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## Different patterns of disease in two inbred mouse strains infected with a clone of *Leishmania mexicana amazonensis*

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### Summary

We have infected BALB/c and C57BL/6 mice with a cloned *Leishmania mexicana amazonensis* population, obtained from the “Maria” strain. Progression of infection and histopathological examination confirmed the extreme susceptibility of BALB/c mice and the resistant pattern of the C57 BL/6. Anti-*Leishmania* antibody titers were higher in BALB/c than in C57BL/6 mice through the period of infection. Tests of delayed type hypersensitivity reaction with *Leishmania* antigens were positive in both strains in the beginning of the infection, but were negative later on in BALB/c mice. Our results are similar to those obtained with mixed parasite populations, and rule out the possibility of selection among different parasite subpopulations as responsible for the divergent course of the disease exhibited by these two strains of mice.

**Key words:** cutaneous leishmaniasis; murine leishmaniasis; *Leishmania*; parasite clones; *L. mexicana*.

### Introduction

Several murine models of cutaneous leishmaniasis have shown a sharp distinct pattern of disease between BALB/c mice and other strains of mice, as the C57BL/6, following infection by *L. tropica* (Bjorvatn and Neva, 1979; Behin et al., 1979; Handman et al., 1979; Nasser and Modabber, 1979; Nacy et al., 1983) or by *L. mexicana* (Perez et al., 1978, 1979; Barral et al., 1983; Andrade et al., 1984; Childs et al., 1984). In these studies the BALB/c mice have displayed an extremely susceptible pattern, exhibiting persistent disseminated and rapidly

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progressive lesions rich in parasites. In contrast, C57BL/6, as well as other strains of mice, develop a local lesion that may or may not ulcerate, depending largely on the inoculum size. Parasite multiplication is contained after a few weeks of infection and positivity can only be detected by culture of the lesion. Upon infection of C57BL/6 mice with *L. mexicana amazonensis* a late metastatic largely ulcerative disease develops after approximately one year of apparent clinical cure (Barral et al., 1983). Such divergent course of the disease has been attributed to genetic and immunological differences among the mouse strains. However, a selection of different parasite populations by the mouse strains could, at least partially, explain the diverse patterns of infection. Such matter can be easily addressed by infecting inbred mouse strains with cloned parasite populations.

We report here the course of the infection by BALB/c and C57BL/6 mice by a clone of *Leishmania mexicana amazonensis*. Antileishmanial antibody titers as well as delayed type hypersensitivity reactions to leishmanial antigen were also evaluated at different periods after infection.

## Materials and Methods

*Mice.* – Female BALB/c and C57BL/6 mice (10 of each strain) were obtained from the CPqGM colony (Salvador, Bahia) at age 8 weeks. The mice were maintained on commercial pellet ration and water ad libitum.

*Parasite.* – The “Maria” strain of *L. mexicana amazonensis* (MHOM/BR/79/Maria) was characterized by isoenzymic pattern (courtesy of Dr. L. Schnur, WHO Reference Center in Israel) and by a panel of monoclonal antibodies (courtesy of Dr. D. Pratt of Harvard School of Medicine). Its behaviour in mice and details of maintenance have been previously described (Barral et al., 1983). The clone used was obtained by limiting dilution in two cycles (cloning and subcloning), as described (Jaffe et al., 1984).

*Infection.* – Animals (10 of each strain) were infected with  $5 \times 10^6$  liver infusion tryptose (LIT)-grown stationary phase promastigotes. Parasites were resuspended in sterile saline (after 5 washings) and injected into the left hind footpad, in a volume of 0.02 ml.

*Measurement of lesion size.* – The thickness of the infected footpad, in millimeters  $\times 10^{-2}$ , minus the measurement of the contralateral uninfected one was taken as the lesion size. Measurements were made with a dial gauge micrometer caliper (C. Starret, Athol, MA).

*Histopathological examination.* – Three animals of each strain were killed at 4, 10 and 14 weeks after infection. Their footpads were fixed in 10% formalin and routinely processed for staining in hematoxylin-eosin.

