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STUDY OF CELL MEDIATED IMMUNE RESPONSE IN TUBERCULOSIS PATIENTS BY IFN-γ ELISPOT ASSAY

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Abstract

Tuberculosis (TB) is primarily a disease of the lung, and dissemination ofthe disease depends on productive infection of this critical organ. Upon infection with Mycobacterium tuberculosis (Mtb), the acquired cellularimmune response is slow to be induced and to be expressed withinthe lungs. IFN-γ is known to play a highly significant role in conferring protective immunity against Mycobacterium tuberculosis. The purpose of the study is to purify and quantify Ag 85A and expose the purified Ag 85A to blood PBMCs of patients and contacts. The IFN-γ levels in Ag 85A stimulated PBMCs of patients and contacts in vitro, were determined by using ELISPOT assay a modification of ELISA. Proteins secreted by mtb are known to be highly immunogenic as they are recognized early in infection. Ag85A is a major secretary protein of mtb. Ag85A expression was induced and was confirmed by SDS-PAGE by visual identification band in induced sample as compared to the uninduced sample.The Ag85A was subsequently purified, quantified and precipitated. The IFN-γ levels in Ag 85A stimulated PBMCs of patients and contacts and the PBMCs were isolated from 4 patients and their 4 contactsafter exposure to Ag85A.The IFN-γELISPOT assay which is the modification of ELISA was done and the individual wellswere visualised under a dissecting microscope and the number of spots in each well were analysed. Based on the higher cell mediated immune response to Ag 85A in patients with advanced disease infection, purified Ag 85A can be used as a sensitive marker for analysis of immune responses in tuberculosis detected by IFN-γELISPOT assay.

Keywords: *Ag85A, CellularImmune Response, IFN-γ, Tuberculosis (TB), T-cell*

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1. INTRODUCTION

Tuberculosis continues to have a detrimental impact on public health worldwide. Indeed, with approximately one-third of the world's population exposed, the 5% of those exposed who eventually develop disease translates into 8 million new cases per year [1].Cell mediated immune response plays an essential role in tuberculosis infection. Tcells are known to have a crucial role in acquired resistance against mycobacterialinfections [2]. Healthy, tuberculin positive volunteers seem to have protective immunity with an enhancement of Th1-cell proliferation response and IFN- γ production,whereas magnitude of such responses is lower in patients with tuberculosis. In animalmodels, a protective immune response against M. tuberculosis depends on theemergence of CD4+ T lymphocytes, which produce cytokines to activate macrophagesto eliminate intracellular mycobacteria [3].At present, the effectiveness of the BCG vaccine introduced in 1921 remains controversial because its protection levels are extremely variable in different population.The *Mycobacterium* cell contains a large number of different proteins; these proteins are classified in major groups with common features (Table 1).e.g. in physical and chemical properties, function, or localization as exemplified by lipoproteins, heat shock proteins, and cytoplasmic, membrane-bound, and actively

secreted proteins, respectively. IFN-γ is an essential protective cytokine which peaks the level when protective immunity is maximally expressed and is produced by CD4+ T lymphocytes, when these cells are in contact with previously infected macrophages (by live mycobacteria or primed with secreted mycobacterial antigens) [3].

1.1 Antigen 85 complexes

The group of secreted proteins is of great current interest in relation to the immune response to infection since these proteins are candidates of particular importance for development of protective immunity as well as clinical symptoms and complications of the disease[4].The proteins of the antigen 85 complex are major secretion products of *Mycobacterium tuberculosis* and *Mycobacterium bovis*BCG [5]. The Ag 85 complex (30-32 kDa Antigen) is a major fraction of the secreted proteins of *M.tuberculosis*and BCG culture filtrate which catalyses the transfer of mycolatesvia a mycolyltransferase exchange process, leading to the formation of Trehalosemonomycolate (TMM) and Trehalosedimycolate (TDM), also known as cord factor [4, 6]. The Ag 85 complex induces a strong T-cell proliferative response and IFN-γ secretion in BCG–vaccinated mice and in most healthy individuals exposed to M. tuberculosis [7].

