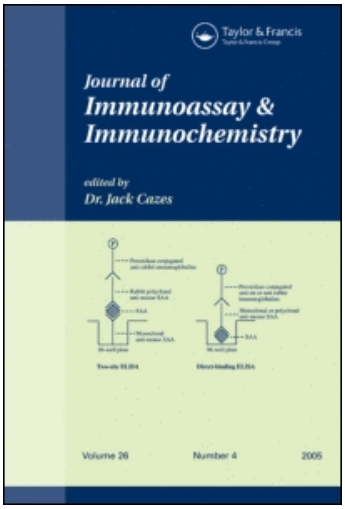


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# A Simple Method of Electroelution of Individual Protein Bands from SDS Polyacrylamide Gels for Direct Study in Cellular Assays

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# A SIMPLE METHOD OF ELECTROELUTION OF INDIVIDUAL PROTEIN BANDS FROM SDS POLYACRYLAMIDE GELS FOR DIRECT STUDY IN CELLULAR ASSAYS

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## ABSTRACT

A very simple and effective procedure which allows simultaneous electroelution of separated proteins from SDS polyacrylamide gel into small quantity of elution buffer is described. Elution parameters have been optimized for maximum possible recovery (50-60%). Protein fractions were

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collected in physiological buffer and an efficient removal of SDS have been obtained, thus fractions collected were suited for direct testing in cell cultures. Method was used to investigate human T-cell responses to purified secreted *M.tuberculosis* H37Rv proteins. Eight low molecular weight (M.w. range 10 kD to 25 kD ) culture filtrate proteins were purified in quantities, sufficient for immunological characterization. Lymphocyte proliferative responses and cytokine release pattern from tuberculosis patients, healthy contacts and healthy controls were studied on stimulation with purified culture filtrate proteins. Immunologically important *M.tuberculosis* proteins were identified by using this method. This approach should be applicable to the rapid identification and characterization of any interesting T cell antigen.
(Key words: Electroelution, Mycobacterium tuberculosis, Culture filtrate proteins, SDS-PAGE.)



## INTRODUCTION

T-cell mediated immune responses are key determinants to the natural course of mycobacterial infection. Thus T-cell activating proteins of mycobacteria continue to generate active interest, particularly in view of their possible role in the design and development of newer and more effective diagnostic tests and vaccines. Several mycobacterial proteins bearing antigenicity for T-cells have been identified (1,2) Nonetheless, since these proteins have mostly been picked up from crude extracts by biochemical means or from recombinant DNA libraries with the help of antibodies, it is quite likely that many predominantly T-cell activating molecules had been missed, as antigens were selected either by their biochemical or by antibody inducing properties, none of which may be correlated to their relevance as T-cell antigens. T-cell Western Blot Method (3,4) has the disadvantage, that proteins blotted can not be quantitated. DMSO and membrane components can interfere with the cell proliferation. To overcome these problems, method of simultaneous electroelution of whole gel was developed by Gulle et al. (5) and

Andersen et al.(6). In both methods a special device was designed. In the first method exceedingly low amount of protein was obtained, which imposes limitations on the application of the method in characterization of stimulating antigens, and in the second method whole gel was divided into 10 fractions hence, individual proteins could not be separated out.

We describe a procedure, which combines the high resolution power of isoelectric focusing followed by gel electrophoresis. Separated individual proteins were directly analysed with peripheral blood mononuclear cells. Proteins from individual bands were electroeluted out simultaneously and no special instrument was required. In this electrophoretic elution method, charged SDS molecules are removed through the dialysis membrane, as their size is smaller than the pore size of dialysis membrane. This is in accordance with the previously reported high efficiency of electrophoretic dialysis in the removal of SDS (6,7). In the present study, the method has been applied to identify T-cell antigens from *M.tuberculosis,* for the possible identification of new T cell antigens of importance for protective immunity.



## MATERIALS AND METHODS

### Mycobacteria

*M.tuberculosis* strain $H_{37}Rv$ were originally obtained from Trudeau Institute, Sarnac Lake, New York, USA and maintained on Lowenstein-Jensen slants in our laboratory. *M.tuberculosis* H37Rv were grown in modified Youman's medium as static surface culture by incubating at 37 °C for 12 to 14 days. Culture supernatant was sterile filtered and concentrated in Amicon assembly having YM3 membrane (Amicon), until protein concentration ranging from 2-3 mg/ml was achieved.

