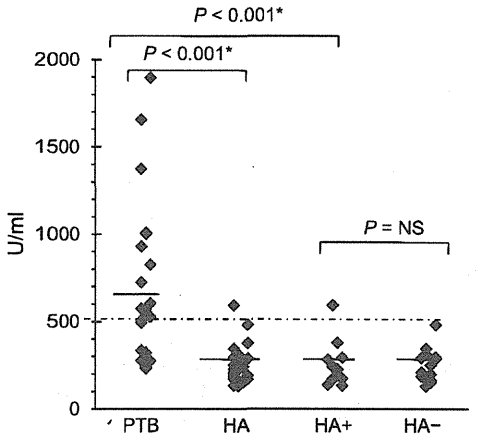

Figure 3 KL-6 titres in adult participants. Dashed line indicates the cut-off value of ≥500 U/ml. Solid bars indicate mean values. *Indicates significant difference ($P < 0.05$). NS = non-significant; PTB = adult PTB patients; HA = healthy adult controls; HA+ = HAs with high TBGL-IgG and -IgA titres;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

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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.

リファンピシン単独耐性結核菌の分子疫学的解析

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要旨：〔目的〕リファンピシン（RFP）単独耐性結核菌の遺伝子解析。〔方法〕初回・既治療を合わせた新規入院患者由来結核菌4,633株中RFPのみ耐性であった15株を用いてhot spotを含む*rpoB*遺伝子シークエンス解析を行い，VNTRによる各菌株の遺伝的異同を検討した。〔結果〕15株中14株にコドン531，526，516，533上の*rpoB*変異が認められた。また，VNTRにより同一パターンを示すグループの存在が明らかとなった。〔考察〕今回全体に占めるRFP単独耐性菌の割合は低いため，これらの株が市中伝播している可能性は低いが，経年的に散発している傾向にあり，RFP単独耐性株の地域拡散が懸念された。

キーワーズ：結核菌，Rifampicin，*rpoB*遺伝子，薬剤感受性検査，VNTR

はじめに

わが国の2009年における全結核患者数は24,170人であり，罹患率は10万対19.0と前年と比べて約2％の減少にとどまっている[1]。また，治療が困難な多剤耐性結核菌（MDR-TB）感染は結核対策上の重要な問題であるが，2002年の結核療法研究協議会が実施した全国調査によると，多剤耐性率は初回・再治療を合わせて1.9%（60/3,122）となっている[2]。2009年結核サーベイランスによる新登録肺結核菌培養陽性患者による登録時薬剤感受性結果から初回・再治療を合わせた多剤耐性率は0.8%（56/6,920）であり，多剤耐性率の減少がみられるが20～40歳代の年代層に耐性結核患者が目立つ[1]。これに対して世界では，2008年には44万人がMDR-TB感染であるとされ，2009年の結核新規登録患者940万人の3.3%がMDR-TBであると推定されている（WHO推計）[3]。世界の結核罹患状況から鑑みて，わが国は中蔓延国ではあるが先進国の中では依然高く，地域的に耐性結核菌の増加が懸念されている高蔓延地域に囲まれている環境上，耐性結核菌の早期発見と適切な治療は重要な意味をもつ。

MDR-TBが生まれる背景として，不充分な服薬や中断による獲得耐性が挙げられ，それらの患者は2～3%の割合で再発すると言われている[4]。一方，不幸にも耐性結核の患者から感染を受けて発病した人は最初から薬剤耐性となってしまう。このような薬剤耐性結核の拡大防止のために，公衆衛生の強化と並んで耐性結核菌の病原性や発症原因に関する研究が従来から行われているが，結核治療において重要な治療薬の一つであるイソニアジド（INH）の単剤耐性結核菌は世界的に高い頻度で分離されているのに比べ，同じく一次抗結核薬であるリファンピシン（RFP）単独耐性の分離頻度は稀であるため，原因究明のための充分な解析がなされていない。本研究では，当センターで分離できたRFP単独耐性結核菌の耐性遺伝子変異と分子疫学的解析を行い，その実態に迫った。

方　法

〔対　象〕

2001～2009年の間，当センターに新規入院患者由来でBACTEC MGIT960システム（日本ベクトン・ディッキンソン）と小川KY培地（セロテック）を用いた培養検査で陽性となり，小川培地上でコロニー性状を確認，さらに自家製PNB培地で複数菌の混在を否定した後，アンプリコア・マイコバクテリウムツベルクローシス（ロ

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シュ・ダイアグノスティックス）とキャピリアTB（タウンズ）を用いて結核菌群と同定された4,633株を対象とした。これらの株は一度も治療歴のない初回治療患者由来の3,644株と過去に治療歴のある既治療患者由来の989株に分類された。これらの株を再培養し，単一コロニーを採取した後，BACTEC MGIT960 AST（日本ベクトン・ディッキンソン）とウエルパック-S（日本ビーシージー）を用いて薬剤感受性検査結果を確認した。MGIT-AST法と小川比率法の結果がともにRFP耐性，INH感受性とされた15株を遺伝子解析の対象とし，MGIT-ASTでINH（0.1 μg/ml）耐性，小川比率法でINH低濃度（0.2 μg/ml）感受性となった3株は対象から外した。

〔RFP耐性*rpoB*遺伝子のシークエンス解析〕

Kimらの方法[5]に準じてRFP単独耐性結核菌のシークエンス解析を行った。プライマーセットはMF（5'-CGACCACTTCGGCAACCG）とMR（5'-TCGATCGGGCACATCCGG）を用い，hot spotの81-bpを含む306-bpの*rpoB*遺伝子領域を3500 Genetic Analyzer（Applied Biosystems）を用いて解析した。得られたデータはBlast解析により，データベースと比較検討した。

〔VNTR解析〕

Supplyら[6]によって提唱された国際標準法である15 MIRU-VNTRと，国内標準法として提唱されているJATA12-VNTR[7]に加えて，わが国で高い分解能をもつ超多変領域（Hypervariable，HV）の3領域[8]を用いて遺伝子型別比較を行った。15MIRU-VNTRとJATA12-VNTRはアガロースゲル電気泳動とキャピラリー電気泳動（Agilent Technologies），HV領域は3500 Genetic Analyzerを用いてPCR産物の分子量を算出し，コピー数の換算を行った。

結　果

〔RFP単独耐性結核菌の分離頻度〕

初回治療患者由来3,644株に占めるRFP単独耐性結核菌の分離頻度は0.4%（13株），INH単独耐性は3.6%（132株），MDR-TBは1.1%（40株）であった。一方，既治療患者由来989株における分離頻度は順にRFP単独耐性0.2%（2株），INH単独耐性は4.6%（45株），MDR-TBは9.1%（90株）であった。

〔*rpoB*シークエンス解析結果〕

RFP単独耐性結核菌15株中14株は*rpoB*遺伝子領域に変異を認めた。これらの株はコドン531に変異を保有する9株と526に変異のある3株，516と533に変異が認められた各1株ずつであった。*rpoB*遺伝子解析で野生型であった1株はMIC測定で32 μg/ml以上と判定されたため，今回解析対象とした*rpoB*遺伝子領域以外に変異をもっていると考えられた（Table）。

