LEISHMANIA (VIANNA) PANAMENSIS - INDUCED CUTANEOUS LEISHMANIASIS IN SUSCEPTIBLE AND RESISTANT MOUSE STRAINS

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SUMMARY

We studied the susceptibility to Leishmania (Viannia) panamensis in strains of mice. The C57BL/6 strain was resistant and showed self-controlled lesion at the injected foot pad. The BALB/c and DBA/2J strains were susceptible and showed a foot swelling that started day 20 post-infection and progressed to a tumour-like lesion in later period of observation. The CBA/H strain was found to be of intermediary resistance. In contrast to other known cutaneous leishmaniasis in mice, the lesion in L. (V.) panamensis-infected mice was restricted to the inoculation site in the skin. In addition, we studied the development of cellular response and antibodies against Leishmania antigen in BALB/c and C57BL/6 strains. The proliferative response of lymph node cells against L. (V.) panamensis antigen was biphasic in both strains. An initial response was seen on day 20, followed by a refractory period between 40 and 80 days and a second response around fourth month post-infection. The response in the latter period was higher in C57BL/6 strain than in BALB/c strain. BALB/c strain presented much higher anti-Leishmania antibody level than C57BL/6 strain. The model and the correlation of immunological variables and the course of the infection are discussed.

KEYWORDS: Cutaneous leishmaniasis; Leishmania (Viannia) panamensis; Experimental model; Mice; Immunology.

INTRODUCTION

Leishmaniasis in man are caused by protozoa of the genera Leishmania. Different Leishmania species cause cutaneous and mucocutaneous leishmaniasis in the New World 19. Leishmania (Viannia) panamensis has been identified as the most frequently occurring species in Panama, being prevalent almost everywhere in Central America 21.

Leishmaniasis caused by Leishmania (Viannia) panamensis have certain distinct clinical characteristics, such as lymph node involvement in chain that resembles sporotrichosis 19,21. This and other features evoke a number of specific questions, including those concerning adequate therapy 2,10.

There are well-established mouse models for cutaneous leishmaniasis using Leishmania (Leishmania) major 5,10 and Leishmania (Leishmania) amazonensis 1,12.
For infection with *L. (V.) panamensis*, however, there is only one report showing that BALB/c mice are susceptible. We have previously described the pathology in *L. (V.) panamensis*-infected BALB/c mice. To further characterize this model and to analyse the susceptibility or resistance pattern in different mouse strains, we analysed four different inbred strains of mice, BALB/c, DBA/2J, C57BL/6 and CBA/HJ with *L. (V.) panamensis*. In addition, the differences in cellular and humoral immune responses were studied in one susceptible, BALB/c, and one resistant, C57BL/6 strain of mice.

The BALB/c and DBA/2J strains were found to be susceptible, the C57BL/6 strain was resistant and the mice of CBA/HJ strain showed intermediate resistance. The lesion was strictly localised in the inoculation site in the skin, with no metastasis to other parts, even in highly susceptible strains. Lymph node cell response to *Leishmania* antigen was higher in the resistant C57BL/6J strain compared with the susceptible BALB/c strain. The antibody titers were higher in BALB/c strain. The model and the correlation with cellular and humoral immune responses during the course of the infection are discussed.

**MATERIALS AND METHODS**

**Mice:** Inbred mice, 6-10 weeks old, males and females of BALB/c, DBA/2J, C57BL/6 and CBA/HJ strains were from the colony maintained at the Department of Immunology of the Karolinska Institute, were used throughout the study.

**Parasites and parasite cultures:** *Leishmania (Viannia) panamensis*, HSJD-1 strain, isolated from a patient in Costa Rica, was characterized by isoenzyme typing by Professor W. Peters, Liverpool School of Tropical Medicine, and by hybridization studies with insert probes of *L. (V.) panamensis* and *L. (L.) amazonensis* at the Research Centre of Parasitic Diseases of the University of Panama by Dr. P. de Carreia. The parasites were maintained in NNN medium with an overlay of RPMI 1640 medium with 10% foetal calf serum (FCS). The same NNN medium was used to isolate parasites from spleen and draining popliteal lymph nodes. The parasite culture was expanded in RPMI 1640 medium with 10% FCS, and grown until stationary phase. After washing, the concentration was adjusted to either 2 x 10^7/ml or 2 x 10^8/ml in 0.01 M phosphate buffered saline, pH 7.2 (PBS) for inoculation into mice.