Gel Electrophoresis

For standardization of elution method, BSA was used as the model protein. BSA or culture supernatant were separated in non-reducing SDS-PAGE (8). 500μg of BSA mixed with sample buffer was loaded on one side of 12% minigel. Electrophoresis was done at constant current of 30 mA, with the system cooled to 4°C during run.

*M.tuberculosis* culture supernatant proteins were purified by preparative two-dimensional gel electrophoresis. Proteins were first fractionated in free solution isoelectric focusing apparatus (Rotofor cell from Biorad). To the 90 mg protein solution (50ml), mixture of non ionic detergents, CHAPS and digitonin were added at 1%w/v concentration to maintain the solubility of focused proteins. Biolytes, pH 3-10 at 2%w/v, glycine 1%w/v and glycerol (5ml) were also added. Constant power (12W) was applied for 4 hrs, with the system cooled to 4°C. Collected fractions containing the proteins of interest were pooled and refractionated to improve the sample purity. Ampholytes attached to protein molecules were removed by dialysing individual fractions against 1M NaCl. Individual fractions having proteins of similar isoelectric point were then run on 15% preparative slab gel (BIO-RAD, Protean II, 18.3× 20 cm) under non-reducing conditions. Electrophoresis was done at constant voltage of 100V for about 15 hours with the system cooled to 4°C during run.

Localization of Protein Bands

In general, the yield of proteins from fixed stained bands is much lower than from unfixed gels. Hence, two longitudinal strips were cut from the right and left sides of the slab gel and were stained with Coomassie blue, while the

rest of the gel was kept on glass plate, covered with cling film at 4°C. Following this, the stained side strips were lined up along the edges of the unstained gel and used as guides to cut out bands from the unstained gel.

Equilibration of Gel

Individual bands cut from the gel were equilibrated in three changes of the 2mM phosphate buffer, pH-6.8 on rocking table for varying periods of time (0 min to 60 min) as gel contain high concentrations of toxic components like SDS, Tris & glycine which have to be eluted out if the fractions are to be used in cellular assays. After equilibration for various periods of time, protein was eluted by electroelution method and concentrated.



Elution of Proteins

Gel strip having single band was cut into small pieces to increase the surface area and sealed in small dialysis bags (M.w. cut off-3,500) with 2mM phosphate buffer, pH-6.8 to just cover the gel slices. 2mM PB was selected for elution experiments, as it provided very effective elution, with minimal generation of heat and is completely non-toxic in cell cultures (6). Dialysis bags were transferred to the platform of horizontal gel electrophoresis apparatus filled with 2mM phosphate buffer. Elution parameters (volt and time) were optimized. Electrophoretic elution was carried out at different volts, keeping the elution time constant. Optimum time for elution was confirmed by keeping the voltage constant.

Significant amount of protein sticking to dialysis bags was desorbed by reversal of current for 1 min. towards the end of main elution period. This

procedure improved the recovery of protein by about 20%. Eluted proteins were concentrated in speed vaccum and were quantitated by the bicinchoninic acid method (9). After optimizing the elution parameters with BSA as model protein, this method was used for purifying the *M.tuberculosis* culture filtrate proteins in quantities sufficient for immunological and chemical characterization.

Cell Cultures

For cell cultures, peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood collected from five categories of subjects. New, untreated, direct smear positive, active pulmonary tuberculosis patients (category I), pulmonary tuberculosis patients after two to three months of chemotherapy (category II), BCG vaccinated healthy professional contacts (category III), BCG vaccinated healthy subjects, without any previous history of known contact with tuberculosis patients (category IV) and non-vaccinated healthy subjects without any previous history of tuberculosis infection or known contact with tuberculosis patients (category V). PBMCs were separated by Ficoll-Hypaque density gradient centrifugation method. Cells were collected at the interphase layer, washed twice, and standardized to $2\times 10^6$cells\ml in RPMI 1640 medium containing 20% heat inactivated AB+ human serum supplemented with 2mM L-glutamine and 100 IU/ml each of Penicillin and Steptomycin. 100µl of suspension containing $2\times10^5$ cells were cultured in 96 well flat bottom plates along with 100µl of stimulating protein antigen. On the basis of initial dose-response studies, antigens were added in the following concentrations: *M.tuberculosis* culture supernatant - 2µg/ml ;