〔VNTR解析〕

15株のうちすべての領域でVNTRのコピー数が一致した2株で構成されるグループAと，3株で構成されるグループBが認められ，残り10株はそれぞれに異なるVNTRパターンを示した。また，今回クラスターを形成しなかった菌株No.207のVNTRパターンは当センターにおける既治療MDR-TB患者2名の由来株と同一であった（Table）。

考　察

多剤耐性結核や慢性持続排菌者となる原因の一つに既に耐性菌感染であることを知らずに治療して，残りの感受性である薬剤を次々に耐性化させてしまうといった人為的な要素が考えられる。現在の結核治療では単剤治療は禁忌であり薬剤感受性検査で耐性薬剤を確認することは必須であるが，迅速に耐性菌を検出するにはRFP耐性株の約95%が変異を有する*rpoB*遺伝子のコドン507からコドン533までの27個のアミノ酸（hot spot領域：81 bp）の遺伝子変異を解析して耐性を確認する方法が有益である[9]。今回の研究結果から，RFP単独耐性結核菌の93.3%に*rpoB*遺伝子変異が認められ，いずれもhot spot内の変異であることが認められた。*rpoB*遺伝子変異の割合は高い順にコドン531（60%），526（20%），533と516（各6.7%）となり，当センターのMDR-TBの割合〔コドン531（62.9%），526（20%），533（5.7%），516（2.9%）：データ未掲載〕とわが国のMDR-TBの割合[10]とほぼ同じとなった。しかし，RFP単独耐性結核菌が少ないためMDR-TBとの間に何らかの有意な違いが示しにくく，さらに患者背景等を考慮せねばならないことから直ちに結論付けることは難しい。

わが国の2002年の全国調査で，未治療患者の8.6%，既治療患者の25.2%は主要な抗結核薬のいずれか一つに耐性をもっており，未治療患者の0.2%，既治療患者の0.5%はRFPにのみ耐性をもっているという[2]。これらのRFP単独耐性率は，INH単独耐性率（未治療群1.2%，既治療群6.2%），MDR-TB率（未治療群0.7%，既治療群9.8%）に比べて低いが，未治療群よりも既治療群のほうが高い。本研究で得られた未治療患者におけるRFP単独耐性，INH単独耐性，MDR-TBの耐性率（順に0.4%，3.6%，1.1%）と既治療患者群の耐性率（順に0.2%，4.6%，9.1%）を比べるとほぼ既治療患者群のほうが高く矛盾しない。唯一，既治療群のRFP単独耐性率が未治療群より低いのは菌株数の少なさに影響されたと思われる。しかし，国際的には既治療患者のRFP単独耐性率は高い傾向がうかがえ[11)~13)]，多剤耐性化を未然に防ぐためにRFP単独耐性結核患者が発見された場合は他の耐性結核と同じく慎重に，適切な治療を施す必要がある。

Table Profiles of variable number of tandem repeat (VNTR) recognized as 15 rifampicin mono-resistant *M.tuberculosis* isolates and H37Rv and distribution of the *rpoB* mutations

Isolates no.	Date (YY/MM/DD)	JATA12 (No./Alias (15MIRU-VNTR))												Hypervariable (HV)			Locus for international comparison*						Identical group	Mutation codon no. (the change in nucleotide sequence)
		J01	J02	J03	J04	J05	J06	J07	J08	J09	J10	J11	J12											
		t4	M10	t21	t24	Q11b	VNTR 2372	M26	Q15	M31	Q3336	Q26	Q4156	Q3232	Q3820	Q4120	M04	M16	M40	EC	t30	t39		
153	2005/02/03	4	3	3	3	6	3	7	4	7	5	8	3	15	19	6	2	4	3	4	4	3		526 (cac→gac)
394	2005/04/15	3	3	4	3	5	3	7	2	5	15	9	2	15	9	8	2	3	2	4	4	3		531 (tcg→ttg)
519	2005/06/07	4	1	3	2	9	4	9	4	5	7	8	5	16	14	12	2	3	3	4	4	3		516 (gac→gtc)
852	2005/09/20	4	8	3	2	7	4	7	4	4	10	8	2	10	12	3	2	2	3	4	2	1	A	531 (tcg→ttg)
210	2006/02/27	4	1	3	2	6	4	7	4	3	8	8	5	30	16	9	2	3	3	4	4	4		526 (cac→ctc)
883	2006/10/13	4	8	3	2	7	4	7	4	4	10	8	2	10	12	3	2	2	3	4	2	1	A	531 (tcg→ttg)
929	2006/10/30	4	3	4	3	6	3	4	4	5	7	8	3	13	20	10	2	3	3	4	4	3	B	531 (tcg→ttg)
1045	2006/12/07	2	3	1	3	4	2	5	1	3	12	6	5	5	5	2	2	3	1	4	2	4		none
441**	2007/06/14	4	3	3	2	7	3	7	4	5	7	9	5	15	12	9	2	3	3	4	4	3		531 (tcg→ttg)
207	2008/03/05	4	3	4	3	4	3	7	4	5	7	8	3	14	14	8	2	3	3	4	4	3	***	531 (tcg→ttg)
413	2008/05/29	4	3	4	3	6	3	4	4	5	7	8	3	13	20	10	2	3	3	4	4	3	B	531 (tcg→ttg)
21**	2008/12/17	5	3	2	2	7	3	7	4	5	7	9	5	15	12	8	2	3	3	4	4	4		526 (cac→tac)
103	2009/02/02	3	3	4	3	5	3	7	2	4	17	9	5	12	9	8	2	3	2	4	4	3		533 (ctg→ccg)
923	2009/11/27	4	3	4	3	6	3	4	4	5	7	8	3	13	20	10	2	3	3	4	4	3	B	531 (tcg→ttg)
739	2009/12/07	4	2	4	3	6	1	7	4	5	7	8	4	15	18	10	2	3	3	4	4	3		531 (tcg→ttg)
H37Rv		2	3	1	4	5	2	3	4	3	8	5	3	4	3	2	3	2	1	4	2	5		

*The six loci composed the international Supply's 15MIRU-VNTR by combination with JATA12-VNTR.
**The patients were classified as previously treated.
***The VNTR pattern were concordant with pattern of MDR-TB strains from 2 patients.

