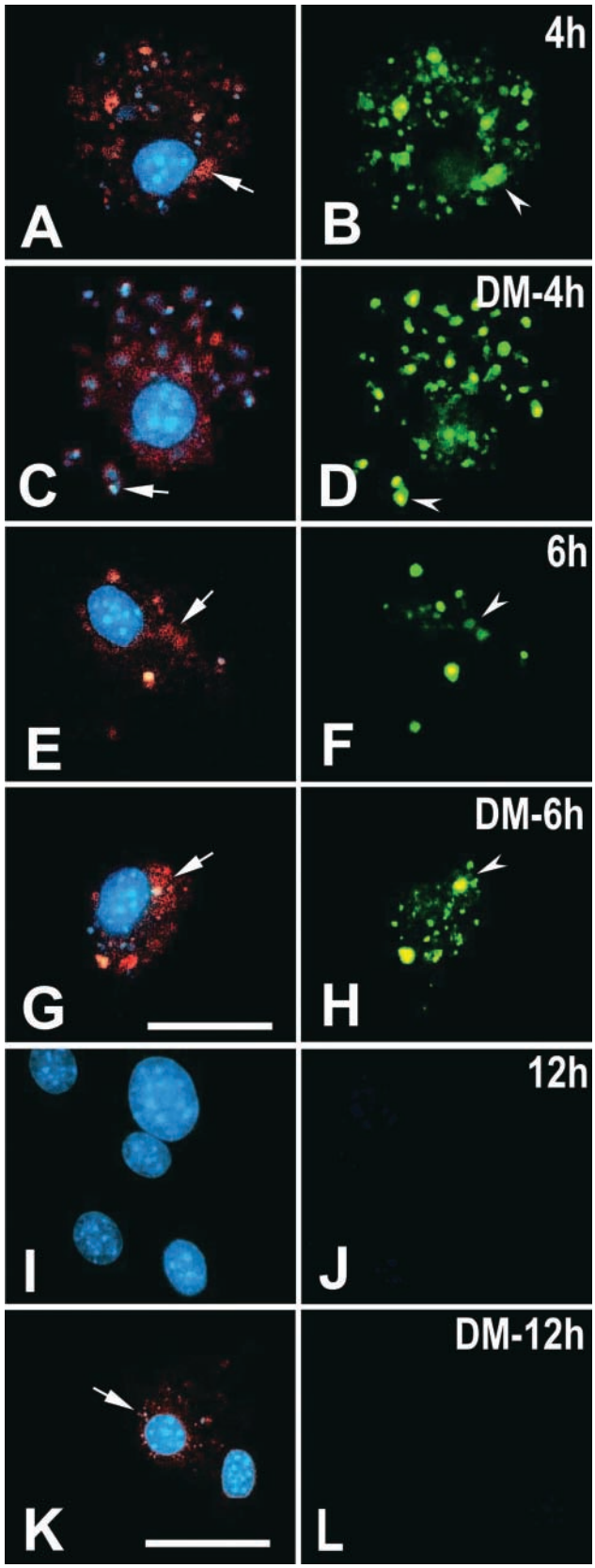

Figure 5 Kinetics of endocytosis of *L. amazonensis* by microglia in control cultures (**A**,**B**,**E**,**F**,**I**,**J**) and DM-treated cultures (**C**,**D**,**G**,**H**,**K**,**L**) at 4-, 6-, and 12-hr interaction periods. In the left-hand column, the nuclei of microglial cells (plus one large nucleus of an astrocyte in **I**) and/or parasites are stained with DAPI, and a *Leishmania*-specific LPG is revealed with 45D3 monoclonal antibody and Cy3-tagged secondary antibody (arrows). The right-hand column shows TUNEL-stained fragmented DNA. In the second, fourth, and sixth rows, the cultures were pretreated with DM, washed, infected for 2 hr, and washed again after 2, 4, and 10 h of infection, respectively (times in the panels indicate additional periods after the removal of non-internalized parasites). Observe that DM-treated microglia contain more parasites or fragments than control cells at 4 and 12 hr (**C**,**K** vs **A**,**I**) and at least as many parasite profiles at 6 hr (**G** vs **E**). Observe that most of the *L. amazonensis* nuclear or kinetoplastic DNA is degraded at intervals up to 6 hr and totally eliminated by the microglia at 12 hr. These results are representative of five separate experiments. Bars: **G** = 20 μm (applies to **A**–**H**); **K** = 25 μm (applies to **I**–**L**).

