Consequences of non-compliance on therapy efficacy and emergence of resistance in murine tuberculosis caused by the Beijing genotype

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Abstract

Despite great effort of health organizations worldwide in fighting tuberculosis (TB), morbidity and mortality is not declining as expected. One of the reasons is related to the evolutionary development of *Mycobacterium tuberculosis* (Mtb), in particular the Beijing genotype strains.

In a previous study, we showed the association between Beijing genotype and an increased mutation frequency for rifampicin. In this study we use a Beijing genotype strain and an East-African/Indian genotype strain, to investigate in our mouse TB model whether the higher mutation frequency observed in a Beijing genotype strain is associated with treatment failure particularly during non-compliance therapy.

Both genotype strains showed a high virulence in comparison to Mtb strain H37Rv, resulting in a highly progressive infection with rapid lethal outcome in untreated mice. Full-compliance treatment was effective without relapse of TB irrespective the infecting strain, showing similar decrease of mycobacterial load in infected organs, and similar histopathological changes. Non-compliance treatment, simulated by a reduced duration and dosing frequency resulted in relapse of infection. Relapse rates were correlated with the level of non-compliance and were identical for Beijing infection and East-African/Indian infection. However, in only Beijing-infected mice isoniazid-resistant mutants were selected at the highest level of non-compliance. This is in line with the substantial selection of isoniazid-resistant mutants *in vitro* in a wide isoniazid concentration window observed for the Beijing strain and not for the EAI strain.

These results suggest that genotype diversity of Mtb may be involved in emergence of resistance and indicates that genotype-tailor-made treatment should be investigated.
Introduction

Resistance to anti-tuberculosis drugs is rapidly emerging worldwide (24) with nearly half a million cases of multi-drug-resistant tuberculosis (MDR-TB) recorded annually. The majority of MDR-TB is found in Former Soviet Union States (FSU) and in Asia. (24) The true magnitude of the resistance problem may be much greater, as in most high TB prevalence settings the laboratory service is underdeveloped and resistance cannot be adequately detected. Moreover, in almost all countries worldwide, extensively drug-resistant TB (XDR-TB) has also been reported (25) and there are already publications on the emergence of totally drug-resistant TB (TDR-TB), e.g. in Iran and India. (23) Because only a minority of the MDR-TB cases can currently be treated according to the guidelines of the WHO, resistant TB may increasingly become an untreatable disease, although there is hope that new drugs may force a major improvement in this situation within some years. (12)

There are multiple factors known that underlie the development of resistance to anti-TB drugs, like unregulated availability of anti-TB drugs, poor quality of drugs, unprofessional prescription, non-compliance, malabsorption in certain subpopulations and large inter-individual variability in pharmacokinetics of anti-TB drugs, and host genetic factors. The combination and/or relative impact of factors that eventually lead to emergence of resistance are not well understood. However, non-compliance is generally considered to be the most relevant factor leading to resistance in TB treatment. (20, 22, 24)

A recent addition to the determinants of resistance to anti-TB drugs is a bacterial one. Certain outbreak-associated strains seem more prone to develop resistance and/or be transmitted as MDR-TB. For example, TB infection caused by the Beijing Mtb genotype strain is strongly correlated to the development and transmission of MDR-TB and even XDR-TB. (11, 19) Regarding transmission of TB in Europe, Beijing strains are significantly associated with transmission of MDR and XDR-TB. (8-9) Regarding development of drug-resistant TB, in multiple studies conducted in (for instance) Vietnam Beijing strains have also been correlated with development of MDR-TB, treatment failures and relapses after initially curative treatment (2, 10, 17), whereas the East-African/Indian (EAI) genotype strains are not associated with these problems. Still both Beijing and EAI genotypes are predominant in Vietnam, both lineages cause about 40% of the TB cases.
each. For this reason, in a recent study we selected strains of these both lineages, compared their intrinsic \textit{in vitro} susceptibility and determined their mutation frequency regarding the development of resistance to four different anti-TB drugs.\cite{7} The results revealed that for the Beijing genotype bacteria a much higher dosage of rifampicin was required to achieve a 100\% killing and that two out of five Beijing strains exhibited a remarkably high frequency of mutations conferring rifampicin resistance.\cite{7} These data underline the importance of the use of different \textit{Mtb} strains in preclinical experimental studies using animal models, as stated by de Groote \textit{et al.} \cite{3} They recommend the use of different \textit{Mtb} strains in the confirmation of treatment efficacy results of novel drugs in the translational phase, in order to strengthen the likelihood of success of novel drug regimens that advance forward into clinical trials.\cite{3}

