Nitric oxide and cellular immunity in experimental cutaneous leishmaniasis

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Summary

We examined the local and systemic production of nitric oxide (NO) and the pattern of cytokine during the course of *Leishmania mexicana* infection in susceptible BALB/c and resistant C57BL/6 mice. NO derivatives were measured in serum, and the expression of inducible nitric oxide synthase (iNOS), interferon (IFN-γ), interleukin (IL-4) and epidermal Langerhans cells (LC) was measured in the lesions by immunohistology. Circulating NO concentrations, iNOS+ cell density, IFN-γ+ Th1 cells and CD205+ Langerhans cells were higher in early lesions of resistant C57BL/6 mice. In contrast, susceptible BALB/c mice developed chronic and progressive lesions with a predominance of IL-4+ Th2 cells. In both susceptible and resistant mice, lesion size and lymph node volume followed a similar course. The early local and systemic production of NO in resistant mice may be related with the premature production of IFN-γ observed, contributing to the resolution of the lesion.

Introduction

Cutaneous leishmaniasis is caused by different species of *Leishmania* in the Old and the New Worlds.[1] In the New World, cutaneous leishmaniasis presents three forms, which embrace a clinical, histological and immune spectrum with different grades of severity.[2,3] The resistant form includes immunoresponder individuals with localized cutaneous leishmaniasis (LCL), who develop a Th1 immune response associated with an effective delayed hypersensitivity reaction. The susceptible form includes nonresponder individuals with diffuse cutaneous leishmaniasis (DCL), characterized by a Th2 immune response associated with the production of nonprotective antibodies.[2–4] Mucocutaneous leishmaniasis (MCL) and chronic or intermediate cutaneous leishmaniasis (ICL) are in the middle of the spectrum; both are characterized by an exacerbated cell-mediated immunity with a mixed pattern of cytokine.[4,5] In mice, depending on the mouse strain, the *Leishmania* strain and the number of inoculated parasites, it has been possible to reproduce the distinct clinical forms observed in humans. Thus, syngeneic strains C57BL/6, CBA, C3H/He and AKR are resistant to *Leishmania major* and *Leishmania mexicana* infection and develop skin lesions that spontaneously resolve, similar to LCL. In contrast, BALB/c, DBA/2 and A/Jax are susceptible and develop chronic and progressive lesions similar to DCL.[6] The Th1/Th2 concept has been shown to be legitimate in the mouse model of cutaneous leishmaniasis.[7,8]

The production of interferon (IFN)-γ drives macrophage activation and the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS),[9] while interleukin IL-4 and IL-10 inhibit NO production by down-regulating iNOS.[10] In the presence of N-metil-L-arginine, an inhibitor of iNOS, the leishmanicide activity diminishes and parasite survival increases, indicating that the NO produced by macrophages may inhibit parasite replication.[10] Recently, it has been demonstrated that NO is a possible regulator of apoptosis in various cell types.[10,11] In this study, we analysed the local and systemic production of NO and its relation to Th1/Th2 responses by characterizing IFN-γ and

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Accepted for publication 30 October 2002

IL-4+ T cells and epidermal Langerhans cells in lesions of *L. mexicana*-infected BALB/c and C57BL/6 mice.

Materials and methods

Mice and parasites

BALB/c and C57BL/6 mice were obtained from Taconic (Germantown, NY) and maintained under standard conditions. Amastigotes of *L. mexicana* (MHOM/BZ/1982/BEL21) were obtained according to Pérez *et al.*[12] Briefly, amastigotes were extracted from footpad nodules of hamsters infected a month earlier. The nodules were aseptically dissected out and washed in phosphate-buffered saline (PBS, pH 7.4) with antibiotics and cut and ground in a Petri dish containing PBS. The suspension was filtered through a sterile sieve to remove debris. The parasites were counted in a haemocytometer and adjusted to 4×10^3/ml.

Leishmania infection

Female BALB/c ($n = 24$) and C57BL/6 ($n = 26$) mice aged 6–8 weeks were inoculated subcutaneously in the left footpad with 1×10^3 amastigotes. The same number of BALB/c and C57BL/6 mice were used as controls, which were injected with PBS. The course of the infection was monitored by measuring the footpad thickness with a dial gauge caliper. Blood samples were taken every week for 12 weeks by heart puncture from two mice in each group. These mice were killed by cervical dislocation and the lesions, the popliteal lymph node and the spleen were dissected out for weighing. As an index of *in vivo* NO generation, serum nitrite concentration was determined by the Griess method. Skin lesion specimens were embedded in Cryomatrix™ resin (Shandon, Pittsburgh, Pennsylvania, USA), snap-frozen in liquid nitrogen and stored at −70 °C until examination. Frozen sections (5 μm) were cut with a cryostat, air-dried overnight, fixed with acetone for 10 min and stored at −20 °C until the immunostaining procedure.

