counsellors together to work on behavior change (Cohen 2006).

Why it is important to do this review

Despite decades of experience with HIV prevention programs, the number of people living with HIV infection continue to rise (Pisani 2003). Some people suggest that behaviours that spread HIV is overemphasized, compared to the social and economic conditions that promote such behaviours (Barnett 2006). The awareness in recent years that social, economic, political, and environmental factors directly affect HIV transmission and risky sexual behaviours has fuelled interest in structural approaches to HIV prevention (Gupta 2008). However, progress in incorporating structural approaches into HIV prevention has been limited due to lack of conceptual and technical consensus on definition and implementation, and limited data on the effectiveness of structural approaches to the reduction of HIV incidence (Gupta 2008).
Behavioral interventions aiming to encourage individual attitude change toward safer sexual practice play an important role in overall HIV prevention efforts. However, behavioral interventions alone are inadequate to produce substantial and lasting reductions in HIV transmission (Coates 2008). As a result, consistent condom use has not reached a sufficiently high level, even after many years of widespread and often aggressive promotion (Potts 2008).
Structural interventions represent a potentially powerful approach to HIV prevention, especially those that promote community mobilization (Blankenship 2010).
Currently, the Cochrane Library has no reviews or protocols focusing specifically on structural and community-level intervention for increasing condom use to prevent HIV and other sexual transmitted diseases both in low-middle income and high-income countries. Program planners and policy makers need descriptions of interventions and quantitative estimates of the effects of various interventions to make informed decisions concerning prevention funding and research (Johnson 2008). This review will help generate knowledge on how structural and community-levels interventions might impacts on condom use.

OBJECTIVES

The objective of this review is to assess the effects of structural and community-level interventions for increasing condom use in the general population and population at-risk, by comparing alternative strategies or by assessing the effects of a strategy compared with a control.

METHODS

Criteria for considering studies for this review

Types of studies

Randomized controlled trials (RCTs), including cluster-randomised controlled trials, that compare either two or more alternative condom promotion strategies, or one condom promotion strategy with a control (i.e., no condom promotion strategy), will be included.

Types of participants

Studies that include the following types of participants will be included in the review:

1. General population (adolescents and adults)
2. STIs patients, including HIV-positive patients and/or sero-discordant couples
3. Drug users, including IDUs as well as other drug-using populations
4. Sex workers (male and female)
5. MSM

Types of interventions

The interventions may include the following types:

1. Interventions focusing on changing condom use behaviours by altering social norms, such as social-cognitive theory intervention, mass media campaigns, the use of public opinion, social marketing strategies, and women empowerment.
2. Interventions focusing on altering economic situations which impact condom use, such as condom taxes or tariffs removal, or condom subsidization (either total subsidization, as in free condoms distributed through the public sector, or partial subsidization, as in social marketing approaches)
3. Interventions which improve condom accessibility, or change or improve distribution, whether through the public sector, the private sector, or NGOs (including social marketing operations)
4. Interventions derived from legislative support for condom use, such as mandating condom use among sex workers
5. Combinations of the above

Types of outcome measures

Primary Outcome:

1. Reported condom use (male or female condoms)
2. HIV incidence / prevalence
3. STI incidence / prevalence

Secondary Outcome:

1. Knowledge about condom use (both self-reported and tested)
2. Attitudes towards condom use (both self-reported and tested)

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3. Condom acquisition/procurement
4. Stated intention to use condoms
5. Negotiation skills around condom use (both self-reported and tested)
6. Changes of other STI risk-related practices, including number of partners
7. Process indicators reporting on satisfaction with and/or acceptability of aspects of the intervention (including the mode of condom distribution as well as the structure and/or content of the condom promotion strategy)
8. Reported harms related to condom use, such as domestic violence or abuse or allergic reactions to latex
9. Comparative costs of interventions

Search methods for identification of studies

Electronic searches

A MEDLINE search will be conducted. The Cochrane search strategy will be used to identify appropriate studies within electronic databases (Higgins 2009). We will not restrict any languages or publication status.The precise search strategy to be employed will be developed with the assistance of the Cochrane HIV/AIDS Review Group. The Cochrane Central Register of Controlled Trials (CENTRAL), EMBASE, Psychological Abstracts and Sociological Abstracts will be searched using a search strategy similar to that outlined for MEDLINE. The sensitivity of the search strategies will be improved if possible by including text and key words from relevant trials accessed by the authors that were not detected by earlier searches. In order to maximize the sensitivity of these searches, searches will be run both with and without keywords referring to study design.