In the present study we assessed the impact of non-compliance and the role of \textit{Mtb} strains as risk factors for the emergence of resistance following TB treatment. More specifically, we investigated in a well-established mouse TB model \cite{4} whether the consequences of suboptimal treatment, simulating non-compliance, would be more pronounced in the outcome of TB caused by a Beijing genotype strain in comparison to TB caused by a EAI genotype strain. Results obtained may help to better understand the large differences in treatment success in Vietnam in TB patients infected by Beijing genotype bacteria versus those infected by EAI genotype bacteria.
Materials and Methods

Bacterial strain. The two Mtb strains used were clinical isolates obtained from Vietnam one of the Beijing genotype and one of the EAI genotype, as described previously.\(^{(7)}\) The strains were provided by the National Tuberculosis Reference Laboratory (Bilthoven, the Netherlands), where they were labelled as Beijing VN 2002-1585 (Beijing-1585) and EAI VN 2002-1627 (EAI-1627) and were cultured as described previously.\(^{(5)}\)

The Minimal Inhibitory Concentration (MIC) of the Mtb genotype strains was determined using the agar proportion method as described by the Clinical and Laboratory Standards Institute.\(^{(26)}\) The MIC’s for both Beijing-1585 and EAI-1627 were 0.125 mg/L isoniazid and 0.25 mg/L rifampicin. MIC determinations were performed in duplicate.

In order to assess stability of isoniazid-resistant mutants, the mutants were subcultured five-times in MGIT\(^{TM}\) medium without isoniazid, with 0.2 mg/L isoniazid or with 1.0 mg/L isoniazid and time to positivity within the MGIT\(^{TM}\) bactec system was used as outcome-parameter.

Infection model. Specified pathogen-free female BALB/c mice were obtained from Charles River [Les Oncins, France]. The experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Institutional Animal Care and Use Committee of the Erasmus MC Rotterdam approved the present protocols. Mice were infected through intratracheal inoculation followed by inhalation, as described previously.\(^{(4)}\) The inoculum of Mtb used for infection contained \(1.3\times10^5\) cfu [range 1.2 - \(1.3\times10^5\)] of Beijing-1585 and \(0.6\times10^5\) cfu [range 0.5 – 0.7\(\times10^5\)] of EAI-1627.

Drug dosing and anti-TB treatment. The mice receiving compliance therapy were treated with dosage and schedules of anti-TB drugs derived from current clinical guidelines. The drugs were injected at once. Isoniazid (Hospital Pharmacy; Rotterdam, The Netherlands), rifampicin (Rifadin\(^{®}\), Aventis Pharma B.V, Hoevelaken, The Netherlands) and pyrazinamide [150 mg/kg; P7136, Sigma Chemical Co, St. Louis, MO] were administered in human pharmacokinetic-equivalent doses, as described previously.\(^{(4, 13)}\) Treatment was started at 2 weeks after infection. The duration of full-compliance treatment was 26 weeks (6 months),
consisting of a 9-week (2 months) initial phase followed by 17-week (4 months) continuation phase, according to clinical guidelines of TB therapy. During the initial phase, animals received a combination of isoniazid [25 mg/kg], rifampicin [10 mg/kg] and pyrazinamide [150 mg/kg]. In the continuation phase, animals received isoniazid and rifampicin. Agents were administered once daily, five days per week, subcutaneously in the neck, to avoid potential damage following long-term daily oral gavage. Proper correction was made for the subcutaneous administration in view of the differences in bioavailability of the drugs after oral administration. In the non-compliance treatment drugs were administered for treatment duration of only 13 weeks (9 weeks isoniazid, rifampicin and pyrazinamide, followed by 4 weeks of isoniazid and rifampicin), simulating premature discontinuation of treatment in patients (defaulting). Also underdosing was simulated by applying a reduced frequency of dosing; mice received treatment either five days per week, or three days per week, or once a week. In the non-compliance treatment, the daily doses were identical to the compliance treatment.

