High Numbers of Interferon-γ-Producing T Cells and Low Titers of Anti-Tuberculous Glycolipid Antibody in Individuals with Latent Tuberculosis

Heinner Guio,¹ Yugo Ashino,¹ Hiroki Saitoh,¹ Umme Ruman Siddiqi,¹ Masako Mizusawa,¹ Peng Xiao,¹ Alonso Soto,³ Andros Theo¹ and Toshio Hattori¹

¹Division of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku University, Sendai, Japan
²Department of Respiratory Diseases, Internal Medicine, Tokyo Metropolitan Fuchu Hospital, Tokyo, Japan
³ALBIOTEC - Asociación Latinoamericana de Biotecnología, Lima, Perú

Latent tuberculosis infection (LTBI) is defined as an infection with Mycobacterium tuberculosis (MTB) without clinical, bacteriological, or radiological findings, and its early diagnosis is essential for eradication of tuberculosis. To identify LTBI, we measured the numbers of interferon-γ-producing T cells, based on the ELISPOT assay, and the antibody titers in the sera to tuberculous glycolipid antigen (TBGL-Ab). Seventeen culture-confirmed TB patients, 13 controls from TB endemic areas (EC) and 13 controls from TB non-endemic areas (NEC) were enrolled. Peripheral blood mononuclear cells (2.5 × 10⁶ per well) were cultured on plates precoated with antibody against interferon-γ. ELISPOT response was defined as positive when the MTB-specific antigen-containing wells showed at least 6 spots and twice numbers of spots than negative control wells. ELISPOT responses were positive in 15 (88%), 8 (62%) and 4 (31%) subjects of TB, EC and NEC groups, respectively. The ELISPOT data differ between TB and NEC groups (p < 0.01) but not between TB and EC groups. In contrast, TBGL-Ab titers were elevated (> 2.0 U/ml) in 12 TB patients (71%), but only in one subject (8%) each from EC and NEC groups. These results indicate the high prevalence of LTBI in EC. In conclusion, LTBI is associated with positive ELISPOT assay and the low titer of TBGL-Ab, while positive results both in ELISPOT and TBGL-Ab assays indicate active TB. The low titer of TBGL-Ab is a helpful marker to identify LTBI in ELISPOT-positive individuals in TB endemic areas.

Keywords: interferon-γ producing T cells/TBGL antibody/active tuberculosis/latent tuberculosis

An estimated 9 million new cases of tuberculosis (TB) and 2-3 million deaths are reported globally every year, making TB the leading cause of death from a single infectious pathogen (World Health Organization 2008). A major challenge in tuberculosis control is the diagnosis of latent tuberculosis infection that is defined as an infection with Mycobacterium tuberculosis (MTB) without clinical, bacteriological, or radiological findings. However, latent TB infected individuals may develop TB disease in the future (American Thoracic Society 2000a). Until recently, the tuberculin skin test (TST) has been used to identify persons infected by Mycobacterium tuberculosis who are at high risk for the progression to active disease. This method has several limitations especially in immunosuppressed individuals such as HIV infected people who may reveal false negative TST response. In addition, the immune-reconstitution in HIV patients after the initiation of anti retroviral therapy makes this problem even more complicated (Okada et al. 2002; Changan-Yasutan et al. 2009). Furthermore, false positive TST responses are also possible in cases of BCG vaccination and/or non-tuberculosis mycobacteria (NTM). (American Thoracic Society 2000b; Jasmer et al. 2002; Barnes et al. 2004).

Recently, the region of difference 1 (RD1), that encodes two highly antigenic proteins ESAT-6 and CFP-10 (Mahairas et al. 1996; Behr et al. 1999; Gordon et al. 1999), was found to be present in all pathogenic strains of mycobacterium TB but not in most NTM species and also deleted from attenuated BCG strains. The ELISPOT method based on the numbers of spots made by interferon-γ producing T cells stimulated by CFP-10 or ESAT-6 (T-Spot.TB; Oxford Immunotec, Oxford, UK) and the ELISA based Quantiferon-TB test (Cellestis Limited, Carnegie, Australia) were developed using these antigens to overcome the false positive result by TST (Codecasca et al. 2006; van Leeuwen et al. 2007), and both were approved by FDA. ELISPOT assay may offer greater sensitivity than the ELISA-based method (Pai et al. 2004; Ferrara et al. 2006).
However, these T cell-based diagnostic approaches failed to differentiate active TB from LTBI.