1.2 Infection and Immunity

The major mechanisms of cell-mediated immunity include CD4 Th1-cell mediated activation of macrophages to destroy intracellular bacterial pathogens. The central role of IFN-γ in the control of tuberculosis has been clearly demonstrated by the susceptibility to mycobacterial infections in mice with a disrupted IFN-γ gene and in humans with mutations in genes involved in the IFN-γ and IL-12 pathways [8].

The $CD4^+$ T-cell–mediated immune response mounted by the majority of people exposed to *M. tuberculosis* thus controls the infection and later protects against reinfection. However, about 10% of individuals infected with *M. tuberculosis* follow a different clinical pattern: the disease progresses to chronic pulmonary tuberculosis or extrapulmonary tuberculosis. This progression may occur years after the primary infection. Both $CD4^+$and $CD8^+$T cells are important for successful immunity to tuberculosis and have redundant effector functions, such as cytolysis and release of potent anti-mycobacterial cytokines such as IFN-γ and TNF-α [8].

1.3 Immunological modification of ELISA to determine cytokine secretion

A modification of the ELISA assay called the ELISPOT assay allows the quantitative determination of the number of cells in a population that are producing antibodies specific for a given antigen or an antigen for which one has a specific antibody.

Subsequent development of the assay by addition of a suitable chromogenic substrate reveals the position of each antigen-producing cell as a point of colored spot. By counting the number of colored spots, it is possible to determine how many cytokine-secreting cells were present in the added cell suspension [2]. Cytokines secreted by Th1 subset which have shown to confer protective immunity against Mtb have been detected by the ELISA and/or ELISPOT assay. The ELISPOT assay which is more sensitive of the two detects secreted cytokine molecules in the immediate vicinity of the cell from which they are derived, while still at a relatively high concentration; each spot in the read-out represents a 'footprint' of the original cytokine-producing cell [9]. Quantitation of these IFN-*γ* spot-forming cells (SFCs) by this technique is highly sensitive; for $CD4^+$ cells. The ELISPOT assay is an order of magnitude more sensitive than other assays like for example the ^{51}Cr-release cytotoxicity assay for detecting CTLs[10].

2. METHODOLOGY

2.1 Patients and Controls

Whole blood was obtained from4 patients in whom pulmonary TB was confirmed by clinical findings, radiography, and sputum smear. Patients with a history of HIV infection, corticosteroid treatment, and autoimmune diseases were

excluded. All Patients were hospitalized at the Sir SayajiraoGaekwad Medical College Hospital, Vadodara – INDIA. Chest X-ray was obtained from each patient at the time of study, and patients were graded for the extent of TB infection and classified as either having active minimal TB, including patients with minimal lesions with slight to moderate density but without demonstrable cavitation, or patients with active advanced TB, including patients with marked cavity lesions in the lungs. The number IFN-γ secreting cells were much higher for PHA which was taken as positive control of the assay and PHA as a strong mitogen and the number of IFN-γ secreting cells in Ag85A exposed wells is 2 fold higher as compared to unexposed controls in both patients and controls suggesting the potential of Ag85A as a vaccine for uninfected individuals or as a diagnostic marker for patients infected by Mtb and suffering from tuberculosis [11].

2.2 Purification of Ag85A

2.2.1 Antigens

Ag85A was purified from culture filtrate of *M. tuberculosis*.Induced Ag85A was preparedat the Department of Microbiology and Biotechnology, The Maharaja SayajiraoGaekwad University, Vadodara from uninduced Ag 85A control and IPTG (1mM). The molecular weight of this protein complex was found by SDSPAGE to be 32 KDa as shown in Fig. 1.Thepellet was resuspended in SDS sample- buffer for protein purification by Nickel affinity chromatography.Nickel slurry was purified using sequential buffer systems including Equilibrium buffer (50 mM TRIS pH 8.5, 300 mMNaCl, 10 mM BME, Glycerol 10%, and 20 mM Imidazole), Wash buffer (50 mM TRIS pH 8.5, 1 M NaCl, 10 mM BME, Glycerol 10%, and 20 mM Imidazole) and elution buffer (50 mM TRIS pH 8.5, 100 mMNaCl, 5 mM BME, Glycerol 10%, and 50 mM Imidazole). The above fractions were analysed for the presence of Ag85A by SDS-PAGE.The molecular weight of this protein complex was foundto be 30-32 KDa by SDS-PAGE as shown in Fig. 2.