RFP単独耐性結核患者のリスクファクターは他の抗結核薬と同様，結核の治療歴の有無，服薬コンプライアンスの低下や不適切な治療による獲得耐性，HIV複合感染患者におけるINH，エタンブトール（EB）の吸収阻害（malabsorption）から生じるRFP耐性化[10]などが挙げられる。今回の対象はすべてHIV検査陰性であり，1名の既治療患者（No.441）以外は過去に結核症や結核以外の感染症でRFPを用いた化学療法を施された形跡はなかった。既治療患者No.441の初回治療時の感受性は不明だったが標準治療が施されており，同じく既治療のNo.21は初回治療時に既にRFP単独耐性であり，仮にRFP耐性化した株に感染したと考えるならば，これらの菌は伝播力が高い株であると思われる。同じVNTRパターンをもつグループAの2患者は，大阪府南部の隣接した市町村に居住していたが菌の分離時期は1年以上離れ，直接の接触歴も認められなかったため，実際同一菌株かどうかははっきりしない。しかし，結核菌が長期的に休眠と再燃を繰り返しながら広範囲に拡散し伝播する病原体であることから，地域で同一のRFP単独耐性結核菌が蔓延している可能性は拭いきれない。グループBの菌株も約4年間に散発しており，グループAと同様に同一クローン株である可能性が示唆され，地域内で感染拡大している危険性は無視できない。また，今回クラスターを形成しなかった菌株No.207と2名の既治療MDR-TB患者由来株は同じVNTRパターンを有していたが，両者に接触歴がないことからNo.207の多剤耐性誘導の可能性が示唆された。したがって，RFP単独耐性を含む耐性結核患者が関与する感染事例が発生した際には，接触者検診対象者に対する潜在性結核治療の徹底が求められる。

結核治療において，過去の結核治療歴や薬剤耐性を有する患者との接触の可能性，その地域での薬剤耐性状況等の情報を把握し薬剤耐性を有している可能性を常に考慮することは自明である。本研究から，上記の項目に加えて，耐性結核菌の分子生物学的特徴の把握と分子疫学解析の継続的な展開の重要性が示唆された。

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Short Report

MOLECULAR EPIDEMIOLOGY OF RIFAMPICIN MONO-RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

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Abstract [Purpose] We aimed to investigate the prevalence and possible transmission routes of rifampicin (RFP) mono-resistant *Mycobacterium tuberculosis* strains.

[Methods] Drug susceptibility testing was used to identify 15 RFP-resistant strains out of 4,633 *M.tuberculosis* isolates. Sequencing of the *rpoB* gene and VNTR analysis were performed to further confirm the genetic classification.

[Results] Resistance-conferring mutations in the RFP resistance-determining region (RRDR) of the *rpoB* gene were found in 14 of the 15 strains with phenotypic RFP mono-resistance. VNTR analysis revealed 2 clusters of 5 identical strains each.

[Conclusions] Although the community prevalence of RFP mono-resistant *M.tuberculosis* is low, the results of VNTR analysis suggested that rather than being recently transmitted, these strains may have been widely transmitted as latent infections in the population.

Key words : *Mycobacterium tuberculosis*, Rifampicin, *rpoB* gene, Drug susceptibility testing, VNTR

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Clinical Study

Frequent Detection of Anti-Tubercular-Glycolipid-IgG and -IgA Antibodies in Healthcare Workers with Latent Tuberculosis Infection in the Philippines

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Anti-tubercular-glycolipid-IgG (TBGL-IgG) and -IgA (TBGL-IgA) antibodies, and the QuantiFERON-TB Gold test (QFT) were compared in healthcare workers (HCWs, $n = 31$) and asymptomatic human immunodeficiency virus-carriers (HIV-AC, $n = 56$) in Manila. In HCWs, 48%, 51%, and 19% were positive in QFT, TBGL-IgG, and -IgA, respectively. The TBGL-IgG positivity was significantly higher ($P = 0.02$) in QFT-positive than QFT-negative HCWs. Both TBGL-IgG- and -IgA-positive cases were only found in QFT-positive HCWs (27%). The plasma IFN-γ levels positively correlated with TBGL-IgA titers ($r = 0.74$, $P = 0.005$), but not TBGL-IgG titers in this group, indicating that mucosal immunity is involved in LTBI in immunocompetent individuals. The QFT positivity in HIV-AC was 31% in those with CD4+ cell counts >350/μL and 12.5% in low CD4 group (<350/μL). 59 % and 29% were positive for TBGL-IgG and -IgA, respectively, in HIV-AC, but no association was found between QFT and TBGL assays. TBGL-IgG-positive rates in QFT-positive and QFT-negative HIV-AC were 61% and 58%, and those of TBGL-IgA were 23% and 30%, respectively. The titers of TBGL-IgA were associated with serum IgA ($P = 0.02$) in HIV-AC. Elevations of TBGL-IgG and -IgA were related to latent tuberculosis infection in HCWs, but careful interpretation is necessary in HIV-AC.

1. Introduction

Although the incidence of tuberculosis has been falling since 2002, there were still 8.8 million incident cases of TB, 1.1 million deaths from TB, and an additional 0.35 million deaths from HIV-associated TB in 2010 [1]. The high rate of latent TB infection (LTBI) is one of the factors that make it difficult to achieve global control and eliminate TB [2]. The recent introduction of the immune-based interferon-γ release assay (IGRA) made a great impact on facilitating the diagnosis of LTBI [3] and clarified the high rate of infection in TB-high-risk populations including healthcare workers (HCWs) [4]. Attempts to detect LTBI in HIV-infected individuals were also facilitated by the development of IGRA, although their higher rates of pseudonegative IGRA response due to low CD4+ T cell counts and diminished Th1 immunity cannot be ignored [5]. Trehalose 6,6-dimycolate (TDM), which constitutes a major part of the mycobacterial cell wall, was identified as the most immunogenic glycolipid and is produced predominantly by virulent MTB as well as by atypical mycobacteria [6]. Tubercular-glycolipid antigen (TBGL) consists of TDM purified from virulent mycobacterial strain H37Rv [7, 19]. The immunoglobulin-G to tubercular-glycolipid antigen (TBGL-IgG) has been proposed to be a useful marker for the serodiagnosis of active pulmonary tuberculosis (PTB) in Japan [7]. However, frequent elevated titers (17%) were also found in healthy elderly control people (age >40 years) in the same study,

and the possibility of LTBI was suggested by Maekura and colleagues [7]. Although IgA antibody to TBGL antigen (TBGL-IgA) was not evaluated earlier as a biomarker, strong association was revealed between the TBGL-IgG and-IgA titers in PTB cases [8]. Frequent positivity for TBGL-IgG (46%) and -IgA (36%) in healthy adults was also observed in our very recent study in Thailand, a TB-endemic country [9]. The TBGL-IgG-positive responses were not related to BCG vaccination [10]. Since both cellular-mediated and humoral immunity are necessary for an effective immune response against MTB, we aimed to clarify the relationship between the TBGL-IgG and -IgA responses with QuantiFERON-TB Gold In-Tube (QFT) assay system, in healthcare workers (HCWs) in a hospital of the Philippines.

Infection of human immunodeficiency virus (HIV) has substantially boosted the occurrence of tuberculosis (TB) disease worldwide [1]. The devastating association between HIV and TB is responsible for one of four TB-related deaths [11]. The East-Asian countries are predominantly TB endemic [1]. Similarly to Sub-Saharan Africa, the rapid, progressive increase of HIV infections in East-Asian countries may further accelerate TB infection in HIV/AIDS patients [12]. To clarify how HIV infection may alter immune responses in LTBI, newly diagnosed, asymptomatic, non-TB HIV-infected individuals were studied.

To understand the health condition of the individuals, we measured two TB-related biomarkers. Leptin, a cytokine-like hormone produced by bronchial epithelial cells and type II pneumocytes in addition to adipose tissue, exhibits a Th1-bias immune response [13]. Osteopontin (OPN) is a member of extracellular matrix proteins that is synthesized within the immune system by activated T cells, NK cells, dendritic cells, and macrophages. Involvement of OPN in Th1 immune responses has been reported [14]. OPN deficiency was found to be associated with the dissemination of mycobacterial disease, and its expression correlated with an effective immune and inflammatory response against mycobacteria in rodents as well as in human [15, 16]. Elevated levels of circulatory plasma OPN [17] and low levels of leptin [18] were reported to be associated with active tuberculosis; these biomarkers served as a negative evidence of active disease.

