多剤耐性結核迅速診断・迅速入院（隔離）法

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研究要旨
多剤耐性結核は世界的に問題となっている。その診断の遅れは、治療失敗につながるのみならず、他者への感染リスクの増大をももたらすため、迅速な診断はきわめて重要である。我々は、多剤耐性結核のスクリーニング法としてのリファンピシン（RFP）耐性迅速診断法の有用性につき検討を行った。従来法の薬剤感受性検査を good standard とした場合の感度は 93.3%、特異度は 99.7%と優れた成績が得られた。本法は RFP 耐性迅速診断、ひいては多剤耐性結核の迅速なスクリーニング法として有用であると考えられた。

A. 研究目的

多剤耐性結核の診断の遅れは、患者本人の治療失敗に加えて周囲への感染拡大につながるため、迅速な感受性検査はきわめて重要である。ジェノスカラーRif-TB は、喀痰中の結核菌に存在する RFP 耐性遺伝子である *rpoB* 遺伝子領域の変異をラインプローブアッセイで検出することによる RFP 耐性迅速診断法であり、24 時間以内に結果を得ることができる。RFP 耐性結核の大部分は多剤耐性結核であるため、本法は多剤耐性結核のスクリーニング法としても期待できる。我々は本法の従来法との相関、有用性につき検討した。

B. 研究方法

結核を疑われて当院を受診した患者で、喀痰検査でアンプリコアマイコバクテリウムによる PCR 検査を行って結核菌群陽性と判定された 331 例を対象とした。対象患者の喀痰を用いてジェノスカラーRif-TB を行い RFP 感受性の有無を判定した。同時に、喀痰検体から培養された結核菌に対して通常の MGIT 及びウェルパックによる小川比率法を用いた薬剤感受性検査を行って、結果を比較検討した。

（倫理面への配慮）

いずれも保険収載されている通常の検査キットを用いた検討であり、また retrospective な検討であり、倫理的な問題はないものと考える。

C. 研究結果

331 例のうち、培養陰性であった 8 例を除く 323 例で比較検討を行った。ジェノスカラーRif-TB で RFP 感受性と判定されたのは 308 例であり、307 例は通常の薬剤感受性検査でも RFP 感受性であったが、1 例は MGIT 法では感受性であったが小川比率法では耐性であった。この例は臨床的には排菌陽性が遷延しており RFP 耐性と考えられた。また、ジェノスカラーRif-TB で RFP 耐性と判定された 15 例中、14 例は MGIT 法・小川比率法いずれも RFP 耐性であったが、1 例は MGIT 法で感受性であった。RFP 耐性の 14 例中 11 例(78.6%)は INH にも耐性を示す多剤耐性菌であった。

D. 考察

本法の感度は 93.3%、特異度は 99.7%と優れた結果が得られており、また本法で RFP 耐性と判定された例の 78.6%は多剤耐性であり、多剤耐性結核の迅速なスクリーニング法としても有用であると考えられた。

E. 結論

ジェノスカラーRif-TB による RFP 耐性迅速診断法は、従来法との相関も優れており、多剤耐性結核の迅速診断法としても有用である。

F. 健康危険情報

なし

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H. 知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

別紙4

研究成果の刊行に関する一覧表

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添付資料

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Elevated anti-tuberculous glycolipid antibody titres in healthy adults and tuberculosis patients in Thailand

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SUMMARY

OBJECTIVE: To evaluate immunoglobulin G (IgG) and immunoglobulin A (IgA) responses to tuberculous-glycolipid antigen (TBGL-IgG and -IgA) in pulmonary tuberculosis (TB) patients and healthy controls in Thailand.

DESIGN: Anti-TBGL antibody titres and other TB related markers were measured in the serum samples of 24 adults with pulmonary TB (PTB), 28 healthy adults (HA), 23 children with TB and 24 healthy children.

RESULT: Both TBGL-IgG and -IgA titres were significantly higher only in adult PTB cases compared to controls ($P < 0.001$ for all). TBGL-IgG was highly sensitive (92%) in PTB patients, but frequent positive proportions of TBGL-IgG (46%) and -IgA (36%) in HAs were the cause of low specificities of TBGL-IgG (54%) and -IgA (64%); that of TBGL-IgG+IgA (75%) was the highest. Antibody titres were positively correlated in TBGL-IgG+IgA double-positive HAs (HA+, 7/28, $P < 0.01$), but not in HA− ($P > 0.05$). Serum IgG and IgA levels were not correlated with TBGL-IgG or -IgA levels ($P > 0.05$). KL-6 and leptin levels were normal and were not different between HA+ and HA−, indicating absence of active TB in HAs.

CONCLUSION: Enhanced TBGL-IgG+IgA responses in HAs could indicate latent TB infection. Careful follow-up studies in HAs could clarify the significance of elevated TBGL antibodies as early disease markers.

KEY WORDS: anti-tuberculosis glycolipid IgG; TBGL; IgA; TB-endemic country; latent TB infection

MYCOBACTERIUM TUBERCULOSIS is a leading global health problem that caused an estimated 9.27 million new cases of tuberculosis (TB) infection and more than 2 million deaths worldwide in 2007.[1] The alarming increase in the incidence of multidrug-resistant TB, particularly among human immunodeficiency virus (HIV) infected patients,[1] and the development of the immune reconstitution syndrome after the initiation of highly active antiretroviral treatment (HAART),[2] have rendered the situation more critical. Conventional microscopy, which has a variable range of sensitivity of 20–60% in detecting tubercle bacilli, is widely used by resource-limited countries,[3] which harbour more than 90% of the world's TB infection.[1] However, approximately 20% of TB cases are not microbiologically proven, even with the more expensive fluorescence microscopy.[3,4] Moreover, a bacteriologically confirmed diagnosis of TB in paediatric groups is much more difficult, as children seldom produce sputum. There is therefore an urgent need to develop an early diagnostic approach to identify both paediatric and adult TB patients.

