Chapter - 5

Discussion
One of the important goals for development of vaccine against tuberculosis is the identification of mycobacterial antigens that induce cellular and/or humoral immune responses during infection and would therefore constitute reagents for developing improved vaccines and/or diagnostic assays. Only live, dividing mycobacteria efficiently induce protective immunity (Bloch, 1995; Orme, 1988).

The choice of antigens to be selected and investigated must be made on the basis of their capacity to interact with a given component of the immune response during a natural infection of immunization and at the same time to play a role during the disease (active protection and/or pathogenesis). Increased resistance against a challenge of virulent *M. tuberculosis* is obtained by prior vaccination with *Mycobacterium bovis* BCG or related mycobacteria like *M. microti* (D'Arcy et al., 1967). On the contrary, when the same bacteria are heat killed and injected alone or with adjuvant only marginal protection on protective response against a virulent challenge is detected (Weiss and Dubo, 1955).
A number of parameters have to be tested and verified to detect immunogenicity: a direct and indirect one through which the immunogenic agent must pass. One of the direct tests to measure the potency of strain is the protectivity test; other indirect tests are the ability of the immunogenic strain to generate the cell-mediated immune response.

We used live *M. habana* to determine the generation of protective immunity to mice and cell-mediated immune responses in mice. The immunization of mice with live *M. habana* induced a substantial level of protective immunity against challenge with *M. tuberculosis* H37Rv thus, there seems to be a reasonable degree of protective efficacy of *M. habana*.

Live vaccines, which can enhance cell-mediated immunity, have the greatest potential for success in prevention of infection and disease. We have chosen mouse model and intravenous infection for the purpose of evaluation of protective efficacy. We have used eight parameters to evaluate our results. All the parameters of study have indicated that live *M. habana* is also offering protection against *M. tuberculosis*. These results are in agreement with several other researchers (Pal and Horwitz, 1992; Andersen, 1994; Hubbard et al., 1992; Collins et al., 1988). Andersen (1994) have used only 4 parameters and Hubbard et al. (1992) have used two assays to evaluate their results. They also reported that there was reduction in viable count of *M. tuberculosis* bacilli in the organs of vaccinated mice when compared to unvaccinated mice.

Pal and Horwitz (1992) also reported protective immunity in excretory protein (EP) immunized animals in several ways. They showed that first, immunized animals were protected from clinical illness reflected by weight loss, lethargy and ruffled fur in control animals. Weight loss was the most readily quantifiable signs of illness. Compared with EP immunized animals, control mice had lost an average of 12.9% of their body weights during the 6 to 9 week observation period. During the present investigation also we have found the same type of observations. There was slow increase in average body weights of vaccinated group of mice, *i.e.* 2.7 g corresponding to *M. habana* vaccinated group after 8
weeks of challenge; whereas control mice lost an average weight of 4 g within 3 weeks of challenge.

Secondly, the immunized animals in Pal and Horwitz's study were protected from death. Only a small percentage of animals exposed to the range of challenge doses of *M. tuberculosis* used in their experiments died during the observation period. However, all deaths occurred in control animals and the difference in survival between *M. habana* immunized mice was highly significant. In our experiment also vaccinated mice were protected from death. About 70% mice were surviving after 8 weeks of challenge in groups immunized with *M. habana*, whereas in control group (unvaccinated), all mice died within three weeks after challenge. Here also the difference in survival between vaccinated and control animals are highly significant. The mean survival time and percent survival of vaccinated group on day 60 was higher than the control. Its value was significant with respect to control, *i.e.*, $p < 0.001$ in the vaccinated group. Vaccinated mice had lesser degree of lesions in their visceral organs and simultaneously the average weight of visceral organs was also lesser than control group. This has led to the observation that live *M. habana* immunization may protect the vaccinated animals against tuberculosis.

Thirdly, on an average, immunized animals in Pal and Horwitz's study has approximately fewer viable *M. tuberculosis* bacilli in their lungs and spleen than did control animals. This difference in the number of cfu of *M. tuberculosis* in the lungs of immunized and controls mice after challenge with *M. tuberculosis* was similar to the difference (1 to 1.5 log units) in cfu represented a life or death difference. More than 40% of vaccinated animals survived on 60 days of challenge, whereas 100% of control animals died within 1 week of challenge with aerosolized *M. tuberculosis* challenge. In their study immunization with EP did not prevent implantation of inhaled bacilli and the number of primary lesions in immunized animals was roughly equivalent to the number in control animals. In our study also immunized group animals had approximately 100-200 fold fewer viable *M. tuberculosis* bacilli in their lungs and 75-100 fold in spleen than did control groups. This difference is similar to 2.0 to 3.0-log difference in case of spleen. Cfu values of the vaccinated group were significant with respect to control. In lungs, it was $p < 0.001$, in
spleen it was $p < 0.01$. in the vaccinated group. *M. habana* appear to limit dissemination of bacilli to the lungs and secondary seeding to the lungs. Similarly, BCG is also reported to exert its effect in animals by limiting the bacillemia that occurs after the development of a primary infection (Smith *et al.* 1970).

