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 aminotransferase [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%

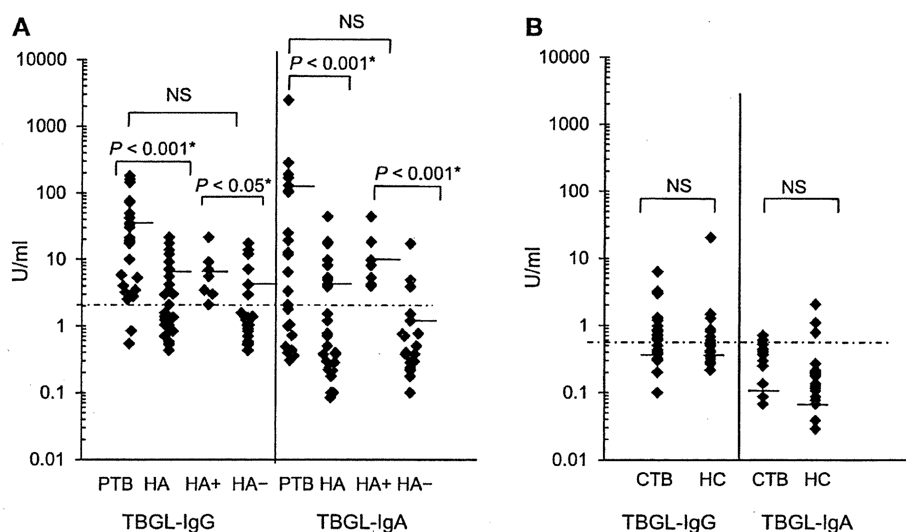

Figure 1 TBGL-IgG and TBGL-IgA titres in **A**) adult and **B**) child participants. Dashed lines indicate the cut-off value of ⩾2 U/ml for both antibodies. Solid bars indicate mean values. * Indicates significant difference. NS = not significant; PTB = adult pulmonary TB patients; HA = healthy adult 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).

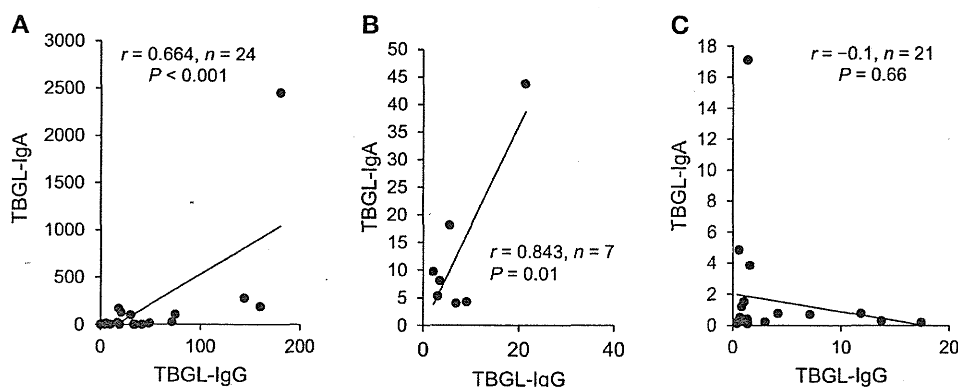

Figure 2 Correlation between TBGL-IgG and -IgA titres. An association was found in **A**) adult PTB patients and **B**) HA+ subjects (healthy adults with high TBGL-IgG and -IgA titres), but not in **C**) HA− subjects (healthy adults with low TBGL-IgG or -IgA titres or both). TBGL = tuberculous glycolipid;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

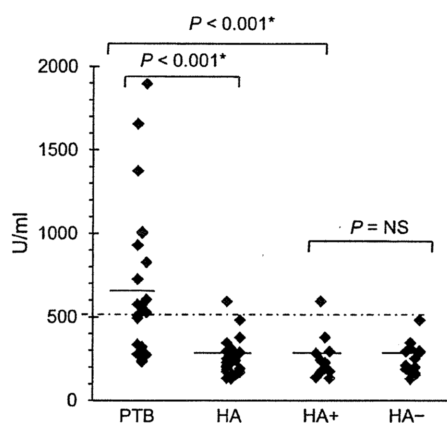

Figure 3 KL-6 titres in adult participants. Dashed line indicates the cut-off value of ⩾500 U/ml. Solid bars indicate mean values. *Indicates significant difference ($P < 0.05$). NS = non-significant; PTB = adult PTB patients; HA = healthy adult controls; HA+ = HAs with high TBGL-IgG and -IgA titres;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

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Quinolone-Induced Upregulation of Osteopontin Gene Promoter Activity in Human Lung Epithelial Cell Line A549

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Quinolones, in addition to their antibacterial activities, act as immunomodulators. Osteopontin (OPN), a member of the extracellular matrix proteins, was found to play a role in the immune and inflammatory response. We found that quinolones significantly enhanced OPN secretion, namely, garenoxacin (220%), moxifloxacin (62%), gatifloxacin (82%), sparfloxacin, (79%), and sitafloxacin (60%). Enhancement of OPN secretion was shown to be due to the effect of quinolones on the OPN gene promoter activity. We also examined the role of quinolones on apoptosis and found that sparfloxacin decreased the late apoptosis of A549 cells, but garenoxacin did not show the antiapoptotic effect. The antiapoptotic effects of quinolones do not appear to be associated with OPN elevation.

Quinolones are synthetic, broad-spectrum antimicrobial agents widely used in clinical and veterinary medicine. They target two essential bacterial enzymes, DNA gyrase and topoisomerase IV (14). Newly developed quinolones especially possess significant *in vivo* bactericidal activity, which makes them attractive therapeutic agents for treatment of tuberculosis, community-acquired pneumonia, and other respiratory tract infections (20). In addition to the bactericidal property, fluoroquinolones (FQs) have been found to elicit an immunomodulatory effect (10).

A number of reports have described the inhibitory effect of FQs on cytokine production. Gatifloxacin (GAT) reduced interleukin-8 (IL-8) release from unstimulated cells of the prostatic cancer cell line PC-3 as well as peptidoglycan-, *Mycoplasma hominis*-, phorbol ester (phorbol myristate acetate [PMA])-, and tumor necrosis factor alpha (TNF-α)-stimulated PC-3 cells but did not significantly reduce the basal level of TNF-α and IL-6 (34). Moxifloxacin (MFX) inhibited IL-8, TNF-α, and IL-1β production in THP-1 cells and in monocytes when preincubated with MFX and stimulated with lipopolysaccharide (LPS) (38). Another report suggested that ciprofloxacin (CIP) may have an immunomodulatory effect on septic patients by attenuating the proinflammatory response, thus decreasing TNF-α, IL-6, IL-1β, and IL-8 levels in patients' serum (15). Levofloxacin at concentrations of 100 μg/ml and higher was found to dose dependently reduce the IL-6 and IL-8 levels in TNF-α-stimulated NL20 human bronchial epithelial cells, but lower concentrations did not alter the studied cytokines (35). Elevated levels of IL-1β, IL-6, and TNF-α in patients with nonbacterial prostatitis became undetectable after treatment with sparfloxacin (SPX) (40).

