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T-Cell-Mediated Protection of Mice against Virulent *Mycobacterium tuberculosis*

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We sought to protect CBA mice against tuberculosis using in vivo transfer of a T-cell line previously shown to be capable of I-A-restricted recognition of peritoneal macrophages infected in vitro with *Mycobacterium tuberculosis*. This line induces total bacteriostasis in vitro. In mice that received 500 rads of irradiation 48 h before infection, the T-cell line caused significant prolongation of life when given intravenously with a challenge dose of 5×10^6 organisms. Similar experiments with two other T-cell lines showed that these lines offered no protection. Bacterial load at the time of death was inversely related to the time of survival. Thus, death occurred at a lower bacterial load in adoptively protected mice, implying the contribution of an immunopathological component in these animals. The protective T-cell line, which was $CD4^+$ $CD8^-$, had no effect on the rate of growth of strain BCG in CBA *nu/nu* mice or *M. tuberculosis* in fully T-cell-deprived mice. This could indicate that $CD8^+$ cells play a role in this system or that there is a need for the recruitment of interleukin 2-producing cells in the recipient. Experiments with monoclonal antibodies to selectively deplete T-cell subsets in normal CBA mice showed that depletion of $CD4^+$ cells strikingly shortened survival, whereas depletion of $CD8^+$ cells did not. However, CD8-depleted mice died with a lower bacterial load than those found in nondepleted controls, and the lesions in CD8-depleted mice were histopathologically distinct. These results suggest that the $CD8^+$ cells either down-regulate bacteriostasis or cause immunopathology in this model and that it is the $CD4^+$ cells that are the major protective subset in long-term protection experiments.

We have reported previously (13) that a T-cell line derived from CBA mice immunized with *Mycobacterium tuberculosis* is capable of recognizing tuberculosis-infected murine macrophages in a I-A-restricted manner and, subsequently, of inducing total stasis of the organisms in these cells. The relationship of this phenomenon to true protection in vivo is unclear for several reasons. First, we have never observed a convincing kill of *M. tuberculosis* in these experiments, and bacteriostasis could result in the establishment of persisting organisms rather than a cure (1). Second, tuberculosis is a disease with a complex pathology, involving tissue damage in the lesions and correlating in humans and guinea pigs with necrotic skin test responsiveness to soluble antigens of *M. tuberculosis*. The necrosis in the lesions is partly due to the unexplained inherent toxicity of *M. tuberculosis* for macrophages and partly to immunopathological mechanisms. The immunopathological mechanisms probably involve locally released macrophage products, including cytokines (14). The study in vitro of control of intracellular *M. tuberculosis* proliferation does not address these aspects of the disease. Moreover, in vivo experiments also fail to do so if the only endpoint measured is the proliferation of the bacilli over a period of a few weeks.

We therefore undertook a series of studies in which infected animals were left until they were moribund, and the time of survival was considered in relation to the bacterial load and the pattern of histopathology at the time of death. The T cells were manipulated by the adoptive transfer of T-cell lines, intravenous administration of cytotoxic rat immunoglobulin G2b (IgG2b) monoclonal antibodies to the major T-cell subsets, T-cell depletion by lethal irradiation, and the use of nude mice.

MATERIALS AND METHODS

Animals. Female CBA/Ca mice were bred in our own animal house and were used at 6 to 10 weeks of age. Homozygous CBA *nu/nu* mice were obtained from the National Institute for Medical Research (Mill Hill, United Kingdom). T-cell-depleted mice were generated by thymectomy, lethal irradiation (900 rads), and bone marrow reconstitution by a standard protocol (5).

Organisms. *M. tuberculosis* H37Rv was used in this study and was a gift from J. Grosset (Paris, France). This strain has been passaged in CBA mice in our laboratory since 1985. The organisms used in the present experiments were cultured from infected spleens and were then subcultured once on Lowenstein-Jensen medium. The organisms were harvested into tissue culture medium and then reduced to a suspension of single organisms and counted as described previously (13). This suspension was stored in aliquots at 2×10^8/ml in liquid nitrogen and was thawed rapidly once before use. The BCG strains from Glaxo Ltd. and the Institut Pasteur were subcultured from the vials provided by the manufacturers and were then prepared and stored as described above.