In our study, supporting evidence of survival (percent survival and MST) and cfu data also came the histopathological observation which proved that immunization with live *M. habana* results in reduced number of acid fast bacilli with complete disappearance of inflammation and restoration of alveolar septa. Contrary to this, lungs of control mice showed marked acute inflammatory reaction (bronchopneumonia) with large number of tubercle bacilli in bunches and macrophages were packed with AFB. Thus, our protection study in all way has shown that live *M. habana* has certainly a protective role to play against *M. tuberculosis* challenge. Protection against intravenous challenge with *M. tuberculosis* H37Rv was monitored by cfu counting over a period of 3 months. Only vaccination with *M. habana* was found to consistently reduce the cfu counts in both spleen and lungs as compared with cfu counts in unvaccinated mice. The number of cfu was 10-30 fold lower in vaccinated than in control mice. The white pulp of the spleen appeared to be disrupted in control mice, but not in the vaccinated group. The livers from vaccinated mice did not show any granulomas in contrast to livers from control mice. The size and number of granulomas in the liver do not appear to reflect bacterial load in this organ.

Other tests to evaluate the efficiency of a vaccine are based on the generation of immune response by the vaccine. It is generally believed that acquired resistance to mycobacteria is a cell-mediated process that starts when sensitized thymus-dependent (T) lymphocytes recognize bacterial antigens on antigen presenting cells. An ideal vaccine would favour the expansion of T-cell population that increases the ability of macrophages to destroy tubercle bacilli over the expansion of T-cell population (Dannenberg Jr. 1989). The specificity of CMI resides entirely in the lymphocytes and not in macrophages. Macrophages kill facultative intracellular microorganisms only nonspecifically.
Greater numbers of T-lymphocytes with specific receptors for the bacillary antigens are present in the blood and lymphoid tissues of individuals possessing DTH and CMI than in blood and tissues of non-immune individuals. These T-cells had clonally expanded during the immunizing process. Therefore, at each site where the bacilli are deposited, greater numbers of specific (and non-specific) T-cells accumulate and produce lymphokines (Mackaness, 1968; North, 1974; Collins, 1982; Dannenberg Jr, 1983).

In the present study, the immunological activity of live _M. habana_ was studied by lymphocyte proliferation test in the mice model of TB infection. The lymphoproliferative test was performed with spleen lymphocytes (SPLC) of previously immunized mice with different antigens _i.e._, live _M. habana_, live _M. bovis_ BCG and heat killed _M. tuberculosis_ H37Rv. PHA-M was kept as nonspecific mitogen control. Transformation by eliciting antigens was compared with control _i.e._, mitogen. Total protein from _M. habana_ produced almost similar degree of proliferative response in all immunized group of animals. This study establishes that total protein from _M. habana_ hold key antigens giving cellular immune responses in animals immunized with homologous (_M. habana_) and heterologous (_M. tuberculosis_ H37Rv and _M. bovis_ BCG) antigens. It also establishes the presence of cross-reactive antigens between them.

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 (Mackaness, 1968; North, 1974; Collins _et al._, 1982). In CMI, T-lymphocytes accumulate at the site of antigen deposition and produce lymphokines.

The induction of CMI responses in individual antigens of microorganisms is often interpreted on the basis of cytokine production. The immunogenicities of individual antigens should also be evaluated in relation to the procedure used for the sensitization of animals, which will influence the sensitizing dose for individual antigens.

Intravenous _M. habana_ administration in mice induced comparable levels of IFN-γ and IL-2 in spleen (systemic) tissues, which are secreted by Th1 cells. These Th1 cells are
known to activate macrophages and to permit the induction of cytotoxic CD8$^+$ T lymphocytes. These CD8$^+$ lymphocytes play an important role in the control of *in vivo* BCG growth (Pedrazzini *et al.*, 1987) and during *M. tuberculosis* H37Rv infection (Flynn *et al.*, 1992; Mazzaccaro *et al.*, 1998). A cytotoxic effect of CD8$^+$ lymphocytes against cells loaded with soluble proteins of mycobacteria has been reported (Denis *et al.*, 1997; Zügel and Kaulmann, 1997). T cells have been divided into subsets based on expression of different surface antigens. CD4$^+$ T cells function as helper cells in antibody formation and mediate DTH, a form of cell-mediated immunity. CD8$^+$ T cells encompass cytotoxic T lymphocytes and antigen-specific T suppressor cells.

