Brief Definitive Report

Internalization of *Leishmania mexicana* Complex Amastigotes via the Fc Receptor Is Required to Sustain Infection in Murine Cutaneous Leishmaniasis

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Abstract

We show here that maintenance of *Leishmania* infections with *Leishmania mexicana* complex parasites (*Leishmania amazonensis* and *Leishmania pifanoi*) is impaired in the absence of circulating antibody. In these studies, we used mice genetically altered to contain no circulating antibody, with and without functional B cells. This experimental design allowed us to rule out a critical role for B cell antigen presentation in *Leishmania* pathogenesis. In addition, we show that mice lacking the common γ chain of Fc receptors (FcγRI, FcεRI, and FcγRIII) are similarly refractory to infection with these parasites. These observations establish a critical role for antibody in the pathogenesis associated with infection by members of the *L. mexicana* complex.

Key words: *Leishmania* • Fc receptor • pathogenesis • antigen presentation • infection

Introduction

Leishmania promastigotes and amastigotes preferentially infect macrophages. Several host cell surface molecules have been proposed to mediate internalization of *Leishmania* into macrophages. The internalization of the promastigote form has been shown to be mediated by the mannose-fucose receptor (1, 2), the fibronectin receptor (3, 4), and the complement receptors CR1 and CR3 (4–7) on the surface of host macrophages. Promastigotes may interact directly with these molecules, or interaction may occur after parasite opsonization by soluble host molecules, as has been elegantly demonstrated in studies with the third component of complement (7, 8). Less is known of the molecules that mediate entry of the amastigote form into host cells, even though amastigotes are responsible for sustaining the infection within the host. In one study, COS cells, which are usually poorly infected by *Leishmania mexicana* amastigotes, could be rendered more susceptible to infection after transfection with FcγRIIb but not the C3 or mannose receptors, as long as the amastigotes were derived from infected tissue, rather than cultured (9). As tissue-derived amastigotes are opsonized with antibody (9–11), this result suggests that antibody might mediate internalization of these amastigotes into host cells. However, this point remains controversial, as certain experimental data suggest no contribution of opsonins to infection of macrophages with amastigotes (10), whereas other studies have demonstrated a role for both Fc receptor and CR3 in macrophage infection by amastigotes in vitro (11). How the mechanism of internalization of *Leishmania* amastigotes affects the pathogenesis of *Leishmania* has not been addressed.

We had noted in antigen presentation studies reported previously (12) that the source of *Leishmania* amastigotes (axenically cultured versus tissue derived) determined whether endogenously synthesized parasite-derived molecules could be presented by infected macrophages to antigen-specific $CD4^+$ T cells. As overnight incubation of tissue-derived amastigotes in low-pH medium minimized the differences between parasites from these sources, we postulated that in the host, parasites are opsonized with host-derived molecule(s), which have an effect on parasite biology in the macrophage. As indicated above, tissue-derived amastigotes are coated with host Ig (9–11). So, to follow up

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on our in vitro observations, we have assessed the role of antibody opsonization in vivo. It has been shown previously that $CD4^+$ T cell activation is required for the initiation of infection by *Leishmania amazonensis* (13). Limited lesion formation was observed in the absence of $CD4^+$ T cells; T cell activation at the site of infection is required to initiate the inflammatory processes that result in macrophage recruitment to the infection site. We hypothesized, therefore, that in the absence of antibody in vivo, parasite entry into macrophages and/or antigen presentation of endogenously synthesized parasite antigens to $CD4^+$ T cells by *Leishmania*-infected macrophages will be impaired. Either mechanism would result in a limited course of leishmaniasis in this murine model.

When the role of antibody has been investigated previously in this and other model systems, the approach has usually been to use mice either treated with anti–μ chain antibody from birth or genetically altered through gene deletion to lack B cells, and hence also circulating Ig. A major limitation of these types of experiments is that one cannot always rule out an effect of the absence of B cells per se. In the current study, we have made use of J_H-deleted (J_HD) mice that lack B cells (14), and J_HD mice reconstituted by transgenesis by Shlomchik and colleagues (15) to contain otherwise functional B cells that either do not secrete Ig (mIgM/J_HD Tg) and/or secrete IgM [(m+s)IgM/J_HD Tg). To further demonstrate the involvement of antibody in the pathogenic process, mutant mice lacking the common γ chain of several Fc receptors were also examined.