2.2.2 Quantification of Protein Estimationby Bradford method

The protein estimation of the purified sample was done by Nickel affinity chromatography was obtained as 1.5 µg/µl at 0.070 O.D. at 595 nm by Bradford method of protein estimation using standard BSA curve is shown in Fig.3.

2.2.3Methanol-chloroform precipitation of purified Ag85A

The purified sample by Ni-affinity was Ag85A was precipitated in presence of methanol followed by chloroform. The upper phase was discarded and to it ethanol was added and the obtained pellet was resuspended in DMSO.

2.3 Exposure of purified Ag85A to blood PBMCs of patients and contacts

Heparinized Whole Blood samples were collected after informing the patients/individuals about the purpose of the study and getting necessary approvals and written consent of patients admitted to Sir SayajiraoGaekwad Medical College Hospital, Baroda. After haematological analysis by cell counter, it was washed, plasma was removed and cells were diluted in RPMI-1640 medium with 10% FCS and adjusted to one million leukocytes /ml.The buffy coat containing the PBMCs was aspirated and collected in fresh labeled conical tubes which were diluted using PBS and decanted after a spin and the pellet was resuspended in RPMI containing 10% FCS. Blood PBMCs were quantified using Neubar`s chamber after observing under inverted microscope. To achieve antigen specific stimulation of T lymphocytes, Ag85A was added in the wells at a concentration of 5µg/ml.Phytohaemagglutinin was used a positive control as it is known to be a potent mitogen and leads to proliferation of blood PBMCs in-vitro.

2.4 IFN-γ ELISPOT assay

For aseptic procedures Functional Grade purified antibody was diluted in sterile ELISPOT Coating Buffer Coat ELISPOT plate with 100µl/well of capture antibody solution andincubated at 4°C overnight and the coating antibody was decanted from plate after 2 ELISPOT buffer washes. The plates were blocked plate with complete RPMI-1640 at

room temperature for 1 hr followed by decanting.The mitogen, antigen or controls were aliquoted to appropriate wells. Aliquot cells at a density of 1×10^5cells/ml and volume of 100µl/wellwere incubated at 37^0C, 5% CO_2 humidified incubator for 24-48 hours.

For Non-Aseptic procedures the cells and medium were decanted from plates followed by 3 washes of ELISPOT Wash Buffer.The biotinylated detection antibodywas diluted in assay diluent and added to plate micro wells and incubated at room temperature for 2 hours. The antibody solution was decanted and washed 4 times with ELISPOT Wash Buffer. TheAvidin-HRP reagent was diluted in Assay Diluent and 100µl/well of AV-HRP was added and incubated at room temperature for 45 minutes. The AV-HRP solution was decanted and plate was washed 3 times with ELISPOT Wash Buffer, and then 2 times with 1X PBS (no Tween-20). The AEC substrate solution100µl /well were added to develop the spots after room temperature incubation for 10-60 minutes. The substrate reaction was stopped by washing wells 3 times with distilled water and the plate was air dried. The individual spots in the wells were visualized under a dissecting microscope, photographed with an Olympus digital camera and spots in the images were analyzed using Image J software.