An ideal vaccine for tuberculosis would contain components of the bacilli that produce more activated (microbicidal) macrophages and less tissue destruction (i.e., more CMI and less DTH). Such a vaccine would favour the expansion of T-cell population that increase the ability of macrophage to destroy tubercle bacilli over the expansion of T-cell population that directly or indirectly produce tissue necrosis. Live *M. habana* also favours CMI response (LTT) with comparatively less tissue destruction as observed by us. Thus these indirect parameters to study the immune responses of live *M. habana* have also indicated the protective efficacy of live *M. habana*. The aim of immunization with *M. habana* is to direct an appropriate CMI response, which will afford subsequent protective immunity following a challenge with virulent *M. tuberculosis* H37Rv. The specificity of the CMI response is derived from T cell recognition of antigens associated with MHC on APC. Proliferation of T cells, although indicative of the CMI response, does not necessarily correlate with protective immunity (Andersen *et al.*, 1992; Orme *et al.*, 1992). It appears from murine studies that protective T cells are characterized by their ability to produce IFN-$\gamma$ (Kawamura *et al.*, 1992).

We could readily measure lymphocyte proliferation and production of IFN-$\gamma$ from PBMC of immunized mice incubated with *M. habana* total protein as seen with *M. bovis* BCG. In the present study, we demonstrated the presence of specific CD8$^+$ cytotoxic cells in the spleen of intravenously immunized mice. Their presence correlated with the increased resistance to a virulent challenge.
This work demonstrates that lymphocytes from *M. habana* immunized mice can secrete IFN-γ when incubated with *M. habana* infected macrophages. In contrast, lymphocytes from control animals produce relatively low levels of IFN-γ when incubated with *M. habana* infected macrophages. The results suggest that the IFN-γ response from immunized animals is mediated through a population of lymphocytes, which specifically recognizes *M. habana* antigens.

In the murine system, T cell-mediated IFN-γ has been shown to play an essential role in anti-mycobacterial activity and stimulation of macrophage with IFN-γ reduces growth of *M. bovis* BCG and *M. tuberculosis* H37Rv (Sypek et al, 1993; Flesch and Kaufmann, 1987). We could readily demonstrate that PBMC from the non-immunized control animals produced considerably less IFN-γ compared with the PBMC from immunized animals.

One possibility is to postulate that mycobacterial antigens presented on the macrophage surface or macrophage surface components themselves (Okragly et al, 1996) are recruiting populations of innate T cells with the ability to activate macrophage. These populations will include γ/δ T cells and NK cells, both cell types known to be involved in the innate immune response (Barnes et al, 1994; Trinchieri, 1990).

Classical models of T cell mediated cytotoxicity involve the release and new synthesis of lytic molecules such as granzymes and perforins following interactions between cytotoxic T lymphocytes and their targets (Janeway and Travers, 1997). CD8⁺ cells are necessary for a protective immune response during mycobacterial infections. It is still uncertain whether CD8⁺ T cells respond to *M. habana* immunization and participate in the host's responses to the mycobacteria. CD8⁺ T cells expand in the lungs after respiratory infection with BCG (Schaible et al, 1999) and BCG-reactive CD8⁺ T cells are present in BCG vaccinated individuals (Smith et al, 1999).

There are inherent differences in the expression of immunity in the lungs and spleen. For example, a smaller dose of *M. tuberculosis* given via the respiratory route resulted in a more severe infection than a larger dose of organisms given systematically (North, 1995).
This may be caused by a delay in the activation of protective Th1 cells in the lungs after aerosol infection (Cardona et al., 1999). Taken together, these results confirm that it is more difficult to protect the lungs than the spleen against mycobacterial infection and highlight the difficulty of preventing aerosol transmission of TB by vaccination induced immunity.

In our experiments, we have used the total protein of *M. habana* and were found to react extensively with different types of antisera raised in mice i.e., anti-*M. tuberculosis* H37Rv, anti-BCG and anti-*M. habana* (sonicate) sera and also with tuberculosis patients’ sera. Reactivity of total protein of *M. habana* with anti-*M. tuberculosis* H37Rv, BCG and *M. habana* sera indicates antigenic similarity of total protein of *M. habana* with respective antigens. Total protein of *M. habana* were recognized by all hyperimmune sera and TB patients’ sera which indicate that these antigens are having epitopes common to respective antigens and may be explored as protective and for diagnostic antigens.