**Experimental protocol:** Promastigotes in 50 µl suspension in sterile PBS were injected subcutaneously in the hind foot pad of four strains of mice. The contra lateral foot pad was inoculated with sterile PBS as control. The mice were observed every 20 days throughout the experiment. Size of the lesion, ulceration in the injected foot pad and the appearance of any other skin lesions were recorded. The thickness of the injected and the non-injected foot pads was measured with a dial calliper (Starrett, Athol, Mass., USA) and the difference between the measurements was considered to be the extent of the swelling. Samples of blood from the retro-orbital sinus in BALB/c and C57BL/6 mice were obtained, and the mice were then killed to obtain the draining popliteal lymph nodes on days 20, 40, 60, 80, 130 and 160 post infection. The experiment was repeated three times.

**Antigen preparation:** Stationary phase culture of *L. (V.) panamensis* was washed and freeze-thawed 10 times to yield a total antigen. This antigen preparation was used for antibody determinations, lymphoproliferative assay and immunization of mice. The antigen concentration was adjusted according to the number of promastigotes.

**Immunization procedure:** Inbred BALB/c mice were inoculated subcutaneously in the footpad with either 50 µl Freund's incomplete adjuvant (Difco Laboratories, Michigan, USA) or with 50 µl of an antigen solution of *L. (V.) panamensis* (10^7 parasites/ml) in Freund's incomplete adjuvant. After 20 days of priming, the mice were killed to obtain draining popliteal lymph node cells, and lymphoproliferation assays were performed.

**Anti-Leishmania antibody determination:** Sera obtained from BALB/c and C57BL/6 mice were maintained at -20°C until analysis. The IgG and IgM anti-Leishmania antibodies were determined by ELISA assay. Maxisorb plates (Nunc-Denmark) were coated with 100 µl of total antigens of *L. (V.) panamensis* (10^7 parasites/ml) in carbonate-bicarbonate buffer, pH 9.6. After an overnight incubation at 4°C, the wells were washed with PBS-Tween, blocked with 5% fat-free milk, and washed again with PBS-Tween, and then 100 µl serum samples were added. Serial dilutions of serum samples from individual animals were
analysed to determine the titer. After 2 hours’ incubation at 37°C, the plates were washed and the conjugate, either rabbit anti-mouse IgG peroxidase conjugate (Dakopatts, Denmark) or rabbit anti-mouse IgM peroxidase conjugate (Dakopatts, Denmark), was added. Finally, 100 μl of 1,2-phenylenediamine (OPD) (Dakopatts, Denmark), 0.67 mg/ml solution was added, the reaction stopped with 2.5 M sulphuric acid and the absorbance determined at 492 nm in a Multiscan (Flow Laboratories, UK). All determinations were performed in duplicate.

**Lymphocyte proliferation assay:** Cell suspensions were prepared from draining popliteal lymph nodes, and the cell concentration was adjusted to 2x10⁶ cells/ml in RPMI 1640 supplemented with 1% fresh mouse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer and 50 μM 2-mercaptoethanol. One hundred microlitres of this cell suspension were added to each well of 96 well flat-bottomed plates (Costar, Massachusetts, USA) and stimulated with either Phytohaemagglutinin - PHA (Flow Laboratories, Michigan, USA), 5 μg/ml, or total L. (V.) panamensis antigen in 100 μl of the supplemented medium. Control cultures had 100 μl of the supplemented medium added. The tests were set up in triplicate. Culture plates were incubated for 72 hours at 37°C in humidified atmosphere with 5% CO₂. The cultures were pulsed with 1 μCi of tritiated thymidine (Amersham, Sweden, 25 Ci/mmole) six hours before harvest. The cell cultures were harvested onto glass fibre paper (Flow Laboratories, England), placed in scintillation liquid (Quickzint 801, Zinsser Analytic, Sweden) and counted in a β-counter (LKB Wallac, liquid scintillation counter, 1218, Finland). The results were expressed as a lymphocyte transformation index: mean cpm of stimulated culture per mean cpm of non-stimulated culture.

**Statistics:** The results were compared using Student’s t-test.

**RESULTS**

1) **Course of infection in BALB/c, DBA/2J, C57BL/6 and CBA/HJ mice**

Three different patterns were observed in the development of lesions in *L. (V.) panamensis*-infected mice (Fig. 1a). The first pattern, represented by the C577BL/6 strain, showed small but significant lesion that stabilized after 60 days. The second pattern, represented by the BALB/c and the DBA/2J strain, displayed progressively growing lesions. The third pattern, represented by CBA/HJ mice, showed more variable course where most of the mice were resistant, but some developed tumour-like lesions similar to those in the susceptible BALB/c and DBA/2J strains.