Assay for NO production

Nitrate formation was estimated by measuring nitrate plus nitrate [NO(x)] using the diazotization method of Griess. Serum samples were deproteinized by adding 20 times their volume of sulphate of zinc (300 g/L), and centrifuged at 10 000 *g* for 5 min at room temperature.[13] The supernatant was incubated for 10 min with 3 times its volume of the Greiss reagent (1 g/l sulphanylic acid, 25 g/l phosphoric acid and 0.1 g/L N-1-naftil-diaminoethane).[14] The absorbance was measured at 570 nm. The concentration of NO_2^- was determined using a standard curve of $NaNO_2$ concentrations.

Antibodies

A rabbit anti-iNOS (Serotec, Oxford, UK) antibody was used at a dilution of 1 : 500 for the enzyme detection in the lesions. Monoclonal antibodies were used for detection of IL-4 (clone 11B.11, 1 : 100) (Biological Resources Branch, NCI, Minnesota, USA) and IFN-γ (Clone 37895.11, 1 : 100) (R&D System, Minneapolis, USA). For the identification of epidermal Langerhans cells, a monoclonal antibody against the CD205 (DEC-205) molecule was used (clone NLDC-145, 1 : 25), kindly donated by George Kraal (Free University, Amsterdam, the Netherlands). A rabbit anti-*Leishmania* sp. produced in our laboratory was used to confirm the presence of the parasite.

Immunoperoxidase staining

The immunostaining was carried out as previously described.[15] The samples were hydrated with PBS, and subsequently incubated for 90 min with the primary antibody, for 30 min with 50 μg/mL biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Burlingame, California, USA), and for 15 min with the Vectastain® Elite ABC kit (Vector Laboratories) at 1 : 100. Incubations were carried out in a Shandon Coverplate™ system (Shandon, Pittsburgh, Pennsylvania, USA), and 5-min washes with PBS were carried out between steps. The reactions were developed for 3 min in Vector® NovaRed™ substrate. The sections were then washed and counterstained with Harris' haematoxylin. Omission of the primary antibody and incubation with an antibody of irrelevant specificity at the same protein concentration were used as controls.

Cell quantification

Cells were counted under a light microscope (Leica, Wetzlar, Germany) connected to a colour video monitor (Panasonic, Tokyo, Japan) calibrated to determine the number of cells/mm^2 in the dermal infiltrate. Only cells with a visible nucleus and a red stain were counted as positive. To obtain a representative sample from the lesion, two nonserial sections of each lesion were immunostained and 20 fields were counted in each section at a magnification of 400×.

Statistical analysis

The results of the immunohistological analysis were expressed as the mean and standard error of the mean (SEM). The means were calculated based on the individual values for each mouse; P-values ≤ 0.05 were considered significant. Comparisons between groups were analysed by the nonparametric tests of Mann–Whitney and Kruskal–Wallis variance analysis. Correlations between variables were analysed using Spearman's rank coefficient. All tests were performed using GraphPad InStat 3.02 (GraphPad Software, San Diego, CA; http://www.graphpad.com).

Results

Cutaneous disease in *L. mexicana*-infected BALB/c and C57BL/6 mice

As previously observed, *L. mexicana*-infected BALB/c mice showed progressive and significant increases of footpad thickness, starting on the 4th week of infection (Fig. 1). These mice also presented a progressive increase in the weight and volume of the popliteal lymph node from the 2nd week of infection, reaching a value of 23 mg in the 11th week. In C57BL/6 mice, cutaneous lesions appeared in the 5th week and progressively increased until the 9th week of infection, when they started to decrease until complete regression was evident. C57BL/6 mice also showed a progressive increase of popliteal lymph node weight from the 4th (weight 1.73 ± 0.01 mg) to the 8th weeks of infection (2.56 ± 0.07 mg), when these measurements began to reduce progressively (2.10 ± 0.07 mg) until the end of the evaluation. In both BALB/c and C57BL/6 mice, the weight of the spleen did not show significant differences during the course of the infection as compared with the control groups (data not shown).

