PROTECTION OF C3HEB/FEJ MICE AGAINST LEISHMANIA AMAZONENSIS CHALLENGE AFTER PREVIOUS LEISHMANIA MAJOR INFECTION

YANNICK VANLOUBBEECK AND DOUGLAS E. JONES
Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa

Abstract. The Th1 response elicited in mice infected with Leishmania major has been used as a model to characterize cellular immune defects associated with L. amazonensis infection. However, it is not known if the immune response associated with the infection by virulent L. major parasites can promote resistance to a subsequent L. amazonensis infection. Our data demonstrate that C3HeB/FeJ mice infected subcutaneously with virulent L. major are resistant to an L. amazonensis challenge. The healing phenotype is characterized by a Th1 response as measured by increased production of interferon-γ and low levels of interleukin-4 in the draining lymph node. Together, this indicates that the Th1 response associated with L. major infection can promote resistance to L. amazonensis infection and that it can be used as a tool to study the immune defects associated with L. amazonensis infection.

INTRODUCTION

Leishmaniasis is a zoonotic disease caused by protozoa of the genus Leishmania, which are transmitted to the host through the bite of an infected sand fly. The disease is characterized by a variety of clinical manifestations, depending on the Leishmania species involved and the type of immune response mounted by the host.1,2 Leishmania major and L. amazonensis are both associated with the cutaneous form of the disease: the lesions induced by L. major are typically localized and self-healing, while the ones caused by L. amazonensis can become diffuse and chronic, sometimes affecting mucous membranes.1,3 Mouse models of leishmaniasis have helped to uncover some of the immune factors involved with resistance and susceptibility to the disease. For example, while C3H and C57BL/6 mice are resistant to L. major, they are susceptible to L. amazonensis. Resistance and susceptibility to L. major are mediated by Th1 and Th2 immune responses, respectively.4 The susceptibility to L. amazonensis is thought to result from an inability to mount a Th1 response.5–9 In particular, it has been shown that the CD4+ T cells from L. amazonensis-infected mice have defective expression of several cytokine and chemokine receptors in comparison to the CD4+ T cells from L. major-infected mice.6,9 However, while the immune response to L. major infection is used as a model in this experimental system of murine cutaneous leishmaniasis, it is not known if the Th1 response elicited by L. major in vivo would provide protection to a subsequent L. amazonensis infection. In fact, it has been reported that even after exogenous administration of interleukin-12 (IL-12) or in the absence IL-10 or IL-4, mice still develop chronic cutaneous lesions upon L. amazonensis infection.6,9,10 However, in mouse studies using L. major, the administration of IL-12 or antibody to IL-4, or the absence of IL-10 was associated with disease control.11–14 Therefore, it is possible that the immune response necessary to promote resistance to L. amazonensis may not be the same as that for L. major.

Cross-protection between different Leishmania species using either Leishmania antigens, attenuated parasites, or live Leishmania promastigotes have been previously described in mice.15–30 Monkeys,31–33 and humans.34,35 However, the effectiveness of cross-protection reported in the literature is variable and several studies have reported its failure, depending on the experimental design and/or the Leishmania spp. involved.15,17,20,32,34,35 Furthermore, the cross-protection induced in mice by a previous Leishmania infection does not necessarily correlate with complete healing. In contrast, it is sometimes associated with the persistence of a smaller lesion or even a slower progressing lesion than the control mice.18,21,29 Finally, the route of immunization appears to have an influence on the subsequent protection, and subcutaneous immunization has provided variable degrees of resistance.18,25 In particular, one mouse study has reported efficient protection to L. amazonensis after a subcutaneous infection with an avirulent strain of L. major.25 However, the study did not characterize the associated immune response nor was parasite quantification determined.

Our data indicate that the Th1 response developed by C3HeB/FeJ mice after infection with wild-type L. major promastigotes does promote resistance to a L. amazonensis challenge, as determined by the development of a transient lesion and decreased parasite load. To our knowledge, the present report is the first to determine the effectiveness of cross-protection using an infectious challenge composed of as much as 5 × 10^6 L. amazonensis promastigotes and using the subcutaneous route of immunization. Our data also confirm that the immune response associated with L. major infection is a good model for the study of the immune defects associated with L. amazonensis infection.

