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ORIGINAL ARTICLE

# A Mouse Model for Latent Tuberculosis

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**The aim of the study was to establish a reproducible murine model for latent tuberculosis. We propose an operational definition of latent murine tuberculosis as a stable Mycobacterium tuberculosis count in lungs and spleens without clinical signs or obvious histopathological changes in the lungs over a long period of time and without spontaneous reactivation of disease. B6D2F1Bom mice were inoculated with a wide range of Mycobacterium tuberculosis doses intraperitoneally or intravenously and followed for a long period to determine suitable conditions to produce latent infection. No anti-tuberculosis drug treatment was used. Microbiological and histopathological studies were carried out. Corticosterone challenge was used to reactivate the latent infection. Mice infected with $4 \times 10^4$ and $4 \times 10^5$ bacilli i.p. were followed up to 107 weeks without spontaneous reactivation. The present model is discussed in comparison with previous latent tuberculosis mouse models as well as the possible mechanisms of shift to stationary phase from multiplying bacilli.**

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## INTRODUCTION

With the challenge of the global tuberculosis situation, it is increasingly recognized that current tuberculosis (TB) control strategies suffer from severe shortcomings. The only available vaccine, Bacille Calmette-Guerin (BCG), has a substantial impact on severe forms of childhood TB (1–3). However, it seems neither to prevent the establishment of latent TB infection nor the reactivation of a latent infection. Chemotherapy also has a limited impact on transmission due to late diagnosis, low compliance (4, 5) and the emergence of drug resistance (6).

The reactivation of clinical TB from latent infection has been difficult to characterize except among elderly people living in countries without ongoing transmission of TB (7–9). Recently a restriction fragment length polymorphism (RFLP) method was applied to study the transmission of TB within groups at high risk of infection (10–15). Some of these studies indicated that recent transmission accounts for approximately one-third of the incidence in San Francisco (14) and 26% (13) to 28% (15) (after subtraction of possible index cases) of incidence in New York City. A higher proportion of clustered strains was seen among TB patients co-infected with Human Immunodeficiency Virus (HIV), poor TB drug compliants, low wages earners, people previously treated for TB, and those living under crowded conditions. This seems to support the assumption that recent transmission contributes a substantial portion of new TB cases in these groups (15, 16) and also in endemic areas (17). However, the studies described above (13–15) showed that most foreign-born TB patients did not carry strains identified in those in close proximity, implying that clinical TB in these patients was most probably due to reactivation of an infection acquired before migration. Today almost one-third of the world population is infected with Mycobacterium tuberculosis (Mtb) (18), constituting a large reservoir for reactivation of latent TB. In the pre-HIV period, an individual infected with Mtb had approximately a 10% lifetime risk of developing clinical TB (19). Reactivated TB appears as pulmonary with cavitary lesions (sputum positive) (20–22), and is therefore the main source of TB transmission in society (19, 23, 24). However, there is no effective preventive measure available.

The basic problem we face is an almost complete lack of knowledge concerning the biology of bacillary dormancy and the relationship between dormant bacilli and host immunity. Characterization of the metabolism and antigens (Ag) expression of Mtb during dormancy could be instrumental for the development of new anti-TB drugs and immune interventions effective against latent infection. There is thus an urgent need to establish simple, reliable and effective animal model for latent TB.

A chronic murine TB model has been described previously (25–31). The differences between our model and the previous models are that (1) it mimics the natural course of latent infection by avoiding the use of anti-TB drugs. (2) We can follow up infected healthy mice for more than 100 weeks post-infection without seeing spontaneous reactivation of disease. Thus this model mimics the natural latency in humans where the bacilli may remain dormant for many years before being reactivated. (3) Relatively high numbers of bacilli in organs of mice in our model offer an opportunity to study the nature of dormant bacilli and immune responses of the mice in the latent period.

The work described here aims at developing a simple and reproducible mouse model for studies of latent tuberculosis.

* Both authors have contributed equally to this study.

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Table I. *Experimental design for Mtb infected mice*

| | Experiment 1 | | | | Experiment 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Groups of mice | I | II | III | IV | I | II | III | IV | V |
| No. of mice | 4 | 6 | 6 | 4 | 30 | 30 | 30 | 30 | 30 |
| Dose of infection | $10^3$ | $10^5$ | $10^5$ | $10^8$ | $4\times10^1$ | $4\times10^2$ | $4\times10^3$ | $4\times10^4$ | $4\times10^5$ |
| Route of infection | i.v. | i.v. | i.p. | i.p. | i.p. | i.p. | i.p. | i.p. | i.p. |

$4\times10^1$–$4\times10^3$ = low-dose infection, $4\times10^4$–$4\times10^5$ = moderate-dose infection, $1\times10^8$ = high-dose infection.

## MATERIALS AND METHODS

### *Mice*

B6D2F1Bom (C57BL/6JBom × DBA/2JBom) mice were purchased from Bomholtgård Breeding and Research Centre Ltd, Denmark. This centre follows the FELASA (Federation of European Laboratory Animal Science Association) recommendations for health control of laboratory animals. The animals were acclimatized for at least 7 d before being included in the experiments. All mice were aged about 17 weeks when they were used in experiments. The average life-span of these mice is 975 d (32). Animals were given standard mouse diet (RM1, SDS, Witham, UK) and water ad libitum. They were kept in MAKIII macorolon cages. The cages were kept in negative pressure plastic film isolators (Plastic Film Industries, UK). The isolators were supplied with temperature (22°C ± 1°C) and humidity (50% ± 10% R.H) controlled air. The room lighting was set at 14/10 light/dark. The experiments were performed with the permission of the Norwegian Experimental Animal Board. Norwegian Law has been harmonized with the European Convention on the Protection of Experimental Animals.

