表 1. HVJ-E の安全性試験の状況

□ ラット　□ カニクイザル

試験項目	動物種	投与経路	投与期間	目的	試験グレード
単回投与毒性	ラット	静脈内	単回	用量設定	GLP試験施設で実施、信頼性基準適合
	ラット	皮下	単回	用量設定	GLP試験施設で実施、信頼性基準適合
	ラット*	皮下	単回	申請用	GLP
	カニクイザル	静脈内	単回	用量設定	GLP試験施設で実施、信頼性基準適合
	カニクイザル	皮下	単回	用量設定	GLP試験施設で実施、信頼性基準適合
	カニクイザル	皮下	単回	申請用	GLP
反復投与毒性	ラット	静脈内	7日間反復	用量設定	GLP試験施設で実施、信頼性基準適合
	ラット**	皮下	2週間間歇	申請用	GLP
	カニクイザル	皮下	2週間間歇	用量設定	GLP試験施設で実施、信頼性基準適合
	カニクイザル	皮下	2週間間歇	申請用	GLP
TK（トキシコキネティク	ラット	皮下	2週間間歇	申請用	GLP（ラット反復皮下投与毒性試験組込）
遺伝毒性（小核試験）	ラット	皮下	単回	申請用	GLP試験
免疫毒性（抗体産生）	ラット	皮下	2週間間歇	申請用	GLP試験
免疫毒性（サイトカイン）	カニクイザル	皮下	2週間間歇	参考	GLP試験で調製した血清使用
安全性薬理試験（コアバッテリー）	ラット 中枢神経系	皮下	単回	申請用	GLP（ラット単回皮下投与毒性試験組
	ラット呼吸器系	皮下	単回	申請用	GLP
	サル 心血管系	皮下	単回	用量設定	GLP試験施設で実施、信頼性基準適合
	サル 心血管系	皮下	単回	申請用	GLP試験実施

＊ 安全性薬理試験(コアバッテリー試験：中枢神経系)を組み込み、 ** TK、抗体産生を組み込み

る重篤な有害事象の発生は認められていない。そのため、HVJ-E の投与による免疫活性化は投与部位の局所反応であり、全身性の重篤な炎症反応を誘導する可能性は低いと考えられた。現在までに臨床治験の開始に必要な GLP 安全性試験データ（一般毒性、特殊毒性、トキシコジェネティクス、安全性薬理）の取得を完了しており、本事業の DNA ワクチンの臨床応用を開始する際にも使用できる状況である（表 1）。

2）大阪大学医学部附属病院が去勢抵抗性前立腺癌を対象とした臨床試験を開始した。これまで実施していた悪性黒色腫を対象とした臨床研究では皮内やリンパ節内の腫瘍内投与が実施されていたが、前立腺癌では前立腺内投与に加え、皮下投与も実施されている。そのため、HVJ-E を含有する DNA ワクチンを皮下、皮内に投与して安全性を評価する場合には、それらの試験結果を考慮する必要があると考えられた（図1）。

3）HVJ-E をアジュバントとして添加することで、Th1 細胞を介する細胞性免疫をより強く活性化出来ることが示唆された（図2、図3）。結核感染の防御には、自然免疫や細胞性免疫を活性化する事が重要であると考えられるため、HVJ-E は結核に対するワクチンのアジュバントに適した免疫活性化能を有する事が示唆された。通常、DNA ワクチンを含む感染症ワクチンの評価では血清中の抗体価の産生が指標とする場合が多いが、HVJ-E のようにより細胞性免疫を活性化するアジュバントの開発では、抗体価に加え自然免疫や細胞性免疫の活性化を評価する必要が有ると考えられた。実際、Th1/Th2 の免疫バランスを考慮した場合、Th1 を介する細胞性免疫の活性化へ免疫バランスがシフトした場合には、抗体価の低減が予測される事から、抗体価を指標とするアジュバント効果の評価は、適切でない場合も考えられた（図4）。一方、DNA ワクチンに適した投与経路の最適化では、皮内投与では筋内投与と比較してワクチンとしての活性が 10 倍程度増強される事を示唆する報告がある。皮内投与での指標は抗体価によるものであるため、より一般的なワクチンの投与系である筋内投与や皮下投与とは免疫活性化の機序が異なる可能性がある。そのため、HVJ-E のアジュバント効果についても種々の投与経路でのアジュバント効果についても評価を実施知る必要があると考えられた。

E．結論

1）DNA ワクチンの構成物質（アジュバント兼デリバリーシステム）である HVJ-E に関する GLP 安全性試験データの取得を実施した。また、臨床での安全性については、大阪大学医学部附属病院が、皮内やリンパ節内の腫瘍内投与に加え、前立腺内投与や皮下投与の安全性評価を開始した。これらの試験結果を考慮して、臨床応用デザインを設定する必要がある。

2）HVJ-E は、結核防御に重要な Th1 を介する細胞性免疫をより強く活性化する事が明らかとなった。DNA ワクチンの投与には、従来の筋内投与よりも皮内投与の方が優位性高い事を示唆する報告があり、今後作用機序を詳細に検討する必要があると考えられた。

G．研究発表

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A. HVJ-Eの臨床応用の概要(1):進行性悪性黒色腫
(HVJ-Eのfirst in human trial)

1. 研究名:
進行性悪性黒色腫患者を対象としたHVJ-E腫瘍内局所注入治療の安全性/忍容性及び腫瘍免疫誘導の評価のための臨床研究(第I/II相臨床研究)

2. 対象:再発、進行性悪性黒色腫
⇒標準治療抵抗性
⇒標準治療が適用
⇒標準治療を拒否
⇒ステージIIIC又はステージ IV

3. 主要エンドポイント: 安全性及び忍容性の確認
4. 副次エンドポイント: 腫瘍免疫誘導能の評価、抗腫瘍効果

5. 試験デザイン: 非ランダム化、オープン、無対照

6. 目標症例数: 6名(最大12名)

7. 責任研究者:大阪大学大学院医学系研究科・皮膚科学教室 片山 一朗 教授

B. HVJ-Eの臨床応用の概要(2):去勢抵抗性前立腺癌
(HVJ-Eの皮下投与のfirst in human trial)

1. 研究名:
去勢抵抗性再燃前立腺癌患者を対象としたHVJ-E腫瘍内投与および皮下投与の 安全性及び有効性の評価のための臨床試験(第I/II相臨床試験)

2. 対象:去勢抵抗性再燃前立腺癌患者
⇒ドセタキセルによる標準治療が適用されない患者
⇒The Prostate Cancer Clinical Trials Working Group 2 (PCWG2)の基準(Scher et al. 2008)で無効である患者
⇒ドセタキセルによる標準治療を拒否した患者

