INFECTION AND IMMUNITY, Apr. 2004, p. 2420–2424 Vol. 72, No. 4
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2420–2424.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

# Effect of Growth State on Transcription Levels of Genes Encoding Major Secreted Antigens of *Mycobacterium tuberculosis* in the Mouse Lung

Lanbo Shi,[1] Robert North,[2] and Maria Laura Gennaro[1]*

*Public Health Research Institute, Newark, New Jersey 07103,[1] and Trudeau Institute, Saranac Lake, New York 12983[2]*

Received 24 September 2003/Returned for modification 28 November 2003/Accepted 5 January 2004

**Arrest of the multiplication of *Mycobacterium tuberculosis* caused by expression of adaptive immunity in mouse lung was accompanied by a 10- to 20-fold decrease in levels of mRNAs encoding the secreted Ag85 complex and 38-kDa lipoprotein. *esat-6* mRNA levels were high throughout infection. The data imply that multiplying and nonreplicating tubercle bacilli have different antigen compositions.**

The seminal finding that live *Mycobacterium tuberculosis* elicit protective immunity more effectively than dead bacilli (6, 24) prompted extensive investigation of proteins that tubercle bacilli secrete as potential targets of protective immune responses. Among the best-characterized secreted proteins are the low-molecular-weight ESAT-6 protein (33), the antigen 85 complex (Ag85A, Ag85B, and Ag85C) (42), encoded by the *fbpA*, *fbpB*, and *fbpC* genes, and a 38-kDa glycolipoprotein (17), the product of *pstS1*. These antigens induce strong immune responses to infection with *M. tuberculosis* or *Mycobacterium bovis*, and they elicit protective immunity in animal models of tuberculosis (for a review, see references 1, 4, and 16). Consequently, experimental vaccines based on ESAT-6 and/or the Ag85 complex have been scheduled for clinical trials (18, 23), ESAT-6 has been proposed for immunodiagnosis of tuberculosis (3, 10, 13, 20, 26), and the 38-kDa protein is included in all serodiagnostic assays for active tuberculosis developed to date. To extend the usefulness of these proteins as new drug targets, the structures of two members of the Ag85 complex (2, 29) and the 38-kDa antigen (40) have been solved by X-ray crystallography and nuclear magnetic resonance has been used to obtain the structure of the cotranscribed ESAT-6/CFP-10 complex (28). Next we need to understand the production of these target antigens at various stages of human infection.

Classic biochemical studies (30) and recent gene manipulation experiments (21) showed that *M. tuberculosis* changes its metabolic state during infection by utilizing alternative metabolic pathways. We have shown (31) that adaptation of *M. tuberculosis* to the expression of adaptive immunity in the lungs of infected mice involves changes in the pathogen's transcription program characteristic of the state of nonreplicating persistence (41). In the present work, we characterize a correlation between the growth state of *M. tuberculosis* and the production of major secreted antigens by measuring levels of *M. tuberculosis* transcripts encoding the Ag85 complex, ESAT-6, and the 38-kDa lipoprotein during *M. tuberculosis* infection of the mouse lung. The data support the view that antigen composition differs between multiplying and nonreplicating tubercle bacilli.

Infection of C57BL/6 mice and isogenic, gamma interferon knockout (IFN-$\gamma^{-/-}$) mice with $\sim 2 \times 10^2$ CFU of *M. tuberculosis* strain $H_{37}Rv$ (Trudeau Mycobacterial Collection strain no. 102) cultivated to mid-log-growth phase in Proskauer and Beck medium containing 0.01% Tween 80 was carried out as previously described (14, 31). At selected times, lungs were harvested from four mice per time point. The number of CFU was determined by spreading 10-fold serial dilutions of homogenates from half of the lung (attached to the left bronchus) on enriched Middlebrook 7H11 agar plates followed by counting bacterial colonies after 3 weeks of incubation at 37°C. As previously shown (19, 22, 31), the lungs of wild-type (WT) mice infected with 100 CFU of *M. tuberculosis* $H_{37}Rv$ sustained exponential growth of *M. tuberculosis* for about 18 days. Further bacterial multiplication was prevented by expression of adaptive, Th1-mediated host immunity leading to chronic infection (Fig. 1, open triangles). In contrast, bacterial cell numbers steadily increased in the lungs of IFN-$\gamma^{-/-}$ mice until the mice died at days 35 to 40 postinfection (Fig. 1, open circles).