Cord factor (trehalose-6-6-dimycolate; TDM), which composes a major part of the mycobacterial cell wall, has been identified as the most immunogenic glycolipid; it is produced mainly by virulent *M. tuberculosis* as well as by atypical mycobacteria.[5,6] Tuberculous-glycolipid antigen (TBGL) consists of purified TDM from H37Rv.[7] The immunoglobulin G (IgG) response to TBGL antigen (TBGL-IgG) has been proposed as a useful tool for TB serodiagnosis (sensitivity and specificity >80%) in Japan, a non-TB-endemic country (incidence rate 20 per 100 000 population).[7,8] Although IgG and immunoglobulin A (IgA) responses to purified TB antigens and a commercial serological assay were demonstrated to have limited significance for the serodiagnosis of pulmonary tuberculosis (PTB) in a meta-analysis and systemic review by Steingart et al., of the lipid antigens, cord factor showed particularly high reactivity.[9,10] IgA responses against the mycobacterial fusion protein MT10.3: MPT64 was recently demonstrated to have higher sensitivity for the diagnosis of extra-pulmonary TB in a TB-endemic country.[11] Although the diagnostic efficacy

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of TBGL-IgA was not evaluated in prior studies, a significant association between TBGL-IgG and -IgA was reported in active TB patients.[12] However, IgG or IgA responses against TBGL antigen have not been evaluated for their diagnostic ability in TB-endemic countries.

As TBGL-IgG titres were found to be associated with C-reactive protein and cavity formation,[12] other markers related to TB pathology, including KL-6 and leptin, could have some role in promoting inflammation in PTB. A high-molecular-weight mucinous glycoprotein expressed on type-II pneumocytes, KL-6 was reported to be elevated in the serum of patients with interstitial pneumonia[13] and PTB with extensive radiographic changes.[14] Leptin, a cytokine-like hormone produced by the bronchial epithelial cells and type-II pneumocytes in addition to adipose tissue,[15] was reported to be low in the serum of PTB patients.[16]

The purpose of the present study was to evaluate IgG and IgA antibody (Ab) responses to the TBGL antigen in adult and children TB patients and healthy controls in Thailand. The relationship of TBGL antibodies to KL-6 and leptin was also assessed.

MATERIALS AND METHODS

Subjects

A case-control study was conducted between April 2007 and October 2008. Adult cases (age >16 years) were 24 newly diagnosed active PTB patients receiving care at the Chest Disease Institute (CDI), Nonthaburi, who were enrolled before or within 2 weeks of receiving anti-tuberculosis treatment. All of the PTB patients were positive for sputum acid-fast bacilli (AFB) stain and culture for *M. tuberculosis*.

Twenty-three children (age ≤12 years) diagnosed with TB and receiving care at the Queen Sirikrit National Institute of Child Health (QSNICH), Bangkok, were enrolled as child TB cases (CTB) before receiving anti-tuberculosis treatment. They were diagnosed with active TB based on the presence of two or more features suggestive of probable TB, including history of close TB contact, positive tuberculin skin test (TST) response (>10 mm diameter), chest X-ray (CXR) findings suggestive of TB, and histopathological features related to TB according to the diagnostic criteria of the World Health Organization (WHO) provisional guidelines for the diagnosis of paediatric TB.[17] Diagnosis was confirmed by positive culture of tubercle bacilli.

Subjects with underlying malignancy, metabolic disorders, HIV/AIDS (acquired immune-deficiency syndrome) or other active pulmonary diseases were excluded from the study.

Healthy adult individuals with no concomitant pulmonary symptoms, normal CXR and negative HIV serology were recruited from among blood donor subjects as healthy adult controls (HA). Volunteer healthy child controls (HC) were selected from among paediatric patients without respiratory symptoms and with normal CXR from the surgical department of the QSNICH.

Blood samples were collected from all enrolled participants. Serum samples were separated and stored in −20°C for further study.

This study was approved by the ethics committees of all the participating institutes in Thailand and Japan. Written informed consent was obtained from all enrolled participants. The study was conducted according to the recommendations of the Helsinki Declaration.

TBGL antibodies

TBGL-IgG and -IgA titres were measured using the Determiner TBGL-antibody ELISA kit (Kyowa Medex, Tokyo, Japan), an in vitro enzyme-linked immunosorbent assay (ELISA) kit for the quantitative measurement of TBGL-IgG and -IgA in serum or plasma. Antibody titres for both antibodies were expressed as U/ml. Samples were classified as TBGL-IgG-positive if TBGL-IgG serum levels were ≥2 U/ml.[7] An arbitrary cut-off value of ≥2 U/ml for TBGL-IgA was used as per the unpublished data of our previous study.[12]

ELISA assay

Serum leptin and sIL-2Rα levels were determined by sandwich ELISA using the Quantikine Human Leptin Immunoassay kit and the Quantikine Human IL-2 sRα Immunoassay kit (both from R&D Systems, Minneapolis, MN, USA) for the quantitative determination of the human leptin and sIL-2Rα concentrations respectively in serum or plasma according to the manufacturer's guidelines. Serum KL-6 levels were measured using an ELISA kit (Sanko-junyaku, Tokyo, Japan).

