Yonsei Medical Journal
Vol. 37, No. 5, pp 339~349, 1996

Isolation of Perchloric Acid Soluble, Heat Stable, Ethanol Extractable Protein from *Mycobacterium tuberculosis*

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The perchloric acid soluble, heat stable, and ethanol insoluble antigen of M. tuberculosis (TB-PBE) was prepared, and antigenicity of this antigen was studied in vivo and in vitro. TB-PBE showed a single band of 60 kDa by SDS-PAGE. Sera from the patients with active pulmonary tuberculosis did not react with this antigen by ELISA. A delayed hypersensitivity skin reaction was induced with this antigen and was correlated with the reaction with PPD. Skin biopsy was performed in this skin lesion induced by TB-PBE and stained by H-E and immunohistochemical methods. TB-PBE induced an inflammatory lesion similar to a lesion induced by PPD. Blastogenic activity of the peripheral blood mononuclear cells stimulated by TB-PBE increased, and showed a peak reaction at 7 days after stimulation. The blastogenic activity changed in a dose-dependent manner. After stimulation with TB-PBE, mononuclear cells were analyzed by FACS. DR+ T cells and CD4/CD8 ratio increased after stimulation by TB-PBE. These cells secreted IL-2, not IL-4 after stimulation with TB-PBE. In the immunofluorescence test, mouse antiserum against TB-PBE showed a positive reaction with M. tuberculosis and showed cross-reactivity with M. bovis and other atypical mycobacteria, but not with S. aureus. With these results, it is evident that TB-PBE is an antigen which can induce cell mediated immunuity in vivo and in vitro.

Key Words: Mycobacterium tuberculosis, 60 kDa heat shock protein

Mycobacteria continue to be responsible for diseases in humans and domestic animals worldwide. Although improved living standards have been accompanied by a progressive decline in cases of tuberculosis in the industrialized countries, the incidence of disease has remained high in the most of the developing countries. The incidence also began to rise in the industrialized countries recently as a consequence of poverty, immigration from higher prevalence countries, the epidemic of the human immunodeficiency virus infection, and limitations in health care services to high risk populations. Also, during the past few years, the incidence of multi-drug resistant disease has increased and the emergence of drug-resistant strains has hampered tuberculosis control efforts (Murry *et al.* 1990; Kochi, 1991).

By way of developing the improved strategies for mycobacterial disease control through vaccination and immunological and molecular diagnosis, many researchers have tried to identify the individual bacterial components involved in interactions with the immune system. *M. tuberculosis* posesses some 1,000 proteins which are all potential T-cell antigens (Young *et al.* 1992). The earliest preparations of mycobacterial antigens

Received September 19, 1996
Accepted November 13, 1996
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used experimentally were Koch's tuberculin. The first of these was old tuberculin, a filtrate of old broth cultures of tubercle bacilli concentrated by evaporation. The initial attempts to separate the soluble components of mycobacterium were reported by Seibert (1949). This preparation was far from ideal because the processes of autolysis, heating and protein precipitation caused considerable denaturation of the antigens, particularly the species-specific ones. A large number of mycobacterial protein antigens have now been defined by many workers using a variety of approaches based on different methods of biochemical fractionation and on immunological screening with monoclonal antibodies, patients' sera, and human and murine T cells. Among these proteins, over twelve antigens have now been identified and their functions as well as their sequences elucidated (Young *et al.* 1992).

In this study, we were able to isolate a 60 kDa protein from *M. tuberculosis* by treatment with perchloric acid, boiling, and with ethanol, and this protein was studied to define the antigenicity in vivo and in vitro.

MATERIALS AND METHODS

Preparation of antigen

Extraction of *M. tuberculosis* with perchloric acid (PCA) was performed as previously described(Kim and Kim, 1993). Briefly, about 600 mg of live *M. tuberculosis* bacteria isolated from patients with active pulmonary tuberculosis and cultured on Ogawa medium was suspended with distilled water to a total volume of 20 ml and an equal volume of 10% PCA was added. The mixture was stirred for 30 min at room temperature and centrifuged for 15 min at 5,000 rpm at 4℃. The supernatant was obtained and dialyzed against cold running tap water for 24 hr. The dialysate was centrifuged again for 30 min at 17,000 rpm at 4℃ and the supernatant was dialyzed for 48 hr against distilled water with 4 changes of dialyses bath. This solution was named TB-P. Then TB-P was boiled for 30 min at 100℃ and adjusted to original volume with diatilled water. After centrifuging for 30 min at 17,000 rpm at 4℃, three volumes of absolute ethanol was added to one volume of this solution and incubated for 24 hr at room temperature. Pellet was withdrawn after centrifugation for 10 min at 3,000 rpm and dissolved with 1 volume of 0.9% normal saline. After repeating this procedure twice, the pellet was lyophilzed and this antigen was named TB-PBE.

