INFECTION AND IMMUNITY, Aug. 1998, p. 3936–3940 Vol. 66, No. 8
0019-9567/98/$04.00+0

Heterogeneous Antibody Responses in Tuberculosis

KONSTANTIN LYASHCHENKO,[1] ROBERTO COLANGELI,[1] MICHEL HOUDE,[2] HAMDAN AL JAHDALI,[3] DICK MENZIES,[3] AND MARIA LAURA GENNARO[1]*

Public Health Research Institute, New York, New York 10016,[1] and BioChem ImmunoSystems Inc.[2] and Montreal Chest Institute,[3] Montreal, Quebec, Canada

Received 5 March 1998/Returned for modification 14 April 1998/Accepted 30 April 1998

Antibody responses during tuberculosis were analyzed by an enzyme-linked immunosorbent assay with a panel of 10 protein antigens of *Mycobacterium tuberculosis*. It was shown that serum immunoglobulin G antibodies were produced against a variety of *M. tuberculosis* antigens and that the vast majority of sera from tuberculosis patients contained antibodies against one or more *M. tuberculosis* antigens. The number and the species of serologically reactive antigens varied greatly from individual to individual. In a given serum, the level of specific antibodies also varied with the antigen irrespective of the total number of antigens recognized by that particular serum. These findings indicate that person-to-person heterogeneity of antigen recognition, rather than recognition of particular antigens, is a key attribute of the antibody response in tuberculosis.

Tuberculosis (TB) is the leading cause of death from a single infectious agent. Worldwide, one third of the population is infected with *Mycobacterium tuberculosis*; each year, 8 million cases of disease arise, and 3 million people die (4, 17). Immunological research on TB has focused largely on cell-mediated immunity because this part of the immune system mediates acquired resistance to TB (8). Much less effort has been placed on the characterization of the nonprotective, humoral immune response. It has often been suggested but never firmly established that different clinical outcomes are related to the fact that some patients show a stronger cell-mediated response and others show a stronger humoral response (12). It has also been conjectured that antibodies and immune complexes play an immunosuppressive role in TB (7, 23). Clearly, a full understanding of immune responses in TB and the use of such information to develop TB control measures require knowledge of humoral immunity in the infected host.

Most of our current knowledge on humoral immunity in TB derives from serodiagnostic studies. In the search for appropriate antigens, it has been repeatedly observed that single-antigen-based assays never achieve satisfactory serodiagnostic performance (reviewed in references 11 and 29), leading to the view that up to 30% of patients with TB are seronegative (15). However, complex antibody binding patterns are usually observed with immunoblot analyses of sera from TB patients (28). Thus, it has not been clear whether the poor performance of serodiagnostic assays reflects a lack of antibody responses in a large number of patients or a lack of appropriate reagents to measure the responses. An answer to this question requires the availability of a broad set of serologically active *M. tuberculosis* antigens.

We looked for antigens that elicit antibody responses in TB by focusing on the extracellular proteins of *M. tuberculosis* (operationally referred to as culture filtrate proteins), since these proteins are known to induce strong immune responses in TB (reviewed in references 1 and 10). Using a panel of 10 culture filtrate proteins purified from recombinant *Escherichia coli*, we found that person-to-person heterogeneity of antigen recognition, rather than recognition of particular antigens, is the signature of humoral immunity in TB.

MATERIALS AND METHODS

Antigens. Ten genes encoding *M. tuberculosis* culture filtrate proteins (Table 1) were cloned in the pQE30 (Qiagen) plasmid vector of *E. coli* as described earlier (19, 20). Recombinant proteins were expressed as NH_2-terminally polyhistidine-tagged fusion proteins and purified from *E. coli* cells to near homogeneity by sequential chromatography with metal chelate affinity, size exclusion, and anion-exchange columns (9).

Sera. Sera were obtained from 139 individuals as follows. Fifty-nine serum samples were collected in the first month of antitubercular chemotherapy from human immunodeficiency virus-negative patients with active pulmonary TB. For 51 of the patients, the diagnosis of TB was microbiologically confirmed by sputum smear microscopy and/or culturing. For the remaining eight patients, the diagnosis of TB was made on the basis of reactivity to the tuberculin skin test, clinical and radiological findings, and response to antitubercular chemotherapy determined with chest X-ray films taken 3 months apart and judged in a blind fashion by reviewers. Eighty control serum samples were obtained from 34 healthy blood donors, from 40 patients with pulmonary disease other than TB, and from 6 patients infected with nontuberculous mycobacteria. Of these 80 individuals, 20 were tuberculin skin test reactive and 24 were skin test negative, and for the remaining 36, skin reactivity to tuberculin was unknown. Of these 80 individuals, 29 had been vaccinated with BCG and 20 had not, and for the remaining 31, BCG vaccination status was unknown.

