INFECTION AND IMMUNITY, June 2000, p. 3720–3723 Vol. 68, No. 6
0019-9567/00/$04.00+0

Gamma Interferon Is Essential for Clearing *Mycobacterium genavense* Infection

STEFAN EHLERS[1]* AND ELVIRA RICHTER[2]

Division of Molecular Infection Biology[1] and National Reference Center for Mycobacteria,[2] Research Center Borstel, D-23845 Borstel, Germany

Received 7 December 1999/Returned for modification 14 February 2000/Accepted 16 March 2000

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 *bcg* 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 10^6 *M. genavense* organisms grown in liquid culture. For infection, 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′-GGGCYCATCCC ACACCGCWAAAG-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

* Corresponding author. Mailing address: Division of Molecular Infection Biology, Research Center Borstel, Parkallee 22, D-23845 Borstel, Germany. Phone: 49-4537-188481. Fax: 49-4537-188686. E-mail: sehlers@fz-borstel.de.

TABLE 1. Detection of *M. genavense* DNA by PCR and sequencing in the spleens of infected mice

Mouse strain	No. of *M. genavense*-positive mice[a] (time postinfection [wk])			
	4	12	20	26
BALB/c	1/4	0/4	ND	0/4
GKO	2/4	4/4	4/4	4/4

[a] Mice were infected with 10^6 *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 mice 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 circumscript quality of granulomas evident in immunocompetent mice infected with other opportunistic 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 $CD3^+$ 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

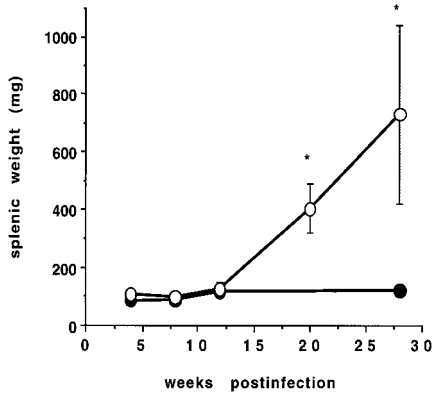

FIG. 1. Course of spleen weight following *M. genavense* infection of BALB/c and GKO mice. Mice were infected with approximately 10^6 *M. genavense* cells, and spleens were removed at indicated time intervals and weighed. Closed circles, BALB/c mice; open circles, GKO mice. Data represent the means of four mice ± standard deviations per time point. *, $P < 0.001$ by Student's *t* test.