### *Mycobacteria*

All experiments were conducted with the H37Rv strain of Mtb. The mycobacteria were first cultured on Lowenstein-Jensen media for 10 weeks.

### *Preparation of inocula*

Stock inocula were prepared by subculturing colonies in Middlebrook 7H9 liquid supplemented by Middlebrook ADC (Difco laboratories, Detroit, MI) (10: 1) and 0.05% Tween-80. The stock culture was incubated at 37°C for 10 d and shaken briefly on alternate days. Small aliquots of the stock bacillary suspension were maintained at −70°C. The desired number of bacilli for inoculation was prepared from the stock suspension after diluting with phosphate buffered saline (PBS). The bacillary suspension for inoculation was sonicated for 5 seconds in a water bath sonicator (Gen-probe®, HF-frequency– 35kHz, Type-T460/H, Germany) before use.

### *Assessment of viability of stock culture by colony forming units (CFU)*

The stock culture was sonicated for 30 s in the bath sonicator. It was then ten-fold serially diluted with medium and plated onto Middlebrook 7H10. This medium was prepared by mixing agar base Middlebrook 7H10 (Difco, Detroit Laboratories, MI, USA) with Middlebrook OADC (Gibco, Life Technologies Ltd., Scotland). The inoculated plates were incubated at 37°C, in a $CO_2$ (5%) incubator. CFU were counted twice weekly after the appearance of pinpoint size colonies.

### *Experimental design, routes and doses of infection*

Two separate experiments were performed to know which route and dose were suitable for establishing chronic infection. In the first experiment Mtb was given in different doses intraperitoneally (i.p.) or intravenouslly (i.v.) to four groups of mice (Table I). A total of 20 mice were used for this experiment. This experiment was terminated at week 15 and 21 post-infection (Fig. 1).

In the second experiment, different doses of infection were given to 5 groups of mice i.p. (Table I). A total of 163 mice were used in this experiment. Of these, 150 mice were infected and 13 were not. Five uninfected mice served as controls for growth and age and were kept in a separate isolator. The 8 remaining uninfected mice were housed with the infected groups as sentinel controls to check for transmission of infection and environmental conditions in the cages. No cross-infection was observed. The time schedule for sacrificing the mice in this experiment is shown in Fig. 4. The experiment was terminated at week 77 post-infection for corticosterone-challenged mice, whereas unchallenged mice were still alive until 115 weeks after infection.

The mice were given a range of inoculum between $4\times10^1$ and $1\times10^8$ bacilli in 0.2 ml PBS. Doses between $4\times10^1$ and $4\times10^3$ were designated "low-dose", $4\times10^4$– $4\times10^5$ "moderate-dose" and $1\times10^8$ "high-dose" inocula.

### *Corticosterone challenge*

By week 53 of infection, the CFU in the organs of infected mice without signs of disease had remained stable for about 12 weeks (see results). At week 53, half of the mice in the low- and moderate-dose groups were given oral corticosterone (Sigma Chemical, St Louis, MO, USA). The remaining mice were left as controls. Corticosterone was dissolved in ethanol and then diluted to a final concentration of 267 mg/ml in 1% ethanol and 0.5% saline. Mice were given this solution in their drinking water. Mice in each group drank from the same bottle. On average, one mouse drank about 1.07 mg of corticosterone per day. The corticosterone challenge started at about 25% of this dose. It was then doubled after week 10, and increased to the final dose 13 weeks after the start of corticosterone treatment.

### *Harvesting of organs*

At the time of sacrifice mice were anaesthetized with 0.06 ml of (1: 1) mixture of Diazepam (Vival™, AL, Norway) and Fentanyl citrate plus Fluanisone (Hypnorm™, Janssen Pharmaceutical Ltd, UK) subcutaneously. After exsanguination by intracardiac puncture, lungs, liver, spleen and femoral bone marrow were removed under aseptic conditions.

### *Preparing of tissues and processing of specimens*

*Histopathology*. About one third of each organ was fixed in buffered formalin and embedded in paraffin for study of histopathology. Sections approximately 5 μm thick were prepared and stained with haematoxylin and eosin (H&E). For detection of Mtb in the tissue specimens, an immunohistochemical method (33, 34) was chosen owing to the results obtained during the first parts of the experiment. Acid-fast staining gave a non-specific background staining in the tissue of these mice. It was not possible to de-stain carbol fuchsin with acid alcohol in mouse tissues. Different batches of reagents, dilutions and staining procedures were used without

achieving a reduction of the unspecified staining. The histochemical method proved more sensitive than acid-fast staining on the positive human controls. Immunohistochemical staining was performed by using anti-BCG antibodies (Ab). For retrieval of Ag, sections were treated with 0.1% trypsin at 37°C for 20 min and microwaved at 750 W for 5 min. Endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ for 5 min. In order to reduce endogenous biotin staining, lung sections were pre-treated with avidin and biotin blocking solution (Vector Laboratories, Burlingame, USA, Cat. no. SP-2001). Sections were incubated with avidin solution for 15 min, followed by a brief rinse with Tris phosphate buffered saline (TBS), and incubation with biotin-blocking solution for 15 min. Incubation was carried out with swine serum for 15 min. Sections were incubated overnight with rabbit polyclonal anti-BCG Ab (Dako A/S, Denmark, code no. B124) (dilution 1: 25 000) and by biotin-labelled swine anti-rabbit immunoglobulin serum (Dako A/S, Denmark, code no.E-353) (dilution 1: 400) for 30 min followed by 30 min with avidin–biotin–peroxidase complex (ABC) (Dako A/S, Denmark, code no.K0355). Location of Ag was visualized by $H_2O_2$ and 3-amino-9-ethylcarbazol containing buffer for 15 min. Sections were washed in TBS for 5 min between each incubation. Slides were slightly counterstained with Mayer's haematoxylin, washed in running tap water for 5 min and mounted with Immunomount (polyvinyl alcohol resin + glycerol). Human lung tissue with active TB was used as a positive control and TBS was used instead of primary Ab as a negative control.

