DISCUSSION
Controversial status of BCG vaccine against tuberculosis (TB) and leprosy has aroused an all round interest for search of some other immunogenic strain(s) of mycobacteria to combat these infections. This has become all the more important due to resurgence of AIDS, emergence of multidrug resistance and appearance of new and unresponsive forms of TB due to *M. avium* *Intracellularare scrofulaceum* complex (MAIS), both in developed and developing countries (Andersen, 1994)

In this endeavour we have identified one strain of atypical mycobacterium; *M. habana* which in comparison to BCG affords better protection against *Mycobacterium tuberculosis* (MTB), *M. leprae* and *M. ulcerans* challenges in animals. *M. habana* also shares some immunologically dominant proteins with MTB and *M. leprae*. This vaccine has been recognised as a potential one and has been granted permission by the Drugs Controller of India for conducting clinical trials against leprosy in human beings. This vaccine candidate has attained the status of broad spectrum antimycobacterial vaccine against TB and leprosy, therefore it is imperative to understand the mechanisms involved in affording protection by this vaccine.

Mycobacteria are obligate intracellular and acid fast bacilli, multiplying mainly in macrophages. MTB resides mainly in lung macrophages (pulmonary TB) and other tissue and wandering macrophages. These cells are mainly involved at all stages of immune response. They act as the third line of defense against an invader with powerful weapons like reactive oxygens and nitrogen metabolites, lysozyme, acid hydrolases and other mediating factors at cellular level (First and second line of defence being intact skin, cilia and other secretions like tears and saliva etc.).
The purpose of this investigation was to ascertain the basic facts about this vaccine regarding its mode of affording protection and/or generation of CMI responses, so that use of this type of vaccine could be advocated in humans.

To test the capacity of an immunogenic strain to generate CMI responses and/or protection some definite criteria have been laid down by WHO working group in mycobacteria:

1. The strain should have some common antigens with MTB or *M. leprae*.
2. Should show positive delayed type hypersensitivity responses with MTB in guinea pig and mice.
3. DTH may be transferable to other recipient passively through cells.
4. Should be able to be quantitated with respect to skin erythema, foot pad enlargement and lymphnode enlargement.
5. Should show protection in mice against virulent challenge.

These tests have already been conducted with *M. habana* vaccine to adjudge its ability to generate protection, CMI responses and sharing of antigens with MTB and *M. leprae* which have been found to be satisfactory, thereby an all round interest in the development of this vaccine for human use have been aroused which has been examined in this study.

Since very little at present is known about the mechanisms of protection provided by the vaccine, it was considered of interest to examine the mechanisms of action in rodents as well as human subjects as a result of *M. habana* vaccination. It has been established beyond doubt that killing of wide
A variety of infectious organisms by the macrophages is mediated by two types of effector mechanisms: the oxygen dependant and oxygen independant (James, 1991; Moncada et al., 1991). Between these two, the former exerts its effect due to inimical actions of its metabolic intermediate to the pathogens and is considered more important in overcoming pathogenic consequences. In the in vitro conditions M leprae is highly susceptible to H$_2$O$_2$ and particularly to its halide products (Sharp et al., 1985; Klebanoff et al., 1984). However, when M leprae is ingested by phagocytic cells, it down regulates the production of superoxide radical and hydrogen peroxide. Many other infectious organisms, including M. leprae and MTB also appear to survive in the host tissue by down regulating the respiratory burst activity (Bogdan et al., 1990). In this context, the enhancement of O$_2^{-}$ and H$_2$O$_2$ production by M. habana induced vaccine provides a strong clue for enhanced bactericidal activity of the vaccine sensitized macrophages leading to protection against live challenge.

