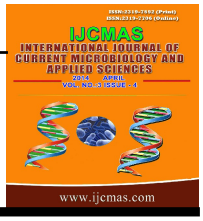
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 4 (2014) pp. 85-95**
http://www.ijcmas.com



Original Research Article

Evaluation of protective immune expression in response to *Mycobacterium tuberculosis* culture filtrate antigens

Sherif Moussa Hussseiny[1]*, Faten Sayed Bayoumi[2] and Ahmed Mohamed Ali[3]

[1]Botany department, Faculty of Women For Art, Science and Education, Ain Shams University, Egypt
[2]Immunogenetics Department, National Research Centre, Dokki, Giza, Egypt
[3]ahmedelymani22@gmail.com
Corresponding author

K e y w o r d s

Mycobacterium tuberculosis, protein, protective response and antigens

A B S T R A C T

Tuberculosis (TB) caused by the intracellular bacterium *Mycobacterium tuberculosis* (MTB), remains a major worldwide health problem where it cause approximately three million deaths annually. New vaccines with better protection rates than Bacillus Calmette–Guérin (BCG) in current immunization program is urgently needed for TB eradication. Secreted proteins, described as culture filtrate proteins (CFP's), are the main targets of the T-cell response in TB. In current study, we used culture filtrate protein fractions collected at different time points from growth phases of MTB culture. Protective immune response was evaluated by measuring mRNA expression of genes encoding Interlukene 12 (IL12), Interferon Gamma (INF□), Tumor Necrosis factor (TNF) and inducible Nitric Oxide Synthase (iONS) in vaccinated mice after using culture filtrate protein. Fraction that secreted at late growth phase (fraction 3) showed highly significant cytokine and iONS expression. Vaccinated mice were aerogenically challenged with approximately 100 *M. tuberculosis* bacilli 30 days after vaccination with CFP fractions. Results showed that fraction 3 provided best protective immune response and best protection against MTB, whereas number of viable MTB significantly reduced over the time in lunge of mice challenged with virulent MTB. Partial characterization of CFP fractions exhibited high concentration of MTB antigens (Ag 85B, 45kDa, CFP-10 and ESAT-6) especially those secreted at late growth phase of MTB culture, this explains high protective immune response provided by that fraction. Thus we concluded that this fractions may be used in near future for development of effective vaccination strategies for improving efficacy of currently available TB vaccine or synergy with the currently available drugs.

Introduction

Tuberculosis (TB) caused by the intracellular bacterium *Mycobacterium tuberculosis* (MTB) remains a major worldwide health problem responsible for approximately three million deaths annually (Boesen *et al.*,1995). Even after

successful control of the primary TB infection, some bacilli remains in a non-replicating or slowly replicating dormant state for the rest of the individual life. This infectious state, termed latent TB infection,it is clinically asymptomatic, and most active TB cases arise as a result of reactivation of dormant bacilli(Parrish *et al*., 1998). Up to one third of the world's population is estimated to carry latent *M. tuberculosis* infection, and hundreds of millions of TB reactivations are anticipated specifically in areas of low or moderate endemicity, where most cases of active TB result from reactivation of latent infection (Parrish *et al*.,1998). The level of protection conferred by only available TB vaccine, Bacillus Calmette Guerin (BCG) is very variable and differs according to the form of pulmonary TB (Dietrich *et al*., 2006). For more than 80 years, no new TB vaccine has successfully been developed (Gupta *et al*., 2007). With TB eradication on the horizon, new vaccines with better protection rates than BCG or improvement in current immunization program is urgently needed. Vaccine candidates currently in clinical trials include improved recombinant BCG vaccines, virus-based recombinant vaccines, and subunit vaccines comprised of dominant secreted antigens (Martin, 2007).

