

The Intravenous Model of Murine Tuberculosis is Less Pathogenic Than the Aerogenic Model Owing to a More Rapid Induction of Systemic Immunity

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The detection of mRNA in the murine model of tuberculosis for key cytokines involved in protective immunity in the lung tissues revealed a much faster emergence of the interferon (IFN)- γ response in the intravenous route than in the aerosol route of inoculation. This slower response in the lungs was associated with a stronger inflammatory response, resulting in large granulomatous structures and eventual tissue damage.

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INTRODUCTION

The use of experimental models in tuberculosis is the first step in testing new vaccines, drugs or other therapies to deal with this disease. Two such murine models consisting of intravenous (i.v.) or aerosol inoculation of mice have been recently compared by North [1], who concluded that aerosol infection was associated with faster development of lung pathology and earlier death than the i.v. inoculation, owing to the rapid generation of acquired immunity in the latter. The results of the present study confirm and extend these earlier findings by demonstrating the emergence in the infected lungs of mRNA for key cytokines involved in the expression of protective immunity.

MATERIALS AND METHODS

Mice. Specific pathogen-free female C57Bl/6 mice, 6–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were provided with sterilized food and water.

Mycobacterium tuberculosis. In order to reproduce as close as possible a human tuberculosis, a strain of *Mycobacterium tuberculosis* from a clinical specimen was used rather than an attenuated reference strain. The strain 511 was isolated in an immunocompetent patient

diagnosed with tuberculosis in Barcelona. It was grown in Proskauer Beck medium containing 0.01% Tween 80 to the mid-log phase. Mice were infected i.v. via a lateral tail vein with an inoculum of 10^5 CFU of *M. tuberculosis* suspended in 0.2 ml phosphate-buffered saline (PBS). For aerosol infection, mice were placed in the exposure chamber of an airborne infection apparatus (Glas-col Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with 10 ml of a suspension of *M. tuberculosis* at a concentration previously calculated to provide an uptake of 100 viable bacilli within the lungs over a 30-min exposure [2]. Four mice were used for each time-point in each experimental group. The numbers of viable bacteria in the homogenates of lung and spleen, and in the broncho-alveolar lavage (BAL), obtained by injecting into the trachea 1 ml PBS and extracted immediately after euthanasia in a CO₂ chamber, was followed against time by plating serial dilutions on nutrient Middlebrook 7H11 agar (Gibco BRL, Gaithersburg, MD, USA) and counting bacterial colony formation after 21 days of incubation at 37 °C in humidified air. The right superior lobe from the lungs of each of the four mice per experimental group was perfused with fresh 10% formaldehyde in PBS. Tissues were embedded in paraffin blocks and sections were made across the widest area of each lobe. Sections were stained with haematoxylin–eosin and using the Ziehl–Neelsen technique.

Analysis of levels of mRNA in infected lungs. For detection of cytokine gene expression in the lungs of infected mice, one lobe was separately homogenized in 1 ml of RNazol (Cinna/Biotecx, Friendswood, TX,

USA) using a tissue polytron and frozen at -70°C . Relative amounts of mRNA for tumour necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-4 and IL-5 were determined by a quantitative reverse transcriptase PCR protocol [3]. Primers, probes and cycles for hypoxanthine phosphoribosyltransferase, TNF- α and IFN- γ were as published [4]. Sense and antisense primer oligonucleotides were for IL-4 primers (s) 5'-GAATGTACCAGGAGCCATATC-3' and (as) 5'-CTCAGTACTACGAGTAATCCA-3' and the probe was 5'-AGGGCTTCCAAGGTGCTGGCA-3'; IL-5 primers were (s) 5'-GACAAGCAATGAGACACGATGAGG-3' and (as) 5'-GAACTCTTGACGGTAATCCAGG-3' and the probe was 5'-GGGGTACTGTGGAAATGCTAT-3'. Thirty-three cycles were used to amplify IL-4 and 32 were used for IL-5. Briefly, total RNA was extracted from lung tissue using cold RNAzol. One microgram of RNA was then reverse transcribed, using a murine Moloney leukaemia virus-derived reverse transcriptase (Gibco BRL, Grand Island, NY, USA), and amplified with *Taq* polymerase (*Thermus aquaticus* polymerase from Promega, Madison, WI, USA). RNA was analysed for hypoxanthine-guanine phosphoribosyltransferase mRNA (a housekeeping gene) as a measure of total readable mRNA in each sample. After amplification, the products were electrophoresed, transferred to Hybond N+ (Amersham, Arlington Heights, IL, USA), and hybridized with the appropriate oligo probe labelled by an enhanced chemiluminescence system (ECL; Amersham). Light output was measured by determination of the pixel value detected on Hyperfilm-ECL (Amersham) analysed on a Microtek Scanmaker Iix.

