CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2008, p. 579–581 Vol. 15, No. 3
1556-6811/08/$08.00+0 doi:10.1128/CVI.00359-07

Humoral Immune Responses of Tuberculosis Patients in Brazil Indicate Recognition of *Mycobacterium tuberculosis* MPT-51 and GlcB▽

Cristina Melo Cardoso Almeida,[1] Arioldo C. Vasconcelos, Jr.,[1] André Kipnis,[2] Ana Lúcia Andrade,[3] and Ana Paula Junqueira-Kipnis[1]*

Departamento de Microbiologia, Imunologia, Parasitologia e Patologia, Laboratório de Imunopatologia das Doenças Infecciosas,[1] Laboratório de Bacteriologia Molecular,[2] and Departamento de Saúde Coletiva,[3] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia-Go, Brasil[3]

Received 3 September 2007/Returned for modification 2 October 2007/Accepted 8 January 2008

The humoral responses to recombinant MPT-51 and GlcB was determined by using an enzyme-linked immunosorbent assay. Levels of immunoglobulin M (IgM) against MPT-51 and IgG against GlcB were higher among tuberculosis (TB) patients than among control individuals. When the MPT-51 and GlcB assays were combined, 90.8% specificity and 75.5% sensitivity were observed. MPT-51 and GlcB were recognized in the humoral responses of Brazilian TB patients.

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis*, is responsible for 2 million deaths per year in the world. Thirty percent of the world population is infected by these bacilli and therefore is at risk of developing tuberculosis, and around 9 million new cases of TB are reported each year (29). Some proteins from *M. tuberculosis* have been shown to be recognized by the immune systems of TB patients and can be correlated with proven human *M. tuberculosis* infection or disease (3–5, 9–12). Among those proteins, we selected MPT-51 (Rv3803c) and GlcB (Rv 1837c), which have previously been demonstrated to be recognized by the humoral immune response in active and subclinical tuberculosis (9, 10, 19). The MPT-51 antigen is a protein of 27 kDa, encoded by the *fbpC1* gene, with 40% homology to Ag85 complex components. It is a new family of noncatalytic α/β-hydrolases, with the ability to bind fibronectin (26, 27). GlcB is an *M. tuberculosis* malate synthase that takes part in the glyoxylate shunt and has been implicated as a virulence factor. The glyoxylate bypass is believed to be important for *M. tuberculosis* survival under adverse conditions, such as low oxygen, nonreplicative states, and the intracellular environment (14, 17).

Regarding active TB, previous reports have demonstrated a humoral immune response for Indian TB human immunodeficiency virus (HIV)-positive patients against the MPT-51 and GlcB antigens; however, this was not observed for all studied individuals (2, 20, 21). Whether these antigens can be recognized in a different setting by immune systems of patients infected with potentially diverse *M. tuberculosis* strains needs to be investigated. These observations prompted us to explore the humoral immune response against the MPT-51 and GlcB antigens in a Brazilian setting. Brazil is among the 22 countries that account for 80% of the world's TB burden. In 2005, Goiânia, in central Brazil, reported 211 new cases, corresponding to an incidence rate of 18/100,000 (8). In this area of endemicity, we investigated the recognition of MPT-51 and GlcB by the humoral immune systems of HIV-negative TB patients.

Patients were recruited from the two reference public health care centers at the municipality of Goiânia. Based on the sensitivity of the gold standard test (bacillus culture), which varies from 30 to 80%, the sample size for the enzyme-linked immunosorbent assays (ELISAs) was calculated to allow detection with a sensitivity of 65% and a 95% confidence interval of 52 to 78%. Based on the incidence of new TB cases in Goiania, 49 patients with active pulmonary TB who were consecutively treated at the TB reference centers were enrolled in this study, corresponding to 23% of the new cases detected in 2005. For comparison purpose, two sex- and age-matched (±6 years) control groups were included, comprising 49 tuberculin-skin-test-negative healthy individuals and 49 individuals with lepromatous leprosy (LL). The latter were selected based on the higher incidence of leprosy in the area (28). The inclusion criteria for active pulmonary TB were clinical manifestations of tuberculosis with an acid-fast smear and positive culture. LL patients were included in this study based on their clinical symptoms. It excluded individuals who were under 18 years old, pregnant, had any chronic disease, or were immunosuppressed (including having HIV-positive status). This work was approved by the Regional Ethical Committee Board of the Federal University of Goiás. Five milliliters of blood were collected from each subject at the time of enrollment, and the serum was stored at −20°C and thawed only once at the time of the serology assays.

