Immunotherapy of airborne tuberculosis in mice via the lung-specific delivery of cytokines

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M DENIS, E GHADIRIAN. Immunotherapy of airborne tuberculosis in mice via the lung-specific delivery of cytokines. Can J Infect Dis 1993;4(1):38-42. The immunotherapeutic potential of interleukin-2 (IL-2), tumour necrosis factor alpha (TNFα) and interferon gamma (IFN-γ) administered by aerosol was examined on mice infected with *Mycobacterium tuberculosis* by the aerogenic route. Infection of BALB/c mice with 10^4 colony forming units (cfu) of *M tuberculosis* led to death of all mice at day 35 post infection after progressive microbial growth in the lungs. Aerosolization of IL-2 (100 μg per mouse) did not promote an increase in resistance to tuberculosis, as seen by growth of *M tuberculosis* in the lungs. Administration of IFN-γ or TNFα (100 μg) by the aerosol route led to a significant reduction in microbial growth in the lungs and a 100% survival of infected mice at day 60. Similarly, aerosolization of TNFα and IFN-γ combined led to a very high degree of tuberculostatic activity in the lungs of infected animals, but not superior to that seen with either cytokine alone. Administration of similar amounts of cytokines by repeated intraperitoneal infusions led to a very marginal improvement in mouse resistance. These results suggest that localized cytokine administration may be beneficial in the treatment of lung diseases.

Key Words: *Cytokines*, Mycobacterium tuberculosis

Immunothérapie de la tuberculose aérogène chez la souris et administration locale de cytokines

RÉSUMÉ: Le pouvoir immunothérapeutique de l'interleukine-2 (IL-2), du facteur de nécrose des tumeurs alpha (TNFα) et de l'interféron gamma (IFN-γ) administrés par aérosol a été étudié chez la souris infectée par *Mycobacterium tuberculosis* par voie aérogène. L'infection des souris BALB/c au moyen de 10^4 unités formant colonie de *M tuberculosis* a entraîné le décès de toutes les souris 35 jours plus tard après une prolifération microbienne progressive dans les poumons. L'administration d'IL-2 (100 mg par souris) par aérosol n'a pas amélioré la résistance anti-tuberculeuse, comme le démontre la prolifération de *M tuberculosis* dans les poumons. L'administration d'IFN-γ ou de TNFα (100 μg) par aérosol a provoqué une réduction significative de la prolifération microbienne dans les poumons et une survie de 100 % des souris infectées au jour 60. De façon similaire, l'administration par aérosol de TNFα et d'IFN-γ combinés a entraîné une activité antituberculeuse intense dans les poumons des sujets infectés, mais pas plus que l'une ou l'autre cytokine employée seule. Des perfusions intrapéritonéales répétées de doses similaires de cytokines n'ont produit qu'une amélioration très marginale de la résistance chez les souris. Les résultats suggèrent que l'administration locale de cytokines pourrait être bénéfique dans le traitement des affections pulmonaires.

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INFECTIONS WITH MYCOBACTERIA STILL POSE A FORMIDABLE health problem both in developed and developing countries (1). The recent acquired immune deficiency syndrome (AIDS) pandemic has exacerbated the problem, as immunosuppressed individuals become highly susceptible to infection with mycobacterial pathogens (2). Although chemotherapy with conventional antituberculous drugs leads to a beneficial effect in most cases (3), drug-resistant strains may present serious problems for clinical treatments. Recent attention has been focused on the use of biological response modifiers (BRMs) for the treatment of infectious and malignant diseases (4). Immunotherapy of mycobacterial diseases thus is still an attractive goal. Infusion of BRMs often is considered in the treatment of infected individuals. However, it is apparent that systemic infusion of cytokines in vivo may lead to considerable toxicity (5). Moreover, distribution of cytokines administered systematically may be problematic to the activation of processes in infectious foci in organs such as the lungs. In that regard, immunotherapeutic measures aimed at treating mycobacterial diseases in the lungs are highly desirable, as mycobacteria thrive in the aerobic environment of the lungs (6). A strategy is described which allows the delivery of BRMs specifically to the lungs of tuberculous mice to modulate the infection positively.

