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Growth of *Mycobacterium bovis* (BCG) in T Lymphocyte-Depleted Mice

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BCG Montreal (10^6 viable bacilli) injected intravenously into adult thymectomized, irradiated, and bone marrow-reconstituted (THXB) C57Bl × C3H F_1 hybrid mice induced a progressive systemic infection which killed 95% of the animals within 60 days. Control mice infected with this dose of BCG did not die. The infected THXB mice failed to develop detectable levels of tuberculin hypersensitivity although they did show considerable Arthus (3 h) reactivity. The BCG-infected THXB mice lost weight progressively, and the root spleen and root lung indices increased substantially as the infection proceeded. None of the THXB mice developed an antibacterial immune response to the systemic BCG infection, and this was reflected by the continued persistence of macroscopic lung granuloma in these animals. The BCG-infected control mice developed as many surface tubercles as did the THXB animals, but the granulomas rapidly regressed in size and numbers in the normal mice. The lung changes correlated with the amount of tritiated thymidine incorporated by the lung cells in the later stages of the BCG infection. T cell depletion depressed the early splenic peak normally seen in BCG-infected controls, but, on the other hand, there was a progressive increase in lung counts in the THXB mice as the infection progressed and this late peak was not seen in the control animals. The significance of these findings is discussed in relation to the development of antituberculous immunity by BCG-infected mice.

Acquired antituberculous immunity is known to be mediated by T lymphocytes since it can be shown that both neonatally thymectomized mice (18) and adolescent thymectomized, lethally irradiated, and bone marrow-reconstituted (THXB) mice (9) are unable to prevent the continued growth of *Mycobacterium tuberculosis* H37Rv in vivo. Infusion of normal syngeneic thymocytes into the THXB host restores this ability to express an effective acquired resistance against a subsequent tuberculous challenge. In another experimental model, *M. leprae* was also shown to multiply more extensively in mice subjected to prior T cell depletion (11, 16). *M. leprae* is an obligate human parasite which can multiply to a limited extent in the footpads of normal mice (15) but which is stimulated several hundred-fold by prior T cell depletion (16). However, even in the immunologically compromised host, this organism has a 12- to 14-day mean generation time so that incubation periods of 14 to 20 months are routinely required before the footpad infection (with some nerve involvement) can be demonstrated (13). In contrast, virulent *M. tuberculosis*, even in normal mice, gives rise to an almost acute infection resulting in death within 1 or 2 months of receiving the intravenous challenge dose of 10^6 viable organisms (5). Such a model is thus unsuitable for studies of the chronic effects of T cell depletion on antituberculous resistance. For this purpose, challenge with a partially attenuated strain of *Mycobacterium* would perhaps be more appropriate.

The attenuated strain of *M. bovis* (BCG Montreal) was selected for the present study mainly because of the extensive background of quantitative information available in this laboratory regarding the characteristics of the infection in intravenously infected mice (2, 4). BCG Montreal produces a self-limiting systemic infection in normal mice which is associated with the development of both tuberculin hypersensitivity and an antituberculous immunity (1). Even massive intravenous inocula of this organism failed to induce a progressive, fatal infection in normal mice (2). On the basis of earlier

studies with virulent tubercle bacilli (9), it could be predicted that T lymphocyte depletion should markedly reduce the ability of the host to limit the growth and persistence of even moderate doses of this attenuated strain of *M. bovis*, resulting in the development of a potentially lethal mycobacteriosis in the immunosuppressed host. The results reported in the present paper uphold this prediction.

MATERIALS AND METHODS

Animals. Four-week-old C57Bl/6 × C3H F_1 hybrid mice were maintained under Isocaps (Carworth Lab Cages, New York, N.Y.) on sterile bedding and were fed sterile, vitamin enriched pellets and chlorinated water ad lib. The mice were thymectomized surgically, exposed to 900 R of whole-body irradiation 10 days later, and immediately reconstituted with 2×10^6 normal syngeneic bone marrow cells (9). The mice were maintained on tetracyclines for 4 weeks, being removed from the drug some 7 to 10 days prior to infection. Control mice were thymectomized only or were sham thymectomized, irradiated, and bone marrow reconstituted (XB) before being tested under conditions similar to those used for test group. Normal untreated mice were always included in each study. At sacrifice, the thymectomized mice were examined to ensure that the thymus had been removed.