2. Materials and Methods

2.1. Study Subjects. A case-control study was conducted between March and October of 2010 in adult participants (age > 18 years) in the Philippines. Thirty-one healthy, adult healthcare workers (HCWs) without any concomitant symptoms or chest radiographic findings relevant to active TB and who had negative HIV serology were recruited from San Lazaro Hospital (SLH), Manila, Philippines. Fifty-six newly diagnosed, asymptomatic HIV carriers (HIV-AC) without any clinical symptoms relevant to tuberculosis were randomly selected from among patients receiving care at the outpatient department of the SLH. None of the subjects took any anti-HIV therapy. Subjects with AIDS-defining events, currently active tuberculosis, or any symptoms relevant to tuberculosis, other than active pulmonary diseases, underlying malignancy or metabolic disorders were excluded from the study. The exclusion criteria for active tuberculosis were based on both clinical findings and chest X-ray (CXR) findings in the HCWs. The study was approved by the ethics committee of SLH and the Tohoku University Hospital. We obtained written informed consent from all the participants. Three mL of blood was obtained directly (one mL in each tube) from each participant to perform the QFT assay. Simultaneously, plasma was separated from blood by centrifugation after treatment with EDTA and was aliquoted to CryoTubes for storage at −80°C until further utilization. All the procedures were conducted in accordance with the Helsinki declaration.

2.2. TBGL-Antibody Assay. TBGL-IgG antibody and -IgA antibodies were measured using the Determiner TBGL Antibody ELISA kit (Kyowa Medex, Tokyo, Japan), an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of anti-TBGL-IgG and -IgA in plasma. This assay employs glycolipid antigens purified from *M. tuberculosis* H37Rv (TBGL antigen) coated on a 96-well plate. The details of the assay were described in our previous study [8]. The antibody titers for TBGL-IgG and -IgA were expressed as U/mL. Positive TBGL-IgG titers were determined according to the cutoff index proposed by Kishimoto et al. [19]. The samples were classified as positive when the serum levels of anti-TBGL-IgG were ≥2 U/mL. An arbitrary cutoff value of ≥2 U/mL for TBGL-IgA was used according to the unpublished data of our previous study [8].

2.3. QuantiFERON-TB Gold In-Tube (QFT). The QFT test was performed using fresh whole blood in accordance with the manufacturer's instruction (Cellestis, Australia). The results were interpreted using specific software provided by Cellestis. The result was scored positive if the IFN-γ concentration in the tube TB-specific antigen containing was >0.35 IU/mL after subtracting the value of the nil control (IFN-γ-nc) and at least >25% of NC value. If the net IFN-γ response (TB Ag minus nil) was <0.35 IU/mL for the antigens and the response to the mitogen-positive control was >0.5 IU/mL, the response was considered as test negative. An intermediate result was recorded if the net IFN-γ response was <0.35 IU/mL for the antigen and <0.5 IU/mL for the mitogen and/or was above 8 IU/mL for the NC.

2.4. Leptin and OPN Elisa Assay. Plasma leptin levels were determined by sandwich ELISA using Quantikine Human Leptin Immunoassay kit (R&D Systems) for the quantitative determination of the human leptin concentrations in plasma according to the manufacturer's guidelines. Plasma OPN concentrations were determined using Human OPN Elisa kit (Immuno-Biological Laboratories, Takasaki, Japan) according to the manufacturer's guidelines, and values were expressed as ng/mL.

2.5. Clinical Data. We measured different laboratory markers including complete red blood cell counts, the number of white blood cells with their differential counts, levels of

hemoglobin, and serum levels of IgG and IgA. The number of CD4+ T cell counts and HIV RNA load of HIV-AC were also determined.

2.6. Statistical Analysis. The data of quantitative variables are summarized as median and range. Categorical variables were computed as frequency and percentage. The data were analyzed using Stat Flex software, version 5 (Artech Co., Ltd: http://www.statflex.net/index.html) and Statcel 2 (OMS Publishing Inc. Saitama, Japan). The ability of each single marker to discriminate HIV from HCW by receiver operating characteristic (ROC) curve and the area under curve (AUC) was also analyzed. The percentage of overall agreement between QFT and TBGL-IgG/IgA ELISA assays was calculated, and a Cohen's Kappa coefficient was used to assess the level of agreement. The significance of association for categorical variables was estimated by Fisher's exact test, whereas correlations between continuous variables were evaluated by Spearman's rank correlation coefficient. The differences in significance between continuous variables were compared by the Mann-Whitney U test. A 2-tailed P value of <0.05 was considered significant.

3. Results

3.1. Characteristics of Study Participants. A total of 31 HCWs and 56 newly diagnosed HIV-AC were enrolled in the current study. Basic demographic and clinical characteristics of the study participants are shown in Table 1. The participating HIV-AC were relatively young ($P = 0.03$) with a significant male predominance ($P < 0.0001$) compared to the HCWs. Although lymphocyte counts were comparable between the two groups, total counts of WBC, neutrophils, and monocytes were significantly lower in HIV-AC.

3.2. QFT and TBGL-Antibody Assays in HCWs. Forty-eight percent (15/31) of the HCWs showed positive reactions in the QFT assay indicating high incidences of LTBI (Table 1). The median age of the QFT-positive responders from among the HCWs were significantly higher than those of the QFT-negative group ($P = 0.002$). TBGL-IgG and TBGL-IgA were positive in 51% and 19% of HCWs, respectively (Table 1).

Eleven of 15 (73%) QFT-positive HCWs had positive TBGL-IgG responses (categorical agreement 73%), whereas 5 of 16 (31%) QFT-negative subjects had positive TBGL-IgG responses (categorical agreement 68.7%). The overall κ value was 0.42, indicating a moderate association between the two assays (overall agreement: 71%; 95% CI: 0.10~0.73). The TBGL-IgG-positive proportions were also significantly different between QFT-positive and QFT-negative groups of HCWs ($P = 0.02$). Although the number of positive TBGL-IgA responders was small in HCWs and failed to show any significant difference ($P = 0.072$), the TBGL-IgG+IgA double-positive response was shown only by QFT-positive HCWs and none of the QFT-negative HCWs had double-positive reactions ($P = 0.043$) (Figure 1) (Table 2).

In addition, significant positive correlation was observed between the concentrations of IFN-γ-nc and TBGL-IgA titers in the QFT-positive group ($r = 0.74$, $P = 0.005$) (Figure 2), but not in the QFT-negative group. There was no such association between IFN-γ-nc and TBGL-IgG levels in HCWs, although a tendency for a positive correlation was observed in the QFT-positive HCWs ($r = 0.43$, $P = 0.11$) (Figure 2). No association was observed in the net IFN-γ concentrations in antigen-stimulated QFT-plasma with TBGL-IgG or -IgA titers (data not shown). The plasma levels of OPN and leptin were not different between QFT-positive and QFT-negative HCWs (Table 2).