The detection of anti-tuberculous glycolipid IgG antibody (TBGL-Ab) in serum, produced in response to the glycolipid antigen trehalose 6, 6′-dimycolate (TDM) has also been found to be useful for the diagnosis of active TB infection (Maekura et al. 1993, 2001; Ashino et al. 2005). It is generally accepted that cell-mediated immunity plays an important role in controlling MTB infection. In contrast, antibody response is believed to have no protective role, although recent studies in B cell-deficient mice showed that antibody responses are essential to contain mycobacterial infection (de Valliere et al. 2005; Abebe and Bjune 2009).

We also believe that the antibody responses in AIDS patients could be detected although their T cell numbers were very low.

Until now, no comparison has been made between ELISPOT assay and TBGL-Ab method for human tuberculosis infection. Therefore, we compared both methods in patients with active pulmonary tuberculosis and in healthy adults from high and low endemic areas for TB to characterize LTBI.

Material and Methods

Subjects and specimens

All participants were recruited prospectively in Tohoku University Hospital and the Metropolitan Tokyo Fuchu Hospital over a 20-month period from November 2004 through June 2006. The diagnosis of pulmonary TB patients was based on the growing of M. tuberculosis from a sputum sample. The BCG vaccination status in the healthy BCG-vaccinated controls was confirmed by the presence of a typical BCG scar, which is a good indicator of BCG vaccination (World Health Organization. 1999, Pereira et al. 2001). In addition, chest X-rays abnormalities, history of TB or TB treatment were the exclusion criteria for this group. All the subjects were underwent voluntary counseling and testing for HIV-1 infection and a positive HIV test. Correlations were evaluated using the Spearman test. A p value of less than 0.05 was considered significant. All the calculations were done using the statistical package STATA version 8.2 (StataCorp LP, College Station, Texas).

Results

We recruited 43 individuals: 25 males (M) and 18 females (F). Seventeen of them were culture-proven pulmonary TB patients (10M/7F). Twenty-six patients were healthy BCG-vaccinated controls. The non-endemic control (NEC) comprised 13 (7M/6F) Japanese, and the 13 endemic controls (EC) comprised immigrants who were born and had lived in countries with a high incidence of TB. Among the 13 EC, 9 (5M/4F) were from Peru, 2 (1M/1F) from China, 1 (1M) from Zambia, and 1 (1M) from Bolivia. No one was less than 16 years old. The mean ± s.d. ages were 54.3 ± 20.9, 56.7 ± 3.17 and 26 ± 9.59 years in the TB, NEC and EC subjects, respectively. Endemic controls were clearly younger (p < 0.05) than others. All the 43 subjects recruited in this study had a BCG scar.

ELISPOT and TBGL-Ab assays were positive in 15/17 (88%) and 12/17 (71%) of TB cases, in 4/13 (31%) and 1/13 (8%) of NEC cases and in 8/13 (62%) and 1/13 (8%) of EC cases, respectively. ELISPOT and TBGL-Ab assays had comparable sensitivity (p = 0.2) to diagnose active TB, but TBGL-Ab had greater specificity than ELISPOT assay to distinguish active tuberculosis (specificity: 92% versus 54% for TBGL-Ab and ELISPOT assays, respectively; p < 0.01). In addition, it should be noted that both of two TB patients who were negative in the ELISPOT assay, were TBGL Ab positive (19.5 and 6.7 U/mL).

TBGL-Ab assay

The serum specimens were assayed without knowledge of the clinical status of the patients. We used TBGL assay kits manufactured using TBGLs consisting of TDM and a minor glycolipid (trehalose monomycolate, diacyltrehalose, phenolic glycolipid 2,3,6-tetraacyltrehalose-2-sulfate, and 2,3,6-trialcyl-trehalose) (Kyowa Medex Co.,Ltd. Tokyo, Japan). Details of this assay were reported previously (Maekura et al. 2001). A value greater than or equal to 2 U/mL was considered to be positive.

