INTRODUCTION
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Tuberculosis (TB) is re-emerging worldwide, and is posing a major challenge to the Public Health due to resurgence of AIDS and emergence of multi drug resistant bacilli. Approximately, half of the world's population is primarily infected with *Mycobacterium tuberculosis* (MTB) and approximately 15 million new cases of pulmonary TB develop annually. It may be the most common cause with highest death from any single infectious agents (Schmidt, 1989). Three million people die of the disease each year which kills more adults than any other infectious disease, even in the industrialized countries, where it was considered to be on the decline. The disease has been registering an increase in recent times. In USA, for example, the disease has increased by 20% between 1985 and 1992, from a declining trend of 6% per year (Mukherjee, 1995). According to WHO report, TB has claimed height of death in 1995 than any other year in the history (Moran, 1996). At present it is estimated that about 1.5% of India's population, or about 14 million persons could be affected with pulmonary TB. It is also known that nearly half of the population in the country is infected with tubercle bacilli, and would break down into active cases in the future (Mukherjee, 1995).

Over a century ago, Robert Koch identified MTB as the causative agents of TB (Koch, 1882). Till date, the only existing vaccine against TB is the Bacille Calmette-Guerin (BCG), an attenuated strain of *M. bovis* (Calmette, 1927) and it is mandatory or officially recommended in 182 countries or territories (Paramasivam et al., 1996). By the year 1970 BCG became the most widely used vaccine in the world being administered to approximately 100 million children each year. About 3 million doses have been given in last four decades, and more than 70% of the world's children have now received BCG
Fig. 1 *Mycobacterium tuberculosis*, 100-1000 bacilli in an average oil immersion field.

Fig. 2 Normal appearance of an intact *Mycobacterium tuberculosis* cell 0.5 x 2μm., the retraction of the cytoplasm is caused by drying in electron microscope.
INTRODUCTION

(Fine 1988; WHO 1989) The BCG vaccine has shown lack lustre performance. The protective efficacy results of BCG vaccine obtained ranged from 0 to 80% (Jacob et al., 1990) although, it has provided consistent protection against tuberculosis meningitis (Singh et al., 1989). There has been virtually no anti TB drug development during the last twenty years due to poor prospect of recovery of R&D investment. The same applies for commercial vaccine development. The knowledge about protective antigens and protective effector mechanisms in TB is rudimentary (Bjune, 1996).

This has necessitated the demand for an alternative vaccine, which could provide protection better than BCG. In search for such an effective vaccine, many mycobacteria have been screened from time to time. Interestingly, a vaccine comprising of atypical mycobacterium M. habana (TMC-5135) has been developed at Central Drug Research Institute, Lucknow which provides consistent protection against MTB H37Rv and indigenous strains of MTB of varying virulence (low, moderate, and high) and M. ulcerans (Gupta et al., 1979; 1984, Singh et al., 1981) by sharing of specific epitopes with M. leprae, MTB and BCG which puts M. habana into the category of broad spectrum candidate mycobacterial vaccine.

The vaccine prepared by gamma-irradiation or by heat inactivation of M. habana cells has been found equally effective in providing protection against leprosy bacillus in mouse foot pad model (Singh et al., 1985; 1989, Gupta et al; 1987). It generates strong CMI response and shares immuno-dominant proteins with MTB and M. leprae.

The Drug Controller of India has accorded permission to initiate trial of the vaccine in different parts of India (phase II). M habana needs further
Fig 3  Estimated Global trends of TB deaths.
exploration with regard to its protective efficacy on the effector cells of the rodents and human subjects. Phagocytosis and subsequent killing of pathogens represent one of the most important defence against disease. The vertebrate effector system or the defence mechanism is an integrated response, involving the cells of immune system as well as those of myeloid series, the phagocytes, primarily the Mφ constitute the first line of defence which destroy the invaders by combination of reactive oxygen intermediates (ROI) reactive nitrogen intermediates (RNI) and lysosomal enzymes.

There is a compelling body of evidence to believe that the failure of the subdued Mφ to kill the bacteria and to initiate cell mediated immunity (CMI) response is mainly due to their inability to express respiratory burst (Sharp et al., 1985, Holzer et al., 1986, Marolia et al., 1987; 1989). These reactive oxygen species (ROS) are deleterious and can damage membranes, nucleic acid and protein sufficient to kill cell or even whole organisms (Babior 1978, Murray et al., 1979, Nathan 1983, Klebanoff 1980). In order to survive in the host, thereafter the cells have to evolve some mechanisms to deal with the toxicity of these species (Sies, 1993). Detoxification of host generated ROS through antioxidant enzymes is regarded as one of the important mechanisms of cell evasion. The hydrolytic enzymes of mononuclear phagocytes are important for many of their metabolic functions (Gordon, 1978). The major hydrolases of mononuclear phagocytes are intracellular acid hydrolases (Acid phosphatase and β-glucuronidase) and constitutive, extracellular glycosidases (lysozyme which is secreted into the extracellular compartment).

Since very little at present is known about the mechanisms of protection provided by this vaccine, it was considered of interest to examine at base level
Fig. 4 Magnitude and dynamics of the world TB problem.
of Mφs, the mechanisms involved in affording protection in BALB/c mice as well as human subjects as a result of *M. habana* vaccine treatment with the following objectives.

- The formation of ROI and RNI by the Mφs and PBM cells.
- Release of cytokines from the activated macrophages.
- Activation of lysosomal and antioxidant enzymes in Mφs and PBM cells.
- Phagocytic index of sensitized Mφs vis a vis normal ones.
- Lymphoproliferative responses of sensitized cells.
- Delayed type hypersensitivity response of *M. habana* vaccine.
- Electron microscopic study of macrophages.

This study may lead to understand the mechanisms involved in generating the protective efficacy of this vaccine.
REVIEW OF LITERATURE
Even after hundred years of Robert Koch's discovery of the tubercle bacillus, tuberculosis (TB) still remains a major unsolved health problem of world wide dimension. Recently resurgence of acquired immunodeficiency syndrome has complicated the problem of tuberculosis control in a big way. Reappearance of hidden cases and disease due to multi-resistant forms of mycobacterium have all the more thrown a challenge in taming the disease.

A dramatic new report sponsored jointly by leading American and International health agencies have confirmed that TB "Hot Zones" are spreading world wide where the new strains are virtually drug resistant which could potentially lead to a "virtually incurable" TB world wide. Among the newly identified "Hot Zones" is India, where Delhi leads with a ratio of 13% of all TB patients who were ill with multi drug resistant (MDR) TB.

The alarming study titled "Anti-TB drug resistance in the world" was conducted by WHO and other leading agencies. According to one leading US authority, Dr Michel Iseman of the National Jewish Medical and Research Centre, it provided the first scientific evidence of what we fear but could not previously prove. The world again faces the spectre of incurable TB and in the developing world MDR-TB is usually a death sentence.

Apart from India, other "Hot Zones" are Russia, Latvia, Estonia, Argentina, The Dominican Republic and Ivory Coast.

Scientists have expressed concern that many of these zones are regional centres of travel emigratory and international activity and that little can be done to prevent people infected with drug resistant TB from travelling and spreading the bacteria to other countries.
REVIEW OF LITERATURE

The report explains that in each of these "Hot Zones" the disease is often resistant to the commonly prescribed drugs-isoniazid and rifampicin. This makes TB an incurable disease for those who do not have access to the most sophisticated and expensive health care system.

Under these prevailing situations, the only answer shall be to develop a vaccine which should be effective enough to provide protection against the disease. A two-pronged strategy has been devised to control/eradication of TB. They are chemo and vaccine-mediated control measures.

2.1.1 Chemotherapy

Major advances have recently been made in short duration chemotherapy. There are now a number of short term course regimens of 6-9 months duration that are very highly effective, of low toxicity and well tolerated. Essential anti-tuberculosis drugs, isoniazid, rifampicin, pyrazinamide, streptomycin, thioacetazone and ethambutal, are recommended, after the revision of the WHO model test of essential drugs. (ICMR, 1979; Katoch et al., 1987). Multidrug therapy is still more expensive, boosts resistance and out of reach of the developing countries and is unable to provide control in the occurrence of new TB cases. Rather, appearance of HIV associated TB cases have greatly emerged in developed countries, where incidence of TB a decade ago was significantly low. Therefore, it appears that chemotherapy alone may not be able to provide control over TB.

2.1.2 Vaccine Therapy

Immunoprophylaxis through vaccination is an established modality for control of infectious diseases. Vaccines act by inducing host immunity, therefore it makes the entire territory (human body) unfavourable for the
growth of mycobacterium. A general principle in microbial vaccinology is to use attenuated organisms that have lost virulence but have retained antigenicity.

Soon after the discovery of TB bacillus by Robert Koch, the search for a suitable immunizing agent against TB started. Three distinct type of vaccines were initially used from time to time.

1. Preparation consisting of significantly small numbers of live Mycobacterium tuberculosis (MTB) (Baldwin and Gardner, 1921, Aichbergan and Von, 1937) which were unable to produce disease but provide protection.

2. Preparations containing non-pathogenic mycobacteria, but pathogenic to other species of animals (Vole bacillus vaccine) (Wells, 1937, Birkhug 1944, Young and Patterson, 1949, Sula, 1955; 1958).

3. Attenuated variants of originally virulent strains of tubercle bacilli pathogenic to man (Weiss, 1959 a, b, c).

Preparations consisting of small numbers of live Mycobacterium tuberculosis never met with success as it proved to be hazardous.

The second type of vaccine was studied with some success. From a trial carried out in 1950 with certain batches of Vole bacillus vaccine, untoward reactions developed and persisted as a skin condition termed Lupus murinus (Maguire, 1968) therefore the vaccine was dropped.

Attenuated type of vaccine was produced by Calmette Guerin under the official name BCG VACCINE which originated from a virulent bovine strain of the tubercle bacillus, that had been isolated by Nocard from udder of a cow with tuberculous mastitis.
BCG is an attenuated strain of *M. bovis*. It was obtained after 239 passages of virulent *M. bovis* in vitro (Calmette, 1927). In 1921 this vaccine was first administered orally on the third, fifth and seventh day to a newborn in hospital Dela Chairite in Paris. (Weill-Halle, 1980). Although the child was at high risk of TB as his mother died of TB and grandmother was suffering from the disease, he remained healthy throughout his life. BCG was recommended by the League of Nations in 1928 for widespread use in the prevention of TB.

2.1.3. BCG VACCINE TODAY

Several BCG vaccine strains are in use today. Three parent strains (Glaxo-1077, Tokyo-172 and Pasteur 173P2) account for over 90% of BCG vaccines in use. Besides these, other BCG strains used are BCG Copenhagen (Davis), BCG Russia, BCG Madras, BCG Tice and BCG Moreau.

BCG has been found to protect against TB, although it has faced serious controversies in recent years. In randomized trials, conducted all over the world, the protection given by BCG strains against TB was found to vary from 80% at Haiti to nil at Chingelput, Madras (TB Prevention Trial Madras, 1980).

A brief summary of 9 trials is as follows:

**Protective efficacy of BCG vaccine against TB**

*(Trials between 1935 to 1975) (Tuberculosis Prevention Trial, 1980)*

<table>
<thead>
<tr>
<th>Population group</th>
<th>Period of intake</th>
<th>Protective efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North American Indians</td>
<td>1935-1938</td>
<td>80</td>
</tr>
<tr>
<td>Chicago infants</td>
<td>1937-1948</td>
<td>75</td>
</tr>
<tr>
<td>Georgia school children</td>
<td>1947</td>
<td>0</td>
</tr>
<tr>
<td>Illinois children</td>
<td>1947-1948</td>
<td>0</td>
</tr>
<tr>
<td>Puerto Rico population</td>
<td>1949-1951</td>
<td>31</td>
</tr>
<tr>
<td>Georgia and Alabama</td>
<td>1950</td>
<td>14</td>
</tr>
<tr>
<td>British children</td>
<td>1950-1952</td>
<td>78</td>
</tr>
<tr>
<td>South Indian rural</td>
<td>1950-1955</td>
<td>31</td>
</tr>
<tr>
<td>Chingleput (South India)</td>
<td>1968-1975</td>
<td>0</td>
</tr>
</tbody>
</table>
Different strains of BCG have also been found to give varying degree of protection (Comstock, 1988, Tendam, 1993). The variable efficacy of BCG has been attributed to several reasons. Previous sensitization of individuals with atypical mycobacteria have shown to reduce protection given by BCG (Palmer and Long, 1966). Differences in antigen expression and genetic differences between different BCG strains have also been reported to interfere with protection given by BCG. Moreover, genetic differences within population have been found to influence the protection given by BCG (Stead et al., 1990, Stead, 1992).

The pathological manifestations imparted by BCG have gain interest in recent years. BCG has always been associated with adverse effect in the vaccinated individuals (Little et al., 198). In case HIV infected individuals the question of safety of BCG vaccine is an area of active interest and evaluation. Few cases of BCGitis have been reported in children which were later found to be HIV seropositive (Vonroyn et al., 1987, Braun and Cauthen, 1992, Weltman and Rose, 1993). WHO has recently recommended BCG vaccination to be excluded in the children showing overt signs of immunodeficiency (WHO, 1987). BCG vaccination has also been reported to cause disseminated BCGitis in case of HIV infected individual. Recently a case was reported in AIDS patient where reactivation of BCG was displayed 30 years after vaccination (Armbruster et al., 1991). Naturally the situation is obvious that at present we have nothing in our hand as vaccine to control TB. The scientists have not developed the courage to discontinue BCG despite its controversial status. BCG although not fully effective against pulmonary TB, certainly has protective efficacy against TB meningitis.
2.2. CANDIDATE VACCINE UNDER STUDY

Tempted with the present day scenario the group working in Central Drug Research Institute, Lucknow have developed another atypical strain mycobacterium designated *M. habana* which has afforded consistent protection against live challenge with indigenous strains of *M. tuberculosis* of varying virulence and also to foreign strains of *M. tuberculosis* H$_3$7, Rv. The mycobacterium protects mice against live *M. leprae* challenge in foot pad and is also protective against *M. ulcerans* (Buruli ulcer in mice) (Singh *et al.*, 1981, 1985, 1989; Gupta *et al.*, 1979). It generates strong cell mediated immune responses recognized by *M. tuberculosis* and *M. leprae* antigens and also shares immunologically dominant proteins with these disease producing mycobacteria.

This mycobacterium was isolated in 1971 from sputum of a tuberculous patient in Cuba (Valdivea, 1971). This strain was procured from Trudeau Type Mycobacterial Culture Collection Centre, Surnac Lake, New York, bearing TMC No 5135.

This mycobacterium belongs to non- tuberculous or atypical group of mycobacteria and is a photochromogen according to Runyon classification (1959). It shows two strong markers and can easily be distinguished from other mycobacteria. The bacteriological studies of *M. habana* are as follows:

1. Colony morphology: Smooth, round and raised colonies in LJ Medium.
3. Pigment: No growth on MacConkey medium upto 4 weeks
5. Catalase test: 45 mm-positive, 68°C pH 7.2-positive.
6. Staining characteristics
   1. Acid fast positive, deep stain.
   2. No cording or particular arrangement.

2.2.1. BASIC FACTS ABOUT *M. habana*
1. *M. habana* protects mice against MTB, arrest the growth and colonization of *M. leprae* in the foot pad of vaccinated mice. This has been confirmed nationally and internationally, National confirmation by JALMA Institute, Agra and international confirmation by RJW Rees of National Institute for Medical Research, Mill Hill, London.
2. It shares immunodominant proteins with MTB, *M. leprae* and BCG.
3. *M. habana* generates strong CMI responses recognized by *M. leprae* and MTB antigens.
4. Crude cell wall preparation of *M. habana* affords protection against live MTB challenge in vaccinated mice.
5. 23 KDA immunodominant protein in *M. leprae* and MTB is also abundantly present in *M. habana* and is a superoxide dismutase.
6. Secretary protein of *M. habana* affords protection in mice against MTB challenge.
7. *M. habana* is non-pathogenic.
8. 65 KDA protein of *M. habana* affords protection against live MTB challenge in vaccinated mice.
9. The vaccine is non-toxic, very little pyrogenic and does not release histamine.
2.2.2. STRATEGIES IN DEVELOPMENT OF ANTITUBERCULOSIS VACCINE(S)

Wide-spread use of BCG has not controlled tuberculosis, and more effective vaccines are clearly needed. Strategies to construct recombinant vaccines against intracellular pathogens have been devised (Stover et al., 1991, Aldovini and Young, 1991) but ultimate development of anti-TB vaccines hinges on an improved understanding of the human immune response to MTB. One can imagine at least two types of vaccine: one to prevent primary infection in tuberculin negative persons and another to prevent reactivation TB in tuberculin reactors. One strategy for developing anti-TB vaccine is to characterize the T-cells and cytokines that mediate clearance of bacilli during the primary immune response as well as the memory T-cell subpopulation and cytokines that protect against TB from exogenous reinfection and endogenous reactivation. Mycobacterial antigens that are recognized by these T-cells and that elicit production of protective cytokines are potentially important vaccine antigens. Because antigen recognition is often linked to expression of specific MHC molecules (Major Histocompatibility Complex) which are highly polymorphic in the population. An effective vaccine would include different antigens that elicit protective immunity, which could be cloned in a live vaccine vector that immunogenic antigen is appealing. Mycobacterial genes coding for protective mycobacterial antigens can be incorporated into a vector organism such as BCG, which will have the potential to secrete multiple immunogenic proteins. Development and wide-spread administration of such a vaccine may play a major role in the elimination of TB throughout the world.
Acquired resistance against TB paradigmatically rests on cell mediated immunity with the major factors being mono-nuclear phagocytes (MP) and T-lymphocytes. The former cells act as a principal effectors and the later ones serve as predominant inducer of protection. MP provides the preferred biotype for MTB and hence play a dual role in TB - the protection against the disease and survival of the pathogen. T-cells produce protective immunity and also contribute to pathogenesis. A co-ordinated cross-talk between Mφs and T-cells, therefore is essential for optimum protection.

Evidences have been obtained that murine Mφs possess anti-mycobacterial function in tissue culture (Lurie, 1942; Suter, 1952). It has been demonstrated earlier that these cells when activated in vitro by supernatant of immunologically stimulated lymphocytes had various degrees of anti-mycobacterial activity (Cahall and Youmans, 1975a, b; Muroak et al., 1976a, b). Soon H₂O₂, one of the reactive oxygen intermediate (ROI) generated by Mφs during the oxidative burst was identified as molecule that mediated mycobactericidal effect of Mφs (Walker and Lowrie, 1981). This binding marked the beginning of much debate concerning the significance of ROI in host defense against MTB. Later, γ-interferon (IFN-γ) was found to be the key endogenous activating agent that triggers the anti-mycobacterial effect of murine Mφs (Rook et al., 1986; Flesch and Kaufmann, 1987). Recombinant DNA technology has facilitated in vitro experiment to evaluate the potential of these interesting molecules in host defense against MTB and have led to the identification of other mediators like cytokines and TNF-α, which play significant role in protection against MTB. TNF-α also appears to play critical role in the control of BCG infection in vivo in mice and monoclonal anti-body
to this cytokine resulted in poor granuloma formation and disseminated BCG infection in mice. Anti-TNF-α-anti-bodies markedly exacerbate disease progression in murine experimental TB (Flynn et al., 1993).

Other cytokines have also been implicated in MΦs defense against MTB. *In vitro* IL-4 and IL-6 have the ability to induce MΦs anti-mycobacterial activity (Kaufmann et al., 1989; Flesch and Kaufmann, 1990a, b) by undefined mechanism.