**Therapeutic efficacy.** Parameters for therapeutic efficacy were (1) Mtb load in pulmonary and extra-pulmonary organs, (2) emergence of drug resistance, (3) clinical signs of illness of mice, (4) relapse of infection during the 13 weeks post-treatment period, (5) histopathological characterization of the infected organs and (6) cytokine concentrations in blood. Treatment success was defined as elimination of the Mtb load from infected organs, prevention of drug resistance and prevention of TB relapse at 13 weeks post-treatment.

**Therapeutic efficacy – Mtb load in pulmonary and extra-pulmonary organs and emergence of drug resistance.** At indicated time points the mycobacterial load in infected organs was assessed. Mice (n=4 per time point) were sacrificed by CO₂ exposure. Subsequently, lung, spleen and liver were removed aseptically and homogenized each in 2 mL PBS. Samples were centrifuged and washed with PBS to prevent carry-over of anti-TB drugs to the subculture plates. From the undiluted tissue homogenate and 10-fold serial dilutions samples of 200 μl were plated onto 7H10 agar. To detect drug-resistant Mtb mutants, subcultures were also performed on rifampicin-containing plates and on isoniazid-containing plates. The concentrations of rifampicin and isoniazid in the subculture plates were 4-fold the “critical concentration”, i.e. 4 mg/L for...
rifampicin and 0.8 mg/L for isoniazid. Colonies of drug-resistant Mtb, were characterized using the GenoType® MTBDRplus assay (Hain Lifescience GmbH, Nehren, Germany), to detect the most common mutations.

Therapeutic efficacy – clinical signs of illness of mice. The behaviour of mice was monitored daily, as prescribed by the animal ethical committee. Clinical parameters were the body weight assessed 3 times per week and daily evaluation of a discomfort score. Mice that displayed severe signs of illness were euthanized by CO₂ exposure.

Therapeutic efficacy – relapse of infection. Mtb load in the lung, spleen and liver of mice (n=4) were assessed 13 weeks after termination of TB treatment. Relapse was defined as Mtb-positive organ cultures, while immediately after termination of treatment organ cultures were Mtb-negative. Emerging drug-resistant Mtb mutants were characterized.

Therapeutic efficacy – statistical analysis. An unpaired Mann-Whitney test was used to analyse differences between (1) the course of infection of the Beijing-infected mice and the EAI-infected mice and (2) the therapy efficacy of the different (under dosing) treatment regimens. Differences were considered statistically significant when the p-value was ≤ 0.05.

Therapeutic efficacy – histopathological characterization of the infected organs. Lung, spleen and liver from sacrificed animals (n=3) at indicated time points (at weeks 1 and 2 of the untreated infection, at weeks 3, 5, 7, 15 and 28 during treatment and at 13 weeks post-treatment (weeks 28 and 41) were processed as described previously. In brief, standard 4 µm haematoxylin-eosin stained sections were prepared from ethanol fixed, paraffin wax embedded lung, spleen and liver tissues. Additionally, a Ziehl-Neelsen staining was performed to detect acid-fast bacilli. Histopathological analysis was performed on tissues from untreated infected mice, from infected mice receiving compliance treatment and from mice receiving non-compliance treatment once per week for 13 weeks. A pathologist, blinded to the experimental conditions, examined the samples.
Therapeutic efficacy – cytokine concentrations in blood. Blood samples were obtained by cardiac puncture from mice at week 2 (start treatment), during compliance treatment at week 28 and 41 and during non-compliance treatment (treatment once a week for 13 weeks) at week 15 and 28. Plasma samples were prepared from EDTA-blood. Quantification of cytokines was performed using a bead-based flow cytometry technique (xMap; Luminex Corporation, Austin, TX, USA). A milliplex map mouse cytokine, 31-plex was used (Millipore Corporation, Billerica, MA, USA) consisting of bead-labelled cytokine receptor against the following biomarkers; Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IP-10, KC-like, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α and VEGF. Tests were performed according the manufacturers' protocol. Samples were tested in duplicate. Results in median fluorescence intensity (MFI) values were converted to pg/mL using MILLIPLEX Analyst software (Millipore) and subsequently averaged.

