Research Article **AJMI 2017 2:1**

American Journal of Microbiology and Immunology
(ISSN: 2474-2910)

The effect of Transfer Factor as Immunotherapy comparing with the effect of BCG in Mice challenged with *Mycobacterium tuberculosis*

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ABSTRACT

Background: Transfer Factor (TF) is an immune modulator which stimulates the cellular arm of the immune system (killer lymphocytes), activates immune cytokine synthesis and regulates immune function (Lawrence, 1955).TF is very effective in those diseases in which CMI plays a relevant role in protection and control of the disease, such as intracellular bacterial diseases (tuberculosis). (Estrada Parra, et al1955). TF are low molecular weight products from immune cells that are able to transmit the ability to express delayed-type hypersensitivity (DTH) and cell mediated immunity (CMI) from sensitized donors to non immune recipients (Kirkpatrick, 2000). **Objectives:**The aim of this experimental study is to determine the protective efficacy of transfer factor (TF) as immunotherapy for mice in comparison to BCG. **Materials and methods:**A total number of 102 mice were examined for their immunopotency and protective efficacy of Transfer factor (TF) comparing to the protective efficacy of BCG single and second repeated dose against challenge dose of *M. tuberculosis* (107 CFU) . A number of 20 mice were immunize with the attenuated strain of M. bovis, Bacillus Calmette-Guérin (BCG). After 21 days of BCG spleens of 10 tuberculous mice were removed aseptically for the preparation of TF. To evaluate the effect of TF 3 groups of inbred BALB/c male mice

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How to cite this article:
Salim et al.,. The effect of Transfer Factor as Immunotherapy comparing with the effect of BCG in Mice challenged with Mycobacterium tuberculosis. American Journal of Microbiology and Immunology, 2017; 2:1

eSciencePublisher
eSciPub LLC, Houston, TX USA.
Website: http://escipub.com/

were injected with TF and challenged with virulent *M. tuberculosis*, followed by another 3 groups of inbred BALB/c male mice which were immunized with BCG single and second repeated dose. All mice with BCG and TF were tested for tuberculin skin test (TST) so as to determine susceptibility and resistance against tuberculosis, susceptible groups of mice were challenged with virulent *M. tuberculosis*.Followed by study of humoral response by immunization of a group of mice with immune serum and challenged with *M. tuberculosis* H37Rv strain. Followed by an experiment of group A and B for the susceptibility and resistance of the strains of mice. A group of dead mice For histological study of the infected lungs were examined. **Results:** After three weeks of observations the mice of experiment(1) were tested for tuberculin skin test and the results were positive. Effectiveness determination of TF as protective efficacy was (83.3%). while effectiveness determination and protective efficacy of BCG first dose and boosting dose were (50%) and (70%) respectively. Humoral immunity response against *M. tuberculosis* showed negative reaction hence mortality rate was 100%, group B mice were resistant for BCG (Swiss white strain) and the results of histological study of the infected lungs showed lung bacilli that was *M. tuberculosis*. **Conclusions:** The results indicated that administration of murine transfer factor (mTF) extracted and prepared from spleen of animal model (mice) as immunotherapy for challenged mice of MTB (H37Rv) showed a better results enhanced immune response in respect to delayed type hypersensitivity , survival rate and mortality rate suggesting that efficacy of mTF as immunotherapy for tuberculosis. Repeat dose of BCG enhanced immune response in respect to delayed type hypersensitivity , survival rate (70%) and mortality rate (30%), suggesting that efficacy of BCG vaccine may improve and give better results if booster doses are given.

Key words: Transfer Factor (TF),BCG ,Mice and *M. tuberculosis*.