MATERIALS AND METHODS

Pathogen-free BALB/c mice weighing between 18 and 25 g were bred in the authors' facilities. Mice were housed in plastic cages and were fed sterile Purina Chow and acidified water ad libitum. *Mycobacterium tuberculosis* H37Rv was grown in 7H9 broth (Difco Laboratories, Michigan) (7). Dispersed cultures were obtained by the addition of 0.05% tween 80. Ampoules containing 1 mL of suspension were stored at -70°C. To produce lung infections, mice were exposed to aerosols of viable mycobacteria using a middlebrook airborne apparatus (Tri-R Instruments, New York). The nebulizer was filled with 10 mL of *M tuberculosis* H37Rv in phosphate buffered saline at $5x10^7$ colony forming units (cfu)/mL which leads to about 10^4 cfu deposited in the lungs of mice in a 30 min exposure. Mice were then exposed to cytokines by the aerosol route immediately after infection.

For this, mice were placed in a nose-only aerosol chamber (Intox Products, New Mexico). Cytokines were dissolved in 20 mL of buffer and aerosolized for 20 mins. Aerosols were generated by an Acorn 2 nebulizer (Marquest Products, Colorado) driven by compressed air at an airflow rate of 15 L/min; the nebulizer delivers a mean aerosol particle diameter of approximately 1.0 mm under these conditions (8).

Groups of mice were exposed to the following aerosolized agents: group 1, buffer control; group 2,100 μg of recombinant mouse interleukin-2 (IL-2) (Cetus, California); group 3, 100 μg of recombinant mouse tumour necrosis factor alpha (TNFα) (Cetus); group 4, 100 μg of recombinant interferon gamma (IFN-γ) (Genentech, California); group 5, 100 μg of TNFα followed by 100 μg of IFN-γ; and group 6, heat-inactivated cytokines (TNFα and IFN-γ). (One hundred micrograms refers to the total amount placed in the nebulizer). Solutions were aerosolized to dryness. Other groups of mice were given repeated intraperitoneal injections of cytokines (5 μg per mouse daily) for 15 days immediately following infection. At predetermined intervals, numbers of viable bacteria in the lungs were determined by plating 10-fold serial dilutions of individual organ homogenate in saline on Middlebrook 7H 10 agar (Difco, Michigan) and counting cfu after incubation for 21 days at 37°C. For each time point, four to five animals were sacrificed. Data are expressed as the log mean number of viable organisms in the lungs. Colony counts were transformed to $\log_{10}$ and subjected to a two-way analysis of variance.

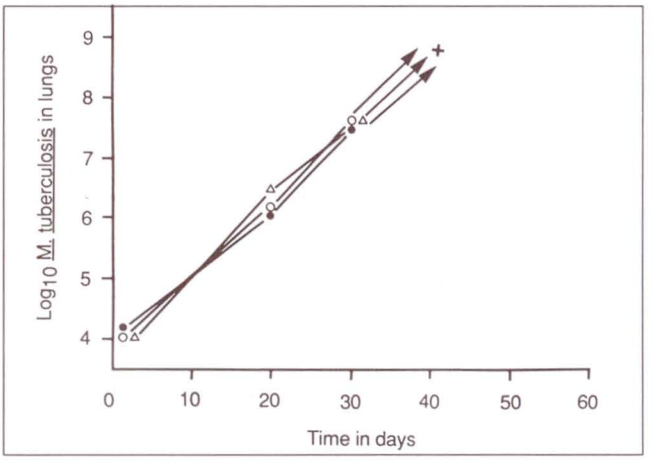

Figure 1) *Growth of* Mycobacterium tuberculosis *in the lungs of infected mice. Mice were infected with* 10^4 M tuberculosis *by aerosol and were exposed to the following aerosolized agents: boiled interleukin-2 (IL-2) only (o), 100 μg of IL-2 at day 0 (•) or 100 μg of IL-2 three times at two-day intervals starting 10 days after infection (Δ). Mibcrobial growth in the lungs was assessed by plating organ homogenates on agar. Standard errors of the means are omitted for clarity (they did not exceed 10% of the means). Results from four combined experiments are shown, with five mice for each point for each experiment. X denotes death of mice. No significant difference between any of the experimental goups (ANOVA test)*

RESULTS AND DISCUSSION

The aerosol inoculum grew progressively in the lungs of untreated mice with no sign of a decrease in the growth until day 25 when mice started dying (Figure 1). All mice were dead at day 35 after airborne infection. This growth pattern is similar to that described by Orme and Collins (9). IL-2 has been found to enhance resistance to infections with virulent mycobacteria, namely *M avium* (10), *M lepraemurium* (11) and *M bovis* (11). Moreover, intradermal application of IL-2 in patients with leprosy led to an elimination of the bacilli