Several studies attempted to elucidate the signaling pathways and transcription factors that regulate the quinolone-induced cytokine modulation. In A549 cells, IL-1β increased the activities of early intracellular signaling molecules, extracellular signal-regulated kinases 1 and 2 (ERK1/2), phosphorylated Jun N-terminal protein kinase (p-JNK), and NF-κB, whose activities were abrogated by MFX (39). Likewise, in human blood neutrophils, it has been reported that grepafloxacin strongly phosphorylates p38 mitogen-activated protein (MAP) kinase (MAPK) but not p44/42 MAPK or JNK (25).

Osteopontin (OPN), a member of the extracellular matrix proteins, is a multifunctional phosphoprotein that is synthesized by a variety of immune and nonimmune cells (31). Basically, there are three major functions of OPN: involvement in tumorigenesis and metastasis, in mineral metabolism and bone remodeling, and in immune reaction and host defense (36). Because the primary structure and distribution of posttranslational modification are highly conserved among species, it is conceivable that OPN plays an indispensable role in the immune system (8, 9, 32). Within the immune system, OPN is a cytokine secreted by activated T cells, NK cells, dendritic cells, and macrophages (37). In the lung, OPN is expressed by alveolar macrophages and bronchial epithelial cells, the passive physiological barrier of the innate immune system (5, 11, 21). Recently, there is increasing evidence that OPN exists in two isoforms, secreted (sOPN) and/or intracellular (iOPN) protein (36). While sOPN affects the target cell functions by binding to their cell receptors, iOPN binds to MyD88, the downstream protein of the Toll-like receptor (29, 30). Furthermore, it has been reported that OPN binding to CD44v downregulated IL-10 production in macrophages, leading to inhibition of the Th2 immune response, but binding to αvβ3 integrin receptor led to the expression of IL-12, facilitating the Th1 response (2).

When hosts are insulted by infections, Th1 immunity plays a central role in the elimination of microorganisms, so it is understandable that elevated plasma OPN levels have been found to be associated with tuberculosis and other lung inflammatory diseases (26). OPN deficiency was found to be associated with dissemina-

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tion of mycobacterial disease, and the expression of OPN correlated with an effective immune and inflammatory response and contributed to resistance against mycobacteria in rodents as well as in human (23, 24). OPN also contributed to protection against rotavirus (28), herpes simplex virus type 1 (2, 7), *Listeria monocytogenes* (2), and *Plasmodium falciparum* (19). These reports suggest that OPN plays a role in defense mechanisms against invading microorganisms, including viruses, bacteria, and protozoa.

However, until now, the effect of quinolones on OPN expression in human lung epithelial cells remained to be elucidated. A recent report demonstrated that a human lung type II epithelial cell line (A549) is a suitable model to study host defense cellular responses (18). Therefore, in this study we chose this experimental model to investigate the immunomodulatory effect of CIP, garenoxacin mesylate hydrate (GRN), MFX, GFL, SPF, and sitafloxacin (STF). The antibiotics chosen were quinolones, which possess activities against various infections. This is the first report showing that quinolones enhance OPN production in lung epithelial cells. Because OPN is thought to elicit antiapoptotic effects (6, 17), we investigated whether quinolone-induced OPN production may increase survival in A549 cells.

MATERIALS AND METHODS

Cells. The human A549 alveolar epithelial cell line was obtained from Riken Cell Bank (Tsukuba, Japan). A549/OPN-luc cells were established by cotransfection of pOPN1-luc (42) with puromycin resistance vector pPUR (Clontech, Mountain View, CA) at a molar ratio of 5:1, followed by selection in the presence of 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO). The cells were maintained in Ham's F-12 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator containing 5% CO_2 in air. For the experiments, subconfluent cultures were harvested by brief trypsinization (trypsin-EDTA solution; Nacalai Tesque, Kyoto, Japan) and resuspended in Ham's F-12 medium supplemented with 2% FBS. Cell viability was determined by trypan blue staining (Sigma-Aldrich, St. Louis, MO).

Antibiotic preparation. CIP (Wako Pure Chemical Industries, Osaka, Japan) and GRN (kindly provided by Taisho Toyama Pharmaceutical, Tokyo, Japan) were dissolved in dimethyl sulfoxide at a concentration of 30 mg/ml, MFX (Santa Cruz, CA) was dissolved in distilled water at a concentration of 10 mg/ml, and GAT, SPX, and STF (Hokkaido University) were dissolved in 0.1 N NaOH at a concentration of 10 mg/ml.

Determination of OPN protein levels. To measure the OPN levels in the culture supernatants, A549 cells were plated in triplicate at 1×10^4 cells per well in 96-well plates. After 24 h of incubation, the cells were washed twice with serum-free Ham's F-12 medium, and fresh serum-free medium was added. Cells were treated by CIP, GRN, MFX, GAT, SPX, and STF at the indicated concentrations. After further incubation for 48 h, the culture supernatant was harvested and stored at −20°C. The OPN protein levels were measured by a human osteopontin Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

Cell viability. To measure the effect of the quinolones on cell viability and proliferation, A549 cells were plated in triplicate at 1×10^4 cells per well in 96-well plates. At 24 and 48 h after the addition of the antibiotics, a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Dojindo, Kumamoto, Japan) was performed according to the manufacturer's instructions. Similarly treated wells without cells served as blanks.

Determination of OPN promoter activity. A549/OPN-luc cells were treated the same as for ELISA. For the luciferase assay, at 24 and 48 h after the treatment, cells were washed twice with phosphate-buffered saline (PBS) and then lysed with a luciferase cell culture lysis reagent (Promega, Madison, WI). The luciferase assay was done with a luciferase assay system (Promega) according to the manufacturer's instructions, and the luciferase activities were measured using a Mithras LB 940 microplate luminometer (Berthold Technologies, Oak Ridge, TN).

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was performed to examine the effect of quinolones on the expression of OPN mRNA. A549 cells were plated at 1×10^6 in a 6-well plate, and after 24 h, the cells were washed twice with serum-free medium. Fresh serum-free medium and quinolones were added at a final concentration of 30 μg/ml, and GRN was added at a concentration of 1, 3, 10, or 30 μg/ml. The cells were incubated for a further 48 h. Cells were lysed with TRIzol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's instructions. After treatment with RNase-free DNase (Promega), the DNA-free RNA (250 ng) was used for synthesis of the first-strand cDNA at 42°C for 60 min using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time quantitative PCR using Power SYBR green PCR master mix was conducted for 40 cycles at 95°C for 15 s and at 60°C for 1 min in a 96-well format on an ABI StepOne real-time PCR system (Applied Biosystems). Primer sequences were as follows: OPN forward, 5′-ACTCGTCTCAGGCCAGTTG-3′; OPN reverse, 5′-CGTTGGACTTG GAAGG-3′; GAPDH forward, 5′-TGATGACATCAAGAAGGTGG-3′; and GAPDH reverse, 5′-TCCTTGGAGGCCATGTGGGC-3′.

shRNA transfection. To diminish OPN expression, A549 cells were transfected with short hairpin RNA (shRNA) targeting OPN or control shRNA (Santa Cruz Biotechnology, Inc.) with Effectene transfection reagent (Qiagen, Valencia, CA) for 48 h. In another setting, cells were transfected with shRNA targeting OPN for 24 h and then washed twice with serum-free medium. The 24-h-conditioned medium from nontreated cells or cells treated with 30 μl/ml of GRN or SPX was added, and the cells were cultured for a further 24 h. Cells were then analyzed by fluorescence-activated cell sorting (FACS).