Selection of isoniazid-resistant mutants in vitro. In order to investigate differences between the Beijing-1585 and EAI-1627 in terms of in vitro selection of isoniazid-resistant Mtb, samples taken after 6 days of exposure to isoniazid at concentrations ranging from 0.015 mg/L to 256 mg/L were cultured on drug-free 7H10 agar plates and isoniazid-containing plates. The concentration of isoniazid in the subculture plates was 4-fold the ‘critical’ concentration, (26) i.e. 0.8 mg/L isoniazid. Only the isoniazid-resistant Mtb were able to grow on the drug-containing subculture plates, whereas both susceptible and drug-resistant Mtb showed growth on the drug-free subculture plates.
Results

Course of untreated TB infection. As shown in figure 1, the course of progressive TB infection caused by Beijing-1585 or EAI-1627 in untreated mice was similar. The Mtb load in the lung from week 1 after infection was not significantly different, and increased at week 2 (the time point at which therapy was started) up to $2.1 \times 10^8$ [1.3 – 3.1$\times 10^8$] cfu Beijing-1585 and $2.7 \times 10^8$ [1.8 – 5.1$\times 10^8$] cfu EAI-1627. In the third week of untreated infection caused by either strain all mice became moribund, as evidenced by their discomfort score and body weight loss (data not shown) or died.

At week 2 histological analysis of lung tissue from untreated mice infected with either strain showed predominantly centrilobular inflammatory infiltrates composed of lymphocytes and histiocytes. The infiltrates were dense and located within the alveoli, sparing the interstitium (Figure 2A and 2F). No differences were observed between the Beijing-1585 or EAI-1627 infected mice at this stage.

Levels of cytokines in plasma were determined in groups of only three mice. Cytokine profile in plasma after 2 weeks of infection revealed only minor differences between the Beijing-1585 and EAI-1627 infected mice. Compared to EAI-1627 infected mice only the levels of Eotaxin and MCP-1 were higher in Beijing-1585 infected mice (supplementary figure 1); whereas the levels of MIP-1$\alpha$ and MIP-1$\beta$ were lower in Beijing-1585 infected mice (supplementary figure 1). As untreated mice died in week 3, their cytokine levels could not be assessed at later time points. Therefore the dynamics of these cytokines in untreated infections could not be assessed further.

Therapeutic efficacy of full-compliance treatment. As shown in figure 1, the therapeutic efficacy of full-compliance treatment as measured by decreases in Mtb load, was similar for the Beijing-1585 infected mice and the EAI-1627 infected mice. The liver was the first organ that became culture-negative, followed by the spleen and finally the lung. After 26 weeks of treatment (week 28) lung, spleen and liver of the Beijing-1585 and EAI-1627 infected mice were culture-negative. Importantly, drug-resistant Mtb mutants were never selected from these mice receiving full-compliance treatment.
Relapse assessment after the 13 weeks post-treatment period, revealed that relapses of infection never occurred. From 2-4 weeks of treatment onwards, the discomfort score and bodyweight of both Beijing-1585 and EAI-1627 infected mice were at the level of the uninfected control mice (data not shown).

During full-compliance treatment, lung tissue from the mice infected with Beijing-1585 initially showed an increase of compact fields of epithelioid histiocytes, which in later stages decreased in intensity (figure 2B). Almost complete resolution at week 41 (26 weeks therapy followed by 13 weeks post-treatment) with only sparse peri-bronchiolar infiltrates was observed (figure 2C-D). Mice infected with EAI-1627 (figure 2I-J) showed essentially similar histopathological characteristics as the Beijing-1585 infected mice (figure 2C-D).

At the end of the full-compliance treatment of EAI-infected mice, the infiltrate diminished to a mild peri-bronchiolar exclusively lymphocytic pattern (figure 2H).

**Therapeutic efficacy at non-compliance treatment.** The non-compliance treatment of mice consisted of only 13 weeks treatment at various dosing frequencies. Results are shown in figure 3. At the end of treatment for five days per week (week 15) half of the mice were Mtb culture-negative in the lung (two out of four mice, for both Beijing-1585 and EAI-1627). However, at 13 weeks post-treatment all mice relapsed and the Mtb load in the lung was $3.1 \times 10^3$ [2.4x10$^2$ – 9.6x10$^3$] cfu Beijing-1585 and $5.1 \times 10^3$ [0 – 8.4x10$^3$] cfu EAI-1627 at week 28. Reduction of treatment frequency to 3 days per week resulted in Mtb culture-positive lungs at the end of treatment (week 15) in all Beijing-1585 infected and all EAI-1627 infected mice, whereas all spleen- and liver cultures were negative. The mycobacterial load in the lung at week 15 was $2.7 \times 10^2$ [1.3 – 3.1x10$^2$] cfu Beijing-1585 and 63 [0.1 – 9.2x10$^2$] cfu EAI-1627. At 13 weeks post-treatment all mice relapsed and the Mtb load in the lung was $9.0 \times 10^4$ [0.3 – 4.3x10$^5$] cfu Beijing-1585 and $2.1 \times 10^4$ [0.3– 2.5x10$^4$] cfu EAI-1627 at week 28.

