Resolution of an Infection with *Leishmania braziliensis* Confers Complete Protection to a Subsequent Challenge with *Leishmania major* in BALB/c Mice

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Both Leishmania major *and* L. braziliensis *induce cutaneous leishmaniasis in BALB/c mice. Whereas BALB/c mice die of infection with* L. major, *they cure an infection with* L. braziliensis. *We report here that after curing an infection with* L. braziliensis, *BALB/c mice are resistant to challenge with* L. major. *When challenged with* L. major, L. braziliensis *pre-treated BALB/c mice mounted a delayed-type hypersensitivity response to* L. major *and produced high amounts of interferon-g (IFN-g) but low amounts of interleukin-4. The IFN-g produced by the* L. braziliensis *pre-infected mice was involved in the protection seen against* L. major *challenge since treating the mice with a neutralizing anti-IFN-g abrogated the protection. This suggests that cross-reactive antigen epitopes exist between* L. braziliensis *and* L. major *and that pre-infection with* L. braziliensis *primes BALB/c mice to epitopes on* L. major *that can elicit a protective Th1 response to the parasite.*

Key words: *Leishmania braziliensis* - *Leishmania major* - mice - cross-protection - cytokines

Organisms of the genus *Leishmania* induce a spectrum of diseases in humans and in experimental animals. Infection of mice with *L. major*, one cause of cutaneous leishmaniasis, is perhaps the best studied model for cutaneous leishmaniasis (reviewed in Bogdan et al. 1993, Liew & O'Donnell 1993, Reed & Scott 1993, Titus et al. 1994, Reiner & Locksley 1995). Most mouse strains cure an infection with *L. major*, however BALB/c mice are a notable exception since they ultimately die of infection with *L. major* when the disease becomes systemic. Considerable work in this model has revealed that mice that are resistant to infection with *L. major* develop a Th1 immune response and its associated cytokine profile [interferon-gamma (IFN-g)^{hi}; interleukin-4 (IL-4)^{lo}]. IFN-g activates *L. major* infected macrophages (MØs) to kill the parasite (Murray et al. 1983, Titus et al. 1984, Nacy et al. 1985). In contrast, susceptible BALB/c mice develop a Th2 response and its associated cytokine profile (IFN-g^{lo}; IL-4^{hi}). IL-4 can block the ability of IFN-g to activate MØs to kill *Leishmania* (Lehn et al. 1989, Liew et al. 1989).

In contrast to infection with *L. major*, *L. braziliensis* induces only a transient cutaneous disease, even in BALB/c mice. This may at least in part be the explanation for why little experimental work has been performed with *L. braziliensis* (Neal & Hale 1983, Childs et al. 1984). We recently reported (DeKrey et al. 1998) that following infection with *L. braziliensis* or *L. major*, BALB/c mice produced similar levels of IFN-g. However, *L. braziliensis* infected mice produced much less IL-4 (approximately 10-fold). In addition, when the *L. braziliensis* infected mice were treated with a neutralizing anti-IFN-g, the animals were unable to resolve their infection. We concluded that BALB/c mice cure an infection with *L. braziliensis* because the low levels of IL-4 they produce are unable to block the ability of IFN-g to activate *L. braziliensis* infected MØs to kill the parasite.

Resolution of an infection with a particular species of *Leishmania* usually confers complete resistance to re-challenge with the same parasite. However, in addition to this, a primary infection with a given species of *Leishmania* can also confer cross-protection against a different species of *Leishmania* (Lainson & Bray 1966, Lainson & Shaw 1977, Alexander & Phillips 1978a,b, Perez et al. 1979, Alexander 1988, Neal et al. 1990, Peters et al. 1990, Melby 1991, Abramson et al. 1995, Gicheru et al. 1997). Cross-protection has been

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shown in several different mammalian hosts; the protection sometimes acts in only one direction (Lainson & Shaw 1977), and in some cases the sex of the host influences the cross-protection seen (Alexander 1988).

Since *L. braziliensis* is unable to trigger a strong Th2 response in BALB/c mice, we hypothesized that following resolution of an infection with *L. braziliensis*, BALB/c mice might be at least partially protected against challenge with *L. major*. We report here that previous exposure to *L. braziliensis* can confer complete protection against a subsequent challenge with *L. major* and that this protection is dependent upon IFN-g production.