Searching other resources

Hand searching of the following will be conducted as available:
1. Proceedings of the International AIDS Conferences (before 1989, as abstracts from 1989-present are included in AIDSLINE)
2. Proceedings of the International Society of STD Research (ISSTDR)
3. Other proceedings, including those of major behavioral studies conferences focusing on HIV/AIDS, STIs, or other reproductive health issues
4. Key journals in this field, including AIDS

Bibliographies of studies and previous reviews will be examined to find other relevant evaluation studies. Professionals in the field (e.g. authors of previous reviews or investigators on prior primary research) will be contacted to identify continuing studies. Internet sites which are concerned with HIV-prevention as well as reproductive health in general, will be explored for condom-promotion intervention information. Other relevant organizations which may possibly involved in condom promotion interventions will be contacted. These may include: international agencies (e.g., UNAIDS, WHO, UNFPA, World Bank), national public health agencies with projects in the developing countries (Centers for Disease Control and Prevention), major NGOs (e.g., International Planned Parenthood Federation, Population Services International, Family Health International), and major academic centres. These contacts will be utilised to assist in identifying existing sources of data as well as the prospective tracking of studies either planned for the near future or still in progress.

Data collection and analysis

Selection of studies

The search for trials will be performed with the assistance of the Cochrane HIV/AIDS Group. The two authors would critically appraise all identified citations independently to establish their relevance for inclusion into the review. Studies will be reviewed for relevance based on study design, types of participants, interventions and outcome measures. Any disagreement will be resolved by discussion or by contacting an independent author. We will give reasons for excluding potentially relevant trials in an excluded studies table.

Data extraction and management

A standardized form will be used for data abstraction. At least two reviewers will independently abstract study characteristics and outcomes including information on:
1. Contexts of study setting, including background attitudes towards and/or levels of use or condoms as well as other forms of contraception, awareness of HIV/AIDS or STIs, and prevalence of HIV/AIDS or STIs
2. Populations involved (including socio-cultural and economic characteristics and possible previous exposure to similar interventions)
3. Puration of exposure (or 'dose') involved in the intervention, such as measurements of total time period over which the intervention took place, and the cost of the intervention per individual or group 'treated' (if available)
4. Type of intervention
5. Outcome measures
6. Study quality
7. Findings

Assessment of risk of bias in included studies

The quality of all selected studies will be assessed independently by both the authors using the following:

1. Allocation concealment

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- Adequate allocation concealment: participants and researchers were unaware of participants' future allocation to condition until after decisions about eligibility were made and informed consent was obtained
- Unclear concealment: allocation concealment measures were not described in detail
- Inadequate allocation concealment: allocation was not concealed from either participants before informed consent or from researchers before decisions about inclusion were made

2. Blinding of participant, assessors and providers of care
   - Yes: assessor blind to condition
   - Unclear: blinding of assessor not reported and information not available from researchers
   - No: assessor not blind to condition

3. Loss to follow up
   - Adequate: losses to follow up were equally distributed between treatment and comparison groups
   - Unclear: information about losses to follow up unavailable
   - Inadequate: losses to follow up in excess of 30% or unevenly distributed between treatment and comparison groups

4. Method of generation of the randomisation sequence
5. The description of the completeness of outcome data for each main outcome

For each trial the authors will categorise the risk of bias as high, moderate, or low. Scores will be allotted to each component, and studies will be categorized to be of adequate, inadequate or unclear quality, depending on the total score calculated. If there is any disagreement between two authors regarding the quality of a particular study, a third reviewer will reconcile the disagreement. The quality of evidence will be evaluated using the GRADE approach (Guyatt 2008).