T-cell depletion with monoclonal antibodies. The preparation, characteristics, and protocol for the use of the rat IgG2b monoclonal antibodies used here have been described in detail elsewhere (3, 4, 8). Monoclonal antibody YTS 191.1 is specific for a monomorphic CD4 determinant that is expressed on murine class II-restricted T cells, and monoclonal antibody YTS 169.4 is specific for a monomorphic

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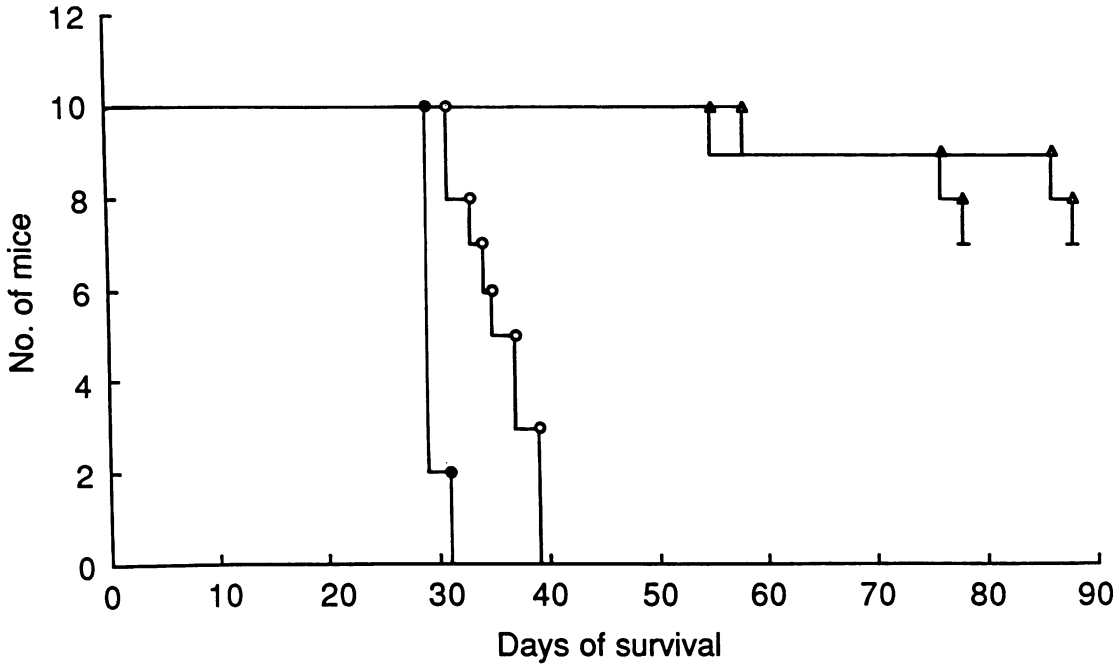

FIG. 1. Survival curves for female CBA mice given 2×10^7 (●), 7×10^6 (○), 2×10^6 (▲), or 7×10^5 (△) H37Rv by intravenous injection 24 h after they were exposed to 500 rads of irradiation.

CD8 determinant. These antibodies are extremely effective at eliminating their respective T-cell subsets in vivo. The depletion persists in thymectomized animals. The protocol used here was performed exactly as described in earlier publications (3, 8). Briefly, female CBA mice were thymectomized at 6 to 7 weeks of age. One month later 400 μg of the appropriate monoclonal antibody was administered intravenously. Three days later they received an additional 400 μg of monoclonal antibody intraperitoneally and were then immediately infected intravenously with 5×10^6 H37Rv.

Eighteen days after the last treatment with the monoclonal antibody, representative mice were sacrificed, and cytospins of the splenocytes were prepared. These were incubated in the same preparations of the rat monoclonal antibodies diluted 1/50 for 45 min. Then, the binding of the monoclonal antibodies to lymphocytes was revealed with a biotinylated rabbit anti-rat immunoglobulin (20 μg/ml for 30 min; Vector, Peterborough, United Kingdom) and then with a Avidin-peroxidase complex (1/500 for 30 min; Amersham International, Amersham, United Kingdom) and diaminobenzidine. Cell counts were performed blind. In spleens from untreated control mice, 14% of the lymphocytes stained with anti-CD4 and 10% stained with anti-CD8. After in vivo depletion with anti-CD4, no $CD4^+$ cells were detectable in the spleen, whereas following CD8 depletion there were still 3% $CD8^+$ cells present. These findings were closely comparable to those found in the study of Muller and colleagues (8), who determined that $CD4^+$ cells were reduced from 16.9 to 2% and that $CD8^+$ cells were reduced from 12.6 to 2.7%.