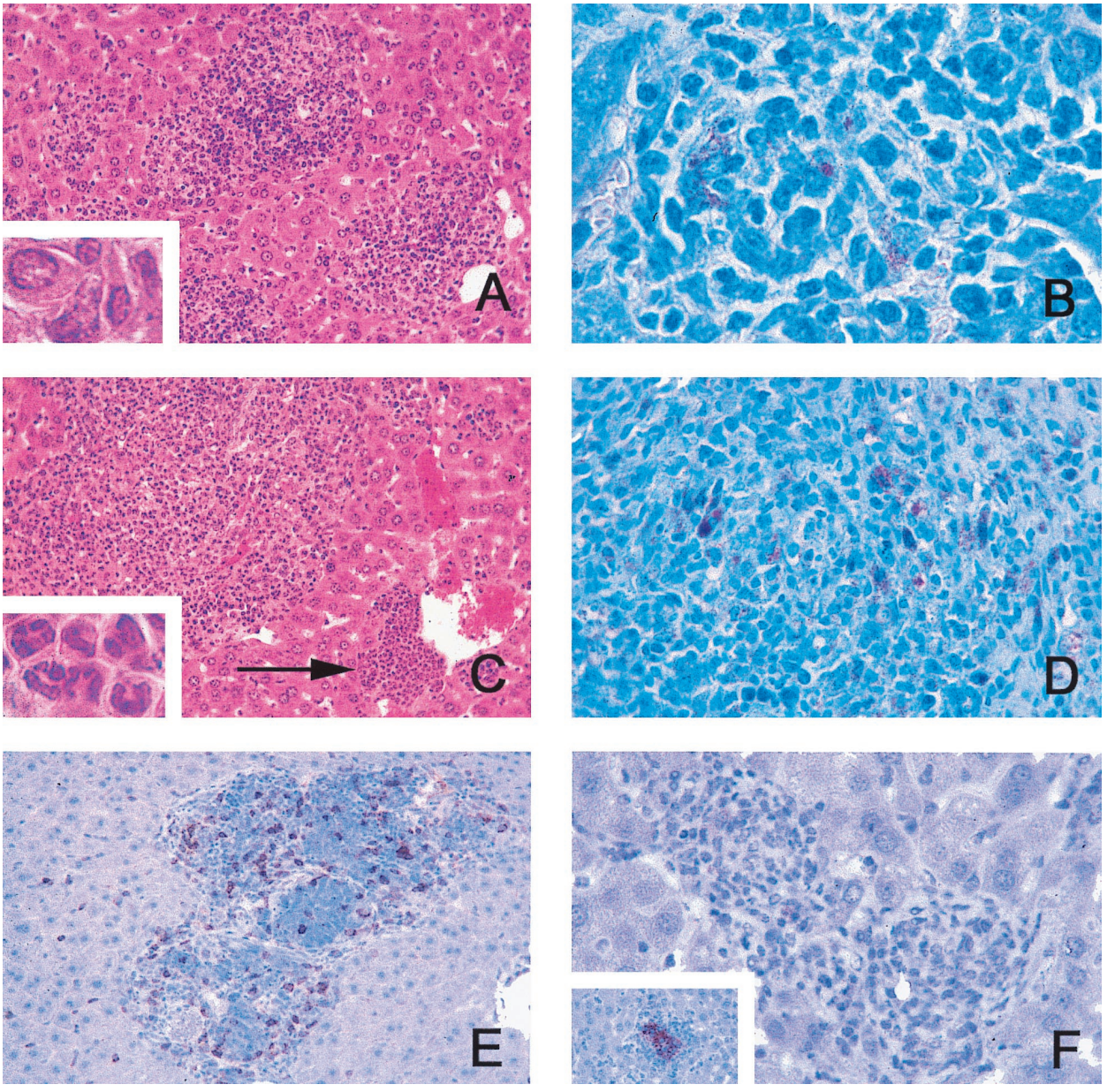

FIG. 2. Granulomatous lesions in GKO mice infected with *M. genavense*. GKO mice were infected with 10^6 *M. genavense* cells, and livers and lymph nodes were removed for histological analysis at indicated times postinfection. (A) Malorganized inflammatory infiltrations in the liver composed predominantly 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 mesenterial 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.

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

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).

We thank Claudia Hahn and Frauke Schaefer for expert technical assistance and Sven Mohr for breeding GKO mice.

