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Virulence Ranking of Some *Mycobacterium tuberculosis* and *Mycobacterium bovis* Strains According to Their Ability To Multiply in the Lungs, Induce Lung Pathology, and Cause Mortality in Mice

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Three virulent strains of *Mycobacterium tuberculosis* (H37Rv, Erdman, and NYH-27) and two virulent strains of *M. bovis* (Ravenel and Branch) were compared in terms of their growth rates in the livers and the lungs of mice, their ability to cause lung pathology, and the time taken for them to cause death. In immunocompetent mice, all strains caused an infection that progressed for 20 days or more and then underwent resolution in the liver but not in the lungs. In the lungs, infection persisted and induced progressive pathology. According to host survival time, Ravenel was the most virulent strain, followed, in decreasing order of virulence, by Branch, H37Rv, Erdman, and NYH-27. The much longer survival times of mice infected with *M. tuberculosis* strains allowed time for lung histopathology to change from a histiocytic alveolitis to a chronic fibroblastic fibrosis that eventually obliterated most of the lung architecture. By contrast, in mice infected with *M. bovis* strains, the alveolitis that developed during early infection was rapid and expansive enough to cause death before chronic lung pathology became evident. In mice depleted of $CD4^+$ T cells, increased growth of all virulent strains induced necrotic exudative lung lesions that rapidly filled most of the alveolar sacs with inflammatory cells. These mice died much earlier than infected control mice did. Attenuated strains had longer population doubling times in vivo and failed to cause progressive disease or pathology in the lungs or livers of immunocompetent mice.

The recent increase in the incidence of tuberculosis in the United States has served to draw attention to the need to develop an efficacious antituberculosis vaccine. To this end, current research in some laboratories is being directed at identifying the immunogens of *Mycobacterium tuberculosis* (12, 30) with a view to producing a subunit or recombinant vaccine. Attention is also being directed toward identifying the genetic basis of mycobacterial virulence with the hope of understanding how the organism survives host defense to cause disease (11, 20). In this connection, a recent paper from this laboratory (19) served to confirm the observations of earlier investigators (22) that in mice, as in most humans, virulent strains of *M. tuberculosis* cause progressive infection in the lungs but not in other organs. It showed with one virulent strain that infection in the lungs progressed despite the generation of $CD4^+$ T-cell-mediated immunity that was capable of resolving infection in the liver, spleen, and kidneys. This was not the case for infection with attenuated organisms which underwent resolution in the lungs and elsewhere under the influence of specific immunity (19). Therefore, the ability to cause progressive disease in the lungs in the face of acquired immunity capable of controlling infection in other organs is a key manifestation of mycobacterial virulence that must be considered when assessing the efficacy of experimental vaccines in animal models.

The study reported here compares three virulent strains of *M. tuberculosis* and two virulent strains of *M. bovis* in terms of the rate at which they multiply in the lungs and the livers of mice, their ability to cause progressive lung pathology, and the time taken for them to kill their host. The results show that according to these criteria, some strains of mycobacteria are considerably more virulent than others toward mice.

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MATERIALS AND METHODS

Mice. Male B6D2F1 (C57BL/6 × DBA/2) mice were obtained from the Trudeau Institute Animal Breeding Facility, Saranac Lake, N.Y., and used in experiments at 7 to 9 weeks of age. CD2F1 (BALB/c × DBA/2) mice were used for the production of hybridoma ascites. All mice were known to be free of common viral pathogens according to routine serological screening performed by the Research Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri.

Immunodeficient mice. Mice were rendered immunodeficient by thymectomy at 5 to 6 weeks of age followed by CD4 T-cell depletion via the administration of purified anti-CD4 monoclonal antibody. This monoclonal antibody was obtained from ascites produced by growing the GK1.5 hybridoma (TIB 207; American Type Culture Collection, Rockville, Md.) in the peritoneal cavity of CD2F1 mice and was purified as previously described (6). The monoclonal antibody was administered to mice in 0.5-mg doses on days 5, 15, 30, and 50 days after inoculation with *M. tuberculosis.*

Bacteria. Most strains of mycobacteria used were obtained from the Trudeau Mycobacterial Culture (TMC) Collection. These included *M. tuberculosis* Erdman (TMC 107), *M. tuberculosis* H37Rv (TMC 102), *M. tuberculosis* R1Rv (TMC 205), *M. bovis* Ravenel (TMC 401), *M. bovis* Branch (TMC 407), and *M. bovis* BCG Pasteur (TMC 1101). All were grown as dispersed cultures in Proskauer and Beck (PB) medium as recently formulated (1), containing 0.01% Tween 80 (PBT), and frozen in aliquots as previously described (19) for use in experiments.

M. tuberculosis NYH-27, also designated the C strain (8), was supplied by Barry Kriesworth, TB Center, Public Health Research Center, New York, N.Y. Before use, it was grown as a surface pellicle on PB medium and then as a dispersed culture in PBT, which was frozen as a seed stock. Working stock cultures were prepared as for other strains.

For each experiment, bacteria were prepared for inoculation by thawing a vial of working stock, diluting it 10-fold in phosphate-buffered saline (PBS) containing 0.01% Tween 80, and subjecting the suspension to sonography for 5 s to break up clumps. The resulting suspension was diluted to the desired concen-