Measured laboratory markers

We assessed the whole blood profile as well as the serum levels of IgG and IgA and hepatic enzymes (aspertate amino-transferase [AST] and alanine amino-transferase [ALT]).

Statistical analysis

Data were analysed using Statcel 2 (OMS Publishing Inc, Saitama, Japan). We compared sensitivity and specificity using the χ^2 test for proportions. Values are presented as median and range. Differences in titres of different variables between two groups were analysed using the Mann-Whitney *U*-test. Correlations between each variable were evaluated using Spearman's rank correlation coefficient. A two-tailed $P < 0.05$ was considered significant.

RESULTS

Subjects

The demographic and clinical characteristics of the enrolled case participants are shown in Table 1.

Table 1 Demographic and clinical characteristics of study participants

Variable	Adult PTB cases (n = 24) n (%)	Healthy adults (n = 28) n (%)	Child TB patients (n = 23) n (%)	Healthy child controls (n = 24) n (%)
Male:female*	23:1	19:9	12:11	19:9
Age, years, median [range]	36.5 [20–50]	35.5 [21–52]	2 [0.5–12]	3.5 [0.6–12]
TST responses (>10 mm/<10 mm/0–5 mm)	ND	ND	19/1/3	ND
Sputum AFB stain and culture positive	24 (100)	ND	1 (4)	ND
Chest X-ray				
Normal	—	28 (100)	—	24 (100)
Pulmonary infiltration	8 (33.3)	—	11 (47)	—
Infiltration+fibrosis	1 (4.1)	—		—
Miliary infiltration		—	2 (8.6)	—
Hilar lymphadenopathy		—	9 (39)	—
Consolidation/cavity/calcification	1/1/1 (4 in each)	—	0/0/3 (13)	—
Diagnosis		—		—
PTB	24 (100)	—	21 (91)	—
EPTB		—	2 (9)	—

*Frequency.
PTB = pulmonary tuberculosis; TST = tuberculin skin test; ND = not done; AFB = acid-fast bacilli; EPTB = extra-pulmonary TB.

Among the 58 adult participants screened, 24 microbiologically confirmed PTB cases with male predominance (96%) and 28 age-matched HA subjects (male 68%) were included in the analysis; six PTB cases were eventually excluded due to HIV co-infection. In contrast, *M. tuberculosis* infection was not confirmed in 23 CTB cases except one; 19 (83%) children had positive TST responses (>10 mm diameter), including 12 who had a history of TB contact through family members. Although the TST response was <10 mm (range 0–10 mm) in the other four cases, they also had a history of TB contact. On CXR, 21 had pulmonary infiltration and/or hilar lymphadenopathy and other abnormalities relevant to PTB. Two others had massive pleural effusion and features of non-necrotising granulomatous pruritis suggestive of extra-pulmonary TB. Twenty-four age-matched children with no TB-related symptoms and normal CXR findings were enlisted for analysis as controls (HC).

Anti-TBGL antibodies and their correlations

In the adult participants, the TBGL-IgG and -IgA titres were elevated in respectively 22/24 (92%) and 17/24 (63%) PTB cases and 13/28 (46%) and 10/28 (36%) HAs. TBGL-IgG and -IgA titres were significantly higher in the PTB group than in the controls ($P <$ 0.001 for both; Figure 1A, Table 2). The sensitivities of the TBGL-IgG and -IgA assay were 92% and 63%

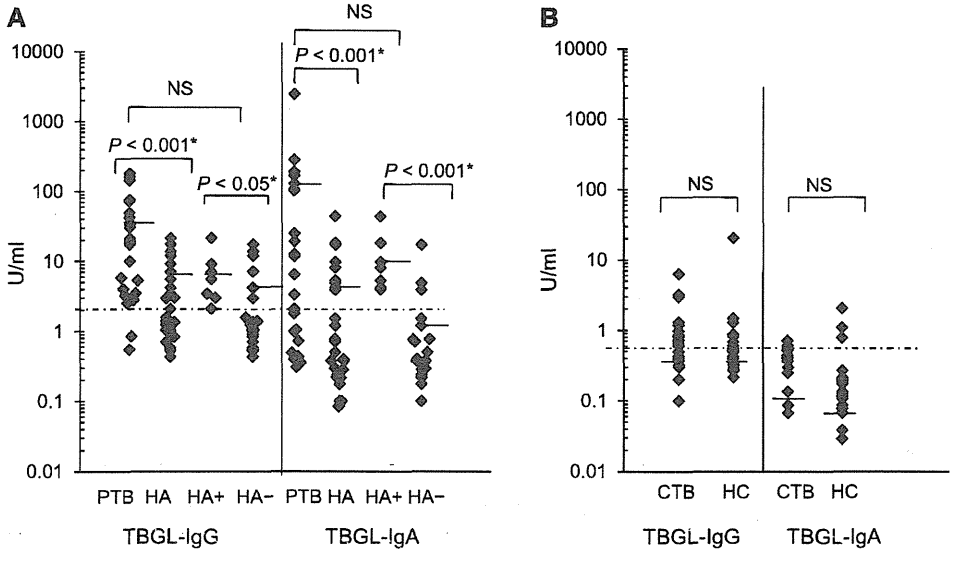

Figure 1 TBGL-IgG and TBGL-IgA titres in **A**) adult and **B**) child participants. Dashed lines indicate the cut-off value of ≥2 U/ml for both antibodies. Solid bars indicate mean values. * Indicates significant difference. NS = not significant; PTB = adult pulmonary TB patients; HA = healthy adult controls; HA+ = HAs with high TBGL-IgG and -IgA titres; HA− = HAs with low TBGL-IgG or -IgA titres or both; TBGL = tuberculous glycolipid; Ig = immunoglobulin; CTB = child TB patients; HC = healthy child controls.

