PROGRESSION OF CHRONIC PULMONARY TUBERCULOSIS IN MICE INTRAVENOUSLY INFECTED WITH ETHAMBUTOL RESISTANT MYCOBACTERIUM TUBERCULOSIS

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Abstract

Purpose: Ethambutol (EMB) is an important first line drug, however little information on its molecular mechanism of resistance and pathogenicity of resistant isolates is available. Present work was designed to study virulence of the EMB resistant M. tuberculosis strains and the host responses in-vivo on infection of EMB resistant M. tuberculosis using Balb/c mouse model of infection. Methods: Three groups of Balb/c mice (female, age 4-6 wk; 21 mice in each group) were infected intravenously with 106 CFU of M. tuberculosis H37Rv and two EMB resistant clinical isolates. Age and sex matched control animals were mock inoculated with Middlebrook 7H9 broth alone. At 10, 20, 30, 40, 50, 60, and 70 days post-infection three animals from each group were sacrificed by cervical dislocation and lung tissue was collected for further analysis. Results: Infection with EMB resistant M. tuberculosis led to progressive and chronic disease with significantly high bacillary load (p=0.02). Massive infiltration and exacerbated lung pathology with increased expression of IFN-γ and TNF-α was observed in lungs of mice infected with EMB resistant strains. The present study suggests that infection with EMB resistant M. tuberculosis leads to chronic infection with subsequent loss of lung function, bacterial persistence with elevated expression of TNF-α resulting in increased lung pathology. Conclusion: These findings highlight that EMB resistant M. tuberculosis regulates host immune response differentially and its pathogenicity is different from drug sensitive strains of M. tuberculosis.

Key words: Balb/c mice, Ethambutol resistance, immunopathology, M. tuberculosis.

Ethambutol (EMB) is an important first line antitubercular drug recommended for the treatment of tuberculosis as well as opportunistic infections by M. avium in AIDS patients.[1] It inhibits linkage of outer lipid layer associated mycolic acid to inner peptidoglycan i.e., synthesis of arabinogalactan and lipoarabinomannan.[2]

The clinical manifestations of tuberculosis are dependent on the cellular immune response to the tubercle bacilli, characterised by the accumulation of macrophages, lymphocytes, and polymorphonuclear leukocytes in tuberculous lesions.[3] Identifying the various components involved in disease processes is central to our understanding of the pathogenesis of tuberculosis. Specific clinical isolates of Mycobacterium tuberculosis may be differentially pathogenic and vary in the ability to cause disease in humans. EMB resistant M. tuberculosis has increased proportion of non-mannose caps and non-mannose capped M. tuberculosis are known to increase cytokine expression.[4] Therefore, the role of EMB resistant M. tuberculosis in influencing host immune response and subsequent disease progression needs to be investigated.

The study of an EMB-mono-resistant clinical isolate in parallel with the MDR and parent EMB -susceptible strains would be particularly useful to better understand the potential activity of EMB in the treatment of tuberculosis caused by EMB-resistant organisms. In the present study, we used BALB/c mouse model of pulmonary tuberculosis to examine the course of infection in terms of survival, lung bacillary load, lung pathology and immune responses induced by ethambutol resistant M. tuberculosis clinical isolates.

Materials and Methods

Bacteria

Two drug resistant strains of Mycobacterium tuberculosis B-3773 and B-3825, previously isolated from patients with pulmonary tuberculosis, and M. tuberculosis H37Rv standard laboratory strain were used in the present study. The strains were epidemiologically unrelated to each other, as shown by IS6110 fingerprinting.[5]

Antimicrobial susceptibility test

Antimycobacterial drug sensitivity for isoniazide (INH),
rifampicin (RIF) and EMB was detected by proportion method at cut-off values 0.1 µg/mL, 40 µg/mL and 2 µg/mL, respectively.[5,6] *M. tuberculosis* H37Rv, standard laboratory strain, was used as control in each set of experiment.

**Animal experiment**

Female Balb/c mice (age 6-8 wk) were procured from Central Drug Research Institute, Lucknow, India. The study protocols was approved by the “Animal Ethics Committee”, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.