3. 主要エンドポイント: HVJ-E調製液の安全性及び忍容性の確認
4. 副次エンドポイント: 腫瘍免疫誘導能の評価、抗腫瘍効果

5. 試験デザイン: 非ランダム化、オープン、無対照

6. 目標症例数: 6名(最大12名)

7. 責任研究者:大阪大学大学院医学系研究科・泌尿器科学教室 野々村祝夫教授

図1. HVJ-Eの臨床研究、臨床試験
(大阪大学医学部附属病院が実施中)

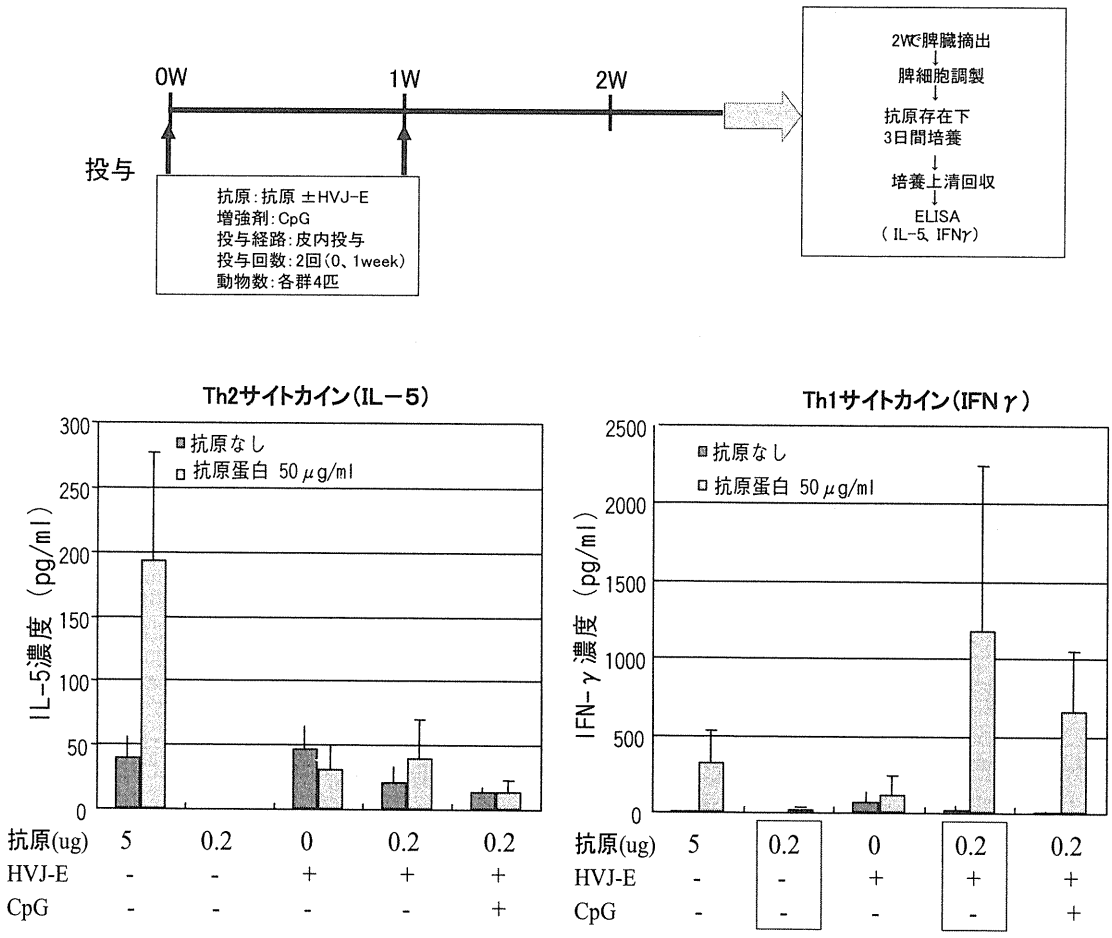
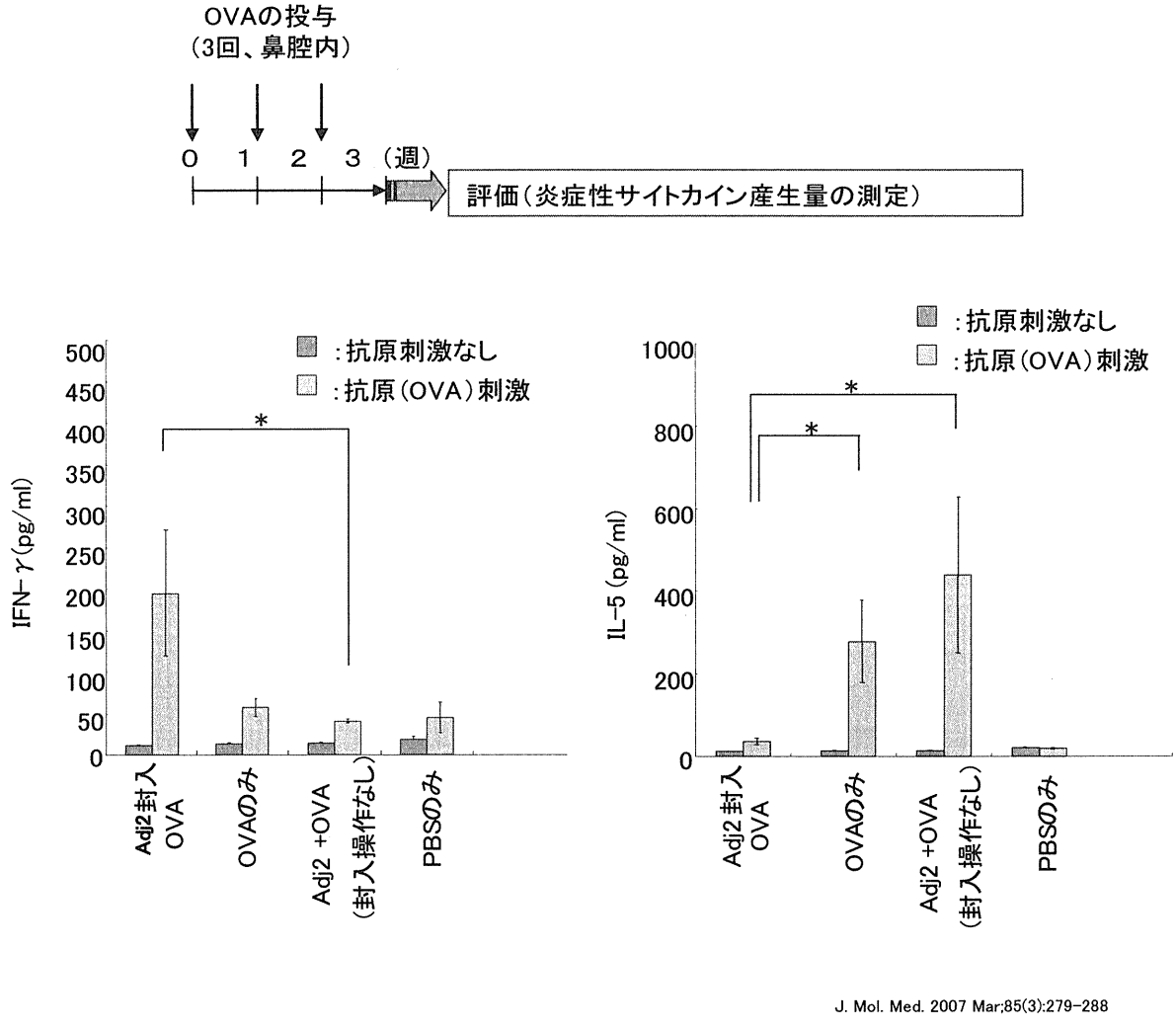