3. RESULTS

3.1 Cellular Immune Response

Lymphocyte cell populations were stimulated in vitro with Ag85A complex, control and PHA as spot forming cells (SFC) as shown in the Fig. 4A and 4B. Cultures without stimulus were considered as negative control for cell proliferation. Proliferative responses were evaluated by titrated thymidine uptake of cells in the well of in vitro stimulation with Ag85A, Control and PHA. While a statistically significant difference was observed between healthytuberculin-positive and negative volunteers regarding the proliferative response to Ag85A, no difference was recorded between thegroups of subjects in response to PHA and RPMI or in the cultures without stimulus.

3.21 IFN-γ ELISPOT assay

The cell proliferation response to Ag 85 complex is significantly different between tuberculin positive patients and tuberculin negative subjects.Production of IFN-γ in response to Ag85A complex was determined by ELISPOT Assay as shown in the figure Firstly the number of spot forming cells (SFC) of IFN-γ is higher in patients as compared to uninfected contacts explaining the $CD4^+$ response in infected individuals. Secondly the number IFN-γ secreting cells are much higher for PHA which was taken as positive control of the assay and PHA as a strong mitogen. Thirdly the number of IFN-γ secreting cells in Ag85A exposed wells is 2 fold higher as compared to unexposed controls in both patients and controls suggesting the potential of Ag85A as a vaccine for uninfected individuals or as a diagnostic marker for patients infected by Mtb and suffering from tuberculosis. The spot forming cells (SFC) were visualised under a dissecting microscope and then were photographed using 3.1 Megapixel Olympus digital camera and the number of spots in each well was analysed using ImageJ software as shown in the Fig. 5A and 5B.

4. DISCUSSIONS

TB causes a staggering burden of mortality worldwide, killing an estimated 1.9 million persons annually. Effective treatment of tuberculosis in developing countries is hampered by the cost of anti-tuberculosis drugs, inability to ensure completion of therapy, and rising drug resistant rates. Vaccination is the most cost-effective strategy to control and eventual elimination of tuberculosis. The current BCG vaccine provides some degree of protection against the most severe manifestations of childhood tuberculosis. However, protection is incomplete, and BCG vaccine does not reduce TB rates in adults.In the present study we analyse the immune response of tb patients with the respective house hold contacts as per the above data giving the number of spots for the corresponding wells the following conclusions can be drawn.

Firstly the spot forming cells (SFC) of IFN-γ is higher in patients as compared to uninfected contacts explaining the $CD4^+$ response in infected individuals. Secondly the number IFN-γ secreting cells are much higher for PHA which was taken as positive control of the assay and PHA as a strong mitogen. Thirdly the number of IFN-γ secreting cells in Ag85A exposed wells is 2 fold higher as compared to unexposed controls in both patients and controls suggesting the potential of Ag85A as a vaccine for uninfected individuals or as a diagnostic marker for patients infected by Mtb and suffering from tuberculosis. This correlates with many studies that have shown the importance of IFN-γ secreting $CD4^+$ cells, and the use of Ag85A to design effective Mtb vaccines.

Patients with an active minimal pulmonary TB infection respond powerfully to secrete antigens of *M. tuberculosis*. Since these antigens are recognized by IFN-γ producing Th-1like cells stimulated during the first phase of infection. Since a basic principle for selecting novel antigen candidates for designing a TB subunit vaccine is based on their ability to induce a protective Th1 response ,this study also confirmed the value of Ag85A as potential vaccine candidate and diagnostic tool based upon specific T cell responses measured by IFN-γ production in patients studied.

In fact, MVA85A a recombinant modified Vaccinia virus Ankara expressing Ag85A, is the first candidate TB subunit vaccine to enter human trials since BCG was first introduced over 80 years ago. Also Ag85A is able to differentiate pulmonary, extra-pulmonary and tuberculosis patients undergoing chemotherapy from healthy individuals by IFN-γ production.