Secreted proteins, regularly described as culture filtrate proteins (CFP's), are the main targets of the T-cell response in TB (Fonseca *et al*., 2009). In recent years, research has been focused on antigens released by live MTB in culture medium; pools of such extracellular antigens have been tested in several laboratories as subunit vaccines with substantial levels of protection in animal models (Ravn *et al*.,1999). The demonstration that non-living vaccines based on secreted proteins could effectively protect against subsequent MTB infection in animal models, has led to the initiation of extensive antigen discovery programs which aimed to identify crucial antigenic molecules in culture filtrates (Gupta *et al*., 2007). Recent data demonstrates that some antigen are expressed in culture filtrates of MTB but absent in *Mycobacterium bovis* BCG and most environmental mycobacterial species investigated (Sorensen *et al*., 1995). These findings have thus increased our interest in these molecules both as potential vaccine and immunotherapy candidates and a novel specific diagnostic reagent. Despite its widespread use, BCG has failed to reduce global burden of TB, hence newer vaccination strategies or focus on new molecules alternate to BCG are needed (Husain *et al*., 2011) In our study, culture filtrates of MTB bacilli isolated at different growth points of MTB culture were used to evaluate protective immune response.

A number of research groups have identified vaccine potential of CFP's. The ESAT-6 antigen purified from strongly stimulatory, low molecular- mass fraction of culture filtrate has attracted considerable interest in recent years, as it is recognized early during infection in several species, including mice (Andersen, 1994). In addition, secreted antigens such as the Ag85A or 85B antigens, and Mtb72F have proven to be promising candidates for BCG-boosting vaccines in mice, guinea pigs, and nonhuman primates (Fonseca *et al*., 2009). Another study done by Lindblad et al. showed that immunization with culture filtrate antigens in the presence of different adjuvant provided protection in mice challenged with MTB, and protection was mediated by gamma interferon (γ-IFN)-Producing CD4 cells(Lindblad *et al*., 2007). The

objective of our present study was to identify and evaluate protective immune mRNA expression in response to secretary antigens isolated at different time periods from MTB culture using BALB/c mice model. In this study, we have also tried to partially characterize the CFP's of MTB culture using an antibody detection assay by ELISA in order to identify antigenic population secreted in different growth phases of MTB culture.

Materials and Methods

Biosafety

The following procedures are conducted at Tuberculosis reference lab , ministry of health central labs, Cairo, Egypt and variations may exist based on Biosafety Committee's recommendations. Common personnel protective equipment will consist of a Tyvek suit, bouffant cap, booties, N95 respirator, eye protection, sleeves, and a double pair of nitrile gloves. Work involving *M. tuberculosis* cultures and/or manipulations with open *M. tuberculosis* containers is performed in the type A2 or B2 biological safety cabinet. Plastic-covered absorbent paper is placed on the working surface. All materials/supplies to be disposed or removed from the facility must be placed in two biohazard bags and decontaminated by autoclaving. Work surfaces and equipment utilized within the cabinet must be disinfected after each work session with 1% Amphyl (tuberculocidal, bactericidal, fungicidal, and virucidal agent). *M. tuberculosis* cultures must be placed under double containment for transportation to larger equipment located outside of the biological safety cabinet such as freezers, incubators, centrifuges, and refrigerators. Centrifugation is conducted with enclosed safety cups and O-ring screw top tubes. For further analysis outside the biosafty laboratory, cell-free extracts are filtered through a 0.2 μm filter or microorganisms heat killed at 95 °C for 15 min.2 Samples are plated to verify the absence of colony-forming units prior to removal from containment.

Animals

This study was performed with viral pathogen-free BALB/c mice were obtained Faculty of veterinary medicine, Banha University, Egypt. Female mice, 12 weeks old, were used in all experiments. The animals were kept under suitable conventional housing conditions. Two days before the challenge with virulent *M. tuberclulosis*, the mice were transferred to biohazard facilities and housed in cages contained within a laminar flow safety enclosure.

Organism

M. tuberclulosis H37Rv was obtained from Tuberculosis reference lab , ministry of health central labs, Cairo, Egypt and grown at 37°C in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose.

BCG was obtained as a freeze-dried vaccine from Egyptian Company for Vaccine and Serum, Giza, Egypt and was rehydrated with sterile saline followed by a brief treatment in a sonication bath to ensure a disperse suspension.