When the mouse model was used to investigate the influence of *M. habana* dose on protective efficacy, it was found that efficacy was sensitive to vaccine dose. A dose of 1 x 10⁶ AFB/mouse induced comparably high levels of protection against *M. tuberculosis* H37Rv infection. Single dose of live *M. habana* administered via intravenous route gave similar levels of protection to a single intradermal route as reported by Gupta et al. (1979). This has important implications when considering oral vaccine for humans.

The increased survival of *M. habana* vaccinated group compared to the appropriate controls and prevention of caseating disease that gradually develops 3 to 4 weeks after intravenous infection. Hence, the live *M. habana* did prevent mortality in a manner to that conferred by BCG, these results generate optimism of using this as vaccine. It was noticeable that bacterial counts in the lungs, liver and spleen of the *M. habana* vaccinated group were significantly reduced below those of controls 4 to 5 weeks after intravenous challenge, also there was evidence of the vaccine causing prolonged survival compared to controls. This may be providing an important lesson, namely, that the short -term reduction in the bacterial counts may be, in fact, be the most important criterion along with the survival / pathology data in the mouse model may in fact give a better picture of the
effectiveness of a vaccine. We believe that the type of lesion produced influences survival data in this model. However, what is of importance is that this study pinpoints the influence of antigen dose on vaccine efficacy and emphasizes the necessity of careful dose response investigations when experimental vaccines are tested. While lesion severity is undoubtedly a critical component of survival, the cytological character of the lesion within the \textit{M. habana} vaccinated group, particularly the degree of lymphocytic infiltration may have a better correlation with survival in this model.

A striking characteristic of immunity to intracellular pathogens \textit{M. tuberculosis} H37Rv and \textit{L. monocytogenes} is the superior ability of live vaccines to induce a specific protective immune response (Mackaness, 1962; Orme, 1988). Murine CD4$^+$ T cells consist of at least two distinct subsets (Mosmann \textit{et al}, 1986). Th1 cells secrete IFN-\(\gamma\) and IL-2 and mediate predominantly DTH, whereas Th2 cells are characterized by the secretion of IL-4 and IL-10, leading to antibody production. Th1 cells are generally believed to be responsible for activation of macrophage, leading to protective immunity against intracellular pathogens. A predominant Th2 response, in contrast, may exacerbate the disease, in analogy with the situation found in Leishmania-infected mice (Scott \textit{et al}, 1988). Recently, however, a more differentiated picture has emerged on the basis of the finding that the levels of IgG1 and IgG2A are controlled by Th2 and Th1 cells, respectively (Mosmann and Coffmann, 1989).

A shift towards a Th2 type of immune response induced by high doses of antigen has previously been reported with different doses of \textit{L. major} (Bretscher \textit{et al}, 1992). On the basis of these findings, Bretscher (1992) has recently proposed a low dose immunization strategy to improve the efficacy of vaccination against \textit{M. tuberculosis} H37Rv. Our results are in agreement with this hypothesis.

Vaccination should at best leave a stable specific immunological imprint that enables the host to mount an accelerated and efficient protective immune response at a given time later in life. The immune surveillance is conducted by memory T cells, which patrol the peripheral tissues for invading pathogens (Mackay, 1991). The present study provides
substantial evidence to support the view that an adequate immune response against *M. tuberculosis* H37Rv can be induced with live bacilli.

In the present study, the fine specificity of the protective T cell response was investigated. The experimental vaccine was found to prime a broad T cell response directed to whole bacilli. The capacity of a live vaccine to induce a protective immune response against *M. tuberculosis* H37Rv and *L. monocytogenes* depends on an initial multiplication of the microorganism (Berche *et al.*, 1997; Orme *et al.*, 1986). The studies described here support the proposition that the dose of live *M. habana* is crucial in determining the Th1/Th2 nature of the immune response, with low doses favouring a predominantly cell-mediat ed, Th1 response, independently of whether live *M. habana* is given i.v. or intradermally.