MATERIALS AND METHODS

Mice and parasites - Young adult female mice were used in all experiments. BALB/c mice were obtained from either the National Cancer Institute (Bethesda, MD) or Jackson Laboratory (Bar Harbor, ME). C57BL/6 were obtained from the National Cancer Institute. Stationary phase promastigotes of *L. braziliensis* (MHOM-BR-79-LTB111) or *L. major* (RHO-SU-59-P) were used. Parasites were maintained as described (Titus et al. 1984).

Infecting mice and determining parasite numbers in cutaneous lesions - Mice were injected with the numbers of promastigotes indicated in the text in one hind footpad and lesion development was followed by measuring the thickness of the infected footpad compared to the thickness of the same footpad prior to infection.

Parasite numbers were determined in infected footpads using a published limiting dilution assay for determining parasite burdens in infected mouse tissues (Lima et al. 1997).

In some experiments mice were treated with a neutralizing anti-IFN-g(XMG1.2) antibody as described in DeKrey et al. (1998).

Determining levels of cytokines in culture supernatants - At various times after infection, 3-5 mice per group were killed for evaluation. Single cell suspensions were prepared from draining lymph nodes (inguinal and popliteal). Cells were adjusted to $5x10^6$/ml in Dulbecco's modified Eagle medium (Maryanski et al. 1982) containing 0.5% normal mouse serum (Harlan Bioproducts, Indianapolis, IN). Cultures were stimulated with 10^6 *L. major* promastigotes/ml and the supernatant of the cultures was harvested 72 hr later (a time determined to be optimal for the cytokines examined) for analysis.

Levels of IFN-g and IL-4 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using techniques published elsewhere (Soares et al. 1997).

Statistical analysis - Significance was determined using an non-paired *t* test. Differences were considered to be significant when $p < 0.05$.

All experiments shown are representative of two to three independent experiments.

RESULTS

To determine whether previous exposure to *L. braziliensis* led to protection against a subsequent challenge with *L. major*, we first experimented with the dose of *L. braziliensis* and the time between infection with *L. braziliensis* and challenge with *L. major*. We found that a large dose of *L. braziliensis* (10^7) administered subcutaneously (s.c.) in one hind footpad led to complete protection against a subsequent challenge with 10^6 *L. major* s.c. in the opposing hind footpad (Fig. 1). Moreover, the protective effect of pre-infecting with *L. braziliensis* was a dose titratable phenomenon. As shown in Fig. 1, a dose of 10^3 *L. braziliensis* led to the least protection against challenge with *L. major* whereas a dose of 10^7 *L. braziliensis* led to the greatest protection. Lesions of *L. major* were the largest in mice pre-treated with 10^3 *L. braziliensis* and only 20% of the mice (see numbers in the legend of Fig. 1) cured these *L. major*-induced lesions; in contrast, lesions of *L. major* were the smallest in mice pre-treated with 10^7 *L. braziliensis* and 100% of the mice cured these *L. major*-induced lesions.

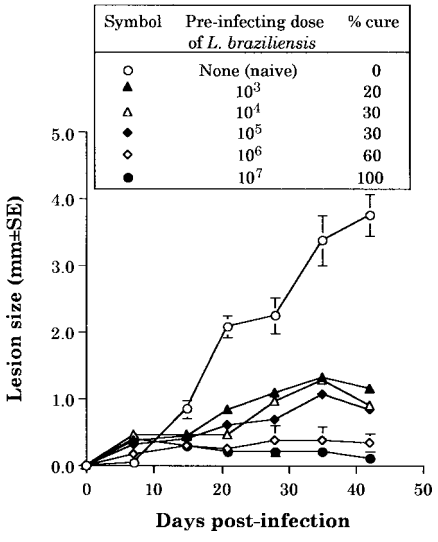

Fig. 1: course of infection with *Leishmania major* in BALB/c mice pre-infected with different concentrations of *L. braziliensis*. Groups of 10 BALB/c mice each were pre-infected with the indicated doses of *L. braziliensis* s.c. in one hind footpad. Twelve weeks later these animals were challenged s.c. in the opposing hind footpad with 10^6 *L. major*. Controls consisted of naive mice infected with 10^6 *L. major*. Lesions were monitored as described in Materials and Methods.