Organisms. *M. bovis* (BCG Montreal; TMC 1012) was obtained from the Trudeau Mycobacterial Culture Bank, Saranac Lake, N.Y. The organism was grown at 37 C for 8 days in modified Sauton medium on a magnetic stirrer (6). The logarithmic growth was diluted 1:10 with fresh Sauton medium, frozen in 1-ml ampoules, and stored at −70 C (8). The viability of the preparation was checked by rapidly thawing one ampoule at 37 C, homogenizing the suspension (3), diluting it suitably in sterile saline, and then plating onto Middlebrook 7H10 agar (Difco). The plates were sealed in plastic bags and incubated at 37 C for 3 to 4 weeks, the resulting colonies were counted, and the number of viable organisms per milliliter was calculated.

Infection. Groups of 100 THXB, XB, and normal control mice of similar age were each infected intravenously with 2×10^6 viable BCG Montreal cells in a volume of 0.1 ml of saline. The viability of the inoculum was checked immediately after injection by plating suitable 10-fold saline dilutions on 7H10 agar plates. Each mouse was weighed prior to sacrifice, and bacterial counts were carried out on selected organs at frequent intervals for up to 3 months.

Enumeration of the in vivo population. Groups of five randomly selected mice were killed, and the test organs were removed aseptically, weighed, and homogenized separately in sterile saline (4). Suitable saline dilutions were then plated on 7H10 agar and incubated in sealed bags for 3 to 4 weeks. A 0.1-ml sample of heart blood was also inoculated onto 7H10 agar. One femur from some of the mice was removed; the marrow plug was washed out with 2 ml of sterile saline, and the homogenized suspension was plated on 7H10 agar.

Cellular proliferation in the host. Proliferation of cells in the lungs and spleens was determined by pulse labeling with tritiated thymidine injected into the mice 30 min before sacrifice. An intravenous injection of 20 μCi of tritiated thymidine ([^{3}H]TdR) with specific activity of 3 Ci/mmol (New England Nuclear Corp., Boston, Mass.) was given (9). Then, known weights of lung and spleen were extracted with trichloroacetic acid, and the [^{3}H]deoxyribonucleic acid (DNA) was counted in a Beckman LS-100 liquid spectrometer (10).

Tuberculin sensitivity. Groups of five mice were injected in one hind footpad with 2.5 μg of purified protein derivative (Lederle Laboratories) in 0.03 ml of diluent, and the increase in foot thickness was measured with a dial gauge calipers (Schnelltäster, Kroplin) at 3 and 24 h (2). An increase of more than 1.8 units (0.18 mm) in foot thickness was significant at the 1% level.

Lung granuloma counts. The surface of the formalin-fixed lung was examined with a 10 × Zeiss plate microscope for macroscopic lesions with the use of incident lighting. The mean number of lesions per unit area ± standard error was determined.

Root mean spleen or lung indices. The organ was removed, blotted dry and freed of macroscopic blood clots, and weighed. The root organ index (17) was calculated as

$$\sqrt{\frac{\text{spleen or lung weight (g)} \times 100}{\text{whole animal weight (g)}}}$$

The average indices for the five organs at any one time point were then plotted.

RESULTS

Growth of BCG Montreal in T cell-depleted mice. After the infection of 100 THXB mice via a tail vein with 2×10^6 viable BCG Montreal cells, there was a progressive increase in the number of viable organisms recovered from the lungs, spleen, and bone marrow over a 50-day period (Fig. 1). The amount of bacterial growth within the liver was minimal, and there was no sign of an immune response in this organ between days 14 and 20 (4). The spleen population increased about 10-fold over the first 20-day period, but then it remained at about 10^6 viable bacilli for the remainder of the experiment. The greatest amount of growth by the BCG population was seen in the lung (Fig. 1). After an initial 7-day lag, there was a near 1,000-fold increase in viable counts over the next 40 days. Eventually the lung population went into a stationary phase which lasted for the remainder of the experiment. One surprising feature of the bacterial growth data was the rapid and extensive increase in the bone marrow population, which reached as high as 10^4 viable bacilli per femur plug by day 50. Bearing in mind that this count was carried out only on a single femur, the number of BCG in the total