It has been known that IL-8 plays a role in granuloma formation by virtue of its ability to act as chemotactic agent for T-cells (Friedland et al., 1993). IL-1 (Dunn et al., 1988; Kasahara et al., 1988), IL-2 (Mathew et al., 1990; Cheever et al., 1992), IL-4 (Chensue et al., 1992) and IFN-γ (Chensue et al., 1992), all have been reported to contribute resistance against MTB because of their capacity to form granuloma.

Recent studies have indicated that IL-12, a recently characterized heterodimeric glycoprotein produced by various immune cells including MΦs (Gazzinelli et al., 1992) may confer resistance to TB in mice. IL-12 plays important role in resistance to *Leishmania major*, *Trypanosoma gondii* and *Listeria monocytogenes* (Gazzinelli et al., 1993). The events triggered by IL-12 help to identify natural killer (NK) cells as a critical cellular component in defense against MTB.

It is now becoming clear that these molecules (cytokines) interact dynamically to form a highly co-ordinated network that is configured by both host and pathogen specific factors, which together influence disease outcome and progression.
Therefore, study of the molecules involved in respiratory burst (ROI, RNI) and cytokines being generated at molecular level in conjunction with granuloma formation may be responsible for generation of protection and examination of these molecules may answer to some extent the protection efficacy of a vaccine candidate and that is what has been done during the present investigations.

2.3. IMMUNOLOGY OF TUBERCULOSIS

The immunology of TB is a subject which has attracted tremendous interest in recent years. Still, there are many aspects which remain poorly understood. TB remains the classic example of a disease that is controlled entirely by CMI involving the MΦs as the effector cell and the lymphocytes (specially T-cell) as the immunoresponsive cell (Mackaness, 1968). This type of immunity is also called acquired cellular resistance. CMI is essentially a local phenomenon carried out by the MΦs that are activated locally at the site of infection by lymphocytes and their lymphokines. Crowle et al., (1983) have summarized the following characteristics of tuberculo-immunity:

1. It is cell mediated.
2. It is expressed by appropriately activated MΦs.
3. The MΦs are appropriately activated to produce immune lymphokines.
4. The immune lymphokines are also made by immune T-lymphocytes which are activated by immunizing antigens of the tubercle bacillus.

2.3.1. CELL MEDIATED IMMUNITY

The immune response is a complex phenomenon involving the differentiation of cells in at least two directions. One is to production of cells specializing in the synthesis and secretion of humoral antibodies. The other
leads to the production of specifically sensitized cells, accepted as being lymphocytes, which are responsible for initiating the events as constituting CMI. The characteristic of CMI reaction, is the sensitization of small lymphocytes derived from thymus (T-cell), to an antigen. Mycobacterial vaccines through their antigenicity bring about the same desired function by stimulating the body to generate sensitized cells which mediates the cellular immunity. Vaccines preparations normally consist of the attenuated or killed form of pathogen itself or some antigenically cross reacting bacteria. There are several ways to screen out such strain but they have to be rigorously tested for human trial. WHO has laid down a definite protocol for testing such strain:

1. These strains should have common antigens with MTB
2. Should show positive DTH.
3. Should be positive in passive transfer of DTH to other animals.
4. Should be quantitated with respect to skin erythema, foot pad and lymphnode enlargement tests.
5. Should show protection in mice against virulent challenge with the causative agents.

Briefly, these tests have been divided for convenience of description into following in vitro and in vivo methods

2.3.2. IN VITRO METHODS
1. Lymphocytes migration inhibition test (LMIT).
2. Lymphocytes transformation test (LTT).
3. Phagocytic activity of sensitized cells.
2.3.3. IN VIVO METHODS

1. Delayed type hypersensitivity skin reactions (DTH)
2. Foot pad enlargement (FPE).
3. Passive transfer of immunity to normal animal.
4. Draining lymphnode enlargement.

2.3.4. Cellular interaction in CMI

The phagocytic cells (macrophages, Langerhan's cells) are known to engulf and process the invading bacilli and soluble products. Also, designated as antigen presenting cells (APC), they are capable of presentation of the processed antigens to the T-cells through their receptor (Editorial, 1993). It has been well documented that, at the initial stages of the CMI response, T-cells cluster around the surface of the APCs before transforming into blast cells. Thereafter, production of interleukin-2 (IL-2) along with the expression of IL-2 are necessary for the replication of these antigen specific lymphocytes for clonal expansion. While such clonal expansion goes on, the cellular interaction further liberates a variety of other interleukins (IL-1, IL-3, IL-4 to IL-8) and lymphokines [granulocytes, monocytes, colony stimulating factors, IFN-γ, tumour necrosis factor-α (TNF-α)], which influence the morphological and functional behaviour of various CMI inducing cells. In general, all these cells consisting of activated mononuclear phagocytes, cytotoxic T-cells, NK cells and lymphokine activated killer cells create the environment of CMI. Effector molecules such as IFN-γ and TNF-α are known to activate Mφs so that they have an enhanced production of toxic ROI and RNI (Chan et al., 1992) in order to more effectively kill both intracellular and extracellular microorganism. Further, IL2 and IL-6 influence T, NK and LAK cells to become
cytotoxic by increasing their perforin (pore forming protein) content and leucotoxin. These IL are also known to modify the function of other cells such as endothelial cells, keratinocytes and Langerhans cells. All these factors collectively influence the cellular function in situ and also further recruit appropriate cells from the circulation. Ultimately, leading to the formation of an immune granuloma for the destruction of the invading micro-organism (Editorial, 1993).

2.3.5. DELAYED TYPE HYPERSENSITIVITY

Delayed type hypersensitivity (DTH) is responsible for the rapid accumulation of lymphocytes and Mφs which became activated wherever tubercle bacilli and their tuberculin like antigens exists in the tissues. DTH, is by the definition, an adverse immunological reaction to components of the tubercle bacilli (Rich, 1951) that is mediated by T cell and their lymphokines. CMI is by definition, a favourable immunologic reaction to bacillary components that also is mediated by T cells and their lymphokines. It has been clearly shown that CMI involves DTH (North, 1974; Collins, 1982). In both CMI and DTH, T lymphocytes accumulate at the site of antigen deposition, produce lymphokines and attract (as well as activate) Mφ and lymphocytes (Collins, 1982).

Why then is CMI beneficial and DTH detrimental, if both are produced by the same immunologic mechanisms? This question has no simple answer, two factors seem to play a major role: the local concentration of the bacillary antigens and the characteristics of these antigens (Collins, 1982).
2.3.5.1. HELPFUL AND HARMFUL EFFECTS OF CMI AND DTH PROCESS:

Low local concentration of the antigens are beneficial in the tuberculin positive host, because they are the stimulus for the development of CMI (local M+ activation) (North, 1974, Collins, 1982). On the other hand, high local concentration of the antigens are very harmful in the tuberculin positive host and cause almost all of the tissue destructions (ceasation and liquefaction) in this disease. For example, tuberculin classically causes marked necrosis when too high a concentration is injected in the skin of such host. The dose of inhaled exogenous tubercle bacilli is always low (only a few bacilli at a time). Therefore, the presence of DTH and CMI is always helpful in preventing exogenous reinfection with inhaled tubercle bacilli (Collins, 1982).

The DTH components of CMI speeds up the accumulation and activation of lymphocytes and M+ at the site of bacillary deposition. The presence of the DTH component is also helpful in destroying haematologically spread (endogenous) bacilli, because only a few of these bacilli are deposited in a given site at a given time. The presence of DTH is harmful, however, whenever tubercle bacilli and their antigens reach high local concentration, then DTH causes cessation and tissue destruction. Such concentration of bacilli and their antigens occur in areas of the lung that are seeded by liquefied caseous material discharging from a cavity into the bronchial tree and within poorly activated M+ of genetically susceptible or immunodeficient host, where the bacilli are able to multiply extensively (Collins, 1982).
2.3.5.2. ANTIgenic COMPOSITION AND VACCINE DEVELOPMENT

An ideal vaccine for TB would contain components of the bacilli that produce more activated (microbicidal) M+is and less tissue destruction (more CMI and less DTH). Such a vaccine would favour the expansion of T cell populations that increase the ability of M+is to destroy tubercle bacilli. Over the expansion of T cell populations that directly or indirectly produce tissue necrosis. An appropriate animal test system for screening new vaccines for such effects is a pre-requisite for rapid progress towards the goal (North, 1974).

2.3.5.3. DURATION AND SPECIFICITY OF CMI AND ITS RECALL UPON RE-INFECTION

Acquired cellular resistance (the number of activated M+is) decreases with time after immunization with BCG. Such resistance, however, can be rapidly recalled to full strength by re-exposure to the BCG immunizing antigens (Dannenberg, 1968). From the first exposure the host retains increased number of T-cells (clonally expanded) with specific receptors for these antigens. Upon re-exposure, these T-cells rapidly produced lymphokines, and an acceleration of M+ accumulation and activation occurs (Dannenberg et al. 1968).

The injections of other types of facultative intracellular bacteria do not recall immunity to tubercle bacilli. However, once M+is have been activated in local sites (by BCG, the specific antigen), these M+is can non specifically destroy a variety of facultative intracellular microorganisms (in the same local sites). The specificity of CMI resides entirely in the T-lymphocytes, not in the
Macrophages kill facultative intracellular micro-organisms only non specifically \( \text{(Daunenberg, 1968)} \).

Mackaness, \( \text{(1968)} \) administered the immunizing (and recalling) BCG intravenously. In this case, most of the bacilli were deposited in the liver and spleen, where many local sites developed that contained activated M\( \text{fs} \). Such M\( \text{fs} \) could rapidly destroy other type of facultative intracellular bacilli that were deposited in the liver and spleen by a subsequent intravenous injection.

The number of specific T- cells in the blood and tissues decreases with time, and the tuberculin reaction may even disappear. These events may occur if the tubercle bacilli and their antigens are eliminated. Nonetheless, both the tuberculin reaction and the large numbers of specific T-cells will be rapidly recalled by re-infection. Tuberculin itself may even have a booster effect in recalling a disappeared skin reaction (Thompson \textit{et al.}, 1979).

2.3.6. LYMPHOCYTE TRANSFORMATION TEST (LTT)

\textit{In vitro} LTT is an alternative method for monitoring CMI responses. It has been widely used in mycobacterial diseases. Clearly immunological information for the CMI response is carried out by lymphocytes and specially the small lymphocytes have been obtained from hypersensitive animals. They affected passive transfer of reactivity to normal recipient. Lymphocytes profuse the tissues and sensitization is likely to occur in the periphery, the lymphocytes migrating from a graft or tissue site to original lymphnodes. Moreover, lymphocytes have been shown to carry immunological memory. This transformation could be initiated in normal lymphocytes or non specifically by antigen like phytohaemagglutinin (PHA) in the \textit{in vitro} system. The fact that
lymphocytes can be transformed at will in vitro by specific antigens or non specific antigen such as PHA, concavalin (Con-A) streptolysins, antilymphocytic sera suggest that this system might be useful for studying the mechanism of regulation and control of mammalian cells. In vitro studies have revealed that antigen activated lymphocytes generate soluble factors to affect the CMI (Touw et al., 1980, Mehnei et al., 1982). Recent work has shown that two of these soluble factors can be produced by the activation of lymphocytes

1. A factor which inhibits the migration of macrophages.
2. A factor which damages mouse fibroblasts in mononuclear culture (lymphotoxin)

There is another evidence that antigen activated lymphocytes generate a third activating factor known as mitogenic factor which stimulates the DNA metabolism of lymphocytes and which may be a soluble mediator of lymphocyte transformation. Ideally, the LTT is an in vitro test for CMI (Standford et al., 1980, Bjune, 1981; Chirmule et al., 1986). Specific immunity or resistance to infective agents can be achieved either by the production of antibodies or by the development of CMI mechanisms or by both. CMI acts by activating macrophages, and releasing soluble factors, collectively termed as lymphokines from specially sensitized lymphocytes. Proliferation in the presence of an antigen indicates that sensitization of that antigen has occurred. The LTT offers several advantages over the skin test. First, it can be performed repeatedly on the same individuals without affecting his response. Secondly, it offers a mean of testing several doses of antigen. And lastly, because proliferation is assessed by thymidine incorporation, the results are quantitative and not subject to any reading bias (Standford et al., 1980)
2.3.7. FOOT PAD ENLARGEMENT

The mouse foot pad model is being described in view of the extensive work carried out by different laboratories and also a wide application of the model. The first experiment from the nasal washings of leprosy patient showed a consistent multiplication of the bacilli in the foot pad. It has now become a standard experimental procedure in a number of laboratories all over the world and it has been established in India since 1965 (Standford et al., 1980).

Foot pad enlargement test is a good correlate of CMI (Lefford 1975, Patel and Lefford, 1978, Shepard et al., 1980). It is widely used in detecting DTH in rodents. Hastings (1981) has shown that foot pad inoculation results in enormous swelling. The present study was initiated to evaluate the immunogenic potency of \textit{M. hahana} vaccine in mice. This was determined by foot pad enlargement. The typical delayed reaction reaches a peak at 24 hours and fades after 72 hours, when an antigen is injected into previously immunised animals.

2.3.8. MODELS FOR VACCINE DEVELOPMENT

If a vaccine for TB that is superior to BCG is to be developed, an appropriate animal model would be required to test its ability to induce both beneficial and detrimental effects in the CMI-DTH response. Lurie (1964) used the rabbit model because both its native resistance and immune responses were, in general similar to those of human (if the appropriate strains of tubercle bacilli were used for challenge). Guineapigs and most monkeys seem to have lower native resistance than do human to both human and bovine strains of tubercle bacilli (Dannenberg, 1984). Rats and mice develop weaker DTH responses and produce much less caseous necrosis than do the other three species.
2.4. SOURCES OF PHAGOCYTIC CELL

Macrophage and macrophage-like cells can be obtained from a variety of vertebrate and invertebrate species. In all vertebrate species M\(^+\)s are readily obtained from the serus cavities (peritoneal, pleural sac), but they can also be isolated from lung, liver, spleen, lymphnode, thymus and synovium (George et al., 1984).

2.4.1. SECRETION OR RELEASE OF ENZYMES AND OTHER PRODUCTS FROM MACROPHAGES

a. ENZYMES HYDROLYZING TISSUE COMPONENTS

1. Acid acting lysosomal enzymes: Proteinase, acid phosphatase and various glycosidases.

b. PRODUCT INVOLVED IN HOST DEFENSE

1. Lysozyme.
2. Complement components.
3. Interferons.
4. Microbicidins: Cationic proteins, \(O_2^-\), \(H_2O_2\).  

c. PRODUCTS MODULATING CELLS

1. Colony stimulating factors that increase the production of phagocytes by the bone marrow.
2. Substances that stimulates the division and differentiation of T and B lymphocytes and also substances that suppress lymphocyte functions.
3. Factors that stimulate the growth of new capillaries.
4. Substances that stimulate fibroblasts
5. Osteoclast activating factor
6 Prostaglandin and c-AMP.
7 Chemotactic factor inactivators.

d. MISCELLANEOUS PRODUCTS
1. Endogenous pyrogens, causing fever and decrease plasma iron and zinc levels.
2. Thromboplastin.
3. Alpha 2 macroglobulin (the protease inhibitor).
4. Transferrin.
5. Transcobalamin II (vitamin B₁₂ transport protein)
6. Ss protein an antigenic euglobulin in serum, that is a product of the histocompatible gene complex.

2.4.2. PHAGOCYTIC ACTIVITY
Phagocytosis is a process by which foreign particles are ingested by leucocytes and by certain endothelial cells of the body. Phagocytosis is a normal function of the body cell, but definitely stimulated by invasion of pathogenic bacteria. The real function of phagocytes in the disease process was shown by Metchnikoff (1907).

2.4.3. INDUCTION AND INDUCING AGENTS FOR MACROPHAGES
Except in mice and rats, the yield of cells from the peritoneal cavity is too low to make the use of untreated animals economical. The yield of M₄s can be increased greatly by the injection of substances into the peritoneal cavity prior to harvesting the resulting peritoneal exudate. The best inducing agents are those that provoke an exudate rich in M₄s and that are readily bio-degradable, leaving no trace in the cultured cell. The use of inducing agent has many drawbacks some of which are:
1. Induced M$^\dagger$ have a different cytochemical profile from that of non-induced M$^\dagger$s, in particular, the levels of hydrolytic enzymes are increased in induced M$^\dagger$s.

2. Depending on the inducing agent used, the isolated M$^\dagger$s may contain phagocytosed granules of the agent, which may interfere with the parameters under study. This is specially true of mineral oil.

3. Some inductants cause clumping of M$^\dagger$s, and corresponding difficulties in counting and culture occur.

2.4.4. MORPHOLOGY

The normal morphology of M$^\dagger$ cultures is illustrated (Fig. 13 and 14).

In general M$^\dagger$s in culture exhibit two basic type of morphology: they are either extended and stellate or rounded cells with abundant cytoplasm. In older culture it is found that the proportion of stellate cells increases with increase in time of the culture. The M$^\dagger$ nucleus may be rounded, indented (reniform) or, more rarely, bilobed (Stuart et al., 1978).

In peritoneal cell cultures from other animals (except guineapig) only one class of M$^\dagger$ appears to be present. After 24hr it can be shown that almost 100% of the glass-adherent cells are actively phagocytic. With increasing time in culture the stellate forms increase (Stuart et al., 1978).

2.4.5. MACROPHAGE ACTIVATION

M$^\dagger$s must be activated before they can destroy tubercle bacilli (Ando et al., 1977). Activation is caused by biologically active mediators (lymphokines) and by the ingestion of necrotic cells and tissues. In the tubercle, the local accumulation of large numbers of M$^\dagger$ and lymphocytes and the activation of many of these cells can restrict the growth of bacilli and arrest the disease (Dannenberg et al., 1968).
Activated MΦs contain many mitochondria and lysosomes and have high levels of oxidative and digestive enzymes. These MΦs metabolize vigorously and can destroy tubercle bacilli more readily than non-activated MΦs. Their microbicidal power is thought to be due to ROI (H$_2$O$_2$, O$_2^-$, $^1$O$_2$ and OH) (Babior, 1978), digestive enzymes (Dannenberg, 1968) and other agents.

2.4.5.1. MICROBICIDAL ACTIVITY OF MACROPHAGES

The tubercle bacillus is a facultative intracellular parasite, i.e., it can multiply intracellularly within the MΦ of the host. Yet these same MΦs are the host's only effective defence against the bacillus. The killing of these organisms by the MΦs was first described by Lurie, (1964). Monocytes in the circulation emigrate to the tissues where they are called MΦs. At first, they are quite immature and incapable of destroying ingested tubercle bacilli. With passage of time in a resistant host, e.g., human beings over one year of age, the local MΦs become activated because of lymphocytes and their lymphokines and other factors. Only then can the MΦ destroy the tubercle bacilli growing in it (George et al., 1984).

With certain bacteria, the hydrolytic enzymes in lysosomes and phagosomes may be lethal. For example, if a lipase could remove the outer lipid coats of tubercle bacilli, lysozyme would be able to hydrolyse the newly exposed peptidoglycan backbone. Tubercle bacilli without their lipid coats are still viable but are more readily destroyed by the host (Bloch, 1950).