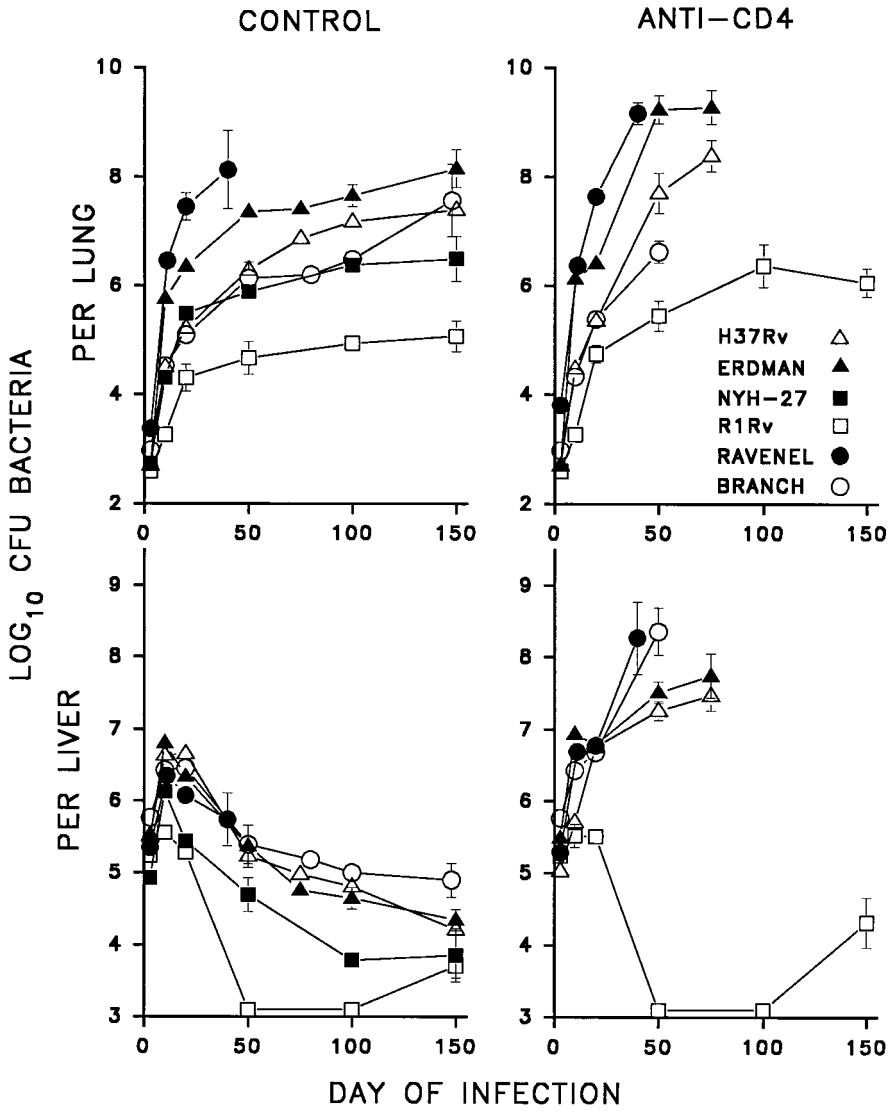

FIG. 1. Comparison of growth of two strains of *M. bovis* (Ravenel and Branch) and four strains of *M. tuberculosis* (Erdman, H37Rv, NYH-27, and R1Rv) in lungs and livers of immunocompetent and $CD4^+$ T-cell-depleted mice infected i.v. with a standard 10^5 CFU inoculum. Infection progressed in the lungs but not in the livers of immunocompetent mice and in both organs of $CD4^+$ T-cell-depleted mice. Results are given as means ± standard deviations for five mice per group.

tration in PBS containing 0.01% Tween 80 and injected intravenously (i.v.) in a 0.2-ml volume via a lateral tail vein. Bacteria were enumerated in the organs of infected mice by plating serial 10-fold dilutions of organ homogenates on enriched agar (Middlebrook 7H11; Difco Laboratories), incubating the plates for 2 to 3 weeks, and counting CFU with a ×4 magnification dissecting microscope. Experiments with three of the strains, *M. tuberculosis* Erdman, *M. tuberculosis* H37Rv, and *M. bovis* Ravenel, were performed twice.

Histology. Lungs and livers were fixed in 10% neutral buffered formalin, washed in running tap water, dehydrated in ethanol, and embedded in paraffin by standard procedures or in glycol methacrylate (JB-4 embedding kit; Polysciences, Inc., Warrington, Pa.) as specified by the manufacturer. Paraffin sections were cut at 5 μm with a rotary microtome (AO 820 microtome; American Optical, Buffalo, N.Y.) and, after being dewaxed, stained for acid-fast bacilli with a modified basic fuchsin strain (7) containing 0.6% (vol/vol) LOC High Suds (Amway Corp., Ada, Mich.). They were then briefly destained with acid-alcohol and counterstained with methylene blue. Sections of glycol methacrylate-embedded tissue 1 to 2 μm thick were cut with glass knives, using a Porter Blum MT-1 microtome (Sorvall, Inc., Newtown, Conn.). The sections were stained at 60°C either with 2% crystal violet containing 0.6% (vol/vol) LOC High Suds followed by brief 95% ethanol decolorization or with water-based MacNeal's tetrachrome stain followed by a brief treatment with distilled water. Photomicrography was performed with a Nikon Microphot-Fx microscope.

RESULTS

Growth of *M. tuberculosis* and *M. bovis* strains in the lungs and livers of immunocompetent and immunodeficient mice. The first experiment compared different strains of *M. tuberculosis* and *M. bovis* in terms of their ability to grow in the lungs and livers of immunocompetent and $CD4^+$ T-cell-depleted mice after the mice were inoculated i.v. with 10^5 CFU. It was found (Fig. 1) that all strains of mycobacteria were progressively inactivated in the livers of immunocompetent mice after an initial 20-day period of growth. In the lungs, in contrast, infection did not resolve. Instead, an initial period of rapid growth was followed, after about day 50, by a substantial reduction in the growth rate. Infection progressed at the lower rate until the mice died or the experiment was terminated. Inactivation of virulent strains in the liver and their reduced rate of growth in the lungs was the result of the expression of T-cell-mediated immunity, in that their growth was progressive

TABLE 1. Population doubling times of different strains of mycobacteria in organs of control and CD4 T-cell-depleted mice

Strain[a]	Anti-CD4 MAb[b]	Doubling time (h)[c] in:			
		Lungs		Liver	
		Days 1–10	Days 20–50	Days 1–10	Days 20–50
M. tuberculosis Erdman	−	21.3	216.9	54.7	R[d]
	+	19.0	76.6	49.8	286.0
M. tuberculosis H37Rv	−	35.9	202.8	64.5	R
	+	36.5	92.4	106.2	409.4
M. tuberculosis NYH-27	−	35.0	541.3	60.2	R
	+	ND[e]	ND	ND	ND
M. tuberculosis R1Rv	−	98.5	602.0	203.2	R
	+	97.2	309.5	232.2	R
M. bovis Ravenel	−	23.4	212.4	74.5	R
	+	28.2	94.5	51.6	91.8
M. bovis Branch	−	41.9	206.3	109.6	R
	+	48.1	176.1	109.6	124.3
M. bovis BCG	−	143.6	R	289.2	R
	+	136.4	195.5	195.1	R

[a] Mice were infected i.v. with a standard 10^5 CFU inoculum.
[b] Some mice were thymectomized before infection and given anti-CD4 monoclonal antibody (MAb) to deplete $CD4^+$ T cells according to the schedule outlined in Materials and Methods.
[c] Population doubling times were calculated from the data points (mean CFU per organ) obtained for days 1, 10, 20, and 50 of the growth curve in Fig. 1.
[d] R, resolving (negative growth).
[e] ND, not determined.

in both organs of mice depleted of $CD4^+$ T cells (Fig. 1). All strains tested grew faster in the lungs than in the livers of $CD4^+$ T-cell-depleted mice, except for *M. bovis* Branch. Moreover, in the livers of these mice, the *M. bovis* strains grew more rapidly than the *M. tuberculosis* strains. The attenuated *M. tuberculosis* R1Rv also grew faster in the lungs of $CD4^+$ T-cell-depleted mice but was rapidly and almost completely inactivated in the livers of these mice, as well as in immunocompetent mice. This strain did, however, reemerge in the livers of both types of mice at a later stage of infection.

An examination of the population doubling times of the different strains during the first 10 days (preimmunity phase) of infection in the lungs of immunocompetent mice showed (Table 1) that the strain with the shortest doubling time in this organ was *M. tuberculosis* Erdman (21.3 h), followed by *M. bovis* Ravenel (23.4 h), *M. tuberculosis* NYH-17 (35.9 h), *M. tuberculosis* H37Rv (35.9 h), *M. bovis* Branch (41.9 h), and *M. tuberculosis* R1Rv (98.5 h). For comparative purposes, the doubling time of *M. bovis* BCG (143.6 h) is also shown, as calculated from growth curves not shown. Similar doubling times were seen in the lungs of $CD4^+$ T-cell-depleted mice during the first 10 days. However, the doubling times in the lungs of these mice after day 10 were much shorter than those measured in immunocompetent mice.