mon tendency for the aggregation of the LPG+ structures in both control and DM-treated cells (Figures 5E–5H). At 12 hr after infection, no LPG+ profiles or TUNEL-labeled particles were detected in control microglial cells (Figures 5I and 5J), whereas rare LPG+ profiles (Figure 5K) but no TUNEL label (Figure 5L) were found in DM-treated cells.

Discussion

Before proceeding to a discussion of our results, a few methodological comments are in order. In our experiments, we first tried to evaluate the infective potential of promastigote forms of *L. amazonensis* in astrocytes and/or microglial cells and verified that the protozoa adhered to and were internalized by microglia only. The use of primary glial mixed cultures was considered advantageous because microglial cells under this condition would be in an activation state different from that in pure cultures and, presumably, more akin to the in vivo situation (Glenn et al. 1989; Lee et al. 1992; Rezaie et al. 2002). It is known that, at least in some species, substances such as cytokines are secreted in mixed glial cultures and are able to cause morphological and phenotypical changes on microglia (Lauro et al. 1995; Liu et al. 1996).

Both LPS and DM treatments resulted in larger percentages of microglial cells with adhered parasites than for controls, possibly attributable to different mechanisms. Because LPS downregulates the mannose fucose receptor (MFR) (Marzolo et al. 1999), which is required for the establishment of intracellular parasitism in mononuclear phagocytes (Wilson and Pearson 1986), enhanced parasite adhesion in LPS-treated microglia may depend on the upregulation of molecules that do not trigger the internalization of parasites (Table 1).

In spite of the verification of a larger number of adhered parasites and increased percentage of cells that internalized parasites among those previously treated by DM compared with control cells, it was observed that apparently viable or intact parasites were no longer stained by the Giemsa method as soon as after 4 hr of interaction. This observation was reinforced by the TUNEL procedure, showing that in both untreated and DM-treated cultures there was fragmentation of the parasite nucleus and kinetoplast DNA as early as after 2–4 hr of interaction. The larger percentage of cells that internalized parasites in DM-treated cultures may derive from either or both of the following effects. First, the MFR could be upregulated by DM treatment, and this upregulation could lead to increased adhesion of parasites to the microglia surface and consequent internalization. This effect would be in agreement with the DM-induced MFR upregulation demonstrated in purified microglial cultures (Marzolo et al. 1999). Furthermore, preliminary studies of ours have shown that the addition of D-mannose to DM-treated mixed glial cultures impairs both microglial infection and adhesion as well as the internalization of the neoglycoprotein mannosyl-BSA (W. Baetas-da-Cruz, unpublished master's thesis). However, the simple alternative that a heavier load of parasites could lead to an apparent delay in the killing and disposal of the parasite can probably be ruled out because the number of internalized parasites per cell at 2 hr of interaction is not different in DM-treated and control cells (Table 1).

A second effect would be DM treatment causing the downmodulation of inducible nitric oxide synthase and, thus, the reduction of nitric oxide synthesis and release. Again, it is known that cortisol (and possibly DM) inhibits the activation of microglia and decreases the production and release of nitric oxide and tumor necrosis factor-α (Drew and Chavis 2000), important cytotoxic and cytotoxicity-inducer molecules, respectively, for *Leishmania* (Roach et al. 1991). At present, we have no evidence that the apparently longer survival of *L. amazonensis* after DM treatment involves either the upregulation of the MFR receptor or the reduction of nitric oxide production by microglia in mixed cultures. The major astrocyte population of these cultures may express the MFR (Burudi et al. 1999) and the endothelial type of nitric oxide synthase (Wiencken and Casagrande 1999), making the identification of the source and/or the quantitative assessment of either MFR or nitric oxide ambiguous or unreliable. Alternative culture models are being tested in our laboratories to approach the questions of the mechanisms involved in phagocytosis and disposal of the parasite by microglia.

