Comparative growth pattern of multi drug resistance versus susceptible isolates of *Mycobacterium tuberculosis* in mice lungs

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Background & objectives: Rise in prevalence of multi-drug resistance (MDR) in tubercle bacilli is a serious cause of concern. As mutations with two house keeping genes rpoB and katG are associated with resistance to two important anti-tubercular drugs rifampicin and isoniazid respectively, there is a need to understand the growth kinetics of organisms with such mutated genes in experimental animals. This study was undertaken to study the growth kinetics of susceptible as well multi-drug resistance *Mycobacterium tuberculosis* isolates in mice.

Methods: Two MDR (having mutations in rpoB and katG) and two drug susceptible isolates of *M. tuberculosis* along with H37Rv were grown in mice after aerogenic infection.

Results: The MDR isolates grew slowly up to 3 wk though the growth was significantly different from sensitive strains. However, after 3 wk, the growth in sensitive as well MDR strains was similar, suggesting that even the mutations in the MDR strains did not have any impact on the growth kinetics.

Interpretation & conclusions: The effect of mutations in other parts of these genes need to be studied. Retention of property of MDR strains to establish infection after aerogenic infection has epidemiological significance in terms of the transmission of MDR tuberculosis.

Key words Aerosol infection - growth pattern - mice - multi-drug resistant (MDR) - *Mycobacterium tuberculosis*

Even after massive efforts to check, global TB incidence is still growing at 1 per cent a year. The multi-drug resistance tuberculosis (MDR-TB) and extensive drug resistance (XDR) tuberculosis have further complicated the situation. A study conducted in South-Africa reported the prevalence of MDR-TB 40 per cent and of XDR-TB 6 per cent and 98 per cent mortality in the patients co-infected with XDR-TB as well as

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HIV. Resistance of Mycobacterium tuberculosis to an antituberculosis drug is usually the result of spontaneous genetic event and worse, “a man made amplification of natural phenomenon.” ² As MDR isolates have alterations in several genes encoding products associated with processes involving transcription (rpoB) and survival in oxygen free radicals (katG), their growth kinetics in animals need to be studied. Such studies could be important to understand the biological differences significant for the pathogenesis, transmission dynamics, alternate modes of treatment, new vaccine development and for better understanding of host parasite relationships. In depth studies on MDR isolates are specially required in animal models to test important treatment modalities/compounds prior to clinical trials and also to understand the effect of immunotherapeutics or immunoprophylactics.

In experimental tuberculosis, different animals models such as guineapigs, mice and rats have been used since a long time³. Mice are commonly used because of types of granuloma generated and also ease of their handling⁴. Most investigators have observed BALB/c (intermediate) mice to be susceptible following intravenous infections, although they are generally more resistant than CBH or CBA mice. After aerosol infection, the survival of BALB/c mice has been reported to be similar to C57BL/6 (resistant) mice⁵. MDR isolates need to be tested in different models specially after aerosol challenge as that will stimulate natural mode of transmission and infection. There is scarcity of data dealing with growth kinetics of susceptible and multi-drug resistant M. tuberculosis isolates, therefore this study was undertaken to study the growth kinetics of susceptible as well multi-drug resistant M. tuberculosis isolates in mice.

Material & Methods

Source of Mycobacterium isolates: This study was undertaken during September-November 2006. M. tuberculosis isolates (n=4) were collected from the National Mycobacterial Repository at National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra. The isolates were arbitrarily selected in terms of geographical source and drug susceptibility profile. The samples were biochemically characterized as belonging to M. tuberculosis complex by nitrile reduction, heat resistant catalase (68°C) and niacin production. Drug susceptibility profile was evaluated by the minimum inhibitory concentration (MIC) method⁶. The drug tested were rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and ofloxacin (OFX). The MICs at which the isolates were considered resistant, were as follows: 64 μg/ml (RIF), 1 μg/ml (INH), 6 μg/ml (EMB), 16 and 32 μg/ml of (STR), and 1 μg/ml (OFX). Four isolates were included in this study, two of the four isolates were sensitive for RIF, INH, EMB, STR, and OFX and two were resistant. All mycobacterial isolates were grown in middle brook (MB) 7H9 medium (Difco, USA) supplemented with 0.5 per cent glycerol, 0.2 per cent tween 80 and 1x albumin dextrose catalase (ADC) (Difco, USA). When required, the following antibiotics and antifungal agents were added at the specified concentrations: amphotericin B (20 μg/ml), carbenicillin (100 μg/ml), cyclohexamide (0.4 μg/ml), polymyxinB sulphate (2x10² units/ml), trimethoprim (20 μg/ml) and vancomycin (10 μg/ml).