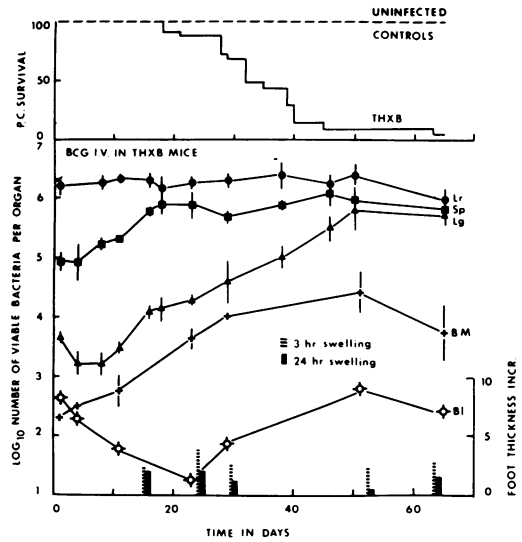

FIG. 1. *Growth curves of BCG Montreal after intravenous inoculation into THXB mice. Lr, liver; Sp, spleen; Lg, lungs; BM, bone marrow; and Bl, blood. The vertical bars represent the standard error of the mean. The broken histograms represent the 3-h footpad swelling, and the solid bars represent the 24-h delayed tuberculin hypersensitivity following injection of 2.5 μg of purified protein derivative in a hind footpad. The top curve represents the percent survival by the infected THXB mice (solid line) and the uninfected THXB controls (broken lines).*

bone marrow compartment must have been very substantial. Counts of the BCG population in the circulating blood during the challenge period initially fell to nearly undetectable levels (Fig. 1). However, as the systemic BCG population developed, so an increasing bacteremia was seen until a maximum of almost 10^3 organism per ml was observed on day 50.

The THXB mice failed to develop detectable levels of tuberculin footpad reactivity although there was some 3-h (Arthus) reactivity (Fig. 1). This early swelling was sufficient to make an assessment of the true nature of the 24-h swelling in the THXB mice very difficult. Much of this swelling probably represented residual Arthus or Jones-Mote reactivity rather than a true tuberculin type of hypersensitivity. Control mice infected with the living BCG developed significant levels of tuberculin hypersensitivity by the fifteenth day of the infection (Fig. 2). There was also considerable 3-h reactivity to the tuberculin injection as the infection progressed and the immune response became established. However, the relative sizes of the 3- and 24-h reactions seen at this time made it clear that considerable delayed-type hypersensitivity existed in these animals.

The BCG growth curves seen in the normal control mice were similar to those described earlier (2, 4). An immune response developed against both the spleen and lung populations, starting about day 16 of the infection (Fig. 2). The lung population increased only about 50-fold before it passed into a stationary phase lasting until day 60, when the viable BCG populations in all three test organs slowly declined. The blood was cleared of viable BCG very rapidly with no subsequent bacteremia. The bacterial population within the bone marrow from the femur showed an approximately five-fold increase with about 10^3 BCG cells per femur plug present on day 25. At this level of infection, the marrow population represented a much smaller component of the overall systemic infection than was the case for the T cell-depleted animal. The corresponding bacterial growth curves obtained in thymectomized but not irradiated controls or in the sham thymectomized, irradiated, bone marrow-reconstituted (XB) animals (Fig. 3) generally resembled those obtained with untreated controls. Both groups of controls developed significant levels of delayed-type hypersensitivity at about the time when the antibacterial immune response to the bacterial population was first noted.

