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PURIFICATION AND CHARACTERIZATION OF PROTEIN ANTIGENS ISOLATED FROM *MYCOBACTERIUM TUBERCULOSIS* (H37Rv STRAIN) AND THEIR EFFECTS ON CELL-MEDIATED IMMUNE RESPONSES IN GUINEA PIGS

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ABSTRACT

Mycobacterium tuberculosis (H37Rv strain) was used in this study. The bacterial cells were disintegrated by sonication. The separation and characterization of the soluble molecules were attempted by various techniques including gel filtration, ion exchange chromatographies and polyacrylamide gel electrophoresis, using SDS and 2ME. Eight protein molecules with molecular weights ranging from 6.3 up to 204 kD were identified. Following reduction of the 204 kD molecule with 2ME, six smaller molecules with 12a, 12b, 21, 29, 45 and 81.5 kD molecular weights were obtained.

All isolated protein molecules were able to induce delayed hypersensitivity skin reaction in sensitized guinea pigs and proliferation of T-cells *in vitro*.

Regarding the fact that an effective protective immunity in tuberculosis is dependent mainly on T-cell response, it is suggested that the molecules isolated in this study may be useful in conceiving a vaccine and/or diagnostic tests for tuberculosis.

Keywords: *M. tuberculosis*, Protein Antigens, CMI Responses, Purification

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INTRODUCTION

Tuberculosis remains a major worldwide health problem, resulting in an estimated 3 million deaths and 8-10 million new cases each year.[1-3] It is estimated that 1.3 million new cases and 45,000 deaths from tuberculosis in developing countries occur annually in children under the age of 15 years.[2] Moreover more than 3 million people are dually infected with the tubercle bacillus and HIV throughout the world.[2] HIV infection is the highest risk factor recently identified which increases the chance of latent infection with tubercle bacilli progressing to active tuberculosis by reducing the protection provided by cell-mediated immunity.[4,36,38,40]

BCG vaccine has been used for a long time all over the world for the prevention of the disease and protection of individuals against TB.[42] However, varying rates of protec-

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tion by this vaccine have been reported in different parts of the world,[42,43] and the need for an effective vaccine for this very contagious disease is increasing.

It is therefore essential initially to identify molecules in the bacterial cell which effectively stimulate the immune system and induce a protective immunity. While tuberculous patients develop high titers of specific *M. tuberculosis* reactive antibodies, there is no evidence indicating these have any role in protection.[10] Cell-mediated immunity is the competent protective immune response in human disease caused by intracellular pathogens.[4,8,9] A number of researchers have attempted to isolate and identify the molecules from *M. tuberculosis* which could effectively activate T-cells.[16-18,21-29,32,41] The present research program aimed at identifying the dominant protein antigens which are directly involved in the induction of the cell-mediated immune responses (CMI) against *M. tuberculosis*. Considering the fact that T-cells recognize and respond mainly to protein antigens,[5] we focused our investigation on the proteins which are present in *M. tuberculosis*. For this purpose, the protein antigens from a standard strain of *M. tuberculosis* (H37Rv) were purified and characterized and their effects on cell-mediated immune responses were evaluated by delayed-type hypersensitivity skin tests in sensitized guinea pigs and the ability of T-cells to recognize and proliferate against these protein antigens *in vitro* were also assessed.

MATERIAL AND METHODS

Bacteria

M. tuberculosis H37Rv (ATCC 27294) and H37Ra (ATCC 25177) were provided kindly by Pasteur Institute of France.

The bacteria were grown for eight weeks as a surface pellicle on modified Souton medium (sodium pyruvate 5 g, glucose 5 g, tween 80 0.5mL, L-asparagine 4.0 g, glycerl 60 g, citric acid 2g, K_2HPO_4 0.5 g, magnesium sulfate 0.5 g, ferric ammonium citrate 0.05 g, distilled water 950 mL, pH 7.0). This medium was sterilized at 121°C, 15 psi for 15 min.

Sonic Extracts

The bacteria were separated from the medium by centrifugation (4000× g for 30 min at 4°C). The pellet was washed three times in tris buffer and resuspended in the same buffer for sonication. The bacilli were sonicated by subjecting to ultrasonic pulses in a Virosonic Cell Disruptor model 16-850 at 4°C, with 5 min rest periods, to alleviate the effect of heat, for a total exposure time of 20 min. The sonicate was centrifuged at 6000× g and subsequently 100,000× g at 4°C for 1 h. Ammonium sulphate (80% saturation) was used to precipitate and concentrate the proteins in the supernatant.