Introduction

Transfer Factor (TF) is an immune modulator. The main function of these peptides in the body is to provide immune protection against microbes (bacteria, viruses, fungi, and protozoa), cancerous cells and other antigens capable of disturbing vital processes in the body. It stimulates the cellular arm of the immune system (killer lymphocytes), activates immune cytokine synthesis and regulates immune function (Lawrence, 1955). TF have been demonstrated to be very effective in those diseases in which CMI plays a relevant role in protection and control of the disease, such as intracellular bacterial diseases (tuberculosis, leprosy) and parasite infections (leishmaniasis, toxoplasmosis) .(Estrada Parra*, et al*1955). TF or leucocyte dialysates are low molecular weight dialysable products from immune cells that are able to transmit the ability to express delayed-type hypersensitivity (DTH) and cell mediated immunity (CMI) from sensitized donors to nonimmune recipients (Kirkpatrick, 2000).In 1955, Lawrence discovered that a dialyzed of viable leukocytes obtained from a healthy donor presenting a positive percutaneous tuberculin test was able to transfer to a healthy receptor the ability to respond to this pathogen. Lawrence named these molecules as Transfer Factor. TF is composed of peptide obtained from lymphocytes ranging from 3500-6000 kDa. T lymphocytes have the ability to express delayed-type hypersensitivity and cell mediated immunity from sensitized donors to non immune recipients. TF plays a vital role in controlling immune over reactions and mistargeted responses in the devel-

opment of autoimmune reactions. TF improves cellular immunity in patients with immune deficits due to their responses are mediated by antigen-specific, inducer, and suppressor/regulatory activities contained in this fraction. Tuberculosis (TB)is primarily a chronic lung infection that is one of the most potent and wide-spread human infections today, and a major cause of death from bacterial pathogens (Lawn, and Zumla;2011).). It affects more young adults and therefore has a high impact on the socioeconomic status of people (Zakham *et al.*, 2012).Although TB is a serious global health problem, several medical advances have been made in the past150 years to facilitate prevention and control of TB. In general, TB mortality started to decrease in most industrialized countries during the 20th century, probably due to a better socioeconomic status including improved nutrition and living conditions (Lienhardt, 2012) TB re-emerged during the 1990s both in developing and several industrialized countries partly due to the HIV/AIDS pandemic and also because of an increased emergence of drug resistant *M. tuberculosis* strains (Dheda*, et al*;2010).

Transfer factor (TF) is shown to be capable of transferring antigen-specific cell-mediated immunity (CMI) to T lymphocytes and therefore TF can successfully be used as treatment for M.TB. and specially for MDR-TB and supplementary for conventional chemotherapy. The BCG based vaccine can provide stimulation of both innate and acquired immunity.(Schreiber, *et al*; 2010). The incidence of TB is high inspite of primary vaccination in neonatal period and therefore requires consideration for repeated immunization of BCG. Researches concerning TF are going on .

Materials and methods

A total number of 102 mice were examined for their immunopotency and protective efficacy of Transfer factor (TF) comparing to the protective efficacy of BCG single and second repeated dose against challenge dose of *M. tuberculosis* (10^7 CFU) . A number of 20 mice were immunize with the attenuated strain of *M. bovis*, Bacillus Calmette-Guérin (BCG). After 21 days of BCG spleens of 10 tuberculous mice were removed aseptically for the preparation of TF.). Three groups of mice were used n= 12 for each group (36mice), the groups were T_1,T_2 andT_3 The mice of group $T_1$and T_2 were given transfer factor (TF) and the mice of group $T_1$and T_3 were challenged with *M. tuberculosis*. Followed by an experiment contains three groups of mice each of ten mice n = 10 (30mice). The first group(A) were immunized with BCG first dose (I.P) for 21 days. The second group (B) were immunized with BCG (I.P) as first dose for 15 days and boosting dose (second dose) for 15 days. The third group(C) were not immunized with BCG. All the three groups were challenged with virulent *M. tuberculosis*. All mice with BCG were tested for tuberculin skin test (TST) so as to determine susceptibility and resistance against tuberculosis.

All the three groups were challenged with (0.5ml) virulent *M. tuberculosis* H37Rv strain (American Type Culture Collection, ATCC 35718) *M. tuberculosis*.10^7(CFU) .

Another group of mice n=6 for the study of humoral response by immunization of mice with immune serum and challenged with *M. tuberculosis* Following by two groups of mice n=10 for each group A and B for the susceptibility and resistance of the strains of mice by immunization of mice with BCG for 21 days and testing by tuberculin skin test (TST). The efficacy was based on a survival rate of challenged mice, mortality rate and bacterial load of *M. tuberculosis* in the lungs of infected mice.