図2．HVJ-Eによる細胞性免疫の増強（1）

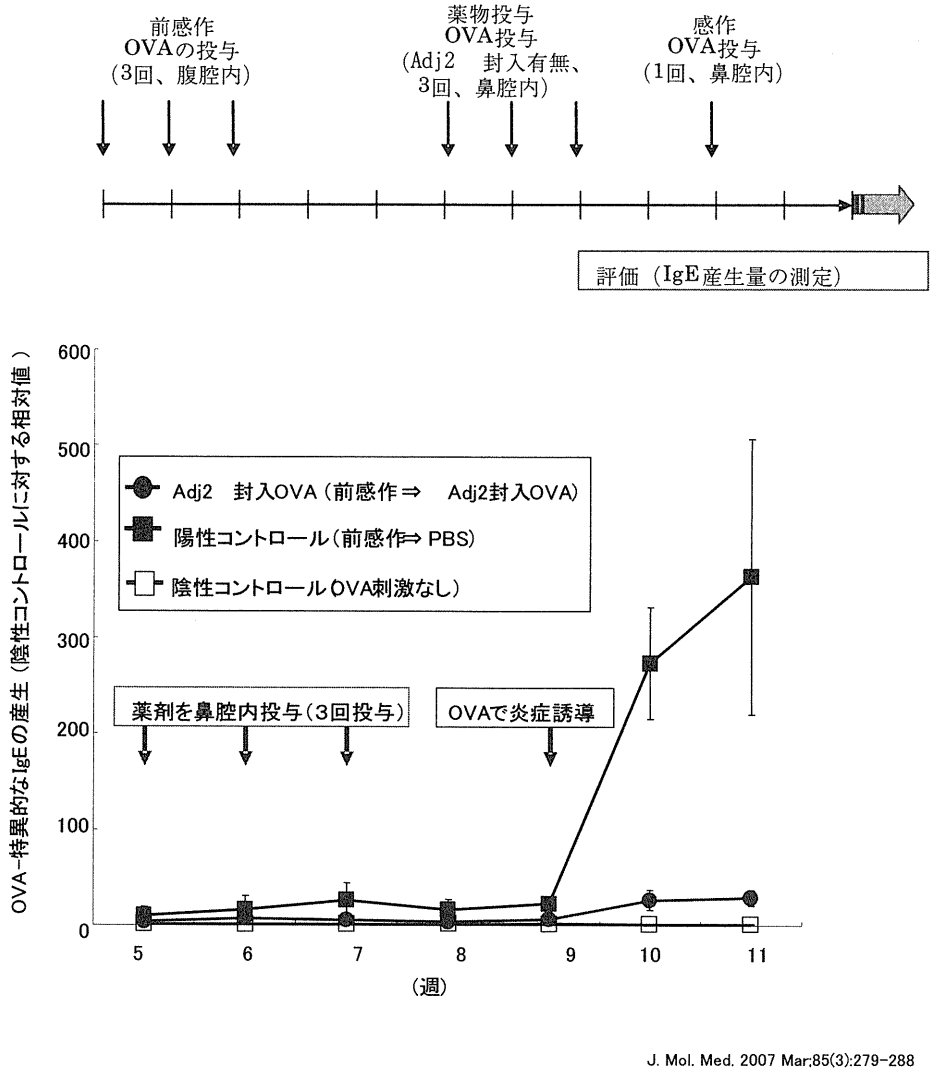
⇒HVJ-Eを添加した抗原蛋白を皮下投与するとTh1サイトカインの産生が優勢になる（細胞性免疫が優勢になる）。



J. Mol. Med. 2007 Mar;85(3):279-288

図3. HVJ-Eによる細胞性免疫の増強（2）

⇒ アレルギー性鼻炎モデルに、アジュバント2を添加した蛋白（OVA）を投与するとTh1サイトカインの産生が優勢になる。



J. Mol. Med. 2007 Mar;85(3):279-288

図4. HVJ-Eによる抗体産生の抑制

⇒アレルギー性鼻炎モデルに、アジュバント2を添加した蛋白(OVA)を投与すると抗体産生(IgE産生)が減少する。

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該当なし

H．知的財産権の出願・登録状況
（予定を含む。）

1．特許取得

該当なし

2．実用新案登録

該当なし

3．その他

該当なし

別紙4

研究成果の刊行に関する一覧表

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添付資料

Microbiology and Immunology

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doi:10.1111/j.1348-0421.2011.00348.x

ORIGINAL ARTICLE

Decreased plasma granulysin and increased interferon-gamma concentrations in patients with newly diagnosed and relapsed tuberculosis

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ABSTRACT

Granulysin and interferon-gamma (IFN-γ) have broad antimicrobial activity which controls *Mycobacterium tuberculosis* (*M. tuberculosis*) infection. Circulating granulysin and IFN-γ concentrations were measured and correlated with clinical disease in Thai patients with newly diagnosed, relapsed and chronic tuberculosis (TB). Compared to controls, patients with newly diagnosed, relapsed and chronic TB had lower circulating granulysin concentrations, these differences being significant only in newly diagnosed and relapsed TB ($P < 0.001$ and 0.004, respectively). Granulysin concentrations in patients with newly diagnosed and relapsed TB were significantly lower than in those with chronic TB ($P = 0.003$ and $P = 0.022$, respectively). In contrast, significantly higher circulating IFN-γ concentrations were found in patients with newly diagnosed and relapsed TB compared to controls ($P < 0.001$). The IFN-γ concentrations in newly diagnosed and relapsed patients were not significantly different from those of patients with chronic TB. However, *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) from patients with newly diagnosed, relapsed and chronic TB with purified protein derivative (PPD) or heat killed *M. tuberculosis* (H37Ra) enhanced production of granulysin by PBMCs. *In vitro*, stimulation of PBMCs of newly diagnosed TB patients with PPD produced greater amounts of IFN-γ than did controls, while those stimulated with H37Ra did not. The results demonstrate that patients with active pulmonary TB have low circulating granulysin but high IFN-γ concentrations, suggesting possible roles in host defense against *M. tuberculosis* for these agents.