Protein in TB-PBE was determined according to Lowry's method (Lowry *et al.* 1951); 1 mg of lyophilized TB-PBE contained 6 μg of protein.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The procedure of Laemmli (Laemmli, 1975) using a discontinuous buffer system with reducing agent was employed. Lyophilized TB-PBE was dissolved in distilled water at a concentration of 10 mg/ml. Twenty microliters either of this solution or of the TB-P sloution was mixed with 20 μl of twiceconcentrated sample buffer consisting of 0.125 M Tris-OH, 4% SDS, 20% glycerine and 0.1% dithiothreitol, pH 6.8. Thereafter, the preparations were boiled for 3 min and applied to 12 % SDS polyacrylamide gel. Molecular weight standards were run simultaneously. After electrophoresis, this gel was stained with neutral silver nitrate (Oakley *et al.* 1980).

Clinical applications

ELISA. ELISA was performed with TB-PBE at 6 μg/ml as a protein concentration or PPD (Statens Serum Institut, Copenhagen, Denmark) at 6 μg/ml as the antigens. The plates were incubated with sera of fifteen patients with active pulmonary tuberculosis diluted to 1:200 and then with peroxidase-conjugated goat antihuman immunoglobulin G (Cappel, Malvern, PA, USA). After adding substrate, optical density was read with a microtiter plate reader. For normal controls, sera from fifteen PPD negative healthy children were tested simul-

taneously.

Delayed Hypersensitivity Skin Tests. TB-PBE was used at the same concentration as protein of intermediate-strength PPD antigen (0.0001 mg/0.1 ml), and one tenth milliliter of TB-PBE and PPD antigen were injected intracutaneously on the volar surface of each forearm simultaneously.

The diameter of induration was determined after 48~72 hrs. Over 10 mm induration reaction was considered positive and an induration reaction of less than 5 mm was considered negative. Doubtful reaction was excluded and results were analysed by Kappa statistics.

The skin specimen of a case with positive reaction to TB-PBE and PPD was stained with hematoxylin-eosin. Also, immunohistochemical staining was done with monoclonal antibodies of CD3, CD4, CD8, CD19, and CD68 (Dako, Carpinteria, CA, USA) with a LSAB kit (Dako) according to the manufacturer's manual.

Peripheral mononuclear cells stimulation tests with TB-PBE

Peripheral mononuclear cells were obtained from the venous bloods of 10 volunteers who had no history of tuberculosis, normal chest X-rays and positive reactions to PPD by centrifugation on Ficoll (Pharmacia Biotech, Uppsaala, Sweden).

Cultures were conducted in 96-well U-bottom polystyrene plate (Costar, Cambridge, MA, USA) in RPMI 1640 medium containing 10% FCS, 5 mM L-glutamate, 50 IU penicillin, and 50 μg streptomycin. Each well (1×10^4 cells per well) was challenged with different concentrations of TB-PBE (10, 1, 0.1, 0.01 μg/ml as a protein concentration) and different durations(1, 2, 3, 4, 7, 9, 10, 14 days). Phytohemagglutinin (PHA, 1 μg/ml, GIBCO, Gaithersburg, MD, USA) and PPD (1 μg/ml) as control antigens were challenged simultaneously. After pulse with [^{3}H]-thymidine for 16 hrs, cells were harvested and read with a β-counter(Beckman Instrument Inc., Palo Alto, CA, USA).

Fluorescence-activated cell sorter (FACS) analysis

Mononuclear cells prepared to the foregoing method were stimulated by TB-PBE with a concentration of 1 μg/ml as a protein concentration for 7 days. Before and after stimulation, these cells were stained by using optimal concentrations of PE-or FITC-conjugated mAbs and isotype-matched control mAbs for 30 min on ice. Monoclonal antibodies CD45/14, CD3/CD19, CD4/CD8, CD3/HLA-DR, and TCR-γ/δ-1 were purchased from Becton Dickinson (San Jose, CA, USA). After fixation with 0.5% paraformaldehyde, cells were analysed by FACStar^{plus} (Becton Dickinson). Results were analysed by paired t-test.

Cytokine measurements

After stimulations of mononuclear cells with TB-PBE, PPD, and PHA at a concentration of 1 μg/ml each as a protein concentration for different days, supernatants were withdrawn. Interleukin-2 (IL-2) and interleukin-4 (IL-4) concentrations of these supernatants were measured by Quantikine Immunoassay Kits (R & D systems, Minneapolis, MN, USA).