purified culture filtrate proteins - 1.2 μg/ml; PHA-1% was used as a +ve control for cell reactivity and viability. After 5 days of incubation at 37°C in humidified 5% $CO_2$ enriched air, the cultures were pulsed with 1μCi of tritiated thymidine (Amersham International UK), incubated for a further 18 h at 37°C and harvested on to glass fiber filters. Incorporated radioactivity was measured in a liquid scintillation beta counter. Proliferative responses were obtained in counts per minute (CPM). Results are expressed as Stimulation Index (S.I.), which is calculated as ratio, mean CPM of antigen containing cultures and mean CPM of control cultures. S.I. value of ≥2 is considered as significant.



Cytokine Assays

Concentration of cytokines IFN-γ and IL-4 in cell culture supernatants were quantitated by commercially available enzyme-linked immunosorbent assay kits (Genzyme, USA). Lymphocyte culture supernatants were harvested from parallel cultures, 72 hr after stimulation with antigens, for the quantification of IL-4 & IFN-γ.

Viability Assay

To check the removal of toxic substances by equilibration in buffer, same amount of the concentrated protein (collected from gel strips equilibrated for different periods of time) were added in human PBMC's culture and incubated for 3 days at 37°C and 5% $CO_2$. After which, percentage of live cells were determined by MTT assay (10). 20 μl of 5 mg\ml stock solution of MTT was added per well of cell suspension. Incubated at 37°C & 5% $CO_2$ for 3 hrs.

Medium was discarded from the wells and 150 µl of DMSO was added per well to dissolve the formazan crystals. Optical density of the dissolved material was measured at 540 nm yielding absorbance as a function of concentration of converted dye, which directly correlates to the number of metabolically active cells in the culture (11,12,13). Quantification of cell number was done by preparing a standard curve, with known number of cells per well and by comparing the absorbance values of sample with standard. Cell number and viability was also determined by trypan blue dye exclusion method using a hemocytometer in parallel culture.



### Statistical Analysis

All data are means of results from triplicate wells. $P \leq 0.05$ was considered as significant.

## RESULTS

### Equilibration of the Gel Before Elution

Polyacrylamide gels, after termination of electrophoretic run were equilibrated in elution buffer for various periods of time and then electroeluted for 30 minutes. Optimization experiments were done using BSA as model protein. Before using BSA as the model protein, its different concentrations were added in cell culture to check if as such it has any toxic effect on cell growth. It was found that even very high concentration of 15-20 µg/ml has no untoward effect on cell proliferation.

Effective removal of toxic components from eluted protein was determined by a sensitive method of quantitating the live cells in cell cultures, where same

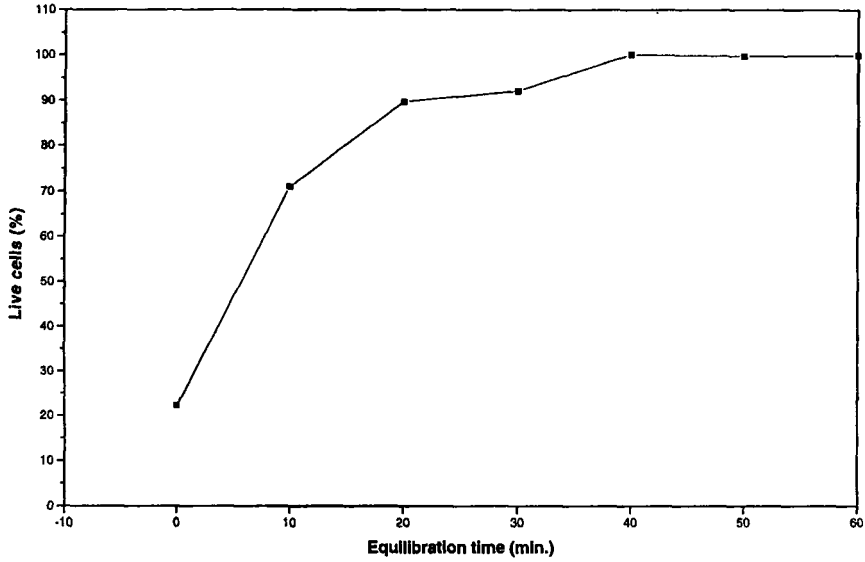


**Figure 1** Effect of equilibration time for which gel strips were soaked in elution buffer to remove the toxic substances, on peripheral blood mononuclear cells in cell culture when the protein eluted from such gel strips was added in cell culture. Cells were incubated with protein for 3 days and after that %age of live cells in culture were determined by MTT assay.