Before experiments, Mycobacterium culture was grown up to log phase in Middlebrook 7H9 broth with 0.04% Tween-80. Three groups of mice (21 animal/group) were infected intravenously (i.v.) with 1x10^6 CFU of *M. tuberculosis* H37Rv and EMB resistant clinical isolates. Age and sex- matched control animals (n=21) were mock inoculated with Middlebrook 7H9 broth alone. Three mice per cage were kept and sacrificed at 10 days interval up to 70 days by cervical dislocation. The lung tissue was collected for microbiological, histopathological and cytokine expression analysis.

**Growth of Mycobacterium tuberculosis in mice lung tissue**

The number of viable mycobacteria in mice lungs was evaluated at designated time points. Tenfold serial dilution of the lung homogenates were plated on Middlebrook 7H10 agar and were incubated at 37°C. The number of viable bacilli was evaluated by counting individual colonies after 2-3 weeks of growth.[7]

**Histology**

At various time points after infection, lung tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for histology. Sections were stained with haematoxylin and eosin and Zeihl-Neelsen for histologic evaluation and photography. Two pathologists studied three lung sections from each mouse, in a masked fashion, to determine area occupied by inflammatory infiltrate with predilection for perivasculer and peribronchial area. Percentage of lung parenchyma infiltrated by epitheloid cell collection, foamy histiocytes and mononuclear cells was evaluated. Necrotic foci and presence of giant cells were also looked for. The extent of severity of each feature was assessed in terms of percentage of area involved.

**Cytokine mRNA levels in infected lungs**

Total cellular RNA from lungs of infected mice was obtained at designated time points following intravenous infection. Tissues were homogenised in 0.75 mL Trizol LS reagent and RNA was extracted according to the manufacturer’s protocol (Invitrogen, USA). For reverse transcription-PCR (RT-PCR) 1µg of RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (MBI, Fermentas). Following cDNA synthesis PCR was performed for IFN-γ, TNF-α, IL-4 and IL-10 cytokines using gene specific primers.[8] RNA replaced by DEPC (Diethyl pyrocarbonate) -water was taken as negative control for PCR. 10 µL of the PCR products were analyzed on 1.8% agarose gel and visualised by UV fluorescence after staining with ethidium bromide. To compare the difference in cytokine gene expression by semi-quantitative method, the ratio of the density of the cytokine gene to the density of β-actin gene was calculated using AlfaEaseFCTM software v 3.2.1 (AlphaInnotech Corporation, USA).

**Statistical analysis**

The data were analysed by SPSS 12.0 (Chicago, IL, USA) using one-way analysis of variance with Bonferroni’s multiple comparison test to compare differences between and within three groups of mice (p<0.05). CFU were subjected to log transformation prior to statistical analysis. Kaplan-Meier analysis was carried out with GraphPad Prism4 ver 4.03 (USA) to determine statistical significance of the differences in survival of mice; 95% confidence indices and the log rank test was used.

**Results**

Strain B-3773 was resistant to EMB only, whereas strain B-3825 was found to be multidrug resistant i.e., resistant to INH, RIF and EMB. *M. tuberculosis* H37Rv standard laboratory strain was sensitive to all anti-tubercular drugs tested.

The course of infection in lungs of mice infected with either strain of *M. tuberculosis* was followed for 70 days and bacterial load in lungs of mice was assessed CFU assay (Fig. 1). In the present study it was observed that during the acute phase of infection (0-30 day post-infection), viable counts in the lung of mice infected with drug resistant strains increased exponentially, more so in animals challenged with MDR strain, B-3825. During the chronic phase of infection (31-70 days post-infection) the CFU count in B-3773 became stable, whereas a decrease was observed in mice infected with strain B-3825. In contrast to drug resistant strains, H37Rv infected mice showed increase in the viable count from 20 days post-infection till the end of the study (Fig. 1).

