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Impaired Resistance to *Mycobacterium tuberculosis* Infection after Selective In Vivo Depletion of L3T4$^+$ and Lyt-2$^+$ T Cells

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The resistance of mice against *Mycobacterium tuberculosis* infection after selective in vivo depletion of L3T4$^+$ and Lyt-2$^+$ T cells was studied. Thymectomized mice were treated with rat monoclonal antibodies against the L3T4 or Lyt-2 molecule to selectively eliminate the respective T-cell subset. In both L3T4$^+$ and Lyt-2$^+$ T-cell-depleted mice, resistance against subsequent infection with *M. tuberculosis* was markedly impaired compared with that in untreated controls, with L3T4$^+$ T-cell-depleted mice showing more pronounced effects. Simultaneous depletion of L3T4$^+$ and Lyt-2$^+$ T cells did not further exacerbate infection. These findings suggest that both L3T4$^+$ and Lyt-2$^+$ T cells are involved in the acquisition of resistance against tuberculosis.

Tuberculosis is a chronic bacillary infectious disease caused by *Mycobacterium tuberculosis*, *M. bovis*, and *M. africanum* (for a summary, see reference 2). These mycobacteria have developed various evasion mechanisms which enable them to persist and replicate in mononuclear phagocytes of the host (for a summary, see reference 18). Acquired resistance against tubercle bacilli depends on specific T lymphocytes (for a summary, see reference 7). According to a widely accepted scheme, activation of increased tuberculocidal and/or tuberculostatic macrophage functions by specific T cells of the helper type which produce multiple lymphokines represents the major step toward acquired resistance against tuberculosis (7). In general, CD4 T cells with helper functions express the L3T4 marker in mice (T4 in humans) and are class II restricted (for summaries, see references 3, 6, and 26). These features distinguish them from cytolytic CD8 T cells which commonly are Lyt-2$^+$ (mice), T8$^+$ (humans), and class I restricted (3, 6, 26). Cytolytic T cells are generally thought to be primarily responsible for anti-viral immunity.

Recently, we have established *M. tuberculosis*-reactive T-cell lines and clones of the L3T4 and the Lyt-2 phenotype and used them for in vitro analysis of the biological functions of either T-cell subset (9, 10). We have concluded from these studies that both L3T4$^+$ and Lyt-2$^+$ T cells participate in the immune response against *M. tuberculosis*. Recently, monoclonal antibodies (MAbs) specific for the L3T4 or Lyt-2 molecule and with potent in vivo activity have been generated (4). Injection of these antibodies leads to marked depletion of the appropriate T-cell subset in mice. We used these antibodies to assess the contribution of L3T4$^+$ and Lyt-2$^+$ T cells to resistance against tuberculosis. We show that selective depletion of either L3T4$^+$ or Lyt-2$^+$ T cells enhances mycobacterial growth and that depletion of both subsets has an effect that is no worse. These findings further support the argument that both L3T4$^+$ and Lyt-2$^+$ T lymphocytes participate in resistance against tuberculosis.

MATERIALS AND METHODS

Mice. Male C57BL/6 and DBA/2 mice raised under specific-pathogen-free conditions in our own breeding facilities were used.

Bacteria. *M. bovis* BCG was kindly provided by R. North, Trudeau Institute, Saranac Lake, N.Y.; and *M. tuberculosis* H37Rv and Middelburg were from J. K. Seydel, Forschungsinstitut Borstel, Borstel, Federal Republic of Germany. Mycobacteria were grown in Dubos broth (Difco Laboratories, Detroit, Mich.) supplemented with bovine serum albumin and Tween 80 at 37°C with shaking. Cultures were centrifuged and washed in phosphate-buffered saline, and CFU were determined by plating 1:10 dilutions on Middlebrook Dubos agar (Difco) as described previously (19).