amount of eluted proteins were added. As shown in Figure 1, percentage of live cells increased steadily until 20 min. of equilibration time. 100% cells were live in the cultures where proteins from gels equilibrated for 40 minutes or more were added, indicating that an effective removal of toxic products had occured. Equilibration period of above 40 minute was found to reduce the final protein yield, and a 40 minute equilibration period was therefore chosen for the rest of the study.

Influence of Elution Conditions (Volt & Elution time)

Proteins from individual bands from the SDS gel were eluted in horizontal

gel electrophoresis apparatus. Elution conditions were standardized using BSA as model protein. Elution of BSA protein from gel was carried out at different volts (20V to 140V) keeping the elution time constant at 30 minutes. As can be seen in Figure 2, total amount of protein eluted in micrograms was almost same from 20V to 60V. On further increasing the voltage, amount of protein recovered decreased. Hence, elution at 40V was done for further studies. Keeping the 40V constant, elution was carried out for different times. Protein recovery was maximum, if elution was carried out for 40 minutes, beyond which it decreased. Hence electroelution at 40V for about 40 minutes was found to give maximum protein recovery. On further increasing the elution time or voltage, loss of protein due to adsorption on dialysis bag occurred. Significant amount of protein which adsorbed on dialysis bags was desorbed by reversal of current for 1 minute towards the end of main elution period.



As shown in Figure 3, different known concentrations of SDS were added in cell culture, incubated for 3 days at 37°C & 5% $CO_2$. Percentage of live cells in the culture were determined by MTT assay. It was found that SDS concentration as low as 0.0003% has inhibitory effect and only 63.3% cells were live.

After optimizing the elution conditions using BSA, mid log growth phase culture of *M.tuberculosis* H37Rv was harvested and culture filtrate proteins were purified. A large number of proteins were identified by the purification and elution method as standardized above. Out of these, eight low molecular weight proteins were characterized immunologically : CFP 1 (M.w-12kD, pI-4.6)., CFP 2 (M.w-11kD, pI-4.8)., CFP 3 (M.w-10kD, pI-3.5)., CFP 4 (M.w-14kD, pI-3.4)., CFP 5 (M.w-20kD, pI-3.7) CFP 6 (M.w-12kD, pI-4.0)., CFP 7