ELISA. For the enzyme-linked immunosorbent assay (ELISA), polystyrene 96-well microtiter plates were coated overnight with antigen at 1.0 μg/ml (0.1 ml per well) or, for the 38-kDa protein, at 0.1 μg/ml, in carbonate-bicarbonate buffer (pH 9.6). Plates were blocked with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T) for 2 h at 37°C and washed extensively with PBS-T. Serum samples were diluted 1:20 in sample diluent (Biochem ImmunoSystems, Montreal, Quebec, Canada), and 0.1 ml of diluted serum was added to antigen-coated wells in duplicate and incubated for 30 min at room temperature. Plates were washed extensively with PBS-T and then incubated for 30 min with 0.1 ml of goat anti-human immunoglobulin G (IgG) labelled with horseradish peroxidase (Tago Inc., Burlingame, Calif.) diluted 1:25,000 in conjugate diluent (Biochem ImmunoSystems) per well. Plates were washed with PBS-T, and 0.1 ml of tetramethylbenzidine substrate (Biochem ImmunoSystems) was added to each well. After the addition of 0.1 ml of 1 N H_2SO_4 to stop the reaction, the optical density at 450 nm (OD_{450}) was measured with an automatic microplate reader (Spectra Shell; Tecan).

Data analysis. For evaluation of antibody responses, cutoff values were calculated for each antigen as the means of OD_{450} values obtained with the sera from 34 healthy donors plus 3 or 6 standard deviations (SD).

RESULTS

We characterized the humoral response during TB by measuring with an ELISA serum IgG antibodies to 10 antigens of *M. tuberculosis* in 59 patients with active TB and 80 control individuals (healthy blood donors, patients with pulmonary pathology other than TB, and patients with non-TB mycobac-

* Corresponding author. Mailing address: Public Health Research Institute, 455 First Ave., New York, NY 10016. Phone: (212) 578-0844. Fax: (212) 578-0804. E-mail: gennaro@phri.nyu.edu.

TABLE 1. Antigens of *M. tuberculosis* used in this study

Antigen	Molecular mass (kDa)	Reference
ESAT-6	6	26
14 kDa	14	27
MPT63	16	20
19 kDa	19	3
MPT64	23	30
MPT51	27	22
MTC28	28	19
Ag85B	30	21
38 kDa	38	2
KatG	80	13

terioses). Antibody responses to each antigen were analyzed with cutoff values equal to the means of OD_{450} readings obtained with sera from 34 healthy individuals plus 3 SD (Fig. 1). In the control group ($N = 80$), the number of sera showing IgG binding to *M. tuberculosis* antigens at or above cutoff values ranged from 1 to 3 per antigen. Thus, the chosen cutoff values were appropriate to evaluate specific antibody responses in TB.

With the criteria outlined above, analyses of antibody responses to 10 antigens of *M. tuberculosis* gave the following results.

Antibody responses to the antigen panel. A total of 88% of sera (52 of 59) from TB patients contained antibodies against at least one antigen (data sorted by antigen are shown in Fig. 1). This observation indicates that, when appropriate reagents are used, specific antibody responses to antigens of *M. tuberculosis* can be measured in the vast majority of individuals with active TB.

TABLE 2. Antigen recognition by serum IgG antibodies in TB patients

Antigen	No. (%) of responders		% of high-level responders among total responders
	Total[a]	High level[b]	
ESAT-6	12 (20)	9 (15)	75
14 kDa	26 (44)	20 (34)	73
MPT63	9 (15)	7 (12)	78
19 kDa	23 (39)	10 (17)	50
MPT64	7 (12)	4 (7)	57
MPT51	5 (8)	0 (0)	0
MTC28	16 (27)	7 (12)	38
Ag85B	10 (17)	2 (3)	20
38 kDa	15 (25)	8 (14)	53
KatG	8 (14)	2 (3)	25

[a] TB patients having antibody levels greater than or equal to the mean OD_{450} plus 3 SD, obtained with negative control sera.
[b] TB patients having antibody levels greater than or equal to the mean OD_{450} plus 6 SD, obtained with negative control sera.