MAbs. The preparation and characteristics of the rat MAbs YTS 191.1, which is specific for a monomorphic L3T4 determinant expressed on murine class II-restricted helper/inducer T cells, and YTS 169.4, which is specific for a monomorphic Lyt-2 determinant expressed on murine class I-restricted cytolytic T cells, have been described previously (4). Both MAbs are of the immunoglobulin G2b (IgG2b) isotype and are highly effective in eliminating their respective T-cell subset in vivo. Ascitic fluid from tumor-bearing, pristane-primed rats were partially purified by precipitation with 50% $(NH_4)_2SO_4$. The total protein concentration of the MAb preparations employed in this study was 10 mg/ml, with approximately 2 mg of active antibody per ml. Mice received two injections of 0.2 ml of antibodies 2 to 4 days apart; the first injection was by the intravenous route, and the second one was by the intraperitoneal route.

Thymectomy. Mice (age, 4 to 5 weeks) were anesthetized by intraperitoneal injection of a mixture of tribromoethanol-amyl alcohol in saline (15). Thymuses were removed through an incision anterior of the sternum by light suction with a cannula made from a Pasteur pipette. After thymectomy the wound was closed with clips, and the animals were allowed to recover for at least 3 weeks.

Analysis of T-cell subsets in MAb-treated mice. Spleen cells

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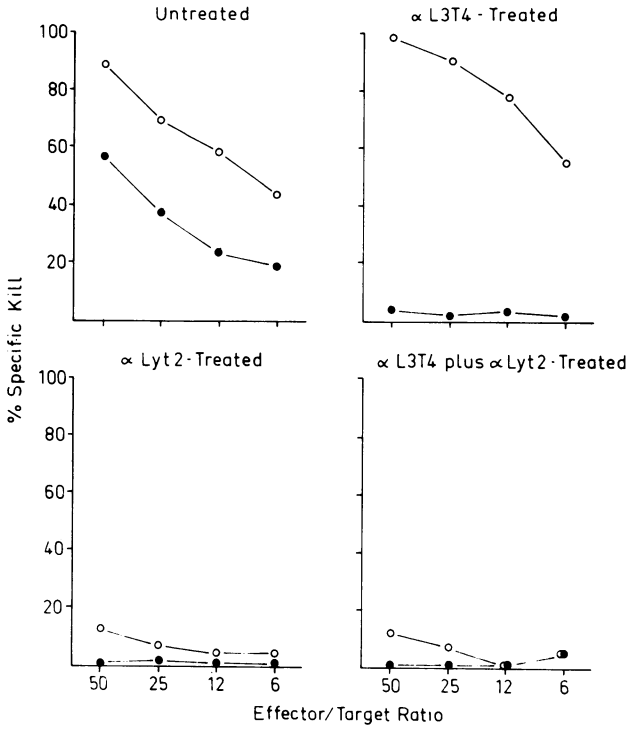

FIG. 1. Effect of in vivo treatment with anti-L3T4 and anti-Lyt-2 MAb on the generation of cytotoxic T cells in mixed leukocyte cultures. C57BL/6 mice were thymectomized and treated with anti-L3T4 MAb, anti-Lyt-2 MAb, or both. Afterward, 6×10^5 spleen cells per ml were cultured with 3×10^6 irradiated DBA/2 spleen cells per ml in the absence (●) or presence (○) of supernatants of concanavalin A-activated rat spleen cells. On day 5, cytolytic T-cell responses were determined by using ^{51}Cr-labeled P815 cells as targets.

from mice treated with anti-L3T4 or anti-Lyt-2 MAbs alone or together were incubated with these MAbs (diluted 1:500) in 200 μl of Iscove modified Dulbecco medium (IMDM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum (GIBCO)–5% selected rabbit serum–0.02% NaN_3 at room temperature for 30 min. After washing, cells were treated with a fluorescein isothiocyanate (FITC)-labeled mouse MAb with specificity for rat IgG2b (NORIG 7.16.2; final dilution, 1:50) for 30 min at room temperature. Afterward, cells were washed three times and analyzed on a cytofluorograph (50 H; Ortho Diagnostic Systems, Westwood, Calif.).