It cannot be ruled out that, as in monocytes (Ma et al. 2004), DM acts through the suppression of interleukin-12 production by microglia. In other words, DM would further decrease any remaining interleukin-12 production by microglia, which is already depressed by astrocytes (Aloisi et al. 1997), in mixed glial cultures. Clearly, additional work is necessary to investigate this issue.

The results obtained with the TUNEL procedure are compatible with the notion that DM treatment delays the killing and eventual disposal of *Leishmania* by microglia. More importantly, they emphasize that the microglial response to the parasite differs from that of peritoneal macrophages. Thus, there is an almost total absence of parasite residues in both DM-treated and untreated microglia after exposure of the cultures to promastigote forms for 12 hr.

In summary, microglial cells are highly effective in the elimination of *Leishmania*, at least in the particular case of mixed glial cultures. This cytotoxicity is apparently slowed by DM, indicating that microglia and

other mononuclear phagocytes present both common and atypical functional features vis-à-vis intracellular parasites that must gain entrance into host cells to successfully complete their cell cycles. The mechanisms responsible for the microglial cytotoxicity of *Leishmania* remain to be determined.

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Literature Cited

Abreu-Silva AL, Calabrese KS, Tedesco RC, Mortara RA, da Costa SC (2003) Central nervous system involvement in experimental infection with *Leishmania (Leishmania) amazonensis*. Am J Trop Med Hyg 68:661–665

Aloisi F, Penna G, Cerase J, Menendez Iglesias B, Adorini L (1997) IL-12 production by central nervous system microglia is inhibited by astrocytes. J Immunol 159:1604–1612

Burudi EM, Riese S, Stahl PD, Regnier-Vigouroux A (1999) Identification and functional characterization of the mannose receptor in astrocytes. Glia 25:44–55

Cunningham AC (2002) Parasitic adaptive mechanisms in infection by *Leishmania*. Mol Pathol. 72:132–141

Drew PD, Chavis JA (2000) Inhibition of microglial cell activation by cortisol. Brain Res Bull 52:391–396.

Dubois-Dalcq M, Altmeyer R, Chiron M, Wilt S (1995) HIV interactions with cells of the nervous system. Curr Opin Neurobiol 5:647–655

Garcia-Alonso M, Nieto CG, Blanco A, Requena JM, Alonso C, Navarrete I (1996) Presence of antibodies in the aqueous humour and cerebrospinal fluid during Leishmania infections in dogs. Pathological features at the central nervous system. Parasite Immunol 18:539–546

Glenn JA, Jordan FL, Thomas WE (1989) Further studies on the identification of microglia in mixed brain cell cultures. Brain Res Bull 22:1049–1052

Kane MM, Mosser DM (2000) *Leishmania* parasites and their ploys to disrupt macrophage activation. Curr Opin Hematol 7:26–31

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312–318

Lang T, Warburg A, Sacks DL, Croft SL, Lane RP, Blackwell JM (1991) Transmission and scanning EM-immunogold labeling of *Leishmania major* lipophosphoglycan in the sandfly *Phlebotomus papatasi*. Eur J Cell Biol 55:362–372

Lauro GM, Babiloni D, Buttarelli FR, Starace G, Cocchia D, Ennas MG, Sogos V, et al. (1995) Human microglia cultures: a powerful model to study their origin and immunoreactive capacity. Int J Dev Neurosci 13:739–752

Lee SC, Collins M, Vanguri P, Shin ML (1992) Glutamate differentially inhibits the expression of class II MHC antigens on astrocytes and microglia. J Immunol 148:3391–3397

Liu J, Zhao ML, Brosnan CF, Lee SC (1996) Expression of type II nitric oxide synthase in primary human astrocytes and microglia: role of IL-1beta and IL-1 receptor antagonist. J Immunol 15: 3569–3576