Mycobacterium tuberculosis strain

Virulent *M. tuberculosis* H37Rv strain (American Type Culture collection) and. *M. bovis* BCG (Bacille Calmette Guerin) Collection strain 1011 were obtained from Kassala TB center (Kassala University) and Kassala Ministry of health.

Animals

Inbred BALB/c (8–12 weeks old) mice were obtained from Veterinary Research Laboratories in Khartoum, Swiss mice (males 20-25grams) were obtained from Professor Hamid Suliman (Un. of Khartoum.) All the mice were maintained in standard cages under sterile conditions and were fed commercial mice chow and water . All animals were housed and maintained in accordance with protocols approved by the Institutional Animal Care.

Preparation of murine transfer factors (mTF)

A number of 20 mice were immunize with the attenuated strain of *M. bovis*, Bacillus Calmette-

Guérin (BCG) by intrapritoneal (i.p) route with 0.5 ml of a 10 fold dilution of BCG. After 21 days success of immunization was confirmed by tuberculin skin test. After 21 days of BCG spleens of 10 tuberculous mice were removed aseptically, chopped and grunt with clean sterile glass beads or sand and cell extract of immunized animals were distributed in 10ml normal saline or phosphate buffer saline (PBS) were used. The cell suspension was centrifuged to remove cellular debris at 1500 rpm for 15 minutes and the upper layer were collected in sterile test tubes. RPMI 1640 media were added to the layer mixed well and then centrifuge at 2000 rpm for 2 hours till the separation of the component and appearance of middle layer. This middle layer which is T mphocyte was collected with Pasteur pipette in test tubes and kept in deep freezer (-70^{0}c) and removed next day or till used. Keep The test tube was thawed by keeping in room temperature before returning to the deep freezer (-70^{0}c).The process of freezing and thawing was repeated 18 times. The suspension was then centrifuged at 40000 rpm for 30 minutes or 5000 rpm for 4 hours. The upper layer which is the transfer factor (TF) was then separated.

Tuberculin skin Test procedure:

Intradermal injection of mice with 0.2 ml of purified protein derivative (PPD) and then induration and swelling were observed in the skin of tested mice after 24-72 hours.

All the three groups were challenged with (0.5ml) virulent *M. tuberculosis* H37Rv strain (American Type Culture Collection, ATCC 35718) *M. tuberculosis*.10^7(CFU) .

Challenge dose:

Preparation of different concentration of *M. tuberculosis* by McFarland 0.5 and colony forming unit (CFU) of 10^2 ,10^3,10^4, upto 10^9.

McFarland standards were made by mixing specified amounts of Barium chloride and Sulfuric acid together. Mixing the two compounds forms a Barium Sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2$•$2H_2O$), with 9.95 ml of 1% sulfuric acid (H_2SO_4).The standard was compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension was too turbid, it was then diluted with more saline. If the suspension was not turbid enough, more bacteria was added.

Preparation of inoculum for LD_{50} determination of *M. tuberculosis*

On the day of inoculation the optical density($O.D._{540nm}$) of bacterial suspension of *M. tuberculosis* was adjusted to 1.35 and 1ml of suspension then serial dilutions (10^0 ,10^1 ,10^2,10^3,10^4, up to 10^9) were prepared in saline, for each dilution 6 mice of each group were inoculated with 0.5ml intrapritoneally.Inoculation of 0.5 ml of different concentrations 10^2,10^3,10^4, up to 10^9 of *M. tuberculosis*. McFarland 0.5 intrapritoneally in a group of 6 mice for each concentration to determine the LD_{50}.The concentration of the suspensions which kills 50% of the mice is the LD_{50}.

The control group received normal saline 0.5ml intrpritoneally. Observations of the two groups were done for one week after the inoculation of different concentrations of *M. tuberculosis* and the normal saline were recorded. Lethal dose (LD_{50}) concentration which kills 50% of the mice was recorded .