Annexin V/7-AAD staining and flow cytometry. A549 cells and cell supernatant were harvested and washed twice with PBS and resuspended in binding buffer containing 10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. A staining mixture consisting of 5 μl annexin V-phycoerythrin and 5 μl 7-aminoactinomycin (7-AAD) (BD Biosciences Pharmingen) was added to 100 μl of cell suspension (1×10^6 cells/ml). Cells were incubated in the dark for 15 min at room temperature and then analyzed on a FACSDiva flow cytometer. Data were analyzed using FlowJo software (FlowJo, Inc.). The cells in early apoptosis were considered to be annexin V positive and 7-AAD negative. Positivity for both annexin V and 7-AAD indicated late apoptosis.

Statistical analysis. Results are expressed as means ± standard errors of the means. The statistical difference was determined by two-sided Student's *t* test, and the correlation between OPN promoter activity and OPN secretion was obtained using simple regression analysis by Statcel2 software (OMS, Tokyo, Japan). A difference with a P value of <0.05 was considered significant.

RESULTS

Quinolones enhance OPN release from A549 alveolar epithelial cells. The A549 cell line, which secretes OPN, serves as a good model to study drug-induced changes in cytokine secretion (13). We found that treatment of the cells with CIP at 30 μg/ml induced a minimal elevation of OPN secretion, while GRN dramatically increased the OPN level in a dose-dependent manner. Treatment with 1 μg/ml to 30 μg/ml of GRN resulted in a 60 to 220% enhancement of OPN secretion. MFX and SPX at doses of 3 μg/ml to 30 μg/ml caused significant OPN enhancements from 23 to 62% and 22 to 79%, respectively. Similarly, 38 to 82% and 23 to 60% elevations of OPN levels were induced by the addition 10 μg/ml to 30 μg/ml of GAT and STF (Fig. 1).

Enhancement of OPN promoter activity. To investigate the mechanisms of enhancement the OPN secretion, A549/OPN-luc

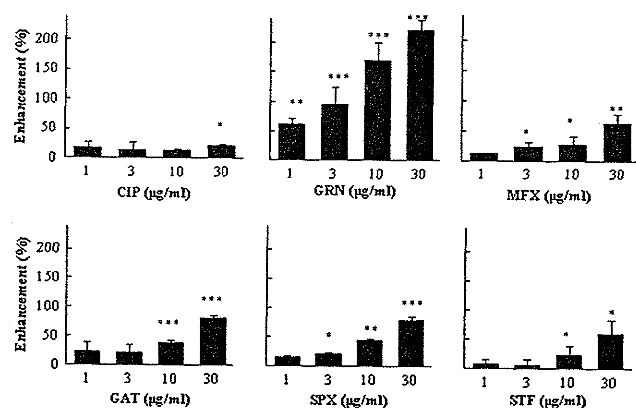

FIG 1 Effect of quinolones on OPN release. A549 cells were plated at 1 × 10^{4} in 96-well plates, and after 24 h, the cells were washed twice with serum-free medium. Fresh serum-free medium and drugs at final concentrations of 1, 3, 10, and 30 μg/ml were added. The cells were incubated for a further 48 h, and the OPN concentrations in the cell culture supernatant were measured by ELISA, as described in Materials and Methods. OPN secretion was normalized by the cell viability optical density (570 to 630 nm) value of the MTT assay and expressed as percent increase above control. Controls are the wells treated only with solvent. The results are expressed as means ± SDs. Representative data of three independent experiments are shown. ***, $P < 0.001$ compared to the control; **, $P < 0.01$ compared to the control; *, $P < 0.05$ compared to the control.

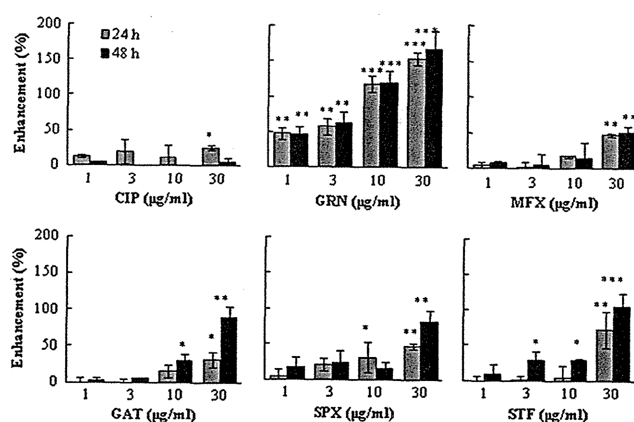

FIG 2 Effect of quinolones on OPN promoter activity. A549 cells were plated at 1 × 10^{4} in 96-well plates, and after 24 h, the cells were washed twice with serum-free medium. Fresh serum-free medium and drugs at final concentrations of 1, 3, 10, and 30 μg/ml were added. Cells were incubated for a further 24 or 48 h, and the OPN gene promoter activity was measured by luciferase assay, as described in Materials and Methods. The OPN promoter activity was normalized by the cell viability optical density (570 to 630 nm) value of the MTT assay and expressed as percent increase above the value for the control. Controls are the wells treated only with solvent. The results are expressed as means ± SDs. Representative data of three independent experiments are shown. ***, $P < 0.001$ compared to the control; **, $P < 0.01$ compared to the control; *, $P < 0.05$ compared to the control.

cells, which stably express OPN promoter/luciferase, were established. Thus, luciferase expression of A549/OPN-luc cells served as a parameter to monitor the level of OPN transcription. CIP at 30 μg/ml induced only a 25% elevation of OPN transcription. In contrast, even 24 h cell exposure to 1 μg/ml to 30 μg/ml of GRN for 24 h induced strong, dose-dependent OPN gene activation, reaching 47 to 160%. A similar finding was observed after 48 h. Enhancement of OPN gene promoter activation was observed with increasing concentrations of MFX, GAT, SPX, and STF. However, the change was not so striking compared to that for GRN (Fig. 2). We also examined the association between OPN promoter activation and OPN secretion and found a statistically significant correlation for GRN, GAT, MFX, and STF (Table 1).

Effect of quinolones on OPN mRNA expression. To examine if quinolones alter the expression of OPN mRNA, we performed quantitative reverse transcription-PCR. First, we screened the effects of CIP, GRN, MFX, and SPX at a concentration of 30 μg/ml and found that GRN and MFX significantly enhanced mRNA expression compared to that for the control (Fig. 3A). Next, we analyzed the dose-dependent effect of GRN. When cells were incubated with various concentrations of GRN, we observed the dose-related increase of OPN mRNA expression, with statistical significance achieved at concentrations of 10 and 30 μg/ml (Fig. 3B).