The half of the lung attached to the right bronchus was used to measure selected *M. tuberculosis* mRNAs and 16S rRNA. The methods used for lung RNA extraction and quantification of bacterial mRNA by real-time reverse transcription-PCR (RT-PCR) have previously been published (31). Briefly, total RNA was extracted from lung tissue by using a guanidinium thiocyanate-based buffer and rapid mechanical lysis of *M. tuberculosis*. RT and quantification of *M. tuberculosis* mRNAs by real-time PCR were carried out by using gene-specific primers and molecular beacons. The nucleotide sequences of the oligonucleotide primers used for enumeration of *fbpA*, *fbpC*, *pstS1*, and *esat-6* transcripts are listed in Table 1. The numbers of copies per lung of four *M. tuberculosis* mRNAs are presented in Fig. 2 (*fpbA* and *fbpC* in panel A; *pstS1* and *esat-6* in panel B). *M. tuberculosis* 16S rRNA was used as a normalization factor to enumerate bacterial transcripts per cell because 16S rRNA levels correlate well with the number of CFU during the course of lung infection, regardless of growth stage ($y = 1.0457x + 3.1093$; $R^2 = 0.954$) (31).

* Corresponding author. Mailing address: Public Health Research Institute, Rm. W250G, 225 Warren St., Newark, NJ 07103. Phone: (973) 854-3210. Fax: (973) 854-3101. E-mail: gennaro@phri.org.

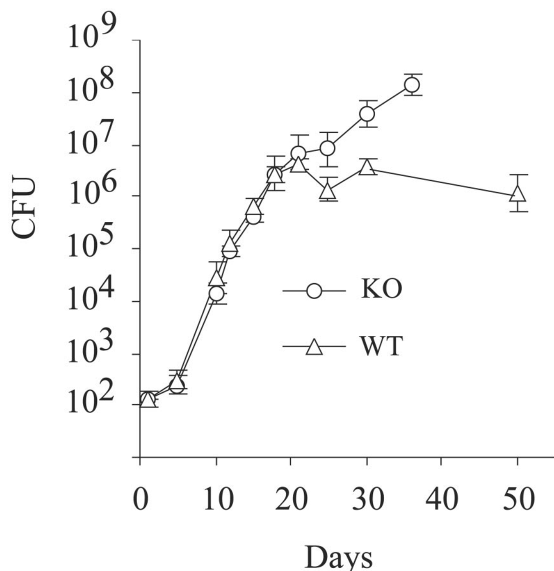


FIG. 1. Course of *M. tuberculosis* infection in mouse lung. Wild-type and IFN-γ $^{-/-}$ (KO) mice on a C57BL/6 background were infected with $2 \times 10^2$ CFU of *M. tuberculosis* $H_{37}Rv$ via the respiratory route. At the times indicated, half of the lung (attached to the left bronchus) was used to determine the number of CFU. The data points represent the mean ± standard deviation (in log units) CFU per lung obtained from four animals per time point per mouse strain during the first 50 days of infection of WT mice and for the entire course of infection of IFN-γ $^{-/-}$ mice.