Indirect immunofluorescence technique

To get mouse antiserum against TB-PBE, six-to eight-week-old BALB/c mice were immunized. One hundred microliters of TB-PBE suspension (1 mg/ml) was mixed with the same volume of incomplete Freund's adjuvant (Sigma Co, St. Louis, MO, USA) and injected intraperitoneally on 4 occasions at 1-week intervals. One week after the 4th injection, the mice were bled and the sera were tested for antibody levels by ELISA.

M. tuberculosis grown on Ogawa medium was fixed on a glass slide with acetone and washed for 10 min with 10 mM PBS and distilled water. After drying by an air stream, the slide was prepared with 1 drop of 1:100 diluted immunized serum of mouse and incubated at 37℃ in a moist chamber for 60 min. After washing and drying, one drop of

FITC-conjugated goat antimouse IgG diluted to 1:50 with PBS containing 1% bovine serum albumin was added. The same washing and drying procedure was performed and then the slide incubated with Evan's blue for 5 min. After washing and drying, the slide was mounted in glycerin and observed with a Leitz Dialux 20 fluorescence microscope. For controls, *M. bovis* BCG, atypical mycobacteria, *M. scroflaceum*, *M. intracellularae*, *M. gordonae*, *Staphylococcus aureus* ATCC 25923 were also stained.

RESULTS

SDS-PAGE

As shown in Fig. 1, PCA soluble *M. tuberculosis* antigen(TB-P) was composed of over 10 proteins. Interestingly, further treatment of TB-P with boiling and ethanol yield[illegible] single band of 60 kDa protein.

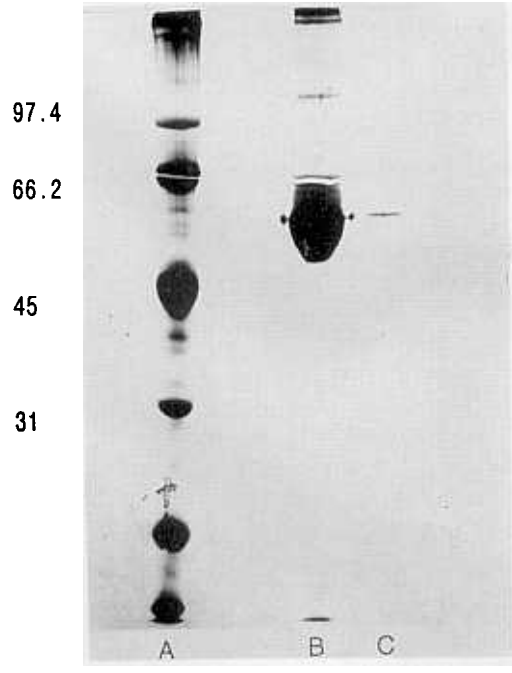

Fig. 1. *SDS-PAGE analysis of TB-PBE. Lane A: Standard mixture of proteins of known MW, Lane B: TB-P, Lane C: TB-PBE.*

Clinical applications

ELISA. Sera from patients with active pulmonary tuberculosis reacted with PPD antigen. However, they showed negative reactions against TB-PBE (Fig. 2). Normal sera showed negative reactions against both antigens.

Delayed Hypersensitivity Reactions. As mentioned in Materials and Methods, TB-PBE was prepared through several treatments. The bioactivity of TB-PBE and PPD to induce a delayed hypersensitivity reaction in normal children vaccinated with BCG, was tested. Seventeen children gave a positive reaction to both antigens and 7 children showed a negative reaction to both antigens.

Table 1. Skin reactivity to TB-PBE and PPD in BCG vaccinated normal children

	PPD positive	PPD negative
TB-PBE positive		
TB-PBE negative		

*$p<0.05$

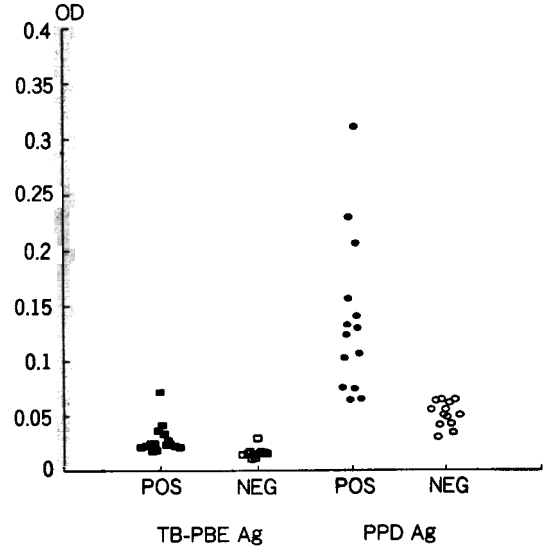

Fig. 2. *OD of Ig G antibody against TB-PBE and PPD antigens in the patients with AFB positive pulmonary tuberculosis (POS) and normal adults with positive skin test (NEG).*

Interestingly, two children who showed a negative reaction to PPD antigen in spite of BCG vaccination showed a positive reaction to TB-PBE (Table 1).