*Preparation of antigen.* – “Maria” promastigotes were grown in LIT medium supplemented with 10% heat-inactivated fetal calf serum, washed five times and submitted to 10 cycles of freezing-thawing. The material was centrifuged ( $200 \times g$ , 5 min at  $4^\circ\text{C}$ ) and the supernatant sterile-filtered. After protein determination (Lowry et al., 1951) the material was aliquoted and stored at  $-20^\circ\text{C}$  until used.

*Assay for delayed-type hypersensitivity (DTH).* – The footpad swelling test was used to assess DTH, as previously described (Barral et al., 1983). Three animals of each strain (at 4, 10 and 14 weeks of infection) were injected with  $75 \mu\text{g}$  protein of leishmanial antigen solution in a volume of 0.015 ml, in the right hind footpad. Measurements of footpad thickness were taken before the injection and 24 h later.

*Anti-Leishmania antibody determination.* – An enzyme-linked immunosorbent assay (ELISA) was used for antibody determination as described elsewhere (Walls et al., unpublished). Leishmanial

antigen (prepared as described above) was diluted in 0.06 M, pH 9.5 carbonate buffer to a concentration of 10  $\mu\text{g/ml}$ . Microtiter plates (Dynatech Laboratories, Alexandria, Virginia) were sensitized with 0.1 ml of antigen solution per well, incubated at 37°C for 3 h and maintained at 4°C until use. Washings between each step were made with phosphate buffered saline (PBS) plus 0.5% tween. Two or three-fold dilutions of sera in PBS (beginning 1:10) were tested. Sera from 5 animals of each strain were tested individually at 2, 4, 6, 10 and 14 weeks of infection. Peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) was used in a dilution of 1:800 in PBS supplemented with 10% newborn calf serum. A solution with 0.04% o-phenylenediamine, 0.012%  $\text{H}_2\text{O}_2$  in phosphate-citrate buffer pH 5.0 served as substrate. After 30 min at room temperature in the dark, 0,05 ml per well of 8 N sulphuric acid was used to stop the reaction. Extinction values were determined at 492 nm on an ELISA spectrophotometer (Titertek Multiskan, Flow Laboratories; McLean, VA).

*Statistical treatment.* – Comparisons of lesion size or DTH values, as well as log transformed antibody titers, between BALB/c and C57BL/6 mice (at each time point) were performed by Student's t test for non-paired data.

## Results

*Course of infection.* – BALB/c mice exhibited a progressive increase in the thickness of the infected footpad reaching values of  $478 \text{ mm} \times 10^{-2}$  above the values of the contralateral uninfected footpad 14 weeks after infection (Fig. 1). The lesions were represented by large and firm nodules, without ulceration but distending the covering skin.

In contrast, C57BL/6 mice developed much smaller lesions, being maximal at 8 weeks of infection, when they reached  $82 \text{ mm} \times 10^{-2}$ . Afterwards there was a constant decrease of lesion size up to the end of the observation period (Fig. 1). The lesions were non-ulcerated and firm.

Differences in lesion size between BALB/c and C57BL/6 mice were always statistically significant ( $p < 0.05$  at 8 weeks of infection;  $p < 0.01$  at all other time points), except at 2 weeks of infection.

*Histopathology.* – At about 4 weeks of infection, in the BALB/c mice, the lesions were represented by large macrophage collections, sometimes resembling fatty tissue, and dissociating muscle fibers. The cells were vacuolated and densely parasitized. Occasionally there were seen areas of coagulative or purulent necrosis with polymorphonuclear leukocytes. A similar aspect, with the prominence of parasitized macrophages infiltrating muscle and dermal structures, and larger areas of purulent necrosis were seen at 10 and 14 weeks of infection. Examination of lesions from C57BL/6 mice, 4 weeks after infection, revealed the prominence of fibroblasts and granulomas as well as parasitized macrophages interspersed in numerous lymphocytes; eosinophils and plasma cells were also present. Areas of fibrinoid necrosis were seen associated with the presence of parasites. At later periods of infection (10 and 14 weeks) there was a decrease in the number of parasitized macrophages, and the fibrotic reaction predominated.