MATERIALS AND METHODS

Parasites. Culture of L. amazonensis (MHOM/BR/00/LTB0016) or L. major (MHOM/IL/80/Friedlin) parasites and the preparation of parasite antigen were performed as previously described.7 Parasite quantification was determined when the mice had healed their L. amazonensis infection, i.e., 10–12 weeks post-L. amazonensis challenge. For the parasite quantification from cutaneous lesions, the infected feet were disinfected with 70% ethanol and the skin was dissected away. The remaining subcutaneous lesion was homogenized using a Tenbrock tissue homogenizer (Kontes, Vineland, NJ), washed twice in phosphate-buffered saline, and resuspended in 2 mL of Grace’s insect cell culture medium (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. A 10-fold serial dilution of each parasite suspension was then performed in triplicate and incubated at 27°C for eight days before parasite quantification was assessed. The lower detection limit of our
were also infected with $5 \times 10^6$ stationary phase $L. major$ promastigotes in their left hind footpad. The resulting lesion size of the footpads was monitored weekly with a dial micrometer (L.S. Starrett Co., Athol, MA), and the results were expressed as the difference between the thickness of the right and left hind footpads. After infection with $L. amazonensis$, the thickness of the left feet (those that had healed a previous $L. major$ infection) remained stable.

Recall responses. The cells from the popliteal lymph node draining the $L. amazonensis$-infected feet were obtained 10–12 weeks after $L. amazonensis$ infection and recall responses were obtained as previously described. The cells were incubated with medium containing 50 μg of $L. amazonensis$ antigen/mL with or without 1 ng/mL of recombinant murine IL-12 (PeproTech Inc., Rocky Hill, NJ). Supernatants were harvested after 72 hours, and interferon-γ (IFN-γ) and IL-4 levels were determined by an enzyme-linked immunosorbent assay (ELISA). Recombinant IFN-γ (Pharmingen, San Diego, CA) and IL-4 (PeproTech Inc.) were used on each ELISA plate to set up a standard curve. The sensitivity of the IFN-γ ELISA ranged between 39 and 78 pg/mL; the sensitivity of the IL-4 ELISA was 39 pg/mL. All ELISAs were performed with commercially available antibodies (Pharmingen), peroxidase-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA) and 2, 2’-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) microwell peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) following the manufacturer instructions.

RESULTS

Resistance of mice infected with $L. major$ to a subsequent $L. amazonensis$ infection. The C3HeB/FeJ mice were infected subcutaneously in their left hind footpad with $5 \times 10^6$ $L. major$ stationary phase promastigotes. The resulting lesion consisted of a discrete cutaneous nodule that occasionally ulcerated and consistently healed over the following 11–15 weeks. At these time points, the mice were re-infected subcutaneously with $5 \times 10^6$ $L. amazonensis$ stationary phase promastigotes in their right hind footpad and the associated lesion size was measured weekly. As a control, naive mice were also infected with $5 \times 10^6$ $L. amazonensis$ stationary phase promastigotes in their right hindfoot. As shown in Figure 1, control mice infected with $L. amazonensis$ developed chronic lesions, whereas in mice previously infected with $L. major$, the $L. amazonensis$ lesion was transient and healed in 10–12 weeks. The chronic lesion of $L. amazonensis$-infected mice contained an average of $10^7$ parasites (Figure 2). In contrast, the healing response of mice infected with $L. major$ and subsequently with $L. amazonensis$ was associated with a significant decrease in parasite load (Figure 2).