RESULTS

Course of infections

The course of tuberculosis infection, followed against time after delivery by the two separate routes, is shown in Fig. 2A,C. In both models, the numbers of organisms deposited in the lung were equivalent as described previously [2]. In animals infected intravenously with a normally sublethal dose (10^5 bacilli) of the strain 511 of *M. tuberculosis*, evidence of control and containment of the infection was clearly evident in spleen and BAL (and with less intensity in lung) by day 10–20.

In mice infected aerogenically with a low dose (100) of bacteria using an aerosol generator, containment of the infection was not evident in all tissues and BAL until day 20. In the lung there was a significant decrease between day 20 and 30, while in the spleen this was not seen until day 30–40. These events were consistent with an earlier report [5] despite the higher virulence of strain 511, compared with the reference Erdman strain (Fig. 1).

Granulomas of the lung become larger in the aerosol infection

The presence of pregranulomas could be detected by day 10 in both infections. No statistical difference between the means of the number of granulomas following i.v. or aerosol infection (Fig. 2B) was seen. The measure of all the granulomas identified in each lobe of each mouse (Fig. 2D) proved that granulomas following aerogenic infection tended to be significantly larger after day 30 compared with those formed after i.v. infection.

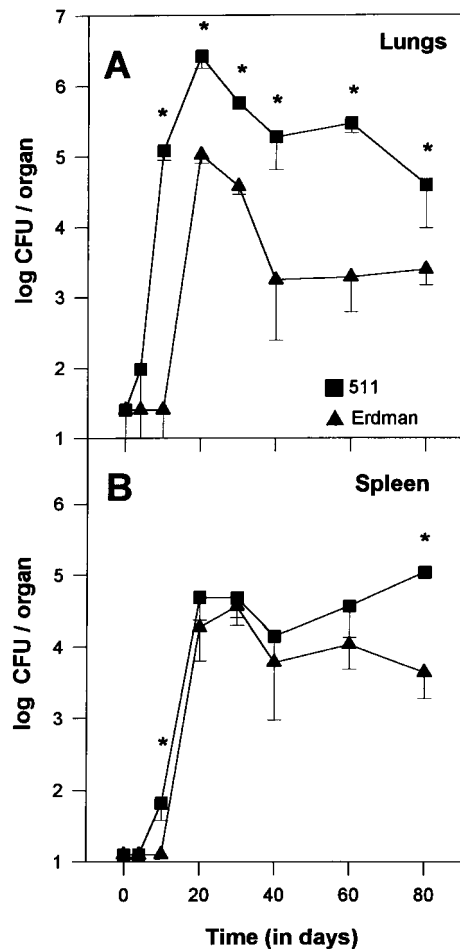


Fig. 1. Growth of *M. tuberculosis* strain 511 (■) and Erdman strain (▲) in lungs (A) and spleen (B) of mice after infection with 100 CFU via aerosol. The data presented are average values and S.D. of four animals at each time point. The difference between the means of both infections was determined using the Student's *t*-test, and were significant when marked by * ($P < 0.05$).

In terms of the cellular composition of the granulomas, the proportion of lymphocytes was always higher in the aerosol model than following i.v. infection. By day 30 foamy macrophages were apparent in both models. A few of these cells contained acid-alcohol-resistant bacilli as revealed by Ziehl-Neelsen staining.

Aerogenic and intravenous infections differ in expression of cytokine mRNA in the lung

The expression of TNF- α and IFN- γ in lungs is shown in Fig. 3A,B. Interestingly a high IFN- γ expression was detected first in the i.v. infection by day 10, which continued to fluctuate with time. Following aerosol infection, peak expression of mRNA message was not seen until day 20, remaining constant after that.

TNF- α production in the aerosol infection was detected from the first day, peaking on day 4. In mice given the i.v. infection

