Original Article

CONSIDERATION OF IMPROVEMENT MEASURES FROM LIMITATIONS OF IMMUNOLOGICAL TESTS—INCLUDING INTERFERON-γ RELEASE AND ANTIBODY-BASED DETECTION ASSAYS—FOR *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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Abstract [Purpose] *Mycobacterium tuberculosis* (MTB) infection should be detected in all patients before progressing to active tuberculosis (TB); however, interferon-γ release assays (IGRAs) and serological assays cannot accurately detect TB infection in all patients. Therefore, we conducted a prospective study to determine whether TB infections in patients with active pulmonary TB could be reliably detected by combined use of both tests. [Methods] We consecutively enrolled 186 patients suspected of having pulmonary TB referred to our institute between October 2008 and March 2010 in this study. All patients underwent IGRA and serological assays at first visit and subjected for differential diagnoses. [Results] MTB infections could be detected in 49 of 50 patients with active pulmonary TB using tests of humoral and cellular immune responses. However, false-positive serological tests and IGRAs using TB-specific antigens were observed in patients with nontuberculous *Mycobacterium* (NTM), old TB, or other respiratory diseases. [Conclusion] MTB infections were detected in nearly all patients with active pulmonary TB using tests of humoral and cellular immune responses. However, these assays need to be improved in order to differentiate the active MTB infection from latent MTB infection or NTM infection using combined other separate antigens.

Key words: *Mycobacterium tuberculosis* infection, Interferon-γ release assay, Serological assay, Tuberculous glycolipid, Lipoarabinomannan polysaccharide

INTRODUCTION

The gold standard method to diagnose active *Mycobacterium tuberculosis* (MTB) disease is either culture-based isolation or the detection of MTB-specific nucleic acids by molecular methods[1)2)]. Active tuberculosis (TB) disease develops in about 10% of infections, mostly within 1–2 years after exposure[3)]. Remaining individuals enter into a state of latency (latent TB infection [LTBI]). Active TB developed as a result of MTB infection; therefore, MTB infection should be detected in all patients before progressing to active TB. Generally, the immunological method of detection for MTB infection can be grouped into two categories: serological (antibody detection) assays based on humoral immune responses and tests of cellular immune response (tuberculin skin tests and *in vitro* interferon [IFN]-γ release assays [IGRAs]). However, MTB infections cannot be detected using individual serological assays and IGRAs in all patients with pulmonary TB[4)]. Commercial serological tests are not recommended for use in the diagnosis of pulmonary TB, and the use of IGRAs is discouraged for active pulmonary TB diagnosis in low- and middle-income countries.

We previously reported that the combined use of serological tests with three separate antigens, i.e., tuberculous glycolipid (TBGL) antigen, lipoarabinomannan polysaccharide (LAM) antigen, and antigen 60, which was prepared from purified protein derivatives, maximizes the effectiveness of serodiagnosis for pulmonary TB[5)]. The sensitivity increased to 91.5% in patients with active pulmonary TB and to 86.0% in smear- and culture-negative patients, and the specificity was 87.5% in the healthy control groups. However, the combined use of IGRAs and serological tests with separate antigens has not been evaluated to determine its effectiveness and limitation for diagnosis of pulmonary TB. So, we conducted a prospective study to determine whether MTB infections in patients with active pulmonary TB could be reliably detected by both serological tests and IGRAs in clinical practice. We also discussed the limitations and improvement measures of

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(Received 17 Feb. 2017/Accepted 16 Jun. 2017)

immunological tests.

MATERIALS AND METHODS

Study patients

We consecutively enrolled 186 patients suspected of having pulmonary TB referred to our institute between October 2008 and March 2010 in this study. Patients who had positive smear tests and/or nucleic acid amplification testing (NAT) documented in their medical records from local physicians were not enrolled. Blood specimens had been obtained at first visit; however, the attending physicians were blinded to the results until confirmation of the diagnosis. The patients received a differential diagnosis and were followed up at our hospital. All patients provided written informed consent to participate in this study, and the study was conducted according to our institutional guidelines. The study protocols were approved by the institutional review board for experimentation on human subjects of National Hospital Organization Toneyama Hospital (approval no.: 0822, dated: 17. 10. 2008).