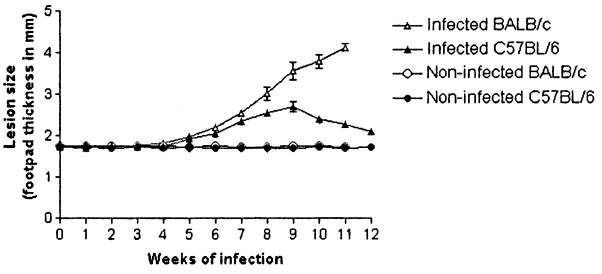

Figure 1 Progression of *L. mexicana* infection in BALB/c and C57BL/6 mice.

Serum nitrate release in *L. mexicana*-infected BALB/c and C57BL/6 mice

The serum nitrate concentration in infected BALB/c mice showed a small increase in the 3rd week of infection, then onwards it diminished to nondetectable levels, increasing again to maximal levels in the 8th week. In contrast, the serum nitrate concentration in infected C57BL/6 mice showed an increase in the 1st week and a further progressive augment from the 2nd week, reaching control levels on the 6th week (Fig. 2).

Leukocyte immunophenotypes in *L. mexicana*-infected BALB/c and C57BL/6 mice

CD205+ epidermal Langerhans cells in the lesions of *L. mexicana*-infected BALB/c mice started to increase in the 4th week of infection (1388 ± 140 cells/mm^2), and decreased to control values during the last week of evaluation (472 ± 111 cells/mm^2). In contrast, infected C57BL/6 mice showed a higher density of CD205+ epidermal Langerhans cells than control animals from the 1st until the 6th week of infection, when the maximum value was reached (1278 ± 163 cells/mm^2) (Fig. 3). Regarding IL-4 detection, infected BALB/c mice showed an increase of IL-4+ cells from the 1st week of

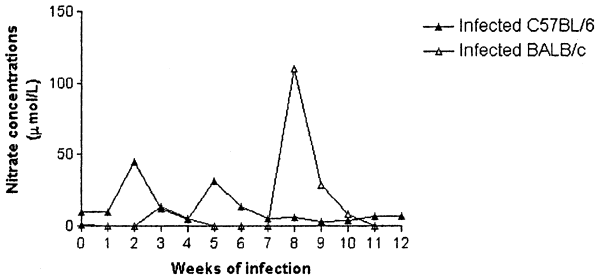

Figure 2 Serum nitrate concentrations in *L. mexicana*-infected BALB/c and C57BL/6 mice during the course of infection.

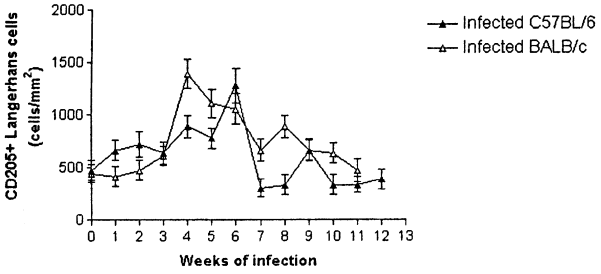

Figure 3 CD205+ epidermal Langerhans cell density in lesions of *L. mexicana*-infected BALB/c and C57BL/6 mice during the course of infection.

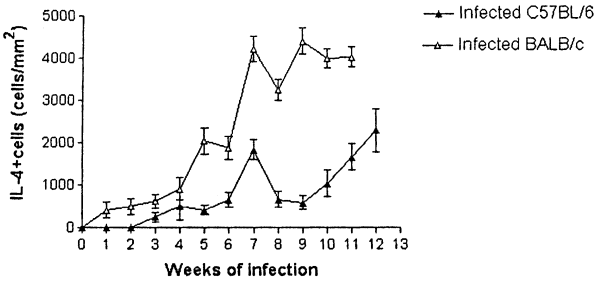

Figure 4 IL-4+ cells in lesions of *L. mexicana* -infected BALB/c and C57BL/6 mice during the course of infection.

infection, with a plateau being reached on the 9th week (4417 ± 315 cells/mm^2). In contrast, fewer IL-4+ cells were observed in the lesions of *L. mexicana*-infected C57BL/6 mice throughout the evaluation as compared with BALB/c mice, although a progressive increase of IL-4+ cells was observed, reaching 2292 ± 513 cells/mm^2 at the end of the evaluation (Figs 4 and 5). Control groups did not show quantifiable numbers of IL-4+ cells.

