PROTECTION AGAINST MYCOBACTERIUM TUBERCULOSIS INFECTION BY ADOPTIVE IMMUNOTHERAPY*

Requirement for T Cell-deficient Recipients

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Analysis of the mechanisms of cell-mediated immunity by the passive transfer of immune cells to appropriate recipients remains one of the most powerful tools in the field of immunobiology. Indeed, the first evidence that hypersensitivity to tuberculin protein was mediated by lymphoid cells was obtained by the passive transfer of such cells from immune guinea pigs to normal recipients, and the subsequent elicitation of a hypersensitivity reaction in the skin after tuberculin challenge (1). However, although the adoptive transfer of such delayed-type hypersensitivity reactions is readily achieved and hence commonplace in the literature, it has proved to be much more difficult to adequately transfer protective immunity against nonacute bacterial infections, for example tuberculosis, which are characterized by progressive growth of the organism over a number of weeks (2-4).

Although it has been shown that acquired immunity to tuberculosis infection is cell mediated (5, 6), the precise mechanisms underlying the generation of such immunity still remains largely undefined. For example, the relationship between the generation of protective immunity, and the generation of cells that possess the capacity to elicit delayed-type hypersensitivity reactions to tuberculin, remains controversial. The exact nature of this relationship takes on added importance with the failure of the recent bacillus Calmette-Guérin (BCG)1 vaccine trial in Southern India (7) in which the immunized individuals expressed an enhanced ability to respond to purified protein derivative but showed no evidence of resistance to subsequent infection. Clearly, therefore, the relationship between skin hypersensitivity reactions and the generation of protective immunity after vaccination procedures should be more rigorously explored. The present report describes an attempt to develop an improved model of virulent tuberculosis infection permitting the study of acquired immunity to the organism while the course of the infectious process was followed in vivo. The experiments described in this report were based on the premise that, if an adequate model of adoptive immunotherapy against virulent tuberculosis infection (both intravenous and

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Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; FCS, fetal calf serum; PBS, phosphate-buffered saline; ThXB, thymectomized T cell-deficient mice.
aerosol) can be constructed, then such a model would serve to facilitate the analysis of the nature and kinetics of the acquired immune response that develops.

The main purpose of the present report, however, is to initially show that passive transfer of protective immunity to virulent tuberculosis infection can be readily achieved, provided that recipient mice are rendered T cell-deficient before an infusion of donor immune cells. Moreover, it will confirm the observations of Lefford (6) who presented evidence indicating that the expression of protective immunity in such recipients is mediated by a population of sensitized T lymphocytes that are acquired in the donor animals in response to the tuberculous infection.

Materials and Methods

**Mice.**  B6D2 (C57BL/6 × DBA/2)F1 and AB6 (A/Tru × C57BL/6)F1 hybrid mice of either sex were used when they were between 8 and 12 wk old. All mice were supplied by the Trudeau Animal Breeding Facility.

**Bacteria.**  *Mycobacterium bovis* BCG (BCG Pasteur, Trudeau Mycobacterium Collection [TMC] strain 1011) was grown in modified Sauton's medium (8) and stored in 1-ml ampules at −70°C. *Mycobacterium tuberculosis,* strain H37Rv (TMC 102) and Erdman (TMC 107) were grown in Proskauer and Beck liquid medium. Dispersed cultures were obtained by the addition to the medium of 0.05% Tween 80, and were stored as above.

**Intravenous Infection.**  Stored ampules were thawed and briefly sonicated (5 s), and bacteria diluted appropriately in cold, sterile, phosphate-buffered saline (PBS). Mice were infected intravenously with an inoculum of 0.2 ml of the bacterial suspension via a lateral tail vein.

**Aerosol Infection.**  Mice were exposed to aerosol infections using a Middlebrook Airborne Infection Apparatus (Tri-R Instruments, Inc., Rockville Centre NY). The nebulizer compartment was filled with 10 ml of *M. tuberculosis* H37Rv in PBS at a concentration of ~5 × 10⁸ ml⁻¹; this procedure deposited ~10⁴ viable bacteria in the lungs over a 30 min exposure period.