*Bacillary counts*. One third of each organ was homogenized with PBS in sterile glass homogenizers (Bellco, Vineland, NJ, USA). Ten-fold serially diluted 50 µl tissue homogenates were plated on Middlebrook 7H10 medium. The number of Mtb CFU per organ was calculated and expressed as $\log_{10}$ per organ. The lowest detectable CFU number in this study was about 200 CFU for lungs and liver and 100 CFU for spleen. When the homogenates of organs from corticosterone-treated mice were cultured, the medium was supplemented with antibiotic and anti-fungal drugs (Carbenicillin 100 µg/ml, Trimethoprim 15 µg/ml, Amphotericin B 10 µg/ml & Polymyxin B 200U/ml). The culture plates were sealed with adhesive tape and incubated at 37°C, in a $CO_2$ (5%) incubator for at least 3 weeks followed by incubation at 37°C in air. The reading was discontinued when the count was stable for 3 weeks. Inoculated culture plates processed from mice in the latent stage should be kept for at least 4 months after inoculation. The reason for this will be discussed in the discussion.

## RESULTS

### *Route and dose related variation in the clinical outcome of Mtb infection*

Mice given the moderate dose of $1 \times 10^5$ CFU/mouse given i.v. or a high dose of $1 \times 10^8$ CFU/mouse given i.p. (Group A), developed clinical disease. Mice given low doses of Mtb either by the i.v or i.p. route and those given the moderate-doses of Mtb by the i.p. route (Group B), remained clinically healthy until challenged with corticosterone.

### *Clinical disease*

Clinical signs of disease appeared by the 7th week after infection in mice from Group A. Mice became less active, lost weight, there was excessive licking and scratching (performed abnormal groaning), they had hunched backs, shallow and rapid respiration and secretions from eyes and nose.

Bacillary counts in lungs and livers of high-dose i.p. infected mice were approximately 1.5 $\log_{10}$ and 3 $\log_{10}$, respectively, higher at the 8th week compared to the 4th week of infection. However, bacillary counts in the spleens at the 8th week was $< 0.5$ $\log_{10}$ lower than at the 4th week post-infection (Fig. 1A). A few bacilli were detected in bone marrow and blood of sick mice from this group. A similar growth pattern was found in the lungs of moderate-dose Mtb i.v. infected mice (Fig. 1B). Bacillary growth was found in the blood of sick mice from this group. The high-dose i.p. infected mice experiment was terminated at the 15th week post-infection due to disease and mortality of the mice, while moderate-dose i.v. infected mice survived until the 21st week of infection. At the 15th and 21st weeks of infection, although the clinical signs of disease were getting worse, the CFU in organs of these mice were decreasing gradually or remained stable compared to the counts at the 8th week post-infection (Fig. 1A, B).

When a moderate-dose of bacilli was given by i.v. route, pinpoint greyish-black nodules were visible in the lungs 4 weeks after infection. Microscopically however, a focal inflammatory cell infiltrate (granuloma) was detectable at 24 h post-infection in one of the infected mice. The cellular composition of the granuloma showed a mixture of polymorphonuclear leukocytes (PMN), large macrophages with pale cytoplasm and big weakly stained nuclei (histiocytes) and few lymphocytes (Fig. 2A, B). After 4 weeks of infection the number of granulomas increased. The granulomas consisted of a mixture of histiocytes and lymphocytes with very few PMN (Fig. 2D, E). At 21 weeks of infection the

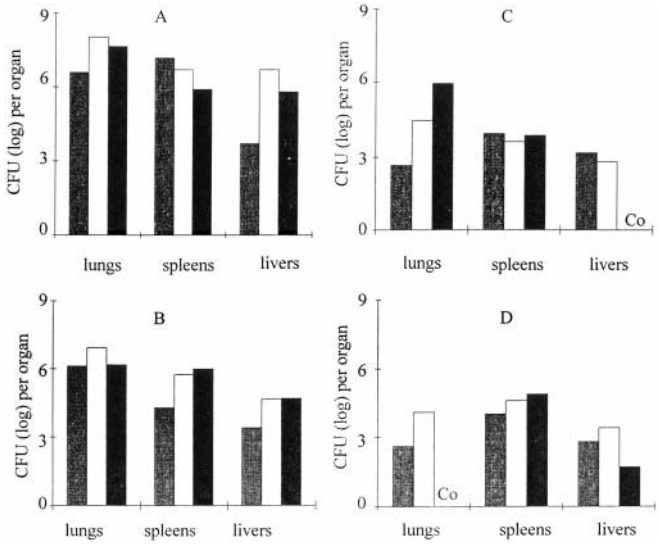


*Fig. 1.* CFU in organs of Mtb infected mice (experiment 1): (A) i.p. high dose; (B) i.v. moderate dose; (C) i.p. moderate doses; and (D) i.v. low doses infected mice. (▨) 4 weeks, (□) 8 weeks, (■) 21 weeks post-infection for (B) and 15 weeks post-infection for the others. Co = Contaminated cultures.