DNA isolation: The isolates were cultured on Lowenstein-Jensen (L-J) medium slants. The colonies were scraped, resuspended in 400 μl of TE [10 mM Tris, 1mM EDTA (pH8)]; and DNA was isolated by the method of Van Embden et al⁷.

DNA sequencing: PCR amplification of rpoB, katG, and inhA genes was done using a set of oligonucleotide primers (Table I) with variation in annealing temperature and product size described elsewhere⁸. The amplicons were resolved in 2 per cent agarose gel and specific bands were excised. DNA was extracted from gel slice with a QIA quick gel extraction kit (Qiagen, DAVE et al: GROWTH PATTERN OF MDR VS SUSCEPTIBLE M. TUBERCULOSIS IN MICE LUNGS 59
Chatsworth, USA) according to the manufacturer’s instructions.

The purified DNA was re-suspended in sterile double distilled water and was used for the sequencing studies. Sequencing of the amplicons was carried out with an ABI Prism 310 Genetic Analyzer, USA and the sequencing output was analyzed and compared with the wild type sequence using Meg Align software (Lasergene; DNASTAR, Inc., Madison, Wis., USA).

Animal studies: The permission of Institute’s Animal Ethical Committee was taken for doing the animal experimentation. BALB/c mice weighing 25-30 g were taken from animal facility of National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra. The animals were individually housed in poly carbonate cages and provided commercial chow in stainless feeders and tap water ad libitum. The mice were placed in the exposure chamber of an air-borne infection apparatus (Glas-col Inc., Terre Haute, Ind., USA). The nebulizer compartment was filled with 10 ml suspension of M. tuberculosis strains (H37Rv, DKU10, TRC-220, JALMA-136 and DRF-186-B) containing 5x10⁸ cfu/ml. Four mice were used at every time point for each strain. The number of viable bacteria in homogenate of left lung at 24 h, 3rd and 6th wk were monitored by plating and serial dilutions on middle brook 7H11 agar and counting bacterial colony after 21 days of incubation at 37°C. Fite Faraco’s staining was done for staining lung tissues.

Results & Discussion

In the present study the identity of already biochemically identified M. tuberculosis isolates revealed mutations in the hot spot regions various loci. Previously reported mutations were identified in these isolates also. In both the MDR isolates viz., JALMA-136 and DRF-86 (B) the mis-sense mutation ser 531 leu and ser 531 trp were observed (Table II). Both the MDR M. tuberculosis isolates exhibited a substitution mutation at 463 position. Mutations at these codons are correlated with high degree of rifampicin resistance. A common mutation in all the isoniazid resistant isolates was the substitution mutation Arg463Leu. Though the codon 463 of katG gene exhibited mutation, inhA gene of the isoniazid resistant isolates did not show any mutation (Table II).

After the inhalation of virulent bacilli by the BALB/c mice (about 20 bacilli), the number of viable bacilli in the lung of the mice increased up to 3 wk but count became more or less constant after 3 wk. While the drug resistant isolates grew slightly slower initially, there was no difference after 3 wk between sensitive and MDR isolates (Fig.1).