**Immune Donor Mice.**  Donor animals were immunized by intravenous infection with 10⁴ viable BCG or 10⁵ viable *M. tuberculosis* Erdman. After 15 or 20 d, respectively, spleen cells were harvested from these mice and used in passive cell-transfer experiments. These time points were chosen since they were associated with the cessation of progressive proliferation of the infectious organism in the spleens and livers of these mice, and hence indicated the presence within the animal of the emergence of an acquired host response.

**Spleen Cell Preparations.**  Prior to their use in passive cell transfers, spleen cell preparations were treated as follows. Single-cell suspensions were prepared from spleens harvested aseptically from donor animals and washed and resuspended in RPMI 1640 medium supplemented with 1 mM glutamine and 2% heat-inactivated fetal calf serum (FCS). Depletion of the bacterial load within the infected spleen cells was carried out by incubating cells (5 × 10⁷ ml⁻¹) in sterile plastic dishes (4030; Lab-Tek, Naperville, IL) for 45 min at 37°C. Nonadherent cells were removed by washing. The cell suspension was then enriched for T cells by the procedure of Mage et al. (9) which depletes B cells by adherence to plastic dishes coated with antisera to mouse immunoglobulin. Plastic dishes (4030; Lab-Tek) were coated with goat anti-mouse IgG (Cappel Laboratories Inc. Cochranville, PA) by adding 20 ml of 0.05 M Tris-HCl buffer, pH 9.5, containing 20 μg antibody ml⁻¹ and incubating dishes at 25°C for 25 min. Unbound antibody was washed away with cold PBS. Spleen cells were added in a volume of 20 ml RPMI plus 2% FCS (2 × 10⁸ cells ml⁻¹) and incubated at 4°C for 45 min. Unattached cells were harvested, washed, and resuspended in cold PBS containing 1% FCS.

**Passive Cell Transfers.**  Unless indicated, age- and sex-matched syngeneic recipient mice were infused intravenously with 8 × 10⁷ nonadherent T cell-enriched spleen cells suspended in 0.8 ml PBS plus 1% FCS. A series of preliminary titrations (I. M. Orme, unpublished data) determined that this number of immune cells possessed the capacity to
transfer detectable levels of protection to appropriate recipients. In the case of intravenous rechallenge experiments, recipient animals were infused with T cell-enriched spleen cells from mice infected with *M. tuberculosis* Erdman 20 d earlier; 1–2 h later they were challenged intravenously with $10^5$–$10^6$ viable Erdman. In aerosol experiments, spleen cells from mice infected with BCG 14–16 d earlier were infused into recipients within 24 h of the aerosol exposure to *M. tuberculosis*.

**Enumeration of Bacteria.** Numbers of viable bacteria in given organs were determined by plating suitable dilutions of organ homogenates in cold saline Middlebrook 7H10 agar (Difco Laboratories Inc., Detroit, MI) and counting colony formation after incubation for 20 d at 37°C. For each time point, four to five animals were sacrificed; data is expressed as the log$_{10}$ mean number of viable organisms per organ (standard error of the mean always <18%).

**T Cell-deficient Test Recipients.** Mice were rendered T cell-deficient by one of two methods: (a) mice were thymectomized at 4 wk of age and 7 d later received 900 rad of whole body gamma irradiation delivered by a $^{137}$Ce irradiator at a dose rate of 29.5 rad/min. Mice were reconstituted intravenously with $2 \times 10^7$ syngeneic bone marrow cells the same day, and used 6 wk later; (b) mice were exposed to 500 rad of gamma irradiation and used 2 d later.

**Treatment with Anti-Thy-1.2 Antibody.** Spleen cell suspensions were prepared as described above, and treated with monoclonal anti-Thy-1.2 antibody as previously described (10). After treatment, cells were resuspended to their initial starting volumes to prevent positive selection of remaining cells.