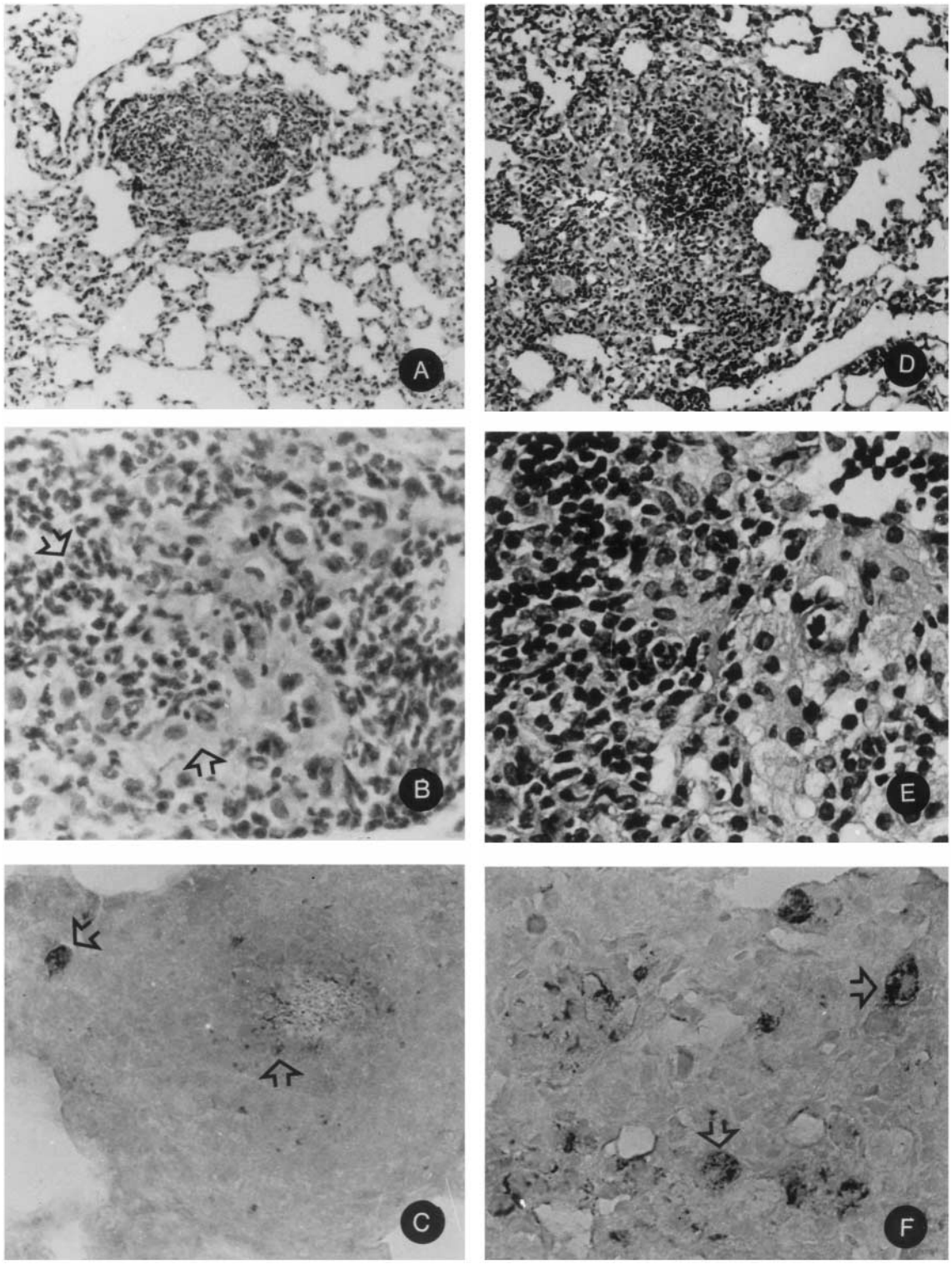


*Fig. 2.* Micrographs of left lung lobe segment of mouse infected with moderate-dose of Mtb i.v. (A–C) 24 h and (D–F) 4 weeks after infection. (A, B) Predominance of PMN and histiocytes (arrows) in the granuloma ( × 150, × 500, H&E). (D, E) Predominance of histiocytes & lymphocytes with very few PMN ( × 150, × 500, H&E). (C, F) Mtb Ag (arrows) are localized in the histiocytes. Ag are more abundant at 4 weeks than at 24 h after infection ( × 500, immunoperoxidase staining with ABC using anti-BCG polyclonal Ab).

inflammatory infiltrate obliterated almost the whole lung parenchyma. Many degenerating cells were observed. Mycobacterial Ag, found mainly intracellularly in histiocytes, were detected in all the lesions by immunoperoxidase stain-

ning with polyclonal anti-BCG antiserum. The amount of the Ag increased with the progression of the disease. The staining pattern was mainly distinctively granular, while some cells exhibited a more diffuse staining. This pattern of Ag staining remained rather uniform throughout the course of infection (Fig. 2C, F).