Skin Biopsy. TB-PBE induced an inflammatory lesion by H-E staining which showed multiple islets of mostly mononuclear cells infiltrating in subcutaneous area. These findings were very similar to a lesion induced by PPD (Fig. 3). Mononuclear cells in in-

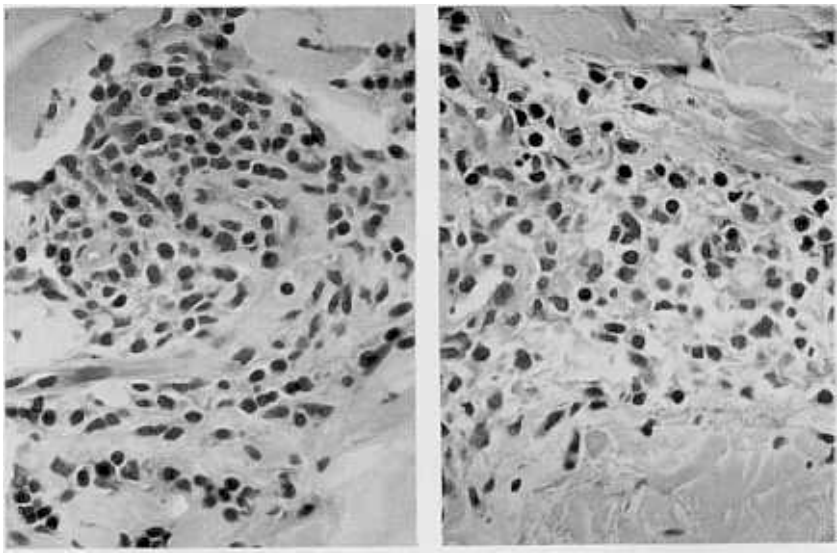

Fig. 3. *H-E staining of the skin biopsy specimens 48hours after inoculation of TB-PBE (left) and PPD (right). Mainly mononuclear cells are seen(× 400).*

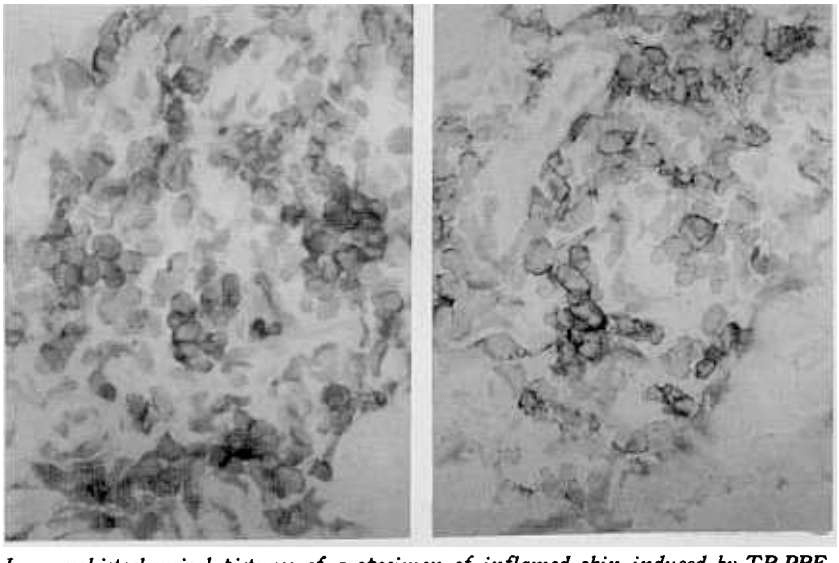

Fig. 4. *Immunohistochemical pictures of a specimen of inflamed skin induced by TB-PBE, by anti-CD3(left) and anti-CD4(right). Infiltrated mononuclear cells are mostly T cells, especially helper T cells(× 400).*

flammatory lesion were mainly CD3 and CD4+ cells(Fig. 4), some CD68+ cells, but not CD19+ and CD8+ cells(data not shown). These cellular components showed almost same pictures as those induced by PPD (data not shown).