Collection and isolation of culture filtrate proteins

Culture filtrate protein was produced as described previously (Andersen et al., 1991). Briefly, M. tuberculosis (8 x 10^6 CFU/ml) was grown in enriched Sauton medium at 37°C on an orbital

shaker. Fractions were collected at different time points (6, 14 and 28 days) from growth phases of MTB culture. For isolation of culture filtrate, cultures were centrifuged (12,000 rpm for 15 min) and supernatants were filtered with a sterile filter and concentrated 100-fold on an Amicon YM 3 membrane (Amicon, Danvers, Mass). Protein content was determined by the bicinchoninic acid assay (Pierce Chemical, Rockford, IL) using a bovine serum albumin (BSA) standard . The collected culture filtrate antigens were divided into three groups. Fraction 1 (collected after 6 days), Fraction 2 (collected after 14 days) and Fraction 3 (collected after 28 days) ,they were stored at 4°C until use.

Methods

Vaccination and challenge test

Immunization was done by injection adult mice with each culture filtrate fraction (fractions 1, 2, 3). 24 animals were used per fraction. 100 µg/ml of protein fraction was emulsified in 0.2 ml Freund's adjuvant (Sigma, St Louis, MO) and then injected subcutaneously into mice. Each animal received three immunizations, each 3 weeks apart. Negative control mice were injected with ovalbumin emulsified in the adjuvant as above, positive control mice were injected with BCG at 10^6 bacilli. Mice were aerogenically challenged with approximately 100 M. tuberculosis bacilli 30 days after vaccination using an aerosol generation device (Glas-Col, TerreHaute, IN).

Real-time RT-PCR analysis of cytokines expression in lung homogenates

Three lungs, right or left, from the same number of animals in each group were used to isolate RNA at 40 day after challenge. Each lung was homogenized in 1 ml UltraSpec (Biotecx, Houston, TX, USA) and frozen rapidly at –80°C. Total RNA was isolated according to the manufacturer's instructions. Reverse-transcribe 1 µg using an Omniscript RT kit (Qiagen, Valencia, CA, USA).The quality and quantity of RNA were evaluated through spectrophotometry (260/280 nm). Reverse transcription (RT) of the mRNA was performed using 5µg RNA, oligo (dT), and the Omniscript kit (Qiagen, CA). Real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems, CA) and Quantitect Sybr green master mix kit (Qiagen, CA). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR run. Specific primers were designed using the program Primer Express (Applied Biosystems, CA) (Table,1).

The following cycling conditions were used: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 34 s. Quantities of the specific mRNA in the sample were measured according to the corresponding gene specific standard. The mRNA copy number of each cytokine was related to one million copies of mRNA encoding G3PDH.

Assessment of *M. tuberculosis* in infected lungs

Right or left lungs from three mice as a replicates (per group) was obtained at 7 times, started 20, 40, 60, 80, 100, 120 and 140 days after last injection. At each time lungs were homogenized with a Polytron (Kinematica, Lucerne, Switzerland) in sterile tubes containing 2 ml of PBS-Tween 80 at 0.05%. Four or five dilutions

of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI) enriched with oleic acid, albumin, catalase, and dextrose-enriched medium (OACD). The number of colonies was counted 21 days later.