After dialysis in 0.1 M Tris-HCl pH 7.3, the sample was passed through a 0.22 μm sterile filter. The protein content of the solution was determined by the method of Lowry et al,[10] and was stored at -70°C.

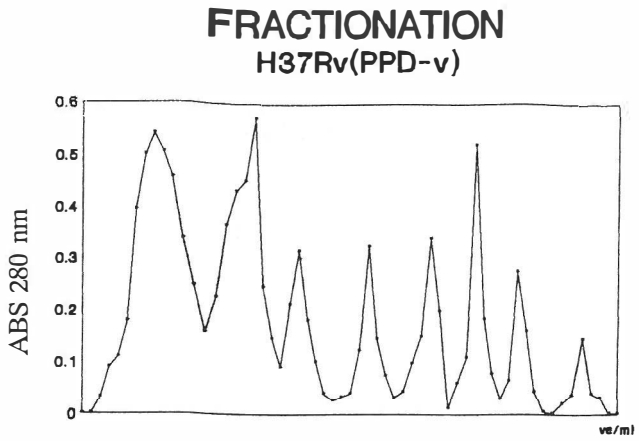

Fig. 1. Elution profile of soluble proteins of *M. tuberculosis* H37Rv. Gel: Sephacryl 200-HR flow rate 2mL/cm² /h, fraction size 3mL/tube, height and diameter of gel: 90×2.5 cm, buffer Tris 0.1 M and sodium chloride 0.5 M pH 8.0. The sample contained 80 mg/5mL buffer.

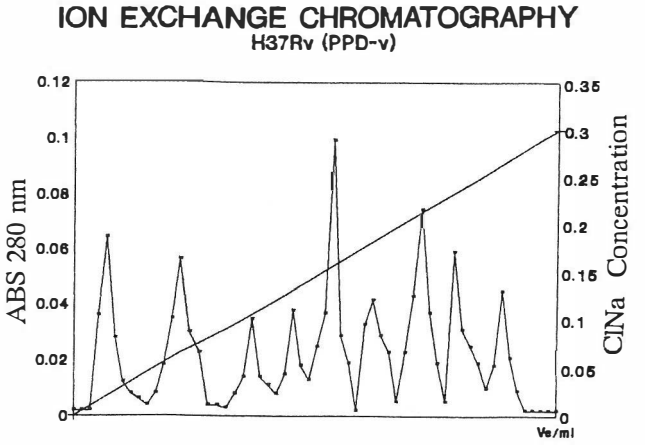

Fig. 2. The elution profile of the soluble proteins of *M. tuberculosis* H37Rv. Column: 20×1.5 cm, elution buffer, 1400 mL of Tris-buffer pH 8.0 with a linear concentration gradient of sodium chloride from zero up to 0.3 M, flow rate 90 mL/h, gel DEAE-cellulose.

Gel Filtration Chromatography

A column (K26/100; LKB Pharmacia, Sweden) was packed with sephacryl 200-HR. Protein solution (80 mg) was then applied to the gel filtration column and eluted under pressure with 0.1M Tris-HCl containing 0.5 M NaCl at a flow rate of 2mL/cm²/hr.

DEAE-Cellulose Chromatography

A DEAE-cellulose column, 1.5 by 20 cm, was equilibrated with 30 mM Tris buffer pH 8 containing a linear concentration gradient of sodium chloride from zero to 0.3