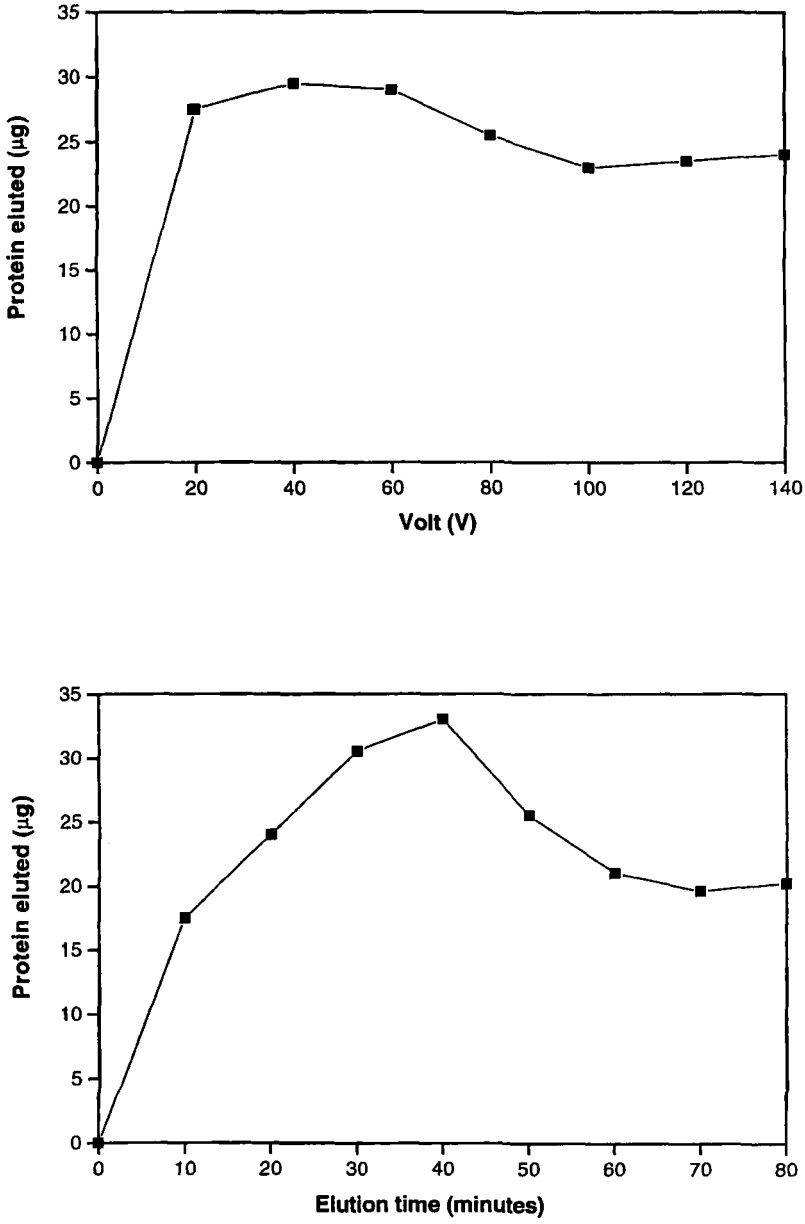


**Figure 2** Optimization of electrophoresis parameters : voltage & elution time, for maximum recovery of protein from gel strips. Amount of protein eluted was determined by BCA method.

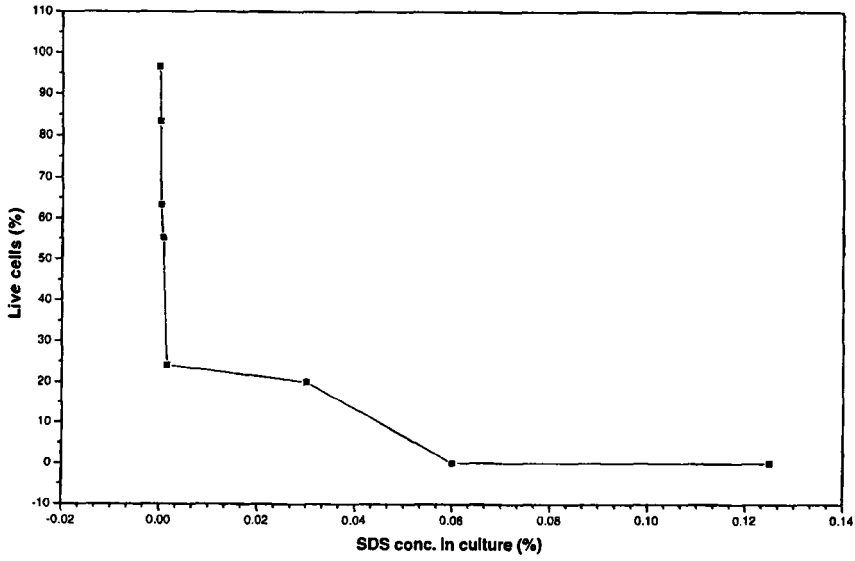


**Figure 3** Effect of SDS concentration in cell culture on %age of live cells after 3 days of incubation.



(M.w-10kD, pI-4.2-4.5) and CFP 8 (M.w-25kD, pI-5.8). As shown in Figure 4, dose response investigation of purified culture filtrate proteins (CFPs) was done. CFPs were used in different concentrations and proliferative responses determined. It was found that proliferative responses increased steadily until 1.25 μg/ml concentration in cell culture, beyond that they had inhibitory effect on cell proliferation. Hence 1.2 μg/ml concentration of CFPs were added in cell cultures for further immunological characterisation.

