Gamma Interferon Is Essential for Clearing *Mycobacterium genavense* Infection

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Factors determining the in vivo replication of the opportunistic pathogen *Mycobacterium genavense* are largely unknown. Following intravenous injection of a patient isolate, *M. genavense* could not be recovered by culture or detected by PCR in the livers or spleens of infected BALB/c mice. In contrast, *M. genavense* was found to chronically persist and multiply in the livers and spleens of intravenously infected syngeneic gamma-interferon-gene-deficient (GKO) mice as evidenced by acid-fast stains of infected tissues and recovery by both PCR and liquid culture following organ homogenization. In GKO mice, *M. genavense* elicited a chronic inflammatory response, resulting in marked splenomegaly and extensive lymphadenopathy. Granulomatous lesions in the livers of GKO mice were diffuse, were composed of monocytes, neutrophils, and CD3+ cells, and were histochemically negative for inducible nitric oxide synthase.

*Mycobacterium genavense* is an opportunistic mycobacterium frequently recovered from pet animals (16) that mainly infects immunocompromised patients, particularly those with AIDS (5, 22, 26). Recently, however, *M. genavense* has also been detected in the lymph nodes of patients with no apparent primary immune deficiency (2, 3, 19). *M. genavense* is difficult to grow in vitro (4, 7, 17). It has a very low growth rate in the standard liquid cultures used for diagnostic purposes, and growth on solid agar media is almost undetectable (7). Long-term incubation of liquid media, particularly under low oxygen tension (23), followed by acid-fast staining and PCR amplification and sequencing of 16S rRNA is needed to ascertain the presence of *M. genavense* in diagnostic specimens (1–3, 5). In vitro studies determining susceptibility to various antibiotic agents are severely compromised due to the very limited growth of this organism (4, 7).

The pathogenesis of *M. genavense* infection is not well understood due to the lack of an animal model of infection. For example, it is not known whether an immune deficiency (and if so, which type of deficiency) is truly necessary for infection to become established. In an effort to develop a robust and reproducible model of *M. genavense* infection in mice, we infected mouse strains (BALB/c or C57BL/6) carrying the susceptible allele of the *bgc* locus, because these mice are frequently used in studies involving other mycobacterial species, such as *Mycobacterium avium* (13, 14, 18). In order to address the question of whether the cytokine gamma interferon (IFN-γ), known to activate macrophages for mycobacteriostasis (6), is important for containing *M. genavense* infection in vivo, we also infected IFN-γ-gene-deficient (GKO) mice.

**Recovery of *M. genavense* from the spleens of infected mice.** *M. genavense* was recovered from the blood of an AIDS patient by liquid culture (BACTEC 13A; Becton Dickinson Microbiology Systems, Cockeysville, Md.). Presence of *M. genavense* was assessed by acid-fast staining and PCR-mediated detection of mycobacterial 16S rRNA followed by sequencing. PCR for ribosomal DNA was performed by using primer A (10) and primer 264 (1) to amplify a DNA fragment of approximately 1,030 bp. Cycle sequencing was done with primer 9 (24) by using the BigDye Ready Reaction terminator sequencing kit (Applied Biosystems, Foster City, Calif.) on an automated DNA sequencer (ABI 377; Applied Biosystems).

C57BL/6, BALB/c, and syngeneic GKO mice (kindly provided by D. Dalton [8]) were raised under specific-pathogen-free conditions at the Animal Facilities of the Research Center Borstel, Borstel, Germany. Mice were infected intravenously with approximately 109 *M. genavense* organisms grown in liquid culture. For culture, mycobacteria were washed by centrifugation at 12,000 × g in an Eppendorf microcentrifuge and were counted microscopically in a Thoma chamber. Spleens and livers of infected mice were removed and subjected directly to PCR and/or liquid culture at different time intervals (Table 1). DNA from mouse tissues was isolated by using the QIAGEN tissue kit (QIAGEN, Hilden, Germany). PCR was performed with a modified nested PCR protocol (24) by using primer 9 and a novel reverse primer (primer 20, 5′-GGGCGCATCCCC ACACCGCWAAG-3′). For culture, specimens were decontaminated by using the N-acetyl-l-cysteine–sodium hydroxide method and were inoculated into liquid media (MGIT 960; Becton Dickinson) and onto Löwenstein-Jensen and Middlebrook 7H10 solid agar supplemented with 10% oleic acid, dextrose, and catalase (Becton Dickinson). Infection experiments were performed at least twice, with similar results.