Identification of antigenic population of CFP using antibody detection assay

Antigenic population of CFP was characterized by antibody detection assay in supernatant of PBMC induced by 100µg of different CFP fractions 1, 2 and 3. Briefly Peripheral blood mononuclear cells (PBMC) were obtained from a healthy BCG vaccinated individuals and separated by Ficoll-Hypaque centrifugation according to Yarchoan *et al*., 1981 . PBMCs were cultured and keeping the concentration at 2 × 10 cells/well cultured. PBMCs were induced with 100µl of different phase fractions of CFP (fraction 1, 2, 3) then characterization was done by antibody detection assay in culture supernatant.100 µl of antigens Ag85B, 45kDa, CFP-10 and ESAT-6 (Bender Med System, Austria) were coated to separate micro titer wells. After overnight incubation, plates were blocked with 0.5% BSA in phosphate buffered saline (PBS-T) for two hrs. After blocking, wells were washed with PBS-T thrice and were kept overnight at 4°C till further analysis. At the day of experiment, 100 µl of cell culture supernatants (1:400 dilutions in PBS-T) were added and incubated for 45 min at 37°C. The wells were washed, followed by addition of peroxidase conjugated secondary antibody (goat anti human IgG-HRP 1:10,000) and were incubated for 45 min at 37°C. For color development, 100 µl of TMB/ H_2O_2 substrate solution was added to the wells and incubated at room temperature for about 10 min. The reaction was stopped using 100 µl of 2.5N H_2SO4 and absorbance of each well was read at 450 nm.

Statistical analysis

Data were expressed as mean. Comparison of Completely Randomized Design (CR) was used for obtaining statistical significance. When difference between two means was more than Latin Square Design (LSD) ,it was considered statistically significant.

Results and Discussion

This study focused on protective immune response to CFPs isolated from different phases of *Mycobacterium tuberculosis* culture using BALB/c mice as a model. BCG-induced acquired resistance to infection with *M. tuberculosis* was used as positive control. In experimental animals, immunization with BCG efficiently induces protective immunity against tuberculosis. In the present study, BCG was used as a reference vaccine for the establishment of an animal model suited for comparative studies of protective immunity.

VI Effects of CFP fractions on expression of cytokines and iONS in the lung

Culture filtrate protein F2 and F3 induced cytokine expression which they showed significance increase in expression of mRNAs encoding IL12, TNFα , INFγ in lunge of mice. On the other hand when they were compared to BCG vaccinated mice fraction3 showed highest cytokine expression, table (2). Fraction 1and 2 did not show high cytokine expression when compared to BCG. Also in case of mRNA

encoding iONS significantly increased with Fraction3 when it compared to BCG (table.2).

Challenge test

Mice were vaccinated with BCG and CFP then were left for 30 days to ensure that the nonspecific inflammatory response to the vaccine itself had stoped then mice were aerogenically challenged with approximately 100 *M. tuberculosis* bacilli. Number of viable bacteria was significantly reduced in lunge of mice vaccinated with culture filtrate fraction 2, 3 and BCG when compared to negative control mice but in case of comparing with BCG vaccinated mice, number of viable bacteria significantly reduced only in F 3 vaccinated mice (table, 3).

Identification of antigenic population in CFP fractions

In this study used antibody detection assay using MTB H37RV antigens (Ag85B, 45 kDa, CFP-10 and ESAT-6) in supernatant of PBMCs culture induced by MTB culture filtrate fractions. Antibody titre levels against MTB H37RV antigens in culture supernatants increased especially in fraction 3. Results in figure1showed high levels of Ag85B ,45 KD and CFP10 MTB antigens in fraction 3 this indicate that levels of these secretory antigens increases with growth phase of MTB culture .

The outcome of infection by *M. tuberculosis* is crucially dependent on the immune response of the host. Most infected individuals high a response that is sufficient to prevent progression to disease but allow persistence of viable bacteria in the form of a latent infection. Ten percent of infected individuals develop clinical tuberculosis (TB) during their life, either as a result of failure to control the initial infection or due to reinfection or reactivation of latent infection(Smith and Ross, 1994). Peter Andersen and his colleagues have pursued this hypothesis in an exhaustive screen of *M. tuberculosis* antigens that are released from bacteria growing in a defined culture medium (Andersen *et al*., 1991). Based on a systematic evaluation of a large number of antigens, they have identified members of two protein families as showing encouraging potential for subunit vaccines (Content *et al*., 1991).