Recently, the microbicidal factors H$_2$O$_2$ and O$_2^-$ have been shown to be produced by activated MΦ (Johnston et al., 1978; Nathan et al., 1979). Since H$_2$O$_2$, O$_2^-$ are released extracellularly by activated MΦs (Johnston et al., 1978; Nathan et al., 1977, 1979), these oxidant may even kill bacteria before, as well
as after, they are ingested by the phagocytes. H$_2$O$_2$ is quite effective against several microorganisms (Nathan et al., 1979). O$_2^-$ probably works synergistically with H$_2$O$_2$ to produce the hydroxylradical (OH) and singlet oxygen (Klebanoff, 1980; Murray et al., 1979) OH and 'O$_2$ are probably the most important microbicidal agent against certain intracellular microorganisms (Murray et al., 1979) and 'O$_2$ apparently can kill BCG in vitro (Ando et al., 1977).

A. TYPE OF MACROPHAGE ACTIVATION

Many types of M$^+$ activation or differentiation exist; e.g., microbicidal, digestive, secretory, antigen-processing, and lymphostimulatory. Some M$^+$ may specialize for some of these functions, other M$^+$ for other functions, and still other M$^+$ may perform two functions at once. Double staining histochemical methods identified M$^+$ with different functions in rabbit dermal tuberculosis lesions produced by BCG (Suga et al., 1980).

In TB, the killing of the bacilli by M$^+$ is usually followed by the hydrolysis (digestion) of their components and the release of antigens for presentation to lymphocytes. Thus, microbicidal and digestive functions would probably occur in the same M$^+$, but other M$^+$s (usually nearby) might be involved in antigen presentation and the regulation of lymphocytes to it. No information is yet available to support or refute these possibilities, although heterogeneity of M$^+$ population is well established (Suga et al., 1980; Walker, 1976).

B. BASIC MECHANISMS OF ACTIVATION

Activation may be excitation and adaption phases of M$^+$. Excitation is the immediate response of these cells to any irritant (including the ingestion
of particles (e.g., phagocytosis) and should be readily reversible. It is characterized by increased oxygen consumption, glycolysis, lipid turnover, and other metabolic phenomena (Oren et al., 1963). These excited cells probably act more faster and ingest particles more efficiently (Oren et al., 1963) than other cells. Adaptation refers to more permanent changes produced in Mφ in response to irritation. It is often characterized by increase in the number of lysosomes and mitochondria and in their enzymes. Adaptation is reversible (Cohn et al., 1965), but is not as rapidly reversible as cell excitation.

Cohn (1978) made thorough studies on factors involved in Mφ activation in vitro (Axline and Cohn, 1970; Bennet and Cohn, 1966; Cohn 1966, 1978). Lysosomal enzymes seem to be made in the endoplasmic reticulum and packaged by the Golgi apparatus into primary lysosomes then fuse with phagocytic vacuoles which contain the material to be digested (Cohn 1966). These secondary lysosomes now rich in enzymes are evidently capable of fusing with more recent phagocytic or pinocytic vacuoles (Cohn, 1966, Gordon et al., 1975; Weissmann and Urh, 1968) and may be more effective than primary lysosomes against recently ingested microorganisms.

C. ACTIVATION BY LYMPHOKINES

Lymphokines are biologically active factors affecting other cells, which are usually T cells exposed to specific antigens. Representative lymphokines are chemotactic factors (CF), migration inhibitory factors (MIF), mitogenic factors (MF) and lymphotoxin. In tuberculous lesions, lymphokines are elicited not only by tuberculin, but also by other antigens of tubercle bacillus.

CF, MIF and MF apparently cause cell infiltration, cell activation and cell division, respectively. The lymphokine type of activation is exceedingly
sensitive, because it involves lymphocytes possessing specific receptors to antigens of the tubercle bacillus. Lymphokines apparently cause the accelerated tubercle formation that is associated with cellular hypersensitivity and cell mediated immunity (acquired resistance). At the site of bacillary lodgement, there is a rapid accumulation and activation of $M\phi$s which limit the multiplication of tubercle bacilli.

2.4.5.2. Non-lymphokine type of activation

The disease begins only when an exceptionally hardy bacillus is ingested by a relatively weak alveolar $M\phi$s. The type of $M\phi$ activation associated with phagocytosis (and pinocytosis) is primitive. Peritoneal $M\phi$s (in culture) increased their content of lysosomal enzymes following the injection of digestible substances and boiled yeast particles (Dannenberg et al., 1963) $M\phi$s entering tuberculous lesions apparently ingest and digest tubercle bacilli and caseous material, and then increase their lysosomal (digestive) enzyme content (Ando et al., 1977). The $M\phi$s enlarge, and not only become rich in lysosomes and digestive, but also in mitochondria and oxidative enzymes (Dannenberg et al., 1963). Thus, the $H_2O_2$, $O_2^-$ induced microbicidal power of these cells is probably enhanced. It is not known whether macrophages activated by the injection of digestible material have microbicidal powers comparable to those of $M\phi$s activated by lymphokines. Different stimuli induce in $M\phi$s for different enzymes and different microbicidal (or inhibiting) function (Morahan et al., 1980). It is also not known whether the arming of $M\phi$s by cytophilic antibodies from B-lymphocytes and/ or by antigen specific components from T-lymphocytes plays an important role in the activation of $M\phi$s and in the development of their microbicidal capacities (George et al., 1984).
2.4.5.3. HYDROLYTIC ENZYMES IN MACROPHAGES OBTAINED FROM ANIMALS WITH ACQUIRED RESISTANCE TO TUBERCULOSIS

A. PERITONEAL MACROPHAGES

Peritoneal Mφ elicited by mineral oil in TB or BCG infected rabbits sometimes show lysozyme (Carson et al., 1965; Mizunoe et al., 1965) and acid phosphatase (Allison et al., 1962) levels more than control Mφs. Both groups show the same levels of proteases, esterases, non specific lipase, deoxy ribonuclease, ribonuclease and certain metabolic enzymes (Carson et al., 1965, Mizunoe et al., 1965; Allison et al., 1962). These enzymes are not specific for tubercle bacilli because they hydrolyse some of the basic constituents of all living things. No enzyme pattern specific for the digestion of tubercle bacilli has yet been described for Mφs from the tuberculous host, but specific enzymes can be induced in Mφs by specific substances (Pearson et al., 1967).

Macrophages washed from the peritoneal cavities of BCG infected mice show elevated acid phosphatase, Beta gluronidase, cathepsin levels and protein contents (Saito and Suto, 1965). Although no irritant was injected intraperitoneally in these mice, the moderate numbers of free Mφs in the peritoneal cavities of normal mice suggest that a constant source of irritation exists there, perhaps from endotoxin diffusing from the gut. Peritoneal Mφs after intraperitoneal injection of tuberculin in BCG infected mice, show marked differences from resident peritoneal Mφ (Edelson and Erbs, 1978). They spread rapidly in culture, have an elevated pinocytosis and show decreased 5'-nucleotidase activities.
B. LYSOSOMAL MECHANISMS

Although increase in lysosomal enzyme content is associated with Mφ activation, this cannot be taken as a cause, and may not even be a consequence, of mycobacterial killing (Lowrie, 1983; Katoh, 1981). Pathogenic mycobacteria and some non-pathogenic ones are resistant to digestion by Mφs lysosomal enzyme, even when they are dead (Andrew et al., 1985). Indeed mycobacterial cell wall components can inhibit some of the lysosomal enzymes. Even when the mycobacterial wall is substantially weakened, the consequences are not necessarily fatal. For example, supplementation of monocyte culture with high concentration of mixture of lysosomal enzymes can cause the appearance of cell wall deficient forms of intracellular tubercle bacilli. Whether lysosomal enzyme attack plays a part in the development of aberrant and non-acid fast forms in normal infection is not clear. M.ovi um loses acid fastness in Mφ despite apparently avoiding exposure to lysosome contents (Crowle et al., 1986).

C. ALVEOLAR MACROPHAGES

The intravenous (or intratracheal) infection of heat killed tubercle bacilli produces granulomas in the lung of mice and rabbits (George et al., 1984). Alveolar Mφs which are washed out of such lungs by bronchial lavage, have increased levels of lysosome, catalase, beta glucuronidase, acid phosphatase (Cohn, 1978; Mizunoe et al., 1965). These cells also have increased bactericidal ability (Evans and Myrvik, 1967, 1968).

In summary, these findings indicate that TB increases the levels of certain hydrolytic enzymes in peritoneal and pulmonary alveolar Mφs. Nonetheless, the study of enzymes and other factors in Mφs washed out of the lungs or
obtained from peritoneal exudates or blood has provided (and will continue to provide) only a small amount of information that is useful in understanding the pathogenesis of TB. This disease is controlled locally by Mφs that are activated within the tuberculous lesion. Mφs obtained from non-infected sites in the body are only influenced by factors absorbed systemically from the lesions, and therefore would not accurately reflect the local acquired resistance within the lesions.

2.5. RESPIRATORY BURST

When a particle attaches to the surface of macrophage, it activates the enzyme nicotinamide adenine dinucleotide phosphate (NADPH). This enzyme catalysed the conversion of NADPH to NADP. When this happens electron are released and these electron combine with a molecule of oxygen to form a superoxide anion (O$_2^-$)

$$\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP} + \text{H} + 2\text{O}_2^-$$

The superoxide anion is destroyed by the enzyme superoxide dismutase (SOD), gaining a second electron and reacting with hydrogen ions to form hydrogen peroxide (H$_2$O$_2$)

$$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

Hydrogen peroxide is converted to highly potent biochemical compounds through the action of myeloperoxidase. Singlet oxygen is a form of oxygen in which one electron is shifted into a high energy orbit. Because of its distorted electron cell singlet oxygen is very unstable and also reacts with bacterial lipids to form toxic hydroperoxidase (Chakraborty et al., 1996).
2.5.1 IN VITRO ACTIVATION OF MACROPHAGES ANTI-MYCOBACTERIAL FUNCTION

Evidence has long existed that murine macrophages have an anti-mycobacterial functions in tissue culture system (Lurie, 1942; Suter, 1952, Mackaness, 1968). Earlier work by various laboratories demonstrated that these cells when activated in vitro by supernatants of immunologically stimulated lymphocytes, had various degree of anti-mycobacterial activity (Patterson et al., 1970; Klun et al., 1973 a, b; Cahall et al., 1975 a, b; Muroak et al., 1976 a, b). Soon hydrogen peroxide, one of the ROI generated by macrophages during the oxidative burst was identified (Iyer et al., 1961; Klebanoff, 1980).

This finding marked the beginning of much debate concerning the significance of ROI in host defence against MTB. Later, it was found to be the key endogenous activating agent that triggers the anti-mycobacterial effects of murine Mφ (Rook et al., 1986; Flesch and Kaufmann, 1987), furnishing a better defined system to examine the anti-mycobacterial effects of Mφs. The ROI play a significant role in host defence against microbes is best exemplified by the frequent infectious complication experienced by chronic granulomatous disease patient whose phagocytes can not mount an oxidative burst (Klebanoff, 1980). The significance of these toxic oxygen species in defence against MTB, however, remains controversial. Since the report that H₂O₂ produced by lymphokine activated murine M kills M. microti (Walker and Lowrie., 1981), much effort has been focussed on testing the role of oxygen radical-dependent killing mechanism in defence against MTB. Such effort, however provided evidence indicating that oxygen radical may not be sufficient to inhibit and/or kill MTB (Flesch and
Kaufmann, 1988; Chan et al., 1992). The validity of these findings has been reinforced by the demonstration of evasion mechanisms employed by the tubercle bacillus to elude the toxic effect of ROI. Of these mechanisms, those that are mediated by mycobacterial components lipoarabinomannan (LAM) and phenoliglycolipid 1 (PGL-1) are among the best studied and characterized (Brennan et al., 1990). LAM, a major cell wall associated, phosphatidylinositol-anchored complex lipopolysaccharide, is produced by MTB in large amount (15mg/g of bacteria) (Hunter et al., 1986). Immunogold staining has demonstrated that LAM exists in a capsular sheath encasing MTB (Hunter et al., 1986). This strategic location places LAM at the front line of attacks directed by the various antimicrobial mechanisms of macrophages. It has now been shown that LAM can incapacitate the oxygen radical-dependent anti-microbial mechanisms at least at two levels:

1. Studies using electron spin resonance spectroscopy and spin-trapping have shown that LAM is an effective ROI scavenger (Chan et al., 1991).

2. LAM can down regulate the oxidative burst by inhibiting protein kinase C (Chan et al., 1991), an enzyme that plays an important role in activation of the oxidative burst in phagocytic cells (Gennaro et al., 1985; Gavioli et al., 1987). In addition, since IFN gamma is a major factor for Mφ's activation (Hamilton et al., 1987; Fan et al., 1988) and has the ability to enhance ROI production by phagocytic cells, it is possible that LAM, by virtue of its ability to inhibit transcriptional activation of IFN gamma inducible genes (Chan et al., 1991) is able to block the expression of an as yet unidentified factor(s) inducible by this cytokine.
that is required for the oxidative burst. These results are in keeping with
the findings that mouse peritoneal MΦs treated with LAM or infected
with *M. leprae* (a LAM producing pathogenic mycobacterium) are not
responsive to IFN-gamma activation as assessed by microbicidal and
tumoricidal activities, O₂⁻ production, and surface la antigen expression
(Sibley *et al.*, 1988) and may partially explain the inability of IFN-
gamma stimulated MΦs from both human and mice to effectively kill

Other mycobacterial components that interfere with the oxygen radical
dependant antimicrobial mechanisms of MΦs are PGL-1 and the sulfatides.
PGL-1 is an oligoglycosylphenolic phthiocerol diesters with species-specific
trisaccharide. Although, universally distributed among *M. leprae*, the
expression of PGI-1 in the various strains of MTB is much restricted (*Daffe et
al.*, 1987; *Brennan et al.*, 1990). In contrast, the sulfatides, derivatives of
multiacylated trehalose 2-sulfate are widely expressed among different strains
of MTB (*Middlebrook et al.*, 1959). Because of its restricted distribution
among tuberculous isolates, the significance of PGL-1 in the pathogenesis of
**TB** remains to be determined. Nonetheless, both PGL-1 and sulfatides have
the capacity to down regulate ROI production in in vitro MΦ culture systems
(*Neill et al.*, 1988; *Pabst et al.*, 1988; *Bozna et al.*, 1991) and PGL-1 directly
scavenges oxygen radical in cell free system. (*Chan et al.*, 1989). Another
mechanism by which MTB could evade the toxicity of ROI is to avoid binding
to MΦ cell surface components, such as Fc receptors, that would provide an
oxidative burst. Instead, the tubercle bacillus mononuclear phagocytes (MP)
via complement receptors CR1 and CR3, molecules of the integrin family.
whose interaction with a ligand does not trigger ROI production (Wright et al., 1983), in resting M\(^{+}\) (Schlesinger et al., 1990).

The \textit{in vitro} delipidified cell component (DCC) of \textit{M. leprae} was used as an immunomodulatory agent in mice. The peritoneal macrophages of these mice were activated to produce increased amount of ROI like H\(_2\)O\(_2\) and O\(_2^{-}\). These macrophages also attained the ability to kill \textit{M. leprae \textit{in vitro}} as shown by several assay systems including the conventional mouse foot pad technique. The increased level of O\(_2^{-}\) seems to be responsible for the killing of \textit{M. leprae} as addition of the enzyme SOD, which breaks down O\(_2^{-}\), resulted in survival of these bacilli inside the macrophages. The increased production of H\(_2\)O\(_2\) does not seem to be responsible for killing of \textit{M. leprae} (Damle et al., 1993 a).

\section*{2.5.2. THE ROLE OF MONONUCLEAR PHAGOCYTES}

When MTB organisms are inhaled into the lung, they are engulfed by alveolar M\(^{+}\)s which performed three important functions. First, the M\(^{+}\) produce proteolytic enzymes and other metabolites that exhibit mycobactericidal effects. Second, M\(^{+}\)s produce a characteristic pattern of soluble mediators /cytokines/ in response to MTB including interleukin -1 (IL-1), IL-6, IL-10, TNF-\(\alpha\) and transforming growth factor-\(\beta\) (Toossi et al., 1991; Barnes et al., 1992).

Cytokines produced by macrophages and by Th1 and Th2 cells are as
follows (Dinarello, 1984).

<table>
<thead>
<tr>
<th>Cytokines</th>
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<tr>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td>IL-2</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ¹</td>
<td>-</td>
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<tr>
<td>Lymphotoxin</td>
<td>-</td>
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<td>IL-4</td>
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<td>IL-5</td>
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<td>IL-3</td>
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<tr>
<td>GM-CSF</td>
<td>+</td>
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<td>TNF</td>
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<td>IL-1</td>
<td>+</td>
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<tr>
<td>IL-8</td>
<td>+</td>
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<tr>
<td>TGF-β</td>
<td>+</td>
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</tbody>
</table>

¹IFN-γ, gamma interferon.
²IL-10 is produced by human but murine TH1 cells.

These cytokines have the potential to exert potent immunoregulatory effects and to mediate many of the clinical manifestation of TB. IL-1 is an endogenous pyrogen that may contribute to produce fever which is characteristic of TB (Dinarello, 1984). IL-6, which enhances immunoglobulin production by activating B-cells (Hirano et al., 1990) may mediate the hyperglobulinemia that is common in TB patients. TNF synergizes with gamma
interferon to increase production of NO metabolites (Ding et al., 1988) and mycobacterial killing (Bermudez and Young, 1988) and is essential for granuloma formation to contain mycobacterial infection (Kindler et al., 1989). On the other hand, TNF may cause the immunopathologic effect such as fever, weight loss, and tissue necrosis that are typical of TB (Beutler et al., 1987). IL-10 inhibits cytokine production by monocytes and lymphocytes (Mosmann et al., 1991) and TGF-β inhibits T-cell proliferation and inhibits M+ effector function (Ding et al., 1990; Palladino et al., 1990). These two cytokines prevent excessive inflammation and tissue damage from an uncontrolled inflammatory response. The third critical function of M+ is to process and present mycobacterial antigens to T-lymphocytes. M+ phagocytose mycobacteria or ingest secreted mycobacterial proteins by pinocytosis and then degrade these proteins into peptides that are expressed on the cell surface in association with self molecules of the major histocompatibility complex (MHC) (Ding et al., 1990).

2.5.3. IMMUNOREGULATORY EFFECTS MEDIATED BY MONONUCLEAR PHAGOCYTES

A significant minority of TB patients have negative tuberculin skin tests and generalized energy associated with failure of T-cells to proliferate in response to MTB antigen (Ellner, 1978). This defect in PPD stimulated lymphocytes proliferation and in IL-2 production is reversed at least in part by depletion of monocytes (Ellner, 1978). These effects were observed specially with PPD but not with mycobacterial antigens. The antigen specific suppressive activity exerted by monocytes from TB patients appears to result from over expression of suppressive factors that are produced when monocytes primed during the
course of TB are stimulated in vitro with PPD. Patients whose lymphocyte proliferation in response to MTB is depressed have elevated percentages of circulating monocytes that are abnormally activated in terms of expression of class II MHC determinants (Tweardy et al., 1994), producing large amount of IL-1 when stimulated with MTB PPD (Fujiwara et al., 1986) and constitutively expressing IL-2 receptors (Toossi et al., 1990). Monocytes production of TNF and IL-6 is also increased in TB patients (Ogawa et al., 1991). Monocyte from TB patients may exert their effects partially through consumption of IL-2 which is a critical T-cell growth factor. In addition, suppressor monocytes may inhibit lymphocyte proliferation through secretion of immunosuppressive cytokines such as IL-10 and TGF- beta. IL-10 mRNA is selectively increased at the site of disease in TB and is expressed predominately by M$^{\dagger}$s rather than T- cells (Barnes, et al., 1993). TGF-beta is produced constitutively by monocytes from patient with TB and production is increased by PPD. The local production of TGF-beta may result in deactivation of M$^{\dagger}$ and immunopathology (Ellner, 1978).