Key words clinical disease, granulysin, IFN-γ, tuberculosis.

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List of Abbreviations: APC, antigen presenting cell; BCG, Bacillus Calmette-Guérin; CTL, cytotoxic T lymphocyte; E, ethambutol; H, isoniazid; IFN-γ, interferon gamma; IGRA, interferon-γ release assay; IL, interleukin; MDR, multi-drugs resistance; MHC, major histocompatibility complex; *Mtb*, *Mycobacterium tuberculosis*, *M. tuberculosis*, *Mycobacterium tuberculosis*; NK, natural killer; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; R, rifampicin; S, streptomycin; TB, tuberculosis; Th1, T-helper type 1; TMB, tetramethylbenzidine; TNF, tumor necrosis factor; TST, tuberculin skin test; XDR, extensively drug resistant; Z, pyrazinamide.

Tuberculosis is a major health problem worldwide, with one third of the world population being infected and approximately 1.1–1.7 million deaths annually (1). Most individuals infected with *Mtb* are asymptomatic. However, 5–10% will progress to active TB during their lifetime, the remainder being resistant to active TB, but remaining infected. Relapse of TB, which is defined as an episode of infection occurring after a previous episode has been treated and considered cured, is possibly due to endogenous reactivation when it occurs in geographical areas with a low incidence of TB infection (2). However, generally the risk of relapse depends on the intensity of exposure to *Mtb*. Other factors that directly affect the clinical course of TB are host factors, including age, immune status, genetic factors and coinfection with HIV, and bacterial factors, including degree of exposure, virulence of strain, MDR and XDR.

Protective immunity against *Mtb* infection involves activated macrophages, antigen-specific T cells and type-1 cytokines such as IL-12, IFN-γ and TNF (3, 4). Inherited defects of the IL-12/IFN-γ pathway appear to result in a variety of changes in mycobacterial susceptibility. People with genetic deficiencies in the type-1 cytokine (IL-12/IL-23/IFN-γ) axis, and those with neutralizing autoantibody against IFN-γ, have been found to be highly susceptible to mycobacterial infections including TB (5–8). In active pulmonary TB, these effectors of the immune response are activated, as evidenced by observation of high circulating IFN-γ concentrations that decrease significantly following two months of therapy (9, 10).

Granulysin can kill extracellular *Mtb* directly, or intracellular bacteria in the presence of perforin (11), expression of granulysin in CD8+T cells being induced upon activation. It has recently been reported that granulysin is strongly associated with diverse activities of NK cells and CTLs in physiological and pathological settings, and might be a useful novel serum marker for evaluating the overall status of host cellular immunity (12). In patients with cutaneous leprosy, the frequency of granulysin-expressing T cells lesions is 6-fold greater than in those with the disseminated lepromatous form of the disease (13). In contrast, adults with active pulmonary TB in a highly TB endemic area in Indonesia had significantly lower plasma granulysin concentrations than did controls, these concentrations increasing after 2 months of anti-TB therapy to values similar to those of controls, and having increased even further after completion of anti-TB therapy. These changes in granulysin concentrations occurred predominantly in patients in whom IFN-γ negative T cells were expressed, suggesting that in TB the cellular sources of IFN-γ and granulysin are partly non-overlapping (14). Similar findings have been reported for Italian children, the lowest concentrations having been found in TB patients who were PPD negative at the time of diagnosis (15), indicating the involvement of granulysin and IFN-γ in curative immune responses against *Mtb*. In chronic pulmonary TB, lung tissue biopsy has shown reduction in amounts of perforin and granulysin in relation to granzyme A, while higher per cell expression of perforin and granulysin is associated with bacteriological control, suggesting that perforin and granulysin could be used as markers or correlates of immune protection in human TB (16). However, effective host mechanisms against *Mtb* infection are not well understood, this lack of understanding being a problem in regard to vaccine development and immunotherapy for TB. Moreover, so far there is limited information regarding the roles of IFN-γ and granulysin in recurrent TB. Therefore, the present study aimed to investigate whether granulysin and IFN-γ responses are associated with clinical disease in patients with newly diagnosed, relapsed and chronic pulmonary TB in northern Thailand, where TB is endemic.

MATERIALS AND METHODS

Subjects

One hundred and fifty-five pulmonary TB patients (aged 9 to 88 years) were recruited from the outpatient and inpatient clinics of Chiang Rai Hospital and Mae Chan Hospital, in the north of Thailand. These included 102 male and 53 female patients with newly diagnosed and previously treated pulmonary TB. Patients with extrapulmonary TB and pulmonary TB/HIV seropositive were excluded. All patients with pulmonary TB had clinical symptoms and a confirmed diagnosis on the basis of presence of acid-fast bacilli in sputum on microscopic examination, positive cultures of *Mtb*, medical history and chest radiographic findings. Patients were categorized according to World Health Organization criteria (1), which include ascertaining whether the patient has previously received TB treatment. The TB drug regimens were based on the recommendations of the National Tuberculosis Program, Ministry of Public Health, Thailand. Standard TB treatment drugs consist of streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). In this study, patients with newly diagnosed TB were defined as those who had never received treatment for TB or had taken anti-TB drugs for less than 1 month prior to enrollment ($n = 84$). Patients with relapsed TB were defined as those previously treated for TB and declared "cured" or "treatment completed", and currently diagnosed as *Mtb* positive by smears and cultures ($n = 35$). Patients with chronic TB were defined as those who had started on a retreatment regimen after having failed previous treatment ($n = 36$). No patients had been reported to be MDR or

XDR cases on the basis of drug sensitivity tests at the time of enrollment in this study.

Thirty three healthy individuals (aged 21 to 54 years old, median = 36 years) recruited from the Blood Bank of Chiang Rai Hospital, Mae Chan Hospital and Phan Hospital were used as controls. They had no history suggestive of TB or other acute infectious diseases or diabetes at the time of enrollment. However, they were not subject to chest X-rays, TSTs or testing for latent TB infection and infection manifesting as active TB by IGRA upon enrollment.

The ethical aspects of this study were approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Ref. No.3/2550) as part of a project studying multiple factors in recurrent TB, and written informed consent was obtained from all subjects.

Blood samples

Before instituting anti-TB therapy, blood was collected aseptically in EDTA Vacutainers. Plasma and packed cells were separated by centrifugation and stored at −80°C.