Cytokines as essential mediators in the immune response were studied. Thus, investigation of TB has been extremely useful in the development of immunology, immunopathology, and many concepts emerging as a consequence of ongoing research of this type will eventually contribute to novel approaches for better control of this significant infectious disease. In this study we have evaluated protective immune response by measuring mRNA expression of gene encoding IFN-γ,IL-12, TNF-α and iONS against CFPs isolated at different time points from MTB culture using BALB/c mice as a model. We found that Fraction 3 particularly induced good cytokine response when compared to BCG vaccine. Fraction 1 and 2 failed to induce good cytokine response, it was lower than that of BCG vaccine. Cellular immune responses are critical for control of MTB infection (Ravn *et al*., 1999). In the experimental murine TB model, the protective response has a distinct Th1 type of cytokine pattern, as demonstrated by manipulation of the immune system through genetic knockout or the administration of specific monoclonal antibodies (Cooper *et al*., 1993; Dalton *et al*.,1993; Flynn *et al*., 1993).

Table.1 Sequences of primers used for amplification of target genes.

Genes and gene product or primer name	Primer sequence	
Target genes	**Forward**	**Reverse**
IL12	5´-GGA AGC ACG GCA GCAGAA TA-3´	5´-AAC TTG AGG GAG AAG TAG GAA TGG-3´
INF-γ	5'-TCA AGT GGC ATA GAT GTG GAA GAA-3'	5'-TGG CTC TGC AGG ATT TTC ATG-3'
TNF-α	5´TGTGGCTTCGACCTCTACCTC-3´	5´-GCCGAGAAAGGCTGCTTG-3´
iONS	5´-AGCGAGGAGCAGGTGGAAG-3´	5´-CATTTCGCTGTCTCCCCAA-3´
G3PDH	5´CATTGTGGAAGGGCTCATGA-3´	5´-GGAAGGCCATGCCAGTGAGC-3´

Table.2 Means of mRNA expression encoding IL-12, IN IL12, TNFα , INFγ (copies/106 G3PDH) in lung of mice

Vaccine	A-IL12	B-INFγ	C-TNFα	iONS
Control	**511.7**	**365.0**	**1060.0**	**21000.0**
BCG	**3040.0**	**2013.3**	**10063.3**	**44566.7**
F-1	**493.3**	**495.0**	**3503.3**	**28433.3**
F-2	**511.6**	**1583.3**	**7310.0**	**35566.7**
F-3	**4226.7**	**2700.0**	**12016.7**	**49166.7**
F value	**16184.2****	**6058.5****	**31037.2****	**849.9****
LSD $_{(0.05)}$	**43.7**	**40.4**	**81.0**	**1242.7**

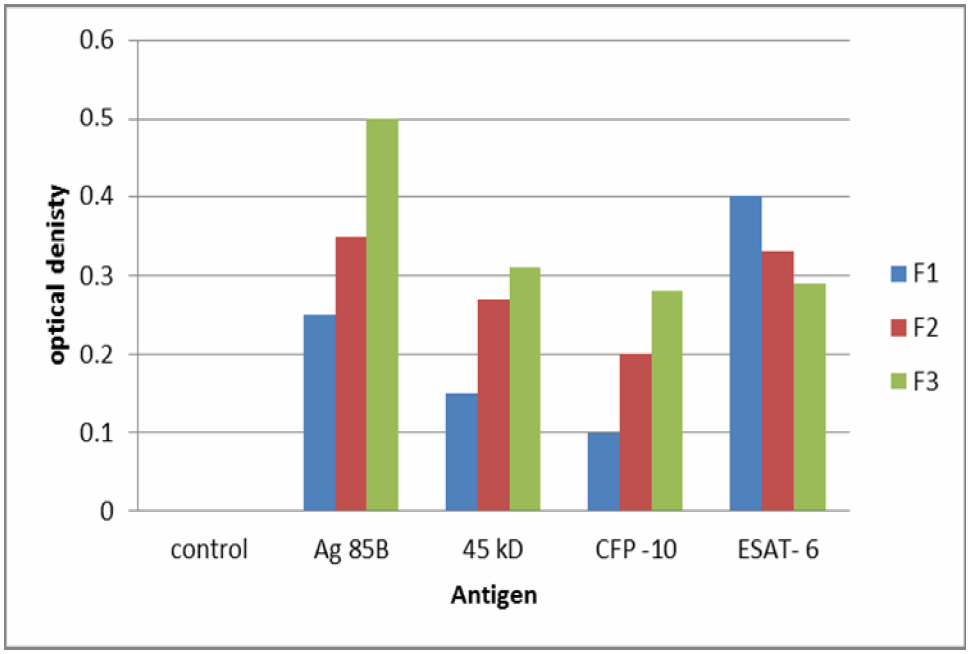
Difference between two means considered significance if they are more than LSD .
** Differences between means is highly significance.