The importance of O$_2^{-}$ has been accentuated by the fact that it may combine with NO, another potent cytotoxic molecule transiently to produce peroxynitrate (ONOO$^-$), which could be more lethal than either of the reactants alone (Radi et al., 1991). Nonspecific stimulators like thioglycolate, protease peptone, endotoxin were also reported to cause similar changes in macrophages and have led to the arrest of infectious organisms (Cohn, 1978; Karnovsky et al., 1978). The increase in cellular content of lysosomal hydrolases brought about by stimulatory agents possibly equips M$^+$ for enhanced phagocytic digestion of engulfed or segregated materials during their interaction. Also, the vaccine treatment markedly enhanced activities of the three lysosomal enzymes studied, namely acid-phosphatase, beta glucuronidase and lysozyme.
Administration of booster dose of the vaccine showed dramatic effect and have induced further rise in the levels of these enzymes. Lysozyme is known to mediate killing by digesting bacterial cell wall. The two enzymes acid phosphatase and β-glucuronidase actively participate in the hydrolytic activity of lysosome where in the invading organisms are digested after phagocytosis.

Evidence indicate that *M. leprae* and *M. tuberculosis* resides in phagolysosomes which are known to contain a battery of hydrolytic enzymes (Marolia et al., 1987). It therefore seems that *M. leprae*, MTB avoids lysosomal attack either by inactivation or down regulation of hydrolytic enzymes. Interestingly, *M. habana* vaccine activates this system at the cellular level and thereby it induced faster digestion of the infectious organisms. This might be one of the mechanisms of action of *M. habana* vaccine which provide protection against mycobacterial infection in mice.

Thus, it could be concluded that, the vaccine aggravates killing machinery of MΦs in both the lysosomal and cytosolic compartments. This would make the phagocytic cells fully active to eliminate any infection, particularly those like MTB, *M. leprae* and *L. donavani* which establishes in these cells types by down regulating their effector system. Therefore, an immunogen and/or vaccine capable of upregulating the effector arms of the macrophages certainly offers better chances of lowering the "takes" of infectious agent(s) and that is what this vaccine is possibly doing in warding off the infectious challenge.

Mycobacterial products obtained from other mycobacteria like delipidified cell components (DCC) of *M. leprae* have served as strong immunomodulatory antigens and have resulted in the increased production of
reactive oxygen intermediates like superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) in DCC activated macrophages. The superoxide has been responsible for intracellular killing of the *M.leprae* since addition of superoxide dismutase (SOD) which destroys the superoxide anion leads to establishment of *M.leprae* in the macrophages. These views have been supported by several investigators (Damle *et al.*, 1993; Mahadevan *et al.*, 1991).

During the present study, the reactive oxygen intermediates like $O_2^-$ and $H_2O_2$ and have been found to increase in the *M.habana* activated macrophages and peripheral blood mononuclear cells (PBMNC). Thus, it may be thought that *M. habana* antigens are also endowed with immunomodulatory properties which leads to increased production of ROI and wards off the infectious challenge and thus our work is corroborating with the earlier findings by other investigators referred as above.

During present investigation we have also seen the increased production of NO by *M.habana* activated macrophages and BMNC in BALB/c mice. NO which has been synthesized from L-arginine has been found to be a potent cytotoxic effector molecule for the invading microorganisms and the tumour cells. Bacterial lipopolysaccharides (LPS) have the property to increase NO concentration *in vivo* and *in vitro* conditions. Mycobacteria including *M.habana* contain these LPS molecules. NO molecule has been known to be produced by several mammalian cell types including macrophages and increased production under antigenic stimulus is responsible for checking the establishment of infectious organism which may explain the role of antigen / vaccine in affording protection. *M.habana* antigens (possibly LPS) might be
doing same functions and thus this pathway could also be responsible for protection being offered by *M. habana* vaccine.

Several workers have shown a good correlation between the capacity of macrophages to kill microorganisms and production of NO. Comparison and production of ROI versus nitrogen intermediates by mouse peritoneal macrophages and their role in killing organisms in various *in vitro* and *in vivo* system have been extensively reported in literature. It has been also reported that toxic activities of NO depends upon co-operation with ROI (Linn et al., 1992). Therefore, dual combination of oxygen radicals and NO can be possible in the immune effector mechanisms against invaders.

*M. habana* antigens have led to increased production of NO and ROI both in activated peritoneal macrophages and in the peripheral blood mononuclear cells. Therefore, the production of these molecules may be of importance both in term of production and induction of host responses (CMI etc.) in the sensitized individuals/animals.