To determine whether there were differences in survival, mice were infected with drug sensitive and EMB mono- and multi-drug resistant strains of *M. tuberculosis*, and monitored for 70 days (Fig. 2). Mice infected with EMB mono-resistant strain B-3773 began to die from day 43 and the median survival time for this group of mice was 57 days. Whereas mice infected with B3825 resistant to EMB, INH and RIF, began to die as early as from day 13 and the median survival time for this group of mice was 35 days. Survival
of mice infected with multi-drug resistant strain B-3825 was significantly shorter than EMB mono-resistant strain B-3773 ($p=0.0008$). In contrast, mice infected with drug sensitive strain H37Rv did not begin to die until day 33 and survived longer than mice infected with drug resistant strains.

On gross examination, the lungs of the mice infected with both the drug resistant strains of *M. tuberculosis* showed relatively more tubercles and caseation necrosis in comparison to mice challenged with drug sensitive strain H37Rv. In the early stages of the infection, scattered tubercles and in later stages coalescent tubercles and caseation necrosis was also seen in mice challenged with drug resistant strains (Fig. 3).

Histologically, it was observed that mice challenged with EMB resistant strains had larger percentage of lung area occupied by inflammatory infiltrates as compared to that infected with H37Rv ($p<0.05$). The mononuclear inflammatory infiltrate were seen in perivascular and peribronchial area covering larger parts of tissue in mice challenged with EMB resistant *M. tuberculosis* (Fig. 4A).

These animals had larger percentage of lung area occupied by histiocytes including loose epitheloid cell collection and mononuclear cells, as compared to mice infected with *M. tuberculosis* H37Rv (Fig. 4B). The B-3773 group developed a progressive disease as the total area occupied by histiocytes including epitheloid cell clusters and infiltration with mononuclear cells was high up to 70 to 80% throughout the study period (Table 1). These animals developed pneumonic areas, consisting of abundant foamy macrophages admixed with lymphocytes (Fig. 4C). Edematous lung in the form of distended alveolar spaces could be seen, suggesting a relative loss of lung function. The pathological response in mice infected with MDR strain, B-3825, was more severe in comparison to EMB resistant infected mice relatively lesser area of lung parenchyma was involved by inflammatory infiltrate in mice infected with *M. tuberculosis* H37Rv (Table 1) and small aggregates of inflammatory cells were seen after 20 days post-infection. However, these started to occupy 50% of

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$p.i. = post-infection$
lung area by day 70 post-infection but did not achieve the size observed in lung of mice infected with both the drug resistant strains.

To visualise the changes in the cytokine-specific mRNA levels over the experimental period, the results were expressed as fold increase over uninfected control assayed at the same time (Fig. 5). It was observed that the expressions of IFN-γ in H37Rv infected mice tend to increase up to 40 days post-infection. Then there was a transient decrease in IFN-γ expression at 50 days, after which it again increased by day 70 post-infection (Fig. 5). IFN-γ expression in mice infected with strain B-3773 was found to be higher throughout the study period in comparison to mice infected with *M. tuberculosis* H37Rv (*p*<0.001). Mice infected with strain B-3825 showed variable expression of IFN-γ. There was a non-significant higher expression at day 30, thereafter, a decreasing trend was observed, even though the expression was significantly higher than H37Rv (*p*<0.05) (Fig. 5). Similarly, TNF-α expression was found to be elevated in mice infected with strain B-3773 throughout the study period. TNF-α levels in mice infected with strain B-3825 was found elevated till day 30 in comparison to other two groups. In the present study we did not find expression of anti-inflammatory cytokines, IL4 and IL-10, in the lungs of mice infected with H37Rv and B-3773. There was a weak expression of IL-4 and IL-10 in the lungs of mice infected with strain B-3825 only at day 30 and 50, but these were not expressed in levels comparable to β-actin, therefore the densitometry could not be performed.

**Discussion**

It is estimated that one-third of the global population is infected with *M. tuberculosis* and that ~8 million new cases of tuberculosis arise annually, with two million people dying from the disease. Yet we know little about the virulence factors responsible for the development of active disease in infected individuals.