In several preliminary experiments, we were unable to recover *M. genavense* organisms from the organs of intravenously infected C57BL/6 or BALB/c mice. In contrast, GKO mice reproducibly had viable *M. genavense* in their livers and spleens 3 and 5 months after infection. A comparison of PCR-mediated detection rates of *M. genavense* in BALB/c and simultaneously infected GKO mice is shown in Table 1. Detection of *M. genavense* in the spleens of infected mice at 4 weeks postinfection was inconsistent in both immunocompetent and GKO mice, in that only one or two out of 4 samples, respectively, showed evidence of the presence of mycobacterial DNA (Table 1). No mycobacterial DNA was detected in any mice at 8 weeks postinfection. However, at 12, 20, and 26 weeks postinfection, all tissue samples (both liver and spleens) taken from GKO mice were positive for *M. genavense* DNA, while all
TABLE 1. Detection of *M. genavense* DNA by PCR and sequencing in the spleens of infected mice

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. of <em>M. genavense</em>-positive mice&lt;sup&gt;a&lt;/sup&gt; (time postinfection [wk])</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>1/4 0/4 ND 0/4</td>
</tr>
<tr>
<td>GKO</td>
<td>2/4 4/4 4/4 4/4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were infected with 10<sup>6</sup> *M. genavense* cells, and spleens were removed at indicated time points and processed for PCR-mediated amplification of DNA encoding 16S rRNA, followed by sequencing of amplicons. Numbers denote positive PCR-mediated detection of *M. genavense* DNA in the spleens of infected animals out of the total number of animals examined per time point. ND, not determined.

tissue samples taken from BALB/c mice gave negative results by PCR (Table 1).

At 12 and 26 weeks postinfection, livers and spleens were also cultured for *M. genavense* growth. All samples from GKO mice showed a positive growth index and positive acid-fast stain in the liquid culture medium, while all the samples from immunocompetent mice remained negative. Organisms retrieved from cultured tissues of GKO mice were processed for 16S rRNA sequencing, and all were found to be *M. genavense*. Thus, live *M. genavense* was reproducibly detected in GKO mice following week 12 of infection.

In contrast to other investigators (27), we have consistently been unsuccessful at quantitating the growth of a number of different *M. genavense* isolates on solid Middlebrook agar supplemented with 10% oleic acid, dextrose, and catalase, even after extended incubation times of 12 weeks. In our experience, *M. genavense* isolates are difficult to expand, even in liquid culture. Since coinfection of AIDS patients with other organisms, such as *M. avium*, is quite common, growth of acid-fast bacilli may easily be mistaken for *M. genavense* (17).

**Histopathology following *M. genavense* infection.** In all GKO mice, significant splenomegaly (splenic weight range at 26 weeks postinfection, 440 to 1,060 mg) and, in some GKO mice, dramatic enlargement of the axillary or mediastinal lymph nodes was noted following week 12 of infection with *M. genavense*. In some cases, enlargement of Peyer’s patches was also evident. In contrast, no gross pathology or increase in spleen weight was observed in BALB/c mice at any point during infection (Fig. 1) or in uninfected GKO mice (data not shown).

When livers, spleens, lungs, and lymph nodes of infected mice were examined histologically, immunocompetent mouse showed only minimal inflammatory infiltrations in the liver and spleen early, i.e., at 4 weeks postinfection, but at no later time point. Acid-fast bacilli were never detected in the organs of BALB/c mice. In contrast, GKO mice showed numerous and increasingly large granulomatous infiltrations following week 12 of infection, particularly in the liver and spleen. These accumulations of inflammatory cells lacked the defined circumscription quality of granulomas evident in immunocompetent mice infected with other opportunist mycobacteria, such as *M. avium* (11, 14), and appeared rather diffuse (Fig. 2A). In the liver, they were composed of granulocytes, foamy macrophages, and other mononuclear cells (Fig. 2A, inset). Frequently, perivascular infiltrations consisting almost exclusively of polymorphonuclear granulocytes were seen (Fig. 2C, arrow and inset), consistent with a previous report on mycobacterium-induced hematopoietic remodelling in GKO mice (21).

Within granulomatous lesions, acid-fast bacilli were always detected following week 12 of infection, but not before that time point (Fig. 2B). Acid-fast bacilli were also prominent in enlarged lymph nodes (Fig. 2D), again suggesting that *M. genavense* had multiplied in GKO mice.

