Restoration of T-Cell Responsiveness by Thymosin: Expression of Anti-Tuberculous Immunity in Mouse Lungs

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Specific pathogen-free, adult thymectomized, irradiated, and bone marrow-reconstituted (THXB) B6D2 mice were infected aerogenically with 1 × 106 to 5 × 106 live BCG Pasteur. Seven days later a group of the mice was placed on a 14-day regimen of 20 mg of calf thymosin per kg per day, and the growth of the BCG in the lungs, spleen, inguinal lymph node, bone marrow, and blood was determined for up to 90 days. The thymosin treatment was followed by a decline in the BCG counts for the lungs and spleens of the THXB mice, whereas the saline-treated controls showed no such decline with time. The thymosin-treated mice did not develop progressive BCG infections in the test lymph nodes or in the bone marrow, both of which became positive in the THXB mice. Spleen cells were harvested from thymosin-treated THXB donors, filtered through nylon wool, and infused three times into BCG-infected THXB recipients. The lung BCG counts declined approximately 10-fold by day 90 compared with THXB mice which received THXB spleen cells. The transferred immune response was only slightly smaller numerically than that seen in THXB mice infused with BCG-immune lymphocytes from normal donors.

Adult thymectomized, irradiated, bone marrow-reconstituted (THXB) B6D2 mice failed to develop detectable levels of anti-tuberculous immunity after aerogenic Mycobacterium bovis (BCG) infection (18). When the immunosuppressed host was infected intravenously with this same attenuated organism, a rapidly fatal, systemic mycobacteriosis developed in the majority of test animals (5). Reconstitution studies with filtered splenic lymphocytes taken from normal syngeneic donors indicated that the adoptively immunized T-cell-depleted mouse could mount an effective anti-tuberculous immune response provided that the transferred lymphocytes were not first treated with antitheta serum and complement (20). Depletion of the host of immunocompetent T-cells was associated with an inability to express a specific cell-mediated immune response against a variety of facultative intracellular parasites (3). However, if the presence of these T-cells in the lymphoreticular organs of the host were reestablished by a means of a thymus graft from a syngeneic donor (22) or by treatment of the host with repeated injections of a thymic hormone such as thymosin (19), the host was able to express both delayed-type hypersensitivity and cell-mediated immune responses to subsequent challenge (7). Studies carried out both in vivo and in vitro indicate that thymosin can bring about the maturation of immunocompetent T-cells in experimentally and naturally immunosuppressed individuals (9, 23).

Recent studies with BCG-infected, T-cell-depleted mice (19) used an arbitrarily established treatment protocol in which massive doses of thymosin (120 mg/kg per day) were injected for 15 days. The previously immunosuppressed host was then able to express an effective anti-tuberculous response against the BCG challenge population such that the mice were protected from lethal effects of the developing mycobacteriosis (5). These experiments were carried out by using intravenously infected mice in which the bulk of the introduced organisms will lodge in livers and spleens, with relatively few bacilli occurring in lungs (8). The liver and spleen infections can be expected to induce a maximum response by the host's cellular defenses in a relatively short time, whereas an aerogenic challenge will not spread beyond the lung for several weeks, thus changing the tempo and extent of the subsequent immune response substantially (15, 17). When living BCG are introduced by the aerogenic route into normal or T-cell-depleted mice, the BCG population within the lung multiplies equally in both groups of animals to a peak of about 105 viable units per lung (18). The only difference which can subsequently be distinguished between the normal and the THXB lung patterns seems to

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be that the latter population persists as an extended plateau almost indefinitely, whereas the control viable curves show a slow decline, which occurs as the cell-mediated immune response to the BCG population in vivo begins to be expressed within a normal lung (18).

The present study examines the ability of a standardized thymosin treatment regimen to restore the ability of T-cell-depleted mice to regain the ability to express an effective anti-tuberculous immune response against an aerogenic BCG challenge infection. Restoration was shown to be due to the presence of immunocompetent splenic lymphocytes in the thymosin-treated spleens that were able to adoptively protect THXB recipients against an aerogenic challenge.