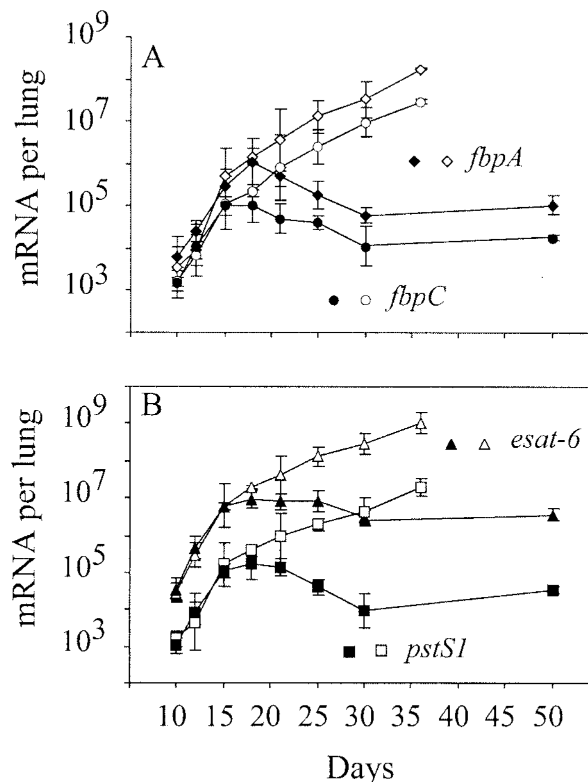


FIG. 2. Determination of *M. tuberculosis* mRNAs of *fbpA*, *fbpC*, *pstS1*, and *esat-6* in the lungs of WT and IFN-γ $^{-/-}$ mice over the course of infection by real-time RT-PCR. The number of copies of selected *M. tuberculosis* mRNAs per lung was determined by molecular-beacon real-time RT-PCR. The means ± standard deviations (in log units) of the results for four mice per time point per mouse strain for *M. tuberculosis* genes *fbpA* and *fbpC* (A) and *pstS1* and *esat-6* (B) are shown. WT mice, filled symbols; IFNγ $^{-/-}$ mice, open symbols.

mRNA levels for *fbpA* and *fbpC* normalized to 16S rRNA levels were stable during exponential growth of *M. tuberculosis* in the lung of WT mice (Fig. 3A and B). Coincident with immunity-induced growth arrest, transcript levels began to decrease on day 21 of lung infection. By day 30, the decrease relative to day 15 was about 10-fold for *fbpA* and *fbpC* mRNAs (Fig. 3A and B). The level of expression of *fbpC* was about fourfold lower than that of *fbpA* at corresponding times of infection (Fig. 3, compare panels A and B), while the levels of *fbpB* (31) were similar or slightly higher than those of *fbpA*. These mRNAs remained elevated throughout the course of lung infection in IFN-γ $^{-/-}$ mice.

*pstS1* mRNA levels normalized to 16S rRNA levels showed only small fluctuations during exponential growth for WT mice (a 3.5-fold increase on day 15 relative to day 10) and essentially no change (only a 1.5-fold increase) for IFN-γ $^{-/-}$ mice (Fig. 3C). *pstS1* mRNA levels decreased in growth-arrested cells from WT mouse lungs (22-fold decrease on day 30 relative to

TABLE 1. Sequences of RT primers, PCR primers, and molecular beacons for measurement of bacterial gene expression

| Gene[a] | RT primer[b] | PCR primer[b] | Molecular beacon[c] |
|---|---|---|---|
| *fbpA* (Rv3804c) | GTTGCAGGTCGGGCTTCATAG | GGATCTGGGTGGCAACAACCT | FAM-CGCCGGTCGAGGGCTTC |
| | | TCGAACACGCCGTTGTGG | GTGCGGACCCGGCG-DABCYL |
| *fbpC* (Rv0129c) | TGAGCACATGCTGGATATCGGC | CGCACCAACCAGACCTTC | FAMGCGAGGGTGGACGCAA |
| | | CAGCTGCTCGTTCCAGTAGGG | CGGGGTGCCTCGC-DABCYL |
| *esat-6* (Rv3875) | GCGAACATCCCAGTGACGTT | CGGAGGCGTACCAGGGTGTC | FAM-GCCTCCACGCCACGGCT |
| | | GACCGGCTTCGCTGATCGT | ACCGAGCTGGGAGGC-DABCYL |
| *pstS1* (Rv0934) | TCCGGGCAGGTTGTAGTTGAC | GCTGCTCTACCCGCTGTTC | FAM-CGGACGTTCTGGTGCCGG |
| | | GCCATATCACCTTCCGACAGA | GATCGCCGTCCG-DABCYL |