Serological reactivity of antigens of *M. tuberculosis*. ELISA measurements of serum antibodies against each of 10 antigens are shown in Fig. 1. The number of serum samples reacting to each antigen with antibody levels greater than or equal to cutoff values (mean OD_{450} of negative control sera plus 3 SD) is presented in Table 2. Antibodies against two antigens (14- and 19-kDa proteins) were detected in more than one third of TB patients (44 and 39%, respectively). Two other antigens (38-kDa protein and MTC28) were recognized by antibodies in approximately one fourth (25 to 27%) of TB patients. Five additional antigens (MPT63, MPT64, KatG, Ag85B, and ESAT-6) elicited antibody responses in a smaller proportion (12 to 20%) of TB patients. While the serological reactivities of

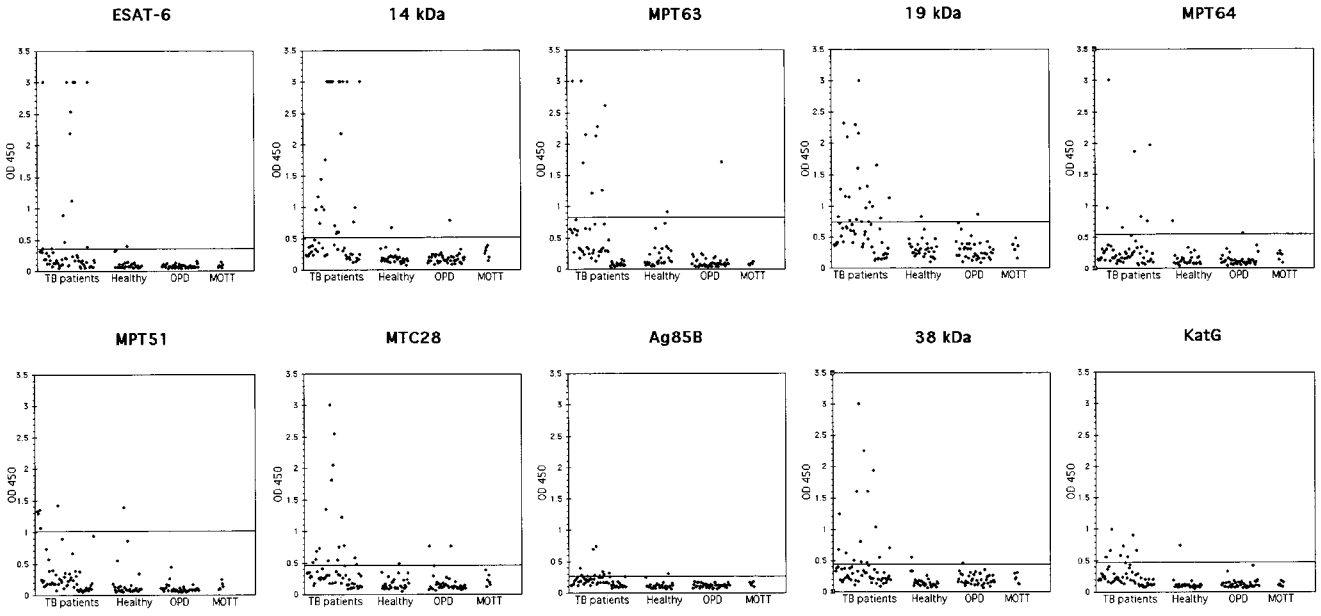

FIG. 1. Antibody responses to protein antigens of *M. tuberculosis*. Levels of serum IgG antibodies to 10 purified antigens of *M. tuberculosis* in TB patients, healthy blood donors (Healthy), patients with pulmonary pathology other than TB (OPD), and patients with mycobacterioses other than TB (MOTT) are shown. Each point represents one serum sample tested by an ELISA. The horizontal bar in each panel denotes the cutoff value determined as mean OD_{450} plus 3 SD by use of negative control sera (see Materials and Methods). An OD_{450} of 3.00 was the highest value obtained under the experimental conditions used. Mean OD_{450} (SD) used for cutoff determinations were as follows: ESAT-6, 0.104 (0.085); 14 kDa, 0.192 (0.109); MPT63, 0.210 (0.209); 19 kDa, 0.318 (0.141); MPT64, 0.140 (0.134); MPT51, 0.180 (0.276); MTC28, 0.166 (0.100); Ag85B, 0.112 (0.051); 38 kDa, 0.157 (0.095); and KatG, 0.119 (0.118). Of 59 sera from TB patients, 52 contained antibodies to at least one antigen. Of the seven nonreactive sera, one serum was sputum smear positive and six sera were sputum smear negative.