Effect of quinolones on early and late apoptosis. To investigate the effect of quinolones on A549 cell apoptosis, we used annexin V/7-AAD double staining for FACS analysis. Treatment of A549 cells with GRN and SPX at a concentration of 30 μg/ml did not affect early apoptosis, but late apoptosis was decreased by SPX (4.42%) and slightly by GRN (9.25%) compared to the control (12.6%). Downregulation of OPN by shRNA resulted in an increase of both early (6.55%) and late (16.7%) apoptosis. When a 24-h supernatant was added to OPN expression-silenced cells, the percentage of apoptotic cells decreased. Surprisingly, the SPX supernatant prevented late apoptosis (5.13%) (Table 2).

DISCUSSION

In this study, we examined the effect of quinolones on OPN synthesis in A549 human lung epithelial cells. We found that GRN, MFX, GAT, SPX, and STF dose dependently enhanced OPN secretion. On the other hand, CIP at a concentration of 30 μg/ml only slightly enhanced OPN. Since our ELISA system cannot distinguish phosphorylated from nonphosphorylated or cleaved forms of OPN, we could not elucidate which form of OPN was enhanced. To elucidate the mechanism of quinolone-induced OPN elevation, we employed a luciferase assay and found that this elevation occurred at the transcriptional level by activation of the OPN gene promoter. The results of qRT-PCR also revealed that MFX and GRN significantly enhanced OPN mRNA expression, but SPX and CIP did not.

Generally, quinolones are thought to attenuate proinflammatory

TABLE 1 Correlation between OPN promoter activity and OPN secretion and maximal serum concentrations of studied quinolones

Drug	Correlation between OPN promoter activity and OPN secretion[a]		Dose (mg)	C_{max}[b] (μg/ml)
	R^2 value	P value		
CIP	0.881	0.061	750 (×2)[c]	3.5
GRN	0.988	0.006**	400 (×1)	5.8
MFX	0.929	0.036*	400 (×1)	3.1
GAT	0.993	0.003**	400 (×1)	4.0
SPX	0.756	0.131	400 (×1)	1.0
STF	0.913	0.045*	200 (×1)	1.9

[a] Luciferase assay and ELISA data from a 48-h quinolone treatment of A549 cells expressing percent increase above the value for the control were assessed by simple regression analysis. **, $P < 0.01$; *, $P < 0.05$.
[b] C_{max}s from previous studies (1, 22).
[c] Data in parentheses represent frequency per day.

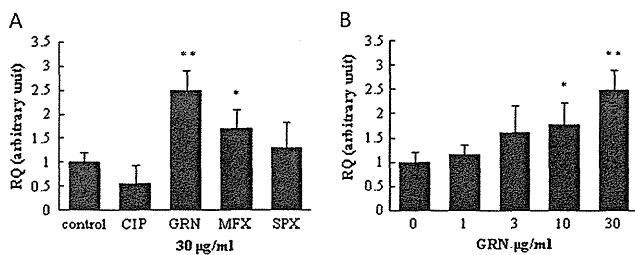

FIG 3 Effect of quinolones on OPN mRNA expression. A549 cells were plated at 1×10^6 in a 6-well plate, and after 24 h, the cells were washed twice with serum-free medium. Fresh serum-free medium and quinolones at a final concentration of 30 μg/ml (A) and GRN at concentrations of 1, 3, 10, and 30 μg/ml (B) were added. The cells were incubated for a further 48 h. Total RNA was isolated, and qRT-PCR was performed as described in Materials and Methods. The results are expressed as means ± SDs. Representative data of three independent experiments are shown. **, $P < 0.01$ compared to the control; *, $P < 0.05$ compared to the control.

cytokine synthesis, though there have been reports that CIP caused an increase in IL-2 secretion from PMA-treated human peripheral blood lymphocytes. In contrast, there was no effect on IL-1 release in the same experimental setting (33). Because the effect of quinolones on cytokine synthesis is not consistent and differs by the cell type and the cytokine examined (13), it is tempting to speculate that the cytokine response to drug treatment is cell type specific and influenced by the microenvironment under which cells exist.

In our study, we investigated the effect of quinolone concentrations above and below the maximal concentrations in serum (C_{max}s) (Table 1). The results showed that GAT, SPX, and STF augmented OPN transcription only at concentrations higher than the C_{max}s, but GRN and MFX elicited the ability to enhance OPN even at their C_{max}s. Since OPN is a multifunctional protein, an elevation of OPN synthesis might have a dual effect. OPN can promote protective immunity through the OPN-dependent induction of the proinflammatory cytokine IL-12 and suppression of the anti-inflammatory cytokine IL-10 (2). However, the excessive OPN production might activate various cell growth signals, finally leading to oncogenesis (26, 42). Zhang et al. observed that the OPN gene is transactivated up to 5-fold by Tax protein of human T-cell leukemia virus type 1 (42). However, in our study, the therapeutic concentrations of quinolones did not enhance OPN more than 2-fold. Despite our observation of a quinolone-induced OPN enhancement, it is conceivable that such an elevation would not lead to oncogenesis.

Several studies confirmed the antiapoptotic activities of FQs. Azuma et al. suggested that tosufloxacin delayed programmed cell death via the activation of phosphoinositide 3-kinase (PI3K)/Akt and/or p38 MAPK (3). Since OPN is a downstream effector of PI3K/Akt (27, 42) and OPN elicits an antiapoptotic effect (6, 17), we speculate that the antiapoptotic effect of the quinolones is caused by the enhancement of OPN synthesis. We examined the effect of quinolones on early and late stages of apoptosis by FACS analysis. During early apoptosis, the phosphatidylserine (PS) changes location from the cytosolic leaflet to the outer leaflet of the cell. This event can be detected by annexin V, which binds to the PS (16). The damage of the cell membrane during late apoptosis and necrosis allows insertion of 7-AAD between the tops of successive cytosine and guanine bases (41). We could not observe significant changes in early apoptosis upon quinolone treatment, but SPX considerably decreased late apoptosis in A549 cells. We found that the cell culture medium from SPX-treated cells prevented late apoptosis in OPN shRNA-transfected A549 cells, but GRN-treated and untreated cell supernatants had only minor effects. Among the studied quinolones, GRN exerted the greatest ability to enhance OPN synthesis and did not significantly alter early or late apoptosis in A549 cells. OPN synthesis was also enhanced by SPX treatment but to a lesser extent than by GRN, but an effect of SPX on late apoptosis was clearly observed. This made us conclude that there might be other factors which prevent apoptosis upon quinolone treatment and that the OPN might have only a supportive effect or the OPN enhancement was not sufficient to exert its antiapoptotic property.

Throughout respiratory infections, apoptosis may be beneficial or detrimental for the host (4). In infections in which pathogens exist within the host cells, apoptosis favors the host. Insufficient apoptosis of alveolar macrophages during tuberculosis infection leads to the chronicity and dissemination of the infection. For extracellular infections, apoptosis of the immune inflammatory cells potentiates the viability of the pathogen and promotes the infection (4). Our *in vitro* finding showed that SPX decreased late apoptosis in A549 cells. The antiapoptotic effect of quinolones on lung epithelial cells *in vivo* remains to be elucidated.