[a] Genes *fbpA*, *fbpB*, and *fbpC* have high homology in their nucleotide sequences (12). However, there is sufficient nucleotide sequence diversity among the three genes to allow the design of gene-specific RT primers, PCR primers, and molecular beacons to obtain gene-specific measurements. The nucleotide sequences of primers and probes specific for the *M. tuberculosis* 16S rRNA and *fbpB* have been published (31).

[b] RT and PCR primers were designed by using the software Oligo 6.6 (Molecular Biology Insights, Cascade, Colo.) and were purchased from Integrated DNA Technologies (Coralville, Iowa). Molecular beacons, which are hairpin-shaped oligonucleotide probes that become fluorescent upon hybridization to their target sequence (36–38), were synthesized at Biosearch Technologies (Novato, Calif.). Hairpin stability of molecular beacons was estimated by using the DNA-folding program available at http://www.bioinfo.rpi.edu/~zukerm.

[c] FAM; an iodoacetamide derivative of fluorescein (5-iodoactamidofluorescein); dabcyl, 4-(4′-dimethylaminophenylazo)-benzoic acid) succinimidyl ester.

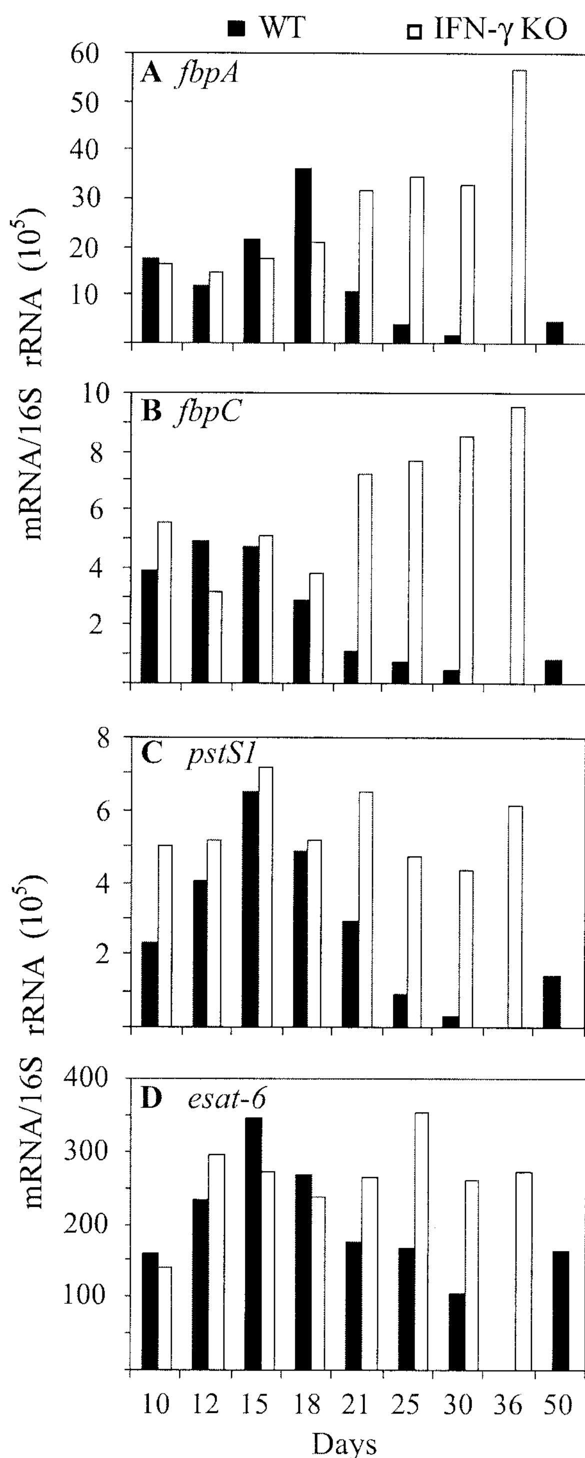


FIG. 3. Numbers of copies of *M. tuberculosis* mRNAs in the lungs of WT and IFN-γ $^{-/-}$ (KO) mice. At each time point and for each mouse strain, normalized mRNA values were obtained by dividing the mean number of mRNA copies per lung (Fig. 2) by the corresponding mean number of 16S RNA copies per lung. Ratios of mRNA to 16S rRNA are shown. Each panel presents results obtained with one gene, as indicated. Normalization of mRNA against CFU gave similar results (data not shown). The difference between day 10 and day 18 of infection for all four genes was not statistically significant ($P > 0.05$ by *t* test). The significance of the slight increase observed on day 50 for all genes needs to be explored by extending the analyses performed in this study to later stages of infection.

day 15) (Fig. 3C), while no decrease was observed at corresponding time points of lung infection in IFN-γ $^{-/-}$ mice (Fig. 3C). The absence of a decrease in *fbp* and *pstS1* transcripts in the lungs of IFN-γ $^{-/-}$ mice provided a strong correlation between down-regulation of these genes and immunity-induced growth arrest.

The numbers of copies of *esat-6* mRNA (Fig. 3D) changed only slightly throughout infection. In the lungs of WT mice, the *esat-6* transcript levels increased only 2-fold on day 15 relative to day 10 and decreased only 3.5-fold on day 30 relative to day 15. No decrease was observed for IFN-γ $^{-/-}$ mice. During exponential growth of *M. tuberculosis* in the lungs of WT mice, the numbers of copies of *esat-6* mRNA were ~10-fold higher than those of *fbpA* mRNA and 60-fold higher than those of *fbpC* and *pstS1* mRNAs. The difference was greater during chronic infection of WT mouse lungs. On day 30, *esat-6* mRNA was ~60-fold more abundant than *fbpA* mRNA, ~210-fold more abundant than *fbpC* mRNA, and ~360-fold more abundant than *pstS1* mRNA.