Materials and Methods

Mice. The FcγR knockout mice (BALB/cByJMTacfBR-[KO]) that are deficient in the common γ chain subunit of FcγRI, FcγRIII, and FcϵRI (16) were purchased from Taconic (model no. 000584-M). Generation of B cell mutant mice has been described previously (14). In this study, we use J_HD mice (15) and the following recombinant derivatives obtained by breeding: VH186.2-(m+s)IgM/J_HD/J_HD transgenics [(m+s) IgM/J_HD Tg] produce soluble IgM, and the VH186.2-mIgM/J_HD/J_HD transgenics (mIgM/J_HD Tg) have functional B cells but no circulating Ig (15). BALB/c and C.B-17 mice were obtained from The Jackson Laboratory.

Parasites and Infection of Mice. Leishmania pifanoi (MHOM/VE/60/Ltrod) amastigotes were maintained at 31°C in F-29 medium containing 20% fetal bovine serum (FBS), as reported previously (17). *L. pifanoi* and *L. amazonensis* (MHOM/BR/77/LTB0016) promastigotes were grown at 23°C in complete Schneider's *Drosophila* medium supplemented with 20% FBS (GIBCO BRL) and 10 μg/ml gentamicin. Parasite infectivity was maintained by regular passage through BALB/c mice. For evaluation of the infection, four to six mice per group were infected in the hind feet with washed 2×10^6 cultured *L. pifanoi* amastigotes, or with low-passage promastigotes harvested in late log phase. The course of infection was monitored by measurement of lesion size using a dial gauge caliper. At designated periods, mice were killed to determine parasite burdens, as described previously (17).

Passive Serum Transfers. Parasite membrane preparations were used to immunize mice for generation of immune serum. These membranes were obtained from cultured *L. pifanoi* amastigotes as described previously (17). BALB/c mice were injected three times at weekly intervals with 50 μg/ml of this preparation, emulsified in CFA (week 1) or IFA (weeks 2 and 3). Serum was collected 4 wk after start of immunization, and was titered. Passive transfers were accomplished by injecting four or five J_HD mice per group intraperitoneally with 100 μl of either immune or normal (Taconic) mouse serum on days 6, 3, and +2 of infection. Success of reconstitution was determined by test bleeding a few mice 1 wk after the last serum administration. Mice were infected with *L. pifanoi* amastigotes, and the course of infection was monitored by measuring lesion development as described above.

Results

Course of L. amazonensis and L. pifanoi Infection in Mice Lacking Circulating Antibody. BALB/c wild-type and B cell–mutant mice were infected with 2×10^6 axenically cultured *L. pifanoi* amastigotes, and lesion development was monitored over a 10-wk period. Some mice were killed at 6 wk after infection, and parasites in lesions (infected feet) were enumerated in limiting dilution experiments. As shown in Fig. 1, wild-type mice develop significant lesions during the period of observation. In contrast, mice lacking circulating antibody, J_HD and mIgM/J_HD mice, barely develop lesions. The course of infection in the (m+s)IgM/J_HD Tg mice, which secrete IgM, was indistinguishable from the mIgM/J_HD mice (data not shown), suggesting very little contribution from circulating IgM to parasite opsonization. Enumeration of parasites in lesions at 6 wk after infection revealed that, consistent with the differences in lesion size, there were >100-fold more parasites in wild-type mice lesions compared with parasites within the putative lesion sites of the B cell–mutant mice.