Mixed leukocyte reactions. Spleen cells from mice treated with anti-L3T4 or anti-Lyt-2 MAb alone or together were purified over nylon wool columns to enrich for T cells, as described previously (14). Afterward, 6×10^5 of these cells were cocultured with 3×10^6 X-irradiated (3,300 rads) DBA/2 stimulator cells in 2 ml of IMDM (GIBCO) supplemented with 10% fetal calf serum–antibiotics–2-mercaptoethanol in macrotiter plates (Nunc, Roskilde, Denmark) at 37°C for 4 days. Cells were added to 2×10^3 ^{51}Cr-labeled P815 tumor cells at the effector to target ratios indicated in Fig. 1, and after 4 h ^{51}Cr release was determined. The percentage of ^{51}Cr released from target cells was determined in triplicate samples and calculated according to the following equation: percent ^{51}Cr released $= 100 \times [(a - b)/(c - b)]$, where a is the ^{51}Cr released from target cells in the presence of cytotoxic T cells, b is the ^{51}Cr released from target cells alone, and c is the maximal ^{51}Cr released from target cells.

***M. tuberculosis* infection of MAb-treated mice.** Mice were treated with anti-L3T4 or anti-Lyt-2 MAb on days −2 and 0. Immediately after the second injection, mice were infected intravenously with 6×10^5 live *M. tuberculosis* organisms. On day 20, spleens were removed and mycobacteria were enumerated by plating 1:10 dilutions on Middlebrook Dubos agar (Difco).

RESULTS

Functional characterization of T cells from anti-L3T4 and anti-Lyt-2 MAb-treated mice. Before analyzing the effects of in vivo treatment with anti-L3T4 MAb, anti-Lyt-2 MAb, or both on the course of tuberculosis, we wanted to determine the efficiency of depletion. Mice were treated with anti-L3T4 MAb, anti-Lyt-2 MAb, or both on days 2 and 0. On day 20, the capacity of T cells from treated and untreated mice to generate an alloreactive cytolytic T-cell response was assessed. Stimulations were performed in the presence or absence of supernatants from concanavalin A-activated rat spleen cells as a source of T-cell factors. In the absence of T-cell factors, only spleen cells from untreated mice were capable of generating a cytolytic T-cell response, whereas spleen cells from mice pretreated with anti-L3T4 or anti-Lyt-2 MAb, either alone or together, failed to do so. In the presence of T-cell factors, spleen cells from both untreated mice and mice pretreated with anti-L3T4 MAb were able to generate cytolytic T-cell responses. In contrast, cells from anti-Lyt-2 MAb-treated mice failed to become cytolytic. This finding is in accordance with many published data (3, 6) that, generally, Lyt-2$^+$ T cells represent the major source of cytolytic activity and that their replication and differentiation in vitro depends on T-cell factors provided by L3T4$^+$ T cells. Hence, in vivo depletion of L3T4$^+$ and Lyt-2$^+$ T cells was effective in a functional sense.

Phenotypic characterization of T cells from anti-L3T4 and anti-Lyt-2 MAb-treated mice. Mice were thymectomized, treated with MAb, and afterward infected with viable *M. bovis* or left uninfected. After 2 weeks spleen cells were collected, washed, and labeled with anti-L3T4 MAb, anti-Lyt-2 MAb, or both. Afterward, cells were incubated with FITC-labeled specific anti-rat IgG2b MAbs, which do not cross-react with mouse immunoglobulin. In this way purification of T cells prior to labeling could be omitted, and the possible selective loss of either T-cell population could be excluded. Pretreatment of mice with anti-L3T4 or anti-Lyt-2 MAb led to a high degree of specific depletion of the relevant T-cell subset (Table 1). This was true not only in uninfected but also in *M. bovis*-infected mice. We found that in nonthymectomized mice given MAb treatment a considerable number of the respective T cells recovered after mycobacterial infection (data not shown). Hence, in the following experiment thymectomized mice were used.