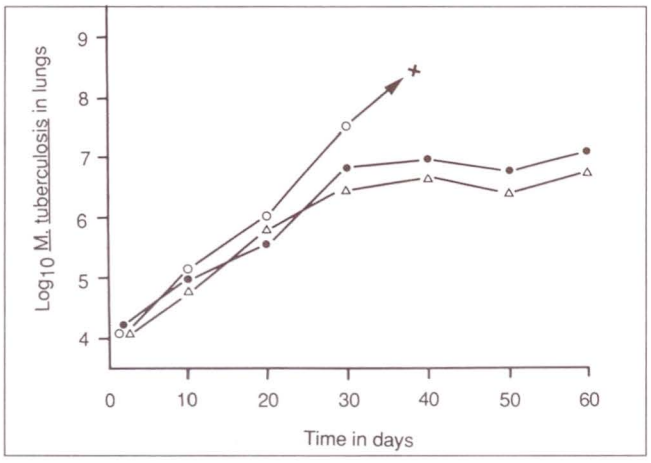

Figure 2) *Growth of* M tuberculosis *in the lungs of infected mice. Mice were infected with* M tuberculosis *and were exposed to the following aerosolized agents: heat-inactivated cytokines only (o), 100 μg of recombinant interferon gamma (r IFN-γ) (D) or 100 μg of recombinant tumour necrosis factor (r TNFα) (•) at day 0. Data expressed as in Figure 1. Data from four experiments*

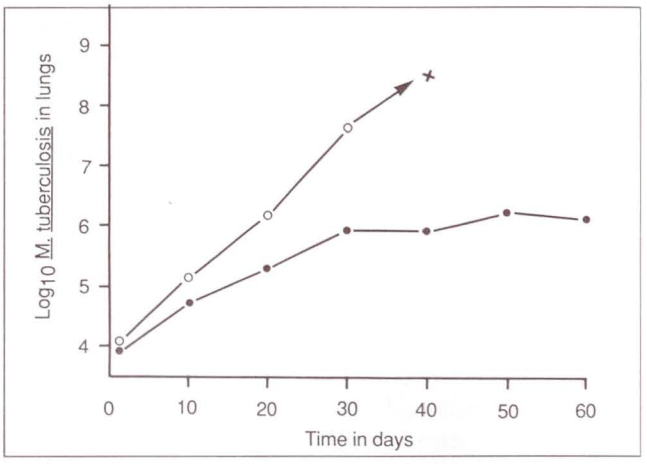

Figure 3) *Growth of* M tuberculosis *in the lungs of infected mice. Mice were infected with* M tuberculosis *and were exposed to the following aerosolized agents: 100 μg of recombinant tumour necrosis factor (r TNFα) and 100 μg of recombinant interferon gamma (r IFN-γ) (•) or the same cytokines heat-inactivated (o). Data expressed as in Figure 1. Data from four experiments. Differences in colony forming units counts are significant (P<0.05 student's* t *test) at days 20 and 30)*

in the lesions (12). Also, IL-2 with its central role as a T cell growth factor (13) and a macrophage-activating molecule (14) has been shown to enhance significantly the resistance against various microbial pathogens (15). In the present study, 100 μg of IL-2 administered by the aerosol route did not lead to any significant increase in resistance of mice to *M tuberculosis* (Figure 1). This suggests that IL-2 may not increase resistance in this aerogenic model. This may be due to the poor expression of IL-2 receptors in normal pulmonary and bronchoalveolar lymphoid tissues. However, treatment with 100 μg of IL-2 three times at two day intervals of already infected mice at a time when mice should express immunity (16) had no beneficial effect on the progression of infection (Figure 1).

TNFα is an important cytokine in host resistance against infections. Depletion of endogenous TNFα significantly increased susceptibility of mice to *M bovis* (17), *Listeria monocytogenes* (18) and *Leishmania donovani* (19). Also, TNFα has been shown to increase resistance against *M avium* strains in vivo. Mice infected with *M tuberculosis* were treated with TNFα shortly after infection. TNFα-treated mice did not show progressive growth of *M tuberculosis*; there was growth up to 7 $\log_{10}$ cfu after which there was bacteriostatic activity in the lungs of infected mice (Figure 2), indicating that local TNFα application may increase resistance to tuberculosis in mice.

Another cytokine considered in this model was IFN-γ. Recognized as a major macrophage-activating molecule, IFN-γ has been shown to increase resistance to a large spectrum of microbial agents (20). In vitro, IFN-γ enhances mouse macrophage resistance to *M tuberculosis* (21). In vivo, IFN-γ may diminish growth of virulent mycobacteria in the organs of infected mice (22), although some studies have shown that virulent strains of mycobacteria may resist IFN-γ in vivo or in vitro (21). The possibility was tested that IFN-γ may increase resistance to tuberculosis. Aerosolization of 100 μg of IFN-γ in infected mice induced a level of resistance similar to that seen with TNFα-treated mice survived up to 60 days with a bacteriostatic activity in the lungs of mice (Figure 2).