MATERIALS AND METHODS

Animals. Four-week-old, specific pathogen-free, B6D2 (C57Bl/6 × DBA-2 F1 hybrid) mice were thymectomized surgically, lethally irradiated (900 rads), and bone marrow reconstituted as described elsewhere (5, 20). The mice were maintained on tetracycline-enriched drinking water for 4 weeks in a laminar flow animal rack. They were fed sterile commercial mouse chow and acidified water ad lib. (6). Sham-thymectomized (XB) and normal age-matched controls were included in most of the studies (5).

Organisms. M. bovis (BCG Pasteur: TMC #1011) was grown in modified Santon's TWEEN albumin broth (8) incubated at 37°C in roller tubes for 14 days and stored in 1-ml ampoules at −70°C, as described previously (6). When required, an ampoule was thawed at 37°C, homogenized in an equal volume of fresh modified Santon TWEEN albumin, exposed for 3 to 4 hr to sonic oscillation to break up the remaining clumps, diluted appropriately in TWEEN saline, and used to generate an aerosol of live BCG in a Middlebrook chamber (Tri-R Industries, New York, N.Y.), as described previously (18). The density of the generating suspension was adjusted so that approximately 5 × 10^3 viable BCG were introduced into the lungs over a 30-min period, using a previously constructed dose-response curve. The size of the infectious inoculum was checked by sacrificing five mice some 30 min after challenge and plating the lung homogenates on Middlebrook 7H10 agar. The plates were incubated in sealed plastic bags at 37°C for 3 weeks before counting.

Thymosin. The thymosin was prepared from fresh calf thymus as described previously (7, 19). The preparation used in the present study was designated lot 2 and corresponded in purity to fraction V of Goldstein et al. (10, 12). The thymosin treatment protocol entailed daily subcutaneous injections of 20 mg of thymosin per kg of body weight per day, which corresponded to 0.5 mg of protein in 0.2 ml of saline per day in a 25-g mouse for 15 days. This treatment was followed by twice weekly injections of 0.5 mg of thymosin throughout the remainder of the experiment. Control mice received 0.2 ml of saline in place of the thymosin. Earlier studies (19) indicated that significant restoration of anti-tuberculous immunity occurred in T-cell-depleted mice treated daily with 3-mg injections of thymosin per mouse (120 mg/kg). Similar injections given to normal mice tended to enhance some cellular reactivity (7), but the overall response was still similar to the saline-treated controls as far as the BCG studies were concerned (19). In the present study, the challenge procedure was changed so that the mice were infected aerogenically with BCG Pasteur 7 days before the thymosin treatment was initiated.

Adoptive transfer experiments. Groups of THXB and normal B6D2 mice were treated with 40 mg of thymosin per kg per day (1.0 mg of protein per mouse per day) for up to 20 days. Another group of normal (non-thymosin-treated) B6D2 mice were infected intravenously with approximately 10^6 viable BCG. The BCG-vaccinated normal donors and the thymosin-treated THXB mice were sacrificed 28 days after initiation of their respective treatments. The spleens were removed aseptically, and the cells were expressed through a stainless-steel grid into cold Hanks balanced salt solution containing 5% fetal calf serum and 5 U of preservative-free heparin (16). The cells were washed three times in Hanks balanced salt solution and were standardized to 5 × 10^6 cells per ml. The viability of each suspension was checked by dye exclusion and was usually more than 80%. Groups of THXB recipients were infected aerogenically with 10^6 viable BCG Pasteur and infused intravenously with 5 × 10^6 filtered lymphocytes on days 20, 28, and 35 of the BCG infection. A smaller number of THXB mice were infused with filtered spleen cells prepared from untreated THXB donors, using the same injection schedule. Viable BCG counts carried out on the filtered spleen cell suspension prepared from the BCG-vaccinated mice indicated less than 1,000 viable BCG were present in the infused cell suspension of 5 × 10^6 cells per ml. BCG counts carried out on lung homogenates prepared from unchallenged THXB mice 24 hr after infusion of the immune spleen cell lymphocytes failed to reveal any mycobacterial colonies on the 7H10 agar plates examined 4 weeks later.