The purified recombinant antigens MPT-51 (rMPT-51) and GlcB (rGlcB), based on the *M. tuberculosis* genome sequence, were kindly provided by John Belisle from Colorado State University (NIH, contract NO1-AI-75320). Serology testing was performed using an ELISA with some modifications (15). Briefly, microtiter plates were coated with the rMPT-51 or rGlcB antigen at 2.5 μg/ml in 0.015 M carbonate buffer, pH 9.6. Blocking was done with buffered carbonate-bicarbonate (pH 9.6)–skim milk (1%), and serum samples were diluted to

* Corresponding author. Mailing address: Rua Delenda Rezende de Melo S/N, Setor Universitário, 74605-050 Goiânia-Goiás, Brazil. Phone: 55 11 62 32096126. Fax: 55 11 62 35211839. E-mail: anapaula@iptsp.ufg.br.

▽ Published ahead of print on 16 January 2008.

TABLE 1. Characteristics of study participants

Variable[a]	Value for group		
	Healthy controls	Active TB patients[b]	Lepromatous leprosy patients
Total no. of subjects	49	49	49
Male/female	39/10	39/10	39/10
Age (yr)			
Mean	42	42	44
Range	18–68	20–74	19–72
BCG (vaccinated/without BCG scar)	38/11	38/11	30/19
PPD (positive/negative/NA)	0/49/0	24/8/17	0/0/49
No. of samples obtained prior to TB treatment		35	
No. of samples obtained after TB treatment		14	
Pulmonary lesions (cavitary/noncavitary/NA)[c]		8/40/1	

[a] PPD, purified protein derivative; NA, not available.
[b] Patients with tuberculosis had acid-fast bacillus-positive sputum smears and positive culture.
[c] Radiographic finding on chest X ray.

1/1,000 in phosphate-buffered saline–Tween 20. Peroxidase-labeled anti-human immunoglobulin M (IgM) (1/15,000; IgM-horseradish peroxidase; ZYMED Laboratories) or anti-human IgG conjugate (1/15,000; IgG-horseradish peroxidase; Bio-Rad) was used. The substrate solution consisted of citrate phosphate buffer (pH 5.1), *o*-phenylenediamine (1 μg/ml; Sigma), and hydrogen peroxide. Sulfuric acid (4 N) was used to stop the reaction, and the optical density (OD) was measured at 492 nm. The cutoff was determined by the receiver operating characteristic (ROC) curve, using a sensitivity of 76%. Student's *t* test was performed for continuous variables, and the chi-square test (or the Fisher test when applicable) was used for dichotomous variables. One-way analysis of variance was applied to compare the variation in ODs from different groups. Differences were considered significant at a P value of <0.05. A ROC curve was performed, and for the MPT-51 ELISA, IgM presented an area (A) of 0.814; for IgG, the A value was 0.628. The area under the curve for the GlcB ELISA was as follows: for IgM, $A = 0.636$; for IgG, $A = 0.738$. The analysis was performed using the SPSS version 11.0 and EpiInfo 6.04 software programs.