The data above show that the arrest of *M. tuberculosis* growth caused by expression of adaptive immunity in mouse lung is accompanied by a drastic decrease in the levels of mRNAs encoding the Ag85 complex and the 38-kDa antigen. *esat-6* mRNA levels varied less, but they were 60- to 360-fold higher than levels of the other transcripts in nonreplicating bacilli. We infer that multiplying and nonreplicating tubercle bacilli have different antigen compositions.

The *M. tuberculosis* transcription patterns defined above are consistent with profiles of immune reactivity to the corresponding antigens during human infection. For example, expression of *pstS1* at a low level and only by multiplying bacilli fits well with the antibody response to the 38-kDa lipoprotein in human infection. This antigen reacts with sera from most patients having multibacillary or advanced pulmonary tuberculosis, but it is poorly recognized in sera from patients having low bacillary counts in sputum and from asymptomatic infected persons (for examples, see references 7, 9, 15, 32, and 43). Likewise, antibodies against the Ag85 complex are associated with smear-positive pulmonary disease and correlate with the extent of disease as determined by radiography, while they are absent in patients with past tuberculosis infections, asymptomatic infections, or recent exposures to *M. tuberculosis* (5, 11, 34, 35). The high-level transcription of *esat-6* in both multiplying and nonreplicating tubercle bacilli also fits well with the notion that ESAT-6 induces cellular immune responses in both active disease and in latent infection (3, 13, 27, 39). Interestingly, an inverse correlation is found between bacillary load and ESAT-6-specific immune responses (25, 32). These observations suggest that ESAT-6 may be a dominant inducer of immune responses only during latent infection or primary infection (when bacillary burden is low), because most other antigens, such as the Ag85 complex or the 38-kDa antigen, are either absent, as in nonreplicating bacteria, or present at low levels when multiplying bacteria are in low numbers. It has previously been shown that, in the mouse lung, levels of the *acr* transcript, which encodes α-crystallin (also called 14-kDa or 16-kDa antigen), are increased (>10-fold) in nonreplicating tubercle bacilli relative to that in multiplying bacilli (31). Accordingly, the serologic reactivity of this antigen has a stronger association