*Anti-Leishmania antibody titers.* – IgG antibody titers anti-*L. m. amazonensis* ("Maria" strain) were determined by ELISA. In BALB/c mice antibody

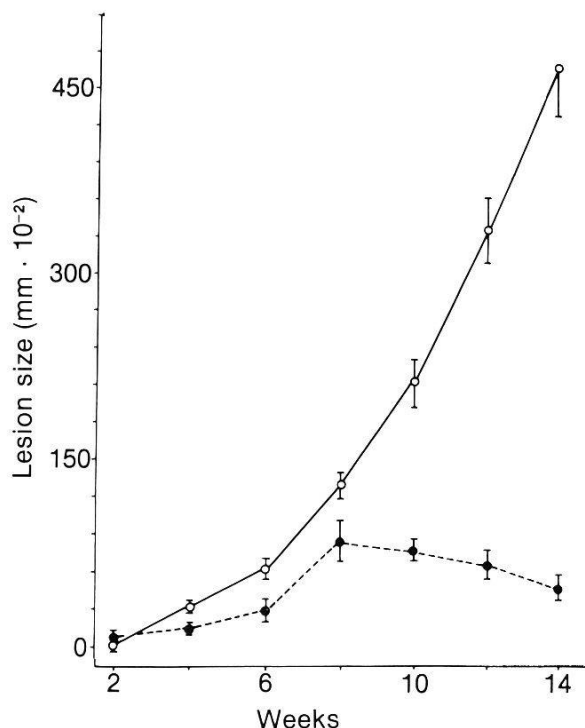


Fig. 1. Time course of primary lesion size of BALB/c (solid line) or C57BL/6 (broken line) mice infected with  $5 \times 10^6$  promastigotes of cloned *L. m. amazonensis* in the left hind footpad. Symbols represent the mean of each group ( $n = 10$ )  $\pm$  S.E.M.

Table 1. IgG anti-*Leishmania* antibodies (ELISA) of BALB/c and C57BL/6 mice at different periods after infection with a clone of *L. mexicana amazonensis*

| Weeks of infection | BALB/c         | C57BL/6        |
|--------------------|----------------|----------------|
| 2                  | 2* (0-10)**    | 0 (0)          |
| 4                  | 7 (0-30)       | 0 (0)          |
| 6                  | 19 (10-30)     | 3 (0-30)       |
| 10                 | 618 (300-1000) | 24 (0-100)     |
| 14                 | 2560 (2560)    | 279 (160-2560) |

\* Geometric mean of titers ( $n = 5$ )

\*\* Range

titers appeared earlier and were higher than in C57BL/6 mice (Table 1). One BALB/c mouse had a positive titer just 2 weeks after infection and at 4 weeks only one out of 5 animals had titer below 10. In contradistinction, only at 6 weeks of infection were there positive tests among the C57BL/6 animals (2 out of 5 animals examined). Even at 10 weeks of infection 3 out of 5 C57BL/6 mice did not have detectable anti-*Leishmania* IgG antibody at the same time the lowest titer exhibited by the BALB/c mice was 300.

*DTH response.* – A positive DTH response to leishmanial antigen was observed in both mouse strains 4 weeks after infection (Table 2). At this time the

Table 2. Time course of DTH reactivity of leishmanial antigen in BALB/c and C57BL/6 mice after infection by a clone of *L. mexicana amazonensis*

| Weeks of infection | BALB/c    | C57BL/6  | p <  |
|--------------------|-----------|----------|------|
| 4                  | 47.3±7.1* | 29.0±7.8 | N.S. |
| 10                 | 9.3±0.3   | 27.3±1.2 | 0.01 |
| 14                 | 6.4±2.1   | 28.8±3.9 | 0.05 |

\* Thickness of the tested footpad 24 h after injection minus values obtained pre-injection. Mean ± standard error in mm×10<sup>-2</sup>.

response was higher in BALB/c mice than in C57BL/6 animals, but the difference did not reach statistically significant levels. The response remained positive (and with similar intensity) in C57BL/6 mice at 10 and 14 weeks of infection. At these same time points the DTH response of BALB/c mice was negative, and differences between the two strains were statistically significant.