T-cell lines. The T-cell lines used here were raised and propagated as described in detail elsewhere (2). PPD9A is a $CD4^+$ T-cell line which recognizes purified protein derivative (PPD) in association with $I\text{-}A^k$ class II molecules.

In order to grow large quantities of the cell lines for cell transfer experiments, portions of the cells stored in liquid nitrogen were washed, suspended in medium (Dulbecco modified Eagle medium; GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum and supplements (2), and seeded at approximately 10^6 cells per 25-cm^2 flask (Nunc, Roskilde, Denmark) with 2×10^7 irradiated (3,000 rads) CBA spleen cells and 25 μg of PPD (Central Veterinary Laboratory, Weybridge, Surrey, United Kingdom) per ml. Cultures were split to two or three new flasks as necessary, and the cells were restimulated after 7 days, as described above. When sufficient growth had occurred, cells either were pooled and washed 10 days after the last restimulation or were restimulated in the usual way at 7 days and harvested 3 days later. The selected number of restimulated or nonrestimulated cells was injected intravenously in 0.2 ml of Dulbecco modified Eagle medium.

Bacterial counts. Organs from strain BCG-infected *nu/nu* mice were homogenized in saline, and serial 10-fold dilutions were plated onto Middlebrook 7H10 medium (Difco Laboratories, Detroit, Mich.) and incubated at 37°C. Colony counts were performed when suitable growth had occurred, which was at approximately 3 weeks.

Organs from mice infected with strain H37Rv were fixed in 10% Formalin in normal saline for at least 1 week. They were then homogenized in a known volume of saline, and 1 μl was spread onto a known area of a glass slide. These preparations were stained with auramine, the organisms were counted blind with a fluorescence microscope, and the counts per organ were calculated.

RESULTS

Selection of the optimal dose of *M. tuberculosis*. Portions of the standard suspension of strain H37Rv were recovered from the liquid nitrogen store, thawed rapidly, and diluted in RPMI 1640 medium. Selected doses were injected intravenously in 0.2 ml of RPMI 1640 medium into female CBA mice (age, 8 to 10 weeks) 24 h after they had received 500 rads. It was noted that there was a sigmoid dose/survival curve (Fig. 1). Thus, at a dose of 2×10^6 organisms there was prolonged survival that was relatively unaffected by an additional threefold reduction in dose. Between 2×10^6 and

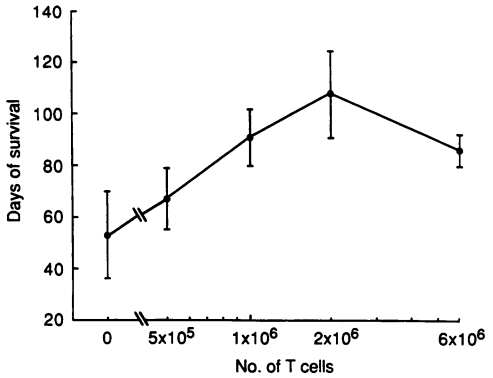

FIG. 2. Mean survival (± standard deviation) of groups of female CBA mice exposed to 500 rads of irradiation and then given 5×10^6 H37Rv, together with the indicated number of T cells of line PPD9A, by intravenous injection.

7×10^6 there was a sudden decrease in survival, while a further increase in the dose to 2×10^7 made little difference (Fig. 1). To check that the critical part of the dose/survival curve fell in the same place when mice given a dose of 500 rads of irradiation were reconstituted with spleen cells, a second experiment was performed in which 8×10^7 normal spleen cells were injected intravenously immediately before infection. The same sigmoid curve was found (data not shown). We concluded that doses between 2×10^6 and 7×10^6 would be most likely to reveal the effects of T-cell manipulations.