Impaired resistance against *M. tuberculosis* infection in T-cell-subset-depleted mice. Thymectomized mice were treated with MAb and afterward were infected with viable organisms of *M. tuberculosis* H37Rv or Middelburg. After approximately 3 weeks, spleens were removed and mycobacterial CFU were determined. Treatment of mice with either anti-L3T4 or anti-Lyt-2 MAb led to marked increase of mycobacterial numbers in the spleen (Table 2). In both experiments depletion of L3T4$^+$ T cells had more pronounced effects than depletion of Lyt-2$^+$ T cells. Infection was no worse following injection of both antibodies simul-

TABLE 1. Specific depletion of L3T4$^+$ and Lyt-2$^+$ T cells by in vivo treatment with anti-L3T4 and anti-Lyt-2 MAb

In vivo treatment (MAb)[a]	% Specific label (% specific depletion) of[b]:	
	L3T4	Lyt-2
Uninfected mice		
None	16.9 (0)	12.6 (0)
Anti-L3T4	2.0 (88.2)	15.0 (−19.0)
Anti-Lyt-2	17.8 (−5.3)	2.7 (78.6)
Anti-L3T4 + anti-Lyt-2	3.5 (79.3)	3.7 (70.6)
Mycobacterium-infected mice		
None	12.6 (0)	13.6 (0)
Anti-L3T4	3.3 (73.8)	12.3 (9.6)
Anti-Lyt-2	12.3 (2.38)	4.4 (67.6)
Anti-L3T4 + anti-Lyt-2	2.4 (81.0)	1.7 (87.5)

[a] Spleen cells from MAb-treated uninfected or *M. bovis*-infected mice were treated with anti-L3T4 or anti-Lyt-2 MAb, followed by treatment with FITC-labeled mouse anti-rat immunoglobulin MAb. Labeled cells were analyzed by flow cytometry.

[b] Percent specific label = percent experimental − percent FITC control. Percent specific depletion = 100 − 100 × (percent specific label of cells from MAb-treated mice/percent specific label of cells from untreated mice).

taneously. In fact, in one of the two experiments shown in Table 2, mycobacterial numbers were higher after depletion of L3T4$^+$ T cells alone than after depletion of both T-cell subsets. We conclude from the results of this experiment that both L3T4$^+$ and Lyt-2$^+$ T cells can participate in the acquisition of resistance against tuberculosis.

DISCUSSION

In this study, mice that were selectively depleted of L3T4$^+$ or Lyt-2$^+$ T cells were used to determine which one of the two major T-cell subsets is involved in the acquisition of anti-tuberculous resistance. Our data indicate that not one but both T-cell subsets participate in the development of effective protection. Our study became possible through the availability of MAbs with potent in vivo activity (4). These MAbs are of rat origin and of the IgG2b isotype. This antibody subclass is capable of destroying cells bearing the homologous antigen in vivo, probably by activating the complement system, antibody-dependent cellular cytotoxicity, or both. Accordingly, in our study a high proportion of the relevant T-cell subset was eliminated in thymectomized mice.

TABLE 2. Mycobacterial growth in anti-L3T4 and anti-Lyt-2 MAb-treated mice[a]

M. tuberculosis strain and group no.	In vivo treatment (MAb)	Median $\log_{10}$ CFU/spleen (range)	$\log_{10}$ increase
H37Rv			
1	None	5.77 (4.00–5.97)	
2	Anti-L3T4	6.78 (6.52–6.96)	1.01
3	Anti-Lyt-2	6.23 (6.02–6.40)	0.46
4	Anti-L3T4 + anti-Lyt-2	6.47 (6.34–6.59)	0.70
Middelburg			
5	None	6.70 (6.54–6.90)	
6	Anti-L3T4	8.07 (7.60–8.57)	1.37
7	Anti-Lyt-2	7.70 (7.18–8.40)	1.00
8	Anti-L3T4 + anti-Lyt-2	8.12 (7.54–8.27)	1.42

[a] MAb-treated mice were infected with viable *M. tuberculosis* H37Rv or Middelburg organisms, and CFU in spleens were determined after approximately 3 weeks. Significant differences for the groups were as follow (Wilcoxon test, $P < 0.05$): 1 versus 2, 1 versus 3, 1 versus 4, 2 versus 3, 3 versus 4; 5 versus 6, 5 versus 7, 5 versus 8.