Another group of mice n=6 for the study of humoral response by immunization of mice with immune serum and challenged with *M. tuberculosis* Following by two groups of mice n=10 for each group A and B for the susceptibility and resistance of the strains of mice by immunization of mice with BCG for 21 days and testing by tuberculin skin test (TST). The efficacy was based on a survival rate of challenged mice, mortality rate and bacterial load of *M. tuberculosis* in the lungs of infected mice.

Preparation of mice tissues for histopathology

For histological study, the lungs from four dead mice after receiving BCG immunization for 21 days and then challenged by *M. tuberculosis* for 3 weeks later, and then were fixed by absolute ethanol, embedded in paraffin, sectioned and stained with haematoxylin and eosin (Hernandez-Pando, et al;1996) In these slides the area of granuloma and the percentage of lung area affected by pneumonia were determined.

Results

Results of tuberculin skin test in BCG imunized mice

Table 1 The results of tuberculin skin test reaction (TST)

Type of mouse strain	No. of mice/group	TST reaction
BALB/c inbred susceptible mouse	10	Positive
Swiss out bred resistant mouse	10	Negative

Table 2 Shows the results of LD_{50} determination

No. of BALB/c mice /group	Bacterial inoculums (CFU)	No. of dead mice	Mortality ratio
10	10^0	0	0%
10	10^1	2	20%
10	10^2	2	20%
10	10^3	3	30%
10	10^4	3	30%
10	10^5	4	40%
10	10^6	4	40%
10	10^7	5	50%
10	10^8	6	60%
10	10^9	7	70%

LD_{50} (CFU (10^7)) is the LD_{50} at which 50% of the mice were killed.

Table(3)The results of Effectiveness determination of murine Transfer Factor in mice challenged with *M. tuberculosis*

Group of mice	Number of BALB/c mice/ group	Dose of T.F./ mouse	Chal-lenge dose of M.tb.	No.of dead mice	Mortality rate%	Protective efficacy%
T_1	12	0.5ml	0.5ml	2	(2/12) 16.7%	(10/12) 83.3%
T_2	12	0.5ml	_	0	0%	100 %
T_3 (control)	12	_	0.5ml	12	(12/12) 100%	0%

A total number of 10 inbred BALB/c mice were tuberculin skin test (TST) positive after immunization with BCG while 10 out bred Swiss white mice were negative for TST.

Results of LD_{50} concentration:

Table(2)shows the groups mice inoculated with different concentrations of *M. tuberculosis*. (10^2,10^3,10^4, up to 10^9) and the normal saline(-control group) for one week showed that the group of colony forming unit (CFU)(10^0) exhibited no death of mice. Mice given (10^1) and(10^2) were exhibited 2deaths out of 10 (2/10) i.e. mortality ratio 20% The groups of (10^3) and (10^4) showed 3 deaths out 10 (3/10)i.e. mortality ratio 30%.In the groups given(10^5) and(10^6) 4deaths out 10 (4/10) mice showed mortality ratio 40% ,In the groups given CFU (10^7) of mice showed 5deaths out of 10 (5/10)with mortality ratio 50%. This showed that the lethal dose (LD_{50}) which killed 50% of the total number of the mice.

Results of the response of murine transfer factor (mT.F) in protection against challenge.

The results of the groups of mice which were injected with mTF were exhibited in Table (3). The control group (T_3) which were not immunized with mTF exhibited 100% mortality after 24 hours of virulent HRv37 with *M .tuberculosis*. The second group (T_2) which received mTF but not challenged with *M. tuberculosis* survived 100%. The third group (T_1) which received mTF and challenged with *M. tuberculosis* 10 out of 12 (10/12) mice survived after 3 weeks of observation and (2/12) were died and hence protection of T.F immunopotency was 83.3%.

Results of vaccination with single and repeated (booster dose) of BCG in mice

Survival mice in group (A) were 50%, group (B) 70% and group(C) 0%.The mortality rates for (A) 50%, (B) 30% and (C) 100%. The immunopotency and protective efficacy of BCG first dose and boosting dose were (50%) and (70%) respectively, and mortality rates of BCG first dose and boosting dose were (50%) and(30%) respectively .For Group(C) which were not immunized 100% mortality and 0% protective efficacy after challenge dose of *M. tuberculosis*.