Mice who received a high-dose of Mtb i.p. developed gross changes as described above at the 7th week of infection. Microscopically their lungs showed granulomatous changes from the 4th week after infection (similar to that seen in the i.v. moderate-dose group at the 4th week). The surrounding lung parenchyma was hypercellular (Fig. 3A, B). After 8 weeks of infection when the mice were sick, the inflammatory foci became larger and more numerous than those in the earlier week of infection. The cellular composition of these foci was similar to those in earlier week of infection. Mycobacterial Ag were found in all the lesions and the staining pattern and distribution was the same as in the i.v. infected mice. The amount of Ag was however less in this group compared to the moderate-dose i.v. group (Fig. 3C, 2F). There were no obvious histopathological changes in livers and spleens of mice from this group.

*Sub-clinical infection*

Mice in group B did not show any signs of disease throughout the course of infection. In the lungs of moderate-dose i.p. infected mice, the number of CFU gradually increased until the 21st week of infection. After that the counts decreased gradually. Between the 21st and 52nd week of infection, the counts appeared stable in the lungs (Fig. 4A). Higher counts were demonstrated in livers and spleens compared to lungs during the first 4 week of infection. In livers, the CFU declined from the 1st week post-infection, and bacilli were not detected between the 13th and 36th weeks post-infection (except for 1 mouse at the 52nd week post-infection) (Fig. 4C). In spleens, the CFU were stable between the 21st and 52nd weeks of infection (Fig. 4B).

There was no detectable bacillary growth in the lungs of low-dose i.p. infected mice. Although bacilli were detected regularly in the spleens, low numbers of bacilli were occasionally cultured from the livers of these mice.

The growth pattern of bacilli in the organs of mice inoculated with low-dose i.v. followed that of moderate-dose i.v. infected mice (Fig. 1D, B), but the number of CFU in their organs was lower than those of moderate-dose infected mice. They did not show any sings of disease at the termination of the experiment (15th week post-infection).

Mice in group B did not show any gross changes in their lungs, livers and spleens. Microscopically there were no pathological changes either. Immunohistochemical staining revealed no mycobacterial Ag.

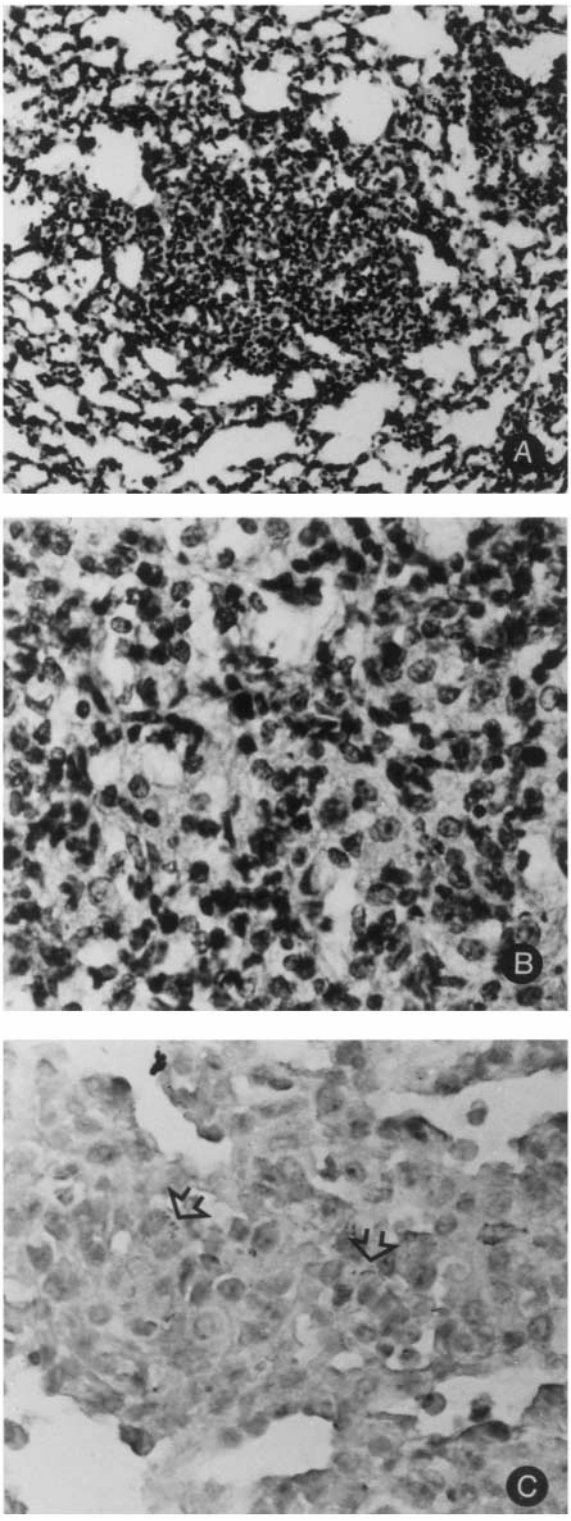


*Fig. 3.* Micrographs of left lung lobe segment of mouse, 4 weeks after infection with high-dose i.p. (A, B) Granuloma shows predominance of histiocytes and lymphocytes ( × 150, × 500, H&E). (C) Mtb Ag (arrows) are localized in the histiocytes ( × 500, immunoperoxidase staining with ABC using anti-BCG polyclonal Ab). Few Mtb Ag-positive cells are found (arrows). Note the higher number in i.v. route at 4 weeks (Fig. 2F).