Differential diagnostic criteria

The criteria for differential diagnosis are presented in Fig. 1. Active pulmonary TB was confirmed when culture and/or NAT of sputum specimens was positive for TB. When smears, cultures, and NAT were negative, clinically active pulmonary TB was confirmed with abnormal chest X-ray findings, which improved after treatment for 3 months with three or four antituberculosis drugs (rifampicin, ethambutol, isoniazid, streptomycin, or pyrazinamide). Improvement was confirmed by unanimous agreement of three independent specialists of respiratory medicine after review of the chest X-ray and computed tomography (CT) scans. Clinically active pulmonary TB was considered class 3 according to the American Thoracic Society (ATS) TB classification[6,7].

The old TB was diagnosed by the presence of stable sclerotic lesions (e.g. nodules with calcification, sclerotic cavity, bronchiectasis or pleural thickenings) on the chest radiogram, persistent culture-negative results for TB, and having a definite history of pulmonary TB. Nontuberculous Mycobacterial (NTM) diseases were diagnosed according to the ATS guideline[8]. Additionally, malignant diseases and other respiratory diseases were diagnosed by chest X-rays and CT scans, consistent with the working diagnosis, physician examination, and other relevant clinical information. Finally, healthy individuals, including TB contacts, showed normal findings on chest X-rays and CT scans and had negative cultures for TB in all sputum specimens.

The results of IGRA and serological tests were compared among the seven groups of patients, as listed in Table. Finally, the remaining seven patients with pleurisy and four undiagnosed patients were excluded from this study. Patients suspected of having human immunodeficiency virus infection based on symptoms and clinical examinations were excluded as well.

Sputum specimens

Sputum specimens for ordinary examination by smear staining and cultivation were obtained for 3 consecutive days as previously described[9]. Identification of MTB and NTM was determined by a DNA-DNA hybridization method with a DDH *Mycobacteria* kit (Kyokuto Pharmaceuticals, Tokyo, Japan). Sputum specimens for NAT were collected

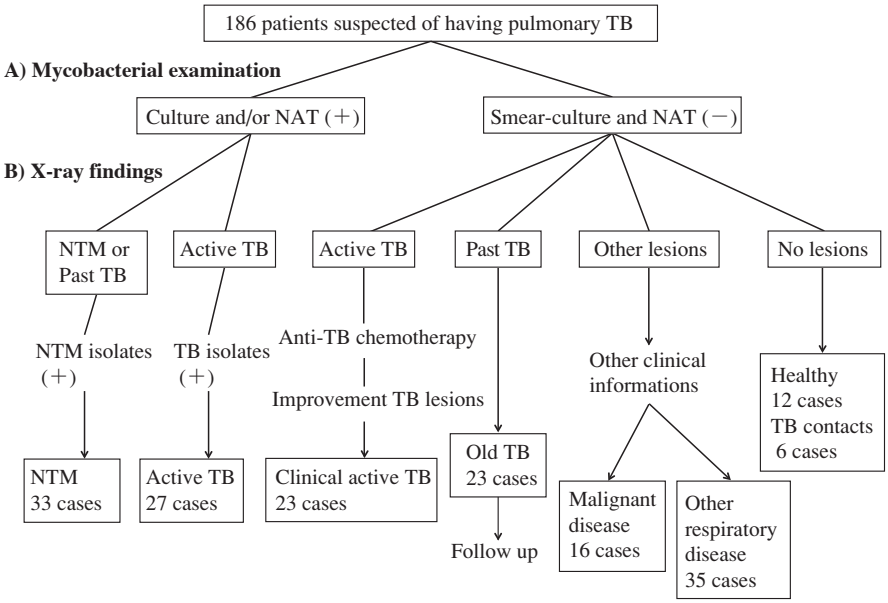

Fig. 1 Study patients and differential diagnosis.
The seven remaining patients with pleurisy and four undiagnosed patients were excluded from this study.
TB: Tuberculosis, NTM: Nontuberculous Mycobacterial diseases, NAT: nucleic acid amplification testing.

separately in special containers from every enrolled patient on the first study day. NAT was performed using the Transcription Reverse-Concerted reaction (TRC) Rapid-TB and -MAC method (Tosoh Co., Tokyo, Japan) according to the manufacturer's instructions[10].