Protection with spleen cells from strain BCG-immunized donors. To confirm that we established optimal conditions for demonstrating prolongation of survival mediated by transferred spleen cell populations, mice irradiated with 500 rads were infected intravenously with 5×10^6 H37Rv immediately after receiving intravenous injections of 8×10^7 cells from normal or BCG-immunized mice. The mice, which were all given 500 rads of radiation 24 h before cell transfer, survived the indicated number of days (mean ± standard deviation) when the following cells (8×10^7) were transferred: normal spleen cells, 60.2 ± 21.4; normal splenic T cells (enriched by passage down nylon wool columns), 75.25 ± 13.8; immune spleen cells (donors received 10^8 BCG [Glaxo Ltd.] intravenously 14 days previously), >110 (6 of 7 mice were still alive at 110 days); immune spleen T cells (enriched by passage down nylon wool columns), >110 (all mice were still alive at 110 days); and BCG spleen cells from cyclophosphamide (25 mg/kg 24 h before BCG administration)-pretreated donors, >110 (all mice were still alive at 110 days). Only one recipient of any of the three preparations of immune spleen cells died by 110 days, when the experiment was terminated. In contrast, all the recipients of normal cells died. Normal spleen cells consistently failed to confer any protection in an additional series of pilot experiments, and they sometimes led to decreased survival times (data not shown).

Prolongation of survival of infected CBA mice with T-cell lines. Using the protocol established above, doses of the PPD-responsive T-cell line (PPD9A) of between 5×10^5 and 6×10^6 were given intravenously with strain H37Rv (Fig. 2). Control mice began to die on day 29, and all were dead by day 72 (mean survival, 52 ± 16.9 days), whereas recipients of more than 5×10^5 PPD9A were protected significantly ($P < 0.01$ by the nonparametric Mann-Whitney U test for 1×10^6 and 6×10^6 T cells and $P < 0.001$ for 2×10^6 T cells). The best protection was achieved with 2×10^6 PPD9A (mean survival, 107.8 ± 16.9 days). All animals in this group were alive on day 72. Some groups of mice were given T cells that were restimulated with antigen before injection, but this did not appear to influence the prolongation of survival.

No protection was achieved with a T-cell line responsive to thyroglobulin or with another PPD-responsive line (PPD13P) (data not shown).

Organ weights and bacterial load at the time of death. It was noted that both the weight of the lungs and the number of bacteria present at the time of death were inversely related to the number of days the mice survived. Thus, the prolonged survival of the PPD9A-protected animals correlated with a lower lung weight and a lower bacterial load, implying that the T-cell line effectively caused inhibition of organism growth and that the immediate cause of death in mice protected in this way was different from the cause of death in unprotected mice.

Failure of the T-cell line PPD9A to protect severely T-cell-deficient mice. Strain BCG (Institut Pasteur) was able to grow progressively in CBA *nu/nu* mice but showed little growth in normal CBA mice. We therefore tested the ability of the T-cell line to inhibit growth of BCG in *nu/nu* animals. *nu/nu* CBA mice received 1×10^6 BCG (Institut Pasteur) intravenously, together with 3×10^6 or 3×10^5 PPD9A. After 30 days the numbers of viable BCG in the spleens, lungs, livers, and kidneys were assessed by colony counting. No effect of the T-cell line was seen (data not shown). Similarly, PPD9A did not influence the survival of thymectomized, lethally irradiated, bone marrow-reconstituted CBA mice after infection with 2×10^6 H37Rv (data not shown). These results indicate that there is a possible requirement for $CD8^+$ cells or interleukin 2-producing cells of host origin.

Effect of selective depletion of CD4 or CD8 cells. Thymectomized CBA mice were depleted of CD4 or CD8 cells as described above. These animals and controls that were thymectomized but not depleted of either cell subset and controls with intact thymuses were infected intravenously with 5×10^6 H37Rv (Fig. 3). Neither thymectomy alone nor thymectomy followed by depletion of $CD8^+$ cells had any effect on the average survival, which was approximately 90 days for these groups of mice. However, thymectomy followed by depletion of the CD4 cell subset resulted in the death of all animals by day 50 (mean survival, 40.2 ± 1.4 days). If the $CD8^+$ cells were also depleted, deaths began 8 days earlier, but the mean survival (37.8 ± 2.5 days) did not differ significantly from that seen following CD4 depletion alone.

Effect of depletion of T-cell subsets on the bacterial load at the time of death. As described above in the experiments with PPD9A, the bacterial load at the time of death showed a striking inverse correlation with the numbers of days survived. This was true in lung (Fig. 4), liver, kidney, and spleen cells (data not shown).

Depletion of $CD8^+$ cells did not influence the numbers of organisms present in CD4-depleted mice. Similarly, thymectomy did not influence the bacterial load at the time of death in mice that were not depleted of either cell subset. However, animals that were depleted of $CD8^+$ cells died with lower bacterial loads than either the thymectomized or the nonthymectomized controls, although these three groups died over the same time period.