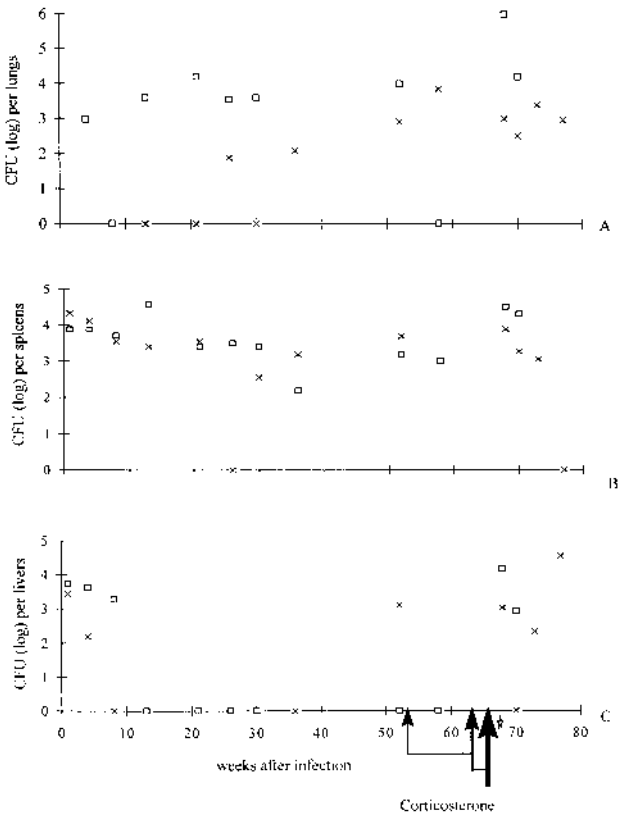


*Fig. 4.* CFU in organs of moderate doses Mtb infected mice (from experiment 2): (A) lung, (B) spleen, and (C) liver. ( × ) $4 \times 10^4$, and (□) $4 \times 10^5$ Mtb CFU given i.p.; (✱) mice started to be sick.

*Clinical disease during corticosterone challenge*

After 53 weeks of infection, half of the remaining group B mice (low- and moderate-dose groups infected i.p.) were given oral corticosterone. 15 weeks after the start of corticosterone challenge (2 weeks after the start of full-dose corticosterone (68th week post-infection), the mice developed signs of disease. The clinical signs, bacillary growth pattern and histopathological changes in the organs of these mice were similar to those found in group A mice.

The CFU from lungs of mice infected with a moderate-dose i.p. remained stable until the mice started to show signs of disease. By 68 weeks post-infection, the counts had increased by approximately 1–2 $\log_{10}$, compared to during the stable period (Fig. 4A). The CFU counts in the livers of moderate-dose infected mice increased from non-detectable levels during the stable period. At 11 weeks after full-dose corticosterone challenge (77 weeks post-infection) the counts were approximately 1.5 $\log_{10}$ higher than 1 week after inoculation (Fig. 4C). The CFU in the spleens at 2 weeks after full-dose corticosterone challenge had increased to a level approximately 1 $\log_{10}$ higher than during the stable phase (Fig. 4B). The bacillary growth in mice infected with low-dose i.p. followed the same pattern.

After 2 weeks with full dose of corticosterone, lungs of mice with the highest initial dose of infection showed macroscopically greyish-black pinpoint nodules. Microscopically the inflammatory foci were similar to those that developed during clinical disease as described above (Fig. 5A, B). Mycobacterial Ag were found in all the lesions and located mainly in the histiocytes. The staining pattern was mainly granular, but sometimes diffuse as well (Fig. 5C). The mice developed disease after corticosterone challenge and showed the above mentioned pathology.

*Long term observation on corticosterone non-challenged mice*

56 mice in the low- and moderate-doses i.p. group who were not given corticosterone treatment stayed healthy until $> 100$ weeks after infection.

Around week 107, mice in the moderate-dose group started to show signs of sickness. Macroscopically nodular lesions were seen in the lungs of 7 mice. In 5 of the sick mice, lobes of the livers were fused. Tumours in the ovaries were seen in some mice. In the lung of one mouse there was a metastatic tumour nodule. Among mice not infected with Mtb signs of sickness were also observed at this age. Retroperitoneal tumours were seen in one of the uninfected control mice and in a BCG infected mouse of the same age. However the rest of uninfected control mice were still healthy.

Six sick mice were studied for CFU numbers. At week 107 post-infection, 2 sick mice had approximately 2 $\log_{10}$, 1 $\log_{10}$ and 1 $\log_{10}$ higher Mtb CFU in their lungs, livers and spleens, respectively (Table II) than the CFU numbers in those organs during the latent phase (Fig. 4). Inflammatory foci were seen in the lungs. Mycobacterial Ag in these foci was much less than in mice with same degree of inflammation and clinical signs in the group A, and barely visible. At 115th week post-infection, the CFU numbers in the lungs, livers and spleens of the rest of the sick mice were still similar to the CFU numbers in those organs during the stable latency period. Foci of inflammatory cells were seen in the lungs, but there were no mycobacterial Ag in these lesions.

## DISCUSSION

We have established a mouse model for latent TB. Our operational definition of TB latency in mice is: stable CFU counts in lungs and spleens without clinical signs of disease or obvious pathological changes in lungs over a long period of time and without spontaneous reactivation of disease. Persistence of bacilli was proven by reactivation using oral corticosterone, and by in vitro growth of Mtb from lungs and spleens.