In the liver, the doubling times of all virulent strains of *M. tuberculosis* were shorter than those of virulent *M. bovis* prior to the expression of immunity. After this, all strains of *M. tuberculosis* and *M. bovis* were progressively inactivated. However, in livers of $CD4^+$ T-cell-depleted mice, the *M. bovis* strains multiplied faster than the *M. tuberculosis* strains after about day 20, indicating that the *M. tuberculosis* strains were subjected to more growth restriction in this organ in the absence of $CD4^+$ T-cell-mediated immunity. The doubling times of all strains were longer in the livers than in the lungs of immunocompetent and immunodeficient mice. During the 2-year period over which these studies were carried out, growth curves and survival data (see below) for *M. tuberculosis* Erdman, *M. tuberculosis* H37Rv, and *M. bovis* Ravenel were obtained again, and the results were found to be identical.

Survival times of immunocompetent and immunodeficient mice infected with different strains of *M. tuberculosis* and *M. bovis*. To determine whether infection with strains with shorter doubling times, as shown in Table 1, results in shorter times of host survival, mice were inoculated i.v. with the standard 10^5 CFU dose of one or other strain of *M. tuberculosis* or *M. bovis* and survival was monitored over a 350-day period. The results in Fig. 2 show that on the basis of survival time of immunocompetent mice, both strains of *M. bovis* were more virulent than the *M. tuberculosis* strains. *M. bovis* Ravenel was the most virulent (median survival time [MST], 60 days), followed by *M. bovis* Branch (MST, 128 days), *M. tuberculosis* H37Rv (MST, 203 days), *M. tuberculosis* Erdman (MST, 244 days), *M. tuberculosis* NYH-27 (MST, 291 days), and *M. tuberculosis* R1Rv (MST, >350 days). This was the case even though *M. bovis* Branch had a much longer initial doubling time than all the *M. tuberculosis* strains except the attenuated R1Rv strain. Therefore, mycobacterial doubling time alone did not determine the duration of host survival.

MSTs were substantially reduced in all mice depleted of $CD4^+$ T cells, but the reduction was greater in mice infected with the virulent *M. tuberculosis* strains. For example, whereas $CD4^+$ T-cell-depleted mice succumbed to *M. bovis* Ravenel infection 20 days earlier than immunocompetent mice did, they succumbed to infection with *M. tuberculosis* Erdman more than 180 days earlier than immunocompetent mice did. This suggests that in immunocompetent mice, T-cell-mediated immunity was more successful in defending against the *M. tuberculosis* strains than against the *M. bovis* strains. Conversely, it suggests that the greater virulence of the *M. bovis* strains as measured by host survival times is based on a superior ability to resist host immunity. $CD4^+$ T-cell-depleted mice did not succumb to infection with *M. tuberculosis* R1Rv during the time course of this experiment. Similarly, a 10^5 CFU i.v. inoculum of *M. bovis* BCG failed to kill mice during the same time. This is in keeping with published evidence (10) which suggests that growth of *M. bovis* BCG can be restricted by a $CD4^+$ T-cell-independent macrophage-based defense mechanism.

Lung histopathology caused by different strains of mycobacteria. The possibility that the ability of the *M. bovis* strains to cause earlier death than *M. tuberculosis* strains was related to their ability to induce more rapid development of lung pathology and thereby to cause earlier loss of lung function was investigated by a histological study of the lungs of mice inoculated 40, 125, or 240 days earlier with one or other of the strains under study. It was found that on day 40, infection had caused an enlargement of the lungs which was more pronounced in mice infected with the bovine strains. Lung enlargement was even greater in mice depleted of $CD4^+$ T cells. This is evident in Fig. 3, which shows low-power micrographs of 10-μm-thick, crystal violet-stained sections through the middle of the left lung lobes of immunocompetent and $CD4^+$ T-cell-depleted mice infected with *M. bovis* Ravenel or *M. tuberculosis* Erdman. It can also be seen from these low-power micrographs that the darkly stained cellular infiltrate in the lungs of immunocompetent mice was more expansive in response to infection with *M. bovis* Ravenel (Fig. 3a) than to

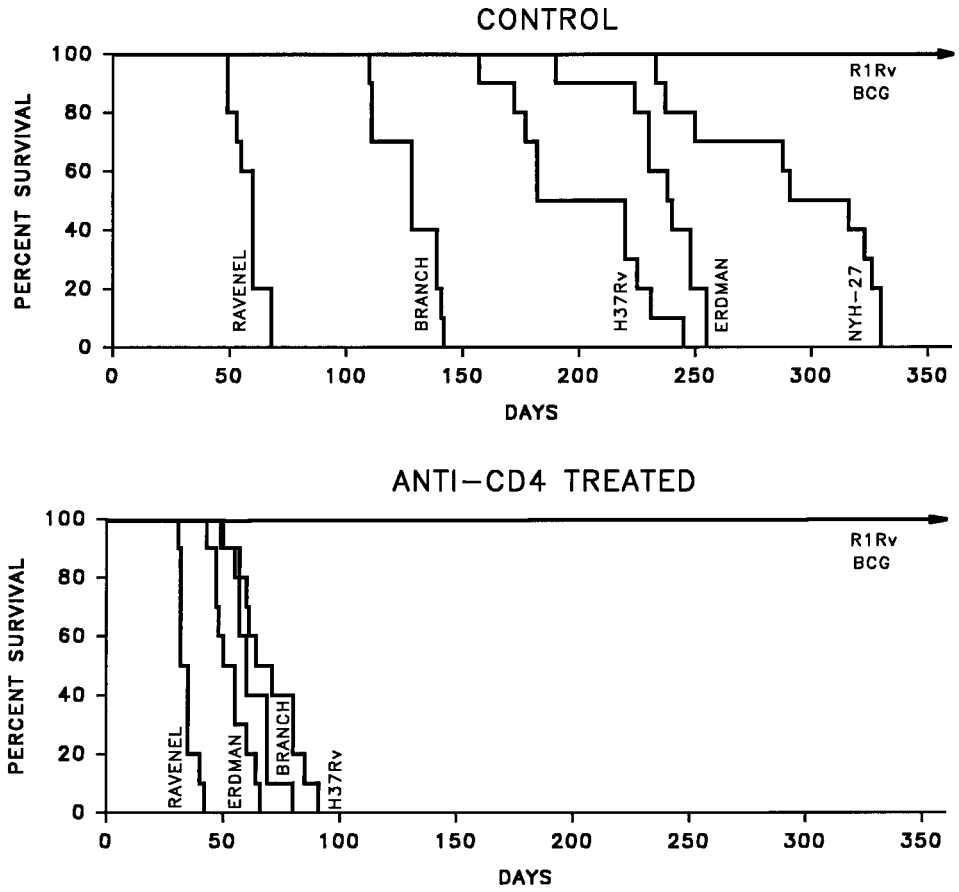

FIG. 2. Survival times of immunocompetent and $CD4^+$ T-cell-depleted mice infected i.v. with the virulent strains used in the experiment in Fig. 1 as well as two attenuated strains, *M. tuberculosis* R1Rv and BCG. MSTs were much shorter in immunocompetent mice infected with the virulent *M. bovis* strains (Ravenel and Branch) than in those infected with the virulent *M. tuberculosis* strains (Erdman, H37Rv, and NYH-27). Attenuated strains (R1Rv and BCG) failed to kill mice during the period studied. $CD4^+$ T-cell depletion reduced the survival time of mice infected with virulent organisms but more so in the case of *M. tuberculosis* strains. Neither BCG nor R1Rv killed $CD4^+$ T-cell-depleted mice over the 350-day period of the study. Results were obtained with 10 mice per group.