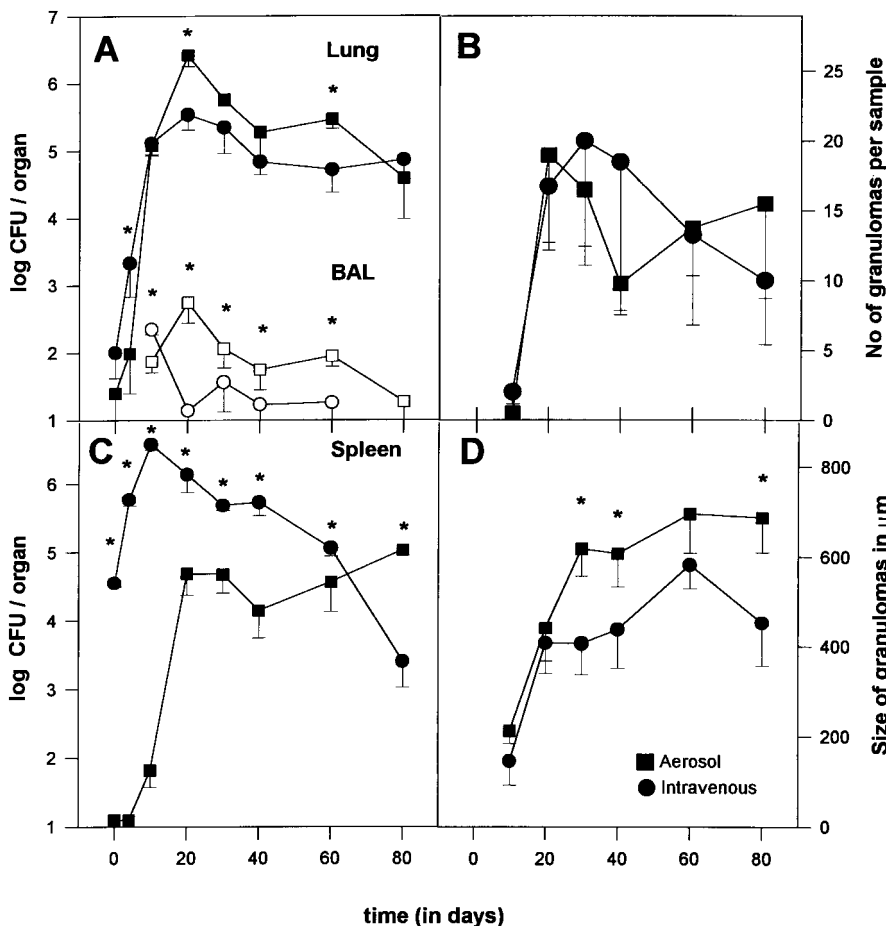


Fig. 2. Growth of *M. tuberculosis* strain 511 in lungs and BAL (open signals) (A) and spleen (C) of mice after infection with 100 CFU via aerosol (■), and 10^5 CFU i.v. (●). Histopathology of the infected lungs. Using $100\times$ magnification, all granulomas of each section were counted (B) and the maximum diameter (D) measured using a micrometer ocular (Olympus WHL $10\times/20$) in the aerosol (■) and i.v. (●) infections. The data presented in A, B and C are average values and S.D. of four animals at each time point. The data in D represents the average of the totality of the granulomas measured in each group and each time with the SE. Difference between means of both infections was determined using the Student's *t*-test, and were significant when marked by * ($P < 0.05$).

much more variation in the response was seen, with two peaks on day 10 and 40.

The detection of cytokines associated with the T helper cell subtype 2 (Th2) production [5] is shown in Fig. 3C,D. IL-4 was first detected by day 40 in the i.v. infection and by day 60 following aerogenic infection. IL-5 was detected in both groups by day 40.

DISCUSSION

The results of this study are consistent with the hypothesis that two separate waves of T-cell-mediated acquired immunity emerge in mice infected with a virulent strain of *M. tuberculosis*. The first wave is evident by day 10 or 20 (i.v. or aerosol model), and is characterized by the appearance of CD4 IFN- γ -secreting T cells, as demonstrated previously [6]. Studies using gene knockout mice have shown that secretion of IFN- γ is essential for the successful retention of the granuloma integrity and for the expression of bactericidal mechanisms [7]. After this primary response has contained the infection, dead bacteria can be processed by macrophages, leading to the emergence of a second wave of Th2-type, IL-4-secreting CD4 cells [5]. Cytokines released at this time support B-cell differentiation and it is quite possible that these may antagonize the ongoing Th1

response resulting in chronic disease [6]; this however, remains to be proven.

Despite the fact that an equivalent amount of bacilli is reached in lungs in both ways of inoculation, in the i.v. model sufficient antigen is presented in the spleen, 10^4 CFU (Fig. 2C), as described previously [2], to drive the rapid emergence of acquired immunity and evidence of control of the infection by day 10–20 (Fig. 2A,C). This is reflected by the differences in lung IFN- γ production in the two models (Fig. 3B). Signal for IFN- γ was detected on day 10 after i.v. infection, interestingly between day 20 and 30 the production of IFN- γ decreased and production of TNF- α was not seen. At day 40, however, the production of IFN- γ increased as evidence of further bacterial clearance was observed.