It seems to be generally accepted that an environmental factor favouring the spread of tuberculosis in crowded living conditions. Most individuals infected with *M. tuberculosis* H37Rv do not become ill (Fine, 1988). It is to be expected, within the context of the findings reported and of the view that only infection is an important factor in determining whether disease develops, with higher number of infectious bacilli leading to disease. It is natural to suppose that crowded living conditions will lead more often to the substantial infection required to establish disease. Second, it might be anticipated that the effective dose of mycobacteria, as perceived by the immune system of a mouse or of a human, would depend not only on the number of bacilli that infect an individual but also on the rate at which the bacilli divide. The greater effectiveness of antigen given i.v. is not surprising as the antigen will reach secondary lymphoid organs more effectively. Our observations are consistent with others reported in the literature showing that antigen given i.v. is more effective as an immunogen (Wortis *et al.*, 1966; Lagrange *et al.*, 1974; Power *et al.*, 1998).

Histopathological examination of the chronically infected lungs clearly showed the establishment of equal numbers of infectious foci in vaccinated and normal animals. The protective effect of *M. habana* was directly related to its ability to induce an accelerated cellular response within the developing tubercles and this was able to quickly limit the further uncontrolled growth of *M. tuberculosis* challenge *in vivo*. In summary, there is a
progression of the granulomatous response in mice infected with \textit{M. tuberculosis}. The results are also consistent with the recurrent theme that virulent strain of \textit{M. tuberculosis} may enhance progression of an overwhelming granulomatous response; perhaps as a means of interfering with the protective response that leads to persistent infection in the host. We observed central rather than peripheral fibrosis in areas rich in activated macrophage (Mφ), often associated with small necrotic foci in these lesions. The association of fibrosis with these necrotic foci suggests that a fibrotic process predominates in the murine response to mycobacteria induced necrosis of pulmonary lesions, an observation which lead us to speculate that perhaps necrotic foci do not undergo central caseation for this reason in mice. The ill-defined granulomata found in the early stages of the infection are characteristic of the host granulomatous response to intravenous challenge routes of delivery (Nibbering et al, 1989; Orrell et al, 1992). The predominant macrophages seen during the development of chronic infection morphologically resembled the epithelioid and foamy macrophage types, of which the former cell type uniquely appears in granulomatous sensitivity reactions (North, 1995). The foamy macrophages appeared to be degenerating as the infection progressed and it was evident that they gave rise to scattered, small necrotic foci in areas that were otherwise thick with activated macrophage and epithelioid cells. However, central caseation of these areas never developed.

Vaccination can be expected to enhance the phagocytosis of the challenge inoculum and this can in fact be demonstrated \textit{in vivo} and \textit{in vitro} (Green, 1969; Wilkinson, 1976). However, what is of importance is that this study pinpoints the influence of antigen dose on vaccine efficacy and emphasizes the necessity of careful dose response investigations when experimental vaccines are tested.

Enumeration of the \textit{M. tuberculosis} population within the lungs, spleens and livers of \textit{M. habana} vaccinated mice reveals, the need for specifically activated macrophages if virulent tubercle bacilli are to be inactivated \textit{in vivo} as reported by Suter (1956) in BCG vaccinated mice. Virulence of the \textit{M. tuberculosis} strain seems to correlate directly with the rate of early growth by the challenge organism within the lungs (Collins and Smith, 1969). Also faster the overall growth rate of the organism \textit{in vivo}, the more intense will be the
resulting cellular response on the part of the host defenses. While some reduction in bacterial numbers can be seen during the peak expression of immunity, many bacilli survive, giving rise to a chronic state of disease, which is eventually fatal (Orme, 1998). These events are associated with the concomitant emergence of a local cellular granulomatous response, characterized by the development of foci of epithelioid type of macrophages and lymphocytes which enter the site of infection from the blood (North, 1970).

Vaccination of mice with *M. habana* generated a substantial level of protective immunity against challenge with *M. tuberculosis* H37Rv as manifested by the protection of immunized mice in terms of weight gain, healthy appearance and activity. Only a small percentage of immunized mice died during the experiment, whereas all deaths occurred, that too with ‘miliary’ infection in the control group. Immunized mice had fewer cfu in their lungs and spleens. Moreover, extensive studies carried out at this institute have shown (unpublished) that the *M. habana* is non-pathogenic as it did not cause tissue pathology after repeated injections in normal and stressed animals (mice).