HIV screening

HIV positive cases were excluded from the study by screening with the particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Champaign, IL, USA) or by ELISA (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

Peripheral blood mononuclear cells isolation and stimulation

Peripheral blood mononuclear cells from 75 pulmonary TB patients and 4 healthy controls were isolated by Ficoll-Hypaque density gradient centrifugation. In brief, 3 mL of whole blood in K_3EDTA (Greiner Bio-One, Bangkok, Thailand) was diluted with an equal volume of PBS, mixed gently and layered carefully over 3 mL Ficoll-paque PLUS (Amersham Biosciences, Uppsala, Sweden). After centrifugation at 1000 g for 20 min at room temperature, the PBMCs were harvested. The supernatant was removed after centrifugation at 700 g for 10 min at 4°C and the pellet adjusted with RPMI 1640 containing 10% FBS. The viable PBMCs were counted in 0.2% Trypan blue. Approximately 1×10^6 PBMCs/mL in RPMI 1640 medium containing 10% FBS and 2-mercapto ethanol were added to each well of a 24 well plate, stimulated either with 20 μg/mL of PPD (Japan BCG laboratory, Kiyose, Japan) or heat killed *Mtb* (H37Ra) (Difco, Detroit, MI, USA) and incubated at 37°C in 5% CO_2. The supernatants were harvested after 40 hr of stimulation, centrifuged at 1200 g for 3 min at 4°C and kept at −80°C. PMBCs stimulated with 20 μg/mL of PPD and not stimulated were used as positive and negative controls, respectively.

Determination of circulating granulysin and granulysin production by peripheral blood mononuclear cell stimulation assay

The granulysin concentrations in plasma and stimulated PBMC supernatant were determined by ELISA according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). The tests were done in duplicate. Briefly, a microtiter plate (Costar, Cambridge, MA, USA) was coated with 100 μL/well of 5 μg/mL monoclonal mouse anti-human granulysin (clone RB1) (MBL International, Nagoya, Japan) in 0.05 M carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with buffered protein solution with ProClin-150 at room temperature for 1 hr. After being washed, the undiluted plasma was added and incubated for 2 hr at room temperature. The bound antigens were detected with 0.1 μg/mL of monoclonal mouse anti-human granulysin biotin (RC8) (MBL International) and avidin-horseradish peroxidase (Av-HRP) conjugate (BD Biosciences Pharmingen) diluted to 1:1000. After incubation for 1 hr, the reactions were developed by coloring with TMB substrate (BD Biosciences Pharmingen) for 20 min in the dark. The reaction was stopped by 2N H_2SO_4 solution (BD Biosciences Pharmingen). Optical densities were measured at 450 nm wavelength by an ELISA reader (ELx808 IU ultra microplate reader, Bio-Tek instruments, Winooski, VT, USA). Granulysin concentrations were calculated from a standard curve using granulysin containing culture supernatant obtaining from Cos7 cell transfected with gene encoding 15K granulysin. The lower detection limit for granulysin was 0.047 ng/mL.

Determination of circulating interferon-γ concentrations and interferon-γ production from stimulated mononuclear cells *in vitro*

Interferon-γ concentrations in plasma and stimulated PBMC supernatant were determined by ELISA according to the manufacturer's instruction (BD Biosciences Pharmingen). The tests were done in duplicate. Briefly, a microplate (Costar) was coated with 100 μL/well of anti-human IFN-γ (diluted to 1:250 in 0.1 M sodium carbonate) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20, blocked with 200 μL/well of buffered protein solution with ProClin-150 and incubated at room temperature for 1 hr. After being washed, 100 μL of undiluted sample was added and incubated for 2 hr at room temperature. The bound

antigen were detected with biotinylated anti-human IFN-γ monoclonal antibody and streptavidin-horseradish peroxidase conjugate (diluted to 1:250 with 10% FBS in PBS) and incubated for 1 hr at room temperature. Then, 100 μL of TMB substrate solution was added and incubated for 30 min at room temperature in the dark. The reaction was stopped by 2N H_2SO_4 solution. Samples were analyzed at 450/550 nm wavelength with a microplate ELISA reader (ELx808 IU ultra microplate reader) and IFN-γ concentrations were calculated from a standard curve using recombinant human IFN-γ. The lower detection limit was 4.7 pg/mL.

Statistical analyses

Statistical analyses were performed by SPSS software version 17.0. IFN-γ and granulysin concentrations in different independent subject groups were compared by Mann-Whitney U test. A P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of subjects

The clinical characteristics of the patients in the study with newly diagnosed, relapsed and chronic TB are summarized in Table 1. Infiltrates without cavitation were found on the chest radiographs of the majority of patients with newly diagnosed (57.1%) and relapsed TB (51.4%). Most patients with newly diagnosed TB (63.1%) were treated with category 1 drug regimens (2HRZE(S)/4HR) whereas relapsed (60%) and chronic TB patients (52.8%) were treated with category 2 drug regimens (2HRZES/1HRZE/5HRE). Treatment success ("cure" or "treatment completed") was achieved in 66.7%, 57.1% and 47.2% of patients with newly diagnosed, relapsed and chronic TB, respectively. Nine chronic TB patients (25.0%) had microscopically positive sputum smears at the end of their treatment course, indicating treatment failure. The median treatment duration was 7 months in patients with newly diagnosed and relapsed TB and 9 months in those with chronic TB.

Table 1. Characteristics and clinical profile of study subjects

Characteristic	Newly diagnosed TB $N = 84$	Relapsed TB $N = 35$	Chronic TB $N = 36$
Sex			
Male	60	27	15
Female	24	8	21
Age (years)			
Median	44	48	49
Range	9–85	28–88	14–82
Chest X-ray findings			
Infiltrate/Non-cavitating	48	18	15
Cavitating	14	8	6
Not documented	22	9	15
Treatment regimens			
2HRZE(S)/4HR[a] (CAT1)	53	10	
2HRZES/1HRZE/5HRE[a] (CAT2)	19	21	19
2HRZ/2HR[a] (CAT3)			
Second line drug (CAT4)	12	4	17
Duration of treatment (months)			
Median	7	7	9
Range	0–26	0–14	5–20
Treatment outcomes			
Cure	51	18	14
Completed	5	2	3
Default	10	5	7
Died	4	6	3
Failure	7	3	9
Not documented	7	1	

[a]The standard code for TB treatment regimens, each anti-TB drug has an abbreviation: streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). CAT, category.