The last decade has witnessed significant advances in mycobacterial genomics and cellular research which have resulted in the development of two new blood tests, ELISpot assay (TSPOT.TB, Oxford Immunotec, Oxford, UK) and ELISA (QuantiFERON-TB Gold In-Tube, Cellestis, Carnegie, Australia). These tests, which are collectively known as interferon gamma release assays (IGRAs), detect latent tuberculosis infection (LTBI) by measuring IFN-γ release in response to antigens present in Mycobacterium tuberculosis, but not bacilleCalmette-Guerin (BCG) vaccine and most non-tuberculous mycobacteria. IGRAs are more specific than the tuberculin skin test (TST) as they are not confounded by previous BCG vaccination. In addition, with active tuberculosis (TB) as a surrogate for LTBI, it appears that the ELISA has a similar sensitivity to the TST, whereas the ELISpot is more sensitive.

5. CONCLUSIONS

The main aim of the study was to expose purified Ag85A to blood PBMCs of patients and analyze IFN-γ release after incubation using the ELISpot assay.Ag85A expression was induced by IPTG (1mM) induction method and the induction was confirmed by 10% SDS-PAGE by visual identification 32kDa band in INDUCED sample as compared to the uninduced sample.The Ag85A was subsequently purified by Nickel affinity chromatography, quantified by Bradford method of protein estimation, precipitated by Methanol-chloroform precipitation and solubilised in DMSO to appropriate concentration.The PBMCs were isolated from 4 patients and their 4 contacts and were exposed to Ag85A; unexposed wells were taken as negative controls while PHA was used as a positive control.The IFN-γ ELISPOT assay was done using kit, the individual wells, were visualised under a dissecting microscope and then were photographed using 3.1 Megapixel Olympus digital camera and the number of spots in each well was analysed using ImageJ software.

6. ACKNOWLEDGEMENTS

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Table:1 Classification of Proteins of M. Tuberculosis and M. Bovis BCG in major groups

Group and mol mass (kDa)	Designation or homolog	MAb showing reaction	CIE antigen no.
Heat shock proteins			
71	DnaK	L7, 51A	63
65	GroEL	TB78	82
12	GroES	SA12	
Lipoproteins			
38	PhoS	TB71, TB72	78
19		HYT6, TB23	
Secreted proteins			
41	MPT32		
31.5	MPT45	HYT27	85C
31	MPT44	HYT27	85A
30	MPT59	HYT27	85B
27	MPT51	HBT4	
26	MPT64	L24.b4	
23	MPB70		70
18	MPT63		
15	MPT53		
Enzymes			
40	EC 1.4.1.1	HBT10	
23	SOD[c]	D2D	62

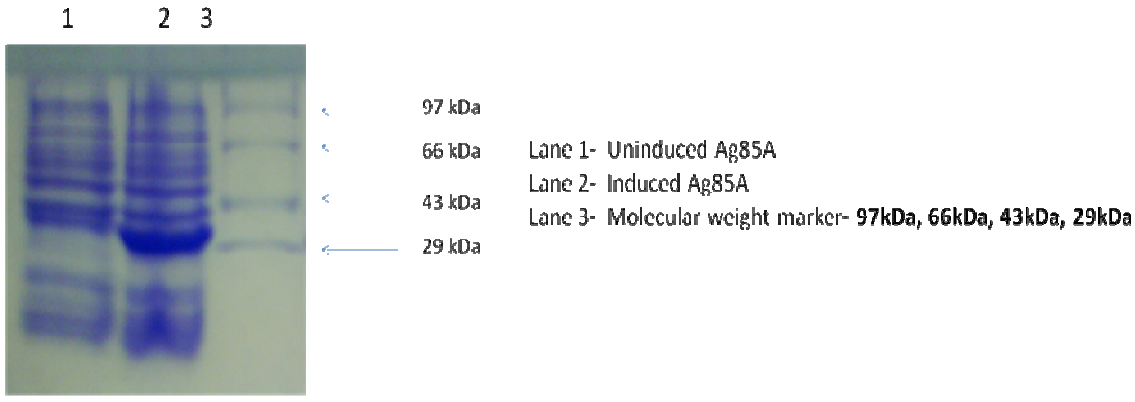

Figure 1: - IPTG induction of Ag85A. The cell extract containing IPTG-induced Ag85A was resolved by 10% SDS-PAGE and stained by Coomassie. The uninduced cell-extract was used as control. The expected band of 32kDa corresponding to Ag85A was visualized.