Protective immunity against mycobacterial infection is mediated by interactions between specifically sensitized CD4⁺ and CD8⁺ T lymphocytes and activated effector cells harbouring the intracellular pathogen. These cells interact with one another by means of a complex network of cytokines. Th1 helper cells are thought to be essential, because their cytokines (e.g., IFN-γ and IL-2) are predominantly secreted in individuals resistant to mycobacterial infection (i.e., healthy humans with *M. tuberculosis* H37Rv and *M. leprae* primary infection and tuberculoid leprosy patients). In contrast, Th2 helper T cell cytokines (e.g., IL-4 and IL-10) have been found mainly in susceptible individuals (i.e., tuberculosis patients with extensive lungs lesion, lepromatous leprosy patients and BCG infected BALB/c mice). Optimal proliferation of Th1 and Th2 subsets is thought to depend on distinct antigen presenting cells and their co-stimulatory factors, such as IL-12 and IL-10 respectively. Furthermore, γ/δ T cells also may influence the initial activation of these two T cell subsets. IFN-γ, a potent activator of macrophages and produced by both CD4⁺ and CD8⁺ T cells, is one of the crucial cytokines in antimycobacterial protection, as demonstrated in knock-out mice that have had either the IFN-γ or the IFN-γ receptor genetically deleted. Finally, CD8⁺ CTLs also play an important role in protection against
mycobacterial disease (Huygen, 1992). Protective immunity against mycobacterial disease is believed to largely result from the induction of a Th1-type cytokines playing an important role in preventing active disease (Orme and Collins, 1994; Zhang et al, 1995).

In order to assess the effect of M. habana vaccination on the level of cell-mediated immunity, cytokine responses to the total 4 antigens were assessed in immunized and naïve animals with ELISA. Since, IFN-γ is a critical cytokine associated with the induction of an anti-mycobacterial protective response (Cooper et al, 1993; Flynn et al, 1993; Newport et al, 1996). We initially focussed on this cytokine response in the vaccinated animals.

There were robust total antigen specific IFN-γ responses, detected in the splenocytes of the vaccinated mice. Immunizations with the total antigens (Ags) all induced spleen cells that responded to specific Ags by producing IFN-γ.

To facilitate assessment of the T-cell phenotype induced by immunization, the splenic IL-4 concentration were also determined by ELISA. Vaccination with the total Ags induced spleen specific IL-4 responses. Consistent with the IFN-γ results, the Ag-specific IL-4 responses were higher in the splenocytes of the animals vaccinated with M. habana. The Th1 bias depicted by the cytokine profile (antibody profile) for the M. habana, is also reflected in the relative concentration of IL-4 and IFN-γ detected in the spleen. The cytokine response in the spleen of unimmunized control mice was assessed 6 weeks later. For live M. habana, the concentration of IFN-γ and IL-2 induced by vaccination exceeded the level of vaccine-induced IL-4 and IL-10. The capacity of live M. habana to elicit protective responses was evaluated 30 days after 1 x 10^6 cfu of intravenous challenge. The vaccine was tested in two to three independent experiments. The control mice were left unvaccinated. In the experiment whose result are shown in Table 5 and in three subsequent studies, 1 x 10^6 cfu of M. habana did significantly reduce the number of cfus seen 30 days after challenge.

Vaccination with M. habana, consistently reduced the cfus by day 30 relative to that seen in the naïve controls (p < 0.05). Immunization with the live M. habana resulted in 70% reduction of bacteria in the lungs compared to controls. Moreover, mice immunized
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with live *M. habana* showed significantly decreased dissemination of the infection in spleen than in lungs.

Control mice were left unimmunized and later challenged with *M. tuberculosis*. Dramatic reduction in the vaccinated group compared to the naïve controls were observed for both lungs (5.1 log$_{10}$) and spleen (4.7 log$_{10}$) cfus. Clearly, the reduction in lungs and spleen cfus in the *M. habana* vaccinated mice was significant.

While live *M. habana* preparation was able to induce a significant immune response, the level of protection elicited by *M. habana* was higher with an optimal dose in the mouse model of human tuberculosis.

A live vaccine is clearly an effective means of generating cell-mediated, humoral and protective immune responses against *M. tuberculosis*. Using intravenous immunization protocols, we have identified that *M. habana* elicit substantial protective activity when evaluated 30 days after a moderate dose (1 x 10$^6$ AFB/ mouse) of intravenous challenge. Because these studies were designed to evaluate the protective efficacy of *M. habana* it should be emphasized that our intravenous challenges were carried out 8 weeks post-immunization, when vaccination should have evoked optimal anti-tuberculous immunity. Obviously much more pre-clinical testing of the *M. habana*, including an assessment of their long-term effects on mouse immune memory and an evaluation of their effectiveness in other animal models, will be needed prior to its consideration as candidate vaccine for human trials.