Lymphocyte proliferative responses of different groups of human subjects were evaluated on stimulation with purified eight culture filtrate proteins. Results are shown in Figure 5. It was observed that category I patients with active disease, before start of chemotherapy showed poor proliferative

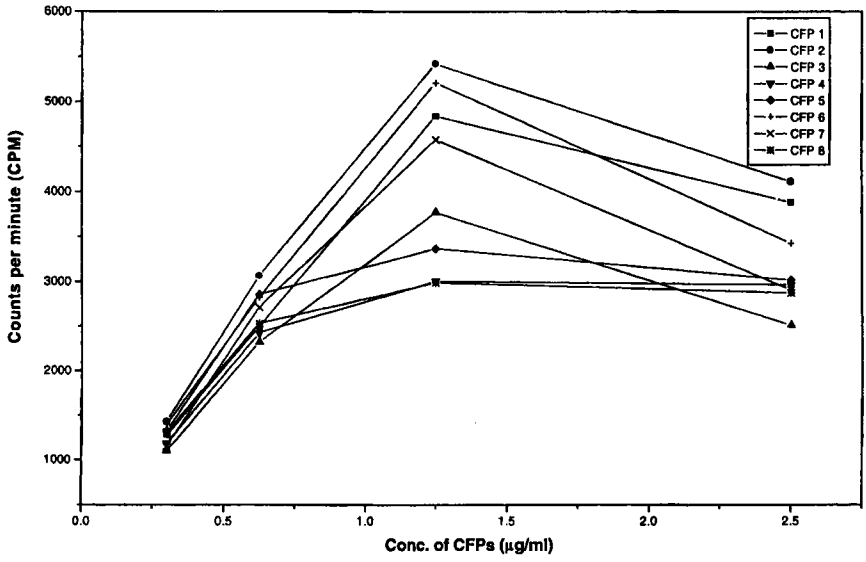


**Figure 4** Dose response curve for estimation of optimum concentration of CFPs to be added in the cell culture. Values shown are mean of response obtained from Cat.III subjects (n=3) to CFPs.



response to all the eight purified culture filtrate proteins studied, whereas patients of category II, after 2-3 months of chemotherapy showed significant proliferative response to all the proteins studied except CFP 8. Marked heterogeneity was observed in proliferative responses, when stimulated with CFPs, and the response was directed to almost all the proteins studied, although to different level. On stimulation with purified CFPs proliferative responses of BCG vaccinated healthy professional contacts were highest as compared to patients of both categories and controls.

Analysis of the proliferative response induced by CFPs in category II patients, recovering from the disease, revealed that mean proliferative