Mortality following BCG infection of THXB mice. The mortality represented in the survival curve shown in Fig. 1 indicated that the T cell-depleted mice were highly susceptible to the lethal effects of the uncontrolled growth of the BCG, showing a mean time to death of only 35 days. By day 65, 95% of the BCG-infected THXB animals were dead, compared with no deaths at all up to day 150 in the normal controls infected with BCG. The uninfected THXB mice showed an overall mortality rate of

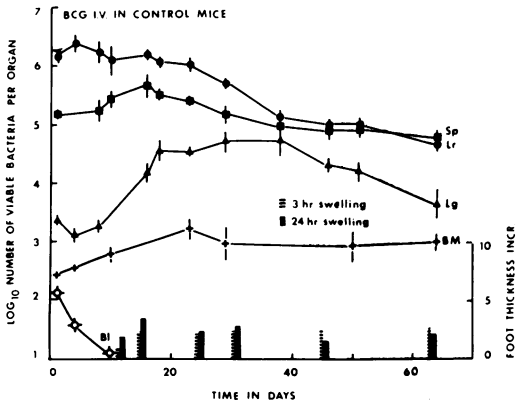

FIG. 2. *Growth curves for BCG Montreal in intravenously infected control mice. (See legend to Fig. 1 for details.)*

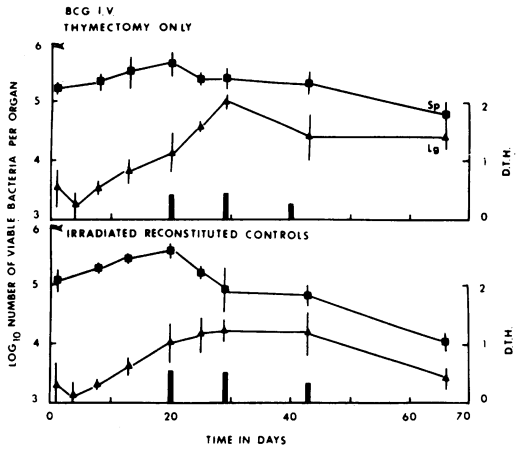

FIG. 3. *Growth curves for BCG Montreal in mice subjected to thymectomy with no irradiation (top) or sham thymectomy, lethal irradiation, and bone marrow reconstitution (bottom) prior to intravenous challenge. The histograms represent 24-h tuberculin reactivity.*

5% over this same time period.

Weight changes in response to the systemic BCG infection. The average body weight gain by uninfected C57Bl × C3H F_1 mice was 8 g during the course of this experiment (Fig. 4). In contrast, the THXB animals gained only about 3 g. The effect of the BCG infection on the normal body weight increase was negligible. In the THXB mice infected with 10^6 viable BCG cells, however, there was an early weight gain of about 1 g by day 18, followed by a steady decline in total body weight, which averaged 6 g by the end of the experiment. The period of the major decline in weight coincided with the maximum mortality seen in these animals (Fig. 1).

Under the influence of the BCG infection, the average spleen weight in the normal mouse increased significantly (Fig. 8). A similar but smaller increase also occurred in the lung. However, because of the changes in total body weight (Fig. 4), such absolute changes may mean relatively little. Several workers have recommended that such organ weight changes be expressed as a root mean organ index (17). When expressed in this form, the spleen weights for the BCG-infected control mice were seen to increase very sharply between days 15 and 25, coinciding with the period of maximum immunity expressed by this organ (Fig. 5). On the other hand, the curve for the THXB mice increased substantially, but the T cell-depleted animal required 50 days to reach a peak, with no subsequent decline equivalent to that seen in the controls. These increases with time were partly due to the declining body weight of the THXB mice but also represented a steady, progressive increase in spleen weight under the influence of the BCG infection (Fig. 9).

The root mean lung index curve for the infected THXB mice (Fig. 6) increased slowly in parallel with that for the controls until day 30, when a sharp peak in the THXB lung curve was observed to day 45. This increase approximated to the time of cessation of growth by the lung BCG population in these animals. The index curve for the normal control lungs showed no such peak at this time but showed a slow gradual increase throughout the bacterial infection (Fig. 6).