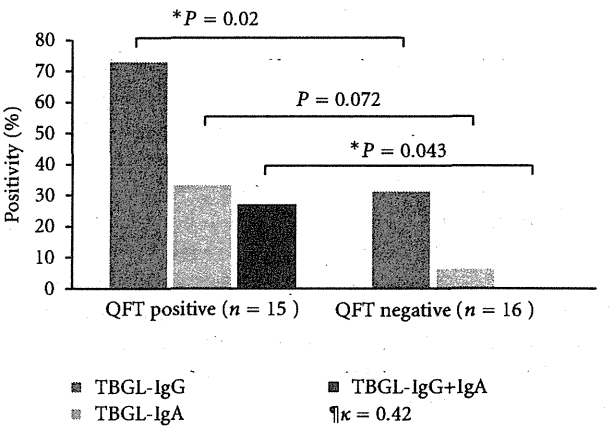

FIGURE 1: Positivity percentage of TBGL-IgG/IgA assay in QFT-positive/negative healthcare workers. The level of agreement between QFT and the TBGL-Ab assay was measured by Cohen's kappa (κ). ¶κ = 0.42; overall agreement 71%; 95% confidence interval: 0.1~0.73.*Significant difference ($P < 0.05$).

3.3. QFT and TBGL-Antibody Assays in HIV-AC. As shown in Table 1, only 13 of 56 (23%) HIV carriers showed positive reactions by QFT assay. The rate of positivity was closely associated with high median CD4+ T cell counts ($P = 0.012$) and younger age ($P = 0.036$) (Table 2). Seven of 56 (12.5%) HIV-AC who had lower mitogen responses (IFN-γ concentrations: median: 1.78 IU/mL; range: 0.38~6.73 IU/mL) than the rest (>10 U/mL) had negative responses by QFT assay. Their median CD4+ T-cell counts were 60/μL (range: 43~425/μL) (data not shown). Thirty-three of 56 (59%) and 16 of 56 (29%) HIV-AC were attributed with positive TBGL-IgG and TBGL-IgA responses, respectively (Table 1). The positive proportions of TBGL-IgG and -IgA responses were not significantly different between QFT-positive and -negative HIV-AC (Table 2). However, 6 of 7 QFT-negative low mitogen responders in HIV-AC were positive for both TBGL-IgG and -IgA assay (data not shown). The TBGL-IgA titers were significantly higher in the TBGL-IgG-positive HIV-AC ($P = 0.041$) (Table 3). In addition, TBGL-IgA-positive HIV-AC had significantly elevated titers of TBGL-IgG ($P = 0.042$), serum IgA ($P = 0.015$), and OPN ($P = 0.03$), (Table 3). Interestingly, the TBGL-IgA-positive proportion was inversely correlated with the CD4+ T-cell counts ($P = 0.018$), and the titers were significantly higher in the HIV-AC with CD4+ T-cell count < 350/μL

Table 1: Demographic and clinical data of study participants.

Variables	HCWs ($n = 31$)	HIV-AC ($n = 56$)	P
Demographic data			
Gender: male; n (%)	16 (51.6)	55 (96.5)	<0.0001*
Age year; median (range)	35 (19~62)	28 (19~48)	0.03*
Laboratory findings†			
Hemoglobin (g/dL)	13.2 ± 2.6	13 ± 1.49	0.36
RBC (million/μL)	4.96 ± 1.6	4.43 ± .55	0.069
WBC ($10^3/\mu$L)	7.5 ± 2.5	5.9 ± 1.9	0.01*
Neutrophil ($10^3/\mu$L)	4.4 ± 2.2	3.3 ± 1.2	0.048*
Lymphocyte ($10^3/\mu$L)	2.4 ± 0.6	2.2 ± 0.9	0.82
Monocyte (/μL)	562 ± 237	338 ± 182	<0.001*
CD4+ T-cell count (/μL)	ND	443 ± 286	NA
QFT assay positive; n (%)	15 (48)	13 (23)	0.03*
TBGL-IgG positive; n (%)	16 (51)	33 (59)	0.9
TBGL-IgA positive; n (%)	6 (19)	16 (29)	0.87
IFN-γ-nc (IU/mL)	0.42 ± 0.96	0.13 ± 0.11	<0.001*
TBGL-IgG (U/mL)	3.12 ± 3.36	3.94 ± 6.63	0.14
TBGL-IgA (U/mL)	1.68 ± 2.56	3.1 ± 6.64	0.012*
Serum IgG (mg/dL)	1409 ± 212	1391 ± 224	0.49
Serum IgA (mg/dL)	246 ± 92	319 ± 138	0.058
OPN (ng/mL)	14.4 ± 11	159 ± 191	<0.00001*
Leptin (ng/mL)	18.6 ± 13.9	7.2 ± 5.4	<0.001*

Abbreviations: HCWs, healthcare workers; HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin; ND, not determined; NA, not applicable.
†values were presented as mean ± SD unless indicated otherwise; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P values for statistical differences between HCW and HIV-AC; *significant differences ($P < 0.05$).

Table 2: Comparison between QFT-positive and QFT-negative HCWs and HIV-AC.

Variables	HCWs			HIV-AC		
	QFT+ ($n = 15$)	QFT− ($n = 16$)	P	QFT+ ($n = 13$)	QFT− ($n = 43$)	P
Age; median (range)	45 (21~62)	23.5 (19~48)	0.002*	25 (19~45)	31 (21~35)	0.036*
Gender: male; n (%)	7 (46.6)	9 (47.4)	0.43	12 (92.3)	42 (97.67)	0.43
Work duration->10 yrs; n (%)	11(73.3)	6 (37.5)	0.098	NA	NA	NA
CD4+ count (/μL); median (range)	ND	ND	NA	611 (148~1466)	356 (13~1125)	0.012*
TBGL-IgG positive; n (%)	11 (73)	5 (31)	0.02*	8 (61.5)	25 (58.13)	0.545
TBGL-IgA positive; n (%)	5 (33)	1 (6)	0.072	3 (23)	13 (30)	0.415
TBGL-IgG+IgA positive; n (%)	4 (27)	0 (0)	0.043*	2(15.4)	10 (23.3)	0.42
IFN-γ-nc (IU/mL)†	0.3 ± 0.4	0.2 ± 0.13	0.9	0.21 ± 0.17	0.1 ± 0.07	0.0087*
Serum IgG (mg/dL)†	1450 ± 188	1368 ± 235	0.2	1306 ± 207	1414 ± 249	0.5
Serum IgA (mg/dL)†	268 ± 81	225 ± 101	0.32	330 ± 130	312 ± 138	0.68
OPN (ng/mL)†	14.5 ± 11.2	14.2 ± 11.2	0.87	115.4 ± 130	173.2 ± 203	0.43
Leptin (ng/mL)†	21.3 ± 13.3	15.9 ± 14.3	0.25	6.46 ± 4.12	7.448 ± 5.68	0.24

Abbreviations: HCWs, healthcare workers; HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin; ND, not determined; NA, not applicable.
†mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P values for statistical differences between QFT-positive and QFT-negative groups; *significant differences ($P < 0.05$).