## Discussion

This study shows that BALB/c mice are extremely susceptible, and C57BL/6 mice are resistant to the infection by a clone of *L. m. amazonensis*. The course of infection, histopathological lesions as well as the pattern of antibody production and cell-mediated immune (CMI) response were remarkably similar to the one observed after the infection of both mouse strains with the uncloned parasite population from which the clone was obtained. When followed by an extended period of time (about 60 weeks post-infection) C57BL/6 mice infected with the same clone used in this report developed ulcerative metastatic lesions in the nasal region and the tail, similar to those described upon infection of the original strain (Barral et al., 1983).

Histopathologically lesions in BALB/c mice can be correlated with an absence of effective response. The large collections of vacuolated macrophages, harboring numerous parasites, had very few lymphocytes. In contrast, the lesions in C57BL/6 animals were predominantly fibrotic, and the presence of parasites was accompanied of fibrinoid necrosis. Also indicative of resistance were the granulomatous reactions observed. Such aspects have been observed previously when these same mouse strains were infected by the original uncloned strain (Barral et al., 1983) and also observed with another strain of *L. m. amazonensis* upon infection of a susceptible and a resistant (A/J) strain of mice (Andrade et al., 1984).

An initially positive anti-leishmanial CMI response became negative later on in BALB/c, whereas it remained positive through the period of infection in resistant C57BL/6 mice. Once more such results are similar to the ones observed



with heterogeneous parasite populations of *L. mexicana* (Perez et al., 1978; Barral et al., 1983; Andrade et al., 1984). It is also noteworthy that the susceptible mouse strain exhibited an earlier and more elevated rise in anti-parasite antibody titers. The association of elevated antibody titers and depressed CMI in susceptible animals, as opposed to the situation on the resistant animals (Howard et al., 1980; Hale and Howard, 1981) has been also observed in many clinical situations (reviewed in Mauel and Behin, 1981). In contradistinction, Perez et al. (1979) have found lower anti-leishmanial antibody titers (haemagglutination tests) in BALB/c mice, compared to other strains, following infection by *L. mexicana*. Another study using *L. m. amazonensis*, however, reported similar specific antibody titers (by immunofluorescence) in BALB/c and in resistant A/J strain (Andrade et al., 1984). Besides from differences in parasite strains and assays it is difficult to explain such discrepancies. Furthermore, BALB/c mice rendered unable to produce antibody by anti- $\mu$  treatment exhibit a more benign course of infection by *Leishmania* than their normal antibody-producers counterparts (Sacks et al., 1984). Specific antibody may be beneficial to the parasite by depressing CMI or by increasing parasite interiorization into macrophages. It has been shown, however, that sera from both susceptible and resistant strains of mice are equally effective in increasing phagocytosis of *L. m. amazonensis* (Reis et al., submitted). It is possible that antibody plays a role in the induction of T suppressor cells (Sacks et al., 1984).

Several reports have shown different courses of disease in different inbred mouse strains upon the infection by *L. m. amazonensis* (Perez et al., 1978; Barral et al., 1983; Andrade et al., 1984; Childs et al., 1984). Such differences have been explained on basis of divergent genetic characteristics, but in no occasion the contribution of a possible selection of distinct parasite populations has been addressed. It is known that parasite populations (or strains) may be represented by a mixture of sub-populations with diverse characteristics (Dvorak et al., 1980; Grimaldi et al., 1982) that may have quite divergent behaviour in the host (Grimaldi et al., 1982; Postan et al., 1983; Handman et al., 1983; Postan et al., 1984). The results presented here, reporting the course of infection with a homogeneous population of *L. m. amazonensis* reinforce the relevance of host characteristics in determining the aspects of the disease. On the other hand, the selection of parasite populations as a factor in determining characteristics of cutaneous leishmaniasis seems to have a secondary role, if any.

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