infection with *M. tuberculosis* Erdman (Fig. 3c). In the lungs of $CD4^+$ T-cell-depleted mice, the extent of cellular infiltrate in response to the two strains (Fig. 3b and d) was greatly increased, although the increase was greater in response to *M. bovis* Ravenel. It was apparent after examining a number of sections that infection of $CD4^+$ T-cell-depleted mice with either *M. bovis* Ravenel or *M. tuberculosis* Erdman caused more than a 50% consolidation of the lung lobes examined by day 40.

Because the cellular composition of day 40 lung lesions induced by all strains was similar, only higher-power micrographs of *M. tuberculosis* Erdman-infected lungs are presented. Figure 4 shows that sites of infection scattered through the lung (Fig. 4a) induce discrete areas of alveolitis (Fig. 4b) dominated by large mononuclear cells, many of which displayed the morphology of epithelioid macrophages (Fig. 4c) and were infected with acid-fast bacteria (Fig. 4d). Multinucleate giant cells were occasionally seen (Fig. 4c) but were not numerous. Each lesion occupied a considerable number of alveolar sacs and consequently resulted in consolidation of an appreciable volume of the lung. Although relatively few neutrophils were present at these sites overall, they were the predominant cells in some alveolar sacs in which it was apparent that bacterial growth was less well controlled (not shown). In the majority of cases, the accumulation of host cells at infectious foci was associated with an expansion of nearby intrapulmonary lymphoid tissue (Fig. 4b) concentrated around blood vessels and bronchioles.

In $CD4^+$ T-cell-depleted mice, the cellular response to infection with virulent *M. tuberculosis* or *M. bovis* in the lungs on day 40 was different. It was much denser than in immunocompetent mice when viewed at low power (Fig. 5a and b); at higher power (Fig. 5c and d), it was visible as a necrotic exudative alveolitis in which the alveoli of many contiguous alveolar sacs were filled with macrophages and neutrophils, the majority of which were dead and degenerating. Individual alveolar sacs were edematous and literally packed with degenerative cells, to the extent that alveoli (outpockets) of the sacs were difficult to recognize. These changes were associated with a pronounced thickening of interalveolar septa (Fig. 5b and c). The number of acid-fast bacilli (Fig. 5c) in many infected air sacs was enormous (>1,000 bacteria per field at ×400 magnification), and it was noted that both neutrophils and macrophages contained mycobacteria. Within each lesion, regions of necrotic exudative inflammation were separated by groups of air sacs occupied predominantly by large foamy macrophages that were only moderately infected with acid-fast bacilli (Fig. 5d). It was apparent that multiplication of *M. tuberculosis* was more restricted in these regions than in those containing necrotic cells. Overall, the inflammation in the lungs of *M. tuberculosis*-infected mice depleted of $CD4^+$ T cells exhibited fea-

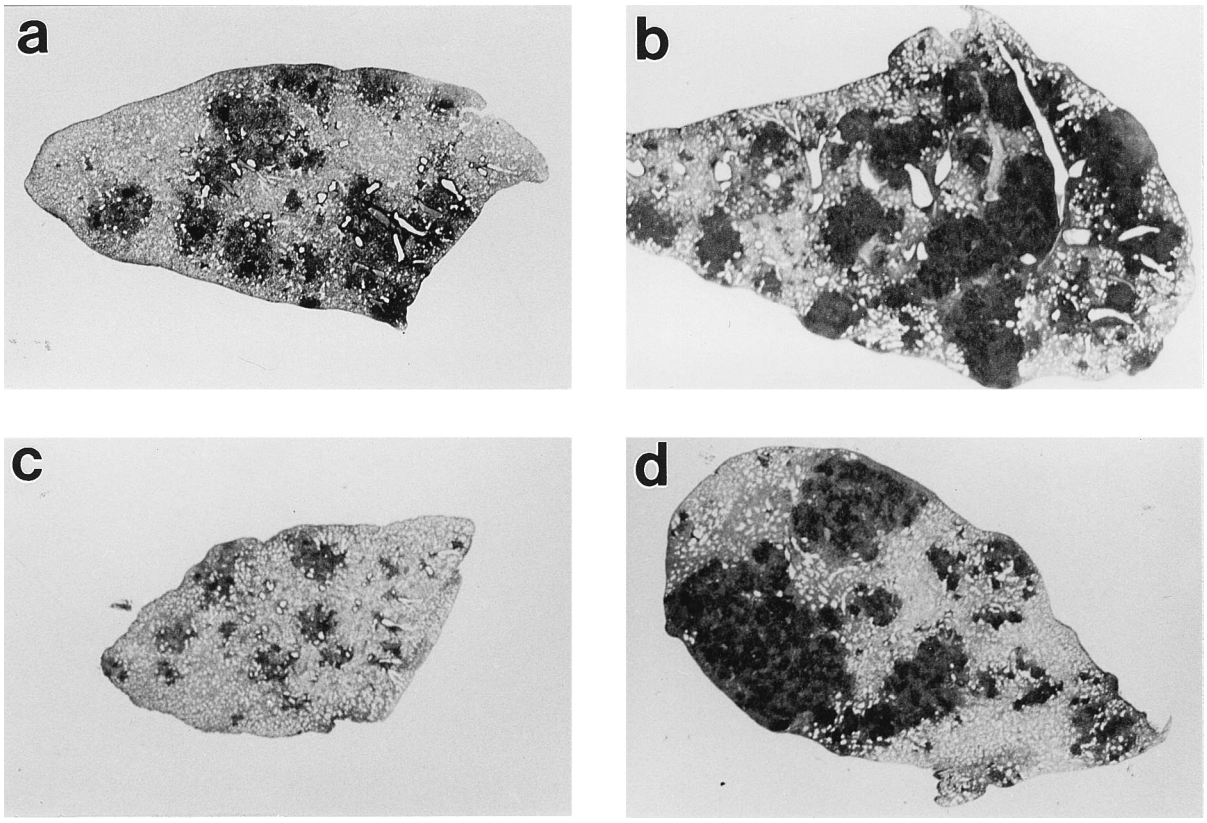

FIG. 3. Low-power micrographs (magnification, ×6.5) of crystal violet-stained sections of the left lung lobe of immunocompetent and $CD4^+$ T-cell-depleted mice infected i.v. 40 days earlier with 10^5 CFU of *M. tuberculosis* Erdman (c and d) or *M. bovis* Ravenel (a and b). Lesions were more numerous and larger in *M. bovis* Ravenel-infected mice (a) than in *M. tuberculosis* Erdman-infected mice (c). In lungs of mice depleted of $CD4^+$ T cells, individual lesions were larger still and in some cases had merged. However, they were more extensive in lungs infected with *M. bovis* Ravenel (b) than with *M. tuberculosis* Erdman (d). Infection also caused an increase in the volume of the lungs, with *M. bovis* Ravenel causing more of an increase than *M. tuberculosis* Erdman, and with both strains causing a greater increase in the absence of $CD4^+$ T cells.

tures similar to those of rapidly expanding bronchopneumonia seen in immunocompromised humans infected with *M. tuberculosis* (27).