Studies of murine host responses against Mtb have so far been focused on acute clinical disease with obvious TB lesions created with high-dose Mtb inoculation. In our model, there was a stable number of cultivable bacilli present in lungs and spleens of moderate-dose i.p. infected mice 26 weeks post-infection and onwards. The Mtb

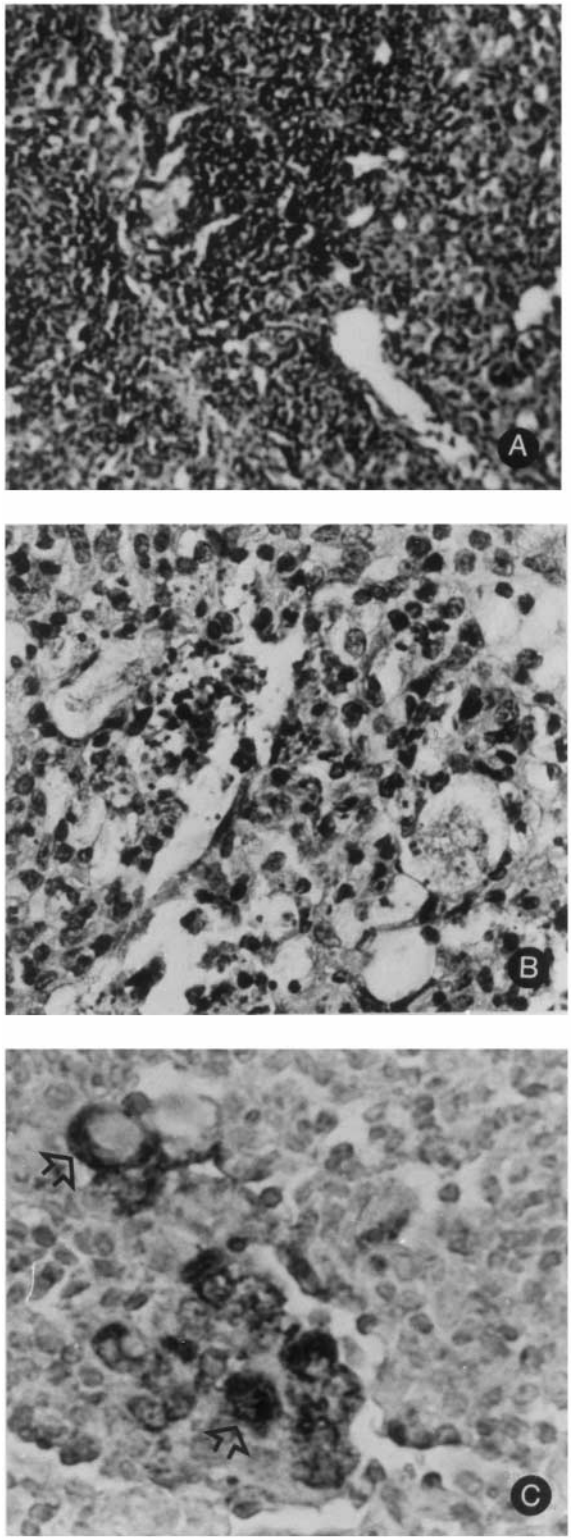


*Fig. 5.* Micrographs of left lung lobe segment of mouse infected with moderate-dose of Mtb i.p., challenged with corticosterone and developed reactivation of latent infection. (A, B) The inflammatory foci are numerous with predominance of histiocytes, lymphocytes, degenerating cells and few PMN ( × 150, × 500, H&E). (C) Abundant Mtb Ag (arrow) are localized in the histiocytes ( × 500, immunoperoxidase staining with ABC using anti-BCG polyclonal Ab).

present in the organs of these mice did not seem to provoke histopathological lesions of TB. The finding of cultivable bacilli in healthy infected mice without lesions is in agreement with earlier findings in subclinical Mtb infection in man (35–37) as well as in mouse (25–27, 38). Opie and Aronson (35) and Griffith (37) studied the recovery of live Mtb from the lungs in autopsies of people who had died from causes other than TB. One interesting finding in Opie et al.'s experiment was that live Mtb was recovered from 31% and 36%, respectively of grossly normal basal and apical parts of lungs with old TB lesions by using a guinea pig inoculation method. These normal looking tissues revealed only a little fibrosis on histopathological examination (35). These authors (35, 37) have frequently been cited in the literature for the finding of sterilization of the necrotic lesions from the primary infection, but their finding that live Mtb is present in normal lung tissue from the same cases does not seem to have been mentioned since 1938 (36).

A chronic murine TB model was first described by Hart and Rees (25–27). Presumably due to the very high dose of inoculum, 80% of their mice died from progressive Mtb infection before they could be examined (26). In our model corticosterone unchallenged mice with moderate-dose i.p. survived more than 100 weeks after inoculation without increasing bacillary numbers in their organs or showing clinical signs of disease. We could detect the bacilli in their organs during latency. This is a prerequisite to explore the host–parasite interaction in situ. Mice in the latent TB model established by Brown et al. received low doses ($10^1$–$10^3$) of Mtb i.v. The bacillary numbers during the latent phase in their model were too low compared to our model (30). McCune et al. provided a latent TB mouse model, the so-called "Cornell Model" (28, 29). Although in lungs and spleens, the bacilli fell below detectable levels after anti-TB treatment, revival of bacilli in these organs occurred in about one third of cortisone unchallenged anti-TB treated mice. This was probably because of relapse due to a too short anti-TB treatment, leaving some few actively growing bacilli behind. The difference of our model from the "Cornell Model" is that our mice were not treated with anti-TB drugs, and a stable state host–parasite interaction was established.