Circulating granulysin concentrations in clinical tuberculosis before anti-tuberculosis therapy

The concentrations of circulating granulysin in patients with newly diagnosed TB (median ± SE = 1.511 ± 0.287 ng/mL, range 0.560–15.600 ng/mL) and relapsed TB (median ± SE = 1.458 ± 0.329 ng/mL, range 0.403–8.110 ng/mL) were significantly lower than those of healthy controls (median ± SE = 2.470 ± 0.186 ng/mL, range 0.662–5.055 ng/mL) ($P < 0.001$, $r = -3.816$ and $P = 0.004$, $r = -2.853$, respectively). Patients with chronic TB (median ± SE = 1.917 ± 0.264 ng/mL, range 0.549–6.970 ng/mL) had lower granulysin concentrations than controls, this difference not being significant ($P = 0.442$, $r = -0.769$). Median concentrations of granulysin were similar in patients with newly diagnosed and relapsed TB, but both were significantly lower than in chronic TB ($P = 0.003$, $r = -2.967$ and $P = 0.022$, $r = -2.294$, respectively) (Fig. 1).

Granulysin production in peripheral blood mononuclear cell stimulation assay

Granulysin production in PBMCs stimulated *in vitro* with PPD and H37Ra were measured in 46 patients with newly diagnosed, 21 with relapsed and 8 with chronic TB. Granulysin production by newly diagnosed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.796 ± 0.071 ng/mL, range 0.208–2.196 ng/mL) and H37Ra (median ± SE = 0.976 ± 0.065 ng/mL, range 0.246–1.823 ng/ml) were significantly higher than those of

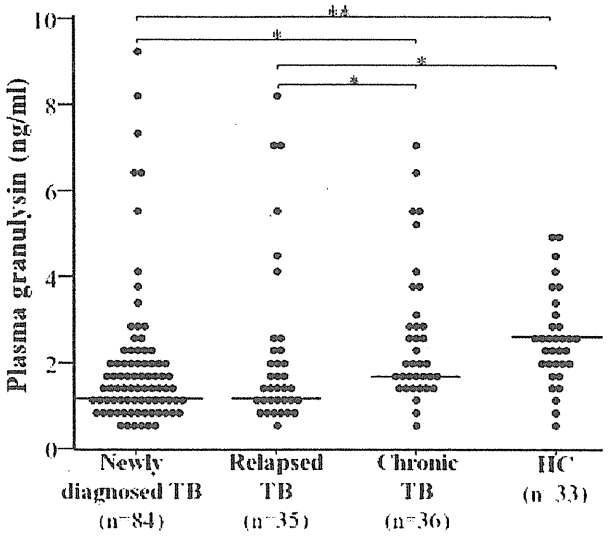

Fig. 1. Circulating granulysin concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represented one individual. The horizontal bars indicate the median of each group. *, $P < 0.05$; **, $P < 0.001$; HC, healthy control.

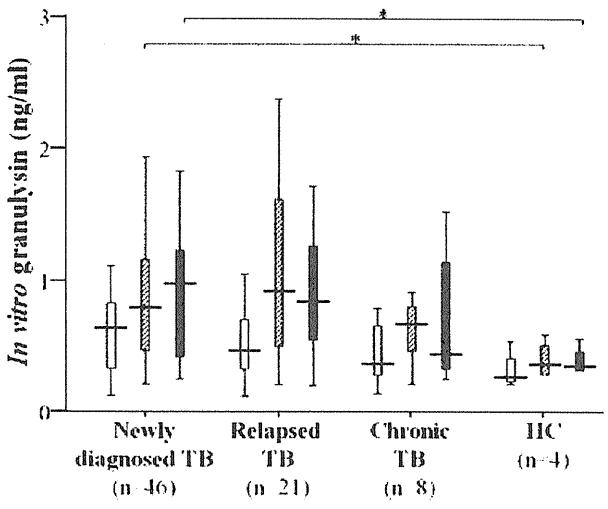

Fig. 2. *In vitro* granulysin production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and heat killed *Mycobacterium tuberculosis* (H37Ra) (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of each group. *, $P < 0.05$.

healthy controls stimulated *in vitro* with PPD (median ± SE = 0.359 ± 0.073 ng/mL, range 0.283–0.591 ng/mL), and H37Ra (median ± SE = 0.348 ± 0.056 ng/mL, range 0.320–0.559 ng/mL) ($P = 0.022$, $r = -2.289$ and $P = 0.032$, $r = -2.146$, respectively). Controls were PBMC supernatants from healthy controls without stimulation (median ± SE = 0.262 ± 0.076 ng/mL, range 0.206–0.542 ng/mL) and PBMC supernatants from newly diagnosed TB patients without stimulation (median ± SE = 0.636 ± 0.051 ng/mL, ranged 0.117–1.665 ng/mL). Although granulysin production by relapsed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.922 ± 0.146 ng/mL, range 0.205–2.374 ng/mL) and H37Ra (median ± SE = 0.841 ± 0.123 ng/mL, range 0.197–2.324 ng/mL) were higher than those of healthy controls, these differences were not significant ($P = 0.054$, $r = -1.927$ and $P = 0.081$, $r = -1.742$, respectively). PBMCs of patients with chronic TB stimulated *in vitro* with PPD (median ± SE = 0.674 ± 0.120 ng/mL, range 0.475–1.345 ng/mL) and H37Ra (median ± SE = 0.435 ± 0.173 ng/mL, range 0.408–1.521 ng/mL) produced greater amounts of granulysin than did healthy controls, the difference not being significant ($P = 0.089$, $r = -1.698$ and $P = 0.497$, $r = -0.679$, respectively). Similar median amounts of granulysin were produced by PBMCs of newly diagnosed and relapsed TB stimulated *in vitro* with PPD and H37Ra but higher amounts by PBMCs of chronic TB, the difference not being significant (newly diagnosed and chronic TB: $P = 0.330$, $r = -0.974$ for PPD and $P = 0.242$, $r = -1.169$ for H37Ra; relapsed and chronic TB: $P = 0.232$, $r = -1.196$ for PPD and $P = 0.380$, $r = -0.878$ for H37Ra) (Fig. 2).

Circulating interferon-γ concentrations in clinical tuberculosis before anti-TB therapy

In contrast to granulysin, the circulating IFN-γ concentrations in patients with newly diagnosed TB (median ± SE = 6.15 ± 4.58 pg/mL, range < 4.7–300 pg/mL) and relapsed TB (median ± SE = 7.93 ± 8.86 pg/mL, range <4.7–310.73 pg/mL) were significantly higher than those of healthy controls (median ± SE = <4.7 ± 0.20 pg/mL, range <4.7–10.13 pg/mL) ($P < 0.001$, $r = -3.923$ and $P < 0.001$, $r = -4.325$, respectively). Circulating IFN-γ concentrations in most chronic TB patients were similar to those of healthy individuals (median ± SE = <4.7 ± 3.76 pg/mL, range <4.7–123.69 pg/mL) ($P = 0.051$, $r = -3.486$). The median concentrations of IFN-γ were similar in patients with newly diagnosed and relapsed TB, but both were higher than in chronic TB, the difference not being significant ($P = 0.395$, $r = -0.851$ and $P = 0.333$, $r = -0.968$, respectively) (Fig. 3).