Statistical analyses

We compared sensitivity and specificity between ELISPOT and TBGL-Ab assays using the chi squared test. We also compared the titers of TBGL-Ab and the ELISPOT between TB patients with and those without active tuberculosis (NEC and EC) using the Mann-Whitney U test. Correlations were evaluated using the Spearman test. A p value of less than 0.05 was considered significant. All the calculations were done using the statistical package STATA version 8.2 (StataCorp LP, College Station, Texas).
In our study, ELISPOT assay showed significant differences between TB and NEC \((p = 0.001)\) but not between TB and EC \((p = 0.1)\) (Fig. 2). There were no statistical differences between spot forming units (SFUs) against ESAT-6 and CFP-10 in all groups. But, the anti-TBGL-Ab titers, depicted in Fig. 3, were significantly lower in subjects with LTBI from both EC \((0.9 \pm 0.26 \text{ U/mL})\) and NEC \((0.7 \pm 0.49)\) groups than active TB group \((p = 0.04 \text{ and } 0.02 \text{ respectively})\). In addition, there was no correlation between anti-TBGL-Ab titers and the SFUs after stimulation either with ESAT-6 \((\text{correlation coefficient} = 0.11, p > 0.48)\) or CFP10 \((\text{correlation coefficient} = 0.10, p > 0.51)\).

**Discussion**

The present study suggests that both positive in ELISPOT and TBGL-Ab assays indicate active TB disease. On the other hand, positive ELISPOT assay with the low titers of TBGL-Ab indicates LTBI.

The incidence of TB per 100,000 inhabitants in Japan is 25, in Peru 206, in China 206, in Zambia 618 and in Bolivia 280 (World Health Organization 2007). Immigrants from TB endemic countries constitute a high-risk population because of the high rates of reactivation (Codecasa et al. 1999; Das et al. 2006). In Japan infection is heavily concentrated in the age group of more than 60 years and 82% of the active TB patients are in the age of more than 40 years (Mori 2000).

In our study, ELISPOT assay showed significant differences between TB and NEC \((p = 0.001)\) but not between TB and EC \((p = 0.1)\) (Fig. 2). There were no statistical differences between spot forming units (SFUs) against ESAT-6 and CFP-10 in all groups. But, the anti-TBGL-Ab titers, depicted in Fig. 3, were significantly lower in subjects with LTBI from both EC \((0.9 \pm 0.26 \text{ U/mL})\) and NEC \((0.7 \pm 0.49)\) groups than active TB group \((p = 0.04 \text{ and } 0.02 \text{ respectively})\). In addition, there was no correlation between anti-TBGL-Ab titers and the SFUs after stimulation either with ESAT-6 \((\text{correlation coefficient} = 0.11, p > 0.48)\) or CFP10 \((\text{correlation coefficient} = 0.10, p > 0.51)\).

**Fig. 1.** Comparison between percentage of positive ELISPOT and TBGL-Ab assays. The percentages of positive results with ELISPOT and TBGL-Ab tests among patients with active tuberculosis \((n = 17)\), non endemic control \((n = 13)\) and endemic control \((n = 13)\) groups were shown. Only in endemic control group, the percentage of ELISPOT positivity differ significantly from the TBGL-Ab positives rates \((p = 0.01)\).

Non endemic controls: Volunteers from Japan

Endemic controls: Volunteers from high TB incidence countries.

**Fig. 2.** Comparison of interferon-\(\gamma\)-producing T cells. Dot plot of individual response to ESAT-6 and CFP-10 expressed in numbers of interferon-\(\gamma\)-spot forming units (IFN-\(\gamma\)-SFUs) per million of peripheral blood mononuclear cells (PBMC). SFUs were compared between ESAT-6 and CFP-10 in TB patients \((n = 17)\), non-endemic control \((n = 13)\) and endemic control \((n = 13)\) groups. Comparisons were also made between TB patients and both non-endemic control and endemic control groups as well.

IFN-\(\gamma\)-SFUs only differ significantly between TB patients and non-endemic control group \((p = 0.001)\).

\(p^*\), \(p\) value between TB patients and Non-Endemic Controls

\(p^{**}\), \(p\) value between TB patients and Endemic Controls

**Fig. 3.** Comparison of anti-TBGL antibody results. Dot plot of individual responses to anti-TBGL antibody (TBGL) for active TB patients \((n = 17)\), non-endemic controls \((n = 13)\) and endemic controls \((n = 13)\). Significant differences were observed between TB patients and both non-endemic control and endemic control groups \((p = 0.02 \text{ and } 0.04, \text{respectively})\).

The dashed line represents the cutoff value of 2 U/mL for TBGL antibody.

In our study, ELISPOT assay showed significant differences between TB and NEC \((p = 0.001)\) but not between TB and EC \((p = 0.1)\) (Fig. 2). There were no statistical differences between spot forming units (SFUs) against ESAT-6 and CFP-10 in all groups. But, the anti-TBGL-Ab titers, depicted in Fig. 3, were significantly lower in subjects with LTBI from both EC \((0.9 \pm 0.26 \text{ U/mL})\) and NEC \((0.7 \pm 0.49)\) groups than active TB group \((p = 0.04 \text{ and } 0.02 \text{ respectively})\). In addition, there was no correlation between anti-TBGL-Ab titers and the SFUs after stimulation either with ESAT-6 \((\text{correlation coefficient} = 0.11, p > 0.48)\) or CFP10 \((\text{correlation coefficient} = 0.10, p > 0.51)\).
ble to the age of our NEC population (56.7 ± 3.17 years old), in which it was found in 31%.