Carbohydrate and glycolipid components of mycobacterial cell wall as well as secreted protein may trigger the immunosuppressive effect of M$^{\dagger}$ in TB patients. Lipoarabinomannan (LAM), a complex heteropolysaccharide, is embedded in the mycobacterium cell membrane and suppresses proliferative response to MTB, perhaps through inducing M$^{\dagger}$ to release immunosuppressive cytokines such as IL-10 (Barnes et al., 1992). LAM inhibits gamma interferon mediated activation of M$^{\dagger}$ and scavanges oxygen free radicals, inhibiting major pathway for destruction of intracellular pathogens (Chan et al., 1991). LAM from virulent MTB does not induce macrophage early activation genes and
does elicit production of large amount of anti-mycobacterial cytokine TNF-α (Chatterjee et al., 1992, Roach et al., 1993). By avoiding M+ activation, LAM from virulent MTB may act as a virulence factor that allows the organism to evade cytokine mediated mechanisms of elimination (Aung et al., 1993).

Secretory proteins of MTB can also induce cytokines production by Mφ and may therefore contribute to systemic immunosuppression and local immunopathology. Both 30-KDa-alpha antigen and 58 KDa constituent of MTB culture filtrates induce a production of TNF by monocytes (Aung et al., 1993). It is likely that carbohydrates, glycolipids and protein of MTB act in concert to modulate expression of the host immune response.

2.5.4. THE CURRENT CONCEPT OF OXYGEN POISONING

It is now widely accepted that the degree of damage produced by hyperexia is determined by the balance between the rate at which ROS are produced and their rate of removal which in turn depends on the activity of the local anti-oxidant defence mechanisms (Dencke et al., 1980; Sies, 1987). The antioxidant defense mechanisms includes the anti-oxy-enzymes, SOD, CAT, GPX together with the enzymes of the pentose monophosphate shunt pathway which regenerate NADPH. Glutathione plays a key part in the anti-oxidant activity and some of the other compounds known to be needed for anti-oxidant activity include vitamin E (tocopherol), vitamin C and selenium (a co-factor for GPX) (Sies, 1978).

In oxygen poisoning, it appears that superoxide is generated in mitochondria, microsomes and possibly other sites at an excessive rate (Jamieson, 1986) and dismutated rapidly to H₂O₂. It has been popularly assumed that O₂- and H₂O₂ then react further in the presence of catalytic
amounts of trace elements notably iron, to produce the highly reactive and thus damaging hydroxyl (OH) radical and possibly singlet oxygen ($^{1}\text{O}_2$). Either of these latter substances is able to initiate lipid peroxidation, which can then continue as a chain reaction (Farber, 1985; Fridovich, 1982; Halliwell, 1986; Freeman et al., 1982, Frank et al., 1980). These reactions have been envisaged thus:

$$O_2 + \text{cellular constituents} \rightarrow O_2 + H_2O_2$$
$$O^2 + Fe^2 \rightarrow O_2 + Fe^2$$
$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2.$$ 
$$Fe^2 + H_2O_2 \rightarrow Fe^3 + OH^- + \text{lipid peroxidation}$$

This is an iron catalysed Haber-Weiss or more correctly a superoxide driven Fenton reaction giving rise to more RO metabolites and leading to lipid peroxidation.

There are many consequences of lipid peroxidation such as membrane damage, inactivation of enzymes. These events are known to occur during oxygen poisoning as described above.

Recently, a transition metal independent pathway to form hydroxyl radicals was proposed (Backman et al., 1990). NO generated by macrophages, hepatocytes, endothelial cells etc. (Spitzer, 1994) can react with $O_2^-$, to form peroxynitrite (OONO$^-$), which after protonation is unstable and decomposes spontaneously to form the hydroxyl radical and nitrogen dioxide (Backman, et al 1990). Because of the large energy gain of the reduction of the hydroxyl radical to water, this active oxygen species will react instantaneously with any biological molecule in its immediate environment by abstracting a hydrogen.
atom (Backman et al., 1990).

\[
\text{OH} + \text{R-H} \rightarrow \text{H}_2 + \text{R}
\]

The resulting more stable radicals are less reactive and therefore, have a longer half-life. Other reactive oxygen species which may be of biological relevance are hypochlorous acid (HOCl) and singlet oxygen. Hypochlorous acid is generated enzymatically by neutrophils and is a strong oxidant. Singlet oxygen is an electronically excited form of molecular oxygen and can be generated by the reaction of hypochlorous acid with \( \text{H}_2 \text{O}_2 \) (Kanofsky, 1989). The pathophysiological importance of singlet oxygen in vivo is still controversial (Kanofsky, 1989).

ROS in particular, superoxide radical and hydrogen peroxide can be generated by many enzymatic sources (e.g., xanthine oxidase and glucose oxidase), subcellular organelles (e.g., mitochondria and cytochrome P-450) or inflammatory cells (e.g., neutrophils and macrophages). Furthermore, environmental factors such as radiation or toxic chemicals can induce the formation of ROS either directly or by causing cell damage and thus activating intracellular sources (Kanofsky, 1989).

2.6. NITRIC OXIDE

The formation of nitric oxide (NO) from L-arginine is now recognised as a ubiquitous pathway involved in the regulation of the cardiovascular, central and peripheral nervous system as well as in other haemostatic mechanisms. These physiological effects of NO are all mediated by the action of a constitutive NO synthase (NOS). In addition, NO is produced in large quantity by an inducible NO synthase (iNOS) during host defense and immunological reactions. It has cytotoxic properties and is generated by activated Mφs.
NO is likely to have a role in nonspecific immunity. The induction of iNOS has recently been demonstrated in human monocytes/ Mφs. After activation with various agents, murine Mφ express high levels of inducible NOS (iNOS), mRNA and protein and produce high amount of NO. In contrast, human monocytes and Mφs have been shown to express iNOS, mRNA and proteins under certain circumstances in vitro, but they produce low level of NO. Whether human cells possess the complete machinery to produce NO is still unknown (Bertholet, 1996).

NO is implicated in a variety of major cellular functions including defence from invasion by microbicidal pathogens. Evidence has been presented suggesting that it is an important mediator of protection in the early nonspecific responses to malaria in mice infected with *P. chabaudi* (Taylor *et al.*, 1993).

Considerable attention has been focussed on multiple functions of a labile inorganic radicle species, NO in a variety of biological systems (Furchaqoh, *et al.*, 1980; Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Shibuli *et al.*, 1991; Moncada *et al.*, 1991). The biological actions of NO are abrogated via a direct reaction with endogenous non haem and haem containing proteins such as, haemoglobins (Moncada *et al.*, 1991) with superoxide anion radicals and with certain organic nitronyl nitroxide radicals that scavenge NO. Among these, O$_2^-$ can react with NO very rapidly, yielding a toxic molecular species, peroxynitratre (ONOO$^-$), whereas, SOD which converts O$_2^-$ to H$_2$O$_2$ and O$_2$ would prolong the biological half-life of NO, because of removal of O$_2^-$ and hence, enhancement of vasorelaxation by endothelium derived relaxing factor [(EDRF) (Miyamoto *et al.*, 1996). (Scheme 1)].
2.6.1. CHEMISTRY AND BIOCHEMISTRY OF NO

NO is a diatomic, free radical gas. NO is relatively non-reactive, for example, in comparison with other biologically important radicals such as hydroxyl or superoxide anion (Stamler, 1994).

The physical properties and chemistry of NO determine its biological and potential sites for interaction. NO is a highly diffusible compound, because of its low molecular weight and reasonable hydrophobicity and solubility. NO is theoretically able to react anywhere within cells and tissues (Feldman et al., 1993). Addition of SOD to experimental systems has been found to preserve greatly the effectiveness of NO including interaction with oxygen and self-association probably can be ignored in physiological systems because of the low NO concentration present in tissues (Stamler et al., 1992). In animal blood, NO is found mostly bound to albumin (Stamler, 1994). This finding is interesting because NO in nitrosothiol form has a much longer half-life and is potentially delivered to targets spatially or temporally distant (Stamler, 1994). In the case of endothelium derived relaxing factor (EDRF), whether this agent is chemically equivalent to NO or a NO-containing compound such as nitrosothiol is still not established.

2.6.2. BIO-CHEMICAL INTERACTIONS

There are multiple sites of NO interaction with biochemical pathways, partly dependant on the NO species encountered (Stamler et al., 1992). Nearly all cell components are possible targets including protein, carbohydrates, lipids and nucleic acids, in addition to various small molecules. Most interactions of NO with enzymes cause inactivation, be it with heme groups, thiols, or other sites, with the notable exception of guanylyl cyclase, which is stimulated by
NO (Arnold et al., 1977). NO can be cytotoxic to cells for a number of reasons. Metabolic dysfunction is caused by NO inhibition of the mitochondrial respiratory chain and enzymes such as dehydrogenases (Nathan, 1992). NO and its oxidation products peroxynitrite and peroxynitrous acid can also indiscriminately damage cell constituents (Nathan, 1992). DNA is subject to strand breakage and deamination of bases, lipids can be oxidized and peroxidized, and protein can be oxidized at any of several groups (Nathan, 1992; Stamler et al., 1992; Stamler, 1994).

NO interacts with metals, especially transition metals, in protein; the major sites of interaction are the iron in heme groups and iron-sulfur centers. A number of mitochondrial enzymes, including complexes of the electron transport chain and the enzymes aconitase are inhibited by NO interaction with their Fe-s centres, which can effectively reduce mitochondrial respiration in cells exposed to NO (Nathan, 1992, Stamler, 1994). Nicholson et al., (1996) observed that high output pathway of NO production helps protect mice from infection by several pathogens— including MTB. However, based on studies of cell cultured from blood, it is controversial whether human mononuclear phagocytes can express the corresponding inducible NO synthase.

2.6.3. REACTIVE NITROGEN OXIDE

The L-arginine dependant cytotoxic pathway of activated Mefs constitutes an important antimicrobial mechanisms against intracellular parasites (Nathan et al., 1991; Liew et al., 1991; Nathan, 1992). Recent studies have demonstrated an association between the antimycobacterial effect of cytokine activated murine Mefs and the activation of the L-arginine dependant cytotoxic pathway (Denis, 1991 b; Chan et al., 1992). Thus, the capability of
Mφs activated by IFN-gamma and *E. coli* lipopolysaccharide or TNF alpha to kill and/or inhibit the virulent Erdman strain of MTB correlates well with RNI production, and nitrogen oxides generated by acidification of nitrite are also mycobactericidal (Chan *et al.*, 1992). *In vitro* studies using the ROI deficient murine Mφ cell line D9 and its ROI generating parental line J774.16 indicate that their antimycobacterial capacities are comparable, and independent of the amount of toxic oxygen species generated, and correlate well with RNI production. These data strongly suggest that L-arginine dependent production of RNI is the principal effector mechanism in activated murine Mφs responsible for killing and inhibiting the growth of virulent MTB. A role of RNI in defense against the tubercle bacillus cannot, however, be entirely excluded. For example, ROI may react with other compounds to generate effective toxic molecules that possess potent antimycobacterial activity. Although not yet tested for its effect on MTB, peroxynitrite anion (ONOO\(^-\)), a product of NO and \(O_2^-\), has been shown to effectively kill *E. coli* (Backman *et al.*, 1990; Zhu *et al.*, 1992; Stamler *et al.*, 1992). Finally, the use of oxygen radical scavengers to probe the significance of ROI in the antimycobacterial function of Mφs can potentially generate misleading information because of non specific effects of these chemicals. Indeed, catalase has been shown to markedly inhibit RNI production by activated Mφs (Li *et al.*, 1992). Although, this inhibitory phenomenon could be reversed by tetrahydrobiopterin (Li *et al* 1992), a co-factor for the Mφ NOS (Knon *et al.*, 1989; Tayeh *et al* 1989). The original observation by Walker and Lowrie (1981) that catalase inhibit the antimycobacterial effect of activated Mφs is probably related to the ability of this \(H_2O_2\) scavenger to suppress production of toxic nitrogen oxides (Scheme 1).
Scheme of cellular defense mechanisms which includes enzymes for the rapid metabolism of ROI, high affinity storage proteins for transition metals (e.g., ferritin) and antioxidants (e.g., vitamin E).
**Formation of Reactive Chlorine**

Formed by one electron step reductions of molecular oxygen
spontaneous reactions with nitric oxide or enzymatic reactions
In the murine system, RNI production by murine Mψs can be down regulated by various cytokines, including IL-4, IL-10 and TGF-beta 1 (Liew et al., 1991; Nelson et al., 1991; Gazzinelli et al., 1992; Oswald et al., 1992). In addition, Mψs digest the microbial antigens, associate them with the class II major histocompatibility complex molecules on the cell surface, and then present the antigen to the T cells. Furthermore, Mψs produce cytokines such as TNF-α, IL-1, IL-6, IL-8 and IL-12, that either induce an inflammatory response or recruit more phagocytic cells and effector molecules to the site of infection (Kuby, 1994).

2.7. ELECTRON MICROSCOPY

The beginning of ultrastructure research on mycobacteria was the result of the wide spread use of the electron microscope on biological specimens. Mycobacteria were among the first microorganism imaged with electron. In 1939 Berries and Ruska published the first quality electron micrographs of mycobacteria. It presents special difficulties for electron microscope investigation because their high lipid content impedes fixation and interferes with the organic solvents used during the embedding process. Intact cell, 0.4-0.5µm thick, are just barely transparent to 80-100KV electrons, and provide only a poorly structured transmission image (Fig 2).

Surface structure of the Mψs immunized with delipidified cell component (DCC) in mice also showed changes like, more ruffled pattern with many folding (Damle et al., 1993b). Similar type of Mψs morphology have been also reported by Horio et al., (1981).

Tripathi et al., (1993) also observed that activated murine Mψs possessed ruffled cell membrane with increase number of fine fillipoda projecting from
the surface. Cytoplasm exhibited prominent and large number of lysosomal like granules and mitochondria, Golgi bodies. The nucleus appeared to be more euchromatic.

The findings reported by the several authors (vide supra) provide enough basis and evidence for the mechanism of protection leading to efficient acquired resistant to MTB.

Thus it is interesting to look for the mechanisms of protection of the vaccine strain *M. habana* which needed an exploration for its mechanisms for affording protection against MTB in mice. In the light of the observations reviewed here, it was thought, proper to study the mechanism of action of the candidate vaccine at macrophage level and PBMN cells of both murine and human origin, stimulated by mycobacterial antigens.
MATERIALS
AND
METHODS
3.1. MYCOBACTERIAL CULTURE

Species of mycobacteria, namely *M. tuberculosis* and *M. haemophilum* were used in the present study. They were obtained from Trudeau Mycobacterial Culture Collection Centre (TMC), Saranak Lake, New York (USA).

### 3.1.1. CHEMICALS

Analytical grade chemicals were procured mainly from SIGMA, Sisco, Hi-Media Lab. and other standard firms dealing with them.

### 3.1.2. MEDIA

Media for mycobacterial culture were prepared according to the description laid down by the International Union Against Tuberculosis (IUAT) (Long, 1958, Sauton, 1912)

#### 3.1.3. SAUTON'S LIQUID MEDIUM

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. L- Asparagine monohydrate</td>
<td>4.00 g</td>
</tr>
<tr>
<td>b. Citric acid</td>
<td>2.00 g</td>
</tr>
<tr>
<td>c. Di-potassium hydrogen phosphate</td>
<td>0.50 g</td>
</tr>
<tr>
<td>d. Magnesium Sulphate</td>
<td>0.50 g</td>
</tr>
<tr>
<td>e. Ferric ammonium citrate</td>
<td>0.05 g</td>
</tr>
<tr>
<td>f. Glycerol</td>
<td>35.0 ml</td>
</tr>
<tr>
<td>g. Triple distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

All the ingredients were taken in a sterilised flask and stirred well to dissolve. The pH was measured and adjusted to 7.2 by adding 40% sodium hydroxide (NaOH) solution drop by drop. This medium was dispensed in sterilised Roux bottles which were autoclaved at 15 lbs pressure for twenty
minutes and allowed to cool down at room temperature, and then used for the cultivation of mycobacteria.

3.1.4. LOWENSTEIN-JENSON MEDIUM

3.1.4.1. Mineral salt solution;

The following constituents were taken in a sterilised flask and dissolved by shaking and gentle heating.

- a. Dipotassium hydrogen phosphate 1.20 g
- b. Magnesium citrate 0.30 g
- c. Magnesium sulphate 0.12 g
- d. L-asparagine monohydrate 1.80 g
- e. Glycerol 6.0 ml
- f. Triple distilled water 300 ml

It was sterilized at 15 lbs pressure for twenty minutes in an autoclave.

3.1.4.2. Malachite green:

2 g of malachite green was initially dissolved in few drops of alcohol to which 100 ml of sterilised lukewarm distilled water was added.

3.1.4.3. Liquid content of eggs;

Fresh eggs were taken and washed with alcohol to sterilise the shell. Liquid contents of the eggs with yolk and albumin were poured in a sterilised wide mouth bottle containing glass beads, and stirred well to mix the contents.

The constituents for complete medium were taken as follows:

- Mineral salt solution 300 ml
- Malachite green solution 11.5 ml
- Liquid contents of fresh eggs 600 ml
All the above ingredients were thoroughly mixed, filtered through sterile muslin cloth in a wide mouth bottle and dispensed in required quantities in sterilised rimless tubes. The tubes were laid down in a slanting position in a hot air oven, at a temperature of 80° C and tyndallised for one hour for three consecutive days.

3.2. ANIMALS

Inbred strain of female BALB/c mice routinely raised in the animal house of the Institute were used, as this strain is sensitive to mycobacterial infection (Skamene 1986). The average body weight of mice ranges between 16-18 g. The age of the mice was 6-8 weeks. The animals in groups of 6-10 were kept in separate cages. They were fed standard pellet diet of Hindustan Lever Ltd. and water ad libitum.

3.2.1. TUBERCULOSIS PATIENTS

Tuberculosis patients attending the out patient clinics of the Rajendra Nagar TB Hospital, Lucknow, were included in this study. Diagnosis was based on the demonstration of acid fast bacilli in sputum or biopsy specimens and was eventually confirmed by positive culture and chest X-ray radiography.

3.2.2. CONTROLS

Healthy volunteer donors from CDRI laboratories were included as control human subjects.

3.3. STAIN

3.3.1. Ziehl-Neelsen stain (David, 1976)

3.3.1.1. Saturated solution of fuschin;

- Basic fuschin 90 g
- Ethyl alcohol 945 ml
MATERIALS AND METHODS

Ethyl alcohol in 300 ml quantity was added to 90 g of basic fuschin and placed in a water bath to boil for two minutes. Solution was allowed to stand for few minutes and supernatant was decanted in a flask. Second time 300 ml of ethyl alcohol was again added to the sediment, stirred well and decanted. Remaining 345 ml of alcohol was added to the sediment and boiled as before. The supernatant was collected and added to the carbol fuschin solution.