The efficacy of T-cell depletion was assessed by phenotypic and functional studies. Interestingly, we observed that in nonthymectomized mycobacterium-infected mice, a significant number of T cells recovered from MAb treatment. Even in thymectomized mice depletion of the T-cell subsets was slightly less impressive in infected as compared with uninfected mice. This effect could have been caused by increased hemopoiesis and by polyclonal T-cell activation in the periphery during mycobacterial infection (1, 17), with the former but not the latter effect having been abrogated by thymectomy. Furthermore, it may be interesting that the proportion of L3T4$^+$ T cells was reduced in the spleens of mycobacterium-infected mice as compared with those of uninfected controls. In lesions of lepromatous leprosy patients, the ratio of T4 to T8 cells is markedly reduced (24). Whether the reduction of CD4 T cells in mycobacterial infections is directly correlated with the observed deficiency in interleukin-2 (IL-2) secretion, as observed with T cells from lepromatous leprosy patients (8) or from mice experimentally infected with *M. bovis* (25), however, remains to be clarified.

Spleen cells from mice pretreated with anti-L3T4 or anti-Lyt-2 MAb failed to generate an alloreactive cytolytic T-cell response in vitro. The addition of exogenous T-cell factors restored the response of L3T4-depleted but not Lyt-2-depleted spleen cells. This finding is in accordance with the notion that the main function of CD4 T cells in the generation of alloreactive cytolytic T-cell responses is the secretion of lymphokines, particularly IL-2, which influence the replication and differentiation of CD8 cytolytic effector T cells (3, 6). Recently, however, evidence for the autonomous generation of Lyt-2$^+$ cytolytic T cells has been obtained in some systems as well (23). We conclude from our findings that the depletion regimen employed was sufficiently specific and efficient and hence could be used for analyzing cell-mediated immunity against *M. tuberculosis* in vivo.

It is generally accepted that acquired resistance against *M. tuberculosis* is mediated by T cells and expressed by activated macrophages. Accordingly, it is thought that T cells of the helper type are of crucial importance for protection (7). In mice, helper T cells are L3T4$^+$. In contrast, Lyt-2$^+$ T cells with cytolytic activity are commonly thought to be responsible for cell-mediated immunity against viral infections. Results of earlier studies performed in the model of experimental infection with the intracellular bacterium *Listeria monocytogenes*, however, have provided strong evidence that not only L3T4$^+$ but also Lyt-2$^+$ T cells participate in antibacterial protection (11–14). This assumption is based on the findings that (i) *L. monocytogenes*-specific T cells of either phenotype can be isolated and propagated in vitro (11, 12) and (ii) that adoptive protection against *L. monocytogenes* depends on both L3T4$^+$ and Lyt-2$^+$ T cells (13, 14).

In the listeriosis model protective effects are determined 2 to 3 days after cell transfer. In contrast, in mycobacterial infections protective effects first become demonstrable after 2 to 3 weeks. Furthermore, adoptive protection against tuberculosis is facilitated by sublethal irradiation of recipients prior to cell transfer. Thus, it is possible that infection with *M. tuberculosis* leads to the stimulation of T cells that are resistant to radiation and leads to nonspecific macrophage activation in recipient mice, which could then contribute to protection. In an attempt to identify the T-cell subset(s) involved in protection against *M. tuberculosis*,