Accordingly, enhanced production of these radicals in the *M. habana* vaccine activated murine macrophages and PBMN cells have led to make these cells very aggressive and potent angry killers for infectious challenges both at intracellular and cellular vicinity level. These findings are in complete agreement with those of Batra et al. (1993) and Singh et al. (1992).

In another parameter of study, the mechanisms of action of *M. habana* vaccine at the cellular level was evaluated by the amount of antioxidant enzymes namely, superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), glutathione peroxidase (GPx) produced by the cells (peritoneal macrophages). Reactive oxygen species are deleterious and can
damage membranes, nucleic acid and proteins of cellular organisms leading to their death and non establishment of infection. On the other hand antioxidant enzymes and other scavengers produced during normal metabolic processes in mammalian and/or other animals are responsible for warding off the harmful effects of oxygen free radicals in biological system (Kugura et al., 1984; Callahan 1988; Batra et al., 1989). These radicals (ROI, RNI) can also bring about a number of reactions which can be harmful to tissues. The production of ROs increases in response to antigens/vaccines or non specific substances like tuftsin etc. leading to offering protection against the infectious organisms.

This is what has been seen in the *M. habana* activated murine macrophages and PBMN cells which have produced increased amount of ROs in addition to RNI and lysosomal enzymes.

The disease spectrum of leprosy and tuberculosis speak that TT, BT, BB, BL, and LL forms does exist in leprosy patients where TT patients have mounted CMI responses and LL patients completely lack in cellular immunity. Tuberculosis patients also fall in the following category of RR, RI, UI and UU, where RR is the reactive form which have mounted CMI responses and UU form is the polar unreactive or anergic form.

It is now established that fundamental immunological defect in LL leprosy patient and unreactive UU TB patient is the lack of CMI to *M. leprae* and *M. tuberculosis*. The major evidence for lack of CMI in LL and UU is that there is absence of granuloma formation in the lesions, lymphnodes show depleted T-cell areas, lepromin and tuberculin delayed type hypersensitivity and DTH skin test are negative. *In vitro* tests like LTT and Leucocyte
migration inhibition tests are negative. Thus it appears most likely and proved by facts that the defect in CMI operating in leprosy and TB is due to the development of an immunodeficiency or immunosuppressive mechanisms.

Therefore, a vaccine for leprosy and/or TB should have the property to boost the cell mediated immune responses and also the vaccine should be endowed with immunomodulatory properties.

For this type of study one *in vivo*-delayed hypersensitivity and one *in vitro*-lymphotransformation test were conducted to assess these responses generated by the vaccine. DTH response is the direct measure of CMI since sensitized animals if receive the same or related antigens in later part of their life exhibit exaggerated responses. This was the basis of intradermal skin test with tuberculin (PPD) and lepromin.

Two distinct phases of reaction can be identified. The first phase is sensitization of T-lymphocytes to a given antigen and migration of the cells to T-cell dependent paracortical zones of draining lymphnodes. A population of T-lymphocytes specially sensitized with antigen is thereby created and can be demonstrated by the lymphoproliferative responses in the areas. Certain cells among these can circulate for several years.

The second is the recognition phase that occurs during re-exposure to antigen. In this phase blast transformation of sensitized T-lymphocytes is associated with the release of mediators that are classified according to their biological activities. They are:

* MIF
* Macrophage activation factors
* Blastogenic factors
* Lymphokines and interferons
When sensitized animals receive the homologous or antigenically related antigens intradermally the lymphocytes and other defensive cells of the body accumulate due to chemotactic factors released by the sensitized cells at the site of inoculation (I.D) and produce a wheel and flare reaction accompanied by induration in 24 to 72 hr (therefore called delayed type). The area is measured and results are concluded with respect to DTH responses and antigenic similarity. The whole sequence of events of wheel and flare and induration is due to the release of these biological mediators mentioned above.