Lung granuloma in THXB versus normal mice. Following the intravenous challenge of the T cell-depleted mice, there was a period of 30 days before any visible change occurred in

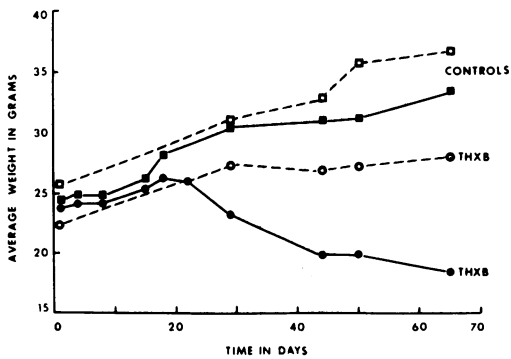

FIG. 4. *Changes in whole body weight in normal uninfected mice (□), BCG-infected controls (■), uninfected THXB mice (○), or BCG-infected THXB mice (●).*

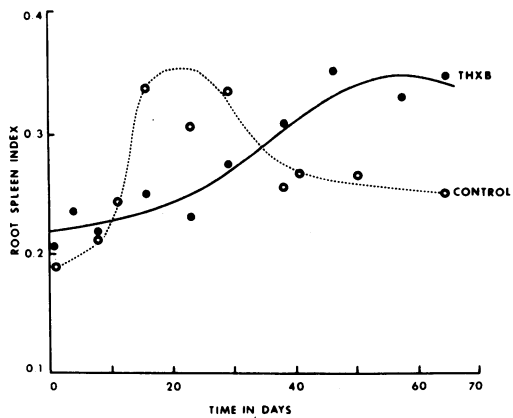

FIG. 5. *Root mean spleen index values for normal control (○) or THXB mice (●) after intravenous BCG infection.*

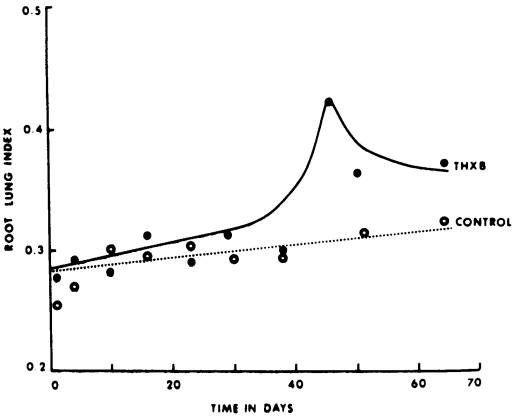

FIG. 6. *Root mean lung index values for control (○) or THXB mice (●) after BCG infection.*

the appearance of the lung surface (Fig. 7). Then a few diffuse white granulomatous patches appeared on the lung surface. The number of lesions increased rapidly to a maximum of 160 per lung lobe on day 33 and then remained constant at this level for the next 30 days. Individual lesions slowly increased in size, and many of them coalesced with time, producing a patchy, raised surface over most of the lung. In the normal control mice, a small number of very small focal lesions were detected on day 14, rising to 150 lesions per lobe on day 24 (Fig. 7). The numbers then fell rapidly to less than 25 per lobe by day 50. By day 65, only a few residual pinpoint lesions could still be observed. Unlike the THXB curve, this decline in the control tubercle counts was not due to coalescence but was indicative of the immune response to the infectious agent.

Proliferative responses to the intravenous BCG infection. The introduction of living BCG into the tissues was followed by a sharp increase in the rate of incorporation of [^{3}H]TdR by cells in both the spleen and the lungs of normal mice (Fig. 8). The peak in splenic incorporation occurred on day 18, coinciding with the maximum spleen weights. Both organ weight and total incorporation thereafter declined to reach near normal values by day 65. When this incorporation rate was expressed as counts per unit organ weight, little change was observed in the uptake of [^{3}H]TdR by the spleen. The [^{3}H]TdR incorporation rate by the BCG-infected lung cells also peaked on day 18 and again on day 28, before declining steadily to near normal values (Fig. 8). The lung weight increased by 50% over the first 20 days or so; the incorporation rate per unit weight of organ represented only a threefold increase in proliferative activity. The first lung peak occurred at the same time as the spleen peak, but it was the second lung peak in [^{3}H]DNA that seemed to coincide with the immune response to the BCG population in that organ (Fig. 2).