with latent infection or recent exposure to *M. tuberculosis* than with active disease (8, 32).

In conclusion, the change-of-growth state of *M. tuberculosis* caused by expression of adaptive immunity in the mouse lung includes changes in bacterial antigen composition. Ongoing studies are working to correlate IFN-γ production, CFU enumeration, and bacterial transcript levels at later times of infection. Establishing the existence of antigen changes during the course of *M. tuberculosis* infection has profound implications for antigen selection in vaccine and immunodiagnostics development and for selection of new drug targets.

We thank Karl Drlica for critical reading of the manuscript.
This work was supported by NIH grants AI-36989 (M.L.G.), AI-37844, and HL-64565 (R.J.N.).

## REFERENCES

1. **Andersen, P.** 1994. The T cell response to secreted antigens of *Mycobacterium tuberculosis*. Immunobiology **191:**537–547.
2. **Anderson, D. H., G. Harth, M. A. Horwitz, and D. Eisenberg.** 2001. An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the *Mycobacterium tuberculosis* 30 kDa major secretory protein (antigen 85B), a mycolyl transferase. J. Mol. Biol. **307:**671–681.
3. **Arend, S. M., P. Andersen, K. E. van Meijgaarden, R. L. Skjot, Y. W. Subronto, J. T. van Dissel, and T. H. Ottenhoff.** 2000. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. J. Infect. Dis. **181:** 1850–1854.
4. **BenAmor, Y., and M. L. Gennaro.** 2002. Genomics as a tool for identifying secreted proteins in bacteria, p. 119–157. *In* A. Danchin (ed.), Genomics of GC rich gram-positive bacteria. Caister Academic Press, Wymondham, United Kingdom.
5. **Benjamin, R. G., S. M. Debanne, Y. Ma, and T. M. Daniel.** 1984. Evaluation of mycobacterial antigens in an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of tuberculosis. J. Med. Microbiol. **18:**309–318.
6. **Bloch, H., and W. Segal.** 1955. Viability and multiplication of vaccines in immunization against tuberculosis. Am. Rev. Tuberc. Pulm. Dis. **71:**228–248.
7. **Bothamley, G. H.** 1995. Serological diagnosis of tuberculosis. Eur. Respir. J. **20**(Suppl. 8)**:**676S–688S.
8. **Bothamley, G. H., J. S. Beck, R. C. Potts, J. M. Grange, T. Kardjito, and J. Ivanyi.** 1992. Specificity of antibodies and tuberculin response after occupational exposure to tuberculosis. J. Infect. Dis. **166:**182–186.
9. **Bothamley, G. H., R. Rudd, F. Festenstein, and J. Ivanyi.** 1992. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. Thorax **47:**270–275.
10. **Brusasca, P. N., R. L. Peters, S. Motzel, H. Klein, and M. L. Gennaro.** 2003. Antigen recognition by serum antibodies in non-human primates experimentally infected with *Mycobacterium tuberculosis*. Comp. Med. **53:**165–172.
11. **Chan, S. L., Z. Reggiardo, T. M. Daniel, D. J. Girling, and D. A. Mitchison.** 1990. Serodiagnosis of tuberculosis using an ELISA with antigen 5 and a hemagglutination assay with glycolipid antigens. Results in patients with newly diagnosed pulmonary tuberculosis ranging in extent of disease from minimal to extensive. Am. Rev. Respir. Dis. **142:**385–389.
12. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, H. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M.-A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