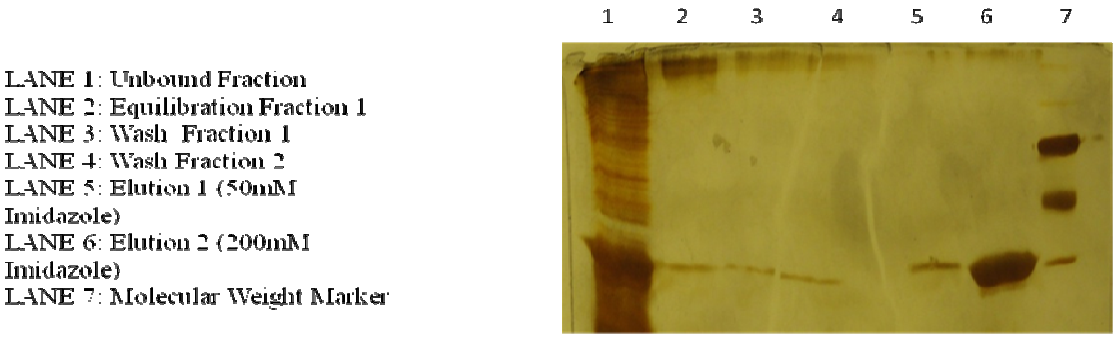

Figure 2: -Purification by Nickel Affinity chromatography. The induced sample was spin down concentrated and purified Nickel Affinity chromatography. The samples were analysed by 10% SDS-PAGE. The expected band of 32kDa corresponding to Ag85A was seen in the Eluted fraction 2.

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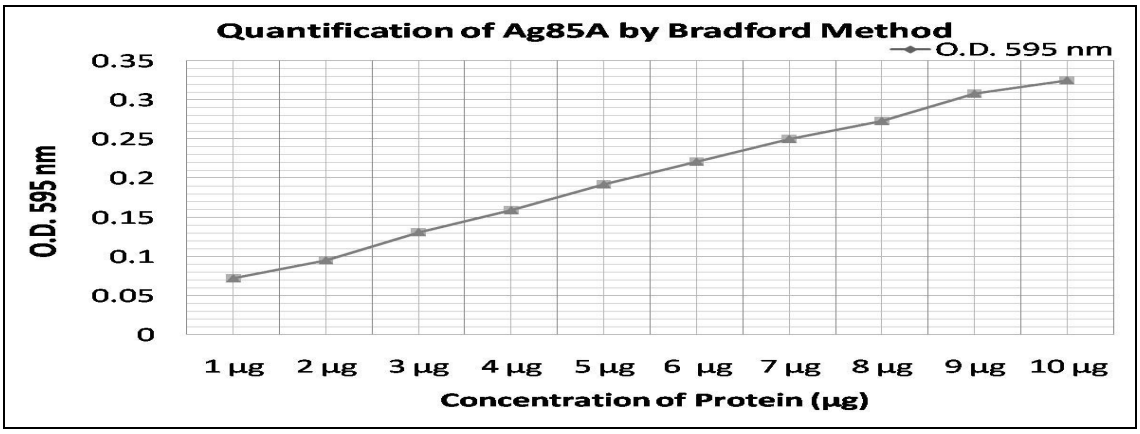

Figure 3: -Quantification of Ag85A by Bradford method of protein estimation using standard BSA curve.