The susceptible strains, BALB/c and DBA/2J, presented swelling from day 20 post infection. After 40 days, the lesions increased exponentially in size to reach a tumor-like lesion after 80 days. These lesions showed no macroscopically observable necrosis at any time, only a crust formation on the skin of the footpad resembling that of traumas. No dissemination of the lesions to other parts of the skin was noted. When fragments of draining popliteal lymph nodes and spleens from infected BALB/c mice were placed in biphasic NNN medium, promastigotes grew in all samples from day 120 (6 mice) and day 160 (9 mice), irrespective of the size of inoculum (10⁹ or 10⁸) used.

The lesion in CBA/HJ or C57BL/6 strains developed without ulceration in any period.

When the susceptible BALB/c and resistant C57BL/6 strains were inoculated with lower doses of parasites (10⁷), the time to the establishment of the lesion was longer in the BALB/c strain (Fig. 1b).

All *L. (V.) panamensis*-infected mice were apparently healthy, without any evident signs of malnutrition and/or cachexia until the end of the experiment.
2) Cell-mediated immune and humoral responses in L. (V.) panamensis-infected BALB/c and C57BL/6 mice.

a - Cell-mediated immune response during infection.

Experiments were first made to find the optimal antigen concentration and stimulation times for the lymphocytes in the Leishmania antigen-induced response. Draining popliteal lymph node cells of mice immunized with L. (V.) panamensis antigen in Freund's incomplete adjuvant showed highest Leishmania antigen-induced lymphoproliferation after 5 day stimulation in vitro (Table 1). In contrast, when L. (V.) panamensis-infected mice were analysed, the highest cellular response to the same antigen was obtained after 3 days (Table 1). On the basis of these initial results, the stimulation of the lymph node cells was done for 3 days in all subsequent experiments. Since the levels of cellular proliferation showed very little variation within the same group of mice (three experiments, n = 9, p = 0.05), lymph node cells of mice from the same group were pooled in the subsequent experiments.

When L. (V.) panamensis antigen-induced lymph node cell proliferation from infected BALB/c and C57BL/6 mice was determined, both strains of mice showed an initial dose dependent response. This was followed by a refractory period, between 40 and 80 days. After this period the response recovered, and found to be higher in the C57BL/6 strain. At the end of the observation period, the response was low or absent in both strains (Fig. 2). As control for non-specific suppression, we analysed the response to PHA and no significant change was observed compared with non-infected control mice (data not shown).

![Fig. 2 - L. (V.) panamensis total antigen (10^5 promastigotes/ml) induced draining popliteal lymph node cell proliferation from 20 to 160 days post infection period in mice infected with either 10^6 (closed symbols) or 10^7 promastigotes (open symbols): a) BALB/c mice; b) C57BL/6 mice. One of three similar experiments is shown. Cells from non-infected mice showed no significant proliferation at any of the antigen concentrations used. For details see Materials and Methods.](image)

![Fig. 3 - Levels of IgG anti-Leishmania antibody in BALB/c (*) and C57BL/6 (●) mice (mean of 6 mice) injected with either 10^6 (closed symbols) or 10^7 promastigotes (open symbols) of L. (V.) panamensis. Frozen and thawed L. (V.) panamensis total antigen (10^7 parasites/ml) was utilized in ELISA assay. One of three similar experiments is shown.](image)

**TABLE 1**

Proliferation (cpm) of lymph node cells from BALB/c mice on day 20 after either immunization or infection, after different period of in vitro culture.

<table>
<thead>
<tr>
<th>In vitro stimulation</th>
<th>Freund's incomplete adjuvant</th>
<th>Freund's incomplete adjuvant</th>
<th>L. (V.) p. Ag (+) treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>days in culture</td>
<td>control</td>
<td>control</td>
<td>control</td>
</tr>
<tr>
<td>3</td>
<td>157 ± 28</td>
<td>282 ± 26</td>
<td>377 ± 29</td>
</tr>
<tr>
<td>5</td>
<td>132 ± 32</td>
<td>121 ± 36</td>
<td>151 ± 10</td>
</tr>
<tr>
<td>7</td>
<td>103 ± 13</td>
<td>116 ± 17</td>
<td>481 ± 60</td>
</tr>
</tbody>
</table>

(1) Freeze-thawed total L. (V.) panamensis stationary phase promastigotes (10^5/ml);
(2) Mean cpm of triplicate culture ± SD

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b- Anti-Leishmania antibodies during infection.

BALB/c mice presented much higher anti-Leishmania antibody level than C57BL/6 mice (Fig. 3). The peak mean level of IgG antibody was 1/7000 in BALB/c compared with 1/1500 in C57BL/6 mice. In BALB/c mice infected with higher dose of parasites showed an earlier initiation of antibody production. After the peak around 60 days' post-infection, the IgG antibody levels decreased. In C57BL/6 mice, IgG antibody levels remained low, with no dramatic increase, during the course of the experiment.