Measures of treatment effect

Measures of effect of the interventions will be assessed according to the outcomes of interest for this review.

Unit of analysis issues

Studies with similar units of analysis will be grouped together for the purposes of analysis. Studies with different units of analysis will not be pooled for analysis.

Dealing with missing data

Should there be missing or inadequate data, three attempts will be made to obtain the data by contacting authors.

Assessment of heterogeneity

We anticipate substantial heterogeneity across studies, and thus meta-analysis of these studies will be undertaken with caution, if at all. For studies that are homogenous with respect to types of populations, the interventions that are compared, and outcome measures, overall relative risks will be calculated and translated into relative risk reductions and NNTs. If there is no statistically significant heterogeneity ($p<0.10$) in results, a fixed effect model (Mantel-Haenszel) will be used. If there is statistically significant heterogeneity we will identify possible explanations and a random effects model (DerSimonian and Laird method) will be used; this will be compared with the corresponding results of the fixed effect model. The following factors will be considered as possible explanations for differences in effects: study quality, patients' gender, age, and the study setting (low-and middle income or high-income countries). To test for robustness of results, sensitivity analyses will be done in which studies using less rigorous methods will be excluded.

Given the likelihood of substantial heterogeneity across studies, and subsequently insufficient data for meta analysis, we intend to include in this review a qualitative overview of the studies and their findings. This may also draw on high-quality studies which did not meet the full selection and/or methodological criteria for various reasons. The main aim of the analysis will be to identify interventions that have been shown to be effective in developing countries, the circumstances under which they have been shown to be effective, and the specific effects that have been measured. We will also identify patterns of intervention strategies which appear ineffective, as well as important gaps in existing knowledge.

Assessment of reporting biases

Funnel plots will be generated to assess for the presence of reporting bias.The presence of asymmetry will suggest that there is reporting bias. We will conduct a thorough evaluation to determine if there are other reasons for asymmetry. If no other reasons for asymmetry are found, we will document the possible presence of reporting bias.

Data synthesis

All eligible studies will be summarized in RevMan. The two authors will extract the data and enter all data into RevMan, and all the entries will be rechecked by both authors. Disagreements will be resolved by discussion. If no consensus is reached, the HIV/AIDS mentor for this review will be contacted.

Subgroup analysis and investigation of heterogeneity

If possible, stratified analysis will be undertaken among participant subgroups. Subgroupings will include age of target population (adolescent versus adult; among general population; or among population at-risk), gender of population, and primary motivation

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for condom use (contraception and prophylaxis versus prophylaxis only), whether individually or cluster. Additional stratified analyses will divide studies according to methodological quality, if sufficient numbers are available.

Sensitivity analysis

If the number of studies and data available allows for sensitivity analysis, we shall perform a sensitivity analysis by using one or all of the following strategies

1. Removing studies with high risk of bias to see if there will be any effect on the results of the meta analysis
2. Studies with missing data may be re-analysed using a reasonable range of missing values
3. Data may be re-analysed using different statistical approaches

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* *Indicates the major publication for the study*

WHAT'S NEW

Last assessed as up-to-date: 21 September 2011.

Date	Event	Description
5 October 2011	New citation required and major changes	New author team, and complete revision of protocol.

HISTORY

Protocol first published: Issue 4, 2001

Date	Event	Description
6 January 2011	Amended	"Clean slate" for new author team
12 November 2008	Amended	Converted to RevMan 5 and re-published without new citation.

CONTRIBUTIONS OF AUTHORS

HN, EO and WW designed, set up, and drafted the protocol. RT, SE, AK and KS commented upon and revised the article. All authors have approved the final protocol.

DECLARATIONS OF INTEREST

We declare that we have no conflict of interest.

SOURCES OF SUPPORT

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Internal sources

- Department of Global Health Policy, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

External sources

- Ministry of Health, Labour and Welfare of Japan, Japan.

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Microbiology and Immunology

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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.