The DTH response is mediated by T-cells and its lymphokines where T-lymphocytes accumulate at the site of antigen deposition and produced lymphokines and attract macrophages and lymphocytes. DTH usually implies a long lasting immunity. DTH to certain antigens often reflects a state of enhanced resistance of the host to these mycobacteria. The essential characteristic of this is the delay in expression relative to the time of eliciting injection. They are depicted by:

* delay in appearance in 24-48 hr
* an indurated or erythematous papule which may be necrotic and/or vesicular in case of partially violent reactions.
* the initial perivascular dermal infiltrate of polynuclear neutrophils, basophils and mononucleated cells followed by progressive appearance of lymphocytes and monocytes.
* it is not transferrable by serum but transferred by cells.

An ideal vaccine for TB would contain components of the bacilli that produced more activated (microbicidal) macrophages and less tissue destruction. Hypersensitivity (DTH) and protection are considered to be
manifestation of one of the same immunologic mechanism. Such a vaccine would favour in expansion of T-cell population that increased the ability of macrophages to destroy tubercle bacilli. Then any demonstration of the existence of hypersensitivity could be taken as evidence of immunity to TB. Any vaccine that produces hypersensitivity would also confer an acquired immunity. Any vaccine that produces a low level of hypersensitivity could be considered as conferring a low level of protection (George et al., 1984).

This way *M. hahana* vaccination in mice has aroused a high level of hypersensitivity (DTH) response against homologous and antigenically related heterologous antigens like PPD and BCG. In concluding the evidences, produced by *M. hahana* vaccine it could safely be interpreted to be the immunogenic property of this vaccine and indirectly DTH response provides the evidences of aggregation of lymphocytes and macrophages at the antigen depository and also release of molecular mediators at the site.

Other vaccinated strains like *ICRC bacillus* and *Mycobacterium W* have also produced high degree of CMI responses and our results are in agreement to those of Deo et al., (1983) and Talwar, (1978).

It is believed that acquired resistance to mycobacteria is cell mediated process that start when sensitized T-cell recognised bacterial antigen presenting cells (APC). An ideal vaccine favours expansion of T-cell population that increased the ability of macrophages to destroy the TB bacilli due to over expansion of T-cell population. It is an established fact that antigenically sensitized lymphocytes if driven in the presence of homologous and antigenically related heterologous antigens multiply enormously which could be measured microscopically (Singh et al., 1985) by studying the cellular and
nuclear changes or by counting of pulses of radio labelled thymidine which gets incorporated in multiplying cells. The later technique is more sophisticated and is followed by most of the researchers. This technique was also followed in this study. During the immunization process the T-cell has clonally expanded when tested in the presence of same or related antigens. These actively multiplying cells produce lymphokines which, when estimated could provide an explanation for one of the basic mechanisms of protection by a vaccine.

In mycobacterial diseases like Leprosy/TB, the level of IL-1, IL-6 and TNF (all produced by macrophages) are reported to be quite low (Yamamura et al., 1991). It is also understood that IL-1, IL-6 are known to induce the production of IL-2, T-cell growth factor, TNF in association with IL-2 on the other hand activates natural killer (NK) cells to release IFN-γ. The subdued production of IL-1β, IL-6 and TNF in the disease like TB lead to decreased production of IL-2 and IFNγ. In general all these cells consisting of activated mononuclear phagocytes, cytotoxic T cells, natural killer (NK) cells and lymphokines activated killer (LAK) cells create the environment of CMI. These effector molecules are known to activate macrophages so that they have an enhanced production of toxic reactive oxygen intermediates (ROI) and also reactive nitrogen intermediates (RNI) in order to more effectively kill both intracellular and extracellular micro-organisms. All these factors collectively influence the cellular functions in situ and also further recruit appropriate cells from the circulation, ultimately leading the formation of an immune granuloma for the destruction of the invading microorganisms.
Interestingly, the release of IL-1β, IL-6 and TNF were markedly increased after *M. habana* vaccination. Thus, the stimulation of Th-1 type of activity by the vaccine would have generated CMI and also considerably high increase in the formation of ROI and RNI. This is what *M. habana* vaccine appears to have done. Thus the vaccine might be useful in controlling the disease like TB.

These findings have largely explained the mechanisms involved in affording protection by *M. habana* vaccine.