Interferon-γ production in peripheral blood mononuclear cell stimulation assay

The median IFN-γ production by PBMCs of newly diagnosed TB patients stimulated *in vitro* with PPD (median ± SE = 535 ± 94 pg/mL, range <4.7–2400 pg/mL) was higher than that of healthy controls (median ± SE = 434 ±

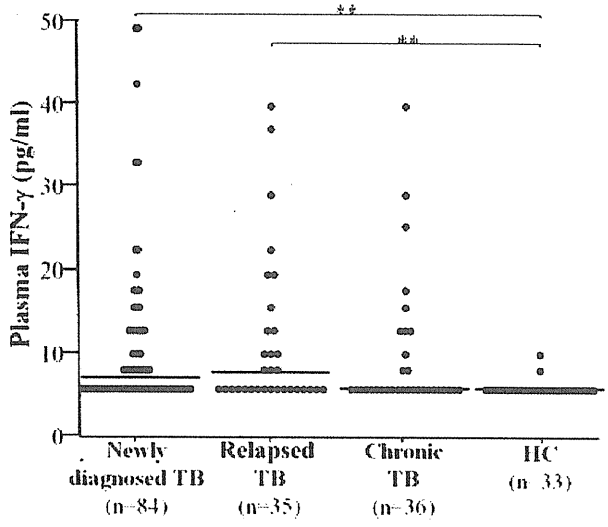

Fig. 3. Circulating IFN-γ concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represents one individual. The horizontal bars indicate the median of each group. **, $P < 0.001$; HC, healthy control.

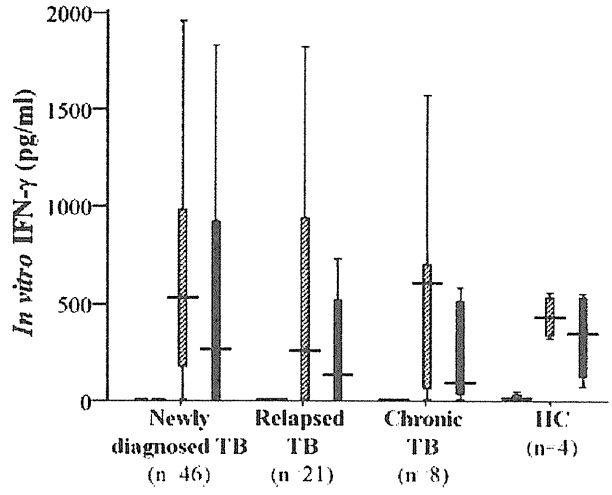

Fig. 4. *In vitro* IFN-γ production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and H37Ra (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of each group.

57 pg/mL, range 326–562 pg/mL) ($P = 0.591$, $r = -0.537$). However, most newly diagnosed TB-PBMCs stimulated *in vitro* with H37Ra produced higher IFN-γ concentrations (range <4.7–8025 pg/mL), but the median was similar (median ± SE = 270 ± 260 pg/mL) to that of healthy controls (median ± SE = 351 ± 120 pg/mL, range 76–556 pg/mL) ($P = 0.914$, $r = -0.107$). Supernatant from PBMCs without stimulation was used as a cell control (median ± SE = 14.29 ± 8.88 pg/mL, range 9.85–48.06 pg/mL), while supernatant from newly diagnosed TB-PBMCs without stimulation was used as a control for IFN-γ production (median ± SE = <4.7 ± 5.08 pg/mL, range <4.7–231 pg/mL). IFN-γ production by PBMCs from half the patients with relapsed TB stimulated either with PPD (range <4.7–4225 pg/mL) or H37Ra (range <4.7–2575 pg/mL) was higher than that of normal controls. However, their medians (median ± SE = 260 ± 258 pg/mL for PPD, and median ± SE = 138 ± 136 pg/mL for H37Ra) were lower than those of healthy controls; these differences were not significant ($P = 0.823$, $r = -0.223$ and $P = 0.412$, $r = -0.821$, respectively). Chronic TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 610 ± 166 pg/mL, range <4.7–1575 pg/mL) produced higher IFN-γ concentrations than did healthy controls, and some PBMCs stimulated *in vitro* with H37Ra also produced higher IFN-γ concentrations (range <4.7–1835 pg/mL) although the median was lower (median ± SE = 95 ± 198 pg/mL) than that of healthy controls ($P = 0.758$, $r = -0.309$ and $P = 0.354$, $r = -0.927$, respectively). Similar median amounts of IFN-γ production by PBMCs of newly diagnosed and chronic TB stimulated *in vitro* with PPD were found, and these were higher than for relapsed TB, the difference not being significant ($P = 0.436$, $r = -0.779$ and $P = 0.928$, $r = -0.091$, respectively). The median amount of IFN-γ produced by PBMCs of newly diagnosed TB stimulated *in vitro* with H37Ra was higher than that for relapsed and chronic TB ($P = 0.202$, $r = -1.275$ and $P = 0.982$, $r = -0.023$, respectively) (Fig. 4).

DISCUSSION

In this study, the correlations of plasma granulysin and IFN-γ concentrations with clinical disease in patients with newly diagnosed pulmonary, relapsed and chronic TB in northern Thailand, where TB is endemic, were evaluated. The effects of *in vitro* stimulation with PPD and H37Ra of PBMCs from these patients were also investigated. The finding of decreased circulating granulysin and increased IFN-γ in patients with newly diagnosed, relapsed and chronic TB before anti-TB therapy indicated involvement of granulysin and IFN-γ in host defense against TB infections.

In patients with newly diagnosed and relapsed pulmonary TB who had not yet received anti-TB therapy, plasma granulysin concentrations were significantly decreased compared to those of healthy individuals. This may be because granulysin is rapidly consumed during active disease, because of an ongoing effector immune response, or because plasma granulysin is reduced during active disease because of a reduction in the T cell subset dedicated to its production (15). However, granulysin

concentrations in patients with chronic TB, which had not been eradicated by treatment with conventional anti-TB drugs, and who had persistent clinical symptoms and progression of disease, were also lower than in healthy individuals. It is possible that persistence of clinical disease is associated with deficient expression of perforin and granulysin at the local site of TB infection (16). Although significant infiltration of T cells (CD3+, CD4+ and CD8+ T cells) is evident in TB lesions in patients with persistent inflammation, there are only small amounts of perforin and granulysin in these lesions, and evidence of severely impaired expression of these cytolytic effector molecules inside the distinct granules (16). Simultaneously, the numbers of granzyme A-expressing cells are increased in TB lesions, suggesting that the down-regulation of perforin and granulysin is selective and not a universal phenomenon involving all cytolytic effector molecules. These results are similar to those of recent studies which demonstrated that circulating granulysin reaches concentrations similar to those of healthy controls during TB therapy and increases further after completion of therapy (14, 15). However, larger sample sizes are necessary to gain better insight into the dynamics of plasma granulysin concentrations.