The course of infection of *L. amazonensis* (another *L. mexicana* complex parasite) promastigotes was also ascertained in mice lacking circulating antibody. As shown in Fig. 2, promastigote-initiated infections develop slowly in

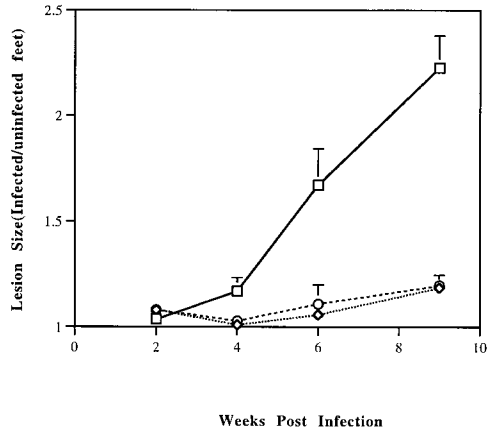

Figure 1. Course of *L. pifanoi* amastigote infection. The course of infection in J_HD (◇), mIgM/J_HD Tg (○), and BALB/c mice (□) was followed after infection with cultured *L. pifanoi* amastigotes. Each data point is the mean from four to six mice.

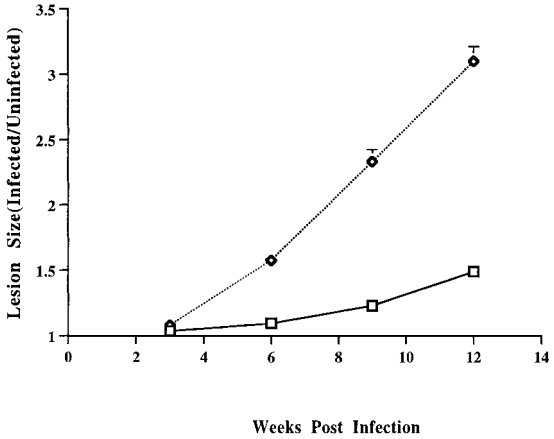

Figure 2. Course of *L. amazonesis* infections. J_HD mice (□) and BALB/c mice (◇) were infected with *L. amazonensis* promastigotes, and lesion development was measured over a course of 12 wk.

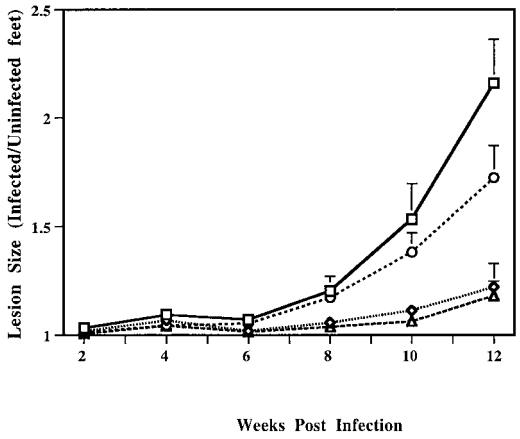

Figure 3. Course of *L. pifanoi* infections in J_HD mice after transfer of normal or immune mouse serum. Culture-derived amastigotes were used to infect BALB/c mice (□), J_HD mice (◇), J_HD mice reconstituted with immune serum (○), or J_HD mice reconstituted with normal mouse serum (△). Each data point is the mean of four to six mice.

the absence of circulating antibody. This may be due to the fact that, although promastigotes may not be internalized via the Fc receptor during the initial stages of the infection, promastigotes transform into amastigotes soon after infection of macrophages.

Effect of Passive Antibody Transfer on the Course of Leishmania Infections. Observations presented thus far have shown that mice lacking circulating antibody are refractory to infections with *L. pifanoi* and *L. amazonensis* parasites. To directly demonstrate that the altered course of infection was the result of the absence of circulating antibody, J_HD mice were passively reconstituted with serum from normal mice or immune serum from mice hyperimmunized with amastigote membrane preparations. The course of infection initiated with *L. pifanoi* amastigotes in these mice was then determined. Fig. 3 shows that transfer of immune serum to mice that lack circulating antibody restores pathogenesis of *Leishmania* amastigotes. Passive transfer of normal mouse serum into J_HD mice did not reverse the refractory state of these mice to amastigote infections. It is of interest that cellular reconstitution experiments in recombination activating gene (RAG)$2^{-/-}$ mice, alluded to earlier, clearly indicated that splenic lymphocytes were significantly more effective in reconstituting pathology than were comparable numbers of purified $CD4^+$ T cells (13). These reconstitution results implicated a second cellular component contributing to pathology associated with infection. The results here indicate that a B cell product, specifically Ig, is critical for the development of pathology.