Peripheral mononuclear cells stimulation tests with TB-PBE

Blastogenic activity of the peripheral mononuclear cells stimulated by TB-PBE increased, showed peak reaction at 7 days after stimulation, and decreased thereafter. The blastogenic action of TB-PBE changed in a dose-dependent manner (Fig. 5). These findings were similar to the reaction stimulated by PPD which was different with those findings stimulated by PHA (Fig. 6). After stimulation with TB-PBE, mononuclear cells were analyzed by FACS. T cell and

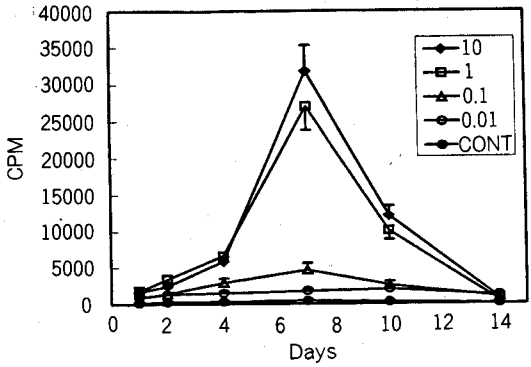

Fig. 5. *^{3}H-thymidine incorporation of human peripheral lymphocytes (1×10^4) stimulated by TB-PBE with different concentrations.*

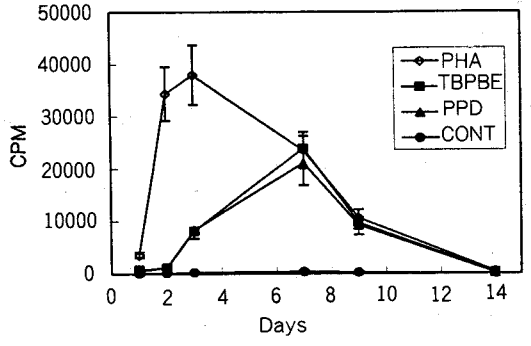

Fig. 6. *Time course of 3H-thymidine incorporation by human peripheral lymphocytes (1×10^4) stimulated by TB-PBE(1 μg/ml), PPD(1 μg/ml), and PHA(1 μg/ml).*

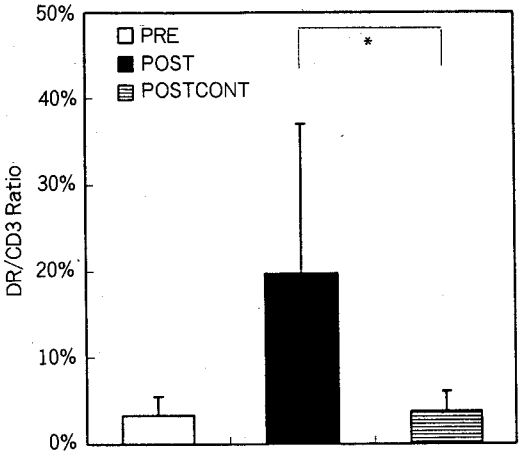

Fig. 7. *Changes of DR/CD3 ratio before and after stimulation with TB-PBE(1 μg/ml) for 7 days (*: $p<0.05$). PRE: before stimulation, POST: after stimulation with TB-PBE, POSTCONT: cultured with medium only.*

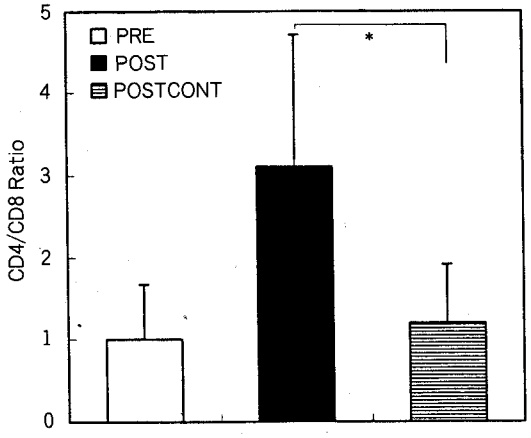

Fig. 8. *Changes of CD4/CD8 ratio before and after stimulation with TB-PBE(1 μg/ml) for 7 days (*: $p<0.05$). PRE: before stimulation, POST: after stimulation with TB-PBE, POSTCONT: cultured with medium only.*