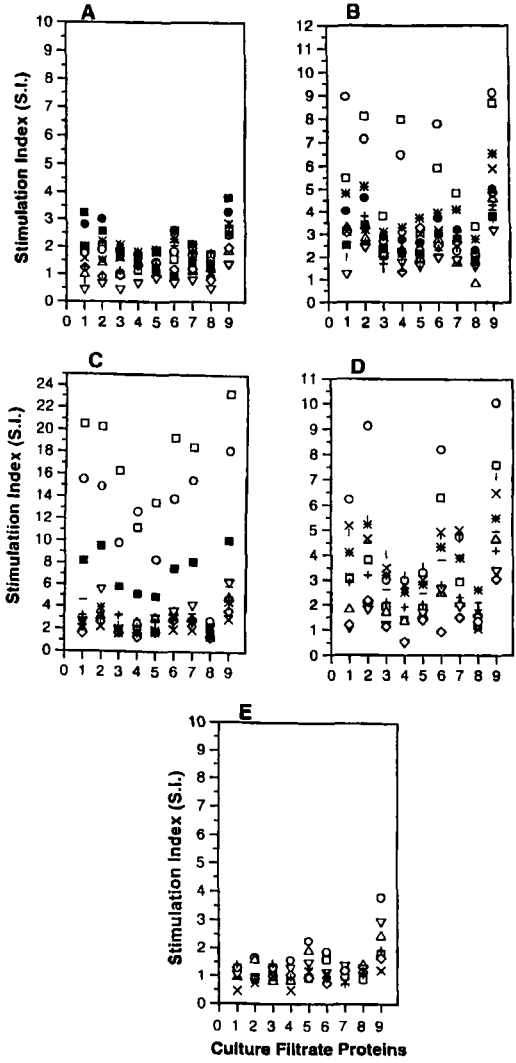


**Figure 5** Proliferative responses of PBMCs from human subjects of different categories on stimulation with CFPs & CS. Cat.I (**A**) is untreated, DS+ve pulmonary tuberculosis patients (n=12); Cat.II (**B**) is pulmonary tuberculosis patients after 2-3 months of chemotherapy (n=12); Cat III (**C**) is BCG vaccinated healthy professional contacts (n=11); Cat.IV (**D**) is BCG vaccinated healthy subjects without any known contact with tuberculosis patients (n=10); Cat.V (**E**) is non vaccinated healthy subjects without any known contact with tuberculosis patients (n=7). Each marker type represents an individual. CFP 9 is total CS proteins.

responses induced by CFP 1, 2 & 6 individually were similar to that induced by total CS proteins (P=0.05) and were higher than the response induced by other proteins studied. Difference was found to be statistically significant (P<0.05).

Cytokine Release Pattern

We evaluated cellular immune responses further by measuring the level of IFN-γ & IL-4 secreted in cell culture supernatants. IFN-γ release is expressed as mean values of triplicate analysis ± standard errors of the mean (SEM). As shown in Figure 6, mean concentration of IFN-γ secreted on stimulation with CFPs was much less in tuberculosis patients, before start of treatment (70 ± 20 pg/ml) as compared to category II patients, after 2-3 months of treatment (571 ± 101pg/ml). IL-4 concentration was less than 15 pg/ml in both categories of patients, hence it is not shown. IFN-γ concentrations were much higher in cell culture supernatant from healthy professional contacts stimulated with CFPs (1405 ± 113 pg/ml) as compared to tuberculosis patients and healthy controls, while IL-4 concentrations were below the detection limit. Mean IFN-γ release in non vaccinated healthy controls was very low (81±15 pg/ml) as compared to BCG vaccinated controls (494 ± 77 pg/ml).

On comparing the mean IFN-γ release by PBMCs from category III professional contacts exhibiting protective immunity, in response to different CFPs, it was observed that mean IFN-γ release in response to CFP 2 was highest followed by CFPs 1 & 6. Mean IFN-γ release on stimulation with CFP 2 was comparable to the amount released on stimulation with total

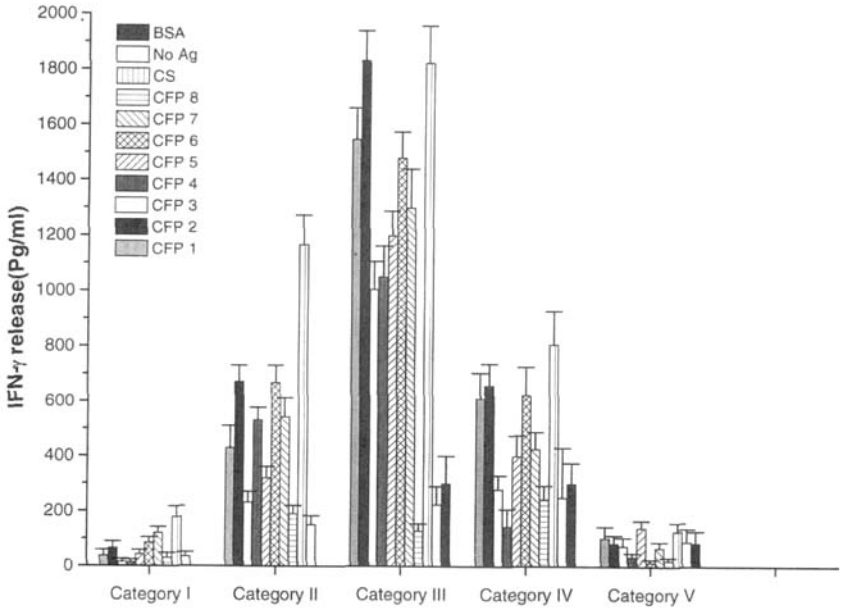


**Figure 6** Mean IFN-γ release and standard error of the mean (SEM) in cell culture supernatants of different categories of subjects on stimulation with CFPs, CS (+ve control), BSA (non specific protein) & no antigen (-ve control).