Characteristics of the 147 enrolled individuals are shown in Table 1. The majority of the TB patients were male, with ages ranging from 20 to 74 years old. From a total of 49 TB patients, 3 presented pleural infection while 46 had pulmonary TB. Most of the TB patients ($n = 33$) had been recently diagnosed, and their blood was collected before the onset of chemotherapy. The control groups, composed of healthy individuals and LL patients, were age and sex matched with the TB group. TB patients presented higher levels of IgM against rMPT-51 (mean OD ± standard deviation [SD], 0.81 ± 0.32) than healthy individuals (0.45 ± 0.16) and LL patients (0.45 ± 0.19) ($P < 0.001$). The MPT-51 IgM ELISA test showed a sensitivity of 77.6%, while the specificity was 76.5% (OD cutoff = 0.560). No difference was observed when the TB group was compared to healthy individuals for IgG against MPT-51, and therefore, the scores obtained with the ROC curve were not used. Analysis of the humoral immune response to rGlcB revealed higher serum IgM levels for TB patients than for the control groups (OD ± SD for the TB group = 0.52 ± 0.20; for healthy controls, OD = 0.44 ± 0.14; for the LL group, OD = 0.43 ± 0.14) ($P < 0.05$). The same pattern was observed when IgG levels against rGlcB were analyzed (OD ± SD for the TB group = 1.22 ± 0.46; for healthy controls, OD = 0.92 ± 0.30; for the LL group, OD = 0.79 ± 0.39) ($P < 0.001$). After a ROC curve development, it was determined that the IgG-GlcB assay had a sensitivity of 75.5% with a specificity was 64.3%, given an OD cutoff of 0.876 (Table 2). Using the cutoff established by the ROC curve, the combined sensitivity and specificity using the MPT-51 and GlcB antigens were superior, 75.5% and 90.8%, respectively. Because of the higher OD values for the control groups and a possible cross-reactivity with environmental mycobacteria, the serum samples were preabsorbed for 1 h at 37°C with *Mycobacterium abscessus* crude protein extract before the ELISA test was performed (data not shown) (24).

Identification of *M. tuberculosis* proteins that induce anti-

TABLE 2. Sensitivities and specificities of the ELISA test for TB diagnosis

ELISA	OD cutoff	No. of patients positive/no. tested		Sensitivity (%)	Specificity (%)
		TB patients[a]	Control groups[b]		
MPT-51 (IgM)	0.560	33/49	3/98	77.6	96.9
MPT-51 (IgG)	1.140	2/49	2/98	4.1	98.0
GlcB (IgM)	0.730	4/49	4/98	8.2	95.9
GlcB (IgG)	0.876	9/49	1/98	75.5	65
MPT-51 (IgM) + GlcB (IgG)	—[c]	37/49	4/98	75.5	95.9
MPT-51 (IgM + IgG) + GlcB (IgM + IgG)	—	38/49	9/98	77.5	90.8

[a] TB patient diagnosis based on *M. tuberculosis* culture and smear positivity.
[b] Controls groups include healthy individuals and leprosy patients.
[c] —, combined cutoff.

body production can help the development of new serum-based diagnostic tests, such as ELISA, which is widely employed (1, 6, 22). However, several problems with specificity and sensitivity have been encountered, limiting its use in areas to which TB is endemic (7, 18, 23, 25). Although the sensitivity of the ELISA for IgM against MPT-51 found in our study was only 77.6%, this was higher than those in other studies with similar population groups, despite the results reported by Steingart et al. (22) and Bethunaickan et al. (6) that combining Igs demonstrated a better performance with HIV-positive TB patients. Although a significant difference was observed when rGlcB was used to discriminate TB patient from other groups (healthy controls and LL group), the assays showed lower sensitivity than that obtained using MPT-51. Other studies evaluating the specific humoral response involving the GlcB antigen also showed higher levels of specific antibodies for TB patients than for healthy tuberculin-skin-test-positive and -negative individuals, although a higher sensitivity was observed in those studies (2, 20).

Some authors suggest the use of more than one antigen in the serum diagnostic assay to elevate the specificity and sensitivity of the reaction (16, 25). Combining the results for use of IgM and IgG against MPT-51 and IgM and IgG against GlcB, the specificity of our results presented a substantial increase, changing from 64.3 to 76.5% to 90.8%. Our results suggest that rMPT-51 and GlcB should be better exploited in their role as candidates for a serological diagnostic assay.

Our study enrolled 23% of the new TB cases confirmed by sputum smear microscopy to be acid fast positive and *M. tuberculosis* culture positive in the year of the study in Goiânia (49 out of 211 new TB cases) (8). Still, our work cannot assure that these results will be comparable when all clinical forms of TB are analyzed. To our knowledge, these results are the first to show that MPT-51 and GlcB are immunogenic in a Brazilian area to which TB is endemic. In conclusion, this work demonstrates that rMPT-51 and GlcB are recognized in immune responses of Brazilian TB patients and could be exploited as a component for a TB diagnosis test.

This work was supported by the National Counsel of Technological and Scientific Development (CNPq).