Mycobacterial drug resistance to various drugs has been attributed to several mechanisms including the presence of complex cell wall membrane proteins acting as efflux pump. But the most striking mechanism responsible for resistance to major anti-tuberculosis drugs is the mutations in the genes encoding the drug target proteins. Over the last one decade different target loci associated with susceptibility/ resistance to rifampicin, isoniazid, streptomycin, ethambutol and other anti-tuberculosis drugs have been structurally and functionally analyzed. In case of RIF, mutations have been found to be associated with resistance in more than 95 per cent of the isolates. Both the isolates used in this study, had a known mutation in codon 531 (rpoB) and. Despite it seemingly simple structure, the elucidation of INH precise mode of action has been difficult although strains possessing low levels of catalase have INH resistance. Further, limited experimental evidence has led to the hypothesis that the katG gene is associated with INH resistance in M. tuberculosis. A common mutation in all the isolates was arg463Leu (substitution mutation). However, this mutation has been shown to have no direct consequence to INH resistance in earlier studies.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Drug susceptibility</th>
<th>Gene</th>
<th>Base change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. JALMA 136</td>
<td>RIF</td>
<td>rpoB</td>
<td>TCG→TTG</td>
<td>Ser 531 Leu</td>
</tr>
<tr>
<td></td>
<td>INH</td>
<td>kat G</td>
<td>CGG→CTG</td>
<td>Arg 463 Leu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inh A</td>
<td>No mutation</td>
<td>No mutation</td>
</tr>
<tr>
<td>2. DRF 86 B</td>
<td>RIF</td>
<td>rpoB</td>
<td>TCG→TTG</td>
<td>Ser 531 Trp</td>
</tr>
<tr>
<td></td>
<td>INH</td>
<td>kat G</td>
<td>CGG→CTG</td>
<td>Arg 463 Leu</td>
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<tr>
<td></td>
<td></td>
<td>inh A</td>
<td>No mutation</td>
<td>No mutation</td>
</tr>
</tbody>
</table>
No acid-fast bacilli (AFB) were found using Fite-Faraco staining at any stage. In histology of lung using haematoxylin and eosin staining, MDR isolates showed more cellular response than H$_{37}$R$_{v}$ (Figs 2 and 3). RIF kills the organism by interfering in transcription process$^{15}$. It is active against growing tubercle bacilli as also stationary phase bacilli with reduced metabolism$^{19}$. INH is active against growing tubercle bacilli but not stationary phase bacilli$^{20}$. It has been reported that complementation with kat$G$ gene not only restores INH susceptibility but also virulence of INH-resistant, catalase deficient strains in mice$^{21}$. Overall, both rpo$B$ and kat$G$ have relationship with survival and growth of $M$. tuberculosis.

It is important to determine whether these genetic changes affect the capacity to infect by aerosol route. In our experiment, the number of viable bacilli of MDR isolates of $M$. tuberculosis in the lungs of the mice was observed to become more or less constant after 3 wk. Though this is in accordance with the findings reported earlier$^{22,23}$, it still remains to be determined whether these isolates had lost the ability to express active kat$G$ and rpo$B$ gene product and whether these changes had any bearing on the virulence of these isolates. Even after mutation, there does not appear to be significant difference in the survival and growth of these organisms by aerosol challenge. Our observations corroborated with the observation of Ordway et al$^{24}$ in C57BL/6 mice. While other mutations may have effect on the in vivo growth of MDR isolates, there could be other unidentified factors, which play a role in allowing the isolates to grow rapidly in the lungs. It would be important to study the pathogenicity of a significant number of MDR isolates in susceptible mouse models like CBA, the infection in BALB/c may be closer to humans most of whom do not get disease after getting infected with $M$. tuberculosis.

The data provided by this study (though limited to 2 sensitive and 2 MDR strains; Table III) clearly indicates that since there growth kinetics are almost same, potential of these MDR isolates to spread the disease in the community may not be significantly different from those of sensitive organisms. Such observations have obvious epidemiological implications. This aspect thus merits further in-depth investigations using large number of MDR isolates in different mice strains and the findings have to be correlated with molecular epidemiology of disease.

Acknowledgment

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