3.3.1.2. Phenolic solution

♦ Phenol crystals (melted) 45 ml
♦ Distilled water 945 ml

3.3.1.3. Carbol fuschin solution

♦ Saturated solution of fuschin 105 ml
♦ 5% Phenolic solution 895 ml

3.3.1.4. Acid alcohol

♦ Ethyl alcohol 97 ml
♦ Hydrochloric acid 3 ml

3.3.1.5 Malachite green

♦ Malachite green 250 mg
♦ Distilled water 100 ml

3.3.1.6. STAINING PROCEDURES

For staining, smears were fixed with gentle heating (passing the slide through a flame). The slide was flooded with carbol fuschin, intermittently heated for five minutes, upto steaming (not boiling). Then slides were washed with running tap water, decolorised with acid alcohol, and counter stained with malachite green, for half a minute. The slides were washed again with tape water dried and examined under oil immersion.
Fig. 5 Female BALB/c mice (sensitive to *Mycobacterium tuberculosis*).
3.4. PREPARATION OF M. habana VACCINE (Gamma -irradiated)

Fresh culture of M. habana grown in Sauton's liquid medium was harvested by centrifugation, washed twice with sterilised normal saline at a concentration of 15 mg/ml (moist weight). The numbers of bacilli in the suspension were counted. Gamma irradiation of M. habana was done with Co-60 source at 300 Krads. Viability and sterility were checked before vaccinating the animals. The vaccine was aliquoted in 2 ml quantities and stored at -20°C for future use.

3.4.1. VACCINATION PROCEDURE

For immunological studies in BALB/c mice, M. habana vaccine was given intradermally. The dose of the vaccine was kept constant at 1.5 mg moist weight of 6.27 x 10^8 AFB or 63.3 μg protein in most cases unless specifically mentioned. The skin of the vaccinating animals was shaved and sterilised with alcohol and vaccinated intradermally (0.1 ml). The animals were kept under observation.

3.4.2. PREPARATION OF HABANIN

M. habana cells were disrupted using a cell disrupter precisely 1.5mg wet weight suspended in 1ml BSS of M. habana was sonicated at 20 KHz/S with short bursts until approximately 90% of the cells were disrupted and protoplasmic contents were liberated (smear examination). The sonicated mixture was centrifuged and filtered through 0.22 μ nitrocellulose filter disc. The protein content of the sonicate was determined by modified Lowry method (Lowry et al., 1951) with B S.A. as reference standard.
3.4.3. PURIFIED PROTEIN DERIVATES (PPD)

PPD of *M. tuberculosis* (MTB) was purchased from Span Diagnostic Ltd, Surat (10TU/0.1 ml).

3.4.4. BCG VACCINE

Freeze dried BCG vaccine, B.P. was procured from BCG vaccine lab., Madras.

3.5. CULTURE MEDIUM FOR CELLS

For culture of different cells RPMI-1640 was used. This medium was supplemented with 25 mM HEPES, 0.01M sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate plus penicillin 100 unit/ml, gentamycin 20 \( \mu \)g/ml, streptomycin 100 \( \mu \)g/ml and nystatin 5 unit/ml and finally supplemented RPMI-1640 was enriched with 10% fetal calf serum/human AB serum (inactivated 56°C for 30 min). This was referred to as complete RPMI-1640 medium.

3.5.1. COLLECTION OF PERITONEAL EXUDATE CELLS (PEC)

PEC from the mice were isolated by the method of Mosier (1984) as adapted in this laboratory (Singh *et al.*, 1992). In brief, the animals were killed rapidly by cervical dislocation and injected intraperitoneally with 5 ml of supplemented RPMI-1640 as well as heparin 5 U/ml. The abdomen was gently massaged and the medium containing PEC was lavaged out and collected in siliconised sterile tubes. The cells were collected by centrifugation and washed with RPMI-1640 medium. The viability of recovered cells was estimated from their capability to exclude trypan blue. The cells were counted in haemocytometer and the adherent cells to the plasticware (tubes and plates) were used for various assays.
Fig. 6 (a,b,c,d) Shows the different stages of collection of murine peritoneal macrophages (a) lifting the skin upwards with forceps to make longitudinal incision 1/2 inch long,(b) stripping back the fur with scissors and forceps (c) injection of the medium (RPMI-1640), (d) needle inserted to the flank so as to form a pocket of fluid.
3.5.2. DISRUPTION OF MACROPHAGES AND MONOCYTES FOR ASSAY OF ENZYMES

For enzyme estimation the macrophages/monocytes were suspended in isotonic KCl (1.15% w/v) and disrupted by sonication 20 KHz for 4 x 20 seconds with one minute cooling intervals. The membrane portion was separated out by centrifugation at 9000 x g for 30 min at 4°C and the supernatant was employed for the assay of different lysosomal and antioxidant enzymes.

3.5.3. ESTIMATION OF PROTEIN

All the cellular fractions were assayed for their protein content according to Lowry et al., (1951) using bovine serum albumin (BSA) as standard. Protein contents were precipitated overnight following addition of 10% TCA (w/v) to each sample. Precipitates obtained were dissolved in 1 ml of 0.1 N NaOH. Aliquots from each sample were made upto 500 μl by adding suitable amount of water and were mixed with 5.0 ml of alkaline copper reagent containing 50 ml 2% (w/v) sodium carbonate in 0.1 N NaOH, 1 ml 2% (w/v) sodium potassium tartrate and 1 ml 1% (w/v) copper sulphate. After 15 min incubation of the mixture at 37°C, 0.5 ml of 1 N Folin Ciocalteu reagent was added, tubes were shaken vigorously and intensity of the blue colour was read at 750 nm against a reagent blank. Protein contents were calculated from a standard curve of BSA and expressed as mg/ml preparation.

3.6. ESTIMATION OF LYSOSOMAL ENZYMES

3.6.1. ACID PHOSPHATASE

Activity of acid phosphatase was quantified using P-nitrophenyl as substrate. (Fiske and Subbarow 1925).
3.6.1.1 Principle

A sample of enzyme solution is added to para-nitrophenyl phosphate and parainitrophenyl is released due to its enzymatic hydrolysis. It imparts yellow colour in alkaline medium which is estimated spectrophotometrically at 500 nm.

3.6.1.2 Reagents

3.6.1.3 Sodium acetate buffer 0.2 M (pH 5.0), prepared by mixing of 0.2 M sodium acetate solution and 0.2 M acetic acid.

a. 0.2 M sodium acetate solution (16.4 g sodium acetate was dissolved in 1000 ml of TDW)

b. 0.2 M acetic acid solution (11.550 ml of acetic acid was dissolved in 1000 ml of TDW)

c. 0.2 M sodium acetate buffer (pH 5.0) 14.8 ml of acetic acid solution (0.2 M) mixed with 35.2 ml of sodium acetate solution (0.2 M)

3.6.1.4 Substrate solution: For 3 mM p-nitrophenyl phosphate 0.0084 gm was dissolved in 10 ml of TDW.

3.6.1.5 Standard p-nitrophenyl: 13.911 mg p-nitrophenyl was dissolved in distilled water to give final concentration of 10 mM. 0.3 ml of this stock was diluted with 11.7 ml of distilled water to make a working stock of 250 M.

3.6.1.6 Sodium carbonate buffer: 10.6 gm of sodium carbonate was dissolved in 100 ml of distilled water.
3.6.1.7. Procedure

Suitable aliquots of enzyme (0.1 ml) were added to 0.4 ml of 0.2 M sodium acetate buffer (pH 5.0) containing 0.1 ml of 3 mM substrate and incubate 30 min. The reaction mixture was stopped by addition of 1 ml sodium carbonate solution (1 M). The intensity of colour produced was measured at 500 nm in spectrophotometer. The intensity of colour is directly proportional to the quantity of substrate transformed. The optical density obtained was compared with standard for para-nitrophenyl.

3.6.2. β-GLUCURONIDASE

Activity of β-glucuronidase was quantified using phenolphthalein mono β-glucuronic acid as the substrate (Fishman 1981). Specific activity of the enzyme has been expressed as nM of phenolphthalein released/ unit time per mg of protein. 3.6.2.1. Principle: A sample of enzyme solution is added to phenolphthalein mono β-glucuronic acid and phenolphthalein is released due to its enzymatic hydrolysis. Phenolphthalein imparts pink colour in alkaline medium which is estimated spectrophotometrically at 550 nm.

3.6.2.2. Preparation of reagents

1. Sodium acetate buffer: (pH 5.0) (Already mentioned)
2. Substrate solution: 2.22 mM substrate, 1.1 mg of this was dissolved in 1 ml of TDW.

3.6.2.3. Standard phenolphthalein

628 mg phenolphthalein was dissolved in 6.28 ml ethanol to give final concentration of 1 mM. This solution was diluted 10 times with TDW to make working stock.
3.6.2.4. Preparation of alkaline buffer: Prepared as followed for 1000 ml.

1. Glycine 1.502 g
2. Na₂CO₃ 5.299 g
3. NaCl 2.922 g
4. NaOH 600 mg

3.6.2.5. Procedure

Suitable aliquots of enzyme (0.25 µl) were added to 50 µl of 0.2 M sodium acetate buffer (pH 5.0) containing 50 µl of 0.002 M substrate. Incubate for 15 min. The reaction was stopped by addition of 100 µl alkaline buffer solution.

The intensity of colour was measured at OD 550 nm in a spectrophotometer. The intensity of the colour is directly proportional to the quantity of substrate transformed. The optical density obtained was compared with standard for phenolphthalein.

3.6.3. LYSOZYME

Lysozyme, responsible for the lysis of complex polysaccharides of the cell wall was quantitated as reported by Smoletis and Hartsell (1955), following the reaction kinetics of disappearance of lysozyme substrate Micrococcus luteus (10 mg/24 ml in 60 mM phosphate buffer, pH 6.2) containing 0.1% NaCl. The decrease in OD is measured spectrophotometrically at 645 nm. Lysozyme activity was defined in terms of U/min/10⁶ cells or U/min/mg protein.

3.6.4. PREVENTIVE ANTIOXIDANTS

3.6.4.1. SUPEROXIDE DISMUTASE (SOD)

The assay of the enzyme SOD was carried out by the procedures of
Kakkar et al., (1984) and Nishikimi et al., (1972). Assay mixture contained sodium pyrophosphate buffer (0.052 M, pH 8.3), phenazine methosulphate (PMS, 6.2 μM), nitroblue tetrazolium (NBT, 30 μM), potassium cyanide (KCN, 1 mM or 10 μM, pH 7.0) and suitably diluted homogenate was pre-incubated for 5-7 min at 37°C prior to addition of nicotinamide adenine dinucleotide reduced form (NADH, 52 μM). Mixture was again incubated at 37°C in water bath for 90 sec and the reaction was stopped by addition of 1 ml glacial acetic acid (17.4 M). The violet colour formed was extracted with 4 ml of n-butanol and absorbancy in butanol layer was measured at 560 nm against blank containing all the reagent except homogenate. Potassium cyanide 10 μM was able to inhibit electron transport chain hence inactivate mitochondrial SOD where as cytosolic form was inhibited by 1 mM KCN. SOD activity was estimated as the inhibition of chromogen formation by SOD due to the NADH mediated PMS dependent reduction of NBT and expressed in terms of U/min/10⁶ cells or U/min/mg protein in cells and homogenate respectively. One unit of enzyme activity was defined as the enzyme concentration required to inhibit the optical density of 560 nm of chromogen production by 50% in one min under assay condition.

3.6.4.2. CATALASE (CAT)

The catalase mediated decomposition of H₂O₂ followed at 240 nm on spectrophotometer (Aebi, 1971). A 100 μM sample was added to 2.9 ml of buffered substrate (50 mM phosphate buffer, pH 7.0 containing 10 mM H₂O₂) and decrease in absorbance was monitored at 37°C for 2.5 min at the interval of 15 sec. The activity was calculated using the coefficient (0.0041) of H₂O₂ at 240 nm and reported in U/min/10⁶ cells or U/min/mg protein.
3.6.4.3. GLUTATHIONE PEROXIDASE (GPx)

GPx activity was measured by the formation of oxidised glutathione and t-butylhydroperoxide (Leopold and Wolfgang 1984). 100 μl of 0.7 mM t-butyl hydroperoxide was added to the assay mixture (100 μl of 100 mM sodium azide, 40 μl of 4 mM GSH, 25 μl enzyme/water 735 μl of 100 mM tris-HCl buffer with 5 mM EDTA pH 8.5), reaction was carried out at 37°C for 20 min and was stopped by adding 200 μl N-ethylmaleimide (NEM, 0.1 M) to prevent oxidation of GSH to GSSG and left for 10 min at 37°C, to this mixture 2.8 ml of 0.1 N sodium hydroxide was added. An aliquot of 200 μl from each tube was taken out and was allowed to react with 200 μl O-phthalaldehyde (OPT, 1 mg/ml (w/v) in methanol) and 3.6 ml of 0.1 N NaOH for 15 min at room temperature. Fluorescence of each tube after 15 min was read at 350 nm (excitation) and 420 nm (emission). Oxidised glutathione at known concentrations (1-40 μg) was used to get a standard curve. Activity of GPx was determined as U/min/mg protein, which is μmole of GSSG formed during standard test condition.

3.6.4.4. MYELOPEROXIDASE (MPO)

It was estimated according to the method of Morris et al., (1966). Briefly 0.4 ml homogenate, 2.2 ml of 0.1 M sodium phosphate (pH 6.2), 0.2 ml 2 μM hydrogen peroxide (H₂O₂ as substrate) and 0.2 ml of 0.55 mM o-dianisidine was incubated at 37°C for 15 min. Enzyme reaction was stopped by adding 0.2 ml of 0.153 M sodium azide in experimental tube, while azide was added to the control prior to the addition of H₂O₂. Formation of orange brown coloured complex was read at 460 nm and used for calculating the MPO activity. Cellular enzymatic activity was expressed as U/min /10⁶ cell.
3.7. REACTIVE OXYGEN AND NITROGEN INTERMEDIATES (ROI, RNI)

3.7.1. SUPER OXIDE ANION (O$_2^-$)

Superoxide release was determined by measuring the SOD inhibitable cytochrome C reduction (Johnston, et al., 1975). Plated cells were washed quickly with RPMI-1640. The second wash was aspirated and replaced with 1.5 ml reaction mixture containing 80 μM ferricytochrome C in HBSS. The standard reaction mixture for stimulated cultures contained, in addition, either opsonized zymosan at final concentration of 1.3 mg/ml or phorbol myristate acetate (PMA) at a final concentration of 2-4 μg/ml. Some assay mixtures contained bovine erythrocyte SOD, final concentration 30 μg/ml, in addition to one of the stimuli. Each reaction was run in triplicate. Control included six cell free dishes, each incubated with HBSS, cytochrome, and opsonized zymosan, PMA, or additional HBSS. After a required time of incubation at 37°C in 5% CO$_2$-95% air, the mixture was removed, placed into iced micro-centrifuge tube, and promptly cleared by centrifugation at 8000 g in an ependorf micro-centrifuge. The optical density of the supernates was determined spectro-photometrically using mixtures from plates without cells as blank and the concentration of cytochrome C reduced was determined using the equation $\Delta \text{E}_{550 \text{ nm}} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

3.7.2. HYDROGEN PEROXIDE (H$_2$O$_2$)

Hydrogen peroxide released by cells was quantified according to Pick et al., (1981). Culture supernatant was used to measure H$_2$O$_2$. Besides these 0.2 ml of 2 unit/ml solution of horse radish peroxidase and 0.2 ml of 0.2 mg/ml solution of phenol red were added to each tube. Sodium hydroxide (1 N-20 μl)
was added to cell culture and blocked production. This served as the basal (cell blank) reading. The absorbance was measured at 615 nm. The level of \( \text{H}_{2}\text{O}_{2} \) expressed as n moles/hr/mg protein which was calculated from a standard curve of OD against concentration \( \text{H}_{2}\text{O}_{2} \).

3.7.3. NITRIC OXIDE (NO)

NO production was determined by measuring nitrite, a stable metabolic product of NO, in cell culture supernatants (Green et al., 1982). Briefly, Griess reagent [1:1 (v/v) mixture of 0.1% N-(1-naphthyl) ethyline diamine dihydrochloride in \( \text{H}_{2}\text{O} \) and 1% sulphanilamide in 5%H\( \text{PO}_{4} \)] was added to 96 well round bottomed microtiter immunoassay plates at 100 \( \mu \text{l}/\text{well} \). An equal volume of cell culture supernatants was then mixed with the Griess reagent, and \( A_{570} \) was determined on a MR 700 microplate reader. The nitrite amount was calculated from an NaNO\(_2\) standard curve.

3.7.4. GENERATION AND ASSAY OF CYTOKINES

The peritoneal cells were suspended to a strength of 2x10\(^6\) cells /ml in complete RPMI-1640 medium. One ml of each cell suspension was transferred to the well of 24 well culture plate and incubated at 37°C in a humid CO\(_2\) incubator. After 6 hr the medium was aspirated and the cells were washed twice with RPMI-1640. One ml of fresh medium (CRPMI-1640) was added to the cells and the plate was return to the incubator. After 48 hr the medium was filtered through 0.2 \( \mu \text{m} \) membrane filters and stored at -20°C until further use.

Concentrations of interleukin -6 (IL-6) and tumour necrosis factor (TNF) in the filtrate were determined by biological assays using hepatoma B-9 and fibroblast L-929 cell lines showing sensitivity to the respective cytokines.(Mosmann, 1983). The level of IL-1\( \beta \) concentration in the sample
was measured by an ELISA kit (GENZYME USA; Hoggquist et al., 1991).

3.7.5. ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMNC)

3.7.5.1. Blood collection

**Animals**

The animals were anaesthetized with ether and after decapitation blood was collected in heparinized tubes for different assays.

**Human subject**

Venous blood from the TB patients as well as from healthy subjects was collected (5 to 10 ml) into preservative free heparin (10 IU/ml) tubes from Rajendra Nagar TB Hospital, Lucknow. Volunteer donors mostly from Central Drug Research Institute, Lucknow were used as control human subjects.

3.7.5.2. SEPARATION OF CELLS

The heparinized blood was diluted 1:1 in RPMI-1640 and separated the cells by sedimentation of histopaque 1119 (density 1.119±0.001 gm/ml) by density gradient centrifugation. The cells were collected at the interphase layer, washed twice with RPMI-1640 and cultured in flat bottom microtiter plates at 10^5 cells per well for different proliferative assays.

3.7.5.3. SEPARATION OF MONOCYTES

PBMN cells were isolated from heparinized blood by density gradient centrifugation method and suspended in complete RPMI-medium. Monocytes were isolated by a modification of the method of Boyum (1968). Briefly, PBMN cells were incubated for 2 hr at a known concentration in complete RPMI-1640 medium in a plastic petri-plates. The supernatants containing non-adherent cells were washed twice in order to eliminate contaminating adherent
cells (monocytes) and were recovered by detaching them with cold RPMI-1640 medium with the help of rubber police man. The cells' viability was checked by trypan blue. The monocytes were seeded in 24 well culture plates at a concentration of 10^6 cells/ml. Approximately 80% of adherent cell population were monocytes as determined by morphology and non-specific esterase staining.