REFERENCES

1. **Abebe, F., C. Holm-Hansen, H. G. Wiker, and G. Bjune.** 2007. Progress in serodiagnosis of *Mycobacterium tuberculosis* infection. Scand. J. Immunol. **66:**176–191.
2. **Achkar, J. M., Y. Dong, R. S. Holzman, J. Belisle, I. S. Kourbeti, T. Sherpa, R. Condos, W. N. Rom, and S. Laal.** 2006. *Mycobacterium tuberculosis* malate synthase- and MPT51-based serodiagnostic assay as an adjunct to rapid identification of pulmonary tuberculosis. Clin. Vaccine Immunol. **113:**1291–1293.
3. **Bahk, Y. Y., S. A. Kim, J. S. Kim, H. J. Euh, G. H. Bai, S. N. Cho, and Y. S. Kim.** 2004. Antigens secreted from *Mycobacterium tuberculosis*: identification by proteomics approach and test for diagnostic marker. Proteomics **4:**3299–3307.
4. **Beck, S. T., O. M. Leite, R. S. Arruda, and A. W. Ferreira.** 2005. Humoral response to low molecular weight antigens of *Mycobacterium tuberculosis* by tuberculosis patients and contacts. Braz. J. Med. Biol. Res. **38:**587–596.
5. **Benabdesselem, C., D. M. Fathallah, R. C. Huard, H. Zhu, M. A. Jarboui, K. Dellagi, J. L. Ho, and R. M. Barbouche.** 2006. Enhanced patient serum immunoreactivity to recombinant *Mycobacterium tuberculosis* CFP32 produced in the yeast *Pichia pastoris* compared to *Escherichia coli* and its potential for serodiagnosis of tuberculosis. J. Clin. Microbiol. **44:**3086–3093.
6. **Bethunaickan, R., A. R. Baulard, C. Locht, and A. Raja.** 2007. Antibody response in pulmonary tuberculosis against recombinant 27kDa (MPT51, Rv3803c) protein of *Mycobacterium tuberculosis*. Scand. J. Infect. Dis. **39:** 867–874.
7. **Conde, M. B., P. Suffys, E. S. J. R. Lapa, A. L. Kritski, and S. E. Dorman.** 2004. Immunoglobulin A (IgA) and IgG immune responses against P-90 antigen for diagnosis of pulmonary tuberculosis and screening for *Mycobacterium tuberculosis* infection. Clin. Diagn. Lab. Immunol. **11:**94–97.
8. **DATASUS/Ministério da Sáude Brasil.** 2005. TUBERCULOSE—Casos confirmados notificados no Sistema de Informação de Agravos de Notificação-SINAN. Departamento de Informática do SUS, Ministério da Sáude Brasil. http://www.datasus.gov.br.
9. **Demissie, A., E. M. Leyten, M. Abebe, L. Wassie, A. Aseffa, G. Abate, H. Fletcher, P. Owiafe, P. C. Hill, R. Brookes, G. Rook, A. Zumla, S. M. Arend, M. Klein, T. H. Ottenhoff, P. Andersen, and T. M. Doherty.** 2006. Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with *Mycobacterium tuberculosis*. Clin. Vaccine Immunol. **13:**179–186.
10. **Gennaro, M. L., M. Affouf, G. V. Kanaujia, P. N. Brusasca, B. Mangura, and L. Reichman.** 2007. Antibody markers of incident tuberculosis among HIV-infected adults in the USA: a historical prospective study. Int. J. Tuberc. Lung Dis. **11:**624–631.
11. **Greenaway, C., C. Lienhardt, R. Adegbola, P. Brusasca, K. McAdam, and D. Menzies.** 2005. Humoral response to *Mycobacterium tuberculosis* antigens in patients with tuberculosis in the Gambia. Int. J. Tuberc. Lung Dis. **9:**1112–1119.
12. **Gupta, S., N. Shende, A. S. Bhatia, S. Kumar, and B. C. Harinath.** 2005. IgG subclass antibody response to mycobacterial serine protease at different stages of pulmonary tuberculosis. Med. Sci. Monit. **11:**CR585–CR588.
13. **Harries, A. D., and C. Dye.** 2006. Tuberculosis. Ann. Trop. Med. Parasitol. **100:**415–431.