Enumeration of the in vivo population. Groups of five randomly selected mice were pulsed with 1 μCi of tritiated thymidine (specific activity, 3 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per g of body weight, injected intravenously some 30 min before sacrifice (5). The animals were killed; the lungs, spleens (18), and inguinal lymph nodes (4) are removed aseptically; weighed and known fractions of each organ were homogenized in cold saline and then were diluted suitably in TWEEN saline and plated on 7H10 agar. Samples of heart blood and bone marrow cells taken from one femur were also plated on 7H10 agar (5). Other portions of each organ were homogenized in cold 5% trichloroacetic acid and the [3H]DNA content was determined by a Beckman LS-100 liquid scintillation counter (20).

RESULTS

Effect of thymosin treatment on BCG
growth in THXB mice. Logarithmic-phase BCG were introduced aerogenically into normal B6D2 mice, after which the inoculum multiplied without an appreciable lag period until it reached a maximum of $2 \times 10^6$ to $5 \times 10^6$ viable bacilli per lung about day 20 (Fig. 1). Thereafter, the lung counts dropped slowly, so that by day 90 the residual lung population represented only about 1% of the earlier maximum. However, the infection also spread from the lungs to the liver and spleen, where the viable counts reached a peak of $1 \times 10^5$ to $2 \times 10^5$ viable BCG by day 28, followed by a decline which roughly paralleled that observed in lungs. Small numbers of viable BCG were recovered from the inguinal lymph nodes (selected because easily accessible and remote from the lung) beginning on day 45, but these counts were sporadic and no accurate estimate of the numbers involved was therefore possible. Blood remained free of detectable BCG throughout the study, as did bone marrow cell cultures.

When the same inoculum was introduced into the lungs of T-cell-depleted mice, the resulting growth curves began similarly to those shown above for normal mice (Fig. 2). The viable counts increased logarithmically to a maximum of $3 \times 10^6$ to $5 \times 10^6$, reached after 20 to 25 days, after which the curve went into a prolonged plateau, with some increase to a maximum of about $10^7$ viable BCG per lung on day 90. There was no sign of a decline in viability over this period corresponding to that seen in normal controls. The infection in the THXB mice also spread to the spleen, where the population eventually reached $10^6$ bacilli by day 90. Similarly, the marrow washouts and the lymph node cultures showed increasing numbers of viable BCG (up to 100 bacilli per sample by day 90). Several of the THXB mice exhibited an emerging bacteremia by day 90.

When the THXB mouse was exposed to multiple doses of thymosin subsequent to BCG inoculation, the early growth in both the lung and spleen appeared unaffected by the treatment regimen. However, after day 30 the lung viable counts went into a definite decline (Fig. 3), followed, on day 35, by the spleen counts. By day 90 the counts had dropped approximately 50-fold compared with the earlier (day 25) maximum, a highly significant difference ($P < 0.001$). The day-90 lung counts for thymosin-treated THXB mice were significantly ($P = 0.01$) reduced compared to those for the THXB controls (Table 1). The later spleen counts for the thymosin-treated mice also declined, although at a slower rate than that seen in the lung (Fig. 3). Thymosin treatment was associated with a lack of viable BCG in the inguinal lymph node homogenates (the few colonies observed were too few to be significant).

![Fig. 1. Growth after aerogenic infection of B6D2 mice with BCG Pasteur. Abbreviations: Lg, lungs; Sp, spleen; LN, inguinal lymph node. The vertical bars represent ± standard error of the mean of five determinations.](http://iai.asm.org/)

![Fig. 2. Growth of BCG Pasteur in THXB mice infected by the aerogenic route. Abbreviations: Lg, lungs; Sp, spleen; LN, inguinal lymph node; BM, bone marrow.](http://iai.asm.org/)