In contrast, in the aerosol model a finite time is needed for the bacterial load to become sufficient to trigger this immunity. For this reason, the peak of IFN- γ is not seen until day 20. It is interesting to note that a low initial peak of IFN- γ is seen at day 4; since this is too early for the generation of specific immunity, this may therefore have resulted from the direct stimulation of local natural killer (NK) cells [5, 8]. In this model, both IFN- γ and TNF- α message is seen throughout the course of infection, in contrast to the i.v. model.

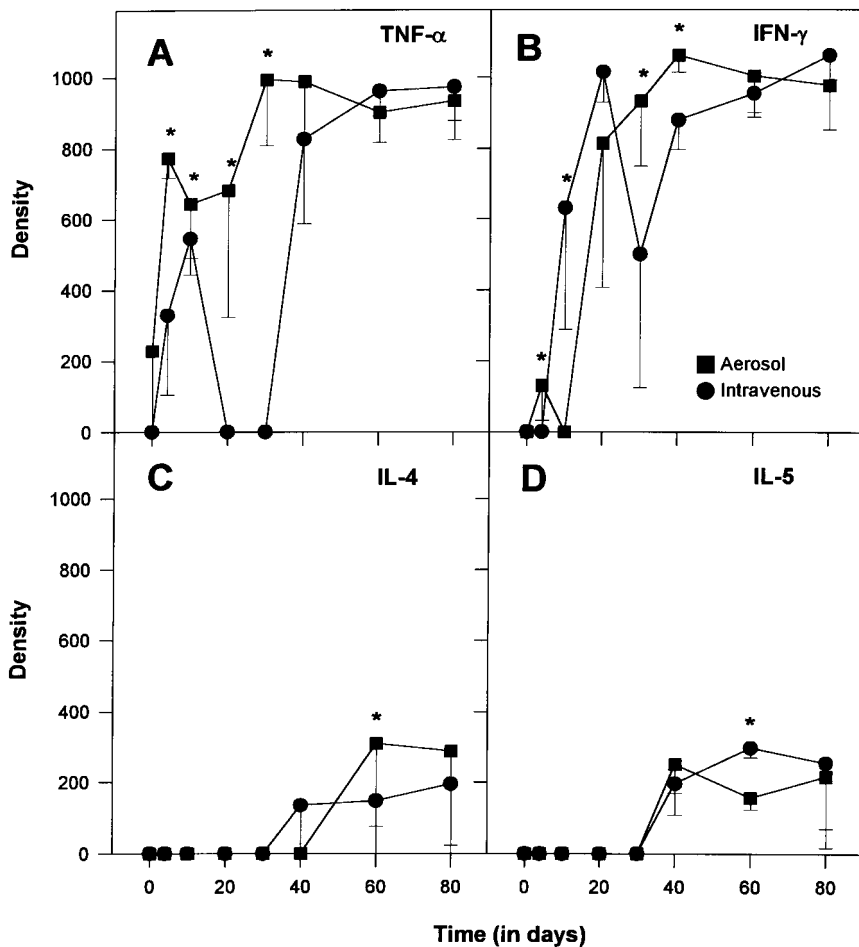


Fig. 3. Lung tissue from aerosol (■) and i.v. (●) infected mice was analysed for mRNA specific for TNF- α (A), IFN- γ (B), IL-4 (C) and IL-5 (D). Amplification was performed with cytokine-specific primers, and the product was blotted onto a nylon membrane and probed with sequence-specific fluorescein-labelled probe. The probe was quantitated by horseradish peroxidase-conjugated antibody. The amount of antibody bound is directly related to the signal induced in a chemiluminescent substrate, which was detected on film. Light output was measured by determination of the pixel value (density) detected on the film analysed on a Microtek Scanmaker IIXe. The data presented are the average values from the analysis of four animals at each time point. Difference between means of both infections was determined using the Student's *t*-test and were significant when marked by * ($P < 0.05$).

Histological examination reflected a clear difference between both models in terms of the size of the granulomas (Fig. 2D). By day 30, granulomas in the i.v. model appeared significantly smaller compared with those seen in the aerosol model in which large volumes of the lung structure can eventually be involved. Again, these smaller granulomas developed when IFN- γ signal decreased and TNF- α was not detected, consistent with the evidence that production of these molecules has a profound influence on granuloma development [7–9].

In summary, the faster emergence of the IFN- γ response in the i.v. model results in the rapid control of the infection and clear evidence of significant levels of bacterial elimination, compared with the aerosol model. In this latter model the cytokine response is more sustained, as is the inflammatory process. Under these conditions the bacteria are eventually contained, but little or no bacterial elimination is seen. The much larger granulomas seen after aerosol exposure may, in the long-term, handicap respiratory functions, as well as contributing to local tissue damage.

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