13. **Doherty, T. M., A. Demissie, J. Olobo, D. Wolday, S. Britton, T. Eguale, P. Ravn, and P. Andersen.** 2002. Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. J. Clin. Microbiol. **40:**704–706.
14. **Dunn, P. L., and R. J. North.** 1995. Virulence ranking of some *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect. Immun. **63:**3428–3437.
15. **Espitia, C., I. Cervera, R. Gonzalez, and R. Mancilla.** 1989. A 38-kD *Mycobacterium tuberculosis* antigen associated with infection. Its isolation and serologic evaluation. Clin. Exp. Immunol. **77:**373–377.
16. **Harboe, M.** 1992. The significance of proteins actively secreted by *Mycobacterium tuberculosis* in relation to immunity and complications of mycobacterial diseases. Int. J. Lepr. Other Mycobact. Dis. **60:**470–476.
17. **Harboe, M., and H. G. Wiker.** 1992. The 38-kDa protein of *Mycobacterium tuberculosis*: a review. J. Infect. Dis. **166:**874–884.
18. **Horwitz, M. A., and G. Harth.** 2003. A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect. Immun. **71:**1672–1679.
19. **Jung, Y. J., R. LaCourse, L. Ryan, and R. J. North.** 2002. Virulent but not avirulent *Mycobacterium tuberculosis* can evade the growth inhibitory action of a T helper 1-dependent, nitric oxide synthase 2-independent defense in mice. J. Exp. Med. **196:**991–998.
20. **Lyashchenko, K., R. Colangeli, M. Houde, H. A. Jahdali, D. Menzies, and M. L. Gennaro.** 1998. Heterogeneous antibody responses in tuberculosis. Infect. Immun. **66:**3936–3940.
21. **McKinney, J. D., K. Honer zu Bentrup, E. J. Munoz-Elias, A. Miczak, B. Chen, W. T. Chan, D. Swenson, J. C. Sacchettini, W. R. Jacobs, Jr., and D. G. Russell.** 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature **406:**735–738.
22. **Mogues, T., M. E. Goodrich, L. Ryan, R. LaCourse, and R. J. North.** 2001. The relative importance of T cell subsets in immunity and immunopathology of airborne *Mycobacterium tuberculosis* infection in mice. J. Exp. Med. **193:** 271–280.
23. **Olsen, A. W., and P. Andersen.** 2003. A novel TB vaccine; strategies to combat a complex pathogen. Immunol. Lett. **85:**207–211.
24. **Orme, I. M.** 1988. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance, in mice inoculated with killed mycobacterial vaccines. Infect. Immun. **56:**3310–3312.
25. **Pathan, A. A., K. A. Wilkinson, P. Klenerman, H. McShane, R. N. Davidson, G. Pasvol, A. V. Hill, and A. Lalvani.** 2001. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in *Mycobacterium tuberculosis*-infected individuals: associations with clinical disease state and effect of treatment. J. Immunol. **167:**5217–5225.
26. **Pollock, J. M., and P. Andersen.** 1997. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J. Infect. Dis. **175:**1251–1254.
27. **Ravn, P., A. Demissie, T. Eguale, H. Wondwosson, D. Lein, H. A. Amoudy, A. S. Mustafa, A. K. Jensen, A. Holm, I. Rosenkrands, F. Oftung, J. Olobo, F. von Reyn, and P. Andersen.** 1999. Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*. J. Infect. Dis. **179:** 637–645.