Results of immunopotency of transfer factor (T.F) comparing with BCG immunization against challenge dose of *Mycobacterium tuberculosis*

The comparison between TF and BCG immunization results are shown in Figure (1).

The results of Humoral response of mice against *Mycobacterium tuberculosis*.

The result was death of the mice within 24hours, and mortality rate of humoral response of mice against *Mycobacterium tuberculosis* was 100% compared with 50% BCG first dose ,30% BCG boosting dose and 16.7% mortality rate of transfer factor (TF) .

Results of lung histopathology of mice

All slides of dead mice stained with haematoxylin and eosin showed lung bacilli that Was *M. tuberculosis*.

Discussion

Since the discovery of Transfer Factor (TF) by Sherwood Lawrence in1955, more than 70 years ago Transfer Factor has been found to be very effective in those diseases in which CMI plays a relevant role in protection and control of the disease , such as viral infections (herpes simplex, varicella zoster), intracellular bacterial diseases (tuberculosis, leprosy) and parasitic infections (leishmaniasis, toxoplasmosis) and some types of cancer. Transfer Factor (TF) are protein that transfer the ability to express cell mediated immunity from immune donors to non-immune recipients. TF treatment were found to selectively affects cytokine production in response to antigenic stimulation (Alvarez and Kirkpatrick; 1996). In this study transfer factor obtained from the spleen of mice which were immunized by BCG intrapritoneally (I.P.) for twenty one days. The peak of immune protection in this animal model reached in day 21.This agree with a study done by Fabre (2004)in Mexico, used Transfer Factor as immunotherapy and supplement of chemotherapy in experimental pulmonary tuberculosis. (Fabre*.et al*;2004). The current study found that the effect of administration of murine transfer factor (TF) on survival of challenged mice was majority (83.3%) comparing with maximum (100%) mortality of the control group, and this result obtained is in accordance with a study done in Mexico 2004 by Fabre (Transfer factors as immunotherapy and supplement of chemotherapy in experimental pulmonary tuberculosis)where they found that the BALB/c mice which were

Table 4 The results of single and repeated (booster doses) of BCG vaccination in mice challenged with M. tuberculosis .

Group of mice	Number of mice/group	No.of surviv-al mice after challenge dose of M.tb.	No.of dead mice	Protective efficacy%	Mortality rate%
(A) immunized with BCG (First dose)	10	**5**	**5**	**50%**	**50%**
(B) immunized with BCG (First dose (15days) and repeated dose for 15days)	10	**7**	**3**	**70%**	**30%**
(C)only challenge dose of M.tb.)	10	**0**	**10**	**0%**	**100%**

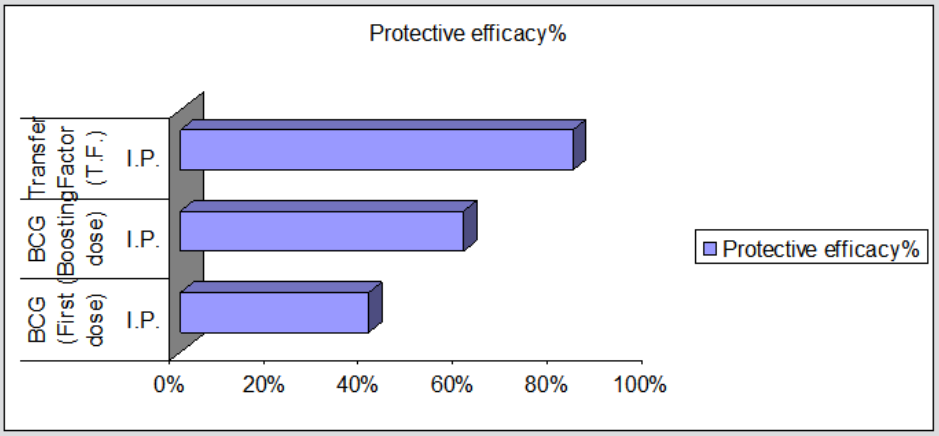

Figure 1 The results of immunopotencyof transfer factor (T.F) comparing with BCG immunization against challenge dose of *M. tuberculosis*