In another parameter of study the, phagocytosis and subsequent killing of pathogens by *M. habana* vaccinated murine macrophages was evaluated. It represent one of the most important early host defence against disease. Furthermore, phagocytosis or engulfment increases after sensitization with foreign antigen/vaccine or in the presence of specific homologous sera containing antibodies.

In our observation, it was clearly seen that after *M. habana* vaccination the phagocytic cells (macrophages) got activated which is a multistep process and have acquired additional power than the normal cells. The destruction or killing of foreign particles as well as intracellular organisms by phagocytosis depends on the cell’s capacity to secrete microbicidal metabolites such as ROI, RNI, lysosomal and antioxidant enzymes and cytokines. On stimulation with appropriate agent *in vitro* these phagocytic cells get activated and result in enhanced production of these metabolites (Wartful *et al*., 1986).

Also, lysosomal enzymes are considered to be one of the constitutive enzymes of mononuclear phagocytes (Gordon *et al*., 1974). Pie *et al*., (1991) have also reported that monocytes treated with LPS, MDP and IFN-γ released
significantly more lysozymes compared to untreated monocytes. Gordon, *et al* (1975) also observed BCG stimulated rabbit alveolar macrophages have high levels of intracellular lysozymes. Likewise, Stanley *et al.*, (1996) reported that murine macrophages exposed to recombinant myeloperoxidase (MPO) exhibited enhancement of the respiratory burst, increased phagocytosis by releasing microbicidal activity like ROI, RNI. Furthermore, MPO stimulated macrophages also secrete various cytokines.

That is what has been seen in the *M. habana* activated murine macrophages and human healthy monocytes when elicited *in vitro* with *M. habana* and other mycobacterial antigens. These *M. habana* activated cells produced increased amount of cytokines in addition to ROI, RNI, lysosomal and antioxidant enzymes. The combined effect of these metabolic intermediates have a joint action (Possibly phagocytosis) on the invading infectious organisms in weakening the armoury of the pathogens in natural infections or during the artificial challenge given after vaccination for evaluating the protective efficacy of vaccine.

On the basis of these studies it can now be safely concluded that more than one pathway is operating in the activation of macrophages/monocytes and release of molecular mediators by *M. habana* vaccine sensitized animals cells. These mechanisms ought to be responsible for affording protection of mice against mycobacterial infections like TB and leprosy.

In this study, we have provided evidences that vaccine activated macrophages have increased metabolic activities and generate several metabolic intermediates leading to increased phagocytic activity. This has been further proved by the electron microscopic studies where we have found
increased number of lysosomal-like granules, more number of Golgi bodies, mitochondria and increase in the cell size of the vaccinated macrophages than the unvaccinated one. The endoplasmic reticulum had become more prominent. The nucleus appeared to be more euchromatic indicating possible increased activity of the nucleus. These findings are in agreement with similar findings by Damle et al., 1993 (b); Horio et al., 1981 and Tripathi et al., 1993 who have reported similar changes in antigen stimulated murine macrophages.

Surface morphology of the macrophages was also studied by scanning electron microscopy where we observed more ruffled surface patterns with many infolding and invagination in vaccine sensitized macrophages than normal ones which had more smoother surface. These features point out towards a better metabolic status of sensitized macrophages in taking care of the invading microorganisms. Similar characteristic changes were observed in IC Eq and IC Ag elicited macrophages by Tripathi et al. (1993).

These observations have finally to establish the basic mechanisms of action of M. habana vaccine and have clearly indicated the role played by M. habana vaccine in affording protection against tuberculosis. There might be some other alternative pathways of action of this vaccine, the possibility of which could not be excluded. Further studies may elucidate if there are other pathways of action of the vaccine in affording protection and is open for further future studies.
CONCLUSION
*Mycobacterium habana*, an atypical mycobacterium has been developed as a vaccine candidate for prophylaxis/therapeutics against tuberculosis (TB) and leprosy. The vaccine has been developed as a whole cell γ- irradiated preparation which provides high degree of protection to vaccinated mice against *M. tuberculosis* (MTB) and *M. leprae* live challenges. The vaccine generates strong cell mediated immune responses recognised by MTB and *M. leprae* antigens and shares several immunodominant proteins with these disease producing mycobacteria.