Table 2 Measured parameters and comparison between adult PTB patients and healthy adult controls

Parameter	Adult PTB cases median [range]	Healthy adults median [range]*	*P* value
TBGL-IgG, U/ml	18.7 [0.5–179]	1.5 [0.4–21.4]	<0.001
TBGL-IgA, U/ml	4.9 [0.3–2448]	0.7 [0.08–43.7]	<0.001
Serum IgG, mg/dl	1961 [1433–2835]	1441 [1032–2051]	<0.01
Serum IgA, mg/dl	519 [411–695]	223 [143–861]	<0.01†
KL-6, U/ml	530 [231–1897]	225 [129–592]	<0.001†
Leptin, ng/ml	0.63 [0.13–5.3]	7.7 [0.3–21.6]	<0.001†
sIL-2Rα, ng/ml	2.8 [0.81–15.5]	0.54 [0.1–0.9]	<0.001†
Haemoglobin, gm/dl	12.5 [9.2–14.9]	13.1 [11.1–17.1]	<0.01†
WBC, 10^3/μl	10 [6.8–16.4]	7 [4.6–10.2]	<0.001†
Neutrophil, 10^3/μl	7.08 [5.04–13.78]	3.7 [2.07–6.9]	<0.001†
Lymphocyte, 10^3/μl	1.74 [0.88–3.2]	2.46 [1.85–3.6]	<0.01†
Monocyte, /μl	580 [248–1096]	393 [222–684]	<0.01†
AST, U/ml	25 [15–158]	21 [15–55]	NS
ALT, U/ml	18.5 [7–67]	15.5 [7–75]	NS

*Healthy adults with high titres of both TBGL-IgG and -IgA.
†Significant difference between the two groups ($P < 0.05$).
PTB = pulmonary tuberculosis; TBGL = tubercular-glycolipid; Ig = immunoglobulin; WBC = white blood cells; AST = aspartate aminotransferase; NS = not significant; ALT = alanine aminotransferase.

Table 3 Comparison between TBGL-IgG, TBGL-IgA and combined TBGL-IgG+IgA for their utility in the diagnosis of active pulmonary TB in adults

	TBGL-IgG %	TBGL-IgA %	TBGL-IgG+IgA %	*P* value*
Sensitivity	92	63	63	0.019†
Specificity	54	64	75	0.057

*Statistical difference between TBGL-IgG and TBGL-IgG+IgA groups.
†Significant difference.
TBGL = tuberculous glycolipid; Ig = immunoglobulin.

for the diagnosis of active TB, and the specificities were 54% and 64% (Table 2). Simultaneous detection of both TBGL-IgG and -IgA improved specificity (75%, $P = 0.057$), although sensitivity was significantly lower ($P = 0.019$) than for TBGL-IgG alone (Table 3). To elucidate the cause of high TBGL antibodies in HAs, we therefore further categorised them into two groups: HAs positive for both TBGL-IgG and -IgA (HA+ 7/28, 25%) and others (HA− 21/28, 75%). TBGL-IgG and -IgA titres in the HA+ group were significantly higher than in the HA− group ($P < 0.05$ and $P < 0.01$, respectively) and were not different from those in the PTB groups ($P > 0.05$ for all, Figure 1A). The levels of two antibodies were positively correlated in the HA+ subjects ($r = 0.843$, $P = 0.01$) and among the PTB patients ($r = 0.664$, $P < 0.00005$), but not in the HA− group (Figure 2). TBGL-IgG and -IgA titres were not correlated with those of serum IgG and IgA in the PTB, HA or HA+ groups ($P > 0.05$ for all). No correlation was observed between TBGL-IgG/IgA levels and KL-6 or leptin levels in patients or controls.

In contrast, among the paediatric subjects, only 3/23 (13%) CTB cases and 1/28 (3%) HC had high TBGL-IgG titres, demonstrating the very limited sensitivity (10%) of the assay for the diagnosis of paediatric TB patients. Neither TBGL-IgG nor -IgA titres were significantly different between paediatric cases and controls (Figure 1B).

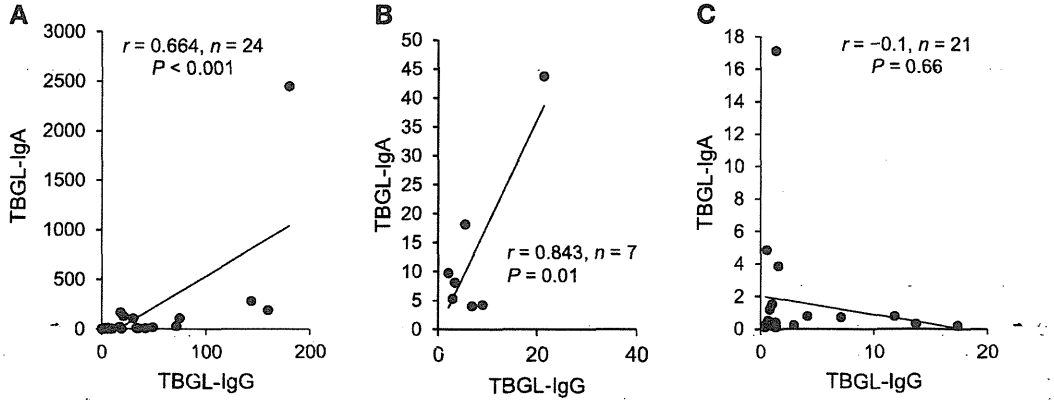

Figure 2 Correlation between TBGL-IgG and -IgA titres. An association was found in **A**) adult PTB patients and **B**) HA+ subjects (healthy adults with high TBGL-IgG and -IgA titres), but not in **C**) HA− subjects (healthy adults with low TBGL-IgG or -IgA titres or both). TBGL = tuberculous glycolipid; Ig = immunoglobulin.