![Fig. 3. Growth of BCG Pasteur in aerogenically challenged THXB mice that received 14 daily subcutaneous injections of calf thymosin (20 mg/kg) beginning on day 7 of BCG infection. Abbreviations: Lg, lungs; Sp, spleen; LN, lymph node. Cultures from the lymph nodes, bone marrow, and blood samples were negative throughout.](http://iai.asm.org/)
TABLE 1. Growth of BCG in the lungs of aerogenically challenged T-cell-depleted mice treated with thymosin or with splenic lymphocyte suspensions

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Normal (1)</th>
<th>Donor mice</th>
<th>Recipient THXB mice infused 3× with 5 × 10⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>THXB</td>
<td>THXB + thymosin</td>
</tr>
<tr>
<td>1</td>
<td>0.044 ± 0.005a</td>
<td>0.044</td>
<td>0.044</td>
</tr>
<tr>
<td>28</td>
<td>5.40 ± 0.22</td>
<td>34.6 ± 2.20</td>
<td>51.6 ± 2.40</td>
</tr>
<tr>
<td>90</td>
<td>0.166 ± 0.048b</td>
<td>54.2 ± 16.3</td>
<td>2.50 ± 0.90b</td>
</tr>
</tbody>
</table>

a Viable BCG per lung (×10⁸) ± standard error.

b Significant difference (P ≤ 0.01) from (2).

sporadic for accurate counting). The blood and bone marrow washout cultures were also negative throughout this study, and this occurrence was in sharp contrast with the increasing counts for the saline-treated THXB controls (Fig. 2). The thymosin-treated normal and thymosin-tREATED XB controls both gave growth curves essentially identical to the normal growth curves shown in Fig. 1, and have not been included for that reason.

Effect of thymosin treatment on tritium uptake by aerogenically challenged animals. Normal B6D2 mice infected aerogenically with live BCG showed an early threefold increase in [³H]DNA levels in lungs over the first 30 days (Fig. 4). This peak could not be seen in the THXB host, but subsequently there was a 10-fold increase in [³H]DNA levels in lung homogenates, peaking on day 45 and then remaining far above normal levels throughout the study. Treatment of the THXB mice with thymosin substantially reduced the tritium incorporation late in the BCG infection, and by day 90 it had returned to near normal limits (Fig. 4).

Adoptive transfer of anti-tuberculous immunity. Suspensions of filtered spleen cells (consisting primarily of T-lymphocytes; 20) were injected intravenously into THXB recipients 20, 28, and 35 days into the BCG infection. The BCG counts for the THXB mouse lungs rose to a maximum about day 20 to 25 and then began to decline slowly, beginning about day 35 of the BCG infection, coinciding with the final infusion of spleen cells from the thymosin-treated donors (Fig. 5). By day 90, the lung counts had dropped by as much as 10-fold compared with the THXB controls (Table 1). This difference was highly significant (P < 0.01) when compared with those for THXB mice that had been the recipients of splenic lymphocytes taken from saline-treated THXB donors (Table 1). In another group of control mice, T-cell-enriched suspensions were prepared from BCG-vaccinated mice. Once again, the difference between the THXB controls and the immune cell recipient lung counts made on day 90 was highly significant. The decline in viability occurred after the third infusion (day 35) of immune lymphocytes, with a 50-fold drop in lung BCG counts by day 90 (Fig. 6). In both the thymosin-treated and the immune spleen cell recipients, a corresponding, though smaller, effect was seen in the spleen. At the same time no viable BCG were detected in the blood, bone marrow, and inguinal lymph node homogenates prepared from the adoptively protected animals, indicating a significant restriction in the dissemination of BCG infection in recipient mice.