Association of resistance to $L. amazonensis$ infection with an enhanced Th1 response within the draining lymph node. In each experiment, the mice were killed 10–12 weeks after $L. amazonensis$ infection, a time at which the associated cutaneous lesion had resolved. The cells from the draining lymph...
whether *L. amazonensis* (Figure 3A). The levels of IFN-γ (Figure 3B) were determined in the culture supernatants. While lymph node cells from both groups of mice produced low to undetectable amounts of IL-4, mice sequentially infected with *L. major* and *L. amazonensis* produced significantly higher levels of IFN-γ than the *L. amazonensis*-infected control mice (Figure 3A). The levels of IFN-γ detected were similar whether *L. amazonensis* or *L. major* antigens were present during the three-day recall response (mean ± SEM = 4.5 ± 1.0 ng/mL and 5.9 ± 1.5 ng/mL with *L. amazonensis* and *L. major* antigens, respectively). To further determine the phenotype of the healing immune response, we also determined the IL-12 responsiveness of the cells from the draining lymph node by measuring the levels of IFN-γ produced in vitro in response to *L. amazonensis* antigen and recombinant murine IL-12. The IL-12 responsiveness is a marker of Th1 responses and as shown in Figure 3B, draining lymph node cells from mice sequentially infected with *L. major* and *L. amazonensis* produced significantly more IFN-γ than the *L. amazonensis*-infected control in response to IL-12. These data indicate that the healing phenotype observed in co-infected mice is associated with an increased Th1 response at the level of the draining lymph node.

**DISCUSSION**

The Th1 immune response elicited after *L. major* infection has been used as a model to describe T cell defects associated with *L. amazonensis* infection.6,9 However, whether the immune response of *L. major*-infected mice represents an appropriate model to study *L. amazonensis* infection, i.e., if the Th1 response of mice infected with virulent *L. major* would promote resistance to a subsequent *L. amazonensis* infection, is not known. Our data indicate that mice previously infected with virulent *L. major* were able to control a *L. amazonensis* infection, characterized by lesion resolution and a decreased parasite load (Figures 1 and 2). The healing phenotype was associated with a Th1 response as determined by low levels of IL-4 and high levels of IFN-γ in the in vitro recall response from the draining lymph node (Figure 3A). This is consistent with previous studies in which the vaccination of mice with parasite antigen or DNA, with or without an adjuvant, was shown to promote resistance to *L. amazonensis* infection.24,27–29,36–38 When determined, the healing response following vaccination correlated with increased production of IFN-γ in the draining lymph node, the spleen, or at the lesion site.24,27–29,37 Furthermore, the adoptive transfer of *L. amazonensis*-specific Th1 cells into C3H mice was recently shown to promote resistance to subsequent *L. amazonensis* infection.9 Altogether, this demonstrates that resistance to *L. amazonensis* infection can be associated with a Th1 response. The IL-12 responsiveness is another marker of Th1 responses that was reported to correlate with the potential of mice to heal Leishmania infection.39–41 Therefore, the increased IL-12 responsiveness of draining lymph node cells from mice with *L. major* and subsequently with *L. amazonensis* further supports the Th1 phenotype of their healing response (Figure 3B).

Previous studies have shown that the exogenous administration of the Th1 cytokines IL-12 or IFN-γ was not able to promote resistance to *L. amazonensis*.6,42 In addition, the neutralization or the absence of IL-4 or IL-10, respectively could not restore resistance to *L. amazonensis*.6,7,9,10 Altogether, this suggests that these cytokines are not, by themselves, altering the course of *L. amazonensis* infection. Rather, resistance may require an array of immune factors that the *L. major*-induced immune response, as a whole, can provide. This would also suggest that some elements of a parasite-specific immune response may be needed prior the *L. amazonensis* challenge to provide protection. Our data confirm that the immune factors necessary to promote resistance to *L. amazonensis* are present after a *L. major* infection. Finally, our data also support a previous study suggesting that subcutaneous vaccination may be effective in promoting resistance to *L. amazonensis* infection,25 even in an experimental system using a high dose challenge.
In conclusion, our data show that the Th1 response of C3HeB/FeJ mice infected with L. major is able to promote protection to a subsequent L. amazonensis challenge. Although leishmanization, i.e., the inoculation of virulent Leishmania promastigotes as a vaccine, has been currently abandoned, this co-infection model confirms that the immune response developed after L. major infection is a good model to study the defects associated with L. amazonensis infection. This model will allow us to better understand and define the immune factors necessary or sufficient to promote resistance to L. amazonensis.

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Authors’ address: Yannick Vanloubbeeck and Douglas E. Jones, Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011-1250, Telephone: 515-294-3282. Fax: 515-294-5423. E-mails: yannick@iastate.edu and jonesdou@iastate.edu.

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