3.7.5.4. MEASUREMENT OF PROLIFERATIVE ASSAY

The proliferative assay was done as previously described by Gheorghiu et al., (1994). Briefly, cells were cultured at 10^5 cells/well in 96 well flat bottom culture plate in the presence of medium and incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Medium alone and mitogen (PHA) were used as negative and positive control, respectively. Eighteen hr prior to termination of the culture 0.5 μCi of [³H] thymidine was added to each well of the proliferating cells. Harvesting was done on a semiautomatic harvester (PHD cell harvester, Cambridge technology Inc. Cambridge, Maryland, USA) using glass fiber filters. Radioactivity was counted in a liquid scintillation counter (Rack beta; LKB, Finland) using a toluene based scintillation cocktail (4 gm 2, 5-diphenyloxazole (PPO), 100 mg 1, 4-bis-5-phenyloxazyl (POPOP), 500 ml methoxy ethanol and 500 ml toluene for 1000 ml of scintillation fluid). The specific DPM value was obtained by subtracting the mean DPM in control triplicate wells from the test wells. The stimulation index (SI) was the ratio of the mean [³H] thymidine incorporation in the test wells to that in wells without antigens.
3.7.5.5. MEASUREMENT OF DELAYED TYPE HYPERSENSITIVITY (DTH)

Mice were divided into two groups of ten animals each, one group received vaccine and other group served as a control. Before 5 days of each experiment of DTH mice were injected intraperitoneally with washed sheep RBC \((10^6/\text{ml})\) suspended in PBS in both groups. After sensitization with SRBC mice were challenged intradermally with 50 \(\mu\)l of washed SRBC in the left hind foot and right hind foot with PBS as control in the both group of mice. The DTH response was measured after 24, 48 and 72 hr (Saiki et al., 1981) using a dial microcalliper (Schnell taster, Kroplin, West Germany) and expressed as the difference in the reading between the foot pad injected with SRBC and one with PBS. The corrected DTH \((\text{mm})\) was finally measured by subtracting DTH of vaccinated mice with non vaccinated mice.

3.8. ASSAY OF PHAGOCYTOSIS

3.8.1. BY RADIO LABELLED \(E\. coli\)

This test was performed according to the method of Leijh et al., (1977). Briefly, unless stated otherwise, PEC were harvested from healthy and vaccinated mice. \(\text{M}\phi\text{s}\) suspended in \((2\times10^6/\text{ml})\) complete RPMI-1640 in well cluster plates \((13 \text{ mm} \times 3\text{ mm depth})\) and incubated at \(37^\circ\text{C}\) for required hr in an atmosphere at 5% \(\text{CO}_2\). The supernatants were removed and \(\text{M}\phi\text{s}\) monolayer formed at the base of the chamber was washed thoroughly with supplemented RPMI 1640. A loopful inoculum of \(Escherichia\ coli\) from nutrient broth slant was subcultured in 5 ml nutrient broth for 18 hr at \(37^\circ\text{C}\), centrifuged \((15,000 \text{ g} \times 10 \text{ min})\) at room temperature, washed twice and suspended in Koser's citrate medium \((\text{KCM})\) containing \(\text{NH}_4\text{Cl}\) \((0.037 \text{ g})\), \(\text{KH}_2\text{PO}_4\) \((0.1 \text{ g})\) \(\text{MgSO}_4\times 7\text{H}_2\text{O}\)
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(0.02 g) and dextrose (5 g) in 100 ml. Sodium citrate was used for pH 6.1. Aliquot of 0.6 ml was subcultured in 4.4 ml of KCM containing 2 μCi of ¹⁴C leucine (0.1% KCM) for 3 hr at 37°C in a shaking water bath. Unlabelled leucine (0.1% KCM) was added. The cells were centrifuged, washed twice with unlabelled leucine and suspended in 2 ml complete RPMI -1640 medium. This suspension was placed (0.2 ml) over the Mφ monolayer for different hr at 37°C containing 2 x 10⁶ cells for assay of phagocytosis. The residual radioactivity of the aspirate from each well was measured in a liquid scintillation counter. The phagocytic activity was expressed as percentage incorporation of ¹⁴C leucine and calculated according to the following formula:

\[
\text{cpm of total labelled } E.\text{coli} - \text{cpm of aspirates from wells with Mφ monolayer} \\
\times 100
\]

\[
\% \text{ Phagocytosis} = \frac{\text{cpm of total labelled } E.\text{coli}}{\text{cpm of aspirates from wells with Mφ monolayer}} \times 100
\]

3.8.2. BY LEIGHTON TUBE METHOD

The method used for phagocytosis by leighton tube was similar to the one used by Damle et al., (1993b). Briefly, peritoneal Mφs culture were set up on cover slip from normal and M. habana immunized mice. After incubation the cells were infected with Mycobacterium habana, with the ratio of 1:40 per leighton tube. Phagocytosis was allowed to take place for 1, 1/2, 2, 6 and 24 hr. Non phagocytic bacilli were washed off and the cover slip containing the cells were fixed with 2.5% gluteraldehyde for 20 min. Mφs culture on coverslip were stained with Ziehl-Neelsen’s carbol fuchsin for 20 min, decolourized with acid alcohol and were dipped 0.1% toluidine blue solution for 1 to 2 sec, rinsed with distilled water and air dried. The number of cell containing acid fast bacilli and the number of bacilli per cells were recorded.
Fig. 7 Desiccator with Leighton's tubes for phagocytic experiments.
The coverslips were mounted on plain slides with DPX mountant, they were kept over night and excess of DPX was removed. The cells were observed under oil immersion lens. The percentage of Mφs containing no bacilli, 1-5 bacilli, 5-10 bacilli and more than 10 bacilli were counted for quantisation.

3.8.3. PHAGOCYTOSIS BY LYSED PHAGOCYTES AND COUNTING COLONY FORMING UNIT (CFU)

The procedure used in this study was similar to that described by Todd et al., (1996). Briefly, 2 x 10^6 Mφs were incubated in 16 wells cluster plate at 37°C. After attachment culture were washed vigorously with warm RPMI-1640 to remove non adherent cells. Subsequently, the monolayer were treated using M. tuberculosis H37Ra at a ratio of 1:40 for different hr to observe the phagocytosis.

3.8.4. PREPARATION OF CULTURE

For preparing mycobacterial culture, fresh growth (about 21 days) of M. tuberculosis H37Ra from L.J. medium is harvested and weighed. The culture is triturated in pestle and mortar (glass) using sterile saline containing 0.05% tween 80 in order to make the bacterial suspension homogenous. One mg of this culture is equivalent to about 1 x 10^9 CFU as determined from a sample of suspension plated to confirm the number of viable bacilli/colony forming units.

3.8.5. LIFE COUNT

After lysis of the Mφs by cold sterile TDW, live count was done on L.J. medium slants. The 10 fold serially diluted growth of the mycobacterium from the original concentration was seeded on the triplicate set of slants and incubated 15-21 days. After appearance of the visible growth, the isolated colonies were counted and the number of live AFB was calculated.
Fig. 8 Schematic representation of the various stages of phagocytosis and intracellular killing of microorganisms.
3.9. ELECTRON MICROSCOPE

3.9.1. TRANSMISSION ELECTRON MICROSCOPY (TEM)

To assess the ultrastructural characteristics of Mφs from control and experimental animals by TEM, the method described by Damle et al., (1993b) was followed. Briefly, Mφs were harvested and fixed in glutaraldehyde (3% v/v), paraformaldehyde (2% v/v) in cacodylate buffer (pH 7.2), 0.1 M for 2 hr at 4°C. These were then washed 2 to 3 times with the osmium tetroxide. The doubly fixed sample were then washed with distilled water and kept overnight at 4°C in aqueous uranyl acetate (1% w/v). These samples were then dehydrated in graded acetone (50%, 70%, 9%) in water and then absolute acetone (two changes) for 10 min each and finally embedded in a mixture of aralite and spoon as described by Damle et al., (1993b). Ultrathin sections were cut on LKB-Ultra microtome using glass knife. The sections were examined under 410 LS Phillips transmission electron microscope at an accelerating voltage of 80 KV.

3.9.1. SCANNING ELECTRON MICROSCOPY (SEM)

This was done according to method Tripathi et al., (1993). Briefly, Mφs from control and immunized (M. habana) cultures were harvested and fixed in glutaraldehyde (3% v/v), paraformaldehyde (2% w/v) in cacodylate buffer (pH 7.2), 0.1M for 2 hr. These were then washed 2 to 3 times with the cacodylate buffer 0.1M, osmicated in osmium tetroxide (1% v/v, in above mentioned cacodylate buffer) and dehydrated with ethanol. Dehydration was achieved by keeping the Mφs in graded alcohols viz 30, 50, 70, 80, 90 and 95 % for 20 min each. This was followed by dehydration in anhydrous alcohol thrice for 10 min each. The samples were dried in liquid CO₂ in critical point drying apparatus.
Fig. 9 Activation of macrophages in the tuberculous lesion. Mononuclear phagocytes are attracted from the blood stream and are activated locally by lymphocytes and their lymphokines (LK) (probably the most efficient mechanism) and also by the ingestion of bacilli, dead cells and tissue debris. Lymphocytes are produced when sensitive lymphocytes (primarily T-cells) are stimulated by bacillary antigens. Only activated macrophages seem to be able to destroy the tubercle bacilli.
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(Balzers union). The dried material was mounted on aluminium tubes coated with gold palladium alloy in metal coater (polaron E 5000) and preserved in vacuum desiccator. Gold palladium alloy coated preparation were examined under 515 Phillips scanning electron microscope.

3.9.2. STATISTICAL ANALYSIS

Significance of difference between the control and experimental samples were calculated by Student's t-test. All experiments were done in triplicate samples. Conclusions were drawn from more than three independant experiment.
RESULTS
The effect of *Mycobacterium habana* vaccine was studied on the murine macrophages (Mφs) and peripheral blood mononuclear cells (PBM C) of vaccinated mice. Four different types of intermediary products namely, reactive oxygen and nitrogen intermediates, lysosomal and preventive antioxidant enzymes along with cytokines were evaluated in these cells.

The effect of habanin, *M. habana* whole cell vaccine, PPD and BCG vaccine on the lymphoproliferative response of peripheral blood mononuclear cells and monocytes in respect to reactive oxygen and nitrogen intermediates of tuberculosis (TB) patient was also studied.

In another study, the DTH response of vaccinated BALB/c mice was seen.

Likewise, the effect of murine Mφs on phagocytosis after *M. habana* vaccination was studied with radiolabelled *E. coli*, *M. habana* and *Mycobacterium tuberculosis* (MTB) H37Ra.

The *in vitro* effect on murine Mφs and healthy human monocytes elicited with *M. habana* and BCG vaccine were also observed. The intermediates namely, reactive oxygen and nitrogen and lysosomal enzymes viz., acid phosphatase, β-glucuronidase and lysozyme were evaluated in these cells.

Furthermore, the ultrastructure of the murine Mφs after *M. habana* vaccination was also examined by transmission and scanning through electron microscopes for any observable changes.
RESULTS

4.1. EFFECT OF VACCINE ON PERITONEAL EXUDATES CELLS (Mφs) OF M. HABANA VACCINATED BALB/c MICE

4.1.1. RELEASE OF REACTIVE OXYGEN AND REACTIVE NITROGEN INTERMEDIATES

Macrophages (Mφs) collected from the peritoneal cavity of mice immunized with M. habana vaccine showed greater production of $O_2^-$, $H_2O_2$ and NO compared to those collected from the untreated animals (Table 1). On 30th day of initial vaccination however, the increase was not statistically significant ($P>0.05$). Stimulation in the production of all the three effector molecules on day 30 of initial vaccination was 14.54 for $O_2^-$, 26.2 for $H_2O_2$ and 1.95 for NO. On day 60 of initial and day 30 of 1st booster the values of these metabolites markedly enhanced. This trend in rise of the effector molecule persisted up to 60 days of initial and 30 days of 1st booster and then declined gradually. A second booster was given on day 60 of initial and 30 days of 1st booster. The values of these metabolites were further increased beyond 90 days. Among these three intermediates, $O_2^-$ showed greater activities in the vaccinated peritoneal Mφs than controls (Table 1).

4.1.2. RELEASE OF LYSOSOMAL ENZYMES AFTER M. HABANA VACCINE

A marked increase in the level of three lysosomal enzymes namely, acid phosphatase, β glucuronidase and lysozyme was observed in the Mφs obtained from mice after vaccination with M. habana (Table 2). However, the increase measured on day 30 of initial vaccination did not register significant values ($P>0.05$). Mφs collected after 60 days of initial and 30 days of 1st booster showed a dramatic increase in the values of these enzymes which were
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statistically significant. The 2nd booster dose was given on the 60th day of initial and 30 days of 1st booster, when these values recorded an increase after showing a declining trend (Table 2).

4.1.3. PREVENTIVE ANTIOXIDANTS AFTER M. HABANA VACCINATION

3.1.3.1. SUPEROXIDE DISMUTASE (SOD) CATALASE (CAT) AND MYELOPEROXIDASE (MPO), GLUTATHIONE PEROXIDASE (GPx):

The level of SOD, CAT, GPx and MPO also recorded after 30 days of prior vaccination. These values were not statistically significant (Table 3). On day 60 of initial and day 30 of 1st booster vaccination, the values of these preventive anti-oxidant increased significantly, although increase in the values of GPx was not significant. On day 90, of initial, day 60 of 1st, and day 30 of 2nd booster vaccination, the values of each intermediary product recorded a moderately significant values (Table 3).

4.2. EFFECT OF VACCINE ON PBM CELLS OF BALB/c MICE

4.2.1. RELEASE OF OXYGEN AND NITROGEN INTERMEDIATES AFTER M. HABANA VACCINATION

Peripheral blood mononuclear cells from M. habana immunized mice showed greater production of $O_2^-$, NO and $H_2O_2$ compared to those collected from the untreated animals (Table 4). However, except $O_2^-$, the increase of NO and $H_2O_2$ was not significant on 30th day of initial vaccination. PBM cells collected after 60 days of vaccination and 30 days of 1st booster vaccination, showed marked enhancement in the release of these metabolites which were statistically significant ($P<0.01$). The 2nd booster dose was given on 60th day of initial and 30 days of 1st booster vaccination, when the values of each
RESULTS

intermediate product recorded a highly to moderately significant values. Among these three metabolites, \( \text{O}_2^\cdot \) showed greater activities in the vaccinated peripheral blood mononuclear cells (Table 4).

4.2.2. RELEASE OF LYSOSOMAL ENZYMES AFTER M. HABANA VACCINATION

Like peritoneal macrophages, PBM cells showed greater production of lysosomal enzymes like acid phosphatase, \( \beta \)-glucuronidase and lysozyme in \( M. \) habana stimulated mice. The result is presented in Table 5. On 30th day of initial vaccination all these three enzymes showed similar degree of activation (\( P>0.05 \)). Administration of first booster dose after 30 days of initial vaccination, showed a dramatic effect in the increase of these enzymes (\( P<0.01 \)). The effect gradually declined with time, but the 2nd booster on 60th day of initial and 30th day of 1st booster vaccination, further increased the activities of these enzymes beyond 90 days. Among the lysosomal enzymes studied, acid phosphatase showed greater activities in the vaccinated mice PBM cells (Table 5).

4.2.3. RELEASE OF ANTIOXIDANT ENZYMES AFTER M. HABANA VACCINATION

Effect on peripheral blood mononuclear cell of Balb/C mice on killing system by the antioxidant enzymes namely SOD, CAT, PMO and GPx is shown in Table 6. The activities of these enzymes was similar to that of peritoneal MΦs shown in Table 3. On day 30 of initial vaccination all the enzymes activities were insignificant (\( P>0.05 \)) where as, in peritoneal exudate cells only SOD and CAT showed significant difference with control. Furthermore, on day 60 of initial and 30 days of 1st booster vaccination, the
values of these enzymes increased significantly ($P<0.05$). Likewise, on day 90 of initial, 60 days of 1st and 30 days of 2nd booster vaccination, the values of each intermediate products record a moderately significant values (table 6).

4.2.4. RELEASE OF CYTOKINES AFTER $M.\textit{HABANA}$ VACCINATION

Macrophages collected from the peritoneal cavity of mice immunized with $M.\textit{habana}$ vaccine showed greater production of cytokines namely interleukin IL-1, IL-6 and INF (table-7). On day 10, of initial vaccination all the cytokines showed greater production which was statistically significant ($P<0.01$). The booster dose given on day 30 of initial vaccine had a dramatic impact on the production of these cytokines, which recorded highly significant values. Among the three cytokines studied IL-6 showed most pronounced acceleration at every stage of measurement (Table 7 and Fig. 11).

4.3. LYMPHOCYTE TRANSFORMATION TEST (LTT)

The proliferative responses of freshly isolated PBM cells from TB patient and healthy subjects were tested $\textit{in vitro}$ by different mycobacterial antigens and known mitogen (PHA). The data is presented in Table 8 and Fig. 12.

The response of TB patient without anti TB drug treatment showed less proliferative responses than that of the treated TB patients and control subjects with all mycobacterial antigens. The LTT responses are expressed at the optimal doses of antigens. Each of the antigen tested showed a dose dependent increased response for PHA, habanin, PPD, $M.\textit{habana}$ and BCG whole cell vaccine.

In healthy subjects, among the mycobacterial antigen tested habanin gave the strongest responses followed by PPD.
RESULTS

In case of TB patient with treatment (2-4 week), *M. habana* whole cell vaccine showed maximum responses in $10^6$ cells/ml dose ($P<0.05$) and minimum response with PPD in 10 $\mu$g/ml dose ($P>0.05$). Also, *M. habana* whole cell vaccine showed strongest responses in TB patients without treatment followed by BCG, which was comparable with *M. habana* vaccine.

The response to *M. habana* vaccine of control subject $\Delta$DPM = 9584±1920 (n=5) was significantly higher than both the group of TB patient, $\Delta$DPM= 3590±800 and 2156±510 with eg treatment and without treatment TB patient, respectively (table 8).

4.3.1. EFFECT ON MONOCYTES

Comparison of O$_2^-$, NO and H$_2$O$_2$ released from the monocytes of healthy subject and TB patient activated *in vitro* with mycobacterial antigens are shown in table 9. Monocytes from TB patient released less O$_2^-$ than the monocytes from the healthy donors in response to mycobacterial antigens. It constantly released significant quantities of O$_2^-$ when incubated with the surface active agent, PMA (Phorbol myristate acetate 10 $\mu$g/ml). Furthermore, among the antigen only PPD (10 $\mu$g/ml) showed significant increase in production of NO, whereas other mycobacterial antigen did not increase production of NO in both healthy as well as in TB patients. Likewise, among the mycobacterial antigens only BCG whole cell vaccine ($10^6$ cell/ml) showed significantly ($P<0.05$) greater release of H$_2$O$_2$ in healthy subject in comparison to TB patients (table 9).

4.4. ASSAY OF DTH RESPONSES IN BALB/c MICE

DTH responses (mm) in immunized BALB/c mice with *M. habana* following the administration of sheep RBC was also studied (table 10). Mice were first vaccinated with *M. habana*. After 30 days of initial vaccination, these
mice were given i/p sheep RBC's and after five days of i/p injection these mice were given challenge with SRBC in one of the foot pad. The foot pad enlargement (FPE) was measured at 24, 48, and 72 hrs intervals after the challenge. It was observed that induration of foot pad after challenge rose to the peak values at 24 hr and gradually started declining. On day 60 of the initial and 30 days after 1st booster vaccination, these mice were again given i/p SRBC and after five days of i/p injection, these mice were also challenged with SRBC in the foot pad. The FPE was measured. The maximum enhancement of DTH response was at 24 hr (P<0.01) which had a trend declining with time. Likewise, on day 90 of initial, day 60 of 1st and day 30 of 2nd booster vaccination, the values of DTH responses recorded further increase beyond 90 days P<0.001 (Table 10).

4.5. PHAGOCYTIC ASSAY
4.5.1. PHAGOCYTOSIS ASSESSMENT BY M. HABANA

The phagocytic ability of the Mφs were higher in the cells from immunized mice (Table II). It was indicated by the observation that the cells (macrophages) from the immunized mice, only 14.87% were having no bacilli and that 48.62% of the cells phagocytosed more than 10 bacilli (M. habana) per cell compared to 32.00% from normal non-immunized mice which was statistically significant P<0.05 (Table 11).