The quinolones used in our study differ in chemical structure as well as in the ability to influence OPN secretion and apoptosis. The fluorine molecule at the C-6 position, which is present in CIP, MFX, GAT, SPX, STF, and other FQs, is thought to improve the antimicrobial properties of these drugs, but the newly developed GRN is lacking this moiety. GRN has fluorine incorporated through a C-8 difluoromethyl ester linkage (1). Manipulation of the group at position C-8 has also been shown to play a role in broadening the spectrum of activity (12). Among the studied quinolones, only CIP is lacking the substituent at C-8. Our results showed that in contrast to CIP, GRN significantly enhanced OPN production. Therefore, we speculate that the ability of quinolones

TABLE 2 FACS analysis showing early and late apoptosis

Treatment[a]	% cells showing[b]:	
	Early apoptosis	Late apoptosis
Control	3.28	12.6
DMSO (5%)	10.9	33.8
GRN	4.82	9.25
SPX	4.74	4.42
shRNA	6.55	16.7
Negative-control shRNA	5.33	13.6
shRNA + control supernatant	4.61	12.2
shRNA + GRN supernatant	4.99	14.3
shRNA + SPX supernatant	4.98	5.13

[a] A549 cells were plated at 1×10^6 cells per well in 6-well plates. After 24 h of incubation, the cells were washed twice with serum-free Ham's F-12 medium and fresh serum-free medium containing 5% DMSO as a positive control or GRN and SPX at a final concentration of 30 μl/ml, or cells were transfected with shRNA against OPN or negative-control shRNA. Cells were then incubated for a further 48 h and analyzed. Other cells were transfected with shRNA against OPN for 24 h. After the medium was removed, cells were washed twice with serum-free medium; 24-h-conditioned medium from untreated (control supernatant), GRN-treated (30 μg/ml), or SPX-treated (30 μg/ml) cells was added; and cells were incubated for a further 24 h. FACS analysis was performed as described in Materials and Methods. Representative data of three independent experiments are shown.

[b] Cells showing early apoptosis are annexin positive and 7-AAD negative. Cells showing late apoptosis are annexin V positive and 7-AAD positive.

to enhance OPN production may be associated with the presence of the C-8 substituent.

In conclusion, we found that quinolones enhance OPN synthesis by activation of the OPN gene promoter. The antiapoptotic effects of quinolones do not appear to be associated with OPN elevation. Our study supports the idea that quinolones have immunomodulatory properties.

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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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showed that the incidence of TB infections examined by IGRA (in most of the cases IGRA ELISA was used) was very low in NTM patients in MTB non-endemic countries (Van Leeuwen et al. 2007; Adams et al. 2008). In contrast, significant percentages of NTM were positive by IGRA in Taiwan and Korea (Wang et al. 2007; Ra et al. 2011). Given the variety of clinical courses of NTM, the effect of LTBI on the clinical and/or laboratory features of NTM should be evaluated.

It is well known that CD4 cells and Th1-mediated signaling molecules and their pathways are important in the defense against NTM infection (Griffith et al. 2007). Recently, it was found that osteopontin (OPN), a pro-inflammatory cytokine secreted by a wide variety of cells including T cells and macrophages and elevated in tuberculosis, is linked with CD4 T helper (Th1) cell lineage stimulation and was reported to regulate *Mycobacterium avium* in cattle (Karcher et al. 2008). It was also claimed that OPN expression correlates with effective immune and inflammatory responses in MAC-infected individuals (Nau et al. 2000). We also measured pentraxin-3 (PTX-3), which is associated with the acute-phase response and involved in innate immunity. PTX-3 is produced by mononuclear phagocytes, dendritic cells, and endothelial cells in response to inflammatory signals. PTX3 binds with high affinity to the complement and activates the classical pathway of complement and facilitates pathogen recognition by macrophages. (Garlanda et al. 2005; Inforzato et al. 2012). The plasma PTX-3 level, which was reported to reflect the degree of inflammation in MTB infection (Azzuri et al. 2005), can be highly expressed *in vitro* on human PBMCs and monocytes stimulated with lipoarabinomannan (LAM) (Vouret-Craviari et al. 1997).

The current study was designed to compare various serological markers that are associated with TB, including OPN, PTX-3, leptin and soluble IL-2 receptor (sIL-2R), in NTM patients with or without LTBI to characterize their status.

Materials and Methods

Study population

All the patients that participated in this study were receiving care at Tohoku University Hospital between January 2008 and July 2010. We enrolled 9 patients (N1-9) who met the 2007 American Thoracic Society (ATS) microbiological criteria for pulmonary NTM diseases (Griffith et al. 2007). Six patients diagnosed as active TB (T1-6) were served as disease controls (Table 1). Among them, 2 patients (N7, T3) were withdrawn themselves from the study. NTM and MTB were confirmed from the site of infection by culture. In addition to culture, we repeatedly tested the samples from patients by PCR (Roche amplicon), and MTB was never detected in the NTM group. All the patients were assessed for clinical features, medical history including prior tuberculosis disease, treatment history and chest CT scan finding. Individuals with HIV/AIDS infection or who were receiving immunosuppressive therapy were excluded from the study. Five healthy volunteers who were without any symptoms relevant to active tuberculosis were enrolled as negative controls. The study was approved by the Ethics Committee of Tohoku University Hospital (2007-136; 2007-257). We obtained written informed consent from all the participants. All work was conducted in accordance with the Helsinki declaration. Plasma was obtained from EDTA-containing blood by centrifugation and was aliquoted to cryotubes and stored at −80°C for future use. Simultaneously, PBMCs were isolated over Ficoll-Paque Plus gradient and suspended in RPMI 1,640 supplemented with 2 mM L-glutamine, penicillin (100 U/ml), gentamycin (5 μg/ml) and 10% heat-inactivated FCS (Sigma) for further assay.

All the laboratory data including blood cell counts, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and sIL-2R were measured at Tohoku University Hospital.

Table 1. Patients profile and result of IFN-γ release assay (IGRA).

Patients ID	Gender	Age (yr)	Diagnosis	Mycobacteria	Duration of illness	IGRA
N1	M	79	NTM	*M. avium*	5 yrs	negative
N2	F	76	NTM	*M. avium*	5 yrs	positive
N3	F	62	NTM	*M. avium*, *M. intracellulare*, *M. fortuitum*	6 months	negative
N4	F	83	NTM	*M. avium*	7 yrs	negative
N5	F	68	NTM	*M. avium*	7 yrs	positive
N6	M	43	NTM	*M. abscesus*	6 months	positive
N8	M	61	NTM	*M. avium*	13 yrs	negative
N9	F	76	NTM	*M. intracellulare*	3 yrs	positive
T1	F	78	ETB	*M. tuberculosis*	2 months	positive
T2	F	71	ETB	*M. tuberculosis*	1 month	positive
T4	M	65	ETB	*M. tuberculosis*	1 month	positive
T5	F	32	ETB	*M. tuberculosis*	1 month	positive
T6	F	57	PTB	*M. tuberculosis*	1 month	positive

NTM, non-tuberculosis mycobacterium; ETB, extra-pulmonary tuberculosis, PTB, pulmonary tuberculosis.