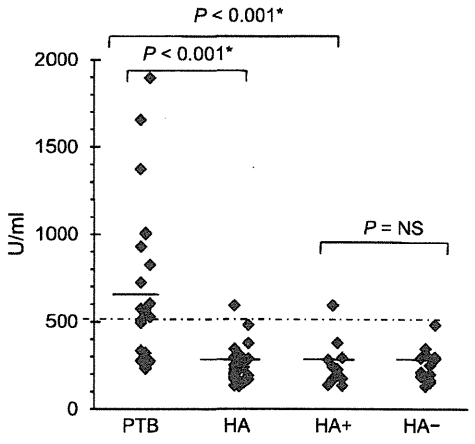

Figure 3 KL-6 titres in adult participants. Dashed line indicates the cut-off value of ≥500 U/ml. Solid bars indicate mean values. *Indicates significant difference ($P < 0.05$). NS = non-significant; PTB = adult PTB patients; HA = healthy adult controls; HA+ = HAs with high TBGL-IgG and -IgA titres; HA− = HAs with low TBGL-IgG or -IgA titres or both; TBGL = tuberculous glycolipid; Ig = immunoglobulin.

KL-6, leptin and sIL-2Rα serum levels, and various laboratory markers

Serum KL-6 levels were significantly higher in PTB cases than in HAs ($P < 0.01$; Figure 3, Table 2) and were elevated (>500 U/ml) in 14/24 (58%) PTB patients. In contrast, significantly lower leptin titres were found in PTB patients than in HA subjects ($P < 0.001$). Serum IgG, IgA, sIL-2α levels and white blood corpuscle and monocyte counts were significantly higher, whereas the lymphocyte count was significantly lower in PTB cases than in HAs (Table 2). There were no significant differences in measured serum IgG, IgA, KL-6, leptin or other parameters between the HA+ and HA− groups (Table 4).

DISCUSSION

We evaluated TBGL-IgG and -IgA levels in paediatric and adult TB patients and healthy controls in Thailand, a TB-endemic country (TB incidence rate 142/100 000 population).[1]

Poor TBGL-IgG and -IgA reactivity was observed in the paediatric TB patients, consistent with previous findings of low antibody responses among child TB suspects against protein antigens, including purified protein derivative (PPD), 38kDa and HSP60.[18] Low TBGL-Ab titres cannot be explained by low serum IgG or IgA, as these were significantly higher in the CTB than in the HC group (data not shown). Although *M. tuberculosis* infection was not confirmed in most of the CTB cases, their clinical and radiological findings were strongly suggestive of active TB, and all responded well to anti-tuberculosis treatment. The cause of the low antibody responses in children is not clear. However, the underdeveloped immune system in young children might play a vital role against the development of specific adaptive immune responses against TB.

In contrast, TBGL-IgG detection in adult PTB patients was revealed to be highly sensitive (92%), in line with a previous report from Japan.[8] However, increased proportions of positive TBGL-IgG in HAs were accountable for the low specificity (54%), and therefore diminished its usefulness as an active TB diagnostic marker in Thailand. The diagnostic ability of TBGL-IgA was also inadequate, showing lower sensitivity and specificity in the current study. However, the specificity was higher than that of TBGL-IgG. Julean et al. also demonstrated high IgA specificity against four trehalose-containing mycobacterial lipid antigens, including cord factor, in a clinical study.[19]

Table 4 Comparison of clinical and laboratory markers between HA+ and HA−

Parameter	HA+ ($n = 7$) median [range]	HA− ($n = 21$) median [range]	*P* value
Male:female*	5:2	14:7	—
Age, years	38 [23–49]	33 [21–51]	—
TBGL-IgG, U/ml	5.5 [2.1–21.4]	1.3 [0.4–17.4]	<0.05†
TBGL-IgA, U/ml	8 [3.9–43.7]	0.3 [0.08–17.1]	<0.001†
Serum IgG, mg/dl	1367 [1281–1943]	1465 [1032–2051]	—
Serum IgA, mg/dl	192 [166–370]	238 [143–861]	—
KL-6, U/ml	227 [132–592]	223 [129–480]	—
Leptin, ng/ml	8.7 [1.14–19.9]	7.5 [0.3–21.6]	—
sIL-2Rα, ng/ml	0.53 [0.1–0.77]	0.55 [0.1–0.9]	—
Haemoglobin, g/dl	13.1 [12.2–5]	13.7 [11.1–17.1]	—
WBC, 10^3/μl	6.4 [5.5–8.1]	7.3 [4.6–10.2]	—
Neutrophil, 10^3/μl	3.46 [2.3–4.5]	4.1 [2–6.9]	—
Lymphocyte, 10^3/μl	2.5 [2–3.1]	2.4 [1.8–3.6]	—
Monocyte, /μl	402 [384–486]	360 [222–684]	—
AST, U/ml	17 [15–23]	21 [15–55]	—
ALT, U/ml	14 [9–24]	16 [7–75]	—

*Frequency.
†Significant difference between the two groups ($P < 0.05$).
HA+ = healthy adults with high titres of both TBGL-IgG and -IgA; HA− = healthy adults with low titres of either TBGL-IgG or -IgA or both; TBGL = tuberculous glycolipid; Ig = immunoglobulin; WBC = white blood cells; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

Elevated TBGL-IgA titres may therefore reflect infection more specifically.