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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

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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

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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

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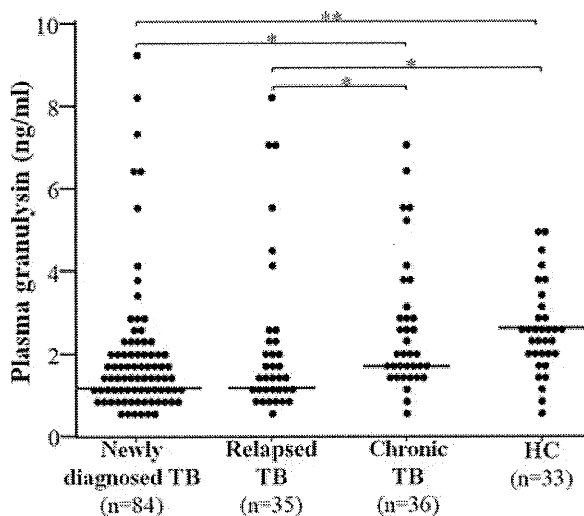

Fig. 1. Circulating granulysin concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represented one individual. The horizontal bars indicate the median of each group. *, $P < 0.05$; **, $P < 0.001$; HC, healthy control.

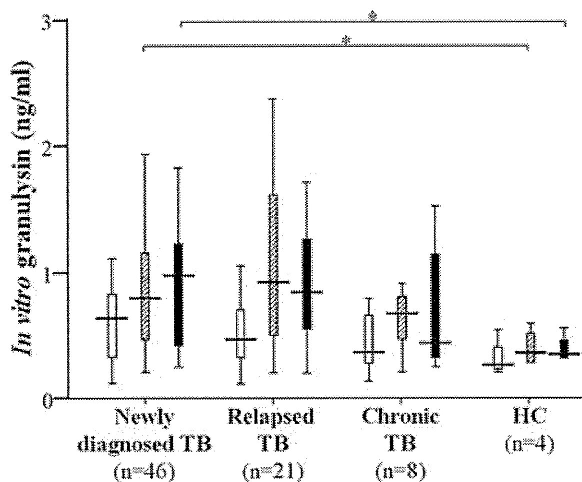

Fig. 2. *In vitro* granulysin production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and heat killed *Mycobacterium tuberculosis* (H37Ra) (black). Supernatant from PBMCs without stimulation was used as controls (clear). 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 ±

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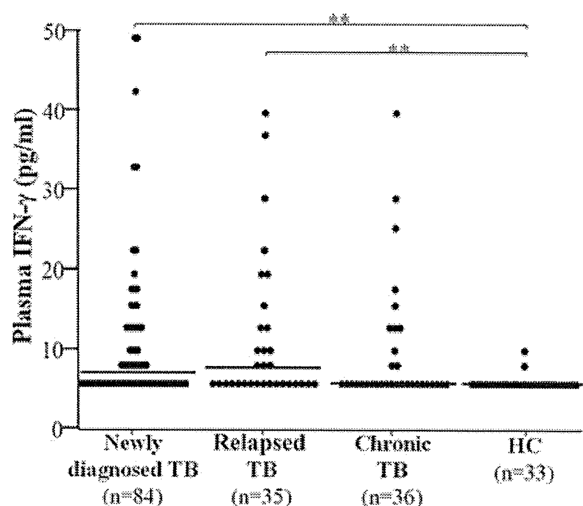

Fig. 3. Circulating IFN-γ concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represents one individual. The horizontal bars indicate the median of each group. **, $P < 0.001$; HC, healthy control.