Necrotic exudative inflammation of the type described above for $CD4^+$ T-cell-depleted mice failed to develop in the lungs of immunocompetent mice, even at an advanced stage of infection, when *M. tuberculosis* numbers approximated those seen earlier in immunodeficient mice. Instead, the longer survival times of *M. tuberculosis*-infected mice allowed time for them to develop a chronic type of lung pathology in response to infection. This pathology was characterized on day 240 of an *M. tuberculosis* Erdman infection, for example, by progressive interstitial fibrosis that almost completely replaced the airspaces of the lung with dense fibrotic tissue that was composed of fibroblasts and histiocytes (Fig. 6a to c). This fibrotic tissue was interrupted by highly conspicuous cysts (Fig. 6a and b) that, in section, gave the lung a honeycomb appearance typical of chronic inflammatory lung disease of humans (33). At this stage of infection, this type of pathology occupied more than 80% of the lung and contained scattered areas of intra-alveolar inflammation containing lymphocytes and neutrophils. It also contained sites of lymphoid cell expansion similar to those seen on day 40.

An examination of sections of lungs taken from day 125-infected mice indicated that the cysts present on day 240 in lungs with advanced fibrosis were caused by the coalescence of groups of alveolar sacs following the breakdown of interalveolar septa. It is apparent (Fig. 6d) that the alveolar sacs destined to form cysts contained epithelioid macrophages that were heavily infected with acid-fast bacilli. It is also apparent that infection-induced death of these macrophages resulted in events that caused dissolution and breakdown of interalveolar septa and loss of alveolar capillaries. The end result of this process and of interalveolar fibrosis was loss of most of the lung structure and function. It was obvious that progressive fibrosis was also associated late in infection with a progressive loss of stainable bacteria. This is in keeping with a 1.5 log reduction in the number of CFU in the lungs between days 150 and 240 of infection (data not shown).

Histological consequences of liver infection. Bacteria were confined to discrete foci in the livers of both immunocompetent and $CD4^+$ T-cell-depleted infected mice. By day 20 in immunocompetent mice (Fig. 7a), the sites were occupied by accumulations of macrophages that formed compact granulomas in which acid-fast bacilli were difficult to find. Lymphocytes and neutrophils were commonly seen at the periphery of these structures. Examination of the livers of these mice at later time points revealed that the majority of granulomas dissociated without trace. This is in keeping with the data from the growth curves in Fig. 1 showing that infection in this organ was undergoing resolution, there having been more than a 95% reduction in bacterial numbers between days 20 and 150. This was not the situation in the livers of mice depleted of $CD4^+$ T cells, in which mycobacteria grew progressively. By day 40, the

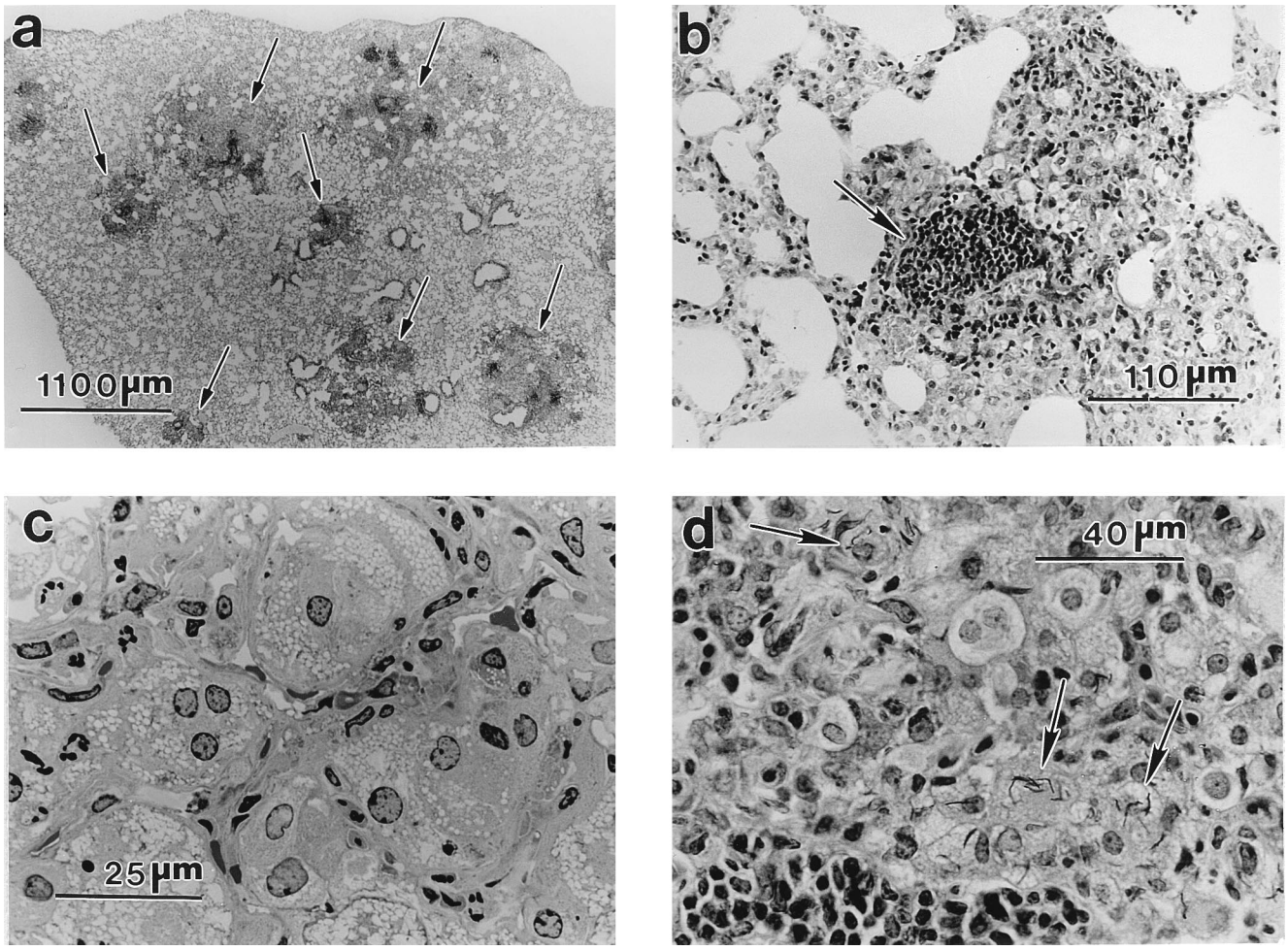

FIG. 4. Histology of the left lung lobe of a 40-day *M. tuberculosis* Erdman-infected immunocompetent mouse. (a) Low-power micrograph shows discrete areas of infection-induced inflammation (arrows) at multiple sites randomly distributed throughout the lung lobe. MacNeal's stain. Magnification, ×18. (b) Micrograph of an individual lesion shows that it is composed of inflammatory cells with an associated lymphoid aggregate (arrow). MacNeal's stain. Magnification, ×180. (c) High-power micrograph of plastic-embedded lung shows that sites of infection-induced inflammation (alveolitis) were dominated by large foamy macrophages. Crystal violet stain. Magnification, ×750. (d) Micrograph of a section stained for acid-fast organisms (arrows) shows bacteria located within foamy epithelioid macrophages of the type shown in panel c. Basic fuchsin stain. Magnification, ×540.