Further reduction of treatment frequency to once per week revealed culture-positive lungs and spleens at the end of treatment (week 15) in all Beijing-1585 infected and all EAI-1627 infected mice. The mycobacterial load in these mice was $9.5 \times 10^5$ [0.3 – 4.5x10$^6$] cfu Beijing-1585 and $2.5 \times 10^4$ [0.3 – 7x10$^4$] cfu EAI-1627 at week 28.
load in the lung at week 15 was $3.8 \times 10^3 [0.5 - 9.2 \times 10^3]$ cfu Beijing-1585 and $1.5 \times 10^5 [1.2 - 1.9 \times 10^5]$ cfu.

EAI-1627. At 13 weeks post-treatment all mice showed relapse of infection, resulting in a mycobacterial load in the lung of $1.4 \times 10^5 [0.5 - 4.0 \times 10^5]$ cfu Beijing-1585 and $6.2 \times 10^5 [0.1 - 2.0 \times 10^6]$ cfu EAI-1627 at week 28.

Mice receiving the non-compliance treatment once per week showed a very high mycobacterial load throughout the entire course of infection, especially in the spleen.

Statistical analysis of the efficacy data obtained with the different treatment entities revealed at week 15 a significantly increasing mycobacterial load in the lung and spleen of both Beijing-1585 and EAI-1627 infected mice together with a decreasing treatment frequency (figure 3A-D). No significant differences in the efficacy of any treatment modality were observed when between Beijing-1585 infected mice and the EAI-1627 infected mice.

Remarkably, only in the Beijing-1585 infected mice, receiving non-compliance treatment once per week for 13 weeks isoniazid-resistant Mtb mutants were selected from lungs and spleens of all mice, at week 20 and week 28 (end of therapy). Isoniazid-resistant mutants at week 20 represented a percentage of 2.2 [range, 0.3-6.4] of the total mycobacterial load in the lung, and a percentage of 1.9 [0.7-2.6] of the total mycobacterial load in the spleen. At week 28, isoniazid-resistant Mtb mutants represented a percentage of 1.7 [1.2-3.4] in the lung and a percentage of 5.1 [3.4-18.5] in the spleen. In the liver drug-resistant Mtb mutants were not found. The selection of resistance was not observed in Beijing-1585 infected mice receiving non-compliance treatment three times per week or five-times per week for 13 weeks nor in any of the EAI-1627 infected mice receiving non-compliance treatment at the three different schedules.

Analysis of the isoniazid-resistant Beijing genotype mutants selected from the lung at week 20 and week 28 showed that the MIC of isoniazid was 128 mg/L, whereas the strain used to infect the mice exhibit an isoniazid MIC of 0.125 mg/L. After a five-time subculture of the isoniazid-resistant mutants in isoniazid-free medium the mutants retained their high MIC of 128 mg/L. Genotypic analysis of these stable isoniazid-resistant Beijing genotype mutants revealed no mutations in the katG, inhA, kasA or ahpC genes. Repeated subculture of these resistant isolates in MGIT™ medium without isoniazid, with 0.2 mg/L isoniazid or with...
1.0 mg/L isoniazid showed similar growth rates in all media, as expressed by their similar time to positivity within the MGIT bactec system (data not shown).

Selection of isoniazid-resistant mutants in Beijing-1585 and EAI-1627 in vitro. We observed a difference between the Beijing-1585 strain and the EAI-1627 strain. For the Beijing-1585 isoniazid-resistant mutants (ranging from 50 to 420 cfu/mL) were selected up to the concentration of 4 mg/L isoniazid, whereas for the EAI-1627 only few isoniazid-resistant mutants (≤20 cfu/mL) were found and only up to the concentration of 0.015 mg/L isoniazid (figure 4).