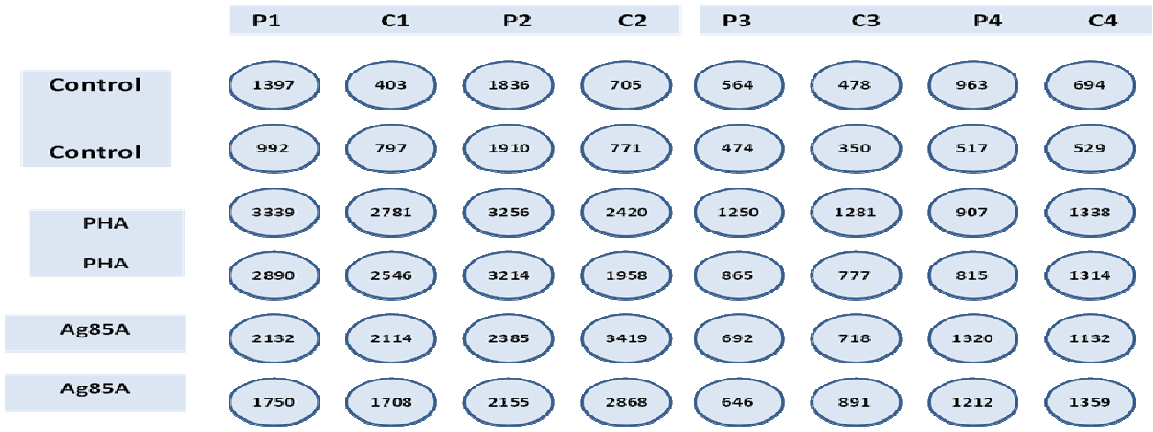

Figure 4A: -Analysis of number of spot forming cells (SFU) in the individual wells of 96 well micro well plate were visualised under a dissecting microscope.

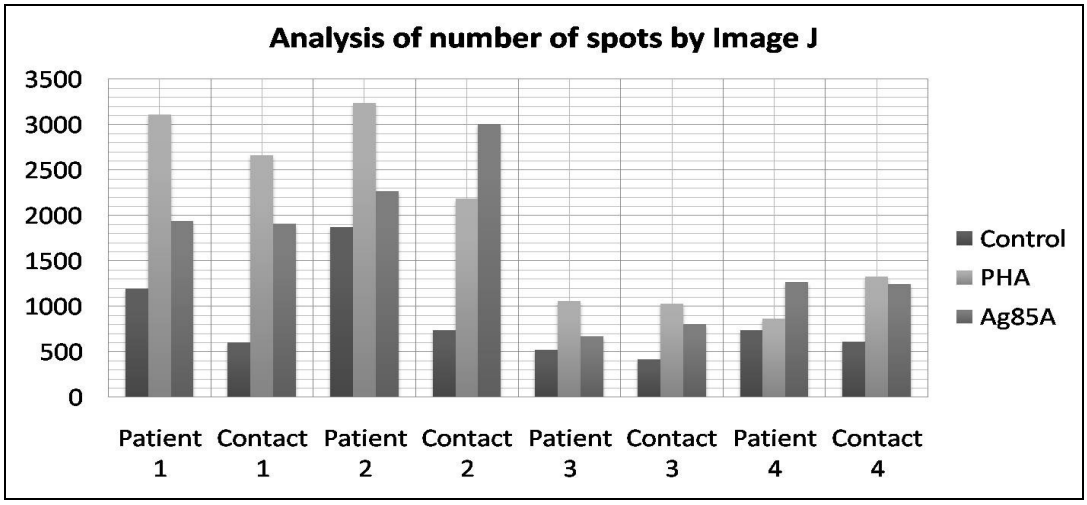

Figure 4B: - Graphical Representation of spot forming cells (SFU) for the determination of Cellular Immune response comparison of Ag85A, PHA and Control for IFN-γ ELISPOT assay of TB patients.