Table.3 Numbers of bacilli log_{10} in the lungs of vaccinated mice and control after challenge infection.

Days / Vaccine	20	40	60	80	100	120	140	Mean (log bacilli)
F-3	**0.00**	**0.48**	**0.79**	**0.98**	**1.23**	**1.02**	**0.55**	**0.72**
BCG	**0.00**	**1.01**	**1.31**	**1.59**	**1.92**	**1.81**	**1.54**	**1.31**
F-2	**0.00**	**1.64**	**2.22**	**3.07**	**3.53**	**4.03**	**5.06**	**2.79**
F-1	**0.11**	**2.35**	**3.12**	**3.51**	**4.25**	**5.06**	**6.03**	**3.49**
Control	**0.53**	**3.07**	**4.13**	**4.31**	**4.70**	**6.03**	**6.54**	**4.19**
Mean (Days) (log bacilli)	**0.13**	**1.71**	**2.31**	**2.69**	**2.93**	**3.59**	**3.94**	

LSD (days) = 0.05 LSD (Treatments) = 0.04 LSD (Days X Treatments) = 0.11

Figure.1 Antigenic population of culture filtrare protein fractions using antibody detection assay



The same cytokine pattern seems to be protective in humans because children with defective receptors for IFN-γ or IL-12 are susceptible to mycobacterial disease (Jouanguy *et al*., 1999; Alcais *et al*., 2005).Culture filtrate antigens secreted during growth phase of MTB culture act has important targets for T-cell response (Gupta *et al*., 2007). In addition to these Th1-type cytokines, TNF-α is also essential for immunity to *M. tuberculosis* in mice as well as in humans (Keane, 2001). These observations further suggest a role for NO, because TNF-α triggers the release of NO from IFN-γ-activated cells. In fact, iNOS expression is essential to control infection in mice (Chan *et al*., 1991) and is also highly expressed in human tuberculous lesions (Schôn *et al*., 2004). Similar abnormalities are also observed in the lungs of Balb/c mice, which have been experimentally infected via the trachea with a high dose of *M. tuberculosis* H37Rv (Hernandez-Pando *et al*., 1996; Brightbill *et al*., 1999)In this model, there is an initial phase of partial resistance dominated by Th1 cytokines plus activated macrophages that produce TNF-α and express iNOS. Also in our study culture filtrate fractions induce iNOS expression, fraction3 induce high iNOS expression compared to BCG. Similar results obtained by Brightbill (1999) among others, the 19- kDa lipoprotein and lipoarabinomanann (LAM) activate macrophages through TLR-2, promoting the inducible nitric oxide synthase and the production of nitric oxide, a powerful microbicidal pathway. In fact, iNOS expression is essential to control infection in mice (Chan *et al*., 1992) and is also highly expressed in human tuberculouslesions (Schôn *et al*., 2004).

Mice were vaccinated with BCG and left for 30 day to ensure that the nonspecific inflammatory response to the vaccine itself had ceased then mice were aerogenically challenged with approximately 100 *M. tuberculosis* bacilli.Our result showed that however only in case of fraction3 has lower number of viable bacteria in lunge when compared to BCG immunized, fraction2 has significantly reduced number of viable bacteria in lung when it compared to control unimmunized mice. These results indicated that proteins secreted at late growth phase fraction3 of mycobacterium culture induce high protective immune response in BALB/c mice models. Similar results obtained by Hubbard et al.,(1991). While the resistance indices for these vaccinated mice were smaller than for live BCG the reduction in growth seen in the lungs (and spleen) was consistently statistically significant (P <005) for the 57, 38 and 10- 14 kD proteins (Hernandez-Pando *et al*., 1998). Under these test conditions, fraction 1 and fraction2 response was not considered sufficient to indicate protection.