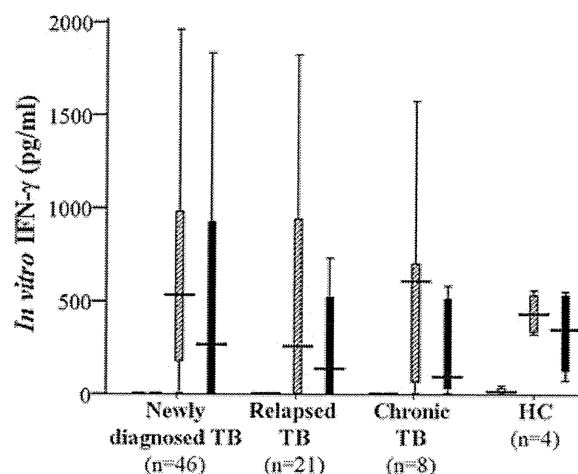

Fig. 4. *In vitro* IFN-γ production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and H37Ra (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of 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 $\pm$ SE = 270 $\pm$ 260 pg/mL) to that of healthy controls (median $\pm$ SE = 351 $\pm$ 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 $\pm$ SE = 14.29 $\pm$ 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 $\pm$ SE = <4.7 $\pm$ 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 $\pm$ SE = 260 $\pm$ 258 pg/mL for PPD, and median $\pm$ SE = 138 $\pm$ 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 $\pm$ SE = 610 $\pm$ 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 $\pm$ SE = 95 $\pm$ 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

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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

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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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ASSOCIATION BETWEEN CIRCULATING FULL-LENGTH OSTEOPONTIN AND IFN-[gam
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ASSOCIATION BETWEEN CIRCULATING FULL-LENGTH OSTEOPONTIN AND IFN-γ WITH DISEASE STATUS OF TUBERCULOSIS AND RESPONSE TO SUCCESSFUL TREATMENT

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Abstract. The T helper type 1 (Th1) immune response plays an important role in protective immunity, pathophysiology and development of tuberculosis (TB). To investigate whether osteopontin (OPN) and other Th1 response-related molecules are associated withTB disease status, including co-infection with HIV, and response to anti-TB treatment, circulating levels of full-length OPN (F-OPN), thrombin-cleaved N-terminal fragment of OPN (N-half OPN), IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10, IL-15 and C-reactive protein (CRP) were measured before and after anti-TB treatment. Patients with newly active pulmonary TB had significantly higher plasma levels of F-OPN, IFN-γ and CRP than healthy controls (HC). F-OPN, N-half OPN, IFN-γ, IP-10, IL-18 and IL-10 levels were higher in patients with extensive TB/HIV co-infection than in patients with a single disease of TB or HIV. Plasma levels of F-OPN correlated well with those of IP-10, IL-18 and N-half OPN among patients with active TB. The F-OPN, IFN-γ, IP-10 and CRP levels decreased significantly after effective anti-TB treatment. These data suggest that circulating OPN and Th1 response-related molecules, including IFN-γ, may be regulated in response to expansion of active TB and could serve as markers of disease activity before and during treatment.

Keywords: osteopontin, IFN-γ, CRP, tuberculosis, HIV/TB

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INTRODUCTION

Tuberculosis (TB) is one of the most important infectious causes of death worldwide (WHO, 2009). Despite its long historical interaction with humans, our understanding of host response to the TB

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pathogen remains incomplete. Investigation of the molecular differences in host immune status between patients with active TB, co-infected with HIV and control subjects may provide a clue to understand the disease process.

In response to *M. tuberculosis*, activated macrophages and CD4$^+$ T lymphocytes produce Th1 cytokines, including IFN-γ, IL-12 and IL-18 (Schluger and Rom, 1998; van Crevel *et al*, 2002). IFN-γ triggers initiation of the major effector mechanism for the Th1 immune response (Flynn *et al*, 1993). IL-12 induction is observed following uptake of *M. tuberculosis* by dendritic cells and macrophages, which drives the production of IFN-γ in NK and T cells (van Crevel *et al*, 2002). Similarly, IL-18 exhibits strong IFN-γ inducing activity synergistically with IL-12 (Dinarello and Fantuzzi, 2003). The expression of IL-10 mRNA has been demonstrated in lymph nodes of TB patients, particularly in those with HIV/TB co-infection (Lin *et al*, 1996). Although IL-10 may down-regulate the immune response to mycobacterial infection (van Crevel *et al*, 2002), the exact role of IL-10 in TB remains controversial. IP-10, an IFN-γ inducible chemokine, is also predominant in active TB lymph nodes and the lung (Ferrero *et al*, 2003). Elevated circulating IP-10 levels have been reported in patients with active TB (Juffermans *et al*, 1999; Azzurri *et al*, 2005; Djoba Siawaya *et al*, 2009) and HIV/TB co-infection (Juffermans *et al*, 1999).