We also determined that the degree of resistance to challenge with *L. major* increased with time after exposure to *L. braziliensis*. Little if any protection against challenge with *L. major* was achieved when the two parasites were injected simultaneously. Some protection was observed when mice were challenged with *L. major* at 6 or 8 weeks after exposure to *L. braziliensis*. However, 100% protection against challenge with *L. major* was consistently achieved only at 12 weeks after exposure to *L. braziliensis* (data not shown). Importantly, at 12 weeks post-*L. braziliensis* injection, we were also unable to detect viable *L. braziliensis* in treated mice by limiting dilution analysis (data not shown). Therefore, for the remaining experiments presented here, mice were treated with 10^7 *L. braziliensis* and challenged 12 weeks later with 10^6 *L. major*.

The experiment shown in Fig. 1 demonstrated that pre-infection with *L. braziliensis* allows BALB/c mice to control the outgrowth of lesions of *L. major* when the mice were challenged with the parasite. To determine whether this was accompanied by destruction of *L. major* in the lesions, we measured the parasite burdens in the lesions. In *L. braziliensis*-naive control mice, *L. major* continued to replicate through day 42 of infection (Table I). In contrast, in mice pre-infected with *L. braziliensis* 12 weeks earlier, *L. major* was destroyed such that by day 42 of the experiment there were approximately 2,000-fold fewer parasites in their lesions compared to control mice (Table I).

We next analyzed the mechanism underlying the protection seen against challenge with *L. major* in BALB/c mice pre-infected with *L. braziliensis*. We first noted that an intense swelling response occurred in the footpads of *L. braziliensis* pre-treated mice when the mice were challenged with *L. major* (Fig. 2). This swelling response was characteristic of delayed-type hypersensitivity (DTH) in that it peaked from 24 to 48 hr post-challenge with *L. major* and it persisted to 72 hr post-challenge (Fig. 2). This observation suggested that cross reactive antigenic epitopes exist in *L. braziliensis* and *L. major* that prime T cell responses. Moreover, since DTH is mediated by Th1-type T cells (Mosmann & Coffman 1989), this also suggested that infection with *L. braziliensis* triggered Th1 T cells in BALB/c mice that could recognize *L. major* antigen(s) when the mice were challenged with the parasite.

To test the hypothesis that cross reactive Th1 T cells were elicited by pre-infection with *L. braziliensis*, we measured the cytokines produced when lymph node cells from *L. braziliensis* pre-infected mice were challenged with *L. major in vitro*. We first harvested the popliteal and inguinal nodes draining the footpad of mice pre-infected with *L. braziliensis* 12 weeks earlier. These cells were stimulated with *L. major* promastigotes *in*

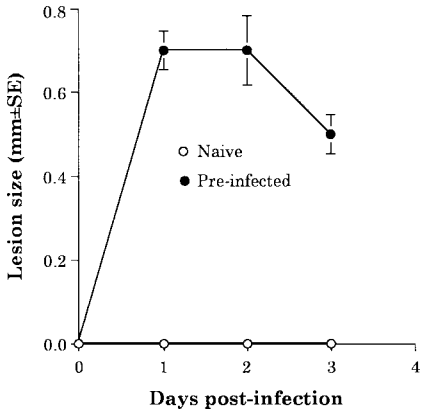

Fig. 2: footpad swelling response of *Leishmania braziliensis* pre-infected BALB/c mice challenged with *L. major.* BALB/c mice were pre-infected with *L. braziliensis* and challenged with *L. major* as described in the legend of Fig. 1.

TABLE I

Numbers of *Leishmania major* in lesions of BALB/c mice pre-infected with *L. braziliensis*

Days post-*L. major* infection	Number of *L. major*/footpad lesion (95% confidence limits)	
	Naive	Pre-infected
3	0.24×10^5 (0.06-0.43)	0.04×10^5 (0.01-0.07)[a]
7	2.85×10^5 (1.06-4.63)	0.40×10^5 (0.10-0.71)
21	35.77×10^5 (9.63-61.90)	3.75×10^5 (1.40-6.05)
42	79.75×10^5 (23.70-135.80)	0.40×10^5 (0.01-0.08)

a: BALB/c mice were infected with 10^7 *L. braziliensis* s.c. in a hind footpad. Twelve weeks later, the mice were challenged in the opposing footpad with 10^6 *L. major.* Controls consisted of age-matched *L. braziliensis*-naive BALB/c mice challenged with 10^6 *L. major.* At the indicated time points after challenge, the footpad lesions from duplicate mice of each group were subjected to limiting dilution analysis to determine the numbers of *L. major* present.

vitro and the supernatants were harvested 72 hr later to determine their content of IFN-γ and IL-4. These lymph node cells produced substantial amounts of IFN-γ (15.48 ng/ml, Table II) but no detectable IL-4. Moreover, when the same lymph node cells were harvested from *L. braziliensis* pre-infected mice at varying times after the mice were challenged with *L. major*, the cells continued to produce substantially more IFN-γ but less IL-4 than control naive BALB/c mice challenged with *L. major* (Table II).