majority of organisms remained confined to sites of host inflammatory cell accumulation that were less compact than those in livers of control mice (Fig. 7b). It was apparent that at sites of infection in T-cell-depleted mice, individual macrophages contained considerable bacterial burdens. This was the case in mice infected with all strains of *M. tuberculosis* and *M. bovis.*

DISCUSSION

In this study, we examined the manifestations of virulence of two strains of *M. bovis* and four strains of *M. tuberculosis* in mice. We showed that according to their ability to cause progressive pathology in the lungs and the time taken for them to kill their host, the two *M. bovis* strains were more virulent than the four *M. tuberculosis* strains. Furthermore, on the basis of host survival time alone, *M. bovis* Ravenel was the most virulent, followed by *M. bovis* Branch, *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman, *M. tuberculosis* NYH-27, and *M. tuberculosis* R1Rv. It is noteworthy that earlier investigators (31) were under the impression that *M. bovis* Ravenel was more virulent for animals than were the *M. tuberculosis* strains they examined. Also of note is the finding that the recently isolated NYH-27 strain (8) of *M. tuberculosis* responsible for many cases of tuberculosis in the New York metropolitan area is of similar virulence to the other *M. tuberculosis* strains tested, which have been subjected to subculturing over many years.

It is shown here that in the lungs of mice, all strains caused a slowly progressive infection, whereas in the liver, infection was almost completely resolved in all mice. This ability to control infection in the liver and slow its progression in the lungs depended predominantly on the acquisition of $CD4^+$ T-cell-mediated immunity. The exception to this rule was infection with *M. tuberculosis* R1Rv, which was rapidly resolved in the liver and progressed only slowly in the lungs in both the presence and absence of $CD4^+$ T cells. This strain of *M. tuberculosis* is considered to be of very low virulence in animals (16, 28, 29). However, it obviously is not as attenuated as *M. bovis* BCG, which, according to previous studies (19, 22), is progressively inactivated in both the lungs and the livers of immunocompetent mice. Taken together, the results presented support the conclusion made in a recent publication (19) that virulent strains of mycobacteria differ from attenuated strains in being able to cause a slowly progressive infection in the

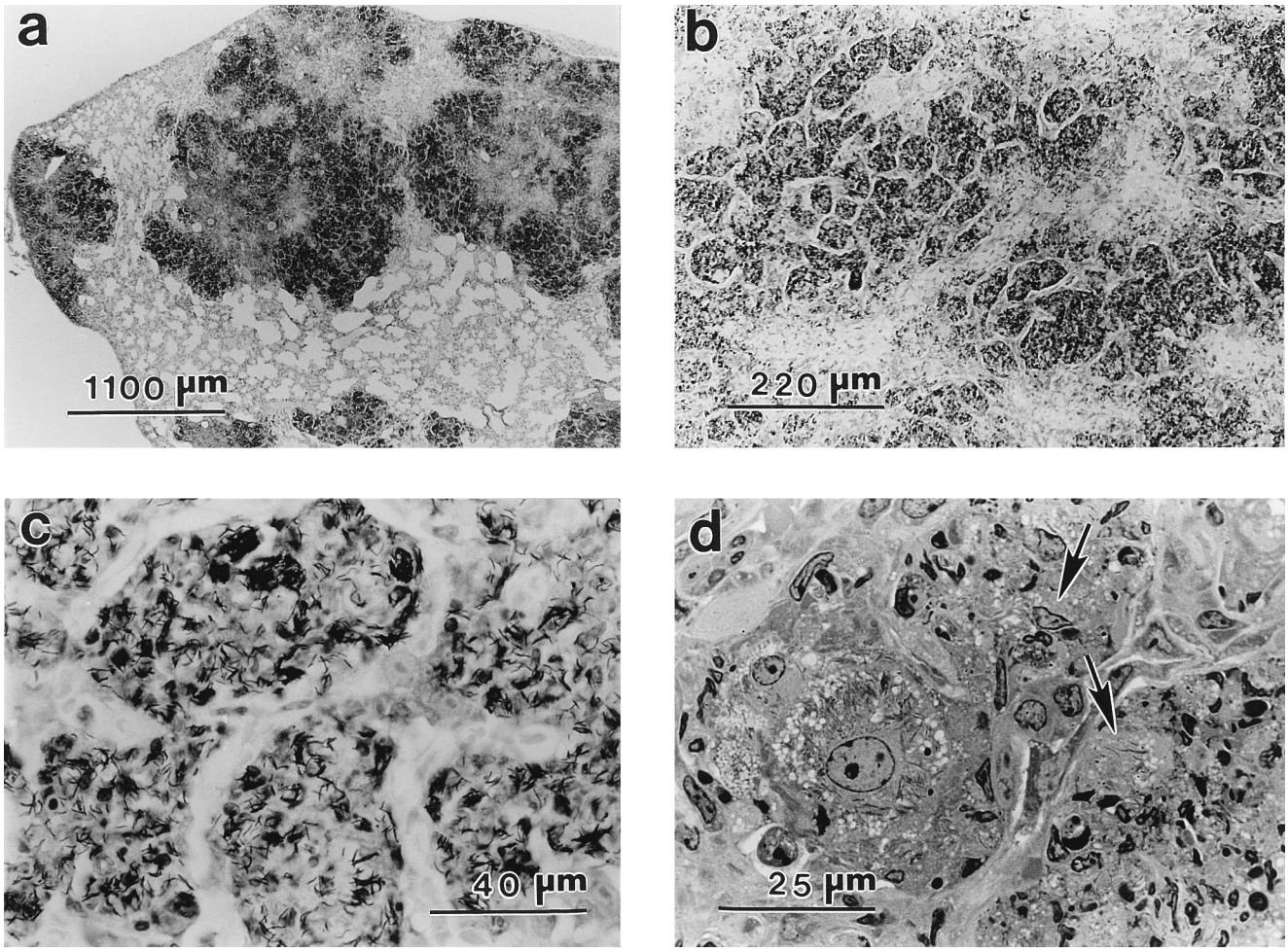

FIG. 5. Histology of the left lung lobe of a 40-day *M. tuberculosis* Erdman-infected $CD4^+$ T-cell-depleted mouse. (a) Low-power micrograph shows extensive, dense cellular infiltration in response to infection. MacNeal's stain. Magnification, ×18. (b) Higher magnification reveals that the cellular infiltrate represents a necrotic exudative pneumonia involving large numbers of contiguous alveolar sacs separated by greatly thickened septa. Basic fuchsin stain. Magnification, ×90. (c) At higher power, the air sacs appear highly cellular, being filled to capacity with cells replete with acid-fast bacteria. Basic fuchsin stain. Magnification, ×540. (d) Micrographs of a section of plastic-embedded lung demonstrate that the alveolar sacs as shown in panel c contain mostly degenerating cells (arrows). An adjacent alveolar sac is occupied by a large, heavily infected macrophage. Crystal violet stained. Magnification, ×750.

lungs in the face of a mechanism of $CD4^+$ T-cell-mediated immunity that is capable of resolving infection in other organs.