Lung tissue of mice infected with Beijing-1585 and receiving non-compliance treatment once per week, initially showed a minor lymphocytic infiltrate (week 15) without epithelioid histiocytes (figure 2E), which became more dense at week 28 and was associated with a mild histiocytic component (figure 2F). In the EAI-1627 infected mice receiving the same non-compliance treatment, the infiltrate initially decreased at week 15 (figure 2K) but became more widespread and denser at week 28 (figure 2L).

Cytokine levels in plasma from mice receiving non-compliance treatment (once per week for 13 weeks) showed that in Beijing-1585 as well as EAI-1627 infected mice, the levels of G-CSF, GM-CSF, IFN-γ, IL-13, IL-17, IL-1α, IL-6, IP-10, KC, MIP-1α, MIP-1β and TNF-α being increased after 2 weeks of infection, were again decreased after 13 weeks of therapy. From these cytokines only the levels of IFN-γ, IP-10 and TNF-α increased again at 13 weeks after termination of therapy, during relapse of infection (figure 1 of the supplementary data).
Discussion

From previous studies in Vietnam, it is known that the outcome in TB patients infected with a Beijing genotype strain is relatively poor compared to TB patients infected with an EAI genotype strain. However, no satisfactory explanation for this observed phenomenon is yet available. Recently, we have shown in our in vitro studies that Beijing genotype strains have an increased mutation frequency for rifampicin, compared to EAI genotype strains. The mutation frequency for rifampicin in Beijing-1585 and EAI-1627 strain were $3.7 \times 10^{-3}$ and $3.5 \times 10^{-6}$, respectively.\(^{(7)}\) Also a relatively large window of rifampicin concentrations within which rifampicin-resistant Mtb mutants were selected, was observed in the Beijing genotype compared to the EAI genotype. In the present study it is investigated whether the high mutation frequency for rifampicin observed in only Beijing genotype strains is associated with treatment failure in Beijing infection. We compared the treatment efficacy of different treatment schedules in a mouse model of TB caused by the Beijing-1585 strain or the EAI-1627 strain. The genotype of Mtb may influence different aspects of the infection. One hypothesis is that the Beijing genotype strain might exhibit mechanisms that modulate the immune response by the host; as such having an advantage over other Mtb strains.\(^{(1, 16)}\) Elaborating on this hypothesis, when mass vaccination with the bacillus Calmette-Guérin (BCG) was introduced in TB endemic areas, the Beijing genotype could use this advantage over other strains and spread more easily.\(^{(1)}\)

In the present study in unvaccinated mice, a potential advantage of the Beijing genotype over the EAI genotype was not observed. It was shown that both Beijing-1585 and EAI-1627 are equal in pathogenic capacity during the progression of TB. In both infections the untreated animals died or became moribund within 3 weeks after infection. Also a similar increase in Mtb load in lung, spleen and liver during the first two weeks was observed. In addition, the histopathological damage inflicted was equivalent and the changes in cytokine levels during the course of untreated infection were similar for both strains. Moreover, the cytokine profile of both strains were in line with previously described cytokine levels in our murine TB model caused by the Mtb strain H37Rv.\(^{(6)}\) The present study shows that the Beijing-1585 and EAI-1627 strains exhibit an increased virulence reflected by a rapid course of TB compared to TB caused by the H37Rv strain that resulted in death of mice not before 22 weeks after infections, as shown in our previous study.\(^{(4)}\) The increased virulence of the Beijing genotype is in accordance with previously described studies.\(^{(14, 18-)}\)
19) The high virulence of the EAI genotype in our mouse TB model is also consistent with the fact that EAI strains, similar to the Beijing strains, do very frequently cause disease in Vietnam (40% of the TB patients). In contrast to the Beijing strains, the EAI-strains in Vietnam are negatively correlated with resistance, and in our previous study (7) and the present study in mice we found clues to explain this.

We observed that the treatment efficacy in conditions mimicking full-compliance is similar for Beijing-1585 infected and EAI-1627 infected mice; both mycobacterial load in infected organs and cytokine levels in blood were equivalent. This finding suggests that if treatment is applied adequately therapy should be successful and not dependent on the causative infecting strain.