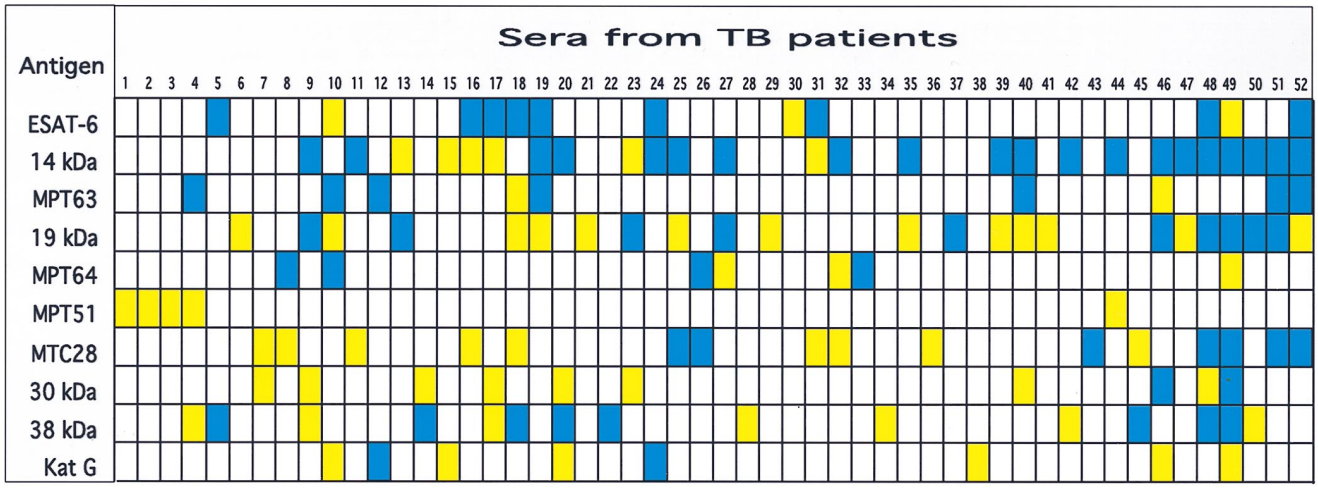

FIG. 2. Heterogeneous antigen recognition by serum IgG antibodies in TB. Each lane represents one serum sample tested by an ELISA. Data for 52 antibody reactors among 59 TB patients are shown. Sera: 1 to 22, sputum smear positive for TB; 23 to 46, sputum smear negative for TB; 47 to 52, culture positive for TB (sputum smear information was not available). Cutoff values were calculated for each antigen as indicated in the text. Yellow boxes indicate OD_{450} values greater than or equal to the mean OD_{450} plus 3 SD, obtained with negative control sera. Cyan boxes indicate OD_{450} values greater than or equal to the mean OD_{450} plus 6 SD, obtained with negative control sera. 30 kDa, Ag85B.

several antigens in our panel (in particular, Ag85B and the 38-, 14-, and 19-kDa proteins) have been well established (reviewed in reference 29), this is the first report of the involvement of MPT63, MTC28, and ESAT-6 in antibody responses in human TB. Seroreactivity to another antigen, MPT51, did not appear to differ significantly between TB patients and control individuals.

The serological reactivity of a given antigen could be evaluated not only by calculating the number of antibody responders among TB patients but also by determining the proportion of high-level responses among sera reacting with that antigen. For example, ESAT-6 and Ag85B reacted with similar numbers of sera from TB patients (12 and 10, respectively) (Table 2). However, even a visual comparison of the data obtained with these two antigens (Fig. 1) indicated that antibody responses to ESAT-6 were much stronger than those to Ag85B. To analyze levels of antibody responses, we counted the number of highly reactive sera (antibody levels greater than or equal to the mean OD_{450} of negative control sera plus 6 SD) (Table 2) and then calculated the proportion of high-level antibody responses among responders to each antigen (Table 2). Some antigens (14-kDa protein, MPT63, and ESAT-6) elicited high-level antibody responses in the majority (>70%) of responders to those antigens. Others (38- and 19-kDa proteins and MPT64) elicited high-level antibody responses in approximately half of the responders (50 to 57%). MTC28 elicited strong humoral responses in only 38% of the responders. Antibody levels against Ag85B and KatG were significantly lower than those against other antigens, with 25% or fewer responders having high-level antibody responses against either antigen.