**Results**

**Course of Infection in Donor Animals.** In an initial experiment the course of the mycobacterial infection was followed in two groups of donor animals (Fig. 1). In the first group, in which mice were originally infected with $10^8$ *M. bovis* BCG, the course of infection was characterized by progressive growth of the organism in the spleen, followed after about 15 d by a gradual reduction in the number

![Figure 1](image-url)

**FIGURE 1.** Growth of mycobacterial infections in donor animals. Mice were infected intravenously with *M. tuberculosis* (strain Erdman) or with *M. bovis* BCG, and the course of the infection in the liver (●), spleen (■), and lungs (▲) followed over time.
of infectious organisms in the spleen and liver. A similar if more acute profile was observed in mice infected with $10^5$ *M. tuberculosis* Erdman, where bacterial numbers ceased to increase by day 20 of the infection. It was reasonable to hypothesize, therefore, that the timing of the abridgement of bacterial proliferation reflected the development in the host of an emerging immune response, and that therefore donor cells should be harvested at those times for use in adoptive transfer of immunity. It should also be noted (with regard to data presented below) that despite the generation of an apparent host response in the spleen and liver of the donor mice, evidence for a protective response in the (intravenously) infected lung was not observable for several more days.

**Demonstration That Adoptive Protection Against Intravenous Challenge with *M. tuberculosis* Requires T Cell-deficient Recipients.** In this series of experiments the growth of *M. tuberculosis* Erdman was followed over time after injection of a challenge inoculum into normal or T cell-deficient mice, and was compared with the growth of the organism in mice infused either with T cell-enriched spleen cells from Erdman-immunized mice or with similar cells from uninfected normal mice (Fig. 2). The results show that the infusion of cells from immunized donors had no protective effect on the course of the challenge infection in the normal recipients, whereas they transferred significant protection against a similar challenge infection in those mice rendered T cell-deficient (500 rad-irradiated or thymectomized [ThXB]). This protection was quickly expressed in the spleen and liver, with significant resistance to the infection present by day 14; in the case of the lungs, however, it was somewhat slower, a result compatible to events

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**FIGURE 2.** Evidence that infusion of T cell-enriched spleen cells from *M. tuberculosis*-immune donor mice has no effect on the course of *M. tuberculosis* infection in normal recipients (top row), but transfers significant adoptive protection to sublethally irradiated recipients (middle) and to ThXB recipients (bottom). (●) Infected recipients alone, (▲) recipients infused with T cell-enriched cells from normal animals, (■) recipients infused with T cell-enriched cells from immune animals.
seen in the lungs of the donor animals. Although the transfer of T cell-enriched spleen cells from normal donors had no effect on the course of infection in normal recipients, there was evidence to suggest that they may have been reconstituting the normal defenses of the T cell-deficient mice, as evidenced by the expression of a minor degree of protection in these mice after 21 d. A similar degree of protection against the Erdman challenge infection could be transferred to either type of T cell-deficient mice if donor spleen cells were obtained from mice immunized with BCG rather than Erdman (data not shown).

Evidence That Cells Which Transfer Protection Against Intravenous Challenge Are T Cells. Evidence that the protection obtained against \textit{M. tuberculosis} challenge was mediated by T cells was obtained by determining whether immune spleen cells from \textit{M. tuberculosis}-infected donors failed to transfer protection to T cell-deficient recipients after the treatment of donor cells with antibody raised against the Thy-1.2 marker plus complement. The results in Fig. 3 show that treatment with anti-Thy-1.2 antibody plus complement completely ablated the capacity of the immune cells to transfer protection against the \textit{M. tuberculosis} challenge in the T cell-deficient mice. This experiment thus demonstrates that passive transfer of protection in this model is T cell-mediated.

Protection of the Lung After Aerosol Infection of \textit{M. tuberculosis}: A Similar Requirement for a T Cell-deficient Host. In these experiments it was determined whether the passive transfer of spleen cells from BCG-immunized donors could protect the host after an acute aerosol challenge with a virulent strain (H37Rv) of \textit{M. tuberculosis}. Again, it was clear that adoptive protection could be transferred only if the recipient was rendered T cell-deficient (Fig. 4). However, in contrast to the intravenous infection model, in which protection was quickly expressed in the spleen and liver, protection against the aerosol infection was not evident within the lungs for at least 20–25 d, after which time the course of the infection was characterized by apparent bacteriostasis, with no further apparent gross changes in the numbers of infectious organisms.