IGRAs and serological tests

IGRAs were performed according to the manufacturer's instructions (QFT-Gold: Cellestis Ltd, Carnegie, Australia) using 10-kDa culture filtrate protein (CFP-10) and 6-kDa early secretory antigenic target (ESAT-6) of MTB as antigens with a cutoff value for a positive test of IFN-γ of 0.35 IU/mL or greater. Anti-TBGL antibodies were measured by enzyme-linked immunosorbent assays (ELISA) with a Determiner TBGL kit (Kyowa Medex Co., Ltd., Tokyo, Japan). Anti-LAM antibodies were detected with a MycoDot kit (Mossmann Associates, Blackstone, MA, USA). Detection of antibodies was achieved by colorimetry, and the intensities of the test reactions were compared with those of the reference. Kits for anti-LAM and anti-TBGL antibody testing were specific for IgG responses. The manufacturer's instructions for test performance were followed for all commercial assays used in this study. The quantitative results of the TBGL test were expressed as U/mL. The LAM test data were expressed using a five-level scale. The cutoff points were 2 U/mL for anti-TBGL antibodies and 1+ for anti-LAM antibodies, according to the standards set by the manufacturer of each test. All specimens were assayed without prior knowledge of the clinical status.

Statistical analyses

Statistical analyses were performed with JMP 9 statistical software (SAS Institute, Cary, NC, USA). The positive rates were compared between IGRA and serological tests in each group using chi-squared tests. Differences were considered significant when the p value was less than 0.05.

RESULTS

Differential diagnosis

Twenty-seven patients were diagnosed as having active pulmonary TB due to either isolation of MTB by culture or detection of MTB-specific nucleic acids, including 19 patients with smear- and culture-negative TB at first visit (Fig. 1). Clinically active pulmonary TB was diagnosed in 23 patients with smear- and culture-negative TB during follow up period. Nine of 23 patients were confirmed by bronchoscopic examination. Thirty-three patients met the ATS guideline for NTM disease. *M.avium* (15 cases), *M.intracellulare* (five cases), *M.kansasii* (four cases), *M.gordonae* (one case), *M.simiae* (one case), and unidentified organisms (seven cases) were isolated from their sputum specimens. Seven patients had past TB lesions on their chest radiogram.

Sixteen patients had malignant diseases, including 12 lung cancers, one malignant mesothelioma, one malignant lymphoma, one lung metastasis from gastric cancer, and one lung metastasis from breast cancer. Other respiratory diseases were observed in 17 patients with bacterial pneumonia, six patients with bronchiectasis, four patients with interstitial pneumonia, four patients with sarcoidosis, and one patient each with pulmonary aspergillosis, lung abscess, sequestration

Table Positive rates of IGRA and Serological tests in each group

	Age	n (male/female)	IGRA (%)	Serological tests			IGRA &/or Sero (%)
				TBGL &/or LAM (%)	TBGL (%)	LAM (%)	
1. Active TB	47.7±20.3	50 (32/18)	33 (66.0)	42 (84.0)	29 (58.0)	31 (62.0)	49 (98.0)
2. NTM	70.4±12.1	33 (11/22)	9 (27.3)*	28 (84.8)	22 (66.7)	22 (66.7)	29 (87.9)
3. Malignant diseases	69.4±12.0	16 (11/5)	0	5 (31.3)	3 (18.8)	3 (18.8)	5 (31.3)
Lung cancer		12	0	5 (41.7)	3 (25)	3 (25)	5 (41.7)
Other malignant diseases		4	0	0	0	0	0
4. Other respiratory diseases	52.5±19.0	35 (24/11)	6 (17.1)	16 (45.7)	15 (42.9)#	6 (17.1)	19 (54.3)
Bacterial pneumonia		17	3 (17.6)	5 (29.4)	5 (29.4)	1 (5.9)	7 (41.2)
Bronchiectasis		6	2 (33.3)	4 (66.7)	4 (66.7)	0	5 (83.3)
Interstitial pneumonia		4	1 (25)	2 (50)	1 (25)	2 (50)	2 (50)
Sarcoidosis		4	0	1 (25)	1 (25)	0	1 (25)
Other diseases		4	0	4 (100)	4 (100)	3 (75)	4 (100)
5. Old TB	66.9±13.9	23 (12/11)	8 (34.8)	15 (65.2)	15 (65.2)$	7 (30.4)	18 (78.3)
6. TB contacts	50.3±25.3	6 (0/6)	4 (66.7)	4 (66.7)	2 (33.3)	2 (33.3)	6 (100)
7. Healthy	50.7±24.6	12 (5/7)	1 (8.3)	3 (25.0)	0	3 (25.0)	4 (33.3)