Effect of $CD8^+$ cell depletion on histological appearance at the time of death. The lower bacterial load seen in CD8-depleted mice at the time of death was apparent in histological preparations and correlated with a less necrotizing pattern of histology. Thus, in mice in which $CD8^+$ cells were

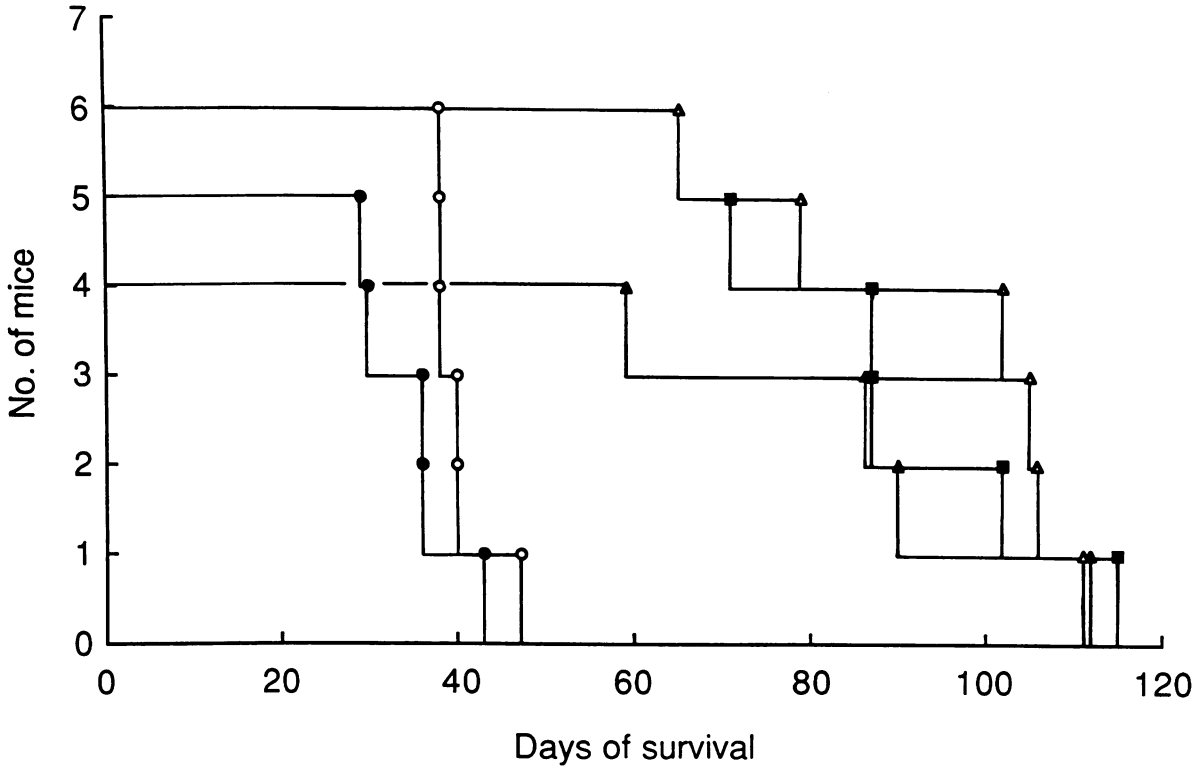

FIG. 3. Groups of female CBA mice were thymectomized and were depleted of $CD4^+$ cells (○) or $CD8^+$ cells (■) or both (●) by using monoclonal antibodies, as described in the text. Controls were nondepleted, thymectomized mice (▲) and nonthymectomized normal mice (△). All animals received 5×10^6 H37Rv by intravenous injection.

not depleted, there was much lung necrosis with foci of degenerating macrophages and polymorphonuclear cells. There were no tuberculoid granulomata (Fig. 5A), and acid-fast bacilli were plentiful (>100 per field at ×400 magnification) (Fig. 5B) and evenly distributed. In contrast, lungs from mice that were depleted of $CD8^+$ cells showed less inflammation, lung necrosis, and polymorph infiltration. Macrophages were focally coalescing to form ill-defined granulomata, and multinucleate giant cells were apparent (Fig. 6A). Acid-fast bacilli were less plentiful (10 to 100 per field) and less evenly distributed (Fig. 6B).