Moreover, the protective effectiveness may be the result of elevated concentration of proteins (Vinita *et al*, 1999). The higher levels of proteins in the host cells should lead to increase secretion of proteins with the elevated uptake by antigen presenting cells and thus, a more generalized activation of the immune system. The higher cytokine titres detected in animals vaccinated with *M. habana* are a reflection of this enhanced immune stimulation. The correlation between antigenic expression level and the extent of immune activation has been reported (Jyothi *et al*, 2000). Moreover, Vinita *et al* (1999) showed that protein expression and immune induction were enhanced in mice inoculated with secretory proteins from *M. habana*. 

Despite these apparent differences in the protein concentrations generated from the live vaccines induced more or less similar levels of IFN-γ, a critical cytokine for antimycobacterial protection (Cooper et al, 1993; Flynn et al, 1993; Newport et al, 1996). The relationship between the dose of the vaccine and cytokine production is often extremely complex. It is also of interest that the protective responses do not directly correlate with the levels of in vitro IFN-γ synthesis. The explanation of this observation may relate to the level of expression of M. tuberculosis antigens in vivo after challenge. The degree of protection does directly correlate with the vaccine induced IFN-γ concentrations in this circumstance leading to generate greater protective immunity (Flynn et al, 1995).

For intracellular pathogens such as Listeria monocytogenes, CTLs are clearly required for protective immunity (Kaji et al, 1994). While the role of mycobacterial CTLs in protection is less certain, recent evidence suggests that CTLs may also be important for establishing long-term protection against tuberculosis (Stenger et al, 1998). We have demonstrated that the live M. habana induce protective activity in the mice model. Vaccination with live M. habana, decreased bacterial cfus in the lungs by approximately 65% following challenge. This was even more apparent in our assessment of the spleen burden at day 30. M. habana was highly effective in reducing dissemination of the lungs infection to the spleen.

The effectiveness of live M. habana has been the major finding by us for the murine model of tuberculosis. The evaluation of immune response elicited by vaccination with the live M. habana suggests that the superior protection afforded by M. habana in this model may be due to its capacity to induce a substantial cell-mediated response with a predominant Th1 phenotype in the spleens of the vaccinated mice. The ELISA data clearly shows that M. habana elicits a Th1-biased immune response, because an elevated IFN-γ response and a relatively low IL-4 response were detected in the spleens of M. habana vaccinated mice. In contrast, immunization with the live M. habana (high dose), generated a mixed Th1-Th2 phenotype. This result suggests that both the phenotype and the intensity of the immune response induced by live M. habana impact the extent of protection observed.
Our results appear to reflect the general rule that for those antigens able to induce both Th1 and Th2 responses, the Th1/Th2 nature of the response depends on the dose of the antigen used for immunization, with lower doses favouring the Th1 component. Instances of this rule were first described after employing protein antigens and examining the immune response in terms of DTH and antibody production (Bretscher et al., 1992; Salvini, 1958) and later extended to xenogeneic erythrocytes (Salvin, 1958; Rosenthal, 1980). Killed mycobacteria have been used in attempts to vaccinate against pathogenic mycobacterial challenge with diverse results (Bretscher, 1996). Given the diversity of the systems used diverse factors are likely to be responsible for the lack of consistent findings. We anticipate that mycobacterial dose will be one of these diverse factors. Indeed, studies with low doses generate a predominantly Th1 response, correlated with increased resistance, whereas higher doses induce a mixed Th1/Th2 response, resulting in increased pathology upon challenge with virulent M. tuberculosis H37Rv. These observations suggest that dead mycobacteria do not inevitably induce Th2 predominant responses, but that the Th1/Th2 nature of the response depends on several conditions of immunization, including antigen dose. The observations reported here and elsewhere (Wiegeshaus et al., 1989; Orme and Collins, 1984) involve the slowly replicating intracellular microorganism, L. major and BCG and an examination of the antigen-dependent generation of Th1 and Th2 cells (Griffin et al., 1995).

It is essential that our vaccination strategy can ensure a protective, Th1 response to protective antigens, without defining their nature, by ensuring a Th1 response to the majority of the components of the microorganisms.

These studies define conditions under which predominantly Th1 responses are generated against mycobacteria. Our current studies are testing the hypothesis that the generation of such responses can establish a Th1 imprint upon the immune system and that such an imprint results in resistance to a pathogenic challenge of virulent mycobacteria.

The aim of vaccination with M. habana is to direct an appropriate CMI response, which will afford subsequent protective immunity following a challenge with a virulent strain. The specificity of CMI response is derived from T-cell recognition of antigens
associated with MHC on antigen presenting cells. Proliferation of T-cells, although indication of the CMI response does not necessarily correlate with protective immunity (Andersen et al, 1992; Orme et al, 1992). Vaccination of mice with $1 \times 10^6$ cfu of *M. habana* has shown to confer protection against challenge with virulent *M. tuberculosis* H37Rv. We therefore used a similar dose immunization regimen to examine the nature of immune response to *M. habana*.