In the course of these experiments it was evident that, despite the procedures to obtain a nonadherent T cell-enriched spleen cell population prior to passive transfer, a contaminating residual level of BCG organisms was present in the passive cell-transfer inocula. By plating suitable dilutions of these cell suspensions

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Evidence that donor spleen cells that adoptively transfer protection are T cells. Recipients were sublethally irradiated, then infected with \textit{M. tuberculosis}. (○) Recipients that received no cells, (■) recipients of T cell-enriched immune cells, (□) immune cells after treatment with antibody to Thy-1.2 plus complement, (▲) immune cells treated with complement only.
Evidence that immune cells can adoptively protect the lungs of T cell-deficient recipients. (a) Normal mice (O), normals infused with normal cells (■), normals infused with immune cells (▲), normals given normal cells admixed with $10^4$ BCG (□). (b) Sublethally irradiated recipients (O), given normal cells (■), immune cells (▲), or normal cells admixed with $10^4$ BCG (□). (c) ThXB recipients (O), given normal cells (■), or immune cells (▲). In all cases cells were nonadherent, T cell-enriched spleen cells and were infused within 24 h of an aerosol-delivered challenge with *M. tuberculosis* HsTRv.

It was determined that each inoculum was contaminated with between $10^5$ and $10^4$ viable BCG. It therefore followed that there was a possibility that this contamination could have induced some *de novo* protection in the recipient resulting in heightened resistance to the aerosol infection. This possibility was unlikely in view of the observation that inocula of immune cells (containing contaminating BCG) did not confer any resistance to normal recipients, but to test this possibility further, normal cells were admixed with $10^4$ BCG and infused into normal or T cell-deficient recipients (Fig. 4). The results show that this admixture had no protective effect against the aerosol infection and thus demonstrate that the observed protection of the lungs was mediated by the passive transfer of immune cells, rather than by the generation of acquired resistance in the recipients to the contaminating BCG.

Evidence That Protection in the Lung Is Mediated by T Cells. The experiment shown in Fig. 5 demonstrates that passive transfer of BCG-immune spleen cells into T cell-deficient mice can adoptively protect the recipient against a progressively growing aerosol infection with *M. tuberculosis*. However, after treatment of the cell population with anti-Thy-1.2 antibody plus complement, this protective activity was completely ablated, demonstrating in this model that protection of the tuberculous lung was T cell-mediated.

Stability of Transferred Protection. It was demonstrated in experiments described above that T cell-deficient mice exposed to a lethal aerosol challenge with *M. tuberculosis* could be protected by the passive transfer of immune T cells regardless of whether the recipients were rendered T cell-deficient by sublethal irradiation (500 rad) or by thymectomy, irradiation, and bone marrow cell reconstitution (ThXB). However, despite an apparent bacteriostasis ($\sim 10^7$ viable organisms) in the lungs of surviving adoptively protected 500-rad recipients, it was clear that continued growth of the infection was occurring from time to time in individual animals, with a resulting steady increase in cumulative mortality...
Evidence that adoptive protection of the lungs was mediated by T cells. ThXB recipients (○) were infused with T cell-enriched immune cells (▲), immune cells treated with antibody to Thy-1.2 plus complement (■), or immune cells treated with complement only (■). Cells were infused within 24 h of an aerosol-delivered challenge with *M. tuberculosis* H37Rv.

Evidence that adoptive protection is much more long-lived in ThXB recipients than in sublethally irradiated (500 rad) recipients. These animals, plus a group of normal mice, were infused with $8 \times 10^7$ *M. tuberculosis*-immune T cell-enriched spleen cells, 24 h after aerosol-delivered exposure to *M. tuberculosis* H37Rv (day 0). 20 animals were tested in each group.

with time (Fig. 6). In contrast, however, very little mortality was observed in ThXB recipients despite similar levels of infections in the lungs of these mice. These results suggest that the passive transfer of adoptive immunity in the lungs was apparently very much more stable in ThXB than in 500-rad recipients, and thus may indicate that mice rendered T cell-deficient by sublethal irradiation are undergoing adaptive changes which may interfere with their subsequent protection and survival.

Discussion

This paper shows that the mouse can be adoptively protected against an acute *M. tuberculosis* infection, after either intravenous or aerosol infection, provided that the infected recipient has been made T cell-deficient by thymectomy and gamma irradiation or alternatively by sublethal irradiation. It shows, furthermore, that the passive transfer of sensitized T cells confers upon such recipients
the ability to control progressive growth of the *M. tuberculosis* challenge, and provides the first evidence that such cells can adoptively protect the animal against an acute aerosol-delivered infection within the lungs. The results obtained in the intravenous infection model confirm and extend the observations of Lefford (6), who demonstrated that T cells from the spleens of BCG-immune donors could adoptively transfer protection into sublethally irradiated recipients. This protection, which was measured as a statistically significant difference in numbers of infectious organisms in the spleen 2 wk after challenge, was conferred equally against BCG reinfection and intravenous challenge with *M. tuberculosis* HsTRv.