RD1 PCR analysis

Mycobacterial isolates from 6 NTM patients were kindly provided by the central research laboratory of TUH. DNA was extracted from the clinical isolates as described previously (Nakajima et al. 2010). Briefly, glass beads (0.1 mm), the mycobacteria sample (500 μl in TE buffer) and 500 μl Chloroform were put in a 2 ml microcentrifuge tube for and oscillated by a minibeadbeater, at 4,800 rpm for 2 min. The aqueous phase was collected immediately upon centrifugation. DNA was isolated by 80% ethanol precipitation, dissolved in 50-μl sterile distilled water and stored in −20°C until assay. DNA samples were amplified for the RD1 region (150 bp) by PCR analysis using the same primers described previously (Parsons et al. 2002). DNA from the H37RV strain of *M. tuberculosis* was used as a positive control. PCR was performed with 5 μl DNA samples in a total volume of 50 μl of PCR mix, 5 μl 10 × buffer, 4 μl of 2.5 nM dNTP, 1 μl of Taq DNA polymerase and 0.2 μl of each primer (50 pmol/μl). The mixture was denatured at 95°C for 5 min and cycled for 45 times at 94°C for 30 s than 62°C for 45 s and 68°C for 45 s followed by a final 10-min extension at 68°C. The PCR product was visualized by UV transilumination of ethidine bromide staining after separation by 2% gel electrophoresis.

IGRA ELISPOT assay

The assay was performed as described previously (Guio et al. 2010). Freshly isolated peripheral blood mononuclear cells (2.5 × 10^5 per well) were cultured on plates precoated with antibody against IFN-γ (IGRA ELISPOT. Oxford Immunotech, Oxford UK). After 18-20 hours stimulation of the cells with Early Secreted Antigenic Target-6 (ESAT-6) and CFP-10, the spots were developed according to the manufacturer's instructions. Spot-forming units (SFUs) were counted with an automated ELISPOT reader (KS ELISPOT Carl Zeiss MicroImaging-Germany). The responses were scored as positive if the test wells contained a mean of at least 5 SFUs more than the mean of the negative control wells and was at least twice the mean of the negative control wells.

Inflammatory markers

Anti-TBGL antibody which recognizes glycolipid (maily trehalose 6,6'-dimycolate) of MTB and NTMs was meausered as described (Mizusawa et al. 2008). The plasma OPN concentrations were determined using Human OPN Elisa kit (Immuno-Biological Laboratories, Takasaki, Japan) as described (Chagan-Yasutan et al. 2009), and the plasma PTX-3 levels were measured in special reference laboratory (SRL, Hachiouji, Japan) (Peri et al. 2000). Plasma levels of leptin were measured as described (Siddiqi et al. 2012).

Statistical analysis

Data were analyzed by statcel2 software (OMS, Tokyo, Japan). Continuous data were compared between groups by Mann Whitney *U* test and significance was considered a p value < 0.05.

Results

Subjects

Profiles of the patients are listed in Table 1. The NTM patients (43 to 83 years old (y.o.)) were infected predominantly by *M. avium complex* (Table 1) and mostly had pulmonary involvement except N6, who had extra-pulmonary involvement (nasal granulomatosis). The duration of illness was variable, ranging from 2 to 148 months. None of them were infected with *M. marinum, M. szulgai* or *M. kansasi,* which are known to possess the RD1 region. All the TB patients included in this study (32 to 78 y.o.) had extra-pulmonary disease except T6 (pulmonary TB). All 5 healthy volunteers (25 to 60 y.o.) were apparently healthy without any TB-related symptoms.

Verification of absence of RD1 region and M. tuberculosis strains

Absence of the RD1 region in the DNA samples from the clinical strains of the 6 NTM patients (N1-N6) was confirmed by PCR analysis. No positive band was observed at the 150 bp position for the RD1 region in any of the clinical isolates (Fig. 1).

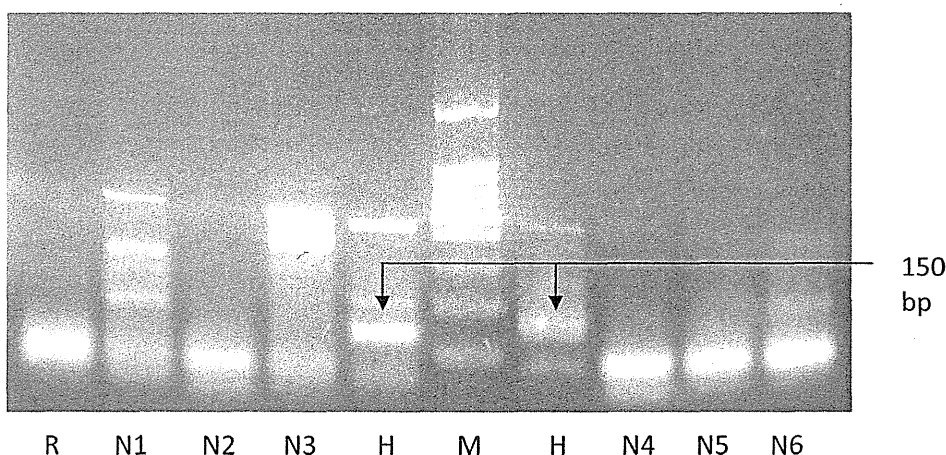

Fig. 1. PCR amplification of RD1 region using DNA obtained from mycobacteria isolated from NTM patients.
Arrows indicate the positive bands at 150 bp. N1 to N6, (DNA from 6 NTM patients); H, DNA from *Mycobacterium tuberculosis* (H37RV); M, Marker; R, reagent only.

IGRA and association with the clinical condition

All healthy controls except one were negative for IGRA. Four of 8 NTM patients and all TB patients were positive by IGRA (Table 1). Repeated examination by culture and PCR showed negative for MTB infection in all NTM patients. We further stratified these NTM patients into two subgroups, IGRA-positive as NTM with LTBI (NTM+) and IGRA-negative as NTM without LTBI (NTM−).