TB: Tuberculosis, NTM: Nontuberculous Mycobacterial diseases (isolated *M.avium* in 15 cases, *M.intracellulare* in 5 cases, *M.kansasii* in 4 cases, *M.gordonae* in a case, *M.simiae* in a case and unidentified organisms in 7 cases)
Malignant diseases: lung cancer in 12 cases and others in 4 cases which were each one patient with malignant mesothelioma, malignant lymphoma, metastasis of gastric cancer or metastasis of breast cancer.
Other respiratory diseases: bacterial pneumonia in 17 cases, bronchiectasis in 6 cases, sarcoidosis in 4 cases, interstitial pneumonia in 4 cases and others in 4 cases which were each one patient with pulmonary aspergillosis, lung abscess, sequestration of lung or benign tumor (inflammatory granuloma).
IGRA : interferon-γ [IFN-γ] release assay, Sero: Serological tests, TBGL: tuberculous glycolipid antigen,
LAM: lipoarabinomannan polysaccharide antigen.
*: $p=0.001$, #: $p=0.0189$, $: $p=0.039$

of the lung, or benign tumors. Acid-fast bacilli were not detected in any patient with malignant diseases and other respiratory diseases.

Twenty-three patients were diagnosed as having old TB and then followed up for 3 years. Finally, 12 healthy individuals and six TB contacts showed normal findings on chest X-rays and CT scans and were culture-negative for TB in their all sputum specimens. These data are summarized in Fig. 1.

Positive rates of IGRA and serological tests in each group (Table)

Positive rates of each test were approximately equal (IGRA: 66%, TBGL: 58%, LAM: 62%) in 50 patients with active TB. Negative results of all three tests were observed in one case of a 43-year-old man with clinically active TB, who underwent anti-TB chemotherapy due to the detection of granuloma (not including caseous necrosis) from pathological specimens obtained by lung needle biopsy under CT. He was diagnosed as having clinically active TB due to the mild improvement of lesions on chest X-ray after anti-TB standard chemotherapy (Fig. 2). However, pathological findings suggested the possibility of sarcoidosis. Thus, this case may have been a false negative. MTB infection in nearly all patients (98%) with active pulmonary TB could be detected using these three tests (Fig. 3).

Positive rates of serological tests (TBGL: 66.7%, LAM:

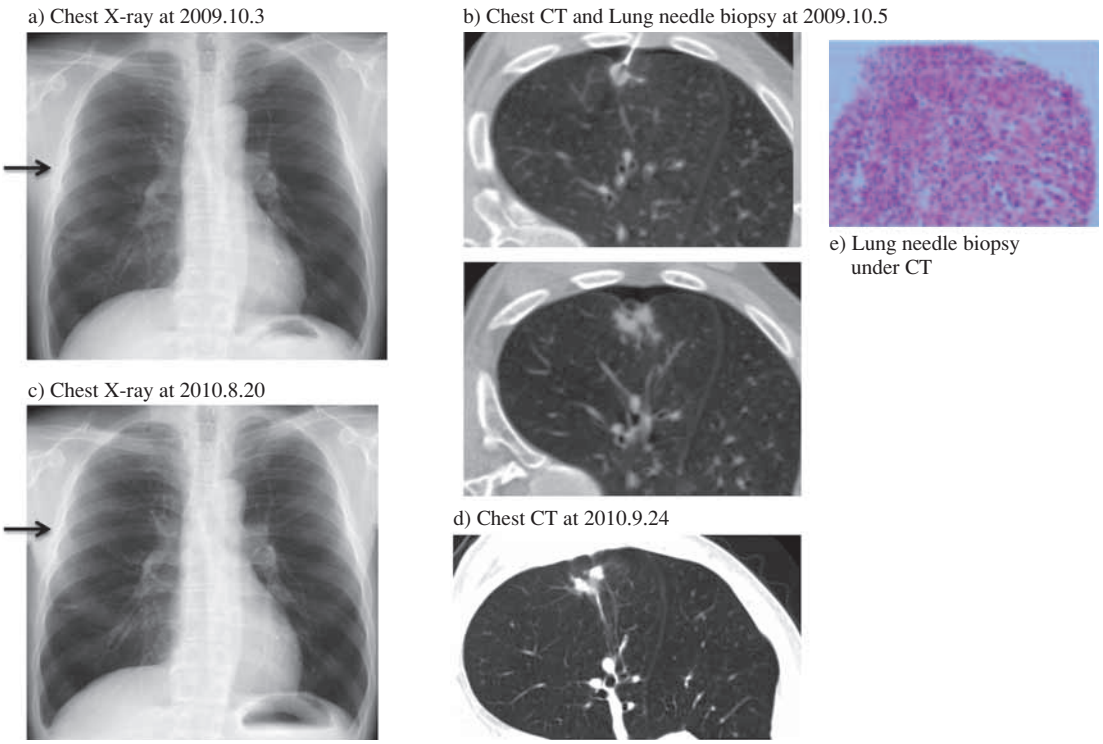

Fig. 2 False-negative result in a 43-year-old man with active TB. The chest X-ray and CT (a, b) before and (c, d) after anti-TB standard chemotherapy. (e) Histological sample stained by HE in lung needle biopsy.

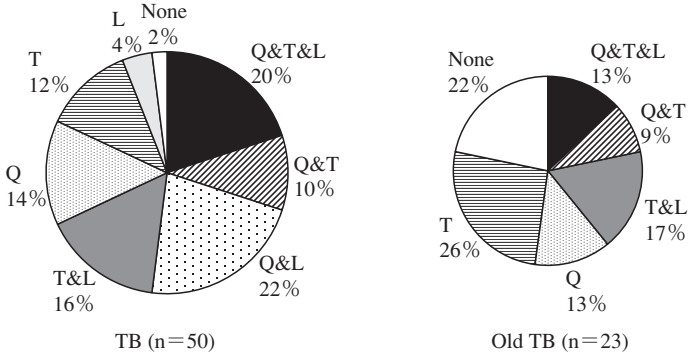

Fig. 3 Positive rates of immunodiagnostic tests, using results from all possible combinations of tests, in patients with active pulmonary TB and with old pulmonary TB.
Q: QFT (IGRA), T: TBGL test, L: LAM test.

66.7%) in patients with NTM disease were approximately equal to those in patients with active TB. The positive rate (27.3%) of IGRA was significantly lower in patients with NTM disease, including patients with *M.kansasii* disease, unidentified NTM disease, or past TB lesions on chest X-rays, compared with that of serological tests.

Six of 35 patients with other respiratory diseases were positive in IGRAs and were assumed to have LTBIs. In three IGRA-positive patients with bacterial pneumonia, cavity lesions or atelectasis was detectable. Thirteen other patients distinct from the six patients listed above showed positive results in serological tests. The positive rate of serological tests for each classification of other respiratory diseases is shown in Table. Most patients had chronic infectious diseases, including bronchiectasis, pulmonary aspergillosis, lung abscess, and sequestration of the lung.

Positive rates of both IGRAs (34.8%) and serological tests were very high in patients with old TB (Table). All tests were positive in three patients, and two tests were positive in six patients (Fig. 3). High TBGL titers (more than 10 U/mL) were found in four patients. Therefore, these patients were followed up. Patient A (63 years old), who had two positive serological tests (TBGL: 10.9 U/mL, LAM: 2+), was diagnosed as having active TB after 18 months (Fig. 4a). Patient B (60 years old) was diagnosed with NTM (*M.gordonae*) from sputum specimens after 3 years (Fig. 4b).