Orme and Collins (21) have used the adoptive transfer system. They found that Lyt-2$^+$ T cells are sufficient for adoptive protection. In contrast, transfer of Lyt-1$^+$ T cells had no effect. (The Lyt-1 marker is preferentially expressed by L3T4$^+$ T cells; however, Lyt-2$^+$ T cells also bear the Lyt-1 molecule, although at a lower density [6, 16].) While these data suggest that Lyt-2$^+$ T cells are involved in the expression of protection against tuberculosis, they do not exclude a role for L3T4$^+$ T cells in the acquisition of anti-tuberculous resistance. Indeed, it is known that helper T cells are generated within 2 weeks after immunization and that they are relatively resistant to radiation. The data presented here suggest that, as for the *L. monocytogenes* model, both Lyt-2$^+$ and L3T4$^+$ T cells participate in the acquisition of resistance against *M. tuberculosis*. Indeed, after completion of this study, Orme (20) reported that L3T4$^+$ T cells are more potent than Lyt-2$^+$ T cells in transferring anti-tuberculous protection.

What could be the function(s) of these T-cell subsets? Recently, we have established a panel of *M. tuberculosis*-specific L3T4$^+$ and Lyt-2$^+$ T-cell clones and characterized their functional activities in vitro (9, 10). In accordance with data obtained in other systems, *M. tuberculosis*-specific T-cell clones of the Lyt-2 phenotype failed to secrete IL-2, whereas those bearing the L3T4 marker were able to do so (9, 10). In most systems growth and differentiation of Lyt-2$^+$ T cells generally requires IL-2, and this notion is further supported by the data shown in Fig. 1. The generation of *M. tuberculosis*-specific Lyt-2$^+$ T cells in vivo could also require IL-2, which could be supplied by L3T4$^+$ T cells. In addition, *M. tuberculosis*-reactive L3T4$^+$ T cells are capable of activating tuberculostatic macrophage functions by lymphokine secretion (10). Thus, L3T4$^+$ T cells could have a dual function in antimycobacterial immunity, and this could account for the fact that treatment with anti-L3T4 MAb exerted more pronounced effects than that with anti-Lyt-2 MAb. Evidence has been presented that mononuclear phagocytes can express the CD4 molecule in the human system (22). Although we are not aware of similar findings in the mouse system, possible effects of MAb treatment on leukocytes other than T cells cannot be excluded by our data, and further studies will be required to determine whether direct effects of anti-L3T4 MAb on macrophage functions further contributed to impaired anti-tuberculous resistance.

Although the role of Lyt-2$^+$ T cells in antituberculous immunity is less clear, we have shown previously (9) that many *M. tuberculosis*-reactive Lyt-2$^+$ T-cell lines produce gamma interferon in vitro; however, this was only after costimulation with antigen plus IL-2 which in vivo could be supplied by L3T4$^+$ T cells. Moreover, *M. tuberculosis*-specific Lyt-2$^+$ T-cell lines could lyse macrophages presenting mycobacterial antigen (9). This finding suggests that lysis of infected macrophages may contribute to protection. We envisage that lysis of parasitized tissue macrophages that are unable to destroy intracellular mycobacteria facilitates subsequent uptake of these pathogens by inflammatory monocytes with a higher antimicrobial potential. The latter phagocytes could then be activated by lymphokines provided by L3T4$^+$ T cells. Effective anti-tuberculous protection would therefore best be accomplished by collaboration between L3T4$^+$ helper T cells that are capable of activating mycobacterium-infected macrophages for increased antimicrobial activities and Lyt-2$^+$ cytolytic T cells which lyse mycobacterium-infected host cells. In the absence of macrophage activation, lysis of infected host cells could lead to mycobacterial dissemination and hence could be harmful rather than beneficial for the host. This assumption could explain why selective depletion of L3T4$^+$ T cells resulted in stronger mycobacterial growth than depletion of Lyt-2$^+$ T cells alone. While it is not clear whether the functional activities of L3T4$^+$ or Lyt-2$^+$ T cells observed in vitro are indeed expressed in vivo, the data presented here suggest that both T-cell populations are involved in the development of resistance against tuberculosis.

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