We hypothesize that IFN-γ production (TH1-dependent) in response to antigenic challenge may be long-lasting in memory cells, while antibody production against mycobacterium glycolipid (TH2-dependent) may be more related to the active replication of mycobacteria, or inflammation that predisposes TH2. In fact the decrease of anti-TBGL antibody after therapy has already been reported (Maekura et al. 2001), but the effect of active TB treatment on IFN-γ responses has shown declining responses in some studies, whereas others have shown unchanging, fluctuating or increasing responses during treatment (Pai et al. 2004; Dheda et al. 2005). This variation was also observed in case of other cytokines (Turgut et al. 2006; Djoba et al. 2009).

TBGL-Ab titers were significantly different between active TB and both NEC and EC. In preliminary studies, ELISPOT (Dosanjh et al. 2008) and TBGL-Ab (Maekura et al. 2001) assays showed sensitivities of 85% and 81% respectively, in diagnosis of active TB. In our study, the sensitivity was 88% for the ELISPOT assay and 71% for the TBGL-Ab assay, confirming the high rates of detection in active TB patients. Moreover, the combined sensitivity for the two methods reached nearly 100%.

The reasons for false-negative ELISPOT assay results in active tuberculosis have not been clarified. Previous studies based on IFN-γ production reported a frequency of 4 to 38% of false negatives (Pai et al. 2004). Kobashi in Japan, using QuantiFERON TB-2G, a method based on a whole blood assay, found 7.1% negative result (Mean age: 66 years old) in TB patient suggesting due to lymphocytopenia caused by aging or no production of IFN-γ for MTB specific antigens (Kobashi et al. 2009). In our study we found 12% (2/17) of false negatives. We think that lymphocytopenia by aging cannot explain this phenomenon since our method is based on the number of IFN-γ producer cells instead of whole blood stimulation, Moreover, these two patients possessed high titers of anti-TBGL-Ab (19.5 and 6.7 U/mL). The mechanism of immune protection provided by humoral immunity is not understood fully. When and how it cooperates with the cell mediated immunity and works in synergy is a topic of future investigations. Recently, in Thailand, we do not have enough evidence that the levels of anti-TBGL IgG are associated with tuberculosis infection in children (unpublished data).

The originality of this study, performed in a country with a low incidence of TB, is that a positive TBGL-Ab can be quite specific for active TB infection in both non-endemic and endemic populations. TDM (the main component of the TBGL-Ab assay) is present in large amounts on the surface of virulent but not avirulent MTB (Blok and Noll 1953) and is also one of the important factors for the granuloma formation among the lipids in the mycobacterial cell-wall fraction (Behling et al. 1993; Geisel et al. 2005). Hunter et al. described that virulent MTB releases large amounts of TDM during growth as a pellicle within cavities, and such growth results in the activation of the toxicity and antigenicity of TDM (Hunter et al. 2006). Recently, we reported the increased synthesis of anti-TBGL IgG in relation to cavity formation in pulmonary tuberculosis and elevated titers of C-reactive protein, which suggest that TBGL IgG could reflect inflammation in active pulmonary TB patients. (Mizusawa et al. 2008). Additionally, the anti-TBGL antibody is not influenced by prior BCG vaccination (Nabeshima et al. 2005). These findings may explain the high titers of TBGL antibody in active TB but not in the control groups.

Therefore, anti-TBGL-Ab could be suitable for screening serum for detection of active TB patients especially in high endemic countries. One of the main limitations in this study is the low number of patients. Therefore, more extensive studies based on the immune status of patients and people at high risk to develop TB should be done in order to confirm our findings.

Acknowledgments

We thank Ms. Yuko Sato for her technical assistance. This work was supported by Grant-in-Aid for Special Educational Grant from Ministry of Education, Culture Sports, Science and Technology and Grant-in-Aid from the Scientific Expenses for Health and Welfare Program from the Ministry.

References


Codecasa, L.R., Porretta, A.D., Gori, A., Franzetti, F., Degli Espositi, A., Lizzioli, A., Carreri, V., Di Proietto, M.C.,