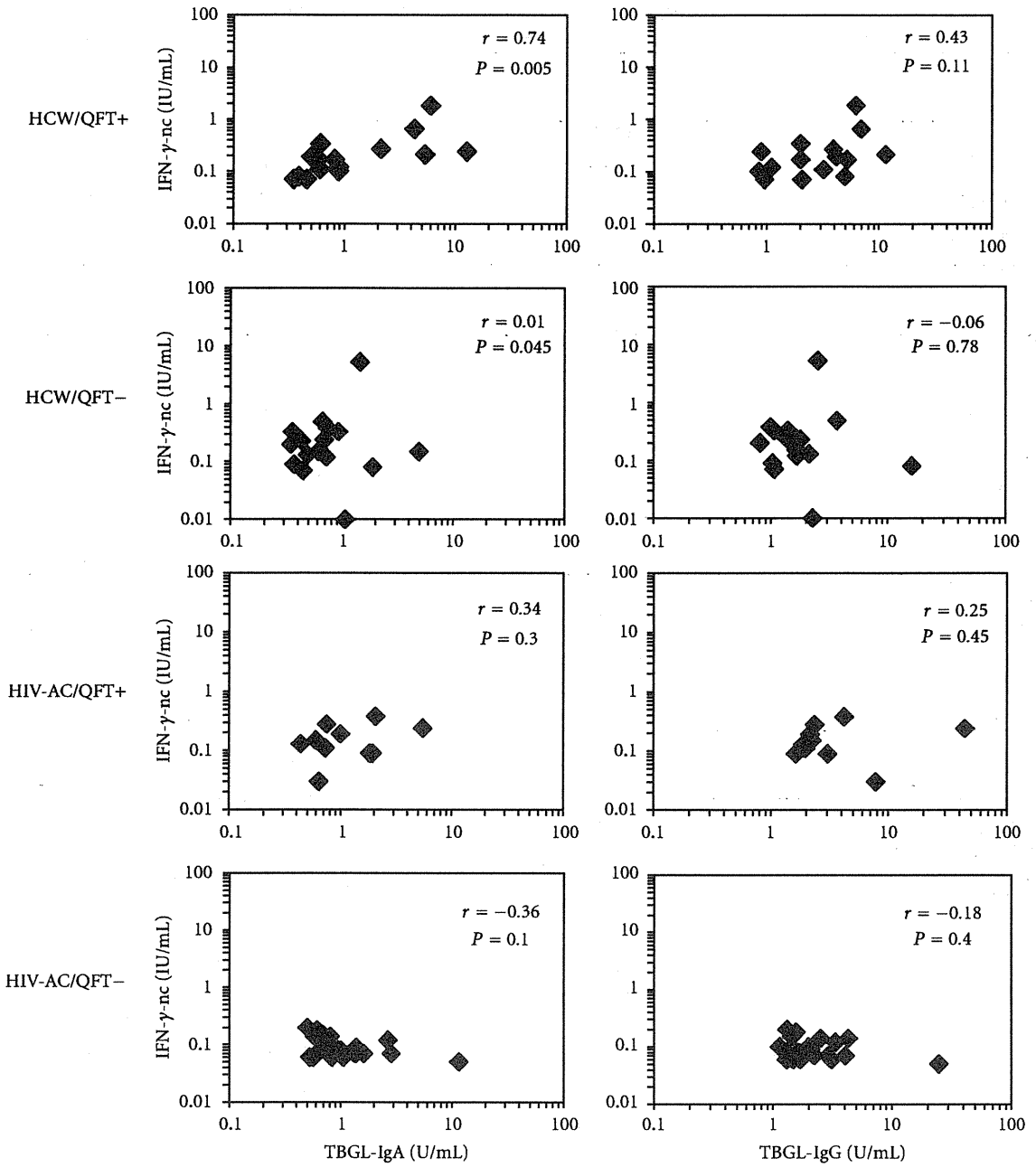

FIGURE 2: Correlations between TBGL-IgA or TBGL-IgG titers and IFN-γ concentrations measured in nonstimulated QFT-plasma samples (IFN-γ-nc) in QFT-positive/QTF-negative healthcare workers (HCWs) and asymptomatic HIV carriers (HIV-AC). The only significant positive correlation was observed between the IFN-γ-nc concentrations and TBGL-IgA titers in the QFT-positive HCW group ($r = 0.74$, $P = 0.005$).

(HIV-LCD) ($P = 0.048$) (Table 4). Furthermore, in the HIV-AC, a relatively higher proportion of double positive (TBGL-IgG+IgA) responders was found in the HIV-LCD group (29%) than in the HIV-HCD group (CD4+ count $\geq$ 350/μL) (16%), although the difference was not statistically significant ($P = 0.32$) (Table 4).

Moreover, the IFN-γ-nc concentrations were significantly lower in the QFT-negative HIV-AC ($P = 0.008$) (Table 2). No association was observed between the IFN-γ-nc concentrations and TBGL-IgG or -IgA titers in any group of HIV-AC (Figure 2). The plasma levels of OPN and leptin were not different between QFT-positive and QTF-negative HIV-AC (Table 2).

3.4. Comparison between the Serum Antibodies and TBGL Antibodies. The TBGL-IgG and -IgA had no correlation with

Table 3: Comparison between TBGL-IgG or TBGL-IgA-positive and -negative HIV-AC.

Variables	TBGL-IgG			TBGL-IgA		
	Positive (n = 33)	Negative (n = 23)	P	Positive (n = 16)	Negative (n = 40)	P
Age; median (range)	28 (19~48)	30 (19~41)	0.18	31.5 (19~48)	28 (19~45)	0.038*
Gender: male; n (%)	33 (100)	21 (91.3)	0.43	16 (100)	38 (95)	1
CD4 count (/μL); mean (range)	436 (13~1466)	450 (60~851)	0.45	346 (46~1125)	480 (13~1466)	0.06
QFT positive; n (%)	8 (24.2)	5 (21.7)	0.545	3 (19)	10 (25)	0.45
TBGL-IgA positive; n (%)	12 (36.4)	4 (17.4)	0.1	—	—	—
TBGL-IgG positive; n (%)	—	—	—	12 (75)	21 (52.5)	0.14
IFN-γ-nc (IU/mL)†	0.13 ± 0.09	0.1 ± 0.05	0.4	0.12 ± 0.09	0.12 ± 0.07	0.9
TBGL-IgA (U/mL)†	4.36 ± 8.4	1.28 ± 1.21	0.041*	—	—	
TBGL-IgG (U/mL)†	—	—		7.5 ± 11.6	2.5 ± 1.5	0.042*
Serum IgG (mg/dL)†	1439 ± 277	1515 ± 677	0.5	1615 ± 404	1355 ± 135	0.46
Serum IgA (mg/dL)†	277 ± 95	279 ± 74	0.37	410 ± 165	313 ± 138	0.015*
OPN (ng/mL)†	176.3 ± 199.9	136 ± 172.5	0.67	280 ± 275	115 ± 109.7	0.03*
Leptin (ng/mL)†	7.33 ± 6.16	7.18 ± 4.12	0.68	7.33 ± 6.16	7.18 ± 4.12	0.07

Abbreviations: HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin.
†mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

Table 4: Comparison between HIV-AC with high§ and low‡ CD4+ T-cell count.