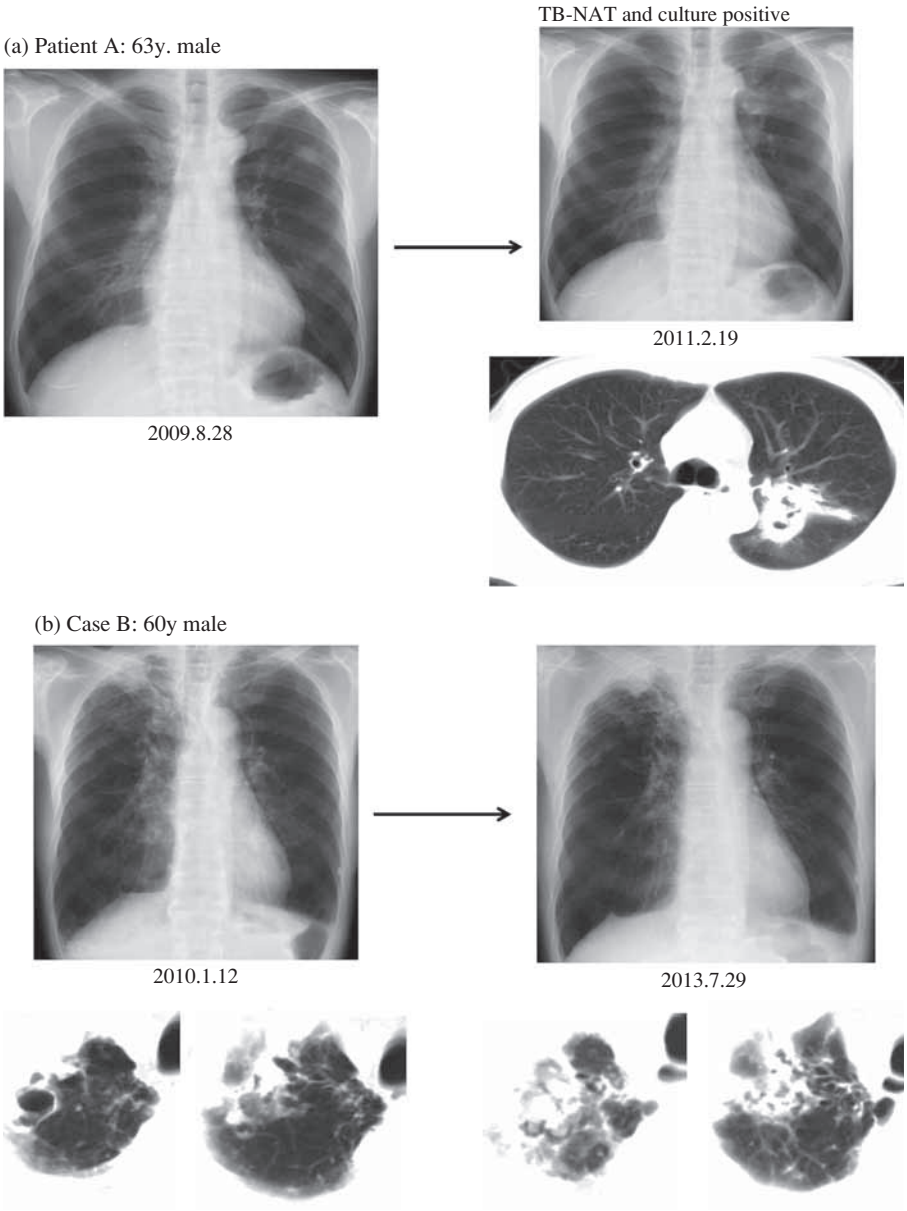

Fig. 4 Courses of the chest X-ray findings in two patients who developed disease. (a) Patient A: TB lesions with a cavity in left upper lung field were detected on chest X-rays on February 19, 2011. NATs and cultures for TB were positive. (b) Patient B: This patient had undergone standard TB chemotherapy in 2002 and complained of bloody sputum. Exacerbation of findings was observed on the chest X-ray, and *M. gordonae* was isolated from his sputum specimens in July 2013.

In this patient, IGRAs and serological tests were positive (TBGL: 18.4 U/mL, LAM: 1+), and the patient had received a hemostatic agent for persistent bloody sputum and past TB lesions with bronchiectasis (Fig. 4b).

Of the 12 healthy individuals with respiratory symptoms, IGRA-positive results were found in one case, and serological tests were positive in three cases. All six TB contacts showed the positive IGRA results or serological tests. The sensitivity for active pulmonary TB was 98% using combined these tests, but the specificity of malignant disease patients and healthy subjects as a control was 67.9%.