The work described here demonstrates that lymphocytes from *M. habana* immunized mice can be phagocytosed when incubated with *M. habana* sensitized macrophages. Long-term immunity to tuberculosis infection requires the generation of antigen specific memory T-cells. This may be achieved by vaccination with *M. habana* that provides the antigen required to stimulate the appropriate CMI response. However, it appears that mice already possess a high level of innate immunity to *M. habana*. This natural immunity might acts as a first line of defence prior to the development of a more potent antigen-specific response. Both responses may be affected at the level of macrophage, but lymphocyte factors are apparently required to induce the antimicrobial activity.

The preferential induction of Th1 or Th2 responses may be due to one or more of the following: the availability of antigen, the location of antigen processing, the type of antigen presenting cell which processes the antigen, the protein half life or the adjuvant properties of the antigen (Seder et al, 1993). When the live *M. habana* was administered, the protective immune response seen in the spleen of vaccinated mice approached the level achieved in animals receiving the live BCG as reported by Bretscher (1992). Another goal of our research has been to evaluate potential protective correlates of immunity for live vaccine. To accomplish this aim, we have focused on the CMI response since the induction of this response is crucial for antituberculosis protection (Flynn et al, 1993; Cooper et al, 1993). However, the cytokine data also supports earlier report that IFN-γ production by the splenocytes *in vitro* does correlate directly with the induction of protective immunity (Li et al, 1999).
Another finding that the more rapid the elimination of vaccine strain from lymphoreticular organs of the host, with its reduced time of exposure to the antigenic stimulus in vivo, the less effective will be the immune response. Predictably, this will also be dependent upon the initial dose of mycobacteria (Lefford, 1970). However, above a certain threshold level, the response by M. habana is likely to be roughly equivalent as observed in BCG (Collins and Miller, 1969). Paradoxically, when the inoculum is increased still further (10^6-10^9 viable units), the bacterial population within the lungs, liver and spleen may actually decline faster than when the inoculum is 100 fold lower. This effect is presumably related to the size of the cellular response induced by the larger antigenic stimulus (Collins and Mackaness, 1970).

One important conclusion from these studies was the finding that none of the vaccination procedures were able to prevent the establishment of intravenous challenge population within the lungs. All that the vaccination procedure can do is to slow the subsequent growth of the challenge organisms within the developing tubercle and so prevent their widespread dissemination throughout the remaining host tissues, a finding that is comparable with other reports (Levy et al, 1961; Fok et al, 1976). However, even in the most effectively immunized host a residual M. tuberculosis population will usually remain within the original lesion and can be recovered from three months or even years after all other traces of the infection have been eliminated from the liver and spleen (Kanai and Yanagisawa, 1955). Persistence of this residual virulent population, despite a fully deployed immune defence system is characteristic of a number of infections caused by obligate and facultative intracellular parasites. But the reason for this so called caseous state is still unclear (Collins, 1979). However, one useful side effect of this microbial persistence within the tissues is the continued generation of memory T cells within the lymphoreticular system, offer for the remainder of the lifetime of the host (Teery and Smith, 1975). Intravenous route of immunization, introduces large number of viable organism into the liver, spleen and lungs in approximately 90:10:1 ratio at time zero (Collins and Montalbine, 1976). To be effective, the vaccine must remain viable and preferably should proliferate within the lymphoreticular organs of the host for a period of several weeks (Collins, 1972).
The generally perceived reciprocal relationship between antibody production and cell-mediated immunity was first described by Parish and Liew (1972). The negative correlation between antibody production and cell proliferation has previously been observed in human tuberculosis (Wilkinson et al., 1997). While our data indicate that the single dose of *M. habana* need further improvement before clinical evaluation can be initiated, the results suggest at least two possibilities for augmenting the effectiveness of tuberculosis live vaccines. Work has to be channelized to shift the immune response induced by these vaccines from a mixed T-helper cell population towards the desired Th1 phenotype, with a potential increase in protective effectiveness. Additionally, intranasal administration of the live *M. habana* formulated in novel delivery systems to enhance expression within the lungs could elevate antigen-specific immune responses against the virulent tubercle bacilli.

In conclusion, single dose of live *M. habana* gave significant protection against experimental infection and disease caused by virulent *M. tuberculosis* H37Rv in the experimental murine model.