Elevated TBGL-IgG levels were also found in healthy older (>40 years, 17%) and younger adults (<40 years, 5%) in Japan (a non-endemic country); the possibilities of latent TB infection (LTBI) in the TBGL-IgG positive group have already been described by Maekura et al.[8] In this study in Thailand, positive proportions of TBGL-IgG were higher in healthy adults (46%, mean age 34 years) and that of TBGL-IgA was also high (36%). As TBGL-IgG and -IgA titres were not associated with those of serum IgG and IgA, high TBGL antibody titres in endemic HAs cannot be explained by non-specific hyperglobulinaemia. Moreover, none of the HAs had a history of TB. Cross-antibody reactions to other respiratory infections can be excluded, as the HAs were free from respiratory symptoms and had normal CXR findings at the time of enrolment, and bacille Calmette-Guérin vaccination status does not influence antibody production against TDM in adults.[20] It was considered that non-tuberculous mycobacteria (NTM) infection may be responsible for the elevated TBGL-Ab titres in HAs. However, TBGL-IgG titres were reported to increase only in active NTM diseases.[8] Although leptin titres were low in some HAs, none of the TB-related markers, including leptin, KL6 and sIL-2Rα, were different between the HA+ and HA− groups, indicating absence of active disease in HA+. Significant elevations of sensitive TBGL-IgG ($P < 0.05$) and specific TBGL-IgA titres ($P < 0.01$) in HA+ compared to HA− subjects, and the correlation between TBGL-IgG and -IgA titres only in the former group, might be suggestive of the enhancement of TB-specific antibody responses in that group. Although we could not confirm LTBI in HA+ individuals by PPD or an interferon gamma (IFN-γ) release assay (IGRA), a significant association between the QuantiFERON®-TB Gold assay (one of the IGRAs) and the TBGL-IgG assay in healthy adults was documented in our very recent study in the Philippines.[21]

Of note, an increased risk of progression to active TB was correlated with high antibody reactivity to some TB antigens in HIV patients[22,23] and with elevated IFN-γ production to early secreted antigenic target-6 in those with household TB contacts,[24] as the adaptive immune system can recognise antigens produced by early *M. tuberculosis* replication that are thought to be initiated months before the development of active TB.[22,23] However, no follow-up study was undertaken in our HA+ subjects to elucidate risk of active TB.

Taken together, we found that reduced specificity of TBGL-Ab in adult TB patients is due to enhanced humoral immune responses against TBGL in HAs, and that the high TBGL-IgG+IgA reactivity in HA+ controls might be specific and indicative of LTBI. Further extensive evaluation of control subjects from different population groups, including healthy subjects and patients with other pulmonary diseases, and careful follow-up studies, may clarify whether HA+ subjects are at greater risk of development of active TB than in HA− subjects. This might be helpful for the identification of potential markers for early TB diagnosis and the prevention of progressive disease.

Acknowledgements

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RÉSUMÉ

OBJECTIF : Evaluer chez les patients atteints de tuberculose pulmonaire (TBP) et chez les sujets-contrôle sains en Thaïlande des réponses en IgG et en IgA à l'égard de l'antigène tuberculeux-glycolipide (TBGL-IgG et -IgA).
SCHÉMA : Les titres d'anticorps anti-TBGL ainsi que d'autres marqueurs liés à la TB ont été mesurés dans le sérum de 24 adultes avec une TBP, 28 adultes sains (HA), 23 enfants avec une TBP et 24 enfants sains.
RÉSULTATS : Les titres tant de TBGL-IgG que de TBGL-IgA ne sont significativement plus élevés que dans les cas de TBP adultes comparés aux contrôles ($P < 0,001$ pour l'ensemble). Le test TBGL-IgG est très sensible (92%) chez les patients TBP, mais des proportions relativement élevées de TBGL-IgG (46%) et de TBGL-IgA (36%) chez les HA sont les causes d'une faible spécificité respectivement de TBGL-IgG (54%) et de TBGL-IgA (64%). La spécificité la plus élevée est celle de TBGL-IgG+IgA (75%). Les titres d'anticorps sont en corrélation positive chez les HA doublement positifs pour TBGL-IgG+IgA (HA+ 7/28 ; $P < 0,01$) mais non chez les HA− ($P > 0,05$). Les taux sériques d'IgG ou d'IgA ne sont pas en corrélation avec les taux de TBGL-IgG ou de TBGL-IgA ($P > 0,05$). Les taux de KL-6 et de leptine sont normaux et ne sont pas différents entre les HA+ et les HA−, ce qui indique l'absence d'une TB active chez les sujets HA.
CONCLUSION : Les réponses renforcées TBGL-IgG+IgA chez les HA pourraient indiquer une infection TB latente. Une étude soigneuse du suivi chez les sujets HA pourrait clarifier la signification du taux élevé d'anticorps TBGL comme marqueur précoce de la maladie.