The early histopathology induced at sites of infection in the lungs of immunocompetent mice, as described here in detail, is in agreement with descriptions of early murine tuberculosis published some years ago by others (21, 23) and for early *M. bovis* infection in rabbits (14, 15). Given the more extensive pathology in lungs infected with the *M. bovis* strains at day 40 of infection, it is reasonable to suggest that this pathology was responsible for the much earlier time to death of mice infected with these strains. On the other hand, the much later time of death of mice infected with virulent *M. tuberculosis* strains was associated with the development of a different type of lung pathology which eventually occupied most of the lung with dense fibrotic tissue riddled with cysts. This chronic-type pathology is similar in appearance to the end-stage honeycomb lung pathology seen in the lungs of humans with any one of several interstitial lung diseases (33) or in the lungs of mice infected with the fungus *Paracoccoidiosis brasiliensis* (24). It is apparent that this chronic type of *M. tuberculosis*-induced lung pathology in mice has not been described previously, probably because earlier studies of mouse tuberculosis (23) used inocula large enough to cause death in 4 to 8 weeks.

The relevance of late-stage, *M. tuberculosis*-induced chronic lung pathology described here for mice to lung pathology in *M. tuberculosis*-infected humans is not obvious. The key histopathological feature in human tuberculosis is caseation at the site of infection, which leads to the formation of cavities (5, 25). However, cavity formation in humans is preceded by perilesional fibrosis and the destruction of infected macrophages and pulmonary tissue within. Therefore, it seems possible that a similar process is set in motion in the *M. tuberculosis*-infected mouse lung, where some air sacs and associated capillaries are displaced by fibrosis while other air sacs that contain heavily infected macrophages merge to form cysts. This latter event could be considered a form of caseation. On the other hand, the lung pathology that developed in response to unrestricted bacillary growth in the absence of specific immunity in $CD4^+$ T-cell-depleted mice bears a close resemblance to that described for immunocompromised humans (27), including those infected with HIV (9, 32). It was seen to consist of a rapidly expanding necrotizing bronchopneumonia that literally filled

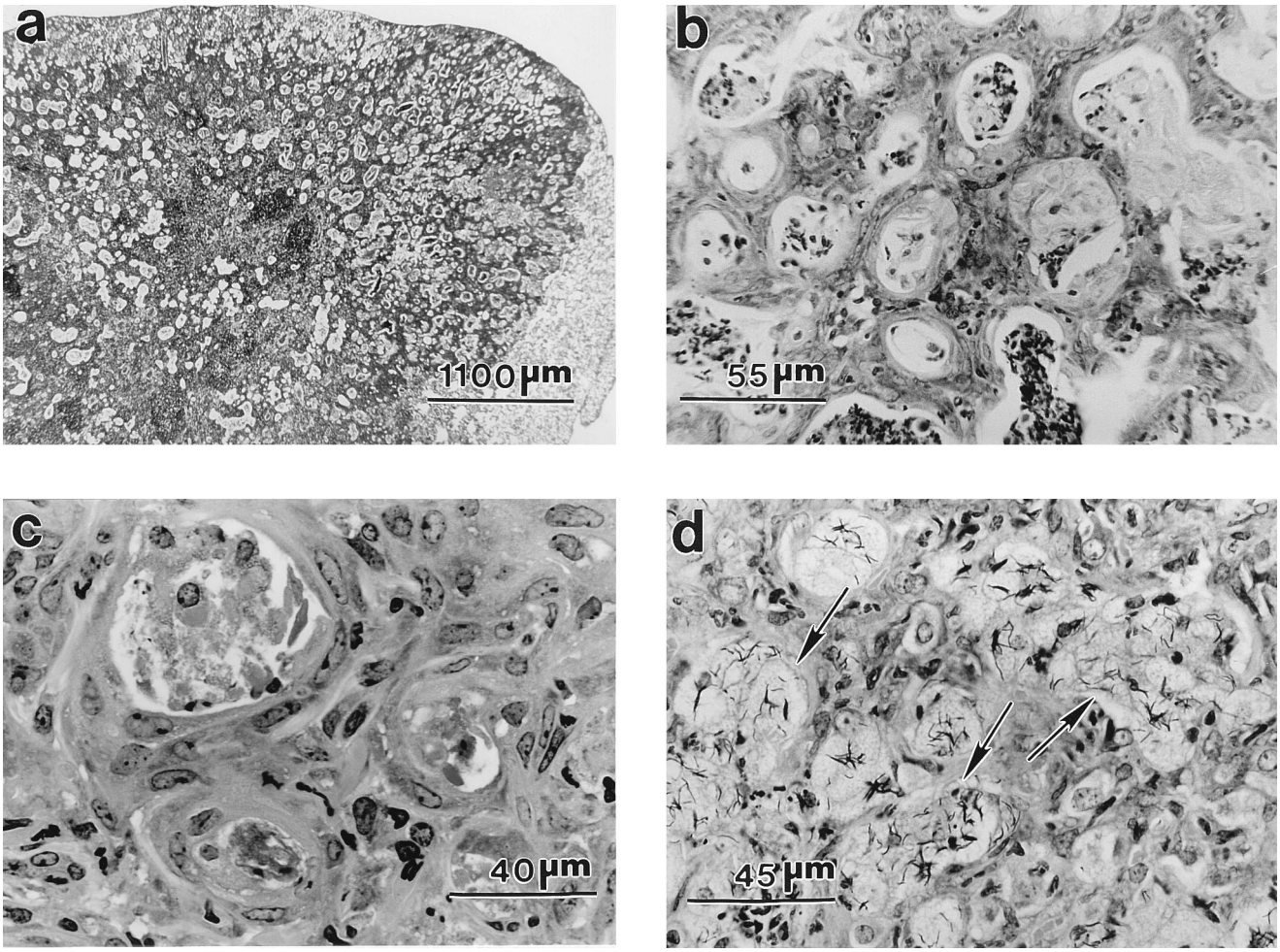

FIG. 6. Histological appearance of the left lung lobe from a 240-day *M. tuberculosis* Erdman-infected mouse with advanced chronic disease. (a) Low-power view shows that most of the lung lobe is occupied by dense fibrosis containing cysts, giving a honeycombed appearance. Crystal violet stain. Magnification, ×18. (b) Higher-power micrograph shows fibrotic tissue containing cystic spaces filled with mucus and degenerating inflammatory cells. Few bacteria were seen in the numerous degenerating macrophages that occupy the cysts. Basic fuchsin stain. Magnification, ×360. (c) High-power view of a thin section of plastic-embedded lung shows an area of expanded interstitial fibrosis and several developing cysts. Crystal violet stain. Magnification, ×540. (d) Micrograph of an earlier stage of development of lung fibrosis (day 125 of infection) shows the process of dissolution of alveolar septa to cause coalescence of alveolar sacs in some areas (arrows). At this stage, cells occupying most of the air sacs were heavily infected. Basic fuchsin stain. Magnification, ×450.

the airspaces with neutrophils and histiocytes, the majority of which underwent degradation. It was noticeable that there was no obvious expansion of intrapulmonary lymphoid tissue at sites of infection or elsewhere in $CD4^+$ T-cell-depleted mice, in spite of their possession of $CD8^+$ T cells and B cells. This is in marked contrast to the situation in immunocompetent mice, in which the cellular response to infection was associated with a conspicuous expansion of lymphoid tissue and its retention around veins, arteries, and bronchioles adjacent to infectious foci. Expansion of this lymphoid tissue persisted until the mice died with chronic pulmonary pathology at day 240 or later.