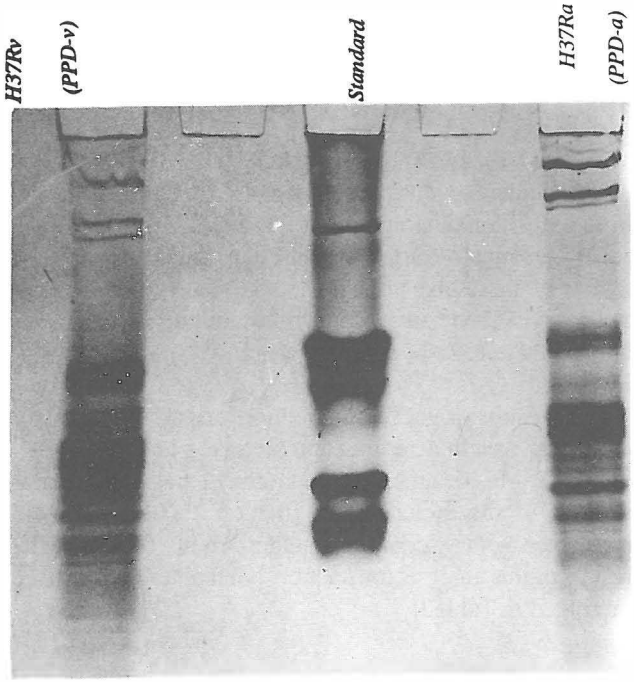

Fig. 3. Non-dissociated PAGE of soluble proteins of *M. tuberculosis* H37Rv and H37Ra. Gel gradient 5-20%, staining coomassie brilliant blue R250.

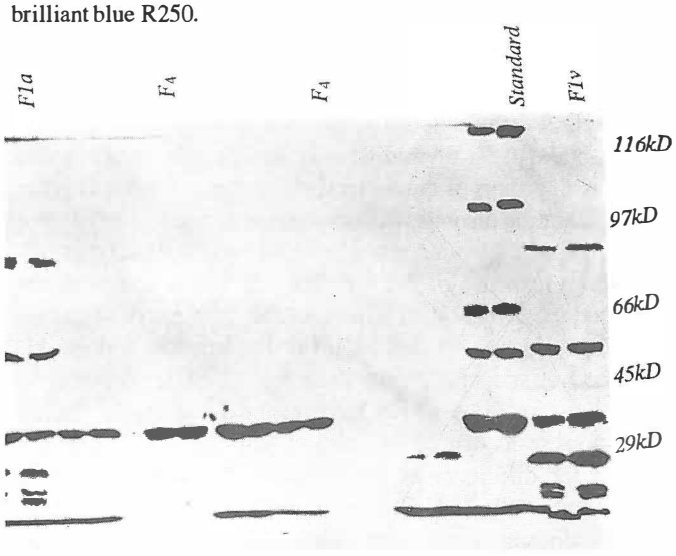

Fig. 4. SDS-PAGE of the 2 ME-treated F1 and F4 fractions. Fraction 1 has six bands and other fractions have only one band. Gel: Acrylamide with a concentration of 12.5%, staining coomassie brilliant blue R250.

M at a rate of 90 mL/h. The fractions were collected and stored

Protein Analysis by SDS-PAGE

SDS-PAGE was carried out on 10, 15 and 5-20 percent gradient of acrylamide gel as described by Laemmli.[13] The gels were stained with coomassie brilliant blue R-250 and it was also silver stained according to Morrisey,[14] or by Sigma AG-25 silver stain kit (Sigma AG25).

Molecular Weight Determination

Two methods were used for molecular weight determination: 1) SDS-PAGE was carried out with Sigma high and low molecular weight standards (Sigma MW-SDS 70L, SDS-64, MW-SDS 175 kit), and 2) molecular sieving was conducted on sephacryl S-200HR. The column was calibrated with the protein standards with relevant molecular weights 2×10^6 - 13.7×10^3.

Immunization of Guinea Pigs

Albino female guinea pigs weighing 400-500 g were purchased from Pasteur Institute of Iran. The guinea pigs were immunized by two injections in the hind legs and backs with 0.5 L of the chosen immunogen.

The route of injections was subcutaneously except for the BCG vaccine. The following immunogens were used: standard live *M. bovis* BCG vaccine (attenuated live bacilli, 1077 strain, Pasteur Institute, France) was injected I.D., killed *M. tuberculosis* H37Rv and H37Ra suspended in incomplete Freund's adjuvant (IFA) were injected S.C. A group of control guinea pigs were injected with the eluate buffer from gel filtration column in IFA. A second group of control guinea pigs were injected with Souton medium.

Skin Test

The animals were skin tested seven weeks after sensitization. Each group of animals was given intradermal injections of 2 µg of each fraction, PPD standard or protein extract from *M. tuberculosis* H37Rv or H37Ra. The two groups of guinea pigs which served as negative controls were injected with PPD standard. Skin reaction from all groups of animals was read from 24 up to 72 h after injection.