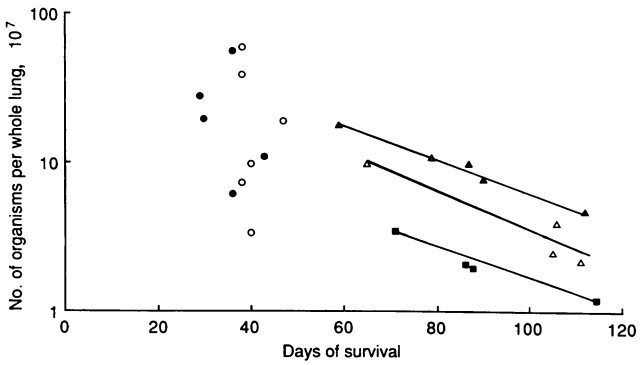

FIG. 4. The number of organisms per lung at the time of death in the groups of mice shown in Fig. 3 plotted against the number of days that they survived. Selectively CD8-depleted mice (■) died over the same period as thymectomized and nonthymectomized controls, but with lower bacterial loads. Symbols are as defined in the Fig. 3 legend.

DISCUSSION

There is disagreement over the role of the major T-cell subsets in protection from *M. tuberculosis* and about the relationship among protection, delayed hypersensitivity as manifested by footpad swelling, and immunopathology. Some of the difficulties have been highlighted by Orme and Collins (11). First, the growth curves of *M. tuberculosis* are different in different organs of mice, and numbers can increase in some organs (spleen and lung) while they decrease in another (liver). Second, the growth curve in the spleen is often biphasic. There is an early phase of rapid growth followed by a fall from about 15 days, when the T-cell response begins. Then, after reaching a trough at 40 days, the counts begin to rise again more slowly and do so progressively until death (11). Therefore, results of experiments based on the assessment of viable counts at a fixed number of days after cell transfer and infection could be misleading (8). A given transferred T-cell type could cause an apparent enhancement or reduction of bacterial growth merely as a result of a shift in the precise timing of the trough.

An additional problem is that from a clinical point of view, protection is not only a question of reducing the growth of bacilli. Regulation of tissue necrosis and cachexia could also help to determine the ultimate outcome of infection.

As confirmed by our results, a degree of protection against *M. tuberculosis* can be achieved by transfer of splenic T cells from BCG-immunized donors into irradiated or T-cell-deficient mice (10). Orme and Collins (11) concluded that transfer of this effect was ablated by depletion of $CD8^+$ cells from the donor population, although the remaining $CD8^-$ cells could still transfer delayed footpad test reactivity. In