The [^{3}H]TdR incorporation curves for the THXB mice was at variance with the normal data in a number of key areas. The splenic peak which had been so obvious in the normal mice

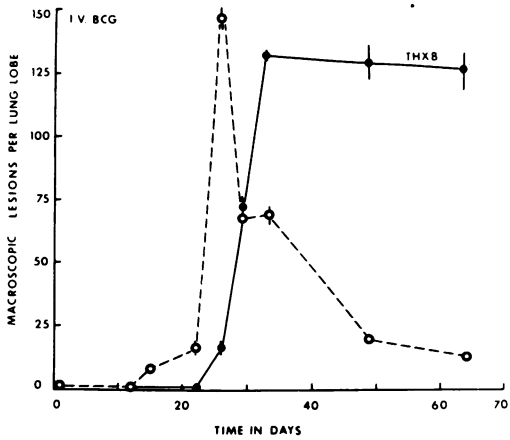

FIG. 7. *Number of macroscopic surface lung lesions in control (○) or THXB (●) mice infected intravenously with BCG Montreal.*

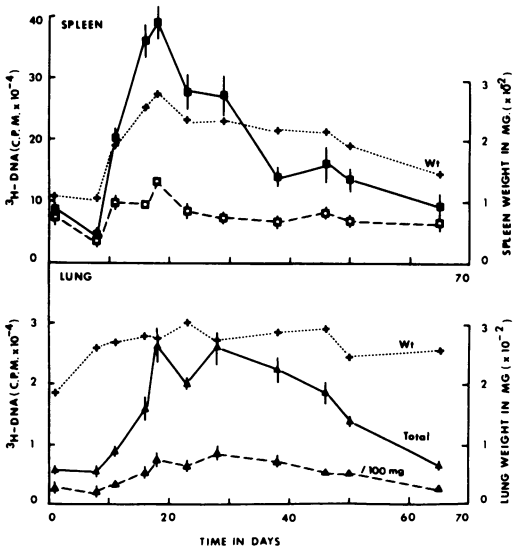

FIG. 8. *^{3}H-TdR incorporation curves for spleen (top) and lung (bottom) in normal mice infected intravenously with BCG Montreal. Closed symbols represent the total incorporation per organ. The open symbols represent the incorporation per 100 mg of tissue. Wt = organ weight in milligrams.*

on day 18 was almost completely ablated in the THXB mice (Fig. 9). There was, however, a minor peak in the THXB spleen about day 48 at the time when the normal [^{3}H]TdR incorporation curve was rapidly returning towards preinfection values. In the lungs of the THXB mice, the [^{3}H]DNA curve showed a double peak, the first on day 18 (corresponding to the small one seen in the THXB spleen), with a somewhat larger one on day 48. Both the absolute spleen and lung weights increased only slightly during the BCG infection, and the incorporation rate per unit weight of spleen remained almost stationary throughout the experiment. The lung incorporation per unit weight increased as much as fivefold, with a plateau from day 18 onwards.

Histological characteristics of the tissue response in the THXB mouse. Microscopic examination of the lungs of the THXB, compared with control mice, when some 20 days into the BCG infection (selected to coincide with the time of emergence of the granulomatous response in the controls; Fig. 7) indicated that few qualitative differences existed between the cellular tissue responses in the lungs of the two groups of animals at that time. The splenic histology of the THXB mice was consistent with that already described earlier for the T cell-depleted animal (9) in which lymphocytes were limited to the germinal centers, with a marked depletion of lymphocytes within the periarteriolar sheaths. The infiltrate which appeared in the lungs of the THXB mice consisted of both polymorphonuclear leukocytes and many large mononuclear phagocytes, most of which aggregated in the lung tissues to give rise to a diffuse granuloma. At this time, lymphoid cells were conspicuous in the infiltrates seen in the control lungs, particularly in the perivascular regions and within the developing granulomas themselves. In the corresponding regions of the THXB preparations, very few lymphocyte-like cells could be detected, but already there was a well-developed plasmacyte response within the granuloma. By day 50, the lungs of the THXB mice contained enormous aggregations of mononuclear cells (with a few polymorphonuclear leukocytes), many of them with the appearance of foamy macrophages and giant cells, together with many mature plasma cells. Very few lymphocyte-like cells could be detected within the diffuse granulomatous lesions at this time. There were, however, signs of an interstitial edema (presumably accounting in part for the sharp increase in lung weight and root lung index shown on day 45 in Fig. 6). In mice surviving for more than 90 days, a diffuse interstitial fibrosis was frequently observed, together with thickening of the basement membrane of the blood vessels in the lung. Ziehl-Neelsen stained sections revealed numerous acid-fast bacilli localized within the lung granulomas. On the other hand, the histopathology of the lungs of the normal control mice had substantially returned to normal by day 50, and no stainable bacilli could be detected within the normal lung sections taken 65 days after the BCG infection.