Infected BALB/c mice showed a progressive increase of IFN-γ+ cells in lesions from the 3rd week of infection, with a maximum value (2833 ± 185 cells/mm^2) on the 7th week. Moreover, C57BL/6 mice showed a higher density of IFN-γ+ cells than BALB/c mice during the early weeks of infection, with a value of 3708 ± 230 cells/mm^2 in the 3rd week, which diminished in the late stage of the infection (Figs 5 and 6).

Both infected BALB/c and C57BL/6 mice showed an increase in iNOS expression after the 1st week of infection with a maximum value on the 5th week. The number of iNOS+ cells was significantly higher ($P \leq 0.05$) in C57BL/6 mice (5250 ± 342 cells/mm^2) than in BALB/c mice (4028 ± 237 cells/mm^2) (Figs 5 and 7). In both cases, iNOS expression decreased after the 5th week of infection.

A positive correlation was found between the densities of iNOS+ cells and IFN-γ+ cells in BALB/c ($r = 0.2465$, $P = 0.4399$) and in C57BL/6 ($r = 0.9157$, $P = 0.0002$) mice using the Spearman correlation test. A positive correlation was also obtained between the densities of iNOS+ cells and IL-4+ cells in BALB/c ($r = 0.6748$, $P = 0.0003$) and in C57BL/6 ($r = 0.4402$, $P = 0.0277$) mice.

Discussion

The results confirmed that C57BL/6 mice are able to resolve *L. mexicana* infection spontaneously, while BALB/c mice develop progressive and chronic lesions.[12]

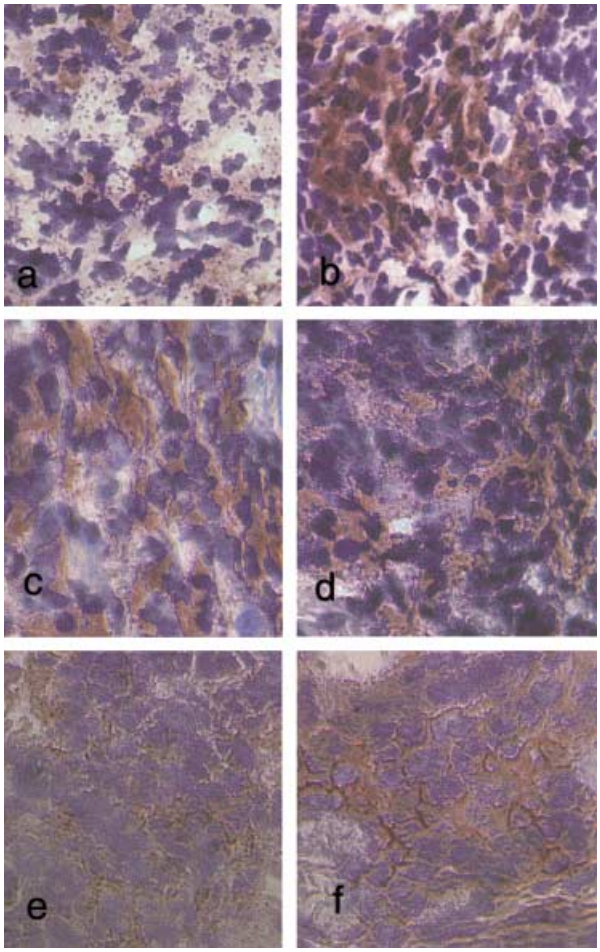

Figure 5 Cytokine and iNOS expression, demonstrating specifically stained cells, in lesions of murine cutaneous leishmaniasis. (a) IFN-γ in *L. mexicana* infected BALB/c mice, week 5; (b) IFN-γ in *L. mexicana*-infected C57BL/6 mice, week 5; (c) IL-4 in *L. mexicana*-infected BALB/c mice, week 9; (d) IL-4 in *L. mexicana*-infected C57BL/6 mice, week 9; (e) iNOS in *L. mexicana*-infected BALB/c mice, week 10; (f) iNOS in *L. mexicana*-infected C57BL/6 mice, week 10. Magnification ×500.

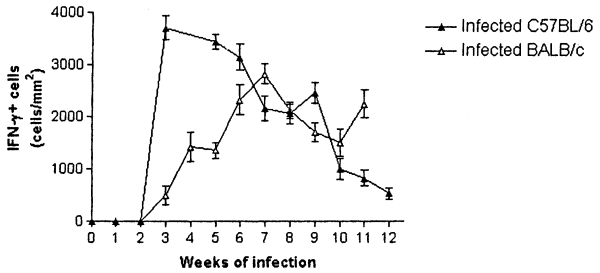

Figure 6 IFN-γ+ cells in lesions of *L. mexicana* -infected BALB/c and C57BL/6 mice during the course of infection.