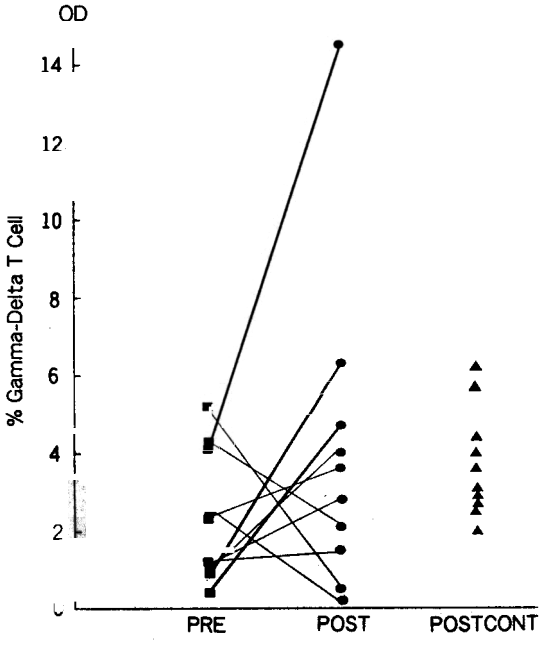

Fig. 9. *Changes of Gamma-Delta T cells before and after stimulation with TB-PBE(1 μg/ml) for 7 days(*: $p>0.05$). PRE: before stimulation, POST: after stimulation with TB-PBE, POSTCONT: cultured with medium only.*

B cell fraction were not changed after stimulation (data not shown), but DR positive cells and CD4/CD8 ratio increased (Fig. 7, 8). γδT cells seemed not to change after stimulation, but 3 out of 10 volunteers showed increased γδT cells after stimulation with TB-PBE (Fig. 9).

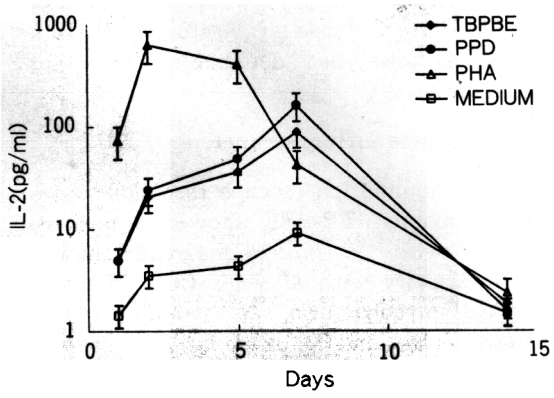

Fig. 10. *IL-2 production from the cultured peripheral mononuclear cells by stimulated TB-PBE(1 μg/ml), PPD(1 μg/ml), and PHA(1 μg/ml).*

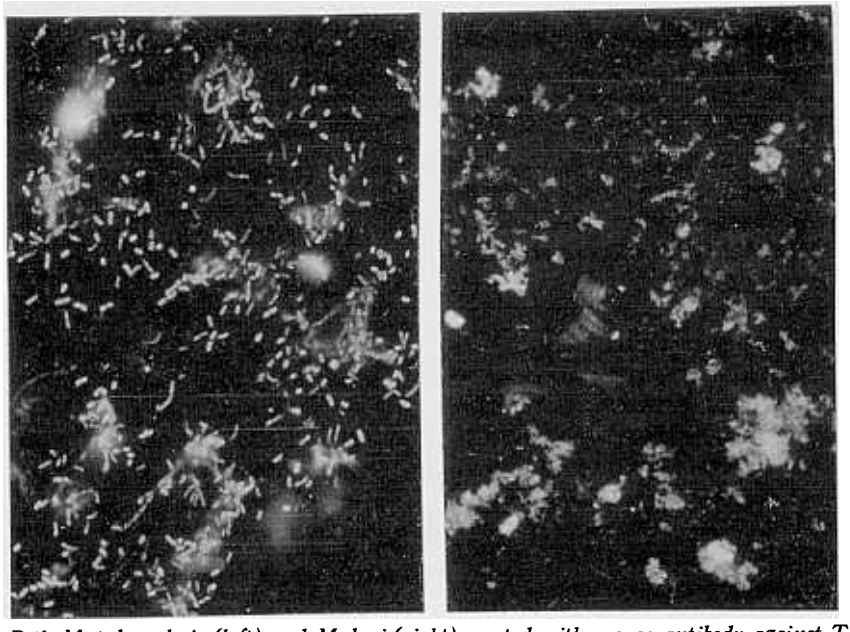

Fig. 11. *Both M. tuberculosis (left) and M. bovis(right) reacted with mouse antibody against TB-PBE by indirect fluoroscent antibody technique(× 400).*

Cytokine measurements

After stimulation of mononuclear cells with TB-PBE on different days, IL-2 and IL-4 production in culture supernatants from these cells were measured. IL-2 secretion from these cells after stimulation by TB-PBE increased with time, showed peak concentration at 7 days after stimulation, and then decreased (Fig. 10). These findings were similar to those of blastogenesis. IL-4 secretions were not detectable (data not shown).