Course of Infection in Fc$\gamma R^{-/-}$ Mice. Antibody-opsonized parasites can be internalized via the Fc receptor. If parasite entry into macrophages via the Fc receptor plays a role in this infection, then it would be predicted that mice lacking the Fc receptor will exhibit a similar course of infection to B cell–mutant mice devoid of circulating antibody. Available to us on the appropriate genetic background were mutant mice lacking the common γ chain of FcγRIII, FcγRI, and FcεR1 (Fc$\gamma R^{-/-}$), obtained by gene deletion (16). Antibody levels in Fc$\gamma R^{-/-}$ mice were similar to those found in normal mice (data not shown). Fc receptor knockout mice were infected with axenically cultured *L. pifanoi* promastigotes and amastigotes as described above, and the course of infection was compared with wild-type mice. Fig. 4 shows a representative experiment. When infected with amastigotes, Fc$\gamma R^{-/-}$ mice develop minimal lesions over a period of 13 wk. Not unlike the infections in mice devoid of circulating antibody, infections initiated in Fc$\gamma R^{-/-}$ mice with promastigotes lag behind comparable infections of wild-type mice, but are more palpable than those initiated with amastigotes. However, it should be noted that there is a somewhat more noticeable in-

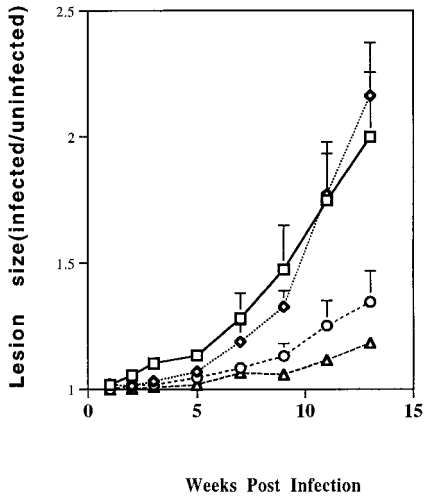

Figure 4. Course of *L. pifanoi* infections in $FcR^{-/-}$ mice. *L. pifanoi* promastigotes (◇, ○) and amastigotes (□, △) were used to infect $FcR^{-/-}$ mice (○, △) and BALB/c mice (□, ◇). Each data point is the mean of four to six mice.

crease in the lesion size of infected FcγR$^{-/-}$ mice than was observed for B cell–deficient mice.

Discussion

We show here that the course of infections with the *L. mexicana* complex parasites, *L. amazonensis* and *L. pifanoi*, are limited in the absence of circulating antibody, and that infections of mice lacking the common γ chain of Fc receptors result in similarly limited lesions. A role for antibody in the pathogenesis of these *L. mexicana* complex parasites is demonstrated using both direct and indirect approaches. We used genetically altered mice that lack B cells, and hence circulating antibody ($J_HD^{-/-}$), mIgM/J_HD Tg mice, and (m+s)IgM/J_HD Tg mice. As was recently reported (15), these mice exhibit important differences in several immunological parameters, including the activation status of naive T cells attributable to B cells but not antibody. However, they are equally refractory to infection with *L. pifanoi* amastigotes and somewhat less so to promastigotes. Reconstitution of the antibody compartment by passive transfer of immune and not normal serum reversed the refractory state of the B cell–mutant mice, thus directly implicating antibody in the pathogenic process. It is therefore clear that the primary defect in these mice that has an impact on *Leishmania* pathogenesis is the absence of circulating antibody. Reversal in the pathogenesis of *L. mexicana* upon passive transfer of immune serum has been shown previously in Ig μ chain–deficient mice (9).