Variations in the protective immune response induced by different *Mycobacterium tuberculosis* culture filtrate fractions may be due to antigen profile of each fraction. In our study we used H37RV antigens Ag 85B, 45kDa CFP-10 and ESAT-6 to characterize antigenic population of MTB CFP fractions 1, 2 and 3 by antibody detection assay by ELISA .Results showed that fraction3 has maximum level of antigens in fraction 3 and this explain high protective immune expression induced by fraction3 of MTB CFP. CFP-10 is major early secretary protein appeared at RD1 region which is absent in currently

available BCG vaccine. Use of such antigen for vaccine development is area on which many scientists are focusing their research on. Based on studies in mice, Dietrich et al., reported that CFP-10 has predominant T-cell response(Dietrich *et al*., 2006) . In another study Huygen et al., showed DNA vaccine encoding Ag85A, B produces elevated IL-12 and IFN- γ response (Huygen *et al*.,1996).

Mycobacterium tuberculosis culture filtrate protein fractions induce protective immune response in mice BALB/c mice model especially those secreted in late phase of growth and this may due to high concentrations of antigens . This fractions may be used in near future for development of effective vaccination strategies for improving efficacy of currently available TB vaccine or synergy with the currently available drugs and therefore can improve their efficacy.

Acknowledgment

Authors would like to thank professor Sabry Mahmoud Yuonis at microbiology department faculty of applied medical science , Dammam university ,KSA for his support and help in this wok. Authors are grateful to professor Adel Mohamed Mahmoud at Agronomy Department, Faculty of Agriculture, Assiut University, Egypt for doing statistics of this work.

References

Alcais, A. Fieschi, C. Abel, L. Casanova , JL.2005.Tuberculosis in children and adults: two distinct genetic diseases. J Exp Med., 202: 1617-1621.

Andersen, P.1994. Effective vaccination of mice against Mycobacterium tuberculosisinfection with a soluble mixture of secreted mycobacterial proteins. Infect Immun., 62: 2536–2544.

Andersen, P. D. Askgaard, L. Ljungqvist, J. Bennedsen, and Heron, I.1991. Proteins released from Mycobacterium tuberculosis during growth. Infect. Immun., 59:1905-1910.

Boesen, H. Jensen, B.N. Wilcke, T. Andersen, P.1995. Human T-Cell Response to Secreted Antigen Fractions of Mycobacterium tuberculosis. Infect Immun., 63: 1491–1497.

Brightbill, H. D. Libraty, D. H. Krutzik ,S. R. et al.1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science, 285: 732-736.

Chan , J. Fan, X. D. Hunter, S.W. Brennan , P. J. Bloom BR.1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect Immun., 59: 1755-1761.

Chan, X. Xing, Y. Magliozzo , R. S. Bloom, B. R.1992. Killing of virulent *Mycobacterium tuberculosis*by reactive nitrogen intermediates produced by activated macrophages. J Exp Med., 175: 1111-1122.

Content, J. de la Cuvellerie, A. deWit, L. et al.1991. The genes coding for the antigen 85 complexes of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG are members of a gene family: Cloning, sequence determination, and genomic organization of the gene coding for antigen85-C of *M. tuberculosis*. Infect Immun., 9:3205–3212.

Cooper, A. M. Dalton, D. K. Stewart, T. A. Griffin, J. P. Russel, D.G. Orme, I. M.1993. Disseminated tuberculosis in interferon gamma disrupted mice. J Exp Med., 178: 2243-2247.

