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Variations in susceptibility to *Leishmania amazonensis* infection in lines of mice selected for high or low immunoresponsiveness

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Summary The degree of resistance to a local *Leishmania amazonensis* challenge has been compared in lines of mice obtained by selective breeding for high or low immunoresponsiveness: High and Low antibody responder mice of Selections I and II (H_I, H_{II} and L_I, L_{II} lines) and high and low responder mice to T mitogen PHA (Hi/PHA and Lo/PHA). The aim of this preliminary study was to focus attention on genetic differences related with well defined immune characteristics. Clear-cut results were obtained, both H_I and H_{II} mice developed large and disseminating lesions, the rate of symptom aggravation being faster in H_{II}, while L_I and L_{II} proved resistant to parasites, only small and transient lesions being observed for them during a 150 days follow up. The outcome of infection also differs in Hi/PHA and Lo/PHA mice, Hi/PHA having a resistant and Lo/PHA a susceptible phenotype.

Keywords: *Leishmania amazonensis*, genetics of resistance, High and Low immunoresponsiveness, mice, *leishmania*

Introduction

Infection by *Leishmania* parasites has many distinct clinical features, and their outcome is thought to be largely influenced by the host's cell-mediated immune (CMI) responses. The most frequent form is single cutaneous leishmaniasis, in which a well balanced, albeit slow CMI response may lead to spontaneous regression. The largely destructive mucocutaneous leishmaniasis may be related to an exacerbation of the production of IL-2, IFNγ

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and lymphocyte proliferation upon stimulation by leishmanial antigen. On the other hand, diffuse cutaneous leishmaniasis and visceral leishmaniasis occur in the absence of measurable parasite-specific CMI responses (Castes *et al.* 1983, Carvalho *et al.* 1985a and b).

Immunity to *Leishmania* has been the subject of many experimental studies, particularly in inbred strains of mice, taking advantage of strain-related differences to identify and map resistance genes (review in Blackwell 1988).

Concerning *Leishmania mexicana*, these genes often modify parasite visceralization more than primary lesion growth, with the exception of recently identified Scl1 and Scl2 genes (Roberts, Alexander & Blackwell 1990).

Another approach has been to compare the outcome of parasite infection in lines of mice obtained by selective breeding for high (H) or low (L) antibody responsiveness. These mice, due to the interaction of several (about 10) independently segregating loci, have extreme phenotypes that are practically never met in natural populations or among inbred lines (Mouton, Stiffel & Biozzi 1985). This makes them useful models for studying the immune factors of resistance to infection, since:

(1) antibody production is higher in H than in L lines whatever the antigen specificity or the immunoglobulin isotype;

(2) expansion of specific T lymphocytes occurs faster in H than in L mice; and

(3) macrophages are the main cause of the interline difference: their stronger catabolic activity in L mice determines a low antibody production, together with a high capacity to control pathogen multiplication (Biozzi *et al.* 1984, Mouton, Sant'Anna & Biozzi 1988).

Resistance to *L. major* was shown to greatly differ in H and L mice. The latter develop but transient lesions, while in H mice unhealing lesions and mortality are consistently observed even after low parasite challenge, in spite of a stronger antibody production to parasite antigens (Hale & Howard, 1981).

This paper describes the outcome of infection by *L. amazonensis* in H or L mice of Selections I and II. Comparing the two high (H_I-H_{II}) and the two low (L_I-L_{II}) lines is important for two reasons: first, because coherent results in the two homologous lines reduce the chance that genes unrelated with immune status, and randomly fixed, interfere with H or L resistance pattern, and second, because H_I or H_{II} lines differ at chromosome 1 cluster gene locus involved in resistance to several infections. H_I mice were recognized as Ity^s, whereas H_{II} as well as L_I and L_{II} mice have Ity^r phenotypes (Plant & Glynn, 1982, Sant'Anna *et al.* 1989). In this cluster gene, the *Lsh* gene was recently shown to have an effect on *L. mexicana* infection (Davies *et al.* 1988).