In order to determine whether GKO mice were deficient in recruiting T cells into granulomatous lesions, immunoperoxidase staining with a rat anti-mammalian-CD3 monoclonal antibody (clone CD3-12; Biotrend, Cologne, Germany) was performed. Inflammatory infiltrations in GKO mice always contained CD3<sup>+</sup> cells, although these were not organized in a lymphocytic cuff around centrally located macrophages, as is the case in *M. avium* and *Mycobacterium tuberculosis* infection in immunocompetent mice (Fig. 2E). In view of the presence of intralesional T cells, we next asked whether granuloma macrophages in GKO mice were adequately activated, as determined by staining with a polyclonal rabbit anti-mouse inducible nitric oxide synthase (iNOS) antiserum (Biomol, Hamburg, Germany). Macrophages in GKO mice were consistently negative for iNOS throughout the observation period (Fig. 2F) (12).

Our study clearly shows that immunocompetent mice are capable of clearing *M. genavense* infection. Furthermore, eradication of *M. genavense* is dependent on the presence of IFN-γ, as mice deficient for IFN-γ develop progressive mycobacterial growth. The mere presence of T cells within the lesions evident in GKO mice is not sufficient to contain *M. genavense* infection, and this may explain why patients with no apparent T-cell defects were also found to be infected with *M. genavense* (2, 3, 19).

IFN-γ has been shown to activate mycobacteriocidal mechanisms in macrophages, one of which is the production of nitrogen intermediates via iNOS (20). GKO mice did not express iNOS protein in their lesions at any point during infection. It remains to be determined whether the generation of nitric oxide via iNOS is necessary to inhibit multiplication of *M. genavense* (as is the case in murine infection with *M. tuberculosis*), because IFN-γ also inhibits bacterial growth in *M. avium* infection, but nitric oxide is not involved (11, 13).

IFN-γ is also a principal mediator of ordered, structured granuloma formation in response to mycobacterial infection. Thus, in GKO and SCID mice treated with a neutralizing anti-IFN-γ antibody and infected with *M. avium*, granuloma formation is greatly delayed, and isolated macrophages and Kupffer cells filled with acid-fast bacteria are frequently found (13, 25). In GKO mice infected with *M. genavense*, we also noted delayed development of inflammatory infiltrations which roughly corresponded to the time required for development of...
splenomegaly. It would seem that M. genavense needs time to establish infection even in GKO mice, and macrophages and/or T cells might only respond by releasing proinflammatory mediators after a certain threshold of bacterial numbers is reached. This would reflect the situation in humans, where a prolonged course of M. genavense infection with minor symptoms is followed by overgrowth in enlarged lymph nodes and spleens, leading to the requirement for medical attention (22, 26). In GKO mice, typically ordered granuloma formation with epithelioid cell differentiation did not occur, again reminiscent of the lesions present in immunodeficient patients with disseminated M. genavense infection (5, 22).

In summary, experimental M. genavense infection in GKO mice may be useful for studying the virulence factors of the microorganism that determine in vivo replication. Furthermore, because M. genavense infection in GKO mice closely resembles infection in immunodeficient patients, it may be a valuable experimental model for evaluating immunomodulatory and chemotherapeutic options. Finally, our studies indicate that in human subjects infected with M. genavense but not

FIG. 2. Granulomatous lesions in GKO mice infected with M. genavense. GKO mice were infected with 10⁶ M. genavense cells, and livers and lymph nodes were removed for histological analysis at indicated times postinfection. (A) Malorganized inflammatory infiltrations in the liver composed predominantly of mononuclear cells at 20 weeks postinfection (hematoxylin and eosin stain; magnification, ×64). Inset, enlargement of intralesional mononuclear cells with little epithelioid transformation (magnification, ×320). (B) Granuloma in the liver with acid-fast M. genavense at 20 weeks postinfection (Ziehl-Neelsen stain; magnification, ×128). (C) Large diffuse mixed infiltrations and perivascular accumulation of predominantly polymorphonuclear cells (arrow) at 26 weeks postinfection (hematoxylin and eosin stain; magnification, ×64). Inset, enlargement of lesion indicated by arrow, demonstrating typical polymorphonuclear morphology (magnification, ×320). (D) Acid-fast M. genavense in mesenteric lymph node at 26 weeks postinfection (Ziehl-Neelsen stain; magnification, ×128). (E) CD3-positive cells in granulomatous lesions at 20 weeks postinfection (immunoperoxidase; magnification, ×64). (F) Granulomatous lesions negative for material reactive with an anti-iNOS-antiserum at 20 weeks postinfection (immunoperoxidase; magnification, ×128). Inset, iNOS-positive control in M. tuberculosis-infected BALB/c mice stained in parallel.
infected with human immunodeficiency virus (HIV) and with no apparent quantitative defect of T cells, some kind of involvement of the IFN-γ pathway (such as a deficiency of the IFN-γ receptor, the interleukin 12 receptor, or interleukin 12 secretion) should be suspected, similar to that recently described for disseminated M. avium infection in HIV-negative patients (9, 15).

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REFERENCES


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