Taken together, analyses of antibody responses to each antigen showed that at least 9 of 10 antigens in our panel reacted with sera from TB patients, indicating that multiple antigens of *M. tuberculosis* are targets of antibody responses to TB. The serological reactivity of a given antigen could be evaluated not only by calculating the number of antibody responders among TB patients but also by determining the proportion of high-level responses among sera reacting with that antigen. These two components of antigen seroreactivity often failed to correlate, suggesting that serological evaluation of antigens must be based on multiple parameters.

Heterogeneity of antigen recognition. Analyses of antigen recognition by individual sera revealed that the pattern of antigens reacting with serum antibodies varied greatly from patient to patient (data sorted by sera are shown in Fig. 2). No single antigen or group of antigens was reactive with all (or even with most) sera from TB patients. The number of antigens recognized also varied markedly, from one (in 16 of 59 sera) to eight (in 1 of 59 sera). An additional element of heterogeneity was that antibody levels to a certain antigen were higher in some responders than in others, irrespective of the total number of antigens recognized. For example, the level of anti-19-kDa antibodies was higher in serum sample 13 (OD_{450}, 2.095) than in serum sample 19 (OD_{450}, 1.052) (Fig. 2), even though serum sample 19 reacted with four antigens and serum sample 13 reacted with only two. Likewise, the level of anti-MPT64 antibodies was higher in serum sample 33 (OD_{450}, 1.960), which reacted only with MPT64, than in serum sample 49 (OD_{450}, 0.644), which reacted with eight antigens (Fig. 2). Thus, in a given serum, the levels of antibodies to one or more antigens did not correlate with the number of antigens targeted. As a result, antigen recognition was heterogeneous at each of the antibody levels analyzed (Fig. 2).

DISCUSSION

The findings described above indicate that serum antibodies are produced against a variety of *M. tuberculosis* antigens in most patients with active TB. The number and type of serologically reactive antigens vary greatly from individual to individual. In a given serum, the level of specific antibodies also varies with the antigen irrespective of the total number of antigens recognized by that particular serum. Thus, the present work establishes that person-to-person variation of antigen recognition, rather than recognition of particular antigens, is a key attribute of humoral immunity in human TB.

Heterogeneous recognition of antigens by serum antibodies in TB probably results from multiple factors. One is the im-

munogenetic background of the infected host. Different patterns of antibody binding to *M. tuberculosis* antigens have been revealed by immunoblot analysis of sera from different mouse strains immunized with BCG (14). For humans, an association between antibody titers against particular *M. tuberculosis*-specific epitopes and certain HLA alleles has been described (6). A second factor in heterogeneous antigen recognition may be the production of different mycobacterial antigens at different stages of TB. Analysis of immune responses in human TB is usually a single snapshot taken for each individual, with the stage of disease varying from individual to individual. The intriguing possibility that antigen recognition varies with stage of disease (also suggested in earlier work [15, 16]) requires rigorous testing with animal models of TB. A third determinant of heterogeneous antigen recognition may be differential gene expression by different strains of *M. tuberculosis* as they cause disease in different patients. We do not favor this explanation, because sera from animals experimentally infected with a single strain (guinea pigs aerosol infected with *M. tuberculosis* $H_{37}Rv$ and cattle infected intratracheally with *Mycobacterium bovis* BM228) also recognized different patterns of mycobacterial antigens in our panel (unpublished observations). A fourth factor in heterogeneous antigen recognition may be bacillary load in the sputum. In the present study, only the 38-kDa antigen reacted more frequently with sera from sputum-smear-positive TB patients (36%; 8 of 22) than from sputum-smear-negative TB patients (16%; 4 of 24), in agreement with the results of previous studies with this antigen (reviewed in reference 29). A correlation between sputum bacillary load and antigen recognition was not observed for any other antigen in our panel. However, definitive information on this question requires sera from a larger cohort of TB patients. A fifth factor to consider is antituberculosis therapy. However, antigen recognition by sera taken prior to therapy was also highly heterogeneous (some examples are serum samples 27 to 35 in Fig. 2). Thus, antituberculosis therapy is unlikely to be an important factor in diverse antigen recognition.