RESUMEN

OBJETIVO: Se buscó evaluar la respuesta en IgG e IgA al estímulo con el antígeno glicolipídico de tuberculosis (TBGL) en pacientes con tuberculosis pulmonar (TBP) y en testigos sanos en Tailandia.
MÉTODO: Se cuantificaron los anticuerpos anti-TBGL y otros marcadores relacionados con *M. tuberculosis* en el suero de 24 adultos con TBP, 28 adultos sanos (HA), en 23 niños con TB y 24 niños sanos.
RESULTADOS: La cuantificación de TBGL-IgG y -IgA dio resultados significativamente más altos en comparación con los testigos, solo en los adultos con TBP ($P < 0,001$ en todos). La determinación de TBGL-IgG fue muy sensible (92%) en los adultos con TBP, pero las frecuentes proporciones positivas de TBGL-IgG (46%) y TBGL-IgA (36%) en los adultos sanos condicionaron una baja especificidad de estas mediciones (TBGL-IgG 54%; TBGL-IgA 64%); la especificidad más alta se obtuvo al combinar ambas determinaciones, TBGL-IgG+IgA (75%). Las concentraciones de ambos anticuerpos se correlacionaron en forma positiva en el subgrupo de adultos sanos con ambos títulos (TBGL-IgG+IgA) positivos (HA+ 7/28; $P < 0,01$) pero no en los HA con uno solo de los títulos positivos (HA−, $P > 0,05$). Ni la concentración sérica de IgG ni la concentración de IgA se correlacionaron con las concentraciones de TBGL-IgG o de TBGL-IgA ($P > 0,05$). Las concentraciones de KL-6 y de leptina fueron normales y no mostraron diferencias entre los subgrupos de HA+ y HA−, lo cual indica la ausencia de TB activa en los HA.
CONCLUSIÓN: Un aumento de las respuestas en IgG e IgA al antígeno TBGL en los HA podría estar en favor de una infección tuberculosa latente. Un cuidadoso estudio de seguimiento de los HA podría definir la significación de una alta concentración de anticuerpos contra el TBGL como marcador temprano de TB.

RESEARCH PAPER

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The study of novel DNA vaccines against tuberculosis

Induction of pathogen-specific CTL in the mouse and monkey models of tuberculosis

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Keywords: tuberculosis, vaccines against tuberculosis, CTL, monkeys, mice, HVJ-Envelope, HSP65 DNA+IL-12 DNA Vaccine, granulysin vaccine, Ksp37

Abbreviations: CTL, cytotoxic T cell; Ksp37, Killer-specific secretory protein of 37kDa; 15K granulysin, 15 kilodalton granulysin; HVJ, hemagglutinating virus of Japan; MDR-TB, multi-drug resistant tuberculosis; TB, *Mycobacterium Tuberculosis*

Results: HSP65 + IL-12 DNA vaccine showed higher protective efficacy compared with BCG in both mouse and monkey models of TB. It induced the TB-specific CTL in the mouse model of TB, while little level of activity was observed after the injection of BCG. It also showed strong therapeutic efficacy against MDR-TB. In the monkey model, the vaccine augmented the production of IFN-γ and IL-2 from PBL and the therapeutic effect was correlated with the level of IL-2. We next evaluated the potential of DNA vaccine encoding a granulysin, which is an important defensive molecule expressed by human T cells. We found that granulysin-encoding vaccine induced the differentiation of the CTL in vitro and in vivo. It also showed therapeutic efficacy against TB in the monkey as well as the mouse model. The DNA vaccine encoding a Ksp37 also induced the TB-specific CTL in vitro and in vivo in the mouse model. It augmented the production of IL-2, IFN-γ and IL-6 from T cells and spleen cells. A synergistic effect on the activation of the TB-specific CTL was observed by the combination of Ksp37 DNA vaccine with granulysin DNA vaccine.

Purpose and Methods: Emergence of the multi-drug resistant (MDR) Mycobacterium tuberculosis (TB) is a big problem in the world. We have developed novel TB vaccines [DNA vaccines encoding HSP65 + IL-12, granulysin or killer-specific secretory protein of 37kDa (Ksp37)] using Hemagglutinating virus of Japan -envelope (HVJ-E). It is suggested that the activity of the TB-specific CTL is one of the most important factor for the resistance to TB and immunity for TB in chronic human TB disease. Therefore, we examined the level of activation of the TB-specific CTL after the administration of these vaccines.

Conclusion: These data indicate that our novel vaccines (HSP65 + IL-12 DNA, granulysin and Ksp37) have a capability to activate the TB-specific CTL and will be very strong protective and therapeutic vaccines against TB.

Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from Mycobacterium tuberculosis (TB) infection. The only tuberculosis vaccine currently available is an attenuated strain of Mycobacterium bovis BCG (BCG), although its efficacy against adult TB disease remains controversial. Furthermore, multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant TB (XDR-TB) are becoming big problems in the world. It has been reported that a cytotoxic T-lymphocyte (CTL) is activated during the induction of host protective immune responses to TB.[1-4] In such circumstances, the development of therapeutic vaccine against TB as well as prophylactic vaccine against TB is expected. Therefore, we have recently developed a novel TB vaccine, a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ). This vaccine