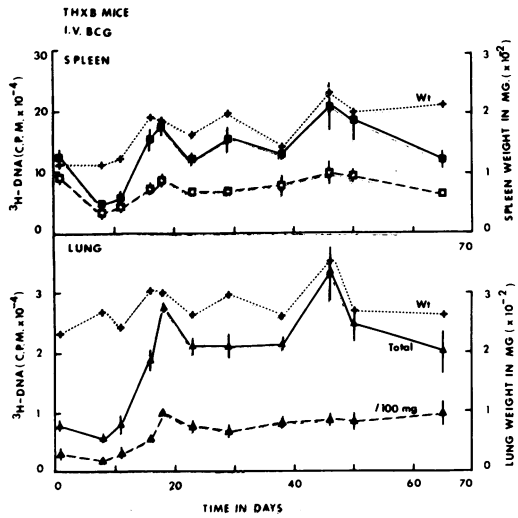

FIG. 9. *^{3}H-TdR incorporation curves for spleen (top) and lung (bottom) in THXB mice infected intravenously with BCG Montreal.*

DISCUSSION

The most interesting finding to emerge from the present study was the ability of BCG Montreal to induce a progressive, fatal infection in the T cell-depleted mice. In fact, the survival curve shown in Fig. 1 resembles that reported earlier for normal mice infected with the highly virulent *M. tuberculosis* strain Erdman (5). The infection caused by the attenuated mycobacteria in the T cell-depleted mouse still interfered with the host's metabolism just as effectively as the most virulent strain of tubercle bacillus, provided that the normal braking action of the cell-mediated immune response on the unrestricted growth of the organism was first removed. The residual T cell population in the thymectomized, nonirradiated controls was still sufficient to give rise to an essentially normal immune response to a BCG challenge (Fig. 3). However, in the absence of these cells, the attenuated organisms rapidly spread to involve all of the lymphoreticular organs of the THXB host, as well as the bone marrow, in an ongoing

bacterial infection. Despite the increasing mononuclear response to the infectious agent, the host was unable to control the increasing bacteremia seen in these animals. The BCG growth curves shown in Fig. 1 indicate that growth of the organisms continued in the lungs of the THXB mice for 40 days after which it suddenly passed into a stationary phase for the remainder of the experiment. At this time, the lung population constituted the major component of the bacterial involvement in vivo. The late drop in viable counts seen in the lung may have been more apparent than real, since it occurred after 95% of the THXB mice had died, and these last few counts may have been carried out on a more resistant subpopulation of mice. The plateau effect was also seen in the spleen and liver counts for these mice. However, the liver counts did not change substantially at any time during the study, and there was no indication that an immune response occurred in this organ in the T cell-depleted host.