Heterogeneous antigen recognition during TB is presumably not unique to humoral immunity. Since IgG antibody responses against proteins are T-cell dependent, heterogeneous antigen recognition by serum antibodies implies that helper T cells also recognize different antigens in different individuals. Indeed, highly diverse patterns of recognition of multiple antigens of *M. tuberculosis* by T cells from TB patients have been described (5, 25).

Heterogeneity of antigen recognition by serum antibodies during TB explains the failure to detect specific antibody responses in up to 30% of TB patients when only a few purified antigens of *M. tuberculosis* were used (15). The present work shows that specific antibodies to at least one of a broad set of serologically reactive antigens of *M. tuberculosis* can be measured in almost 90% of TB patients. The use of additional seroreactive antigens of *M. tuberculosis* should lead to the detection of specific antibodies in almost all patients with active TB. Thus, an important practical implication of patient-to-patient variation in antigen recognition is that serodiagnostic assays for TB must be based on a rational design of antigen combinations to achieve high diagnostic accuracy.

Heterogeneous immune responses may be common to infection by a variety of pathogens that rely on an array of parasite-produced factors. For example, immunoblot analyses of serum antibody reactivities in patients with visceral leishmaniasis revealed different antibody binding patterns (24) suggestive of diverse antigen recognition. The complex life cycle of *Plasmodium* spp., the causative agents of malaria, is associated with the expression of different antigens at different phases of infection, presumably inducing highly diverse immune responses (18). Thus, the heterogeneity of antigen recognition in humoral immunity established above for TB may also be characteristic of other important infectious diseases.

ACKNOWLEDGMENTS

We thank Clemence Desputeau and Stephane Francoeur for excellent technical assistance. We also thank Tom Daniel, Karl Drlica, Sam Kayman, and Carol Lusty for comments on the manuscript.

This work was supported in part by NIH grant AI-36896 (to M.L.G.). R.C. was the recipient of an AIDS training fellowship from the Istituto Superiore di Sanitá, Rome, Italy.