The two lines of mice selected for H or L responsiveness to PHA mitogen (Hi/PHA and Lo/PHA) (Stiffel *et al.* 1977) were also checked for resistance/susceptibility to *L. amazonensis*. The genetic difference between the two lines was also expected at CMI level, by mixed lymphocyte (*in vitro*) or graft versus host (*in vivo*) reactions, but did not significantly affect Ab production.

The results reported here are a bare evaluation of the impact of genetic control of immune function upon the capacity to resist *L. amazonensis* infection. Our interest in the immunity to this parasite is reinforced by the capacity of *L. amazonensis* to cause different forms of clinical disease (Barral *et al.* in press), and by the impressive clinical results recently obtained by Badaro *et al.* (1990), who succeeded in curing visceral Leishmaniasis patients who were refractory to chemotherapy by using IFN γ combined with antimony.

The selected lines of mice could constitute experimental models for investigating the role of cytokines in innate resistance to infection, and for screening cytokine treatments that are liable to be efficient and harmless whatever the individual immune potentiality.

Materials and methods

MICE

H_I and L_I mice are from F_{70} of Selection I. H_{II} and L_{II} mice are from F_{50} of Selection II. Both Selections were carried out for antibody production to heterologous erythrocytes. Hi/PHA and Lo/PHA are from F_{40}. The selection was carried out for responsiveness to mitogens in an *in vitro* assay (Stiffel *et al.* 1977). The three Selections were carried out starting from distinct foundation populations and were maintained at Institut Curie. All experiments were made on 2–4 months old mice.

L. AMAZONENSIS INFECTION

L. amazonensis (Josefa) was obtained from Salvador (Brazil) and cultivated at Institut Curie.

Two 10^7 promastigotes of *L. amazonensis* from stationary phase cultures were injected in left hind footpad under a 10 or 20 μl volume. Pads thickness was measured weekly with a gauge callipser and results expressed as difference between control and infected footpad thickness. $\bar{x}$ and s.e. were calculated in groups of 8 mice. Results of repeated experiments were remarkably constant and pooled as indicated in legends to the figures.

ANTIBODY TITRATION

ELISA titrations were made using for coating a crude parasite extract prepared as described in Carvalho *et al.* 1988. Plates were coated with 50 μl/well of a 3 μg protein/ml solution, incubated at 37°C for 2 h and then washed and saturated with 1% ovalbumin solution. ELISA test was run as usual. Phosphatase-conjugated rabbit anti-mouse Ig and anti-IgG1, as well as anti-IgG2a isotypes, were purchased from Zymed, California. Results are expressed as $\log^2$ serum dilution giving OD = 30% plateau level.

HISTOLOGICAL EXAMINATION

10 weeks after infection, the animals were sacrificed, and infected footpads were excised. The material was fixed in formalin, decalcified in EDTA and processed for paraffin embedding and HE staining.

Results

OUTCOME OF *L. AMAZONENSIS* INFECTION IN H AND L ANTIBODY RESPONDER LINES OF MICE

Results on *L. amazonensis* infection in H and L. antibody responder mice of Selections I