DISCUSSION

In this study, MTB infection in nearly all patients with smear test-negative and NAT-negative active pulmonary TB could be detected using both serological tests (TBGL and LAM) and IGRA (CFP-10 and ESAT-6) with separate antigens. This was possible because these methods with combined antigens (protein, glycolipids, and polysaccharides) detected both humoral and cellular immune responses owing to the wide variety of antigenic materials in the cell wall of acid-fast bacilli and to individual specificity in serum antibody production by acid-fast bacterial infection[5)11)12)]. However, serological tests and IGRA revealed a number of positive results in groups of patients with conditions other than active pulmonary TB.

In particular, serological antibodies were detected at high rates in patients with NTM disease, similar to those of active TB, because TBGL and LAM are common components of cell wall in acid-fast bacilli. For differentiation between TB infection and NTM infection, it is necessary to use species-specific substances as antigens, such as TB-specific CFP-10, ESAT-6, and secreted antigen MPB-64[11)13)~15)], which were used in IGRA rather than in serological tests, as well as *M.avium-intracellulare* complex-specific glycopeptidolipid[16)]. Although many TB-specific antigens have been found, it is necessary to identify other antigenic substances specific for NTM (e.g., *M.kansasii* and *M.abscessus*, etc.) in the future. In patients with NTM, IGRA showed positive results in some cases albeit in fewer cases than serological tests, because *M. kansasii*, which expresses CFP-10 and ESAT-6 in the cell wall, was detected in sputum specimens and because seven patients with past TB lesions on chest X-rays were included.

The positive rate of serological tests (particularly TBGL) was higher in patients with old TB than those reported in previous studies[17)18)], likely because there was an uneven distribution of cases with suspected active pulmonary TB due to respiratory symptoms and past TB lesions on chest X-rays. Eight patients had positive results in IGRA in patients with old TB and were considered to have LTBI. A previous report showed that TB bacilli in the dormancy and exponential growth phases were present within an immune-mediated granuloma lesion in a patient with tuberculoma[19)]. Additionally, antibodies against dormancy- and growth-related antigens were detectable in serum samples from patient with old TB. The patients, who had shown positive results on serological tests, were also considered to be at high-risk of TB recurrence. Indeed, one of these patients, who had positive TBGL and LAM results, progressed to active TB disease during follow up. Anti-TBGL antibody titers decreased to the normal levels at more than 3 years after bacterial negative conversion by TB chemotherapy[20)]. During stable asymptomatic infection or after vaccination with *M.bovis* BCG, tests for antibody production are largely negative[17)21)22)]. In contrast, antibody titers to MTB antigens increase prior to TB progression[23)24)]. Serological tests may also be useful to identify individuals at higher risk of reactivation of LTBI in patients with respiratory symptoms and past TB lesions. This antibody response in the absence of disease may reflect the control of an incipient TB infection by antituberculous prophylaxis. Moreover, for another patient, *M.gordonae* was detected in sputum specimens. The high positive rate of serological tests could be explained by LTBI and/or co-infection with NTM in patients with old TB.

The positive rate of TBGL tests was also higher in patients with other respiratory diseases than those in previous studies[16)]. In particular, antibody titers (mean: 9.1 U/mL) were high in patients with chronic infections diseases, such as bronchiectasis, pulmonary aspergillosis, lung abscess, and lung sequestration. These lesions were exposed to environmental indigenous NTM bacilli and were likely to be easily infected. In serological tests using common components as antigens, differentiation from NTM infection is also required in patients with other respiratory disease. For the differentiation between TB infection and NTM infection, it is also necessary to use species-specific substances as an antigen.

The positive rates of IGRAs and serological tests were clearly different between healthy individuals and TB contacts whose abnormal lesions were not detectable on chest CT. These false positives were also considered as potential indication of LTBI and/or NTM infection. However, neither test could prove this, although immunological tests based on IGRAs and antibody assays to MTB antigens have been established[25)]. The detection of antibodies against antigens related to dormant TB infections (e.g., MDP-1: mycobacterial DNA-binding protein 1, Acr: alpha-crystallin like protein, and HBHA: heparin-binding hemagglutinin)[19)23)24)] and/or NTM-specific antigens (glycopeptidolipids)[16)] may be required in the future.