Infections initiated with promastigotes rather than amastigotes might not be expected to be affected by the absence of circulating antibody, as other opsonins and surface molecules have been implicated in promastigote entry into macrophages (1–7). However, we observed that even though promastigote infections in mice without circulating antibody were somewhat more progressive than those initiated with amastigotes, these infections still lagged significantly behind comparable infections in intact mice. In vivo, promastigotes quickly enter macrophages and transform into amastigotes or are cleared from infected mice. Therefore, the implication is that the effect of infection by the promastigote would be transitory; the subsequent course of infection would be determined by the amastigote, which is dependent upon antibody for perpetuation of infection.

Although amastigote entry via the Fc receptor of macrophages has been shown to occur, its impact on *Leishmania* pathogenesis had not been fully appreciated (10). We therefore assessed the course of *Leishmania* infections in mice lacking the common γ chain of the Fc receptors FcγRI, FcγRIII, and FcεRI, which mediate the internalization of IgG1, IgG2a, IgG2b, and IgE isotypes (18). Parasites in FcRγ$^{-/-}$ mice should be opsonized with antibody; however, they will not be internalized via the Fc receptors in the absence of the common γ chain. The course of infection initiated by promastigotes or amastigotes in FcRγ$^{-/-}$ mice was similar to that in B cell–mutant mice.

It is possible that the interaction of opsonized parasites with Fc receptors on the surface of macrophages might lead to differential cytokine production, and hence explain differences in disease progression. NK cells express the FcγRIII and can be activated to secrete cytokines by antibody cross-linking, so we cannot rule out the possibility, though unlikely, that impaired activation of NK cells might contribute to our observations. It should be noted that the role of NK cells in leishmaniasis has been shown to be insignificant in the absence of activated T cells (19–21). In addition, several studies have shown that entry of amastigotes into macrophages poorly activates respiratory burst and cytokine production (22, 23). Most of these studies employed tissue-derived *Leishmania* amastigotes that have been shown to be coated with antibody (10, 11). Consequently, the differences in pathogenesis observed in Ig-deficient and FcR$^{-/-}$ mice (compared with wild-type mice) are not likely to be the result of differential macrophage cytokine production.

Recent investigations of the mechanism by which antibody modifies the presentation of antigens to T cells have suggested that internalization via the Fc receptor affects endocytic transport of the internalized molecule, which leads to enhanced presentation of certain epitopes (24, 25). Studies using particles opsonized with antibody and internalized via Fc receptors have shown that these particles transit through a compartment uniformly surrounded by the cytoskeletal proteins, talin, vinculin, and paxillin (26, 27). Tissue-derived *Leishmania* amastigotes, a great proportion of which are antibody coated, would be expected to transit through such compartments when internalized via Fc receptors. Indeed, it was recently demonstrated that some vacuoles containing internalized tissue-derived amastigotes are transiently surrounded with paxillin and talin (10).

Antibody opsonization of *Leishmania* amastigotes may increase the efficiency of parasite internalization, and/or qualitatively modify the host's response to infected macrophages. In vitro studies have shown that macrophages minimally present endogenously synthesized parasite antigens to CD4$^+$ T cells if these macrophages are infected with axenic amastigotes (nonopsonized parasites [12, 28]). This contrasts with observations that macrophages infected with promastigotes can transiently present endogenously synthesized parasite antigen to CD4$^+$ T cells. In addition, previous studies with *L. amazonensis* had demonstrated that T cell activation at the putative lesion site is required for the establishment of cutaneous lesions (13). It would therefore be of interest to further elucidate the effect of FcR-mediated uptake on antigen presentation and T cell activation at the site of infection on the pathogenesis caused by these parasites.

We have shown here that circulating antibody plays a critical role in the pathogenesis of the *L. mexicana* complex parasites, *L. amazonensis* and *L. pifanoi*. It is known that disease presentation upon infection with these parasites is variable (ranging from relatively controlled cutaneous to uncontrolled diffuse cutaneous disease). It is quite likely that this variability is in part determined by the nature of the host antibody compartment. In addition, it can be inferred from the proposed mechanism by which antibody exerts its

role that predetermining the receptor by which these parasites gain access to cells might be a viable strategy for disease control.

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