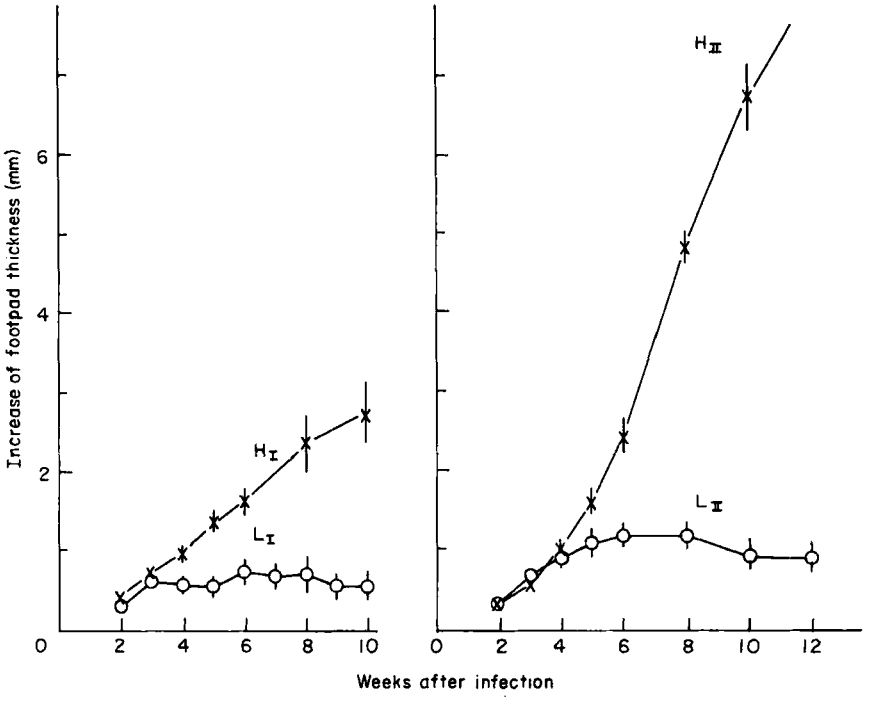

Figure 1. Increase in footpad thickness, in the course of *Leishmania amazonensis* infection, in H and L antibody responder mice of Selections I and II.

and II are shown in Figure 1, as the mean increase of footpad thickness during 10–15 weeks post infection. L_I and L_{II} mice are resistant to the high 2×10^7 promastigotes challenge, since regression of footpad lesions occurs in 100% animals. In contrast, the two H lines are susceptible to parasite infection. Of interest is the faster increase of infected footpads thickness consistently observed in H_{II} compared with H_I. The mean increases per week, calculated in 3 different experiments on the linear part of the kinetics shown in Figure 1, are 0·30 mm $\pm$ 0·06 in H_I versus 1·06 mm $\pm$ 0·07 in H_{II}. As a result, maximal measurable lesions ($\geqslant$ 8 mm) were observed in most (70%) H_I mice after 18 weeks, while similar lesion sizes were attained in 90% of H_{II} mice within 11 weeks only.

Long-term observations were made in the four lines: diffuse lesions appeared first in H_{II} (day 150 post infection), on uninjected pads and on the nose. By that time, only few H_I mice had signs of nose lesions; however extension to uninjected pads was noticed in about 50% H_I mice by day 200 post injection.

The two L lines were much more resistant to dissemination: respectively 20% and 40% of L_I and L_{II} mice had inflammation signs on one forepaw only, on day 320 post injection. A residual inflammatory reaction persisted at the site of injection, never exceeding 0·1 mm size.

OUTCOME OF *L. AMAZONENSIS* INFECTION IN HI/PHA AND LO/PHA MICE

Results in the two lines of mice selected for high or low responsiveness to the T mitogen

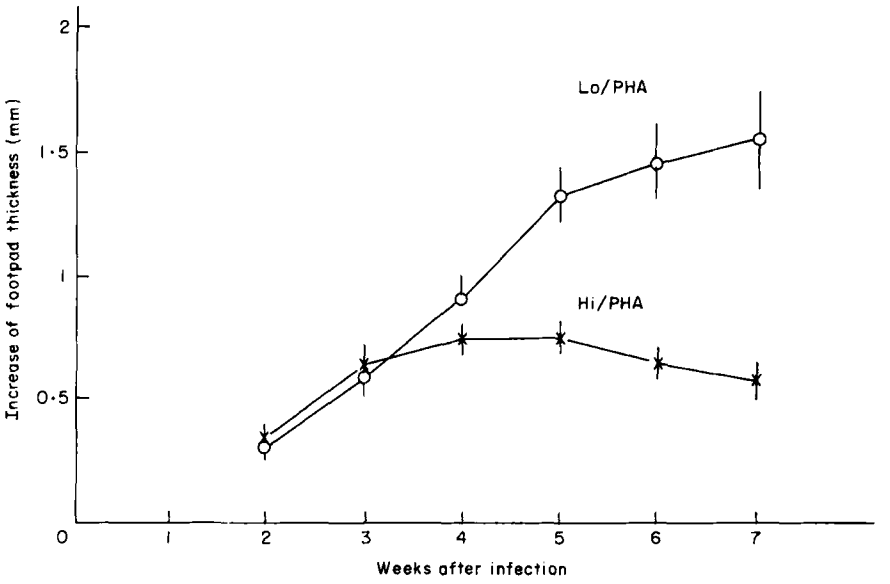

Figure 2. Increase in footpad thickness, in the course of *Leishmania amazonensis* infection, in HI/PHA and Lo/PHA mice.