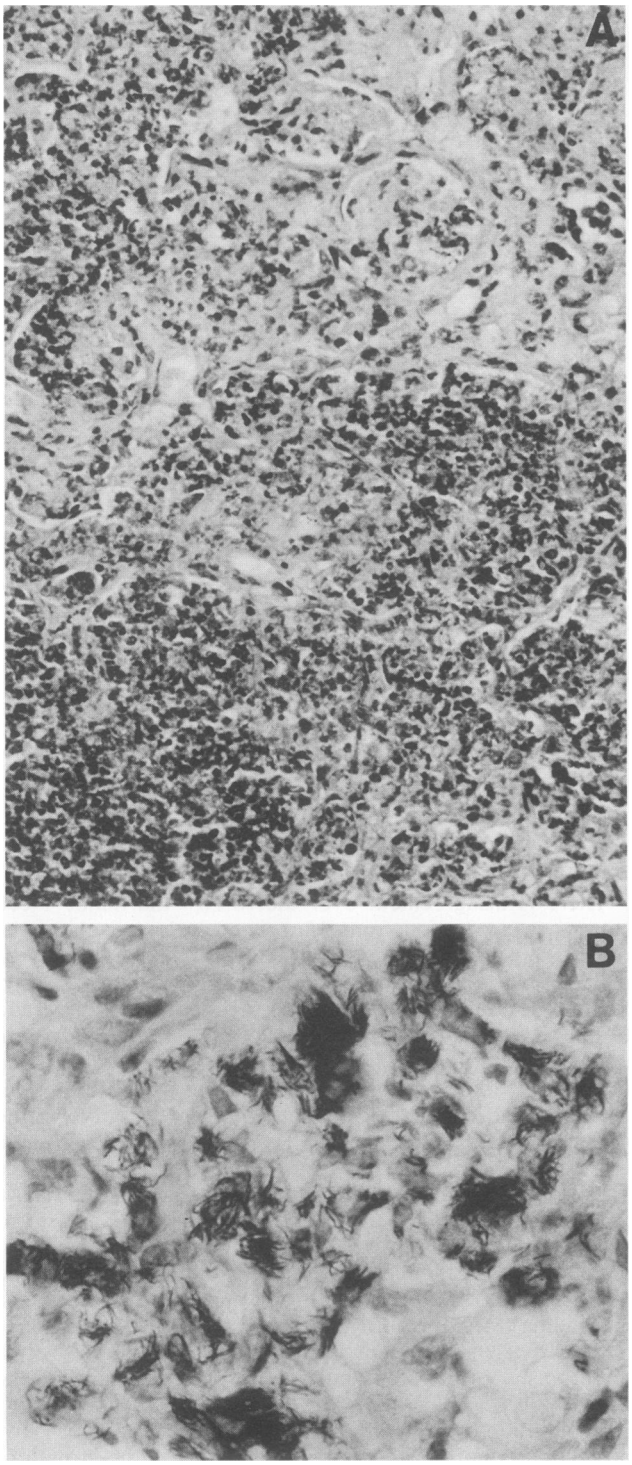

FIG. 5. Histopathology of the lung of an infected, thymectomized mouse. (A) Necrosis and nuclear debris from the intense polymorphonuclear infiltration. No granulomas were present. Hematoxylin and eosin stain was used. Magnification, ×400. (B) Ziehl-Neelsen stain of the lung section in panel A showing abundant acid-fast bacilli. Magnification, ×1,000.