Osteopontin (OPN), a phosphorylated acidic glycoprotein associated with inflammation and tissue repair, is abundantly produced in the early stage of macrophage and T cell activation in granulomatous inflammation (O'Regan and Berman, 2000). OPN may polarize early Th1 cytokine responses through induction of IL-12 and suppression of IL-10 in macrophages (Ashkar *et al*, 2000). In a mouse model, a protective role of OPN in mycobacterial infection has clearly been demonstrated through experiments using OPN-null mice in which clearance of *M. bovis* BCG was reduced (Nau *et al*, 1999). In humans, OPN accumulates in well-formed granulomas with local mycobacterial infection, whereas OPN is absent or low in histologically ill-defined granulomas with disseminated infection (Nau *et al*, 2000). OPN is considered to play an active role in effective granuloma formation, inducing a Th1 response at an early stage of mycobacterial infection. Circulating OPN has also been measured in patients with active TB and their levels are generally high initially and decrease after anti-TB treatment (Koguchi *et al*, 2003; Inomata *et al*, 2005). Although these OPN levels have been reported to be correlated with Th1 cytokines, IFN-γ and IL-18 (Yamada *et al*, 2000; Inomata *et al*, 2005), the results of measuring circulating Th1 cytokine levels in human TB patients have often been inconsistent or unclear (Yamada *et al*, 2000; Morosini *et al*, 2003; Deveci *et al*, 2005; Inomata *et al*, 2005; Aktas *et al*, 2009). Immune reconstitution syndrome occurs after commencement of highly active antiretroviral therapy (HAART), at a stage when the *M. tuberculosis*-specific Th1 response is partially restored (Lawn *et al*, 2005). In HIV infected individuals, elevated OPN levels are found in cerebrospinal fluid and plasma and correlate with neurocognitive abnormalities (Burdo *et al*, 2008). OPN is the only pro-inflammatory cytokine found to increase after 1 month of HAART in lymph nodes (Li *et al*, 2004) and persists for 6 months of HAART (Chagan-Yasutan *et al*, 2009). OPN is susceptible to proteolytic fragmentation and a thrombin-cleaved N-terminal fragment of OPN (N-half OPN) is known to

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affect its biological activity (O'Regan and Berman, 2000).

In this study, we attempted to address three questions unsolved by previous studies: 1) is OPN associated with TB even with HIV co-infection (CD4+ T cell-depletion) in which granulomatous formation is generally poor? 2) is the N-half form, presumably cleaved by thrombin at the site of disease, more accurately connected with parameters of disease activity? 3) Do a variety of Th1-related molecules all coordinate with OPN levels? We investigated the concentrations of both full-length and N-half OPN, cytokines and a chemokine, including IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10 and IL-15, in the plasma of patients with newly active pulmonary TB, HIV/TB co-infection, HIV single infection and healthy controls and their levels within and between groups were compared. OPN and Th1 response-related molecules in patients with newly active pulmonary TB were also evaluated before and after anti-TB treatment. C-reactive protein (CRP) was simultaneously measured as a marker to monitor response to anti-TB treatment and an indicator of inflammation (Sahiratmadja *et al*, 2007; Peresi *et al*, 2008).

MATERIALS AND METHODS

Subjects

Twenty-three patients with pulmonary TB and 6 HIV/TB co-infected patients without highly active antiretroviral therapy (HAART) (HIV+TB+HAART) were recruited from the outpatient and inpatient clinics of Mae Chan and Chiang Rai hospitals, Chiang Rai Province, northern Thailand. HAART was defined as the regular use of two nucleoside reverse transcriptase inhibitors, NRTI [Stavudine (d4T) and Lamivudine (3TC)] plus a non-nucleoside reverse transcriptase inhibitor, NNRTI [Nevirapine (NVP) or Efavirenz (EFV)]. The patients with TB and HIV+TB+HAART-(HAART-) were all newly diaganosed pulmonary TB patients with sputum smears positive for acid-fast bacilli and confirmed by positive cultures for *M. tuberculosis* and abnormal chest radiographic findings. The patients had never received anti-TB treatment or had taken anti-TB drugs for less than 7 days at the time of enrollment. They had never received any immune-suppressive drugs or other immunomodulators. None of them had diabetes mellitus or other acute infections. On enrollment, the HIV/TB co-infected patients had not previously received antiretroviral therapy but were positive for HIV antibodies detected by particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Abbott Park, Ill) followed by a confirmation test using enzyme-linked immunosorbent assay (ELISA) (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