Indirect immunofluorescence

In the immunofluorescence test, mouse antiserum against TB-PBE showed a positive reaction with *M. tuberculosis* and showed cross-reactivity with *M. bovis* BCG (Fig. 11). Atypical mycobacteria, *M. scroflaceum*, *M. intracellularae*, and *M. gordonae* also showed cross-reactivity, but not S. aureus (data not shown).

DISCUSSION

A 60 kDa protein antigen from cultured M. tuberculosis was isolated by perchloric acid (PCA) treatment, boiling, and ethanol treatment, which could stimulate cellular immune response in vivo and in vitro. This antigen could be found not only in *M. tuberculosis*, but also in atypical mycobacteria, but not in *S. aureus*.

Many investigators attempted to fractionate the antigens of *M. tuberculosis* by sophisticated techniques, such as differential precipitation, gel filtration, ion-exchange chromatography, preparative electrophoresis, ammonium sulfate precipitation and acrylamide gel electrophoresis (Daniel and Janicki, 1978). But no reagent suitable for clinical use had been developed. Recently, the establishment of recombinant expression libraries has made it possible to characterize the antigenicity. Several such antigens have now been identified and their functions as well as their sequences elucidated(Kauffman, 1993). However, none of these antigens has so far been shown to actively induce protective immunity.

Classically, PCA has been used for the extraction of glycoprotein from samples such as carcinoembryonic antigen (Abeyounis and Milgrom, 1972) and some bacterial proteins (Kim *et al.* 1989). In our previous report, 65 kDa, possibly a heat shock protein, was isolated from *M. bovis* BCG by PCA treatment (Kim and Kim, 1993). In this study, we performed the PCA treatment on *M. tuberculosis* to see whether the 65 kDa protein were isolated, like in *M. bovis* BCG. However, as shown in Fig. 1, over 10 antigens were isolated with the treatment of PCA to *M. tuberculosis* (TB-P) which was different from those with the treatment of PCA to *M. bovis* BCG.

TB-P was then boiled to isolate heat stable antigens. Ethanol treatment was conducted to exclude the lipid soluble fraction, because *M. tuberculosis* contains a large amount and a wide range of lipids. After these two simple procedures, a single antigen of 60 kDa (TB-PBE) was isolated. This antigen was thought to be a HSP because it was prepared from boiling and a strong acid of PCA.

Heat shock proteins (HSP) were coined because they are synthesized in dramatically increased amounts following a brief exposure of cells to an elevated temerature. Also the particular constellations such as anoxia, ethanol, and heavy metal ions can induce HSP in nearly all cells. The HSP are molecular chaperones to help guide the folding of a protein. Among these molecular chaperones, two components were related to HSP60 and HSP70. The HSP70 proteins act early, recognizing small patches on a protein's surface. The HSP60-like proteins appear to act later and form a container into which proteins that have still failed to fold are transferred.

The name of HSP70 is DnaK, and HSP60 is GroEL (Georgopoulus and Welch, 1993). *M. tuberculosis* contains several HSPs such as DnaK and GroEL. A 60-kDa protein that we isolated in this article is thought to be

GroEL. Further conduction of molecular cloning and sequencing of this protein is required.

Our interest was extended to find out whether this protein also induced immunological responses in vivo and vitro. As shown in Table 1, TB-PBE had a stimulatory activity on the cellular immune response. The skin reactivity to TB-PBE correlated well with PPD. Two children, who had a negative reaction to PPD in spite of BCG vaccination, showed a positive skin reaction to TB-PBE. With this result, we think that TB-PBE may be an appropriate antigen as a skin test reagent after BCG vaccination to evaluate the efficiency of vaccination. However, it is necessary to study a larger population.

In our country, BCG vaccination is routinely done for all neonates. So, clinicians can easily be confused whether the PPD skin reactivity is from BCG vaccination or from a primary infection of *M. tuberculosis*. With this motive to differentiate these two reactions, we tried to get an antigen which is specific for *M. tuberculosis*. However, the results of indirect immunofluorescence technique showed that TB-PBE was not specific for *M. tuberculosis* and is thought to be distributed throughout the mycobacterial genus.

PPD stimulates not only a cellular immune response but also a humoral immune response. We carried out tests to see whether TB-PBE also could stimulate both cellular and humoral immune responses. Contrary to our expectations, TB-PBE had no stimulatory effects to produce antibody against this antigen in vivo. With these results we postulate that TB-PBE stimulates only the cellular immune response in man.