The stable CFU counts of bacilli in the latent period in vivo might be due to a fine balance between host killing and bacillary multiplication. The increasing bacillary counts after corticosterone challenge (Fig. 4) could thus simply be an effect of any reduction in the host's capacity to kill Mtb. This hypothesis has been challenged by Hart and Rees (27). Their results have suggested that when stable CFU counts were observed in the tissues, Mtb had rather switched to a vegetative, non-multiplying stage and survived in a "dormant" state. This was also suggested by Wayne (39). He noticed that growth of Mtb may adapt to anaerobic or microaerophilic conditions by transforming into a non-multiplying stage. Further experiments have

Table II. *CFU numbers in organs of corticosterone challenged and unchallenged $4 \times 10^5$ CFU i.p. infected mice*

| Organs | Weeks after infection | Unchallenged ($Log_{10}$ CFU) | Challenged ($Log_{10}$ CFU) |
|---|---|---|---|
| Lungs | 58 | 4 | 0* |
| | 70 | 0* | 4.2 |
| | 107 | 5.7 | ND† |
| | 115 | 4.8 | ND† |
| Livers | 58 | 2.8 | 0* |
| | 70 | ND† | 2.9 |
| | 107 | 3.8 | ND† |
| | 115 | 2.3 | ND† |
| Spleens | 58 | 2.8 | 3 |
| | 70 | 2.6 | 4.3 |
| | 107 | 3.6 | ND† |
| | 115 | 2.8 | ND† |

* Only one third of lung and liver tissues from 1 mouse in each time point were used for culturing.
† Experiments for challenged mice were terminated at 77 weeks after infection due to mortality of the mice.

indicated that this dormant stage could revert to a multiplying stage when the cultures were re-aerated (40–43). In our model, dormant bacilli seemed to lodge largely in normal lung tissue, implying that low oxygen tension might not be the only way to initiate this transformation. A recent study has given support for a common mechanism for transforming to a stationary phase of bacteria (44). The authors found sequence homology between the sigma factor gene (*sigF*) of Mtb and sporulation sigma factors (SigF) from Streptomyces coelicolor and Bacillus subtilis and a stress–response sigma factor (SigB) from B. subtilis. *sigF* mRNA of Mtb was identified during stationary phase of bacillary growth and when bacilli were exposed to various stresses. mRNA of *sigF* was not detected during exponential-phase of bacillary growth.

We noticed no regular correlation between high Mtb numbers in the organs and severity of clinical signs of disease in sick mice (Fig. 4). However the degree of histopathological changes of TB lesions seems to correlate to severity of clinical signs of disease in our studies. Earlier investigators (45–49) have also noticed these findings. We also noticed that a few CFU in cultures from mice with latent TB started to grow $> 2$ months after inoculation. Bacillary growth from specimens collected from closed tuberculous cavities of humans has been detected at periods varying from 3 to 10 months after inoculating onto the media (50). This might indicate a prolonged lag phase for bacilli recovered from a dormant stage.

The choice of mouse, route and dose of Mtb infection has been shown to be important in establishment of latent TB infection in mice. We employed an F1 hybrid to reduce the likelihood of seeing immune defects of a recessive genetic origin compared to inbred strains. In addition, F1 hybrids are genetically homogenous within the group, are more resistant to infections and have a longer life-span (51). We chose to use the F1 hybrid of one strain (C57BL/6J) carrying the *Bcg*-susceptible gene ($Bcg^s$) and the other (DBA2) with *Bcg*-resistant gene ($Bcg^r$) (52). Airborne infection is undoubtedly the most relevant route of infection for mimicking human TB in an animal model. Orme established a latent TB model in mice with 5–10 CFU given by aerosol route (31). It is, however, difficult to obtain complete control over the desired dosage in mice when giving TB bacilli through the aerosol route (45). It poses a hazard to the researchers, and it is necessary to have sophisticated and expensive facilities (53). The results reported here showed that route of infection can modify the outcome of infection in mice. Mice infected with moderate-dose i.v. developed clinical disease and started dying by week 20 after infection, whereas mice infected with same dose i.p. stayed healthy in spite of high numbers of bacilli in the lungs and spleens (Fig. 4). Costello et al. also noticed that Mycobacterium bovis (BCG) growth patterns in organs of mice could vary with the route of inoculation (54). Different routes of inoculation could lead to involvement of different Ag presenting cells (APC) (55, 56). Very low or high doses of bacilli in the inoculum can affect the development of protective immunity (45) and thus mortality (38, 57). In our experiment no. 1, mice infected with high-dose i.p. developed clinical disease and started to die in early phase of infection. On the other hand, mice infected with low-dose i.v. or i.p. neither suffered from clinical disease nor did they have identifiable histopathological changes in the organs, even though lungs and spleens revealed about 1,000–10,000 CFU during early phase of infection. We therefore assume that moderate-dose by the i.p. route is suitable for the studying of latent infection in mice because there are higher numbers of bacilli in the organs throughout the course of infection compared to those infected with low-dose i.p.

We conclude that it is possible to establish a stable asymptomatic infection with moderate-doses ($4 \times 10^4$–$4 \times 10^5$) of virulent Mtb bacilli given i.p. to F1 hybrid mice. The biological characters of latent Mtb bacilli from our

model and in vitro Wayne's model will be explored in the future. Whether this model will prove to be relevant for latency of TB infection in humans is presently under investigation.

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