To what extent the pathogenesis of tuberculosis depends on the infecting organism, and is independent of the host factors, is an important issue in tuberculosis research. Previously it has been shown that specific clinical isolates of *M. tuberculosis* may be differentially pathogenic and vary in the ability to cause disease in humans. In the present study the pathogenesis of drug resistant strains of *M. tuberculosis* and host immune responses against EMB mono- and multi-drug resistant mycobacterial infection has been explored.

In the present study, infection in Balb/c mice with EMB mono- and multi-drug resistant clinical isolates and H37Rv was implanted via intravenous route. It was observed that AFB score (data not shown) and CFU count was higher in mice infected with EMB resistant *M. tuberculosis* than H37Rv. In agreement with the study of Mogues *et al.*, our results show that implantation of drug resistant *M. tuberculosis* in the lungs of mice was followed by ~4 week period of progressive *M. tuberculosis* growth after which bacterial growth was controlled and infection was held at an approximately stationary level. It is generally accepted that the more virulent strains of *M. tuberculosis* have faster generation times, and it has been proposed that

**Figure 4:** Lungs of mice infected with ethambutol resistant *M. tuberculosis* showing varying degree of pathology (H&E stain). (A) Massive perivascular and peribronchial infiltration (x100); (B) Dense infiltration in lungs of mice infected with *M. tuberculosis* B-3773 (x200); (C) Pneumonic areas accompanied by foamy macrophages admixed with lymphocytes (x200); (D) Extensive tissue necrosis in mice infected with *M. tuberculosis* B-3825 day 50 post-infection (x100)

**Figure 5:** Cytokine mRNA levels in lungs of mice infected with *M. tuberculosis* B-3773, B-3825 and H37Rv. Results are expressed as fold increase over specific mRNA obtained from uninfected control lungs, normalized to the amount of β-actin mRNA.
this speed may be responsible for their progressive growth *in vivo.*\[10,12\] Considering the growth rate of mycobacteria to be an indicator of virulence, Manca *et al.*\[7\] have also reported that virulent strains of mycobacteria grows faster in the mouse lungs over the initial period of infection. With regard to the isolates that grew very quickly in the mouse lungs over the initial period of infection, our results confirm that drug resistant strains grow faster in mice than the laboratory strain, H37Rv (*p*=0.02). We also observed that drug resistant strains are more pathogenic as mice infected with both the EMB and multi-drug resistant strains had lower mean survival time than the mice infected with drug sensitive strain H37Rv.

In the mouse model of tuberculosis, the chronic phase of infection is characterised by progressive lung pathology despite relatively low number of viable bacteria.\[11\] Macroscopically and microscopically, lungs are the only organ that show the signs of progressive disease during the course of infection. On gross examination, we observed that the lungs of the mice infected with both the drug-resistant strains showed tubercles and varying degrees of necrosis. In one mouse each infected with B-3825, which died at day 40 and 50, it was observed that the expansion and coalescence of lesions eventually lead to consolidation of the large areas of lungs and loss of respiratory function. Therefore, it is apparent that the mice died because of respiratory insufficiency. Similar type of lung pathology has also been reported by Dunn *et al.*\[14\]

Histological analysis revealed that mice infected with drug resistant strains had larger percentage (70 to 90%) of lung area (perivascular and peribronchial) occupied by inflammatory infiltrates as compared to those infected with *M. tuberculosis* H37Rv (*p*<0.05). Edematous lung in the form of distended alveolar spaces was observed, suggesting a relative loss of lung function. Thus it can be said that that infection with EM resistant *M. tuberculosis* led to progressive and chronic disease with higher bacillary load, large foci of inflammatory infiltrate and the increased bacillary load was accompanied by progressive pneumonia and mortality.

The human immune response to *M. tuberculosis* is involved in protection against the disease but can be at the same time detrimental and largely responsible for the lesions seen during tuberculosis. Hence, the different clinical picture of pulmonary tuberculosis is the result of a complex series of interactions among immunocompetent cells and their secreted cytokines such as TNF-α and IFN-γ. IFN-γ and TNF-α play a pivotal role in imparting a protective immune response to *M. tuberculosis* but functions and roles of these have not been well elucidated. Presence of pro-inflammatory cytokines leads to immune activation, which may exacerbate lung pathology via TNF-α induced inflammation without reducing bacillary load.