Ma W, Gee K, Lim W, Chambers K, Angel JB, Kozlowski M, Kumar A (2004) Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated human monocytic cells by down-regulating the activity of c-Jun N-terminal kinase, the activation protein-1, and NF-kappaB transcription factors. J Immunol 172: 318–330

Marzolo MP, von Bernhardi R, Inestrosa NC (1999) Mannose receptor is present in a functional state in rat microglial cells. J Neurosci Res 58:387–395

McGeer PL, McGeer EG (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Rev 21:195–218

Medana IM, Hunt NH, Chan-Ling T (1997) Early activation of microglia in the pathogenesis of fatal murine cerebral malaria. Glia 19:91–103

Medana IM, Chan-Ling T, Hunt NH (2000) Reactive changes of retinal microglia during fatal murine cerebral malaria: effects of dexamethasone and experimental permeabilization of the blood-brain barrier. Am J Pathol 156:1055–1065

Morillas-Marquez F, Martin-Sanchez J, Acedo-Sanchez C, Pineda JA, Macias J, Sanjuan-Garcia J (2002) *Leishmania infantum* (Protozoa, kinetoplastida): transmission from infected patients to experimental animal under conditions that simulate needle-sharing. Exp Parasitol 100:71–74

Moura Neto V, Mallat M, Alliot F, Pessac B, Prochiantz A (1985) Astrocytic cerebellar cell clones synthesize the beta′ isoforms of the beta-tubulin protein family. Neuroscience 16:333–341

Nieto CG, Vinuelas J, Blanco A, García-Alonso M, Verdugo SG, Navarrete I (1996) Detection of *Leishmania infantum* amastigotes in canine choroid plexus. Vet Rec 139:346–347

Pimenta PF, Saraiva EM, Sacks DL (1991) The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*. Exp Parasitol 72:191–204

Prasad LS, Sen S (1996) Migration of *Leishmania donovani* amastigotes in the cerebrospinal fluid. Am J Trop Med Hyg 55: 652–654

Rezaie P, Trillo-Pazos G, Greenwood J, Everall IP, Male DK (2002) Motility and ramification of human fetal microglia in culture: an investigation using time-lapse video microscopy and image analysis. Exp Cell Res 274:68–82

Roach TI, Kiderlen AF, Blackwell JM (1991) Role of inorganic nitrogen oxides and tumor necrosis factor alpha in killing *Leishmania donovani* amastigotes in gamma interferon-lipopolysaccharide-activated macrophages from Lshs and Lshr congenic mouse strains. Infect Immun 59:3935–3944

Rozenfeld C, Martinez R, Figueiredo RT, Bozza MT, Lima FR, Pires AL, Silva PM, et al. (2003) Soluble factors released by *Toxoplasma gondii*-infected astrocytes down-modulate nitric oxide production by gamma interferon-activated microglia and prevent neuronal degeneration. Infect Immun 71:2047–2057

Sacks D, Sher A (2002) Evasion of innate immunity by parasitic protozoa. Nat Immunol 3:1041–1047

Sasaki A, Hirato J, Nakazato Y (1993) Immunohistochemical study of microglia in the Creutzfeldt-Jakob diseased brain. Acta Neuropathol (Berl) 86:337–344

Streit WJ (2002) Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40:133–139

Suzuki Y (2002) Host resistance in the brain against *Toxoplasma gondii*. J Infect Dis 15:58–65

Vannier-Santos MA, Martiny A, de Souza W (2002) Cell biology of *Leishmania* spp.: invading and evading. Curr Pharm Des 8:297–318

Vinuelas J, Garcia-Alonso M, Ferrando L, Navarrete I, Molano I, Miron C, Carcelen J, et al. (2001) Meningeal leishmaniosis induced by *Leishmania infantum* in naturally infected dogs. Vet Parasitol 101:23–27

Wiencken AE, Casagrande VA (1999) Endothelial nitric oxide synthase (eNOS) in astrocytes: another source of nitric oxide in neocortex. Glia 26:280–290

Wilson ME, Pearson RD (1986) Evidence that *Leishmania donovani* utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. J Immunol 15:4681–4688

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