4.5.2. PHAGOCYTOSIS ASSESSMENT BY RADIO LABELLED E. COLI

Phagocytic activity of Mφs obtained from the peritoneal cavity of mice immunized with M. habana vaccine was not impressive than normal mice (Table 12) after 6 and 24 hr of incubation with radio labelled E. coli (P > 0.05)
The phagocytic response was 91.77 and 88.71% in vaccinated and unvaccinated cells, respectively in 6 hr incubation. Furthermore, in 24 hr incubation it was 93.77 and 90.25% in vaccinated and non-vaccinated Mφs, respectively (P>0.05) (Table 12).

4.5.3. ASSESSMENT OF IN VITRO ATTACHMENT/PHAGOCYTOSIS OF MYCOBACTERIUM TUBERCULOSIS (MTB) H₃₇,Ra

Attachment/phagocytosis of MTB H₃₇,Ra by activated murine Mφs was shown in Table 13. Increased phagocytosis suggested the immune competency of the macrophages in the in vitro conditions. Maximum phagocytosis/attachment at 12 hr was observed in 1:10 of the original concentration of Mφs monolayer (2x10⁶ cells/ml) recovered after lysing the Mφs by cold sterile triple distilled water. The colony forming unit (CFU) of mycobacteria (H₃₇,Ra) recovered from the lysed macrophages followed by incubation on LJ medium was measured. The CFU was 23.33 in immunized mice cells (macrophages) against 15 in control macrophages at 12 hr in 1:10 of original concentration. After 24 hr of incubation it was observed that the killing of mycobacteria had started which was observed more in immunized macrophages by reduction in the number of CFU (10.00) than the control macrophages (12.00) in 1:10 of original concentration of macrophage monolayer (P<0.01). Likewise, the killing of mycobacteria as assessed by reduction in the number of CFU were more in immunized and less in control macrophages at 1:100 of original concentration also (Table 13).
RESULTS

4.6. **IN VITRO EFFECTS ON MURINE MΦs BY *M. HABANA* VACCINE**

4.6.1. **EFFECT ON $O_2^-$**

Stimulation of vaccinated and normal murine macrophages with *M. habana* and BCG vaccine ($10^6$ bacilli each) and LPS ($10\mu$g/ml) was studied *in vitro* for the production of $O_2^-$ radical. The data has been presented in Table 14. In immunized mice the production of $O_2^-$ was greater than non-immunized MΦs in both 6 and 24 hr treatment. However, maximum release of $O_2^-$ was observed in immunized MΦs on *in vitro* stimulation with LPS followed by *M. habana* and BCG both in 6 and 24 hr treatment periods, respectively (Table 14).

4.6.2. **EFFECT ON $H_2O_2$**

Release of $H_2O_2$ (n mol/hr/10$^6$ cells/mg protein) by MΦs in normal and immunized mice on *in vitro* exposure of mycobacterial antigens (*vide supra*) were shown in Table 15. In the immunized mice the production of $H_2O_2$ was higher than non-immunized mice. However, maximum release of $H_2O_2$ was observed in immunized MΦs on *in vitro* stimulation with LPS followed by *M. habana* and BCG in 6 and 24 hr treatment periods, respectively from the control nonimmunized MΦs (Table 15).

4.6.3. **EFFECT ON NO**

Release of Nitric oxide (NO) (nm/hr/10$^6$ cells /mg protein) by normal and immunized murine macrophages on *in vitro* stimulation by LPS (10$\mu$g/ml), *M. habana* ($10^6$ cells/ml) and BCG ($10^6$ cells/ml) shown in Table 16. The ability to generate production of NO is higher in immunized MΦs compared to control MΦs in both 6 and 24 hr. Furthermore, when these MΦs were stimulated *in vitro*, the generation of NO was also higher with LPS, *M. habana* and BCG vaccine respectively with control in 6 and 24 hr of treatment periods (Table 16).
4.6.4. EFFECT ON LYSOSONAL ENZYMES

Lysosomal enzymes released from peritoneal exudate cells (PEC) of immunized (*M. habana*) and non-immunized mice and on *in vitro* effect of *M. habana*, BCG vaccine and LPS is presented in Table 17. Also, all the three lysosomal enzymes viz., acid phosphatase, β-glucuronidase and lysozyme were found to have increased secretion in immunized mouse cells as compared to non-immunized Mφs in 6 and 24 hr of incubation periods. Furthermore, in immunized mice, the production of lysosomal enzymes was found to be more when stimulated *in vitro* with LPS followed by *M. habana* and BCG vaccine at both between 6 and 24 hr treatment periods (Table 17).

4.7. *IN VITRO* EFFECT ON HUMAN HEALTHY MONOCYTES OF *M. HABANA* VACCINE

4.7.1. SUPEROXIDE ANION (O$_2^-$)

Monocytes (10$^6$ cells/ml) without stimulation incubated in medium produced 9.15 and 11.43 nmoles/hr/10$^6$ cells/mg protein of superoxide radicals at 6 and 24 hr, respectively (control). Monocytes treated with LPS (10 μg/ml), *M. habana* (10$^6$ cells) and BCG (10$^6$ cells/ml) vaccines when incubated for 6 and 24 hr produced significantly higher amounts of this radical (P<0.05). It was observed that 24 hr treatment of monocytes was most effective in the release of superoxide radical (Table 18).

4.7.2. EFFECT ON H$_2$O$_2$ (Table-19)

A significant increase in H$_2$O$_2$ (n moles/hr/10$^6$ cells/mg protein) production was observed in monocytes treated with LPS, *M. habana* and BCG in 6 and 24 hr of treatment, when compared to untreated monocytes. Whereas, untreated monocytes the yield of H$_2$O$_2$ remained low, the production of H$_2$O$_2$
when treated with LPS for 24 hr period recorded a considerably higher level (Table 19)

4.7.3. EFFECT ON NO

Release of NO (n moles/hr/10⁶ cells/mg protein) by healthy human monocytes on in vitro stimulation of different mycobacterial antigen is shown in Table 20. When these monocytes were elicited with mycobacterial antigen in vitro they showed the highest generation of NO with LPS (10 μg/ml) followed by M. habana and BCG vaccines. In the untreated monocytes, the generation of NO remained low at both 6 and 24 hr of incubation. The NO production by the monocytes in the presence of LPS was significant both at 6 and 24 hr, respectively. However, production of NO in the presence of M. habana and BCG vaccines was not at all significant at both the time intervals of 6 and 24 hrs (Table 20).

4.7.4. EFFECT ON LYSOSOMAL ENZYMES

Lysosomal enzymes namely, acid phosphatase, β-glucuronidase and lysozyme released from the healthy monocytes of human subjects after treatment with mycobacterial antigens and without treatment is shown in Table 21. Also, except β-glucuronidase, two other enzymes, namely acid phosphatase and lysozyme were found to have increased secretion in mycobacterial elicited monocytes as compared to non treated monocytes in 6 and 24 hr of incubation. Maximum production of lysosomal enzymes were found to be more when stimulated in vitro with LPS 10 μg/ml (Table 21).
4.8. ULTRA STRUCTURAL ANALYSIS

4.8.1. TRANSMISSION ELECTRON MICROSCOPY (TEM)

To determine the precisely defined ultrastructure characteristics of activated MΦs, transmission electron microscopy (TEM) studies were done. Thin sections of resident and *M. habana* vaccinated MΦs are shown in Fig 13 (a,b,cd). *M. habana* elicited mouse peritoneal macrophages (MPM) depicted ruffled cell membrane with increased number of fine fillipoda projecting from the surface. Cytoplasm exhibited prominent and large number of cytoplasmic vacuoles and more Golgi bodies. There was definite increase in the number of lysosomal like granules and mitochondria. The endoplasmic reticulum had become more prominent. The nucleus appeared to be more euchromatic indicating possible increased activity of the nucleus. When compared to the unstimulated MΦs, it appeared that there was a fairly good amount of increase in the cytoplasmic mass, there by changing the nucleus/cytoplasmic ratio with the stimulated macrophages. These changes resulted in bigger size of activated MΦs than the control untreated ones (Fig 13 a,b,c,d).

4.8.2. SCANNING ELECTRON MICROSCOPY (SEM)

Surface morphology of murine MΦs as determined by scanning electron microscopy is presented in Fig 14 (a,b,c,d). The difference between the macrophages from the normal mice and the immunized mice is evident. In the *M. habana* vaccinated mice, the surface had a more ruffled pattern with many infolding and invaginations than in the cells from the normal mice which had more smoother surface.
TABLES
TABLE 1

Effect on murine macrophages in the release of ROI and RNI after *M. habana* vaccination.

<table>
<thead>
<tr>
<th>Period (days)</th>
<th>Group</th>
<th>( \text{nmole/hr/10}^6 \text{ cells/mg protein} )</th>
<th>( \text{O}_2 )</th>
<th>( \text{H}_2\text{O}_2 )</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days after initial vaccination</td>
<td>Control</td>
<td>12.23±2.91</td>
<td>24.81±2.62</td>
<td>1.78±0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaccinated</td>
<td>14.54±3.10</td>
<td>26.20±4.02</td>
<td>1.95±0.08</td>
<td></td>
</tr>
<tr>
<td>30 days after initial and 60 days after 1st booster dose.</td>
<td>Vaccinated</td>
<td>15.27±2.62*</td>
<td>26.66±3.95</td>
<td>1.90±0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaccine + 1st booster</td>
<td>24.89±4.20***</td>
<td>33.42±3.21**</td>
<td>2.66±0.08**</td>
<td></td>
</tr>
<tr>
<td>90 days after initial, 60 days after 1st and 30 days after 2nd booster dose.</td>
<td>Vaccinated</td>
<td>14.87±1.80*</td>
<td>26.21±4.87</td>
<td>1.96±0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaccine + 1st booster</td>
<td>18.81±1.05*</td>
<td>29.72±4.03*</td>
<td>2.42±0.10*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaccine + 1st &amp; 2nd booster</td>
<td>23.12±2.98**</td>
<td>31.36±3.89**</td>
<td>2.58±0.09*</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SD of 4 set of experiments (* P<0.05, ** P<0.01, *** P<0.001 when compared with control.

\[\text{Page 3}\]
TABLE 2

Effect on murine macrophages in the release of lysosomal enzymes after *M. habana* vaccination.

<table>
<thead>
<tr>
<th>Period (days)</th>
<th>Group</th>
<th>Acid phosphatase</th>
<th>β-Glucuronidase</th>
<th>Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 Days after initial vaccination.</td>
<td>Control</td>
<td>8.2±1.120</td>
<td>73.09±5.82</td>
<td>20.68±2.80</td>
</tr>
<tr>
<td></td>
<td>Vaccinated</td>
<td>10.0±0.95*</td>
<td>80.40±16.21</td>
<td>22.98±1.98</td>
</tr>
<tr>
<td>60 Days after initial and 30 days after 1st booster.</td>
<td>Vaccinated</td>
<td>9.91±0.87</td>
<td>82.8±6.12*</td>
<td>23.42±3.12</td>
</tr>
<tr>
<td></td>
<td>1st booster</td>
<td>16.00±1.76***</td>
<td>127.18±8.2***</td>
<td>38.39±2.78***</td>
</tr>
<tr>
<td>90 Days after initial, 60 days after 1st and 30 days after 2nd booster dose.</td>
<td>Vaccinated</td>
<td>9.66±0.80</td>
<td>81.87±6.76</td>
<td>24.13±1.03*</td>
</tr>
<tr>
<td></td>
<td>1st booster</td>
<td>13.33±2.0**</td>
<td>102.33±7.0*</td>
<td>31.03±2.58**</td>
</tr>
<tr>
<td></td>
<td>2nd Booster</td>
<td>15.0±1.90***</td>
<td>116.10±6.12**</td>
<td>34.48±4.10</td>
</tr>
</tbody>
</table>

Data are mean ±SD of 4 set of experiments. *P<0.05, **P<0.01, ***P<0.001 when compared with control.
TABLE 3

Effect of murine macrophages on the oxidant killing system in peritoneal exudates cells of BALB/c mice after *M. hahana* vaccination.

<table>
<thead>
<tr>
<th>Period</th>
<th>Group</th>
<th>SOD^a</th>
<th>CAT^b</th>
<th>MPO^b</th>
<th>GPx^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days after initial</td>
<td>Control</td>
<td>2.59±0.07</td>
<td>3.40±0.08</td>
<td>48.05±4.21</td>
<td>0.072±0.006</td>
</tr>
<tr>
<td>vaccination</td>
<td>Vaccinated</td>
<td>2.90±0.15</td>
<td>3.95±1.08</td>
<td>50.60±4.80</td>
<td>0.074±0.007</td>
</tr>
<tr>
<td>60 days after initial and</td>
<td>Vaccinated</td>
<td>2.42±0.08</td>
<td>3.50±0.11</td>
<td>52.68±5.10</td>
<td>0.072±0.008</td>
</tr>
<tr>
<td>30 days after 1st booster.</td>
<td>1st booster</td>
<td>3.04±0.10*</td>
<td>4.00±1.02*</td>
<td>57.25±4.19*</td>
<td>0.089±0.006</td>
</tr>
<tr>
<td>90 days after initial, 60</td>
<td>Vaccinated</td>
<td>2.35±0.10</td>
<td>3.30±1.00</td>
<td>51.69±3.00</td>
<td>0.072±0.007</td>
</tr>
<tr>
<td>days after 2nd and 30 days</td>
<td>1st booster</td>
<td>3.00±0.20</td>
<td>4.04±1.06</td>
<td>52.79±3.80</td>
<td>0.081±0.008</td>
</tr>
<tr>
<td>after 1st booster.</td>
<td>2nd booster</td>
<td>3.05±0.09*</td>
<td>4.17±1.11*</td>
<td>56.76±4.88*</td>
<td>0.085±0.003*</td>
</tr>
</tbody>
</table>

The data are mean ± SD of 4 set of experiments. Unit/mg protein (1U=50% inhibition of auto oxidation of epinephrine at pH 10.2). ^b nmole/min/mg protein. ^p<0.05 . Control Vs treated.
TABLE 4

Effect on murine PBM cells in the release of ROI and RNI after *M. habana* vaccination.

<table>
<thead>
<tr>
<th>Period (days)</th>
<th>group</th>
<th>( \text{O}^2 )</th>
<th>( \text{H}_2\text{O}^2 )</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days after initial vaccination.</td>
<td>Control</td>
<td>8.03±0.692</td>
<td>20.11±1.09</td>
<td>1.21±0.08</td>
</tr>
<tr>
<td></td>
<td>Vaccinated</td>
<td>10.11±0.82*</td>
<td>22.19±2.00</td>
<td>1.30±0.09</td>
</tr>
<tr>
<td>60 days after initial and 30 days after 1st booster vaccination.</td>
<td>Vaccinated</td>
<td>9.86±0.80</td>
<td>21.69±1.89</td>
<td>1.29±0.07</td>
</tr>
<tr>
<td></td>
<td>Vaccinated with 1st booster</td>
<td>14.21±1.10**</td>
<td>29.00±2.80**</td>
<td>2.00±0.09***</td>
</tr>
<tr>
<td>90 days after initial, 60 days after 1st and 30 days after 2nd booster vaccination.</td>
<td>Vaccinated</td>
<td>9.00±0.60</td>
<td>21.00±1.66</td>
<td>1.26±0.09</td>
</tr>
<tr>
<td></td>
<td>Vaccinated with 1st booster</td>
<td>12.62±1.00**</td>
<td>24.61±2.01*</td>
<td>1.55±0.07*</td>
</tr>
<tr>
<td></td>
<td>Vaccinated with 1st and 2nd booster</td>
<td>13.22±0.67**</td>
<td>26.28±5.80**</td>
<td>1.75±0.09*</td>
</tr>
</tbody>
</table>

The data are mean ± SD of 4 set of experiments. *P<0.05, **P<0.01, ***P<0.001 when compared with control.
TABLE 5

Effect of murine PBM cells in the release of lysosomal enzymes after *M. habana* vaccination

<table>
<thead>
<tr>
<th>Period(days)</th>
<th>Group</th>
<th>Acid phosphatase</th>
<th>β-glucuronidase</th>
<th>Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days after initial vaccination</td>
<td>Control</td>
<td>6.82±1.00</td>
<td>50.92±3.00</td>
<td>16.20±3.00</td>
</tr>
<tr>
<td></td>
<td>Vaccinated</td>
<td>7.98±1.21</td>
<td>54.69±4.21</td>
<td>18.62±3.91</td>
</tr>
<tr>
<td>60 days after initial and 30 days after 1st booster</td>
<td>Vaccinated</td>
<td>7.12±1.19</td>
<td>52.81±2.96</td>
<td>18.00±3.66</td>
</tr>
<tr>
<td></td>
<td>1st booster</td>
<td>12.91±2.11***</td>
<td>79.90±4.12**</td>
<td>25.69±4.10*</td>
</tr>
<tr>
<td>90 days after initial, 60 days after 1st and 30 days after 2nd booster</td>
<td>Vaccinated</td>
<td>7.10±1.18</td>
<td>51.90±3.00</td>
<td>17.62±2.85</td>
</tr>
<tr>
<td></td>
<td>1st booster</td>
<td>10.89±2.00*</td>
<td>69.21±5.60*</td>
<td>22.62±2.79*</td>
</tr>
<tr>
<td></td>
<td>2nd booster</td>
<td>11.68±2.17**</td>
<td>74.00±45.31*</td>
<td>23.76±4.00*</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 4 set of experiments. *P<0.05, **P<0.01, ***P<0.001.
(Compared with Control)
### TABLE 6

Effect on murine PBMN cells in the release of anti-oxidant enzymes after *M. habana* vaccination.

<table>
<thead>
<tr>
<th>Period (Days)</th>
<th>Group</th>
<th>SOD(^a)</th>
<th>CAT(^b)</th>
<th>MPO(^b)</th>
<th>GPx(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days after initial vaccination.</td>
<td>Control</td>
<td>2.80±0.09</td>
<td>3.40±0.09</td>
<td>51.05±3.00</td>
<td>0.085±0.006</td>
</tr>
<tr>
<td></td>
<td>Vaccinated</td>
<td>2.89±0.10</td>
<td>3.16±1.00</td>
<td>53.06±2.81</td>
<td>0.90±0.006</td>
</tr>
<tr>
<td>60 days after initial and 30 days after 1st booster.</td>
<td>Vaccinated</td>
<td>2.86±0.08</td>
<td>3.51±0.11</td>
<td>52.11±2.75</td>
<td>0.087±0.007</td>
</tr>
<tr>
<td></td>
<td>1st booster</td>
<td>3.10±0.10*</td>
<td>4.00±0.09*</td>
<td>67.00±3.10*</td>
<td>0.103±0.009*</td>
</tr>
<tr>
<td>90 days after initial, 60days after 1st and 30 days after 2nd booster.</td>
<td>Vaccinated</td>
<td>2.85±0.10</td>
<td>3.48±0.06</td>
<td>51.82±3.11</td>
<td>0.088±0.005</td>
</tr>
<tr>
<td></td>
<td>1st booster</td>
<td>3.00±0.20</td>
<td>3.81±0.09*</td>
<td>60.88±4.00</td>
<td>0.98±0.004*</td>
</tr>
<tr>
<td></td>
<td>2nd booster</td>
<td>3.06±0.12*</td>
<td>3.9±0.07*</td>
<td>62.97±3.19*</td>
<td>0.100±0.009*</td>
</tr>
</tbody>
</table>

The data are mean ± SD of 4 set of experiments. \(^a\)P<0.05, \(^b\)Unit/mg protein (1U=50 % inhibition of autooxidation of epinephrine at pH 10.2) \(^c\)nmole/min/mg protein.
### TABLE 7

**Effect of murine macrophages in the release of cytokines after *M. habana* vaccination**

<table>
<thead>
<tr>
<th>Period (days)</th>
<th>Group</th>
<th>TNF (IU / million cell)</th>
<th>IL-1β (% proliferation of B-9 cell line)</th>
<th>IL-6 (IU / million cell)</th>
<th>IL-6 (% proliferation of B-9 cell line)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>18±1.29</td>
<td>12.24±0.9</td>
<td>48.23±3.78</td>
<td></td>
</tr>
<tr>
<td>10 days after</td>
<td>Vaccinated</td>
<td>38.21±3.98</td>
<td>22.42±2.8</td>
<td>107.24±12.41</td>
<td></td>
</tr>
<tr>
<td>vaccination</td>
<td></td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>30 days after</td>
<td>Vaccinated</td>
<td>22.32±2.65</td>
<td>14.56±1.76</td>
<td>54.65±7.52</td>
<td></td>
</tr>
<tr>
<td>vaccination</td>
<td></td>
<td>*</td>
<td>***</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>30+10 (booster)</td>
<td>Vaccinated + booster</td>
<td>52.65±5.56</td>
<td>40.52±2.00</td>
<td>242±42.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

Data are mean ±SD of 5 set of experiments. * P<0.05, **P<0.01, ***P<0.001 when compared with control.
TABLE 8

Lymphocyte proliferation on exposure to PBM cells of TB patients with mycobacterial antigens.