The finding that sublethal irradiation of recipients before passive transfer of immune cells may facilitate the transfer of adoptive cell-mediated immunity has been noted elsewhere (6, 11). It was clear in the present study however, that mice given sublethal irradiation before the *M. tuberculosis* challenge but which did not receive a passive transfer of cells had themselves started to express some degree of resistance to the challenge by the 3rd wk of infection. It is reasonable to hypothesize, therefore, that these animals were beginning to be able to re-express their own cell-mediated immunity to the infection. Although the nature of the radiosensitive barrier that prevents the transfer of large numbers of immune cells into normal recipients remains unknown (12), it is quite possible that this blocking mechanism is subsequently reestablished in the sublethally irradiated recipients during their recovery from exposure to this irradiation. It is quite possible, therefore, that any interaction between such a mechanism and the presence in the recipient of passively transferred immune cells may affect the stability or longevity of the transferred immunity. Evidence in support of this possibility is provided by the observation that adoptive protection against the aerosol infection in the lung was achieved in the 500-rad irradiated recipients, but consisted of prolonged survival rather than absolute protection over a long period of time. In contrast, in ThXB mice, in which there was no evidence for the emergence of cell-mediated immunity to the infection, passive transfer of immune cells protected in the lungs and resulted in the long-term survival of these animals (only 15% mortality at day 120). It follows that in models of chronic tuberculosis infection (resulting from passive transfer of protection against progressive infection) the ThXB mouse should be the recipient of choice, in that the expression of adoptively transferred immunity apparently remains stable over a long period of time. On the other hand, it is possible that the less stable immunity in the 500-rad recipient may serve as an appropriate model of the reemergence of progressive tuberculous infection in the lungs that may follow as a result of any interaction between cells which mediate protective immunity and normal negative regulatory mechanisms. This latter possibility remains to be investigated.

It has previously been suggested that adoptive immunity could not be expressed within the lung (13) perhaps due to the defective migration of lymphocytes in this organ. The more recent findings of Lefford (14), however, and the results of the present study refute this suggestion, and as previously suggested (14), it is clear that adoptive immunity to tuberculosis can be expressed in the lungs provided that a sufficiently large inoculum of immune cells, taken from the
donor at the height of the primary immune response, is intravenously transferred. This requirement, plus the need for T cell-deficient recipients, and because of the considerable delay before protection can be detected in the aerosol-infected lung, may help to explain the earlier failures reported in this area (2, 3).

It is concluded, therefore, that the expression of adoptive protection by the passive transfer of sensitized T cells into \textit{M. tuberculosis}-infected, T cell-deficient recipient mice may serve as a basic model for the analysis of the nature and kinetics of the acquired immune response to aerosol-delivered and intravenous \textit{M. tuberculosis} infection, and may further our understanding of the development of immunity to the naturally acquired human disease. The characteristics of sensitized T cells that are acquired in the host at the height of the primary immune response to \textit{M. tuberculosis} are currently under investigation in this laboratory.

Summary

The results of this study demonstrate that spleen cells taken from mice at the height of the primary immune response to intravenous infection with \textit{Mycobacterium tuberculosis} possess the capacity to transfer adoptive protection to \textit{M. tuberculosis}-infected recipients, but only if these recipients are first rendered T cell-deficient, either by thymectomy and gamma irradiation, or by sublethal irradiation. A similar requirement was necessary to demonstrate the adoptive protection of the lungs after exposure to an acute aerosol-delivered \textit{M. tuberculosis} infection. In both infectious models successful adoptive immunotherapy was shown to be mediated by T lymphocytes, which were acquired in the donor animals in response to the immunizing infection. It is proposed that the results of this study may serve as a basic model for the subsequent analysis of the nature of the T cell-mediated immune response to both systemic and aerogenic infections with \textit{M. tuberculosis}.

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