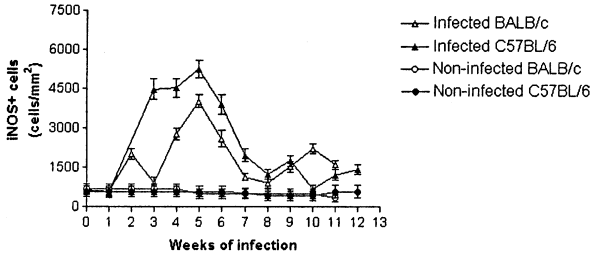

Figure 7 iNOS+ cells in lesions of *L. mexicana*-infected BALB/c and C57BL/6 mice during the course of infection.

The higher density of IL-4+ cells observed in *L. mexicana*-infected BALB/c mice confirmed the presence of a Th2 immune response, whereas C57BL/6 mice develop a Th1 response.[7,8] However, the increase of IL-4+ cells during the last weeks of infection in C57BL/6 mice suggests that Th2 cell clones may appear to counterbalance the vigorous Th1 response associated with immunopathology.[8,16]

Our results demonstrate that both susceptible and resistant mice produced NO during the course of infection with *L. mexicana*, as observed in *L. major* infection.[10,17] Susceptible mice showed high concentrations of circulating NO during chronic infection when the parasite load is very high and NO is probably unable to act. Thus, NO has an important role in parasite elimination during the early stages of infection.[18]

Also, C57BL/6 mice had more iNOS+ cells in lesions than BALB/c mice during the course of the infection. Interestingly, the high density of iNOS+ cells during the first 5 weeks of infection coincides with the previously described decrease in parasitic load in C57BL/6 mice.[10,18,19] In contrast, the numbers of iNOS+ cells in the lesions of susceptible BALB/c mice were insufficient to control parasite proliferation, thus confirming that local and early production of NO is necessary to control the infection in resistant mice.[20]

The present study shows that, in susceptible and resistant mice, the increase of iNOS+ cells parallels an increase of IFN-γ and IL-4 in lesions. The current paradigm, derived from *in vitro* studies, states that IFN-γ drives macrophage activation and iNOS expression, while IL-4 and IL-10 down-regulate iNOS and NO production, thus promoting disease.[10,21] However, the density of iNOS+ cells was also correlated positively with the production of IL-4. This suggests that *in vivo* all mechanisms are active simultaneously and the effect of the predominant factor prevails.

The density of CD205+ epidermal Langerhans cells increased with the *L. mexicana* infection of both susceptible and resistant mice. The later decrease in the density of Langerhans cells was related to the increase of the weight of the popliteal lymph node. This may reflect the migration of Langerhans cells to the paracortical area of the lymph node, where they present the antigen to naive T cells and trigger the clonal expansion of the memory T cells, initiating the effector phase of the immune response against *Leishmania*.[22] The increase of the epidermal dendritic cell density during the 1st week of infection in resistant mice could be important in driving the early initiation of the specific immune response in favour of the elimination of the parasite.

The positive correlation observed between the density of CD205+ cells and IL-4 production in susceptible mice suggested that an increase in antigen presentation to naive T cells initiates the Th2 response, thus increasing IL-4 production. This did not occur in the resistant mice. In resistant mice, a positive correlation was obtained between the CD205+ cell density and the production of IFN-γ. This confirms that the immune response generated after antigen presentation is mainly Th1 and is related to low IL-4 and high IFN-γ production which favours macrophage activation and intracellular parasite elimination by nitric oxide.[14,23]

In conclusion, early production of local and systemic NO plays an important role in the resolution of infection by *L. mexicana* and is related to the type of cellular immune response developed, where the dendritic cells participate as immunomodulators. The present study contributes to the understanding of leishmaniasis, relating aspects of capture and parasitic death with the induction of the immunological process. Future studies will clarify these aspects and elucidate the role of the dendritic cells in innate and acquired immunity.

Acknowledgements

We are grateful to Dr Marian Ulrich for reading and commenting on the manuscript. This study was supported by Fondo Nacional de Ciencia, Tecnología e Innovación (FONACIT) Proyecto S1-98000041, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, European Commission INCO Programme, and CDCH- Universidad Central de Venezuela.

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