Lymphocyte Proliferation Assay

Lymphocytes were isolated in a density gradient and washed twice in culture media. The cell suspension was adjusted to 2×10^6 viable cells per mL containing 5mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 20% inactivated fetal calf serum (GIBCO). The cell suspension was distributed in wells of U-shaped 96-well microculture plates (Nunc, Denmark). In each well 2×10^5 cells in a volume of 200 µL was added and an appropriate amount of antigen was added according to the following scheme: either 5-10 µg/mL of one of the fractions or 10µg/mL of PPD (Serum Institute-Denmark, batch RT 146) or 10 µg/mL of concanavalin-A (Sigma).

Following 4 days of incubation at 37°C in a humidified 5% CO_2 incubator, the cultures were pulsed with 1 µCi of tritiated thymidine (Amersham International, U.K.). The cultures were subsequently incubated for a further 16 h at

at 4°C until us

Table I. The fractions characteristics by gel filtration chromatography.

Fraction number	ve*(mL)	Kav*= $\frac{ve-vo}{vt-vo}$	Molecular weights (kD)
1	221	0.114	204
2	254	0.200	124.4
3	293	0.315	66
4	338	0.447	31.0
5	353	0.490	23.4
6	377	0.560	15.8
7	398	0.620	11.2
8	434	0.720	6.3

*ve and Kav and molecular weights of fraction obtained from sephacryl S200-HR column.
vo= void volume, vt= total volume, ve= elution volume.

Table II. Molecular weights of the molecules in the fractions as determined by gel filtration and SDS-PAGE.

Fraction number	Molecular weights (kD)	
	Gel filtration	SDS-PAGE
1	204	81.5, 45, 29, 21, *12
2	124.4	118
3	66	66-67
4	31	30.2
5	23.4	**
6	15.8	**
7	11.2	12
8	6.3	**

* Two molecules with very close molecular weights.
** Electrophoresis was not possible due to inadequate amount of the sample.

37°C, and harvested on to glass fiber filters.The incorporated radioactivity was measured in a beta scintillation counter (Pharmacia, Wallack β-counter). The results were expressed as mean counts per minute ± standard error of the means.

Statistical Methods

Statistical analysis was attempted by ANOVA test using SPSS package.

RESULTS

Chromatographic Analysis

Fig. 1 illustrates the peaks obtained from gel filtration chromatography of soluble proteins from *M. tuberculosis* (H37Rv). Eight fractions were obtained after elution from sephacryl 200-HR column. Fig. 2 shows the peaks and fractions obtained from DEAE-cellulose ion exchange chromatography of the same soluble proteins from *M. tuberculosis* (H37Rv). Nine fractions were collected after elution from the column. Fraction 5 was found not to be a protein. It could probably be derived from other components of the bacterium.

Molecular Weight Determination

Molecular weights of fractions were determined by gel filtration chromatography and SDS-PAGE. In order to choose a suitable gel for gel filtration chromatography, a sample of the soluble protein was analysed by non-dissociated PAGE. The results indicated that the native proteins of the sample were in the approximate range of 5-250 kD (Fig. 3).

The results of gel filtration chromatography regarding molecular weight determination are given in Table I. Following 2 ME treatment of each fraction, the molecular weights of the molecules in fraction 1 (F1) yielded 6 molecules with various molecular weights (Fig. 4). The results of this analysis for fraction 1 and other fractions are presented in Table II.

T-Lymphocyte Proliferation

Blood lymphocytes from different groups of guinea pigs immunized to killed *M. tuberculosis* H37Rv or BCG were exposed to either PPD, Con-A or each fraction, *in vitro*. The cells were incubated at 37°C for four days. ^{3}H-thymidine was then added in each well and the uptake of ^{3}H-thymidine by cells was measured 16 hours later. The results as assessed by uptake of ^{3}H-thymidine indicated that the lymphocytes from all groups of immunized guinea pigs showed a higher proliferation response *in vitro* in comparison to the lymphocytes derived from control groups ($p<0.0001$). The proliferation response of the lymphocytes which had been exposed *in vitro* to either whole soluble proteins of *M. tuberculosis* H37Rv, PPD standard or the fraction with 66 kD were not statistically significant ($p<0.0001$). Among the various fractions which were tested *in vitro*, the lowest response was that of the fraction with 6.3 kD MW. Moreover the difference of proliferation response of the three fractions with 204, 23.4 and 15.8 kD molecular weights were not statistically significant ($p<0.0001$) (Fig. 5).