Variables	CD4+ high§ (n = 32)	CD4+ low‡ (n = 24)	P value¶
Age; mean (range)	25.5 (19~45)	25 (22~48)	0.018*
Gender: male; n (%)	31 (97)	23 (98)	1.0
CD4+ count (/μL); median (range)	618 (356~1466)	201 (13~349)	<0.001*
QFT-positive; n (%)	10 (31)	3 (12.5)	0.12
TBGL-IgG positive; n (%)	16 (50)	16 (67)	0.27
TBGL-IgA positive; n (%)	5 (16)	11 (46)	0.018*
TBGL-IgG+ IgA positive; n (%)	5 (16)	7 (29)	0.32
IFN-γ-nc (IU/mL)	0.14 ± 0.12	0.13 ± 0.09	0.9
TBGL-IgG (U/mL)†	4.6 ± 8.4	3 ± 2.8	0.59
TBGL-IgA (U/mL)†	1.55 ± 2	5.16 ± 9.6	0.048*
Serum IgG (mg/dL)†	1352 ± 185	1549 ± 380	0.5
Serum IgA (mg/dL)†	265 ± 89	423 ± 149	<0.001*
OPN (ng/mL)†	119 ± 126	214 ± 246	0.19
Leptin (ng/mL)†	7.7 ± 6	6.6 ± 4.9	0.5

Abbreviation: HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN: osteopontin.
§High: CD4+ T cell count ≥350/μL; ‡low: CD4+ T-cell count <350/μL; †mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the non-stimulated QFT-plasma samples; P values for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

the serum IgG and IgA in HCW and HIV-AC except for the association between the serum IgA levels and the TBGL-IgA titers in HIV-AC (P = 0.02) (data not shown).

3.5. Comparison of Biomarkers between HCW and HIV-AC. The levels of IFN-γ-nc ($P < 0.001$) were significantly higher in HCWs than in HIV-AC. However, the titers of TBGL-IgA (P = 0.012), but not -IgG, were significantly higher in HIV-AC than in HCWs. Similarly, the serum IgA levels were also higher (P = 0.058). The OPN levels were significantly higher ($P < 0.0001$), and the leptin levels were considerably lower ($P < 0.001$) in the HIV-AC compared to the HCWs (Table 1).

ROC curve analysis was used to discriminate HIV from HCW groups using the net IFN-γ, leptin, and plasma levels

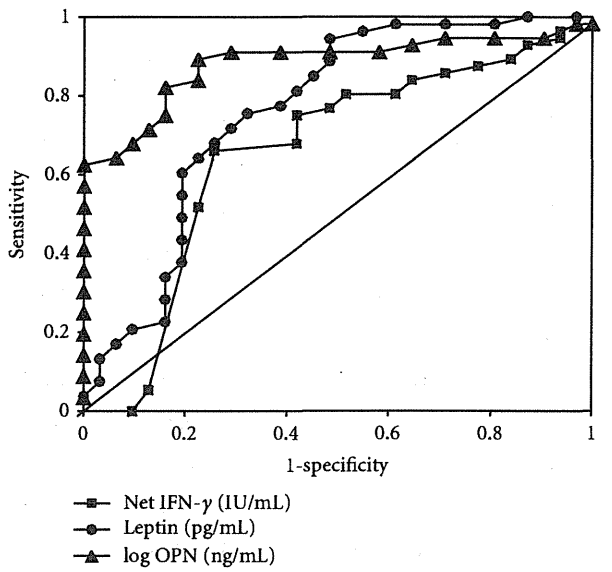

FIGURE 3: Receiver operating characteristic analysis for comparison of biomarkers between healthcare workers and asymptomatic HIV carriers. The result showed that the OPN plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCW based on the AUC (0.883), followed by leptin (0.763) and net IFN-γ (0.648).

of OPN (log) as biomarkers. As shown in Figure 3, the plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCWs based on the AUC (0.883), followed by leptin (0.763) and net IFN-γ (0.648). However, QFT assay as well as TBGL-IgA and -IgG did not show such profiles (data not shown).

4. Discussion

In our data, the application of QFT assay to HCWs in the Philippines demonstrated a high incidence (48%) of LTBI, which was comparable to other already published data in HCWs in TB-endemic developing countries [4]. The increased risk of LTBI among HCWs was confirmed by the recent introduction of IGRA [20, 21]. In our country, a higher incidence of LTBI in HCWs was reported in high-risk groups for TB, such as homeless areas [22], compared to other areas [23].

We aimed to clarify the relationship between the TBGL-IgG and -IgA responses and that of IFN-γ in the QFT assay in LTBI. The rate of TBGL-IgG positivity was significantly higher in the QFT-positive than QTF-negative group of HCWs. The significant association between the two assay systems indicated by the κ value in HCWs demonstrated the TBGL-IgG in LTBI. However, about 30% of QFT-positive populations from among the HCWs lacked TBGL-IgG, and 30% of those of the QFT-negative group have elevated TBGL IgG antibody, and the discordant cases were higher in TBGL-IgA. However, the reasons for such discordances between the two systems in HCWs are not clear. It is possible that the generation of antibody requires larger amounts of antigens than does the generation of T-cell responses. Although associated immunosuppressive conditions were found as risk factors for false-negative QFT responses [24], such cases were excluded from HCWs in our study.

The mechanism of the synthesis of anti-TDM antibody is not clear, though TDM is known to bind to Mincle (macrophage-inducible C-type lectin) that is present on macrophages [25], and upon the activation, on T cells [26]. It was found that Mincle is specific for the ester linkage of a fatty acid to the trehalose, which explains the strong binding of TDM, but not trehalase-treated TDM, soluble trehalose, or purified mycolate [26]. The conversion of TDM into glucose monomycolate (GMM) upon mycobacterial infection might be the mechanism by which mycobacteria escape from the Mincle-mediated immunity. However, the immune system possesses other tools to monitor and eliminate live mycobacteria through CD1 molecules expressed on the activated macrophages and dendritic cells, which are different from MHC I, II molecules. Recently, GMM but not TDM was demonstrated to interact with CD1b and may induce adaptive immunity [27]. Although it is not known whether the adaptive immune system leads to antibody synthesis, the generated antibody may recognize both TDM and GMM because the two molecules are structurally very similar.

Interestingly, the IFN-γ-nc levels that were observed to have a significant association with the TBGL-IgA titers in LTBI of HCWs. IgA is a typical marker of the mucosal immune response. An elevated serum IgA has been proposed to have a protective role in IFN-γ-positive immunocompetent LTBI individuals [28]. Frequent exposure to tubercle bacilli can possibly stimulate the mucosal immune system in TB-endemic countries. It is also known that commensal bacteria on the mucosal surface induce IgA in an NO-dependent manner [29], although it is not known whether MTB in LTBI has a similar effect in lung mucosa. Circulating glycolipid immune complexes might lead to nonspecific stimulation of T cells, but a component of TBGL, TDM, could also enhance the in vivo production of IL-12p40 and IFN-γ in mouse model [30]. IgA antibody and IFN-γ induce TNF-α and NO production, which mediated the inhibitory mechanism for *M. tuberculosis* infection in mouse model [28]. Furthermore, there is strong evidence of a synergic effect between IgA and IFN-γ in bactericidal activities against MTB infection [31]. Therefore, the association between anti-TBGL-IgA and IFN-γ may indicate protective, mucosal immune activities in LTBI in HCWs.