Dalton , D. K. Pitts-Meek, S. Keshav, S. Figueri, I.S. Bradley, A. Stewart, T. A.1993. Multiple defectsof immune cell function in mice with disrupted interferon gamma genes. Science, 259: 1739-42.

Dietrich, J. Weldingh, K. Andersen, P. 2006. Prospects for a novel vaccine against

tuberculosis. Vet Microbiol., 112: 163–169.

Flynn, J. L. Chan, J. Triebold, K. J. Dalton, D. K. Stewart, T. A. Bloom, B. R. 1993. An essential role ofinterferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med.,178: 2249-2254.

Fonseca, D. M. Silva, C. L. Wowk, P. F. Paula, M. O. Ramos, S. G. et al. 2009. Mycobacterium tuberculosis culture filtrate proteins plus CpG oligodeoxynucleotides confer protection to Mycobacterium bovis BCG-primed mice by inhibiting interleukin-4 secretion. Infect Immun., 77: 5311–5321.

Gupta, U. D. Katoch, V. M. McMurray, D. N. 2007. Current status of TB vaccines.Vaccine, 25: 3742-3751.

Hernandez-Pando, R. De La Luz Streber, M. Orozco, H. et al.1998. The effects of androstenrediol and dehydroepiandosterone on the course of tuberculosis in Balb/c mice. Immunology, 95: 234-241.

Hernandez-Pando, R. Orozco, H. Sampieri, A. et al. 1996. Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology, 89: 26-33.

Hubbard , R. D. Flory, C. M. Collins, F. M. 1992. Immunization of mice with mycobacterial culture filtrate proteins. Clin. exp. Immunol., 87: 94-98.

Husain, A. A. Kashyap, R. S. Kalorey, D. R. Warke, S. R. Purohit, H. J. et al. 2011. Effect of repeat dose of BCG vaccination on humoral response in mice model. Indian J Exp Biol., 49: 7-10.

Huygen, K. Content, J. Montgomery, D. L. Yawman, A. M. Deck, R. R. et al.1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nat. Med., 2: 893–898.

Jouanguy, E. Doffinger, R. Dupuis, S. Pallier, A. Altare, F. Casanova, JL.1999. IL-12 and IFNgamma in host defense against mycobacteria and salmonella in mice and men. Curr Opin Immunol., 11: 346-351.

Keane, J. 2001.Tuberculosis associated with infliximab a tumor necrosis factor alpha-neutralizing agent. N Engl J Med., 1098-1104.

Lindblad, E. B. Elhay, M. J. Silva, R. Appelberg, R. Andersen, P. 2007. Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infect Immun., 65: 623-629.

Martin, C. 2007. New Vaccines against Tuberculosis. Tuberculosis book, Chapter 10.

Parrish, N. M. Dick , J. D. Bishai, W. R.1998. Mechanism of latency in *Mycobacterium tuberculosis*. Trends Microbiol., 6: 107-12.

Ravn ,P. Demissie, A. Eguale, T. Wondwosson, H. Lein, D. et al. 1999. HumanT Cell Responses to the ESAT- 6 Antigen from Mycobacterium tuberculosis. J Infect Dis., 179: 637–645.

Schôn, T. Kimberger, G. Nagese, Y. Hernàndez –Pando, R. Sundqvist, T. Britton, S. 2004. Local production of nitric oxide in patients with tuberculosis. Int J Tuberc Lung Dis., 8:1134-1137.

Smith, P. G. Ross, A. R.1994. Epidemiology of tuberculosis. In: Bloom BR, ed. Tuberculosis: Pathogenesis, Protection, and Control. Washington, D.C.: American Society of Microbiology.

Sorensen, A. L. Nagai, S. Houen, G. Andersen, P. Andersen, A. B. 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by Mycobacterium tuberculosis. Infect Immun., 63: 1710–1717.

Yarchoan, R. Murphy, B. R. Strober, W. Schneider H. S. and Nelson D. L.1981. specific anti-influenza virus antibody production in vitro by human peripheral blood mononuclear cells.J. Immunol., 127(6):2588-2594.