*M.tuberculosis* culture supernatant (1825 ± 132 pg/ml), which represents mixture of secreted proteins.

Our data demonstrates that CFP 2, CFP 6 & CFP 1 are immunologically active, in that they elicited a high release of IFN-γ from PBMCs isolated from healthy professional contacts, vaccinated controls and patients who were recovering from the disease.

## DISCUSSION

We describe a simple method by which, individual protein antigens can be

separated from complex protein mixtures by simultaneous electroelution of separated bands from polyacrylamide gels. Proteins were obtained in quantities sufficient for further immunological characterization and the procedure was used for identification of potential T-cell antigens.

Different strategies and methods have been used to identify T-cell antigens. Monoclonal antibodies (MoAbs) had been widely used as tools to select mycobacterial proteins for purification (14,15) or to isolate genes for cloning. In this approach, antigens were selected by their antibody inducing ability, hence relied on their immunogenicity in the few inbred strains of mice. Moreover, bacteria comprise hundreds of different proteins which could be potential T-cell antigens and this number by far exceeds the number of available antibodies. Further, dominant B-cell antigens and T-cell antigens need not necessarily be identical. T-cell Western Blot Method (3,4) has the disadvantage that membrane components interfere with the cell proliferation. To overcome this problem Gulle et al. (5) transferred the proteins separated by 2D gel electrophoresis to 400 holes of a master plate by electroelution. Each hole contained 100μl of buffer, hence the quantity of protein obtained was very less. Andersen et al. (6) designed a special device and divided the whole gel into 10 fractions, each containing several bands, and electroeluted the proteins from each fraction, but individual proteins could not be eluted out in their method.

In our study, culture filtrate proteins were purified by preparatory 2-D electrophoresis method. Single bands of low molecular weight proteins were cut out and proteins were electroeluted out by a simple and effective approach. Different proteins were eluted out simultaneously and no special device was

required. Only horizontal gel electrophoresis apparatus, which is commonly available in labs and 3 kD M.w. cutoff dialysis bags to retain the proteins of low molecular weight were required. Elution parameters were optimized for maximum possible recovery (50-60%). Proteins were eluted in 2mM phosphate buffer, which is completely non toxic, provides very effective elution and heat generated during elution is very minimal.

For using the proteins in cell culture, SDS should be removed completely as presence of even small amount of SDS causes loss of biological activity of isolated proteins (16) and is toxic to cell culture (17). Various procedures for removing SDS from proteins have been devised, but they are laborious and inefficient and do not generally remove the toxic effects of SDS completely (18,19,20). Concentration of SDS as low as 0.001% were found inhibitory in cell cultures (6). Previous attempts by Andersen et al. (1) to remove SDS from protein fractions were based on extensive pressure dialysis and repeated passage through detergent binding affinity columns, procedures which yield an SDS removal of varying efficacy and are extremely time consuming, when processing a large number of fractions. Andersen et al. (6) later on used electrophoretic dialysis in a specially designed device for removal of SDS. Proteins recovered by the method standardized by us could be directly used in immunological studies after concentration, as in this electrophoretic elution method, charged SDS molecules are also removed through the dialysis membrane by selective migration, in accordance with the previously reported high efficiency of electrophoretic dialysis in the removal of SDS (7). We have observed that the end product of the purification method has virtually unchanged stimulatory potency, if compared to the protein which is not

subjected to SDS-PAGE. Proteins were obtained in quantities sufficient for immunological and chemical characterization by this method.

The current research focus of many laboratories is, developing a vaccine alternative to BCG, as the currently used BCG vaccine is of questionable efficacy. T lymphocytes are of crucial importance for protection against the disease and a future subunit vaccine should possibly be composed of T cell antigens, which induce a protective immune response. In the present study, 2D electrophoresis method along with electroelution has been used to identify immunologically active secreted mycobacterial proteins. Proteins which elicited powerful immune response in PBMCs isolated from healthy professional contacts and patients after 2-3 months of treatment seems to be immunologically important and are being studied further for possible development of future immune interventions.



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