T-lymphocytes are vital for the acquisition of protection against tuberculosis. All three known T cell populations, CD4+ $\alpha\beta$ T cells, CD8+ $\alpha\beta$ T cells, and double-negative $\gamma\delta$T cells, seem to participate in the acquisition of optimal protection (Kauffman, 1993; Orme *et al.* 1993). The $\alpha\beta$ T cells, comprising over 90% of all peripheral T cells in man, are central to immunity against tuberculosis. Among these $\alpha\beta$ T cells, the CD4+ T cells are more important. Thus, adoptive transfer of selected CD4+ T cells with specificity for *M. tuberculosis* minimizes tuberculosis in recipient mice, and in contrast, depletion of CD4+ T cells with monoclonal antibodies exacerbates the disease (Orme and Collins, 1984; M ller *et al.* 1987). In line with the idea that CD4+ T lymphocytes have a central role in immunity, it has been found that mycobacterium specific T cells isolated from *M. tuberculosis* immune mice and humans consistently express the CD4+ phenotype (Kauffman and Flesch, 1986; Ottenhoff *et al.* 1988; Kauffman, 1993). CD8+ T cells specific for mycobacteria have also been isolated from *M. tuberculosis*-immune mice, but have only rarely been identified in tuberculosis patients (DeLibero *et al.* 1988; Rees *et al.* 1988). Interestingly, T cells that infiltrated in hypersensitive subcutaneous tissue induced by PPD and TB-PBE were mostly CD4+ cells. These results were very similar to Tsicopoulos' study (Tsicopoulos *et al.* 1994). In vitro, total T cells stimulated by TB-PBE had not changed. However, the number of activated(DR+) T cells and the CD4+/CD8+ ratio had increased, which means that TB-PBE stimulated the T cells, especially CD4+, but not CD8+.

Recently, cumulative evidence for the participation of $\gamma\delta$ T cells in protective immunity to tuberculosis have been found. $\gamma\delta$ T cells accumulate in lymph nodes and lung after subcutaneous or aerosol immunization of mice with mycobacteria (Augustin *et al.* 1989; Janis *et al.* 1989; Balaji *et al.* 1995). Increased numbers are found in tuberculous lymphadenitis, but not in chronic lymph node granulomas of tuberculosis patients (Falini *et al.* 1989; Tazi *et al.* 1991). Furthermore, human $\gamma\delta$ T cells are activated by in vitro stimulation with various mycobacterial components (Pfeffer *et al.* 1991; Tsukaguchi and Boom, 1994). We conducted a similar study to observe whether TB-PBE could stimulate $\gamma\delta$ T cells in vitro. Three out of 10 samples of peripheral mononuclear cells definitely showed increased numbers of $\gamma\delta$ T cells 7 days after stimulation with TB-PBE, but the other 7 samples failed to stimulate

them. In vivo, T cells that infiltrated in skin lesions induced by TB-PBE were stained with monoclonal antibody against $\gamma\delta$ T cells, but the result was negative (data not shown). It was believed that TB-PBE can also stimulate $\gamma\delta$ T cells in some cases but not all. Whether TB-PBE can induce $\delta\gamma$ T cells in vivo and vitro need further clarification.

There is a dichotomy among CD4+ T cells into Th1 and Th2 subsets which was originally seen among the murine CD4+ T cell clone (Mossman *et al.* 1986). However, in humans, the dichotomy among CD4+ T cell clones is less clear-cut (Mossman and Sad, 1996). When the cytokine profiles of mycobacterial antigen-specific CD4+ T cell clones are studied, a consistent pattern is observed in that the majority produce large amounts of IFN-γ and IL-2, and little or no IL-4 or-5 (Haansen *et al.* 1991; Mutis *et al.* 1993). Even though there is some argument on this matter (Surcel *et al.* 1994), this pattern of cytokine production is consistent with the so-called Th 1-like phenotype of T cell clones.

We observed a pattern of cytokine production after stimulation of peripheral mononuclear cells with TB-PBE by measuring IL-2 and IL-4. As shown in the results, IL-2 secretion was observed after stimulation with TB-PBE which was consistent with that of blastogenesis of peripheral mononuclear cells. However, IL-4 secretion was not observed. It was thought that TB-PBE preferentially induced CD4+ Th1 like cells.

With these results, we conclude that PCA soluble, heat stable and ethanol extractable antigen of *M. tuberculosis* (TB-PBE) is an antigen which can induce cell-mediated immunity in vivo and in vitro. We will conduct a purification of the 60 kDa protein and research on the antigenicity of this protein, since TB-PBE is composed of more than a 60 kDa protein.

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