Truitt and Mackaness (19) commented on the decreased ability of lung macrophages to express an effective immunity against a number of bacterial agents compared with that routinely observed in the corresponding liver and spleen cells. Macroscopic examination of the lung surface in BCG-infected mice revealed a significant difference in the rate of development, persistence, and ultimate regression of visible surface tubercles in the THXB and control animals. The total number of lesions per lobe in both groups was essentially the same, but there was a significant difference in the timing of the peak responses (Fig. 7). Perhaps more significantly, the number of lung tubercles in the THXB animals remained elevated throughout the remainder of the experiment, whereas the control counts quickly fell to zero. Microscopic examination of the chronically infected THXB lungs showed evidence of an extensive edema and fibrotic consolidation, and this correlated with a progressive loss of lung buoyancy in water. This picture bears a striking resemblance to that described by Gray et al. (7) working with normal C57Bl mice heavily infected with virulent *M. tuberculosis* H37Rv. Here, death was ascribed to the extensive granuloma formation and consolidation (20) seen in such lungs, which progressed to the point where the vital capacity of the lung was sufficiently reduced that death by asphyxiation ultimately occurred. It was possible that death was due to an unsuspected intercurrent infection in the immunosuppressed animals. To check this possibility, lung homogenates were prepared from moribund animals and plated on a wide variety of media, including one suitable for culturing mycoplasmas. Examination of the plates 7, 14, and 28 days later indicated that BCG was the only microorganism present in significant numbers in these preparations.

One noteworthy feature of the intravenous BCG infection in both THXB and normal mice was the size of the resulting bone marrow populations. The BCG plate counts for the marrow cultures for the control animals rose only about fivefold, but in the THXB mouse the total contribution by the bone marrow population of BCG constituted a substantial portion of the total BCG population present within the tissues. The effect of such a progressive infection within the bone marrow itself is not presently clear, but it might well be expected to contribute to the general state of debilitation and weight loss noted as the infection progressed. The bacteremia and bone marrow involvement is reminiscent of the rapid and extensive hematogenous spread by tubercle bacilli during miliary tuberculosis (14). Bacterial counts were also carried out on a number of lymph nodes in the THXB mice, and the results of these counts were also consistent with a rapidly disseminating type of fulminating disease progressively involving all of the host tissues.

From the pulse labeling data, it can be seen that T cell depletion markedly depressed incorporation by BCG-infected spleen cells, in a fashion analogous to that reported by North (9) for the livers and spleens of H37Rv-infected mice. In the normal animal, the peak incorporation occurred about the time that the emerging immune response was first observed. However, in the THXB animal there was no developing acquired resistance, and this correlated with the 40 to 50% reduction in the rate of incorporation of [^{3}H]TdR by the liver and spleen cells. This reduction also correlated with the smaller number of highly labeled lymphocytes seen within the developing lesions in the THXB mouse. As the incubation period continued, a peak in spleen incorporation was seen around day 40 to 50 of the BCG infection in the THXB mice. A similar increase in [^{3}H]TdR incorporation in the THXB lung cells was also seen during this later phase of the infection. The slow, almost imperceptible increase in uptake per unit weight of lung tissue contrasted strikingly with the corresponding decline seen in the control lungs (Fig. 8). This peak difference was as much as fivefold and, combined with the unresolving granuloma seen in the THXB lungs, was presumably due to the almost complete absence of immunocompetent T cells in these animals.

The presence of increasing numbers of mature macrophages containing five or more acid-fast bacilli per cell in both spleen and lung indicated that the mere presence of increasing numbers of mononuclear phagocytes in the infectious lesion was not sufficient to control the further growth of this attenuated microorganism in vivo. It is only with the introduction of syngeneic immunocompetent lymphocytes (9, 13) or by the reimplantation of an intact thymus (12) into the THXB host that a complete restoration of the ability to mount a cell-mediated immunity can be again observed. It is apparent that T cell depletion does not increase the tempo of the chronic infection appreciably. The BCG infection involved other organs of the body, but it was the increasingly severe tissue reponse seen in the lungs which eventually led to the fatal outcome of the disease. There are obviously considerable differences between the infections caused by an intravenous dose of BCG and by *M. leprae* growing in the footpads of T cell-depleted mice. Perhaps the most striking difference is the absence of extensive tissue changes in such central organs as the lung and spleen in the *M. leprae* mice. Despite this difference, however, the present experimental model does offer a real opportunity to study the effect of immunosuppressive manipulations on the tissue responses to a chronic intracellular parasite which is known to require a fully developed cell-mediated type of immunity before the infection can be brought under effective control.

ACKNOWLEDGMENTS

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