During the present investigation, the mechanism of action of the vaccine was studied with regards to its immune-antibody and CMI response leading to the changes affected by the vaccine at cellular level.

It has been established beyond doubts that killing of a wide variety of organisms is mediated through macrophages by generating a wide variety of molecular mediators. Therefore, changes produced at the macrophage level in vaccinated animals vis-a-vis normal ones were studied which comprised of the following:

- Reactive oxygen and nitrogen species
- Lysosomal enzymes
- Anti-oxidant enzymes
- Molecular mediators involved in CMI response
- Phagocytosis by the vaccine sensitised macrophages
- Observation of electron microscopic changes in sensitised cells

*Mycobacteria* are obligate intracellular acid fast bacilli residing mainly in macrophages. These cells are mainly involved at all stages of immune
responses. They act as the third line of defence against an invader with powerful weapons like oxygen and nitrogen intermediates, lysozymes, acid hydrolases and other mediating factors at cellular levels. Hence, these intermediary substances were examined at cellular level both in vaccine sensitised murine and human macrophages and PBMN cells.

It was observed that the intermediary products like reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), acid hydrolases (acid phosphatase, β-glucoronidase ) and lysozyme production were increased in vaccine sensitised macrophages. Their level was further increased on booster doses of vaccination. This increasing trend has also been found in peripheral blood mononuclear cells (PBMNC). Both NO and ROI have synergistic effect in mycobacterial killing and both the products were increased in vaccine sensitised murine macrophages. Thus, the vaccine aggravates the killing machinery of the macrophages in both the lysosomal and cytosolic compartments.

Anti-oxidant enzymes like superoxide dismutase, catalase, myeloperoxidase and glutathione peroxidase were also increased after vaccination. These are responsible for warding off the harmful effect of oxygen free radicals and also damage the membrane and nucleic acid and proteins of pathogenic organism and ward off infection. Thus, the vaccine is considered to provide killing by ROI, RNI and other free radicals side by side it also provides a brake over the excessive production by anti-oxidant enzymes.

An ideal vaccine for TB would contain components of the bacilli that produce more activated (microbicidal) macrophages and less tissue destruction. Any vaccine that produces hypersensitivity, would also confer an acquired
immunity. *M. haemophilum* vaccination has aroused high level of hypersensitivity responses against homologous and heterologous antigens, this could be considered as an ideal vaccine.

Acquired resistance to mycobacteria is cell mediated and an ideal vaccine favours expansion of T-cell population which was examined in vaccine sensitised lymphocyte population. There was enormous multiplication of the cells under antigenic influence, hence the vaccine seems to have this property and the possible mode of action of the vaccine could also be attributed to this.

The hypersensitivity and lymphocyte transformation are mediated by release of molecular mediators and when examined in vaccine sensitised cells the level IL-1β, IL-6 and TNF were markedly increased. Thus, the generation of TH-1 type of activity by the vaccine has led to the production of CMI and high increase in the formation ROI and RNI leading to offering of protection and the possible mechanisms of action of the vaccine.

The vaccine sensitised macrophages have acquired greater engulfment and digestive power than the normal macrophages. This has led to establish another pathway of action of vaccine in warding off infection and providing protection.

The electron microscopic studies of vaccine sensitised cells have also established beyond doubt that there are markedly increased activity in these cells. This has been evidenced by increased number of lysosomal like granules, more number of Golgi bodies, more number of mitochondria and increase in the cell size. The nucleus in the cells were more euchromatic possibly due to increased activity which ultimately exhibited in the form of protection offered by these activated cells.
In scanning electron microscopic studies, the vaccine sensitised cells had more ruffled surface patterns with many infoldings and invaginations. This indicates better metabolic status of sensitised macrophages in taking care of the invading micro-organisms and elucidates the possible mode of action of the vaccine.

Thus the study of these pathways of action of vaccine points out towards possible mechanisms involved at cellular level, although other possibilities of action of the vaccine cannot be ruled out and could be explored in future studies, and is suggested further studies.


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