PHA are shown in Figure 2. An interline difference is clearly observed after an initial increase of footpad thickness up to 3–4 weeks after parasite inoculation. Lo/PHA mice are much more susceptible to infection than Hi/PHA mice. The $\bar{x}$ rate of footpad increase after 2×10^7 inoculum is 0·34 mm/week in Lo/PHA measured during the 2–5 weeks interval, and 0·21 mm/week in Hi/PHA, measured during the 2–4 weeks interval. A long-lasting observation (over 18 weeks) showed that only 30% Lo/PHA and 14% Hi/PHA mice had a steady increase of lesions, while in others, stabilization or even regression occurred.

The interline difference is somewhat lower after injection of a smaller parasite number (5×10^6), but it is still significant, the $\bar{x}$ increase being 0·27 mm/week in Lo/PHA in the 2–6 weeks interval and 0·16 mm/week in Hi/PHA in the 2–4 weeks interval (data not shown).

ANTIBODY RESPONSES TO *L. AMAZONENSIS* ANTIGENS

The production of antibodies to parasite antigens during the course of infection was studied on pooled sera, using an ELISA titration. Representative results from one experiment in H_I, H_{II} and Hi/PHA, Lo/PHA mice are summarized in Table 1.

In the two H lines, the Ab IgG rise is similar; maximal values are found on day 28 (occasional measurements on days 65 and 85 gave no higher values). The isotype repartition of antibodies in the 28th day serum was compared in the two lines, mainly as regards IgG1 and IgG2a. Both isotypes are produced in H_I and H_{II} mice, however the IgG1/IgG2a ratios are 1·69 and 1·26 in H_I and H_{II} respectively, indicating a relatively lower IgG2a response in H_I.

The Ab responses, measured in L_I and L_{II} mice, are about 1 log lower than in their H

Table 1. Antibody response To *L. amazonensis* antigen

	Serum titre (log2) Days post infection				
	7	14	28		
			IgG	IgG1	IgG2a
H_I	5·00	5·90	6·50	6·50	3·90
H_{II}	4·20	4·90	6·70	6·70	5·30
Hi/PHA	<4	4·90	6·50	n.d.	
Lo/PHA	<4	5·00	6·50		

Table 2. Histological findings in mice selected for high or low immunoresponsiveness 10 weeks after infection by *Leishmania amazonensis*

	General pattern			Intensity of parasitism		
Group	Susceptible	Intermediate	Resistant	I	II	III
H_I	5*	1	2	2	2	4
L_I	0	2	6	8	0	0
Hi/PHA	2	2	4	5	1	2
Lo/PHA	4	3	1	3	5	0

* Number of animals in the group with each pattern.

counterpart throughout the whole course of infection (day 7 to day 90), IgG1 and IgG2a isotypes being produced in similar amounts (data not shown). Such a small interline difference is commonplace for long-lasting antigenic challenges.

The kinetics of Ab responses do not differ in Hi/PHA or in Lo/PHA mice either.

HISTOLOGICAL EXAMINATION

Histological data are given in Table 2. The general pattern of susceptibility is characterized by an extensive macrophage infiltration, and such cells are highly vacuolated, large vacuoles being predominant. There are foci of coagulative necrosis and neutrophils infiltration, as well as muscle fibre destruction and extensive epidermis ulceration. The resistance pattern is composed of extensive fibrinoid necrosis, extending up to the epidermis and related to the ulceration. The inflammatory infiltrate, mainly

mononuclear, is concentrated around the neuro-vascular plexus and also extends to the muscle.