There were several limitations to this study. First, this study was carried out at a single facility. Additionally, the number of cases in the healthy group was small because we focused on recruiting patients with suspected active TB. However, this study was carried out using routine clinical methods with Japanese health care insurance; thus, we believe that our findings provided a good representation of the actual situation of medical care in high-income countries.

The population of Japan is rapidly aging, as also seen in

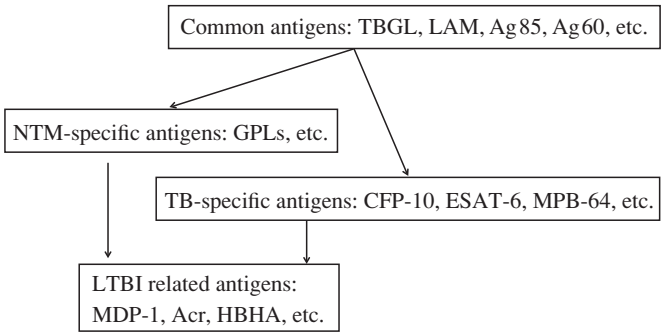

Fig. 5 Mycobacterial infection (including TB) detection kits using the antigens corresponding to infected bacterial species and its growth phases.

Acr: alpha-crystallin like protein (also called HspX) (ref. 19, 23, 24), Ag85: antigen 85 complex proteins (mycolyltransferases) (11, 13−15, 19), CFP-10: 10 kDa culture filtrate protein (11, 13−15), ESAT-6: 6 kDa early secretory antigenic target of *Mycobacterium tuberculosis* (11, 22), GPLs: Glycopeptidelipids (16), HBHA: heparin-binding hemagglutinin (23, 24), LAM: lipoarabinomannan polysaccharide (5), MDP-1: mycobacterial DNA-binding protein 1 (also called Mt-HLP, HupB, LBP) (19, 23, 24), MPB-64: Secreted antigen MPB-64 (11, 13−15), TBGL: tuberculous glycolipid (5, 12, 16−18, 20).

other developed countries. The number of people living in sanatorium, medical and nursing care facilities and group homes for the elderly is increasing. The frequency of emergency transportation to hospitals due to sudden illness is also increasing. The delays of diagnosis for TB have become a problem in these facilities, because respiratory specialists are hardly working. We purport that three immunological tests—IGRA, TBGL, and LAM—may improve the delays in TB diagnosis. For example, when positive results of MTB infection are obtained by immunological examinations from blood specimens, even general physicians not specialized in respiratory diseases can perform appropriate examinations, such as sputum specimens and chest X-ray examinations, and will not miss pulmonary tuberculosis. If results of all three tests are negative, it can be determined that no *M. tuberculosis* infection exists.

In summary, although the sensitivity of the immunological test for active pulmonary tuberculosis was improved as expected by employing immune responses and separate antigens, the specificity decreased. Especially, false-positive results of IGRA using TB-specific antigens were observed in patients with NTM, old TB, or other respiratory diseases. These findings were considered to be a result of co-infection of NTM and/or LTBI. Accordingly, an important role of the immunological test is the more accurate detection of subclinical TB infection status progressing from LTBI to active TB disease[26]. When this detection becomes possible, the outcome of preventive therapy will be more effective and disease incidence will be reduced, paving the way for the eradication of TB. Furthermore, the diagnosis of clinical active TB becomes easier, possibly leading to an economic effect owing to the decrease in cases requiring invasive examination (e.g. bronchoscopy). Furthermore, it is required to be able to differentiate between patients with clinical active TB and pulmonary NTM infection at an early stage when pulmonary lesions are observed on a chest X-ray but acid-fast bacteria are not detectable from sputum specimens, which is significant because the incidence rate of pulmonary NTM disease exceeded that of TB for 2014 in Japan[27]. We consider addressing these issues in the future because the development of Mycobacterial infection (including TB) detection kits, which use the humoral and cellular immune responses as an assay method with combined separate characteristic antigens (mycobacterial common antigens, species' specific antigens, and dormancy related antigens), currently requires accurate differential diagnosis for Mycobacterial infection (Fig. 5). Furthermore, the limitations of IGRAs and serological assays and their consequent improvements for the diagnosis of MTB infection in respiratory clinical practice will need to be addressed in the future.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases).

Conflict of interest: None to declare

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