Since *L. braziliensis* pre-infected mice produced elevated levels of IFN-γ but lower levels of IL-4 compared to naive mice when the mice were challenged with *L. major* (Table II), we next tested the hypothesis that the IFN-γ was involved in the protection seen against challenge with *L. major*. *L. braziliensis* pre-infected BALB/c mice were treated with a neutralizing IFN-γ antibody as described in the Materials and Methods and challenged with *L. major*. As can be seen in Fig. 3, treating with anti-IFN-γ abrogated the protection seen when the mice were challenged with *L. major*.

Finally, since *L. braziliensis* pre-infected BALB/c mice were protected against challenge with *L. major*, we tested whether resistance to infection with *L. major* was enhanced in a mouse that normally cures an infection with the parasite. C57BL/6 mice were pre-infected with 10^7 *L. braziliensis* and 12 weeks later were challenged with 10^6 *L. major*. As can be seen in Fig. 4, these mice showed markedly increased resistance to challenge with *L. major*.

TABLE II

Cytokines produced by lymph node cells from *Leishmania braziliensis* pre-infected BALB/c mice following challenge *in vitro* with *L. major*

Days post-*L. major* infection	Levels of cytokine produced by	
	Naive	Pre-infected
IFN-γ[a] (ng/ml±SD)		
0*	None detected	15.48±0.47c
3	23.12±6.26	16.38±2.14
7*	16.39±9.64	80.30±7.47
21*	23.23±1.95	45.78±3.02
42	8.05±5.24	14.09±0.54
IL-4[b] (pg/ml±SD)		
0	None detected	None detected
3	None detected	60.74±25.77
7	751.63±250.59	280.95±61.73
21*	326.57±27.93	98.24±38.27
42*	484.10±22.62	69.11±3.44

a: interferon-γ; *b*: interleukin-4; *c*: groups of BALB/c mice were infected with 10^7 *L. braziliensis* s.c. in a hind footpad. Twelve weeks later, the lesion-draining popliteal and inguinal lymph node cells from some of the mice were harvested and restimulated with *L. major* promastigotes as described in Materials and Methods. The remaining mice were challenged in the opposing footpad with 10^6 *L. major.* Controls consisted of age-matched *L. brasiliensis*-naive BALB/c mice challenged with 10^6 *L. major.* At the indicated time points after challenge, cells from lymph nodes draining the *L. major*-challeged footpad were stimulated with *L. major in vitro.* Seventy-two hr later, the levels of IFN-γ and IL-4 present in the supernatants of the cultures were determined by ELISA as described in Materials and Methods. The asterisks in the table indicate a statistically significant difference ($P<0.05$) between groups.

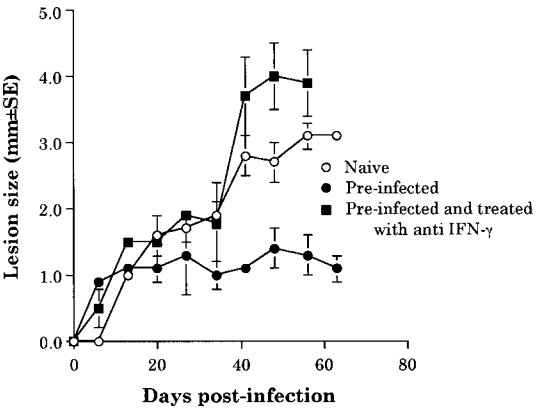

Fig. 3: *Leishmania braziliensis* pre-infected BALB/c mice do not resist infection with *L. major* when the animals are treated with anti-IFN-γ antibody. Groups of BALB/c mice were pretreated with 10^7 *L. braziliensis* and 12 weeks later the mice were challenged with 10^6 *L. major.* One of the groups of pretreated mice was also injected with a neutralizing anti-IFN-γ antibody as described in Materials and Methods. Controls consisted of age-matched *L. braziliensis*-naive BALB/c mice challenged with *L. major*. Lesions were monitored as described in Materials and Methods.