The intensity of parasitism is graduated as follows:

Grade I is for slight and mainly local parasitism, Grade II for moderate parasitism with initial stage spreading, and Grade III for the stage when heavily parasitized cells are widespread in tissue sections. It is important to note that the presence of parasites in the epidermis was observed in 5 out of 8 H_I mice, and never in L_I. Similarly in PHA selection, this occurs in 5 out of 8 Lo/PHA mice versus one out of 8 Hi/PHA.

As a whole, the histological examination confirms, for both I and PHA Selections, the interline difference observed at the level of footpad increase.

Discussion

Mice selected for high or low immunoresponsiveness clearly show a distinct pattern of resistance/susceptibility to *L. amazonensis* infection.

Large interline differences were observed by measuring footpad thickness increase after local parasite injection. They were verified through histological examination; the resistant lines showed lymphocyte infiltration together with typical fibrinoid necrosis, whereas macrophage and neutrophil infiltration as well as coagulative necrosis were observed in the susceptible lines of mice. These findings reinforce the correlation of histological data with the resistance/susceptibility status previously reported in inbred mouse strains, even if the within-group-variation in histological features is somewhat larger in the selected lines of mice than in inbred strains (Barral-Netto, Cardoso & Barral, 1990, Barral-Netto, Freitas & Andrade 1987).

Both H_I and H_{II} mice selected for high antibody production are susceptible, whereas their L_I and L_{II} counterparts are resistant to *L. amazonensis* infection. The concordance of results in both Selections argues in favour of a genetic correlation between the control of infection outcome and the mechanisms regulating quantitative Ab production. From these results, no effect can be attributed to genes at Ity locus on chromosome 1.

Among possible mechanisms, special attention should be given to macrophages for their potential role in determining the general capacity of L mice to cope with intracellular pathogens (Biozzi *et al.* 1984).

Results in Figure 1 also show that local lesions develop more rapidly in H_{II} than in H_I mice, which was confirmed by a more severe dissemination of lesions late in the course of infection. Up to now, there is no definite explanation available for this finding. The hypothesis of a different Th1/Th2 subset frequency in the two lines is not supported by data on IgG1/IgG2a antibody isotypes ratios. Th1 cells, which preferentially help IgG2a production, are thought to induce an IFNγ-mediated protective effect on infection, while the role of Th2 cells, IgE and IgG1 helper cells, is more controversial (Müller & Louis 1989), some authors claiming that they might accelerate lesion development (Scott *et al.* 1988). In fact, our results indicate that H_I mice, the less susceptible, have a relatively lower IgG2a response than the hypersusceptible H_{II} mice.

Clear-cut results were also obtained by comparing the degree of resistance to *L. amazonensis* infection in the lines of mice selected for high or low responsiveness to PHA. The Hi/PHA mice, as shown in Figure 2, are much more resistant to parasite infection than Lo/PHA mice, even if lesion size is smaller in the latter than in susceptible

H_I and H_{II} mice due to unknown background effects. Accordingly, infection has a less severe outcome in Lo/PHA mice within the observation duration (18 weeks) limits. The higher susceptibility to *L. amazonensis* of Lo/PHA versus Hi/PHA mice fully corroborates the resistance pattern of these two lines to another intracellular pathogen, *Listeria monocytogenes*, recently reported by Berche *et al* (1989). In both infections, it should be mentioned that DTH responses to pathogens were stronger in Hi/PHA than in Lo/PHA mice. In fact, DTH reaction to *L. amazonensis*, measured at day 70 of infection, is 3 times higher in Hi/PHA than in Lo/PHA mice (data not shown). Also to be considered is the possible role of $CD8^+$ T cells in the resistance to *L. amazonensis*, since this subset was predominantly activated in Hi/PHA during *L. monocytogenes* infection (Berche *et al*, 1989).

These preliminary data underline the importance of the selected lines as models for studying the resistance to *L. amazonensis* in mice: differences between high and low Ab responder mice particularly depend on the macrophage defensive role, while a comparison of Hi/PHA and Lo/PHA mice might be helpful to define T cell involvement.

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