Regarding non-compliance a strong dose-dependent efficacy of the therapy was observed. Most likely, caused by reduced blood concentrations of the anti-TB drugs and thus reduced area under the concentration-time curve (AUC) over MIC-ratio, which is the pharmacokinetics / pharmacodynamics determinant for therapeutic efficacy. It was demonstrated that an increasing level of non-compliance resulted in decreasing Mtb killing in the lung, as well as in the spleen and liver at the end of the 13 weeks treatment period. The results obtained in Beijing-infected and EAI-infected mice in this respect are strikingly identical.

From the cytokines selected in this study an increased level of IFN-γ, IP-10 and TNF-α could serve as a biomarker of treatment failure after non-compliance, resulting in relapse of infection. As these data are obtained in inbred mice, it should be noted that in patients changes in cytokines may also be influenced by other factors not related to the TB infection. In the TB-infected mice the cytokine analysis was not helpful in differentiating between the two endemic strains causing the TB-infection.

Another factor possibly contributing to the emergence of MDR-TB might be variability in pharmacokinetics of anti-TB drugs among patients. Gumbo et al. investigated in their hollow fiber model both bactericidal and sterilizing effects of anti-TB drugs, and predicted that approximately 1% of TB patients following full-compliance therapy would still develop MDR-TB due to interindividual variability in pharmacokinetics of
anti-TB drugs, causing variable and low drug concentrations in a subset of patients. Differences in pharmacokinetics of anti-TB drugs are expected to be marginal in inbred mice as used in our TB-model. The most prominent observation of this study is the selection of isoniazid-resistant mutants exclusively in the Beijing-infected mice and only during the most severe non-compliance treatment (once per week treatment) resulting in relapse of TB. Emergence of resistance is possibly related to a substantial period of low-drug concentrations in blood following once weekly dosing, as such creating a mutant selection window. Remarkably, from week 20 to week 28 (5 and 13-weeks post treatment, respectively) the percentage of isoniazid-resistant mutants increased in the spleen, whereas the percentage of isoniazid-resistant mutants in the lung remained at the same level. Possibly the spleen serves as a reservoir for these resistant mutants and provides a niche (e.g. the large numbers of macrophages) within which the resistant mutants can divide and thereby re-infect other organs via haematogenic transmission. The stability of these isoniazid-resistant Beijing-genotype mutants was demonstrated by repeated subculture of the mutant in drug-free medium, indicating that the isoniazid resistance was conserved even without isoniazid pressure, suggesting that genotypic drug-resistant mutants were selected. In EAI-infected mice at all non-compliance conditions resistant Mtb mutants were never found, although the treatment responses at the various non-compliance conditions were similar in EAI infection and Beijing infection. Similar observational data are available from clinical studies in endemic areas (2, 10, 17). As a result the present model could serve in future studies to further unravel the mechanism(s) by which Beijing genotype strains facilitate the selection of resistant mutants. In a previous in vitro study, focusing on rifampicin we have shown that Beijing genotype strains exhibit an increased mutation frequency for rifampicin. However, in the present study in Beijing infection in vivo no selection of rifampicin-resistant mutants was observed. In contrast, isoniazid-resistant mutants were found only in the Beijing-infected mice during the most severe non-compliance treatment. Given that in patients isoniazid-resistance is most often observed and is regarded as precursor for MDR-TB, our in vivo results are well in line with clinical reality. The fact that isoniazid-resistant mutants were only isolated from the Beijing-
infected mice and not from the EAI-infected mice may be explained by our in vitro data. We assessed the time-kill kinetics and selection of isoniazid-resistant mutants using the Beijing-1585 and EAI-1627 strains exposed to isoniazid for 6 days. Whereas for the EAI strain only few isoniazid-resistant mutants were cultured and could survive isoniazid concentrations ≥0.015 mg/L, for the Beijing strain much higher numbers of mutants were found in an isoniazid concentration selection window of 0.015 to 4 mg/L. In an attempt to understand these observed differences one could argue that mutations in the DNA-repair genes of the Beijing genotype strain resulted in the increased resistance mutant selection range. Possibly, an increase in isoniazid dosage could prevent the selection of isoniazid-resistant Beijing mutants, and thereby prevent the formation of MDR-TB. Further studies to explore this hypothesis are needed.

In the present study we compared only one Beijing genotype strain and one EAI genotype strain. In general, it should be noted that studies with Mtb genotypes are complex because there are many different outbreak-associated strains from many different geographical locations. This diversity in strains hinders generalized conclusions upon two genotypic strains.