Ten HIV patients not taking HAART (HIV+HAART-) and 17 HIV patients receiving HAART (HIV+HAART+) were recruited from the HIV Care and Treatment Project (Daycare clinic), Mae Chan Hospital. These patients had no previous history of TB. One patient who was HIV+HAART+ was taking isoniazid preventive therapy (IPT) for active TB on enrollment. Their sputum smears were negative for acid-fast bacilli and the cultures were negative for *M. tuberculosis*. They were negative (induration<5 mm) for tuberculin skin test and had no concomitant active AIDS-related opportunistic infections during the 30 days prior to enrollment. None had diabetes mellitus or

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Table 1
Baseline characteristics of study subjects.

Characteristics	HC n=25	TB n=23	HIV+ HAART- n=10	HIV+ HAART+ n=17	HIV+TB+ HAART- n=6
Age, median (range), years	35.0 (21-52)	46.0 (18-64)	37.5 (31-53)	39.0 (27-52)	43.0 (30-47)
Sex, number of males/females	15/10	15/8	6/4	8/9	5/1
WBC x 10^3, median (range), cells/µl	6.80 (3.64-11.20)	9.60 (3.10-15.80)	5.21 (3.31-6.06)	5.48 (2.82-9.11)	8.62 (5.70-12.80)
$CD4^+$ T cell count, median (range), cells/µl	1,050 (451-1,580)	564 (226-1,081)	274 (30-789)	437 (104-843)	146 (19-344)
≤ 200, No. (%)			3 (30.0)	4 (23.5)	4 (66.7)
201-500, No. (%)	1 (4.0)	10 (43.5)	5 (50.0)	7 (41.2)	2 (33.3)
>500, No. (%)	24 (96.0)	13 (56.5)	2 (20.0)	6 (35.3)	
CXR findings, No. (%)					
Normal	23 (92.0)		9 (90.0)	17 (100.0)	
Infiltrate /non-cavitary		20 (87.0)	1 (10.0)		5 (83.3)
Cavitary		3 (13.0)			1 (16.7)
No definite infiltration	2 (8.0)				
Site of TB infection by member					
Pulmonary		22			3
Extra-pulmonary					
Both		1			3

HAART, highly active antiretroviral therapy; HC, healthy control; TB, patients with tuberculosis; HIV+HAART-, HIV patients without HAART; HIV+HAART+, HIV patients with HAART; HIV+TB+HAART-, HIV/TB co-infected patients without HAART.

was receiving immune-suppressive drugs or other immunomodulators during the 90 days prior to enrollment.

Twenty-five Thai healthy controls (HC) were recruited through the blood bank at Mae Chan Hospital and served as controls. They had no previous history of TB or risk factors for TB. Their chest radiographs were normal. They had no latent TB infection detected by interferon-γ release assays [QuantiFERON®-TB Gold In-Tube (QFT), Cellestis, Victoria, Australia]. None of them had diabetes mellitus. All were negative for hepatitis B surface antigen, hepatitis C antigen and HIV antibodies using particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or ELISA (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

The baseline characteristics of this patients and healthy controls are summarized in Table 1. Patients with TB had significantly higher white blood cell (WBC) counts (p<0.05) than HC; patients with HIV+TB+HAART- tended to have higher WBC counts. Patients with HIV+HAART- had significantly lower WBC counts than HC (p<0.01). The $CD4^+$ cell counts in TB patients were significantly higher than in HIV+HAART- patients (p<0.01), but were not significantly different from those with

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