In another set of experiments, the possibility was investigated that IFN-γ in combination with TNFα could increase resistance in an additive or synergistic fashion. Administration of cocktails of cytokines in vivo or in vitro has often been shown to result in greater increases in resistance to infectious agents than seen with individual factors (4).

Notably, combinations of IFN-γ and TNFα enhance resistance to mycobacteria, listeria and *Schistosoma mansoni* in vitro to an optimal degree (23,24). Both IFN-γ and TNFα were applied by aerosol sequentially after infection, and the progression of the infection followed as described above. Figure 3 shows results obtained with combined treatments. Mice treated with IFN-γ and TNFα exhibited a high degree of antituberculous resistance, as seen by the reduced growth of the microbes in the lungs. *M tuberculosis* grew to only approximately 6 $\log_{10}$ cfu in the lungs of TNFα/IFN-γ-treated mice. This enhancement of resistance was not superior to that seen with either cytokine used alone (Figure 2, P>0.1). The protection obtained with TNFα/IFN-γ was dependent upon the route of exposure. Infusion of cytokines, such as TNFα and IFN-γ (5 μg each cytokine per mouse daily), by the intraperitoneal route for 15 days increased the resistance only by a marginal degree (P<0.05 at days 20 and 30); however, all mice were dead by day 40 (Figure 4). This set of experiments

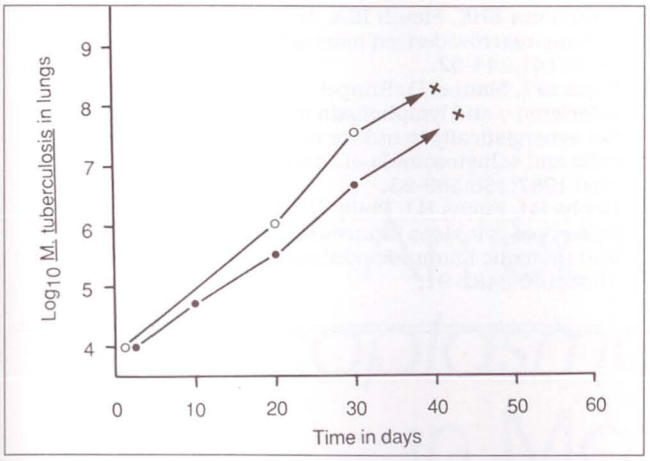

Figure 4) *Growth of* M tuberculosis *in the lungs of mice infected by the aerogenic route treated with cytokines by the intraperitoneal route. Mice were infused with 5 μg of interferon gamma (IFN-γ) and 5 μg of tumour necrosis factor (TNFα) daily (•) by the intraperitoneal route, or heat-inactivated cytokines (o). Differences in colony forming unit (cfu) counts between groups is significant at days 20 and 30 (P<0.05). Data expressed as in Figure 1. At day 30 there is a significant difference in log cfu (P<0.05, student's* t *test)*

shows that repeated intraperitoneal infusion with large doses of cytokines was ineffective at modifying the resistance/susceptibility of mice to tuberculosis.

Previous results have shown that aerosolized IFN-γ and/or TNFα induced a high level of alveolar macrophage activation, as seen by enhanced IA expression, tumour cytotoxicity and IL-1 expression (25). The present results also show that aerosol infusion of such cytokines may enhance antimicrobial activity in the lungs. Follow-up studies have shown that alveolar macrophages from cytokine-treated mice have elevated levels of superoxide anion release after phorbolmyristic acetate triggering, indicative of an increase in cellular effector functions (unpublished data). It is sill not clear what is the actual distribution of the cytokines in the lungs of infected mice. Administration of potentially toxic agents by site-specific delivery has been proposed as a way to concentrate the cytokine at the infectious foci (8) and to reduce the toxicity of systemic administration (25). It remains to be determined how the cytokines were acting on the progression of the disease in the present study. As mentioned above, there is evidence that IFN-γ and TNFα are involved in antimycobacterial resistance. TNFα may be involved in promoting granulomatous lesions which are bacteriostatic (17). IFN-γ may promote mouse macrophage antimycobacterial functions directly (21).

In summary, the present results indicate that aerosolized cytokines may endow infected hosts with strong resistance against *M tuberculosis* airborne infection. The authors are currently investigating this system of lung-specific delivery of cytokines in other infectious models.

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