F2, F3, F4 with molecular weights of 118, 66, 30.2 kD respectively, as well as the soluble protein extracts of *M. tuberculosis* H37Rv (PPD-v) and H37Ra (PPD-a) induced the highest *in vitro* response ($p<0.0001$) (Fig. 5).

Regarding the dose of Con-A, there was no difference between the *in vitro* response to 5 and 10 μg/mL of Con-A (the positive control).

Skin Reaction

All groups of guinea pigs which were skin tested showed a positive delayed type skin reaction to the intradermal injection of the antigen in comparison to that of the negative controls ($p<0.0001$) (Table III).

Table III. The results of induration due to intradermal injection of various fractions, soluble extracts of *M. tuberculosis* H37Rv (PPD-v), H37Ra (PPD-a) and PPD standard in each group of guinea pigs.

Antigens	204 kD F1	118 kD F2	66 kD F3	30.2 kD F4	23.4 kD F5	15.8 kD F6	112 kD F7	6.3 kD F8	PPD-V (H37Rv)	PPD-a (H37Ra)	PPD-S*	PPD-S**	PPD-S***	PPD-S****
Diameter of Induration (mm) (mean±SD)	6.2±1.3	8.8±2	8.44±2.18	8.5±1.8	6±1.3	6.2±1.47	5.7±1.15	5.1±0.99	8.3±1.41	9.1±1.79	7.4±0.84	7.7±1.82	2±0.63	0.45±0

* sensitized to *M. tuberculosis* H37Rv
** sensitized to BCG
*** sensitized to culture medium plus incomplete adjuvant
**** No previous sensitization

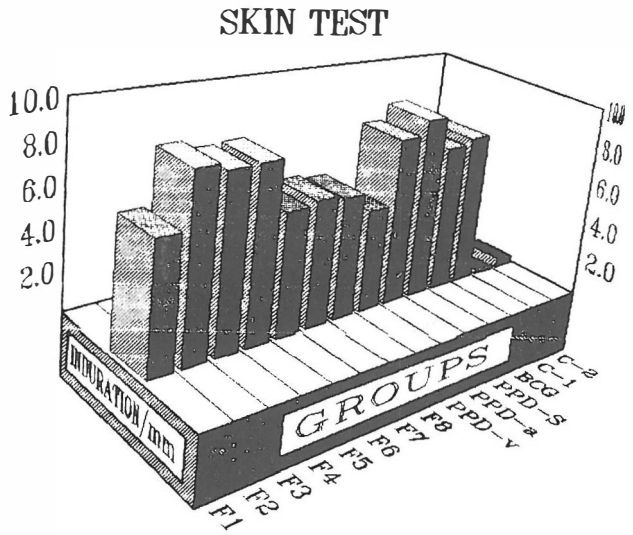

Fig. 5. The *in vitro* response of peripheral guinea pig lymphocytes to various fractions, PPD and Con-A. No antigen was added to the controls (C1-C3).

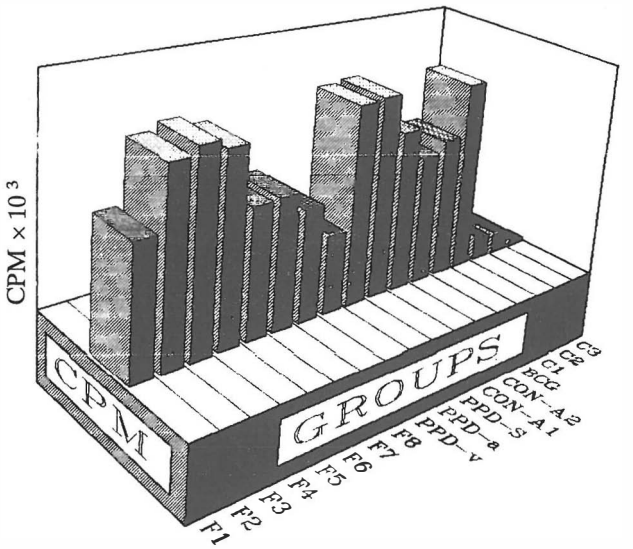

Fig. 6. The results of induration due to intradermal injection of various fractions and PPD in different groups of guinea pigs (n= 10).