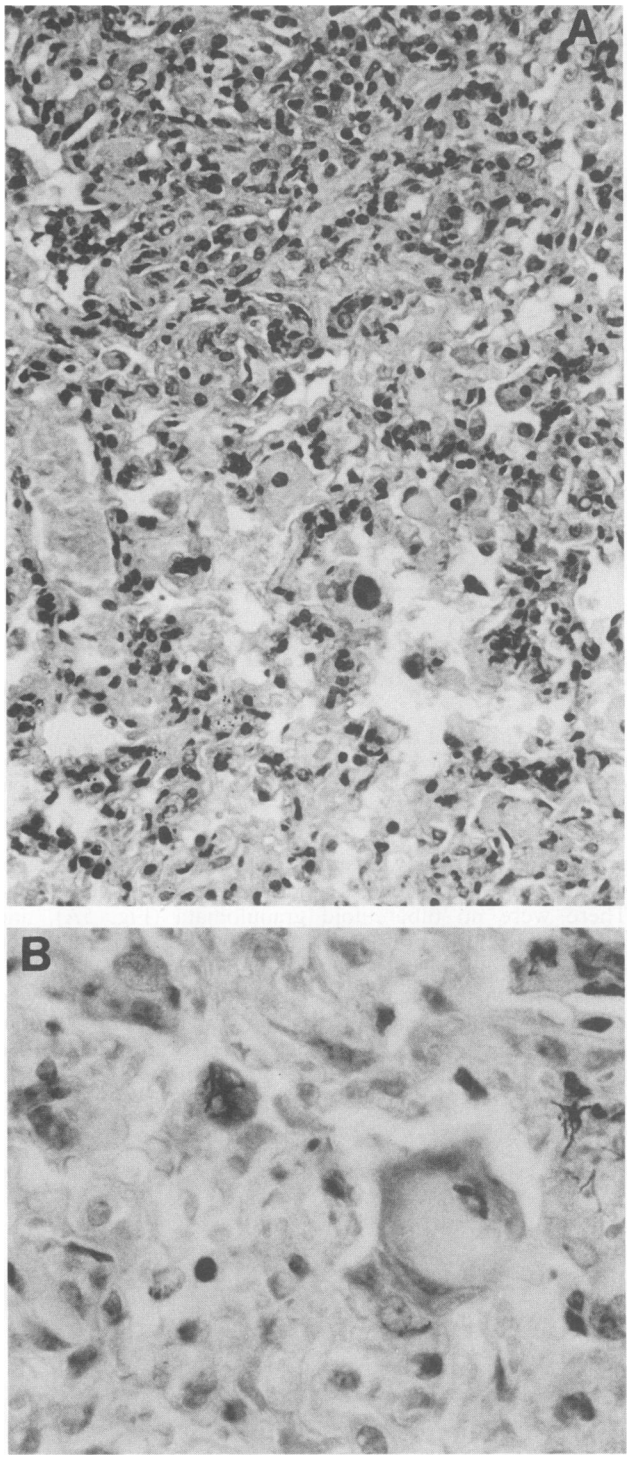

FIG. 6. Histopathology of the lung of an infected, CD8-depleted thymectomized mouse sacrificed at 87 days. (A) Epithelioid cells forming poorly defined granulomata. Hematoxylin and eosin stain was used. Magnification, × 400. (B) Ziehl-Neelsen stain of the lung section in panel A showing fewer acid-fast bacilli than in non-CD8-depleted mice (Fig. 5) and a macrophage giant cell. Magnification, ×1,000.

subsequent studies in which a panning technique was used to enrich immune donor cells for $CD4^+$ or $CD8^+$ populations, both subsets proved to be able to reduce the rate of replication of *M. tuberculosis* in recipients that were sacrificed 10 days after challenge (9). The peaks of bacterial growth in the donors were followed by peaks in the activity of CD4 cells and then, roughly 10 days later, by peaks in the activity of $CD8^+$ cells.

Muller and colleagues (8), using the same monoclonal antibodies and protocol exploited in our study and achieving a very similar degree of depletion, reported that depletion of either $CD4^+$ or $CD8^+$ T cells results in greater proliferation of *M. tuberculosis* during the first 3 weeks after infection. These observations are compatible with those of Orme (9). In contrast, Pedrazzini et al. (12) concluded that depletion of $CD8^+$ cells does not have important effects on the growth of BCG in mice and that only the CD4 cells were protective in their system.

From the results of the present study we have cast further light on the relative roles of the two cell subsets by studying the long-term effects of T-cell manipulations. We found that a $CD4^+$ T-cell line that has already been shown to be capable of I-A-restricted recognition and activation of tuberculosis-infected macrophages (13) can cause significant prolongation of the lives of irradiated CBA mice. This effect was only seen if the mice were infected with a dose of *M. tuberculosis* that neither killed the animals within a few days with an overwhelming infection nor killed them so slowly that the T cells of the recipient could recover their function and obscure the effect of the injected cells. The same T-cell line failed to inhibit growth of BCG in CBA *nu/nu* mice or of *M. tuberculosis* in thymectomized, lethally irradiated, and bone marrow-reconstituted mice. These findings could imply that the cell line can only offer protection if $CD8^+$ cells are also present, or that the cell line failed to become established in fully T-cell-deprived recipients in the absence of a recruitable pool of interleukin 2-producing cells.

The effect of the cell line in normal irradiated mice was to limit the growth of the bacilli. A striking finding was that the bacterial load at the time of death was inversely related to the time of survival. Therefore, the ultimate cause of death in the controls differed from that in the protected mice, in which chronic immunopathology and cachexia may have been important factors.

Similar conclusions were drawn from the experiments in which $CD4^+$ cells were depleted with the rat IgG2b monoclonal antibodies. Death was rapid in these animals and occurred with a higher bacterial load than that in nondepleted controls. Depletion of $CD8^+$ and $CD4^+$ cells had no additional effect. This result is in conflict with that of another report (8) based on viable counts at 3 weeks after infection, but as pointed out above, the previous result (8) can be explained by a shift in the trough and does not reliably indicate the true significance of $CD8^+$ cell depletion. It is also possible that the role of this cell subset changes at different times after infection. Thus, it is interesting that other investigators (12), using similar monoclonal antibodies followed by intravenous infection with BCG, also concluded that the $CD4^+$ cells were the protective ones but nevertheless noted a significant effect of CD8 depletion at 15 days that was not present at 30 or 45 days.

Although depletion of $CD8^+$ cells had no effect on survival, subtle roles for these cells were apparent when the bacterial load and histology at the time of death were examined. The absence of $CD8^+$ cells correlated with less bacterial growth and less necrotizing histology. This could imply that these cells normally decrease the efficacy of the CD4-mediated bacteriostatic mechanism, perhaps by acting as cytotoxic cells killing infected macrophages, as suggested by De Libero and colleagues (6, 7). This could account for the increased tissue damage. On the other hand, they could merely act as regulatory cells, and the necrosis could be a consequence of the presence of more bacilli in the nondepleted animals.

ACKNOWLEDGMENTS

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