Patients in the NTM+ group had pulmonary pathology related to NTM disease based on clinical and radiographic features: consolidation and pulmonary infiltration in N2, both cavity and nodule in N5 and N9 had a combination of bronchiectasis, cavity, consolidation and nodules. Patients of the NTM− group also had typical radiographic features related to NTM infection. The patients of the NTM+ group had symptoms for longer periods (36 to 84 months), whereas the NTM patients had symptoms for variable durations (2 to 148 months), although the difference was not significant. No differences in age or clinical presentation were observed between the NTM+ and NTM− groups. Only patient N5 from the NTM+ group had a history of prior TB infection at the age of 18 that was cured by anti-TB therapy. Among all the NTM patients, only 2 NTM+ patients (N5, N9) had been receiving treatment for NTM infection because of clinical and radiological severities. Patient N5 was treated with rifampicine (RFP), clarithromycine (CAM), ciprofloxacin (CPFX) and ehtambutol (EB) for 6 months starting from July 2001. From May 2004, he was treated again with RFP and CAM, and treatment was continued until October 2005. Since then, he has been treated with different quinolone derivatives including gatifloxacin, CPFX, levofloxacin until June 2006. Finally, the patient was on combined therapy of RFP, CAM and a quinolone derivative until the time of the assay. Patient N9 was also treated with RFP, EB and CAM for 2 months before enrolling in the study. All the other patients hadn't received any drugs for NTM infection. Patients, N5 and N9 had died before the writing of this paper, though detailed information was not available.

Laboratory markers

Laboratory findings are listed in Table 2. There were no significant differences in the conventional markers between the NTM− and + groups. The levels of leptin were apparently lower in the NTM+ and TB groups than in the NTM− group, but no significant differences were found among the groups (Table 2, Fig. 2F).

Inflammatory markers

The data of inflammatory markers are shown (Fig. 2). The TBGL antibody levels are elevated in the NTM+ group and such elevations were unexpectedly not seen in the MTB group, probably because the most of the TB patients were the mild, extra-pulmonary type (Fig. 2A). Patients of the NTM+ group had increased levels of OPN (859 to 1,499 ng/ml) (normal value: < 820 ng/ml; according to Chagan-Yasutan et al. 2009) and the levels were significantly higher

Table 2. Clinical characteristics of patients enrolled in the study.

Laboratory data	Ref. range	Median		
		NTM−	NTM+	TB
RBC (10^6/ul)	3.93-5.03	3.915	3.74	3.76
WBC (/ul)	3.2-9.6	5,250	5,650	4,100
Neutrophil %	31-73	62	58.5	69
Eosinophil %	0-7	1	2	1
Basophil %	0-3	0.5	0.5	0.005
Lymphocyte %	18-51	27	30.5	5
Monocyte %	1-12	8	7.5	5
Platelets (10^3/ul)	155-347	232.5	229.5	277
Hb (g/dl)	11.7-14.8	12.9	11.2	11.7
CRP (mg/dl)	0-0.2	1.2	2.45	0.5
Albumin (g/dl)	4.2-5.3	4.1	3.6	3.7
IgG (mg/dl)	748-1,694	1,320	1,542	1,564
IgM (mg/dl)	33-254	86.5	103	115.5
IgA (mg/dl)	91-391	281	336	295
ESR (30 min) (mm)	(−)	8	11	23
ESR (1 hr) (mm)	(−)	22.5	25	53
KL-6 (U/ml)	105-435	310	315.5	347.5
Leptin (pg/ml)	4,700-32,500	8,105	3,437	4,013

NTM−, IGRA negative non-tuberculosis mycobacterium patients; NTM+, IGRA positive non-tuberculosis mycobacterium patients; TB, tuberculosis patients.

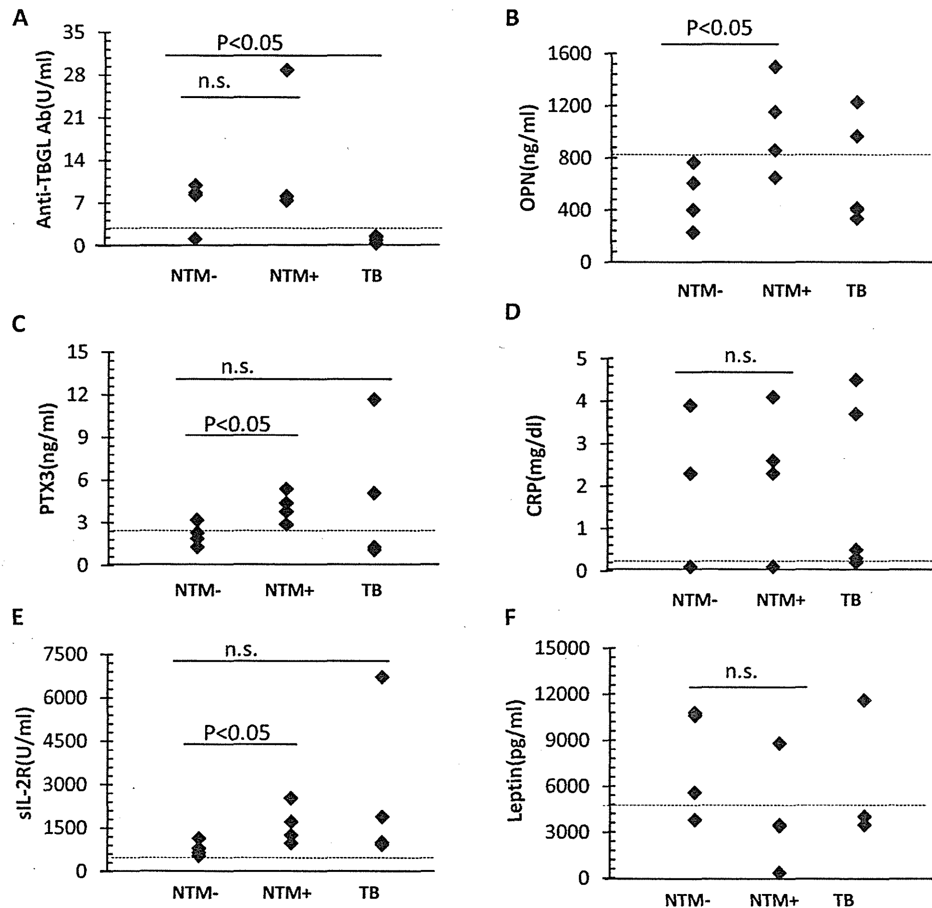

Fig. 2. Comparison of plasma inflammatory molecules.
NTM−, NTM patients without LTBI (n = 4); NTM+, NTM patients with LTBI (n = 4); TB, active TB patients (n = 5).

than those of the NTM− ($p < 0.05$) (Fig. 2B). The OPN titers in patients N5 and N9 were very high 1,150 ng/ml and 1,499 ng/ml, respectively (Fig. 2B). The CRP levels were not different between the NTM+ and NTM− groups, but the PTX-3 levels (normal value: < 2.3 U/ml) and sIL-2R (normal value: 122-496 U/ml) were elevated in all NTM+ patients and the difference from the NTM− group was significant ($P < 0.05$) (Fig. 2C, D and E).