In conclusion, this study shows that the genotypic diversity of Mtb in terms of selection of resistant mutants occurred not only in vitro but also in vivo in experimental TB in mice. Only isoniazid-resistant Mtb mutants were selected in Beijing-infected mice during severe non-compliance treatment and not in EAI-infected mice. This might justify genotype-tailor-made therapy, in order to prevent the selection of resistance in Beijing-infected patients.
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References


Non-compliance and emergence of resistance in TB


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Figure 1. TB infection and efficacy of compliance treatment in mice infected with Beijing-1585 or EAI-1627. The mycobacterial load in lung (A), spleen (B) and liver (C) of the untreated mice, infected with Beijing-1585 (black bars) or EAI-1627 (chequered bars). Mice receiving therapy started 2 weeks after infection and continued for 26 weeks are indicated for the BE-1585 (diagonally stripped bars) and for the EAI-1627 (open bars). Results are expressed as median ± range (error bars) of the colony forming units (CFU) per organ, n = 4 per time point. Numbers above bars are the numbers of culture-positive mice out of total numbers of mice at that time point. † = 4 out of 4 untreated mice died due to TB infection before this time point.

Figure 2. Histology. Lung tissue from mice infected with Beijing-1585 or EAI-1627. A-F: mice infected with Beijing-1585. G-L: mice infected with EAI-1627. A, G: week 2, start of treatment. B, H: week 28, end of compliance treatment. E, K: week 15, end of non-compliance (once per week) treatment. C, D, I, J: week 41, 13 weeks post-compliance treatment. F, L: week 28, 13 weeks post-non-compliance (once per week) treatment. A (original magnification 100x): Extensive intra-alveolar accumulation of histiocytes is observed, with a peri-bronchiolar lymphocytic component. (25x): Peri-bronchiolar infiltrates, predominantly composed of lymphocytes are seen, admixed with a minor histiocytic component. C, D (50x): Images from single lung with highly variable histology, ranging from large areas of normal lung tissue (C) to minor areas of residual densely inflamed lung (D). E (100x): A moderately dense peri-bronchiolar predominantly lymphocytic infiltrate is present, no intra-alveolar or interstitial inflammation is seen. F (100x): A combination of an intra-alveolar histiocytic infiltrate is present with a moderately dense peri-bronchiolar lymphocytic component; the pattern is similar, however less extensive to the pattern observed in A. G (100x): Intra-alveolar histiocytes are seen with a centri-lobular distribution, combined with a minor lymphocytic component. H (50x): A mild to moderately dense lymphocyte predominant infiltrate is present; a mild increase in alveolar macrophages is also seen. I, J (25x): Images from single lung with highly variable histology, ranging from large areas of normal lung tissue (I), (50x) to minor areas of residual densely inflamed lung (J).K (50x): A mild to moderately dense peri-bronchiolar lymphocytic infiltrate is present. L (25x): A dense lympho-histiocytic infiltrate is present.
Figure 3. TB infection and efficacy at non-compliance treatment in mice infected with Beijing-1585 (A, C and F) or EAI-1627 (B, D and E). Mycobacterial load in lung (A-B), spleen (C-D) and liver (E-F) of untreated mice (black bars). Therapy started 2 weeks after infection continued for 13 weeks. Mice received treatment five times per week (chequered bars), three times per week (open bars) or once per week (diagonally stripped bars). Results are expressed as medians ± range (error bars) of the colony forming units (CFU) per organ, n = 4 per time point. Numbers above bars are the numbers of culture-positive mice out of total numbers of mice at that time point. † = 4 out of 4 untreated mice died due to TB before this time point. * indicates the presence of isoniazid-resistant mutants. The p-values of the statistical evaluation of differences between the non-compliance groups were indicated in the figure if p-values were ≤0.05.

Figure 4. Selection of isoniazid-resistant mutants in vitro. The Mtb genotype strains Beijing-1585 (A) at a density of 7.7x10^5 CFU/mL and EAI-1627 (B) at a density of 2.3x10^5 cfu/mL, were exposed to isoniazid at fourfold increasing concentrations for 6 days at 37°C. After 6 days of exposure quantitative cultures were performed on subculture plates containing 0.4 mg/L isoniazid (diagonally stripped bars) or subculture plates without isoniazid (black bars).