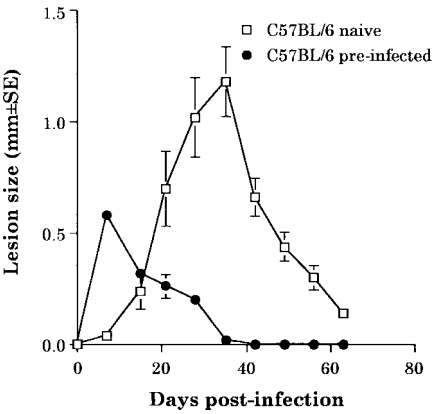

Fig. 4: pre-infecting with *Leishmania braziliensis* enhances the resistance of C57BL/6 mice to challenge with *L. major.* C57BL/6 mice were pre-infected with 10^7 *L. braziliensis* promastigotes and 12 weeks later the mice were challenged with 10^6 *L. major.* Controls were *L. braziliensis*-naive C57BL/6 mice challenged with *L. major*. Lesions were monitored as described in Materials and Methods.

DISCUSSION

We recently reported that cutaneous lesions develop on BALB/c mice following infection with either *L. major* or *L. braziliensis*; however, while BALB/c mice ultimately die of infection with *L. major*, the mice resist infection with *L. braziliensis*, kill the parasite, and heal their cutaneous lesions (DeKrey et al. 1998). Further analysis of this system revealed that following infection with *L. major*, BALB/c mice developed a Th2-biased immune response. In contrast, following infection with *L. braziliensis*, the mice developed a Th1-biased response (DeKrey et al. 1998). Since a Th1 response mediates cure in mice infected with *L. major*, we hypothesized that BALB/c mice that had cured an infection with *L. braziliensis* would resist challenge with *L. major*. To our knowledge, no one has examined whether pre-infecting mice with *L. braziliensis* confers protection against challenge with *L. major*.

We report here that pre-infecting BALB/c mice with *L. braziliensis* confers full protection against challenge with *L. major*. That is, whereas *L. braziliensis*-naive control mice were susceptible to infection with *L. major*, *L. braziliensis* pre-treated mice resolved cutaneous lesions of *L. major* (Fig. 1) and destroyed *L. major* parasites within the lesions (Table I).

Full protection against *L. major* required pre-infection with a high dose of *L. braziliensis* (10^7, Fig. 1). The reason such a high dose of *L. braziliensis* was required is not known. However, *L. braziliensis* is known to be poorly infective for laboratory mice (Samuelson et al. 1991). Since the metacyclic form of *Leishmania* is the infective form of the parasite [the form that survives and efficiently infects MØs in the vertebrate host (da Silva et al. 1989, Puentes et al. 1990)], it is possible that conversion to metacyclics by *L. braziliensis* is very inefficient using standard culture conditions. Thus, a large dose of *L. braziliensis* is required to successfully infect antigen presenting cells such as MØs in the host which in turn stimulate an effective immune response.

In addition to requiring a large dose of *L. braziliensis* to achieve full protection against challenge with *L. major*, it was also necessary to wait 12 weeks before challenging with the parasite, a time when *L. braziliensis* parasites could not be detected in the mice. Indeed, little if any protection was achieved when *L. braziliensis* and *L. major* parasites were injected simultaneously. Taking these observations together, in conjunction with the fact that *L. braziliensis* pre-treated mice produced large amounts of IFN-g when challenged with *L. major* (Table II), suggests that protection against challenge with *L. major* was not mediated by either concomitant immunity or a non-specific inflammatory response against *L. major*. Rather, the data suggest that there are antigenic epitopes shared between *L. braziliensis* and *L. major*. When a BALB/c mouse is infected with *L. braziliensis*, these epitopes elicit a protective Th1 T cell response such that the mice mount a Th1 response when challenged with *L. major*. The nature of the cross-reactive epitopes of *L. braziliensis* and *L. major* are unknown but are currently under investigation. It would be interesting if these cross-reactive epitopes were found to be expressed at low levels on *L. braziliensis* and/or to stimulate rare T cell clones. If this were the case, it would offer an alternative explanation for our observations that a large dose of *L. braziliensis* was required to achieve full-protection against *L. major* challenge and that it required 12 weeks for this protection to develop.

In conclusion, the data presented here confirm and further characterize previous reports that demonstrated cross-protection between different species of *Leishmania*. In addition, the data show that BALB/c mice can be induced to mount a protective Th1 response against a normally lethal infection with *L. major*, and that this can occur in the absence of intervention with cytokines or anti-cytokines.

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