Discussion

In the current study, 4 of 8 NTM patients (50%) were found to be LTBI, and their average age was 74.5 y.o. In addition, PCR analysis showed that all of our NTM isolates lacked the RD1 region. An age-dependent increase of LTBI has already been described in Japan, where 9.8% for those aged 60-69 were IGRA ELISA positive (Mori et al. 2007). The excellence of IGRA ELISA to differentiate NTM and TB infection in children in non endemic countries was reported (Detjen et al. 2007). In Japan, only 1-8% of IGRA ELISA positive rate in Japanese patients with MAC disease was reported (Kobashi et al. 2006; Kobashi et al. 2009). However 34-49% IGRA ELISA positive cases were reported in NTM in endemic countries (Ra et al. 2011). The reasons of high rates of IGRA-positive rates in this study could be explained by IGRA ELISPOT assay employed here. It is known that IGRA ELISA tends to show false negative results among thin elderly people, presumably due to decreased immune levels, whereas IGRA ELISPOT assay might have detected LTBI more sensitively in the elderly patients with NTM disease. It was reported that the long-lasting positive IFN-γ response to antigenic challenge continues for 5 to 10 years following anti-TB therapy (Adams et al. 2008).

NTM+ patients had significantly higher titers of inflammatory markers such as OPN, PTX-3 and sIL-2R, though CRP did not show significant differences (Fig. 2). We also observed sustained high levels of OPN after treatment in NTM+ patients. It is possible that anti-NTM therapy was ineffective because two patients died. Alternatively, persistent elevations of OPN after chemotherapy were already reported by us in AIDS patients treated by anti retroviral therapy (Chagan-Yasutan et al. 2009). Additionally our recent study supports the idea of immune-modulator effect of quinolone which enhance the

production of OPN in human lung epithelial cell line A549 *in vitro* (Shiratori et al. 2012). We assume that the quinolone treatment may be one of the factors of persistent OPN elevation. The increased plasma OPN in TB patients contributed to the disease pathology by activating the IL-12 mediated Th1 immunity (Koguchi et al. 2003). It was also found that OPN expression correlates with an effective inflammatory response and contributes to human resistance against MTB (Nau et al. 2000). In cattle, it was proposed that OPN is a key regulator against *M. avium* (Karcher et al. 2008). Immune responses by *M. avium complex* preferentially depend on the phase of infection in human. Early acute infection causes increased IFN-γ secretion, while the chronic phase has been reported to be associated with copious IL-10 production (Azouaou et al. 1997) with an inclination toward Th2 cytokines (Vouret-Craviari et al. 1997) that may provide protection against chronic diseases. We have already reported that the plasma levels of IFN-γ, OPN and leptins did not show any significant changes between LTBI and non-LTBI health care workers (HCW). Though only LTBI HCWs showed the association of TBGL-IgA antibody titer and serum IFN-γ (Siddiqi et al. in press). Our finding may imply that NTM co-infection with LTBI can synergistically induce large amounts of OPN. The synergistic effect could be explained by the natural resistance associated macrophage protein 1 (NRAMP1) because it was reported as host genetic factor for development of both tuberculosis and NTM, however the involvement of NRAMP1 in OPN production was not studied (Li et al. 2011; Sapkota et al. 2012).

It is also interesting that the PTX-3 levels were significantly higher in the NTM+ group while the CRP levels did not differ. It was documented that 5 of 220 TB contacts who developed active TB within 5 to 12 months of follow-up had elevated levels of PTX-3 (Azzuri et al. 2005). The PTX3 haplotype frequencies significantly differed in TB cases compared to controls, and a protective effect against MTB was found in association with a specific haplotype (Olesen et al. 2007). Hence, Th1 mediated PTX-3 production in mycobacterial infection also warrants further investigation.

In conclusion, frequent LTBI was detected in aged NTM patients, and these patients expressed higher levels of inflammatory markers than NTM without LTBI patients. The low number of patients is the main limitation of this study, but careful observation and extensive therapeutic intervention appear to be necessary in NTM with LTBI patients.

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Conflict of Interest

The authors declare no conflict of interest.

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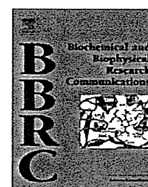

A simple, rapid, and sensitive system for the evaluation of anti-viral drugs in rats

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ABSTRACT

The lack of small animal models for the evaluation of anti-human immunodeficiency virus type 1 (HIV-1) agents hampers drug development. Here, we describe the establishment of a simple and rapid evaluation system in a rat model without animal infection facilities. After intraperitoneal administration of test drugs to rats, antiviral activity in the sera was examined by the MAGI assay. Recently developed inhibitors for HIV-1 entry, two CXCR4 antagonists, TF14016 and FC131, and four fusion inhibitors, T-20, T-20EK, SC29EK, and TRI-1144, were evaluated using HIV-1_{IIIB} and HIV-1_{BaL} as representative CXCR4- and CCR5-tropic HIV-1 strains, respectively. CXCR4 antagonists were shown to only possess anti-HIV-1_{IIIB} activity, whereas fusion inhibitors showed both anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities in rat sera. These results indicate that test drugs were successfully processed into the rat sera and could be detected by the MAGI assay. In this system, TRI-1144 showed the most potent and sustained antiviral activity. Sera from animals not administered drugs showed substantial anti-HIV-1 activity, indicating that relatively high dose or activity of the test drugs might be needed. In conclusion, the novel rat system established here, "phenotypic drug evaluation", may be applicable for the evaluation of various antiviral drugs *in vivo*.

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1. Introduction

Numerous antiviral agents have been developed to suppress infection with viruses such as human immunodeficiency virus type 1 (HIV-1) [1], and have successfully provided excellent outcomes *in vivo*. However, the emergence of drug-resistant HIV-1 variants is a major concern in HIV therapy. Therefore, the development of novel drugs with sustained activity to resistant variants is desirable. Drugs, especially those targeting HIV-1 entry, have been recently developed and approved, such as a CCR5 antagonist, maraviroc [2], and a fusion inhibitor, enfuvirtide (T-20) [3], where both drugs effectively suppress HIV-1 in the patient even resistant to previous drugs [4,5].

In addition to CCR5, which is a main co-receptor for clinical HIV-1 strains, CXCR4 can also act as a co-receptor for HIV-1 (X4-tropic HIV-1), such as that seen for the vast majority of laboratory-adapted HIV-1 strains [6]. Thus, CXCR4 is also considered an important therapeutic target. We previously identified a β-sheet-like 14-residue peptide, T140 [7,8], and its down-sized analog, a cyclic pentapeptide FC131 (Fig. 1) [9], as potent and specific CXCR4 antagonists. Both T140 and FC131 were proved to inhibit X4-tropic HIV-1 infection *in vitro*. T140 has been further modified to TF14016 (4F-benzoyl-TN14003; BKT140) that shows more potent inhibitory effect [10].

The first fusion inhibitor, T-20, efficiently inhibits replication of HIV-1 resistant even to inhibitors for reverse transcriptase and protease [11,12]. However, the genetic barrier to overcome suppression by T-20 seems to not be high since a 1–2 amino acid(s) substitution in gp41 appears to be sufficient for resistance [13–15]. Therefore, we developed T-20EK [16] and SC29EK [17] as novel and potent fusion inhibitors that sustain their inhibitory effects on T-20 resistant HIV-1 stains. A series of systematic replacements with hydrophilic glutamic acid (E) or lysine (K) was introduced (EK motif) at the solvent-accessible site to enhance the α-helicity of the peptides by possible intrahelical electrostatic interactions [18]. T-20EK/S138A [16] was synthesized

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