<table>
<thead>
<tr>
<th>Eliciting mycobacterial antigens</th>
<th>PHA(µg/ml)</th>
<th>PPD(µg/ml)</th>
<th>Habanin(µg/ml)</th>
<th>M.haban cells/ml</th>
<th>BCG cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>5</td>
<td>2</td>
<td>0.2</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>52610±8321</td>
<td>41845±6531</td>
<td>8231±981*</td>
<td>11260±1202</td>
<td>6308±1210*</td>
</tr>
<tr>
<td></td>
<td>22.47</td>
<td>17.87</td>
<td>3.51</td>
<td>4.84±2.69</td>
<td>1.79</td>
</tr>
<tr>
<td>SI</td>
<td>18450±3042**</td>
<td>8171±764*</td>
<td>4457±1901*</td>
<td>2517±2018±5</td>
<td>1659±308</td>
</tr>
<tr>
<td>TB1</td>
<td>10.88</td>
<td>4.82</td>
<td>2.62</td>
<td>1.48±1.19</td>
<td>0.97</td>
</tr>
<tr>
<td>SI</td>
<td>16120±490**</td>
<td>8091±810*</td>
<td>3939±810*</td>
<td>1592±1504±6</td>
<td>980±1201±6</td>
</tr>
</tbody>
</table>

Stimulation index(SI)=Average DPM of experimental value(5*)=average DPM of control value. Mean value of healthy control, TB patient with treatment and T1 patient without treatment(without antigens) are 2341±890, 1695±612,1501±340 respectively.*P<0.05,**P<0.01,***P<0.001 as determined by student t test H=Healthy. TB1=patient with treatment. TB2=without treatment. DPM=Disintragration per minute.
TABLE 9

Generation of $O_2^-$, NO and $H_2O_2$ from the monocytes of TB and Healthy human subjects

<table>
<thead>
<tr>
<th>Stimulus used</th>
<th>n mole/hr/mg protein</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Super oxide ($O_2^-$)</td>
<td>H$_2O_2$</td>
<td>Nitric oxide (NO)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>H</td>
<td>TB</td>
<td>H</td>
</tr>
<tr>
<td>PHA (2 µg/ml)</td>
<td>4.07±0.19</td>
<td>8.65±1.00</td>
<td>20.66±2.77</td>
<td>25.22±1.07</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>**</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>PPD (10 µg/ml)</td>
<td>3.62±0.40</td>
<td>5.90±0.61</td>
<td>16.11±2.25</td>
<td>19.77±2.68</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>habanin (10 µg/ml)</td>
<td>3.86±0.13</td>
<td>6.00±0.50</td>
<td>15.67±2.00</td>
<td>20.01±1.80</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>BCG (10^6 cells/ml)</td>
<td>3.60±0.90</td>
<td>6.92±0.88</td>
<td>19.00±2.48</td>
<td>23.66±3.01</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>M. habana (10^6 cells/ml)</td>
<td>3.90±0.14</td>
<td>7.08±0.73</td>
<td>16.77±1.00</td>
<td>24.11±2.31</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 3 set of experiments *P<0.05, **P<0.01 when compared with control (without antigen). The mean control value of $O_2^-$ was 3.00±0.11 and 4.40±0.30, $H_2O_2$ 15.77±3.72 and 18.22±2.82 and NO 2.62±0.05 and 2.88±0.08 in TB and healthy control subjects. H=healthy subjects, TB=tuberculosis patients.
**TABLE 10**

DTH responses (mm) in immunized and control BALB/c mice following administration of sheep RBC.

<table>
<thead>
<tr>
<th>Period (days)</th>
<th>Group</th>
<th>24 (hr)</th>
<th>48 (hr)</th>
<th>72 (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days after initial vaccination.</td>
<td>Control</td>
<td>0.56±0.16</td>
<td>0.38±0.07</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td></td>
<td>Vaccinated</td>
<td>1.35±0.28**</td>
<td>0.78±0.16*</td>
<td>0.46±0.09</td>
</tr>
<tr>
<td>60 days after initial and 30 days after 1st booster.</td>
<td>Vaccinated</td>
<td>0.67±0.23</td>
<td>0.40±0.11</td>
<td>0.42±0.09</td>
</tr>
<tr>
<td></td>
<td>Ist booster</td>
<td>1.72±0.19**</td>
<td>1.02±0.10**</td>
<td>0.49±0.19</td>
</tr>
<tr>
<td>90 days after initial, 60 days after 1st and 30 days after 2nd booster.</td>
<td>Vaccinated</td>
<td>0.60±0.10</td>
<td>0.41±0.11</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td></td>
<td>Ist booster</td>
<td>1.45±0.18**</td>
<td>0.87±0.06**</td>
<td>0.47±0.05</td>
</tr>
<tr>
<td></td>
<td>2nd booster</td>
<td>1.63±0.19***</td>
<td>1.01±0.09**</td>
<td>0.62±0.10*</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 5 set of experiments. *P<0.05 , **P<0.01 *** P<0.001 when compared with control.
**TABLE 11**

Percent of macrophages from normal (N) and *M. habana* vaccinated (I) mice phagocytosing intact *M. habana* cells.

<table>
<thead>
<tr>
<th>Time</th>
<th>No bacilli</th>
<th>1 to 5 bacilli</th>
<th>5-10 bacilli</th>
<th>&gt;10 bacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>I</td>
<td>N</td>
<td>I</td>
</tr>
<tr>
<td>90 min</td>
<td>60</td>
<td>42</td>
<td>56</td>
<td>63</td>
</tr>
<tr>
<td>3hr</td>
<td>20</td>
<td>7</td>
<td>88</td>
<td>32</td>
</tr>
<tr>
<td>6hr</td>
<td>18</td>
<td>0</td>
<td>53</td>
<td>21</td>
</tr>
<tr>
<td>24hr</td>
<td>21</td>
<td>0</td>
<td>42</td>
<td>12</td>
</tr>
<tr>
<td>Average</td>
<td>14.87</td>
<td>6.12</td>
<td>29.87</td>
<td>16</td>
</tr>
<tr>
<td>SD ±</td>
<td>10.1</td>
<td>10</td>
<td>9.88</td>
<td>11.11</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 4 set of experiments. *P < 0.05 when compared with control.
Phagocytosis responses of immunized \((M. \ hahana)\) and non-immunized BALB/c mice macrophages with radiolabelled \(E. \ coli\).

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean CPM of non vaccinated</th>
<th>Mean CPM of vaccinated</th>
<th>Statistical Significance vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>4460±1021 (88.71)</td>
<td>3249±812 (91.77)</td>
<td>(P &gt; 0.05)</td>
</tr>
<tr>
<td>24 hr</td>
<td>3850±940 (90.25)</td>
<td>2461±610 (93.77)</td>
<td>(P &gt; 0.05)</td>
</tr>
</tbody>
</table>

Mean of total count: 39514±3610.
Data presented here are mean ±SD of 3 set of experiments.
Data in the parenthesis denote % of phagocytosis of \(E. \ coli\) by the murine macrophages.
'\(P\)' value \(< 0.05\) than considered as non-significant.
TABLE 13

Colony forming unit (CFU) of macrophages recovered mycobacteria on LJ medium

<table>
<thead>
<tr>
<th>Hour</th>
<th>Concentration of macrophages from original concentration</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1:10</td>
<td>15±2.82</td>
<td>23±1.15**</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>4±2.15</td>
<td>8±1.57*</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1±0.57</td>
<td>2±0.57</td>
</tr>
<tr>
<td>24</td>
<td>1:10</td>
<td>12±2.80(20)</td>
<td>10±1.85(56.52)b</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>3±1.90(25)</td>
<td>3±2(62)\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1±0.39</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are mean ±SD of 5 set of experiments. \textsuperscript{*}p<0.05, \textsuperscript{**}p<0.01 when compared with control, \textsuperscript{a} p<0.05, \textsuperscript{b} p<0.01 when compared with 12 hr of incubation. Data in parenthesis denote % change of CFU over 12 hr (CFU= colon forming unit).
TABLE 14

Superoxide production by murine Mφs from normal and M. habana immunized mice on *in vitro* stimulation with mycobacterial antigens.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Source of macrophages</th>
<th>(nmole/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With macrophages without stimulant</td>
<td>Macrophage + LPS (10 μg/ml)</td>
</tr>
<tr>
<td>6 hour</td>
<td>Control</td>
<td>10.87±0.81</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>14.10±0.98*</td>
</tr>
<tr>
<td>24 hour</td>
<td>Control</td>
<td>11.25±0.11</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>16.18±1.20*</td>
</tr>
</tbody>
</table>

Data are mean ±SD of 4 set of experiments. *P<0.05, **P<0.01, ***P<0.001 over the value of macrophages without stimulant.
TABLE 15

Release of $\text{H}_2\text{O}_2$ by the macrophages of normal and immunized mice on in vitro exposure to mycobacterial antigens.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Source of macrophages</th>
<th>With macrophage without stimulant</th>
<th>macrophage+LPS (&lt;10μg/ml)</th>
<th>macrophage+M. habana (&lt;10⁶ cells/ml)</th>
<th>macrophage+BCG (&lt;10⁶ cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hour</td>
<td>Control</td>
<td>25.00±0.81</td>
<td>30.31±2.00**</td>
<td>27.61±1.20</td>
<td>27.87±1.21</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>29.61±0.76*</td>
<td>38.1±1.22</td>
<td>34.00±1.00*</td>
<td>34.60±2.00*</td>
</tr>
<tr>
<td>24 hour</td>
<td>Control</td>
<td>29.28±1.27</td>
<td>34.00±2.00</td>
<td>30.84±1.26</td>
<td>28.00±0.61</td>
</tr>
<tr>
<td></td>
<td>Immunized</td>
<td>36.91±0.88*</td>
<td>46.80±1.22***</td>
<td>39.61±1.77**</td>
<td>36.41±0.89**</td>
</tr>
</tbody>
</table>

Data are mean ±SD of 4 set of experiments. *P<0.05, **P<0.01, ***P<0.001 when compared with the value of macrophages without stimulant.
TABLE 16

Release of nitric oxide (NO) by macrophages from immunized and non-immunized mice on *in vitro*
exposure of mycobacterial antigens.

<table>
<thead>
<tr>
<th>Incubation of macrophages with vaccine</th>
<th>Source of macrophages</th>
<th>(nmole/hr/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Macrophase without stimulant</td>
<td>Macrophase+LPS stimulants (10μg/ml)</td>
</tr>
<tr>
<td>6 hour</td>
<td>Control</td>
<td>1.79±0.10</td>
<td>2.42±0.19</td>
</tr>
<tr>
<td></td>
<td>immunized</td>
<td>2.11±0.09</td>
<td>3.66±0.08**</td>
</tr>
<tr>
<td>24 hour</td>
<td>Control</td>
<td>2.10±1.18</td>
<td>2.62±0.20</td>
</tr>
<tr>
<td></td>
<td>immunized</td>
<td>2.61±0.21*</td>
<td>4.17±0.21***</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 4 set of experiments. *P<0.05, **P<0.01, ***P<0.001 when compared with control macrophages (without stimulant)
TABLE 17
Mean level of lysosomal enzymes in macrophages obtained from normal and immunized balb/c mice on in vitro of *M. habana* and BCG vaccine

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Incubation of macrophages with vaccine</th>
<th>Source of macrophage</th>
<th>(nmole/hr/mg protein)</th>
<th>Macrophage leth system (10^6 cells/ml)</th>
<th>Macrophage + LPS stimulant (10^6 cells/ml)</th>
<th>Macrophage + M habana stimulant (10^6 cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>6 hr</td>
<td>control</td>
<td>6.81±2.72</td>
<td>10.88±11.21</td>
<td>9.00±5.00</td>
<td>8.32±4.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunized</td>
<td>8.00±3.76</td>
<td>13.76±10.21*</td>
<td>11.67±4.76</td>
<td>10.76±6.00*</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>control</td>
<td>9.62±4.00</td>
<td>12.88±4.11</td>
<td>11.66±3.00</td>
<td>10.21±4.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunized</td>
<td>12.22±3.66*</td>
<td>19.28±4.86**</td>
<td>16.00±6.21*</td>
<td>14.66±5.10*</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>6 hr</td>
<td>control</td>
<td>74.21±7.89</td>
<td>84.62±10.21</td>
<td>82.88±16.19</td>
<td>81.92±17.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunized</td>
<td>89.69±4.62</td>
<td>106.00±19.00*</td>
<td>95.69±10.62*</td>
<td>99.48±13.14</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>control</td>
<td>77.62±12.12</td>
<td>91.00±16.13</td>
<td>87.62±8.45</td>
<td>88.29±11.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunized</td>
<td>95.92±15.17*</td>
<td>120.29±27.00**</td>
<td>96.61±19.10*</td>
<td>104.69±16.16</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>6 hr</td>
<td>control</td>
<td>20.21±3.00</td>
<td>30.27±6.01</td>
<td>24.69±8.62</td>
<td>26.25±7.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunized</td>
<td>24.11±4.20</td>
<td>39.68±12.00**</td>
<td>30.13±9.79*</td>
<td>34.00±9.00*</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>control</td>
<td>23.21±5.00</td>
<td>34.69±14.00</td>
<td>27.84±4.20</td>
<td>28.22±9.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunized</td>
<td>31.11±5.00*</td>
<td>49.00±23.00**</td>
<td>36.00±7.00*</td>
<td>33.89±12.11*</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 4 set of experiments *P<0.05, **P<0.01 (compared with the values of macrophages without stimulant).
**TABLE 18**

Superoxide production by the monocytes of healthy human subjects on
_in vitro_ stimulation with _M. habana_ and BCG vaccine.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>(nmole/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>monocyes without stimulant</td>
</tr>
<tr>
<td>6 hr</td>
<td>9.15±0.08</td>
</tr>
<tr>
<td>24 hr</td>
<td>13.43±0.19</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 3 set of experiments. *P<0.05, **P<0.01, ***P<0.001 when compared with the value of monocytes without stimulant.
Release of $H_2O_2$ from the monocytes of healthy human subjects elicited with mycobacterial antigens

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>(nmole/hr mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With monocytes without stimulant</td>
<td>monocytes+LPS (10μg/ml)</td>
</tr>
<tr>
<td>6 hr</td>
<td>10.61±1.00</td>
<td>19.62±1.10**</td>
</tr>
<tr>
<td>24 hr</td>
<td>14.39±0.89</td>
<td>22.69±1.88*</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 3 set of experiments *P<0.05, ** P<0.01, *** p<0.001 when compared with the value of the monocytes of without stimulant.
**TABLE 20**

Release of nitric oxide (NO) by human healthy monocytes on *in vitro* stimulation with mycobacterial vaccine namely *M. habana* and BCG

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>(nmole/hr/mg protein)</th>
<th>(nmole/hr/mg protein)</th>
<th>(nmole/hr/mg protein)</th>
<th>(nmole/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>monocytes only</td>
<td>monocyte+LPS (10 μg/ml)</td>
<td>monocytes+<em>M. habana</em> (10⁶ cells /ml)</td>
<td>monocytes+BCG (10⁶ cells /ml)</td>
</tr>
<tr>
<td>6 hr</td>
<td>1.23±0.24</td>
<td>2.89±0.24 **</td>
<td>1.69±0.20 *</td>
<td>1.71±0.28 *</td>
</tr>
<tr>
<td>24 hr</td>
<td>1.80±0.84</td>
<td>3.00±0.60 *</td>
<td>1.96±0.80</td>
<td>1.98±0.66</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 3 set of experiments. *P<0.05, **P<0.01 when compared with the value of monocytes without stimulant.
<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Time of incubation</th>
<th>monocytes only</th>
<th>monocytes+LPS(10 µg/ml)</th>
<th>monocytes+M.habana(10^5 cells/ml)</th>
<th>monocytes+BCG(10^6 cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>6 hr</td>
<td>3.41±0.12</td>
<td>7.60±0.31**</td>
<td>4.58±1.00*</td>
<td>5.00±0.81*</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>5.10±0.18</td>
<td>9.22±0.81**</td>
<td>6.80±1.12*</td>
<td>6.90±1.18*</td>
</tr>
<tr>
<td>ß glucuronidase</td>
<td>6 hr</td>
<td>50.82±3.66</td>
<td>65.66±4.00*</td>
<td>56.32±2.70</td>
<td>57.00±2.81</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>60.39±3.00</td>
<td>72.56±2.56*</td>
<td>67.60±2.11</td>
<td>68.12±2.01</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>6 hr</td>
<td>16.69±2.40</td>
<td>25.21±1.80*</td>
<td>21.42±1.00*</td>
<td>22.10±1.11*</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>19.22±3.18*</td>
<td>27.60±2.00*</td>
<td>23.77±0.85*</td>
<td>24.00±0.92*</td>
</tr>
</tbody>
</table>

Data are mean ± SD of 3 set of experiments. *P<0.05, **P<0.01 when compared with the value of monocytes (without stimulant)
ILLUSTRATIONS
The bar diagram represents DTH response (mm) in immunised and non-immunised mice, following the administration of SRBC at 24, 48 and 72 hr, after different doses of vaccine (*M. habana*). Representative values are mean ± SD of 4 set of experiments (* P < 0.05 **P < 0.01 ***P <0.001) when compared with control.
Fig. 14. Histogram represent the effect of *M. hahana* vaccine on cytokines (IL-1B, IL-6 and TNF) production by mouse peritoneal macrophages. Representative values are mean ± SD of 5 set of experiments. *p < 0.05, **p < 0.01, ***p < 0.001 when compared with control.
Figure 12: The histograms (ABCDEF) represent the proliferative responsiveness of PBM cells of TB patients as well as healthy human subjects by PHA, PPD, habitin, M. habana and BCG whole cell vaccines. The representative values are mean ± SD of 5 set of experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 when compared with the values of without stimulants (mycobacterial antigens).

[Graphs showing stimulation indices for different antigens and cell concentrations]
Fig. 13 (a,b,c,d). Immunised murine peritoneal macrophages showed prominent mitochondria (M), distinct vacuole (v), more Golgi apparatus (G), increased cell size and lysozymes (L) in transmission electron microscope (TEM).
Immunised murine peritoneal macrophages showed ruffled pattern of the surface and bigger in size. However, in low magnification (x 500), difference could not be observed.