In contrast to granulysin, the concentrations of circulating IFN-γ in patients with newly diagnosed and relapsed TB were significantly higher than those of healthy controls, suggesting that IFN-γ plays a role in the regulatory and effector phases of the immune response to *Mtb* infection. In general, IFN-γ is synthesized from CD4+T cells that have been activated by recognition of mycobacterial antigen on APCs (9), as well as by CD8+ T cells from both mice and humans specific for mycobacterial antigens (17).

However, when recurrent TB was analyzed in this study, including both relapsed and chronic TB, granulysin concentrations were found to be significantly lower ($P = 0.038$, $r = -2.071$), whereas IFN-γ concentrations were significantly higher, than in controls ($P < 0.001$, $r = -4.180$, respectively), the concentrations being similar to those found in newly diagnosed TB, which is possibly due to patients with recurrent TB becoming as active as those with newly diagnosed TB. In this study, the proportional decrease in granulysin and increase in IFN-γ concentrations in newly diagnosed TB was not significantly different from that found in relapsed TB. Possible explanations are that: (i) both types of TB were active at the time of enrollment; and (ii) patients with relapsed TB had lost their immunity to *Mtb* and become active in the same way as newly diagnosed TB (because the relapsed TB patients had previous histories of newly diagnosed TB [their first episodes], re-exposure [second episode] and were registered as relapsed TB on enrollment in this study with a duration of 1–180 months [median 12 months]) between their initial treatment success and diagnosis of relapse. It is not possible to ascertain whether the episodes of relapse represented reactivation of previously inadequately treated TB, or reinfection with a new *Mtb* strain. The present results are similar to previous findings that plasma IFN-γ concentrations are significantly higher in patients with active pulmonary TB than in healthy controls and decrease after treatment. These findings might be because circulating IFN-γ comes from both local production and spill-over of IFN-γ from activated lymphocytes sequestered at the site of *Mtb* infection, as previously described (9, 14, 18). In chronic TB, circulating IFN-γ concentrations did not increase in most patients. Clearly, substantial CD4+ T cell responses occur in patients infected with *Mtb*. Failure of that response to eliminate bacteria may be partially at the level of recognition and activation of infected macrophages. *Mtb* is known to be equipped with numerous immune evasion strategies, including modulation of antigen presentation to avoid elimination by T cells. There is evidence that *Mtb*-infected macrophages have diminished ability to present antigens to CD4+ T cells, apart from IFN-γ production, which would contribute to the inability of the host to eliminate persistent infection (19).

In contrast, when PBMCs from newly diagnosed, relapsed and chronic TB were stimulated *in vitro* with PPD or H37Ra, they produced more granulysin than did stimulated controls, a finding which is in contrast to the median and individual concentrations of circulating granulysin. Possible explanations for this discrepancy are that: (i) during *in vivo* stimulation during active disease, granulysin might be rapidly consumed because of the ongoing effector immune response; (ii) *in vivo* serum granulysin is reduced during active disease because of a reduction in the T cell subset dedicated to its production (15); or (iii) when PBMCs that possibly contain primed T cells (indicated by high plasma concentrations of granulysin) are re-stimulated *in vitro* with either PPD and H37Ra, they may produce more granulysin in the supernatant. A related phenomenon has been reported in which stimulation with PPD *in vitro* PBMCs from healthy tuberculin skin test positive individuals results in increased granulysin expression in PPD-stimulated CD4+ and CD8+ T cells, compared to that of unstimulated cells (20). Moreover, it has been reported that, after stimulation *in vitro* with *Mtb* including H37Ra, both CD4+ and CD8+ T cells up-regulate mRNA expression for granulysin, granzyme A and B, perforin and CD95L (Fas ligand), and are able to lyse *Mtb* infected target cells, this being mediated primarily through the granule exocytosis pathway (21).

Median and individual concentrations of circulating IFN-γ in patients with newly diagnosed and relapsed TB were significantly higher than in healthy controls. Similar

results, namely greater IFN-γ production than in stimulated healthy controls, were seen with *in vitro* stimulation with PPD and H37Ra of PBMCs from most patients with newly diagnosed and half of relapsed TB patients, although some stimulated PBMCs from these patients produced less IFN-γ. However, the median IFN-γ production with *in vitro* stimulation of PBMCs from relapsed TB patients is lower than that of healthy controls. Surprisingly, PBMCs from healthy individuals stimulated *in vitro* with PPD and H37Ra in this study did induce significant IFN-γ production. However, these four healthy individuals were recruited from the Blood Bank of a provincial hospital in Chiang Rai where TB is endemic, and did not undergo chest X-ray, TST and any testing for latent TB infection and infection manifesting as active TB by IGRAs. At the time of recruitment, based on their histories, these individuals were thought to be healthy blood donors. However, we cannot be sure that they had never been exposed to *Mtb* and remained asymptomatic, or been vaccinated with BCG. It is known that 5–10% of those infected with *Mtb* will progress towards active TB during their lifetime, whereas the remainder are resistant to active TB, but remain infected. In fact, most Thai people are vaccinated with BCG since child. Therefore, it is possible that these healthy individuals had been exposed to *Mtb* in their lifetime, and that this had caused the high production of IFN-γ after stimulation *in vitro* with PPD and H37Ra. More normal healthy individuals from non-endemic TB areas who have been confirmed negative by chest X-ray and TST, and tested for latent TB infection and infection manifesting as active TB by IGRAs, should be included in future studies.

IFN-γ is produced from T cells (both CD4+ and CD8+T cells) and NK cells and activates bactericidal mechanisms in macrophages (3). It has been demonstrated that during the course of chronic and fatal TB infection, CD4+ T cells are absent even though CD8+ T cells can produce large amounts of IFN-γ. This supports the hypotheses that CD4+ T cells have important, non redundant roles in control of *Mtb* in addition to IFN-γ production, that CD4+ T cells assist in the development of cytotoxic CD8+ T cell populations and that the cytotoxicity exerted by effector CD8+ T cells might be an important component of anti-mycobacterial immunity (22). The present results indicate that patients with newly diagnosed and relapsed TB have low circulating granulysin but high IFN-γ concentrations before anti-TB therapy, suggesting that granulysin and IFN-γ may act in concert or in synergy in host defense against *Mtb* infection.

In conclusion, patients with active pulmonary TB have low circulating granulysin but high IFN-γ concentrations before treatment indicating their possible role in controlling *M. tuberculosis* infection.

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