Regarding the diameter of the induration, F2, F3, F4, PPD-v, PPD-a and PPD-S induced the highest and F8 induced the weakest skin reaction ($p<0.0001$). Statistically there was no difference between the diameters of the induration induced by F1, F5, F6 and F7 ($p<0.0001$) (Fig. 6).

DISCUSSION

Purification of eight antigens from *M. tuberculosis* H37Rv was achieved by simple procedures using gel filtration and ion-exchange chromatographies. These antigens consisted of proteins with molecular weights of 204, 124.4, 66, 31, 23.4, 15.8, 11.2 and 6.3 kD. All isolated proteins with the exception of the protein with the molecular weight of 204 kD appeared to be single chain polypeptides since they migrated to the same position in the gels under reducing and non-reducing conditions. Following 2ME treatment, the 204 kD molecule was fragmented into smaller molecules with molecular weights of 81.5, 29, 21 and 12 kD (Fig. 4). SDS-PAGE also revealed a somewhat different molecular weight for one isolated molecule. This particular molecule was the 124.4 kD molecule as determined by gel filtration chromatography, which by SDS-PAGE was shown to have a molecular weight of 118 kD. 2ME treatment of this molecule could have split a very small fragment of it, which did not seem to be a protein since no trace of a visible band was seen in polyacrylamide gel following SDS-PAGE.

In recent years several antigens of *M. tuberculosis* have been identified by using monoclonal antibodies and other procedures.[17,18,20,24,26] Regarding chemical nature and molecular weights, the antigens identified by these researchers are to a great extent similar to the antigens isolated and identified in the present study. However, in the present study two relatively large protein antigens were isolated with molecular weights of 118 and 204 kD which to our knowledge have not previously been reported. It seems by using mild procedures as well as high resolution sephacryl-200, these two molecules were isolated without fragmentation. It seems probable that the molecule with 204 kD MW is labile to reduction and fragmentation as the result of certain procedures and the procedures such as those mentioned in the present study are able to isolate and identify it. The immunological relevance of the purified antigens were

clearly demonstrated by positive skin test and lymphocyte transformation assay in guinea pigs previously sensitized to BCG or killed *M. tuberculosis* (H37Rv). It is therefore inferred from these results that the chains of these protein molecules comprise sequences of amino acids which structurally make up the epitopes recognized by T-cells. Since the CMI immune responses against these antigens were quantitatively unequal, it is reasonable to suggest that these proteins bear epitopes which regarding structure and or the number of epitopes in the polypeptide chains, are different. It was found that the proteins of 118, 66 and 31 kD which appear to be the major antigens of *M. tuberculosis* H37Rv were very immunogenic. They were able to induce a pronounced delayed hypersensitivity reaction and promote a significant level of lymphocyte proliferation in sensitized guinea pigs.

The antigenicity of protein molecules with molecular weights in the range of those reported in this article has previously been demonstrated by skin test and lymphocyte proliferation assay in guinea pigs or mice sensitized with BCG or *M. tuberculosis*.[10,16,24,25,26,28,29,33,34,41]

In the present investigation, an attempt was made to minimize denaturation of protein molecules. Moreover, the immune response of each individual guinea pig against each antigen was evaluated by skin test as well as lymphocyte proliferation assay. The results of skin tests and those of lymphocyte proliferation assays agreed closely with each other.

The results reported by other researchers on this line,[19,24,26,28,29,41] as well as the results of the present study indicated that the majority of protein molecules in *M. tuberculosis* are able to activate and to induce CMI responses as assessed by delayed hypersensitivity skin test and *in vitro* proliferation of T-lymphocytes. However, it is quite unlikely that each protein anti
a protective immune response against tuberculosis; these two tests may not be able to trace and identify the protective antigens in the bacterial cell. Quantitative measurement of certain cytokines such as IL-2 and γ- interferon produced by the subsets[44] and enumeration of T-cell subsets including γδ T-cells which increase in mycobacterial infection[45] may also be needed to provide a vivid profile of CMI responses for any test antigen. The protective antigen isolated from a pathogen should be able to protect the susceptible immunized host against a challenging dose of live pathogen.

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