REFERENCES

1. **Andersen, Å. B., and P. Brennan.** 1994. Proteins and antigens of *Mycobacterium tuberculosis*, p. 307–327. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. ASM Press, Washington, D.C.
2. **Andersen, Å. B., and E. B. Hansen.** 1989. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. **57:**2481–2488.
3. **Ashbridge, K. R. A., R. J. Booth, J. D. Watson, and R. B. Lathigra.** 1989. Nucleotide sequence of the 19kDa antigen gene from *Mycobacterium tuberculosis*. Nucleic Acids Res. **17:**1249.
4. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
5. **Boom, W. H., R. S. Wallis, and K. A. Chervenak.** 1991. Human *Mycobacterium tuberculosis*-reactive $CD4^+$ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. Infect. Immun. **59:**2737–2743.
6. **Bothamley, G. H., J. S. Beck, G. M. T. Schreuder, J. D'Amaro, R. R. P. de Vries, T. Kardjito, and J. Ivanyi.** 1989. Association of tuberculosis and *M. tuberculosis*-specific antibody levels with HLA. J. Infect. Dis. **159:**549–555.
7. **Campa, M., P. Marelli, and G. Benedettini.** 1986. B and T lymphocytes regulated by idiotype anti-idiotype interactions inhibit delayed-type hypersensitivity to BCG in mice. Cell. Immunol. **98:**93–103.
8. **Chan, J., and S. H. E. Kaufmann.** 1994. Immune mechanisms of protection, p. 389–416. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. ASM Press, Washington, D.C.
9. **Colangeli, R., A. Heijbel, A. Williams, C. Manca, J. Chan, K. Lyashchenko, and M. L. Gennaro.** Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of *Mycobacterium tuberculosis*. J. Chromatogr., in press.
10. **Cooper, A. M., and J. L. Flynn.** 1995. The protective immune response to *Mycobacterium tuberculosis*. Curr. Opin. Immunol. **7:**512–516.
11. **Daniel, T. M.** 1988. Antibody and antigen detection for the immunodiagnosis of tuberculosis: why not? what more is needed? where do we stand today? J. Infect. Dis. **158:**678–680.
12. **Daniel, T. M., M. J. Oxtoby, E. Pinto, and E. Moreno.** 1981. The immune spectrum in patients with pulmonary tuberculosis. Am. Rev. Respir. Dis. **123:**556–559.
13. **Heym, B., Y. Zhang, S. Pulet, D. Young, and S. T. Cole.** 1993. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. J. Bacteriol. **175:**4255–4259.
14. **Huygen, K., L. Ljungqvist, R. T. Berg, and J.-P. V. Vooren.** 1990. Repertoire of antibodies to culture filtrate antigens in different mouse strains infected with *Mycobacterium bovis* BCG. Infect. Immun. **58:**2192–2197.
15. **Ivanyi, J., G. H. Bothamley, and P. S. Jackett.** 1988. Immunodiagnostic assays for tuberculosis and leprosy. Br. Med. Bull. **44:**635–649.
16. **Jackett, P. S., G. H. Bothamley, H. V. Batra, A. Mistry, D. B. Young, and J. Ivanyi.** 1988. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. J. Clin. Microbiol. **26:**2313–2318.
17. **Kochi, A.** 1991. The global tuberculosis situation and the new control strategy of the World Health Organization. Tubercle **72:**1–6.
18. **Maddison, S. E.** 1991. Serodiagnosis of parasitic diseases. Clin. Microbiol. Rev. **4:**457–469.
19. **Manca, C., K. Lyashchenko, R. Colangeli, and M. L. Gennaro.** 1997. MTC28, a novel 28-kilodalton proline-rich secreted antigen specific for the *Mycobacterium tuberculosis* complex. Infect. Immun. **65:**4951–4957.
20. **Manca, C., K. Lyashchenko, H. G. Wiker, D. Usai, R. Colangeli, and M. L. Gennaro.** 1997. Molecular cloning, purification, and serological characterization of MPT63, a novel secreted antigen of *Mycobacterium tuberculosis*. Infect. Immun. **65:**16–23.
21. **Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada.** 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α-antigen. J. Bacteriol. **170:**3847–3854.
22. **Ohara, N., H. Kitaura, H. Hotokezaka, T. Nishiyama, N. Wada, S. Matsumoto, T. Matsuo, M. Naito, and T. Yamada.** 1995. Characterization of the gene encoding MPB51, one of the major secreted protein antigens of *My-*

cobacterium bovis BCG, and identification of the secreted protein closely related to the fibronectin binding 85 complex. Scand. J. Immunol. **41:**433–442.
23. **Orme, I. M., and D. N. McMurray.** 1996. The immune response to tuberculosis in animal models, p. 269–280. *In* W. N. Rom and S. M. Garay (ed.), Tuberculosis. Little, Brown & Co., New York, N.Y.
24. **Santos, J. I. D., M. G. Morgado, and B. Galvão-Castro.** 1987. Human visceral leishmaniasis: analysis of the specificity of humoral immune response to polypeptides of *Leishmania donovani chagasi*. Am. J. Trop. Med. Hyg. **37:** 263–270.
25. **Schoel, B., H. Gulle, and S. H. E. Kaufmann.** 1992. Heterogeneity of the repertoire of T cells of tuberculosis patients and healthy contacts to *Mycobacterium tuberculosis* antigens separated by high-resolution techniques. Infect. Immun. **60:**1717–1720.
26. **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.
27. **Verbon, A., R. A. Hartskeerl, A. Schuitema, A. H. Kolk, D. B. Young, and R. Lathigra.** 1992. The 14,000-molecular-weight antigen of *Mycobacterium tuberculosis* is related to the alpha-crystallin family of low-molecular-weight heat shock proteins. J. Bacteriol. **174:**1352–1359.
28. **Verbon, A., S. Kuijper, H. M. Jansen, P. Speelman, and A. H. J. Kolk.** 1990. Antigens in culture supernatant of *Mycobacterium tuberculosis*: epitopes defined by monoclonal and human antibodies. J. Gen. Microbiol. **136:**955–964.
29. **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, England.
30. **Yamaguchi, R., K. Matsuo, A. Yamazaki, C. Abe, S. Nagai, K. Teresaka, and T. Yamada.** 1989. Cloning and characterization of the gene for immunogenic protein MPB64 of *Mycobacterium bovis* BCG. Infect. Immun. **57:**283–288.

Editor: S. H. E. Kaufmann