IgM antibody levels were generally low and it was only detected in BALB/c mice on day 20 post infection with a titer of 1:120 (data not shown).

DISCUSSION

The susceptibility of inbred mouse strains to infection with Leishmania parasites covers a wide spectrum. Several reports have shown that BALB/c and DBA/2J mice are susceptible to different Leishmania parasites; for example, both are susceptible to L. (L.) major, and only the former to L. (L.) amazonensis. On the other hand, CBA and C57BL/6 have been shown to be relatively resistant to all tested Leishmania species.

In this study with L. (V.) panamensis, BALB/c and DBA/2J mouse strains were susceptible, C57BL/6 strain, resistant, and CBA/HJ strain showed intermediate resistance. Most interesting and uniquely, the lesions did not metastasize to other parts of the skin during the whole period that, for all tested strains, exceeded six months. We were able to isolate Leishmania parasites from spleen of BALB/c mice; however, as reported previously, this was not associated with any pathological alterations either in spleen or liver. This particular characteristic distinguishes it from infection of BALB/c strain with L. (L.) major and L. (L.) amazonensis where dissemination to skin and viscera has been reported. In our study, CBA strain showed varied susceptibility. Most of them were resistant, but some of them presented tumour-like lesions like in BALB/c and DBA/2J strains. This is in contrast to previous report where no development of lesions in CBA mice was observed. This difference could be attributed to the intra-species variation of the L. (V.) panamensis. Finally, none of the strains of mice presented ulcers or necrotic lesions, as seen in L. (L.) major, and L. (L.) amazonensis infection, respectively.

We conclude that L. (V.) panamensis model in mice is unique, strictly cutaneous and it allows the study of the mechanisms that control the dissemination of the infection.

In order to link the pattern of susceptibility or resistance to Leishmania-driven immunological responses, lymphoproliferative response and antibody production were studied in one resistant, C57BL/6, and one susceptible strain, BALB/c.

Proliferation of L. (V.) panamensis antigen-stimulated lymph node cells of infected mice was shown to be biphasic in both strains. An initial response was seen on day 20, followed by a refractory period between 40 and 80 days and a second response around fourth month post infection.

The initial cellular response detected on day 20 was similar in the resistant and susceptible strains. A similar early response has been reported in L. (L.) major susceptible BALB/c mice around day 20 post infection.

This period of initial responsiveness was followed by a refractory phase. Similar absence of specific cell mediated immune response is a common finding in several other types of trypanosomatid infections, such as acute Trypanosoma cruzi infection, the active phase of human visceral Leishmaniasis, human disseminated cutaneous Leishmaniasis, and L. (L.) major-infected BALB/c strain. This feature has been related to the progression and the dissemination of the infection. However, in our model, a recovery of the cellular immune response followed this unresponsiveness. This finding suggests that it could be a phase of diversion of the immune response occurring in both mouse strains. A consequence of this diversion was an exponential increase in the size of the lesion with increasing number of parasites in BALB/c strain. In contrast, we observed the stabilization of the lesion in C57BL/6 strain. Another consequence of this diversion could be seen in the anti-Leishmania antibody production profile in the BALB/c and C57BL/6 strains. The BALB/c strain showed an exponential and dramatic increase in the titer of antibody starting during the period of cellular unresponsiveness, in contrast to the antibody titer in C57BL/6 strain that remained low and stable throughout whole period of observation.

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In the following period, when the cellular immune response was recovered, L. (V.) panamensis antigen-stimulated lymph node cell proliferation was higher in C57BL/6 strain than in BALB/c strain.

In murine cutaneous leishmaniasis two different sub populations of CD4 cells are involved in the healing or progression of L. major infection: TH1 sub population producing essentially IL-2 and γIFN, is predominantly responsible for healing, and TH2 producing IL-4, for progression of the infection. From the course of infection and the immunological parameters, we can speculate on possibility that BALB/c strain is developing predominantly the TH2 type and C57BL/6, the TH1 type of response.

It is important, however, to stress that the mechanism which governs the development of infection could be different in the skin and in the draining lymph node. In fact, we have previously reported diverse tissue responses comparing the draining lymph nodes and the skin. In the forth-fifth month of infection in the BALB/c strain, the draining lymph nodes showed granulomatous response suggestive of control of infection, whilst the skin showed no sign of control of the process, only homogeneous macrophage infiltrates containing many amastigotes. Therefore, future studies will be concentrated in the analysis of subsets of lymphocytes and lymphokines present in the early and the late immune responses both in the skin lesions and in the draining lymph nodes. This will provide more complete understanding of the processes involved in the progression of the infection, as well as those leading to an immune state of the host.

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