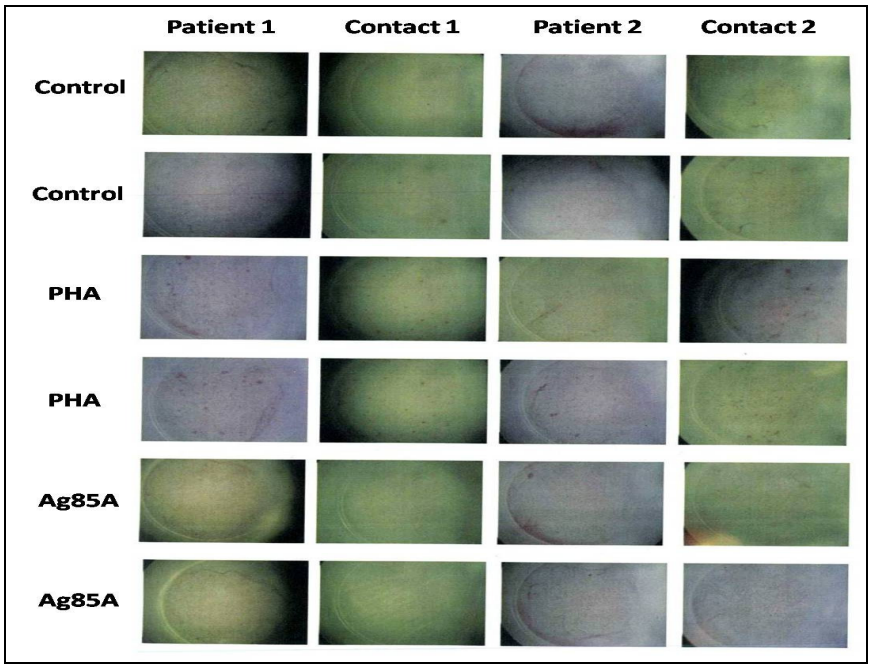

Figure 5A: - Photographic images of SFU in IFN-γ ELISPOT assay of individual wells visualised under a dissecting microscope and were photographed using 3.1 Megapixel Olympus digital camera and the number of spots in each well for patients 1 and 2 with their contacts was analysed for AG85A, Control and PHA.

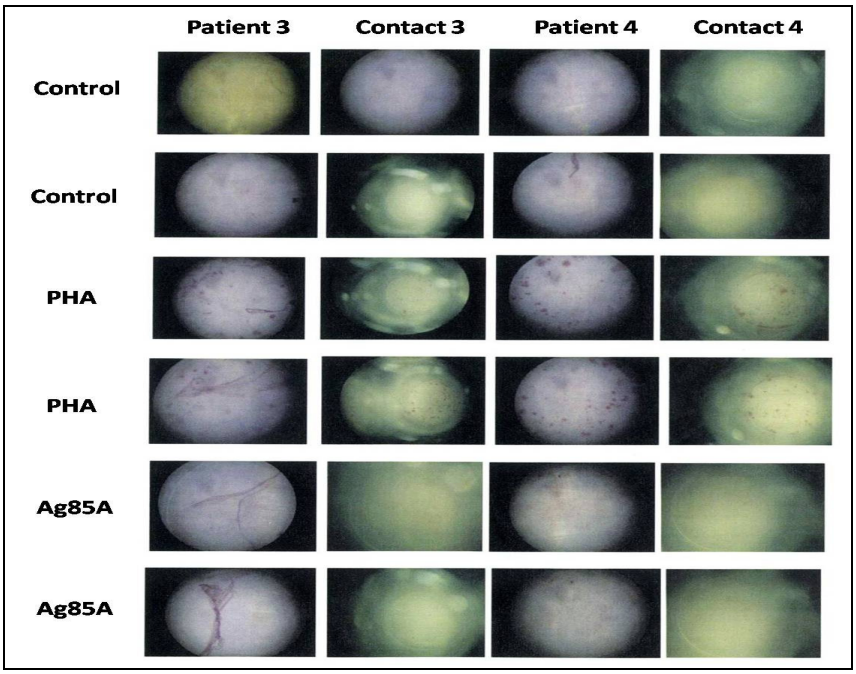

Figure 5A: - Photographic image of SFU in IFN-γ ELISPOT assay of individual wells visualised under a dissecting microscope and were photographed using 3.1 Megapixel Olympus digital camera and the number of spots in each well for patients 3 and 4 with their contacts was analysed for AG85A, Control and PHA.

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