14. **Kinhikar, A. G., D. Vargas, H. Li, S. B. Mahaffey, L. Hinds, J. T. Belisle, and S. Laal.** 2006. *Mycobacterium tuberculosis* malate synthase is a laminin-binding adhesin. Mol. Microbiol. **60:**999–1013.
15. **Laal, S., K. M. Samanich, M. G. Sonnenberg, S. Zolla-Pazner, J. M. Phadtare, and J. T. Belisle.** 1997. Human humoral responses to antigens of *Mycobacterium tuberculosis*: immunodominance of high-molecular-mass antigens. Clin. Diagn. Lab. Immunol. **4:**49–56.
16. **Lyashchenko, K. P., M. Singh, R. Colangeli, and M. L. Gennaro.** 2000. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. J. Immunol. Methods **242:**91–100.
17. **Munoz-Elias, E. J., and J. D. McKinney.** 2005. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. Nat. Med. **11:**638–644.
18. **Raja, A., K. R. Uma Devi, B. Ramalingam, and P. J. Brennan.** 2004. Improved diagnosis of pulmonary tuberculosis by detection of free and immune complex-bound anti-30 kDa antibodies. Diagn. Microbiol. Infect. Dis. **50:** 253–259.
19. **Sartain, M. J., R. A. Slayden, K. K. Singh, S. Laal, and J. T. Belisle.** 2006. Disease state differentiation and identification of tuberculosis biomarkers via native antigen array profiling. Mol. Cell Proteomics **5:**2102–2113.
20. **Singh, K. K., Y. Dong, J. T. Belisle, J. Harder, V. K. Arora, and S. Laal.** 2005. Antigens of *Mycobacterium tuberculosis* recognized by antibodies during incipient, subclinical tuberculosis. Clin. Diagn. Lab. Immunol. **12:**354–358.
21. **Singh, K. K., Y. Dong, L. Hinds, M. A. Keen, J. T. Belisle, S. Zolla-Pazner, J. M. Achkar, A. J. Nadas, V. K. Arora, and S. Laal.** 2003. Combined use of serum and urinary antibody for diagnosis of tuberculosis. J. Infect. Dis. **188:**371–377.
22. **Steingart, K. R., M. Henry, S. Laal, P. C. Hopewell, A. Ramsay, D. Menzies, J. Cunningham, K. Weldingh, and M. Pai.** 2007. Commercial serological antibody detection tests for the diagnosis of pulmonary tuberculosis: a systematic review. PLoS Med. **4:**e202.
23. **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.
24. **Verma, R. K., and A. Jain.** 2007. Antibodies to mycobacterial antigens for diagnosis of tuberculosis. FEMS Immunol. Med. Microbiol. **51:**453–461.
25. **Wang, B. L., Y. Xu, Z. M. Li, Y. M. Xu, X. H. Weng, and H. H. Wang.** 2005. Antibody response to four secretory proteins from *Mycobacterium tuberculosis* and their complex antigen in TB patients. Int. J. Tuberc. Lung Dis. **9:**1327–1334.
26. **Wilson, R. A., W. N. Maughan, L. Kremer, G. S. Besra, and K. Futterer.** 2004. The structure of *Mycobacterium tuberculosis* MPT51 (FbpC1) defines a new family of non-catalytic alpha/beta hydrolases. J. Mol. Biol. **335:**519–530.
27. **Wilson, R. A., S. Rai, W. N. Maughan, L. Kremer, B. M. Kariuki, K. D. Harris, T. Wagner, G. S. Besra, and K. Futterer.** 2003. Crystallization and preliminary X-ray diffraction data of *Mycobacterium tuberculosis* FbpC1 (Rv3803c). Acta Crystallogr. D Biol. Crystallogr. **59:**2303–2305.
28. **World Health Organization.** 2003. Brazil's leprosy research and control efforts enhanced. Tropical Disease Research Programme, World Health Organization, Geneva, Switzerland. www.who.int/tdr/research/finalreps/no60.htm.
29. **World Health Organization.** 2007. Tuberculosis. World Health Organization, Geneva, Switzerland. http://www.who.int/globalatlas/dataQuery/reportData.asp?rptType=1.