28. **Renshaw, P. S., P. Panagiotidou, A. Whelan, S. V. Gordon, R. G. Hewinson, R. A. Williamson, and M. D. Carr.** 2002. Conclusive evidence that the major T-cell antigens of the *Mycobacterium tuberculosis* complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6/CFP-10 complex. Implications for pathogenesis and virulence. J. Biol. Chem. **277:** 21598–21603.
29. **Ronning, D. R., T. Klabunde, G. S. Besra, V. D. Vissa, J. T. Belisle, and J. C. Sacchettini.** 2000. Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines. Nat. Struct. Biol. **7:**141–146.
30. **Segal, W.** 1984. Growth dynamics of *in vivo* and *in vitro* grown mycobacterial pathogens, p. 547–573. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria. A sourcebook. Marcel Dekker, Inc., New York, N.Y.
31. **Shi, L., Y.-J. Jung, S. Tyagi, M. L. Gennaro, and R. North.** 2003. Expression of Th1-mediated immunity in mouse lung induces a *Mycobacterium tuberculosis* transcription pattern characteristic of nonreplicating persistence. Proc. Natl. Acad. Sci. USA **100:**241–246.
32. **Silva, V. M. C., G. Kanaujia, M. L. Gennaro, and D. Menzies.** 2003. Factors associated with humoral response to ESAT-6, 38kDa and 14kDa antigens in patients with a spectrum of tuberculosis. Int. J. Tub. Lung Dis. **7:**478–484.
33. **Sørensen, A. L., S. Nagai, G. Houen, P. Andersen, and Å. B. Andersen.** 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infect. Immun. **63:**1710–1717.
34. **Stroebel, A. B., T. M. Daniel, J. H. Lau, J. C. Leong, and H. Richardson.** 1982. Serologic diagnosis of bone and joint tuberculosis by an enzyme-linked immunosorbent assay. J. Infect. Dis. **146:**280–283.
35. **Turneer, M., J. P. Van Vooren, J. De Bruyn, E. Serruys, P. Dierckx, and J. C. Yernault.** 1988. Humoral immune response in human tuberculosis: immunoglobulins G, A, and M directed against the purified P32 protein antigen of *Mycobacterium bovis* bacillus Calmette-Guerin. J. Clin. Microbiol. **26:**1714–1719.
36. **Tyagi, S., D. P. Bratu, and F. R. Kramer.** 1998. Multicolor molecular beacons for allele discrimination. Nat. Biotechnol. **16:**49–53.
37. **Tyagi, S., and F. R. Kramer.** 1996. Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. **14:**303–308.
38. **Tyagi, S., S. A. Marras, and F. R. Kramer.** 2000. Wavelength-shifting molecular beacons. Nat. Biotechnol. **18:**1191–1196.
39. **Ulrichs, T., P. Anding, S. Porcelli, S. H. Kaufmann, and M. E. Munk.** 2000.

Increased numbers of ESAT-6- and purified protein derivative-specific gamma interferon-producing cells in subclinical and active tuberculosis infection. Infect. Immun. **68:**6073–6076.
40. **Vyas, N. K., M. N. Vyas, and F. A. Quiocho.** 2003. Crystal structure of *M. tuberculosis* ABC phosphate transport receptor: specificity and charge compensation dominated by ion-dipole interactions. Structure (Camb) **11:**765–774.
41. **Wayne, L. G., and C. D. Sohaskey.** 2001. Nonreplicating persistence of *Mycobacterium tuberculosis*. Annu. Rev. Microbiol. **55:**139–163.
42. **Wiker, H. G., and M. Harboe.** 1992. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. Microbiol Rev. **56:**648–661.
43. **Wilkins, E. G. L.** 1994. The serodiagnosis of tuberculosis, p. 367–380. *In* P. D. O. Davies (ed.), Clinical tuberculosis. Chapman and Hall Medical, London, United Kingdom.

*Editor:* S. H. E. Kaufmann