In the present study we observed large foci of inflammatory infiltrate in lung parenchyma in mice infected with EMB resistant strains in comparison to mice infected with *M. tuberculosis* H37Rv. The massive infiltration was accompanied with high TNF-α expression and this leads to chronic infection-induced pathology i.e., lung consolidation. We also observed that in spite of the small differences in the volume of the cellular aggregates induced in the lungs of mice infected with B-3773 and B-3825, TNF-α mRNA expression was higher in the mice infected with B-3825 for the first 30 day post infection and this could be a reason for the vigorous pathological response. Our results are in concordance with Moreira *et al.*\[15\] where mycobacterial antigens induced TNF-α caused a significant increase in granulomas in lungs, increased inflammation and accelerated mortality without affecting bacillary load.

Several studies including those of Zhang *et al.*\[16\] and Lin *et al.*\[17\] have shown that IFN-γ is a key cytokine to play a protective response but alone is insufficient to control *M. tuberculosis* infection. We also observed a high concentration of IFN-γ in the mice infected with EMB resistant clinical isolate in comparison to *M. tuberculosis* H37Rv and strain B-3773 produced higher levels of this cytokine with respect to strain B-3825. Earlier Florido *et al.*\[18\] have shown the acceleration of the induction of lung pathology associated with enhanced mycobacterial loads and exacerbation of the immune response, namely IFN-γ response. In the present study we have also observed that IFN-γ levels were always higher in all the three groups of the mice in addition to TNF-α and high bacillary load. These findings suggest that increased expression of IFN-γ in mice infected with EMB resistant *M. tuberculosis* may further contribute to chronic disease without controlling bacillary load.

In the present study we did not find significant expression of IL-4 and IL-10 cytokines. This could be due to two reasons.\[16,17\] First, IL-4 is biologically active at much lower concentrations than IFN-γ, and has a correspondingly lower mRNA copy number. Secondly, rapid early production of IFN-γ can suppress Th2 cytokine release. We observed presence of IL-4 and IL-10 cytokines and necrotic lesions in the lung of the B-3825 group of mice, which died in the early stages of infection. This is similar to previous study of Hernandez-Pando *et al.*\[19\] who in the Balb/c mouse model of pulmonary tuberculosis infection have shown that appearance of IL-4 in the lung lesions coincides temporally and spatially with the appearance of areas of pneumonia and necrosis, leading to rapid clinical deterioration and death. The data reported here suggest that the differential cytokine-inducing capacity of EMB mono- and multi-drug resistant *M. tuberculosis* appears to be a property of that strain. Previously Manca *et al.*\[7\] have shown that when crude lipid fractions of the drug resistant bacilli CDC1551
were tested in monocytes, they induced significantly higher TNF-α and IL-12 production and higher CD14 expression than similar fractions prepared from H37Rv. This suggests that polar and/or apolar lipids of *M. tuberculosis* may be responsible for the differential response. It is still not clear which molecule(s) may be responsible for the differences. Studies have shown that lipoarabinomannan, a major cell wall component of *M. tuberculosis*, induces monocyte cytokine production possibly via a CD14-dependent pathway.[20,21] However, other lipids may signal monocyte cytokine production through a CD14-dependent pathway.[22]

Therefore, it appears that the possible reason of the greater immunogenicity of the EMB resistant strains may be the mycobacterial cell wall component lipoarabinomannan (LAM), as comparison of LAM from laboratory strain of *M. tuberculosis* with EMB resistant clinical isolate revealed significantly higher proportion of nonmannose capped arabinan termini in EMB resistant strain and nonmannose capped LAM stimulate monocytes to "express early immediate genes"- tissue factor and TNF-α.[23]

In summary, the results presented here provide experimental evidence that EMB resistant *M. tuberculosis* strains are more virulent and immunogenic as they induce a rapid and vigorous cytokine response which leads to chronic progressive disease in mice without controlling bacillary load.

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**References**


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