REFERENCES

1. **Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
2. **Bogdan, C., P. Kern, E. Richter, A. Tannapfel, S. Rüsch-Gerdes, T. Kirchner, and W. Solbach.** 1997. Systemic infection with *Mycobacterium genavense* following immunosuppressive therapy in a patient who was seronegative for human immunodeficiency virus. Clin. Infect. Dis. **24:**1245–1247.
3. **Bosquée, L., E. C. Böttger, H. De Beenhouwer, P. A. Fonteyne, B. Hirschel, L. Larsson, W. M. Meyers, J. C. Palomino, L. Realini, L. Rigouts, M. T. Silva, A. Teske, P. Van der Auwera, and F. Portaels.** 1995. Cervical lymphadenitis caused by a fastidious mycobacterium closely related to *Mycobacterium genavense* in an apparently immunocompetent woman: diagnosis by culture-free microbiological methods. J. Clin. Microbiol. **33:**2670–2674.
4. **Böttger, E. C., B. Hirschel, and M. B. Coyle.** 1993. *Mycobacterium genavense* sp. nov. Int. J. Syst. Bacteriol. **43:**841–843.
5. **Böttger, E. C., A. Teske, P. Kirschner, S. Bost, H. R. Chang, V. Beer, and B. Hirschel.** 1992. Disseminated *Mycobacterium genavense* infection in patients with AIDS. Lancet **340:**76–80.
6. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
7. **Coyle, M. B., L. C. Carlson, C. K. Wallis, R. B. Leonard, V. A. Raisys, J. O. Kilburn, M. Samdadpour, and E. C. Böttger.** 1992. Laboratory aspects of *Mycobacterium genavense*, a proposed species isolated from AIDS patients. J. Clin. Microbiol. **30:**3206–3212.
8. **Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart.** 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science **259:**1739–1742.
9. **Dorman, S. E., and S. M. Holland.** 1998. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. J. Clin. Investig. **101:**2364–2369.
10. **Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Böttger.** 1989. Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:**7843–7853.
11. **Ehlers, S., S. Kutsch, J. Benini, A. Cooper, C. Hahn, J. Gerdes, I. M. Orme, C. Martin, and E. T. Rietschel.** 1999. NOS2-derived nitric oxide regulates the size, quantity and quality of granuloma formation in *M. avium*-infected mice without affecting bacterial loads. Immunology **98:**313–323.
12. **Ehlers, S., and U. Seitzer.** 1998. Measuring immune responses in vivo, p. 365–387. *In* S. H. E. Kaufmann and D. Kabelitz (ed.), Methods in microbiology. Academic Press, London, United Kingdom.
13. **Florido, M., A. S. Gonçalves, R. A. Silva, S. Ehlers, A. M. Cooper, and R. Appelberg.** 1999. Resistance of virulent *Mycobacterium avium* to gamma-interferon-mediated antimicrobial activity suggests additional signals for induction of mycobacteriostasis. Infect. Immun. **67:**3610–3618.
14. **Hänsch, H. C. R., D. A. Smith, M. E. A. Mielke, G. B. Bancroft, and S. Ehlers.** 1996. Mechanisms of granuloma formation in murine *M. avium* infection: the role of CD4+ T cells. Int. Immunol. **8:**1299–1310.
15. **Jouanguy, E., F. Altare, S. Lamhamedi, P. Revy, J.-F. Emile, M. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, and J.-L. Casanova.** 1996. Interferon-γ-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. N. Engl. J. Med. **335:**1956–1962.
16. **Kiehn, T. E., H. Hoefer, E. C. Böttger, R. Ross, M. Wong, F. Edwards, N. Antinoff, and D. Armstrong.** 1996. *Mycobacterium genavense* infection in pet animals. J. Clin. Microbiol. **34:**1840–1842.
17. **Kirschner, P., U. Vogel, R. Hein, and E. C. Böttger.** 1994. Bias of culture techniques for diagnosing mixed *Mycobacterium genavense* and *Mycobacterium avium* infection in AIDS. J. Clin. Microbiol. **32:**828–831.
18. **Leitzke, S., W. Bucke, K. Borner, R. Müller, H. Hahn, and S. Ehlers.** 1998. Rationale for and efficacy of intermittent treatment with liposome-encapsulated amikacin in chronically lethal *M. avium* infection. Antimicrob. Agents Chemother. **42:**459–461.
19. **Liberek, V., C. Soravia, B. Ninet, B. Hirschel, and C. A. Siegrist.** 1996. Cervical lymphadenitis caused by *Mycobacterium genavense* in a healthy child. Pediatr. Infect. Dis. J. **15:**269–270.
20. **MacMicking, J., R. North, R. LaCourse, J. Mudgett, S. Shah, and C. F. Nathan.** 1997. Identification of NOS2 as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA **94:**5243–5248.
21. **Murray, P. J., R. A. Young, and G. Y. Daley.** 1998. Hematopoietic remodeling in interferon-gamma-deficient mice infected with mycobacteria. Blood **91:**2914–2924.
22. **Pechere, M., M. Opravil, A. Wald, J. P. Chave, M. Bessesen, A. Sievers, R. Hein, J. von Overbeck, R. A. Clark, E. Tortoli, et al.** 1995. Clinical and epidemiologic features of infection with *Mycobacterium genavense*. Swiss HIV Cohort Study. Arch. Intern. Med. **155:**400–404.
23. **Realini, L., K. De Ridder, J. Palomino, B. Hirschel, and F. Portaels.** 1998. Microaerophilic conditions promote growth of *Mycobacterium genavense*. J. Clin. Microbiol. **36:**2565–2570.
24. **Richter, E., U. Greinert, D. Kirsten, S. Rüsch-Gerdes, C. Schlüter, M. Duchrow, J. Galle, H. Magnussen, M. Schlaak, H.-D. Flad, and J. Gerdes.** 1996. Assessment of mycobacterial DNA in cells and tissues of mycobacterial and sarcoid lesions. Am. J. Respir. Crit. Care Med. **153:**375–380.
25. **Smith, D. A., H. C. R. Hänsch, G. B. Bancroft, and S. Ehlers.** 1997. T cell independent mechanisms of granuloma formation in *M. avium* infection: the role of TNFα and IFNγ. Immunology **92:**413–419.
26. **Tortoli, E., F. Brunello, A. E. Cagni, D. Colombrita, D. Dionisio, L. Grisendi, V. Manfrin, M. Moroni, C. Passerini Tosi, G. Pinsi, C. Scarparo, and M. T. Simonetti.** 1998. *Mycobacterium genavense* in AIDS patients, report of 24 cases in Italy and review of the literature. Eur. J. Epidemiol. **14:**219–224.
27. **Vrioni, G., C. Nauciel, G. Kerharo, and P. Matsiota-Bernard.** 1998. Treatment of disseminated *Mycobacterium genavense* infection in a murine model with ciprofloxacin, amikacin, ethambutol, clarithromycin and rifabutin. J. Antimicrob. Chemother. **42:**483–487.

Editor: S. H. E. Kaufmann