DISCUSSION

Thymosin can be conveniently assayed in terms of the restoration of delayed hypersensitivity to sheep erythrocytes measured in a mouse footpad (7). Based on this assay procedure, a standardized protocol, using 15 daily injections of calf thymosin (20 mg/kg per day), was
adopted in mice infected 7 days previously with live BCG. This dosage was considerably lower than that used initially to study the immune response to an intravenous challenge infection (19), but it was nevertheless sufficient to induce a highly significant restoration in cellular reactivity against the developing lung infection. Since treatment of the THXB mouse was not initiated until a week after the lung infection had been established, it was not surprising that the early growth behavior of the BCG population within the lung was similar in both treated and control groups. However, by day 28 a highly significant change in survival rates for the BCG population in the thymosin-treated lungs was already being observed. This decline continued in both lungs and spleen throughout the remainder of the study. During this time, the mice continued to receive a maintenance regimen of twice weekly doses of thymosin. No attempt was made to determine whether animals not given this continued treatment underwent a later relapse or not. The anti-mycobacterial immune response within lungs of the treated mice was associated with a dramatic alteration in the level of tritium incorporation by lung cells compared to the saline-treated THXB controls. The sharp drop in cellular proliferation within the lung seemed to correlate with the presence of increased numbers of lymphocyte-like cells within the lung lesions, particularly during the time visible tubercles were resolving (18).

There is considerable evidence that the level of anti-tuberculous immunity expressed within lungs can vary extensively depending upon the route of administration and dose of immunogen used (2, 15). Furthermore, the immune response seen in adoptively immunized mice is often more readily demonstrated in spleens of recipients compared to that seen in lungs (14). In the present study, excellent levels of anti-tuberculous immunity were detected in lungs of aero-genically infected mice infused with splenic lymphocytes taken from thymosin-treated THXB donors (Fig. 3). However, in this case the mice were infected aerogenically before the lymphocytes were infused intravenously into the recipients so that a substantial proportion of the immunocompetent T-cells may have trafficked through the lung rather than lodging in the spleen, as they were more likely to do in an uninfected recipient. This may well explain the enhanced anti-mycobacterial activity seen within the lungs of these adoptively immunized mice compared to that observed for the spleens.

The successful adoptive transfer of immunity to the T-cell-depleted recipient by the T-cell-enriched suspension present in the nylon-wool-filtered spleen cell suspension occurred at least 7 days after the final injection of thymosin had been given to the THXB donors. This makes it unlikely that any residual thymosin could have been transferred to the recipients with the splenocytes, since the half-life of this agent in mouse serum is known to be quite short (1). Furthermore, the successful adoptive transfer of cellular reactivity to the THXB recipients makes it unlikely that any of the transferred cellular reactivity was caused by nonspecific macrophage activation by contaminating factors (possibly endotoxins) present in the relatively crude thymosin preparations. Filtered spleen suspensions prepared from saline-treated THXB donors were without protective value under these conditions (Table 1). T-cell-depleted mice infused

![Fig. 5. Growth of BCG Pasteur in aerogenically infected THXB mice which received intravenous infections of 5 x 10^8 filtered splenic lymphocytes harvested from thymosin-treated THXB donors on days 20, 28, and 35. Abbreviations: Lg, lungs; Sp, spleen; LN, lymph node.](http://iai.asm.org/)
with specifically sensitized T-cells prepared from BCG-vaccinated B6D2 donors also resulted in significant anti-mycobacterial activity within the lungs of the THXB recipients. The transferred activity could not have been due to active immunization by residual viable BCG in the immune spleen cell suspensions. First, the nylon-wool filtration effectively removed most of the infected macrophages from spleen cell suspensions (21). This was confirmed by the small number of viable BCG present in the filtered spleen cell suspension compared with that for the spleen cells before harvesting (approximately 10^6 viable BCG per spleen). Second, the injection of 10^6 viable BCG intravenously into THXB mice failed to induce any de novo immune response (even in normal B6D2 mice) over a 90-day period (F. M. Collins and W. E. Woodruff, unpublished data). Thus, the transferred anti-tuberculous immunity could only be ascribed to T-lymphocytes introduced in the spleens of the THXB mice as a result of repeated thymosin injections. Such data are consistent with the proposed role of polypeptide thymic hormones, such as thymosin (11), in maturation of immunocompetent T-cells within lymphoreticular organs of a normal host and also have considerable significance with respect to the possible use of such hormones in the immunotherapy of advanced tuberculosis and leprosy in humans.

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LITERATURE CITED