In HIV carriers, the QFT-positive responses were significantly lower than in HCWs and were greatly dependent on the high CD4+ T-cell counts in the present study. Much evidence suggests that the baseline CD4+ T-cell count is a determining factor for a positive QFT response in HIV infection [32]. Since HIV infection is a disease of immune deficiency, immune deprivation may be less prominent in relatively young QFT-positive cases because IFN-γ could be synthesized properly by stimulation with the appropriate signals. In contrast, the response could be altered in advance immune-deficiency state, as indicated by low CD4+ T-cell counts. Therefore, it is expected that

significant numbers of false-negative reactions are present in QFT-negative HIV carriers. The relatively low IFN-γ levels by mitogen stimulation in some of the QFT-negative responders also support this possibility. Therefore, for TB diagnosis in advanced immunosuppression, the ratio of the IFN-γ response/CD4+ T-cell count Elispot assay was suggested to improve the sensitivity of the assay [33].

It is not clear why HIV infection does not diminish the TBGL antibody titers. It is known that the CD-1 presentation pathway persists in patients with HIV, but antiglycolipid antibodies were found to have no relationship with the TST results [34] or bacillary yield [35]. Similarly, we did not find any correlation between the QFT result and anti-TBGL antibodies. It is also possible that concomitant non-TB mycobacterium infection may stimulate the TBGL antibody synthesis in HIV-AC [7]. Significant numbers of HIV carriers have antibodies to TBGL, but we could not confirm if they indicate LTBI or not.

The increases of serum IgA in advanced HIV infection and of IgG in the early stage were already reported [36]. Although specific antibody titers in HIV infection are decreased by some infectious agents including hepatitis B virus but not in hepatitis A virus, probably because of alterations in the immune systems in advanced HIV infection [37], it is not known whether nonfunctional or functional IgA was synthesized in our cases. The main limitation of the current study is the small number of study subjects and the lack of a follow-up study for estimating the risk of developing active tuberculosis.

Finally, to determine the correlations between biomarkers in infected states, we evaluated data by ROC curve analysis (**Figure 3**). In this study, the plasma levels of OPN were most specific to HIV and the levels were not elevated in LTBI HCWs (**Figure 3, Table 2**). Therefore the levels can be a good marker for active TB in non-HIV individuals, because the OPN is known as a marker of active TB [17]. In HIV-AC, the OPN plasma levels are already elevated as described here, and it was already reported that the levels further increase when they developed active TB [38, 39]. It is also known that interferon-inducible protein-10 (IP-10) and IL-18 were elevated in HIV/TB patients than in HIV patients and suggested to be helpful in monitoring the treatment for patients [38]. All these biomarkers were mainly produced by macrophages, and it was also reported that OPN is synthesized by macrophages as well as CD4+ T cells in HTLV-1-induced lymphoma [40, 41].

In this study we noted elevations of anti-TBGL antibody in LTBI in HCWs, but no link between the elevations with LTBI in HIV-AC was confirmed, probably due to the inflammatory conditions in HIV.

5. Conclusion

We have found the elevation of TBGL-IgG titers in LTBI in HCWs. In addition, the association between TBGL-IgA and IFN-γ in HCWs was found, and it was hypothesized that the mucosal immunity is involved in LTBI in HCWs. We could not find any relationships between QFT and TBGL in HIV-AC. Low CD4+ cell count was associated with inflammatory conditions as represented by high OPN in HIV-AC, which may be the reason for ambiguous results.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

US and PL did experiments using the samples and contributed equally as the first author. HCY and BS contributed to the experimental system and statistical analysis. HS and YA and YS contributed to the planning and proposal of the work. TH and ET coordinated the work.

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Distinct Clinical Features in Nontuberculous Mycobacterial Disease with or without Latent Tuberculosis Infection

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Nontuberculous mycobacteria (NTM) diseases are in the face of a progressive increase even in immune-competent subjects, and the clinical features of NTM diseases are heterogenous. The decision to institute treatment of the patients should be made after a period of follow up, because therapy is often prolonged, and frequently ineffective. The reasons why some patients develop severe NTM diseases are not clear. Here we observed the involvement of latent tuberculosis infection (LTBI) in clinical and laboratory features of NTM diseases. We evaluated various tuberculosis-related inflammatory markers including osteopontin (OPN), pentraxin-3 (PTX-3), and soluble IL-2 receptor (sIL-2R) in NTM infected patients with or without LTBI. Eight NTM and 5 tuberculosis (TB) patients, and 5 healthy subjects were enrolled. Polymerase Chain Reaction (PCR) analysis confirmed the absence of tuberculosis specific gene (RD1 region), among clinical isolates from NTM patients. Interferon-γ (IFN-γ) release assay (IGRA) using Early Secreted Antigenic Target-6 (ESAT-6) and CFP-10, the RD1-encoded protein, was employed for determining LTBI. IGRA was positive in 4/8 NTM (NTM with LTBI, 50%) and 5/5 TB patients. Only 2 of 4 NTM with LTBI were under chemotherapy among all NTM patients, and others were followed up. The plasma levels of OPN, PTX3 and sIL-2R were significantly higher in NTM patients with LTBI than in those without LTBI ($P < 0.05$). The two patients under therapy showed the highest OPN levels that persisted after treatment. The increased inflammatory levels in NTM patients with LTBI indicate enhanced inflammatory reaction. Extensive therapy may be necessary in such patients.

Keywords: interferon-γ release assay; latent tuberculosis infection; nontuberculous mycobacterial disease; osteopontin; pentraxin-3
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Infection caused by nontuberculous mycobacterium (NTM), especially by *Mycobacterium avium complex* (MAC), has been increasing in both immunocompromised and immunocompetent individuals in Japan and worldwide (Prince et al. 1989). MAC may cause progressive lung diseases leading to morbidity and mortality in previously healthy patients as well as in patients with pre-existing lung diseases and immunodeficiency (Griffith et al. 2007). Recently, it was reported that progressive lung disease due to MAC is associated with specific variable number of tandem repeat (VNTR) genotypes (Kikuchi et al. 2009) or with the clinical features of cavity formation (Ito et al. 2012). Lung disease due to NTM occurs commonly in structural lung diseases, such as chronic obstructive pulmonary disease (COPD), bronchiectasis, cystic fibrosis, pneumoconiosis, prior tuberculosis, pulmonary alveolar proteinosis, and esophageal motility disorders (Griffith et al. 2007). The detection of both *Mycobacterium tuberculosis* (MTB) and NTM by PCR in a patient was reported in Japan (Takeda et al. 2008) and most patients with MTB and MAC co-infection reported in USA were foreigners (Khan et al. 2010). NTM infection in Japan is frequently observed in elderly people, and elderly people are more frequently latently infected with MTB (LTBI) than young people in Japan and the Philippines (Siddiqi et al. in press), as confirmed by IFN-γ release assay (IGRA) and in Thailand, as confirmed by anti-tuberculous glycolipid (TBGL) assay (Siddiqi et al. 2012). Recently, several studies in different countries

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