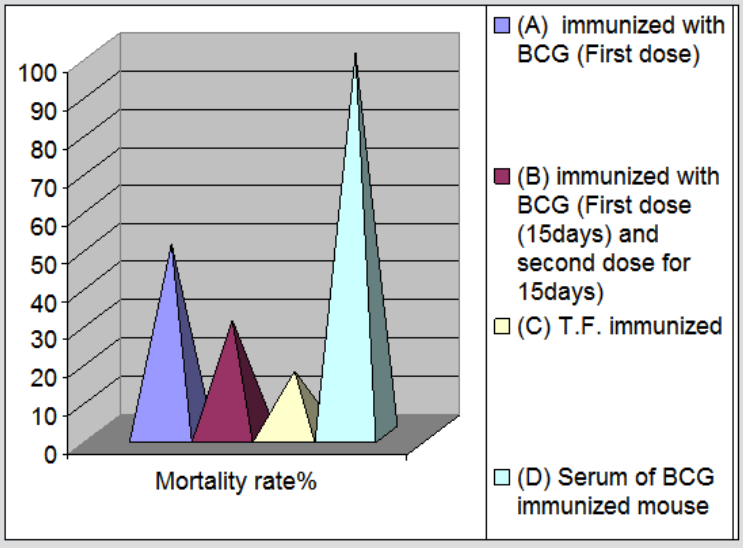

Figure 2 Shows Humoral response of mice against Mycobacterium tuberculosis compared with BCG first dose , BCG boosting dose and transfer factor (TF) mortality rate.

infected via the trachea with *Mycobacterium tuberculosis* H37Rv and treated with mTF showed a significant 95% survival when compared with the control group, which exhibited 100% mortality. Mexican team of investigators have shown that treatment of mice infected with *M. tuberculosis* with a murine tuberculosis- specific TF restored expression of the Th1 cytokine pattern and resulted in inhibition of bacterial proliferation, significant increase of DTH, and animal survival (Fabre, *et al*., 2004) The efficiency of TF was highly depended on the dose and, to its source (murine ,human or bovine),colostrums is rich in transfer factor.4lifeTransfer Factor, American company is famous and well known in the production of TF.

The efficacy of BCG against challenge dose of *M. tuberculosis* was 50% and mortality rate was 50% The obtained results are similar to previous studies done by Turner; *et a*l in 2001.Who demonstrated used low-dose aerosol infection of *M. tuberculosis* to compare chronic tuberculosis.

Susceptible mice, which are often able to contain bacterial growth in the liver and spleen, are unable to restrict growth in the lung. While granulomas in resistant mice are well organized, consisting of aggregated lymphocytes and macrophages, lesions in susceptible mice are often poorly organized, necrotic and contain few lymphocytes. This implies that susceptible strains have a defect in recruiting or retaining lymphocytes in the lung. The production of cytokines crucial for the control of tuberculosis ,such as IFNg, is usually diminished in susceptible mice, resulting in a general delay in the effect or phase of the adaptive immune response. In many cases, susceptible mice are deficient in maintaining a single dose.(Flynn *et al*.,1995).

Conclusion

The results indicated that administration of murine transfer factor (mTF) extracted and prepared from spleen of animal model (mice) as immunotherapy for challenged mice of *M. tuberculosis* (H37Rv) showed a better results enhanced immune response in respect to delayed type hypersensitivity , survival rate was (83.3%) and mortality rate (16.7%), comparing with (50%) and (70%) survival rate of BCG single and repeated dose respectively suggesting that efficacy of mTF as immunotherapy for tuberculosis.The results obtained in this current study in animal model (mice) suggest that repeat dose of BCG enhanced immune response in respect to delayed type hypersensitivity , survival rate (70%) and mortality rate (30%), suggesting that efficacy of BCG vaccine may improve and give better results if booster doses are given.

Inbred strains of mice exhibit varied patterns of susceptibility following infection with virulent *M tuberculosis*. Susceptible mice have progressive fulminate disease resulting in their premature death; in contrast, resistant mice are able to control bacterial replication, limit lung injury and survive longer.

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