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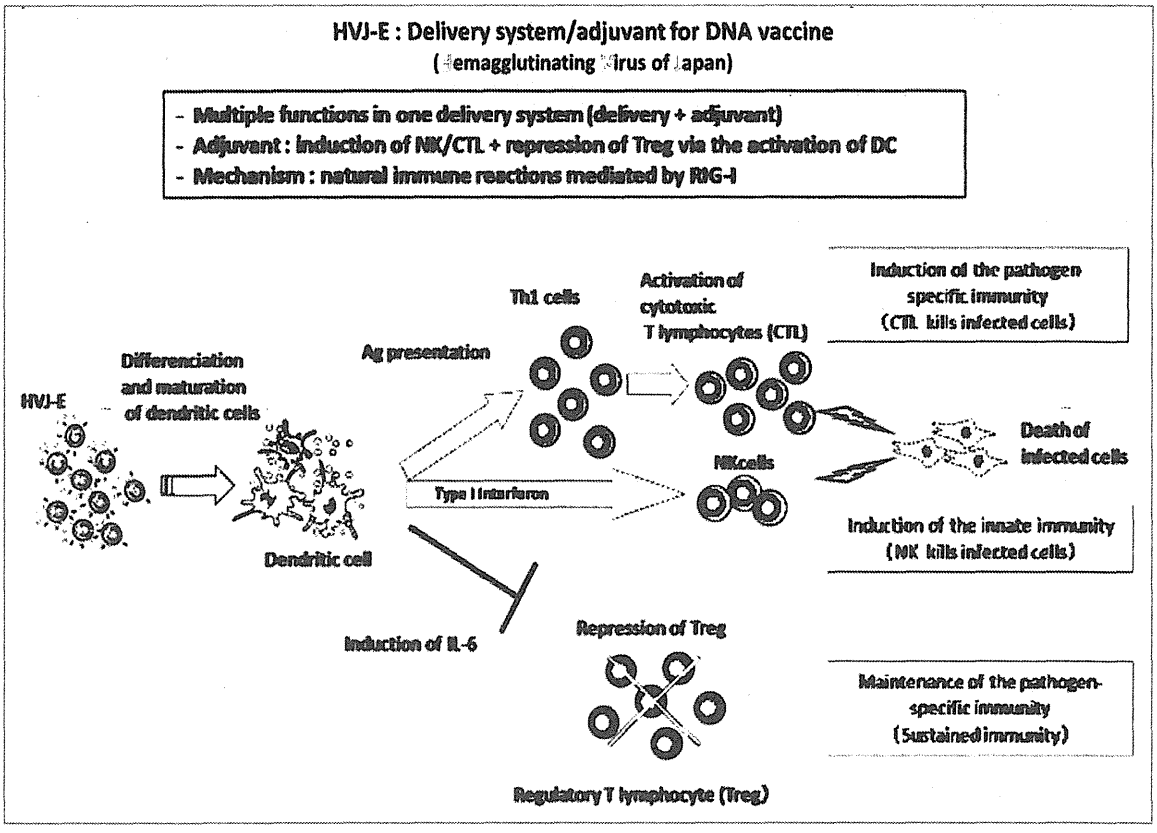

Figure 1. HVJ-Envelope exerts strong adjuvant activity by the induction of CTL/NK and repression of regulatory T cells via the activation of dendritic cells.

was 100 fold more efficient than BCG in the murine model on the basis of the elimination of M. tuberculosis, which is mediated by the induction of CTL.[1,5,6] Furthermore, the HSP65 + IL-12 vaccine delivered by HVJ-envelope was 10,000 fold more efficient than BCG in the murine TB-prophylactic model. This vaccine induced a strong activity of CTL against TB, while BCG vaccine induced little activity of CTL in the same model. It is considered that CTL is most important lymphoid cells for the immunity to TB in chronic human TB diseases. In the present study, we analyzed CTL activity and IFN-γ production after the vaccination with our vaccines. We also evaluated the prophylactic effect in the cynomolgus monkey and mouse models of TB. A nonhuman primate model of TB is an excellent model of human tuberculosis and provides a lot of information for vaccine development. In fact, we previously evaluated the protective effects of HSP65 + IL-12/HVJ vaccine in the cynomolgus monkey model and obtained a data indicating the synergistic effect of the HSP65 + IL-12/HVJ and BCG injected by a prime-boost method.[5-7] The combination of the two vaccines showed a strong prophylactic efficacy in the monkey model infected with M. tuberculosis (100% survival). We have previously obtained a similar data in the monkey model of TB.[5,6,8] In the present study, we examined the production of cytokines (IFN-γ and IL-2) from PBL and revealed the correlation of cytokine levels and efficacy in the monkey model of TB. We also compared the production levels of these cytokines between the combinatorial vaccination (BCG prime and HSP65+IL12/HVJ vaccine boost) group and BCG vaccination group. We also evaluated the potential of other novel vaccines (DNA vaccines encoding granulysin or Ksp37), which were expected to induce the differentiation of CTL against TB. Granulysin is a protein secreted from T cells and NK cells and has an antibacterial effect on TB. Killer-specific secretory protein of 37 kDa (Ksp37) vaccine also showed anti-TB efficacy mediated by the induction of CTL. Synergistic effect on the activation of CTL in vitro was observed by the simultaneous administration of Ksp37- and granulysin-based vaccines. In the present study, we further demonstrated the correlation of the activation of CTL and the efficacy of these novel vaccines (HSP65 + IL-12/HVJ-E DNA vaccine, granulysin vaccine and Ksp37 vaccine) in the mouse and monkey models.

Results

Induction of CTL by HSP 65+IL-12/HVJ DNA vaccine in the mouse model of TB. The advantage of HVJ-Envelope vector is shown in **Figure 1**. (1) HVJ-Envelope is efficient delivery system and functions as an adjuvant for DNA vaccine, (2) It induces CTL and NK cell, (3) It induces a production of IL-6 which suppresses the regulatory T cell (T reg) and (4) It activates the innate immunity by the stimulation of RIG-I signaling pathway.

Mice were immunized three times with the DNA vaccine using HVJ-Envelope every three weeks. Four weeks after last