It was to be expected that the absence of T-cell-mediated immunity would serve to greatly reduce the MST of mice infected with any one of the virulent strains of mycobacteria tested. However, survival time was reduced to a much larger extent in mice infected with less virulent organisms. This had the effect of bringing all MSTs closer together and indicated that specific immunity had a more restrictive influence on disease progression caused by the less virulent organisms. This was not due to the ability of immunity to restrict the multiplication of the less virulent strains to a greater extent, because after day 20 of infection, immunity was capable of restricting the rate of growth of all strains examined to approximately the same degree. It can be argued, therefore, that immunity was less protective against more virulent strains because it was less restrictive of the development of the pathology they caused. In other words, more virulent organisms are more capable of inducing lung pathology in the presence of immunity. This might result from an altered capacity of these strains to induce the production of proinflammatory cytokines and chemokines by the cells they infect, which might depend, in turn, on the quantity of metabolites that these strains secrete in their host cells.

One such metabolite that would have to be considered in inducing lung pathology is lipoarabinomannan, a cell wall constituent of mycobacteria that is considered to be a key virulence factor (2) and that when ingested by macrophages, induces them to produce tumor necrosis factor alpha (3, 18, 26). It can be suggested that acquired immunity, as expressed through the activation of infected lung macrophages, is more capable of suppressing the metabolic activity of and consequently influencing the secretion of products like lipoarabino-

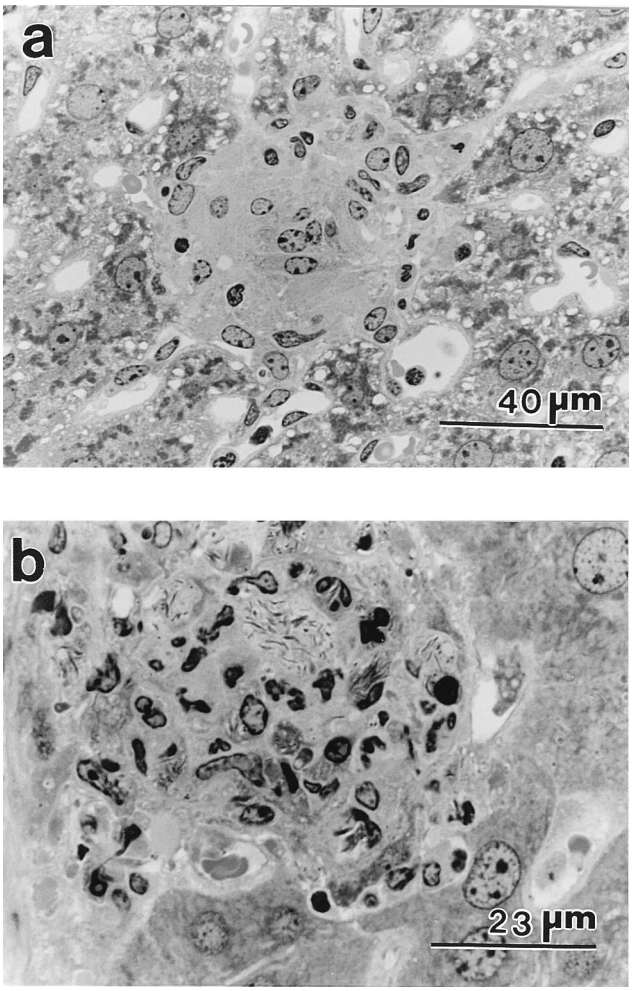

FIG. 7. Sites of Erdman infection in the livers of 20-day-infected immunocompetent (a) and 40-day-infected $CD4^+$ T-cell-depleted (b) mice. In both cases, the lesions are well demarcated and surrounded by intact hepatocytes. (a) In immunocompetent mice, the lesions were compact and contained mostly epithelioid macrophages. Acid-fast bacteria were difficult to find. Crystal violet-stained sections of plastic-embedded liver. Magnification, ×540. (b) The liver lesions of $CD4^+$ T-cell-depleted mice were less compact, and epithelioid macrophages were rarely evident. Instead, the lesions were populated by neutrophils and mononuclear cells with a lymphoid appearance. Macrophages present were often heavily infected with acid-fast bacteria. Crystal violet stain. Magnification, ×900.

mannan from less virulent than the more virulent strains of mycobacteria. There is also evidence (2) that lipoarabinomannan can inhibit the activation of macrophage microbicidal function by inhibiting the transcription of gamma interferon-inducible genes. Thus, in lung macrophages, this mycobacterial product, and perhaps others, might activate the transcription of inflammation-inducing cytokine genes on the one hand, yet suppress transcription of genes necessary for the expression of mycobactericidal function on the other.

Another factor to consider in explaining the faster progression of disease in the lungs of mice infected with *M. bovis* strains, however, is that a larger number of the former organisms were implanted in the lungs following i.v. inoculation of a standard number of bacilli. For example, it was determined during this study that 2.8% ± 1.3% of an i.v. inoculum of *M. bovis* Ravenel, in contrast to 0.48% ± 0.26% of the same-sized inoculum of *M. tuberculosis* H37Rv, was implanted in the lungs by 24 h postinoculation. The possibility must be considered, therefore, that the *M. bovis* strains were more virulent partly because their i.v. inoculation resulted in the establishment of more sites of infection in the lungs, the only organ in which disease develops. It will be important, therefore, to compare virulence of the *M. bovis* and *M. tuberculosis* strains in mice infected with the same number of bacilli by aerosol.

According to this study, mycobacterial virulence as gauged by host survival time was not a function of mycobacterial doubling time, in that *M. bovis* Branch, with a doubling time in the lungs of 41 h, was more virulent than *M. tuberculosis* Erdman and *M. tuberculosis* H37Rv, with doubling times of 21 and 36 h, respectively. It is obvious, on the other hand, that the preimmunity doubling time for each strain determined its number in the lungs on day 20 of infection, when immunity began to be expressed. Because of this, there were much larger numbers of *M. tuberculosis* Erdman and *M. bovis* Ravenel bacilli in the lungs at day 20 than of *M. tuberculosis* H37Rv and *M. tuberculosis* NHY-27 bacilli and a considerably larger number of the last two strains than of *M. tuberculosis* R1Rv. In spite of this, however, the restricted rate of progression of infection after day 20 was similar for all organisms, with the exception of *M. bovis* Ravenel, whose infection progressed somewhat faster. Assuming that a larger number of bacilli in the lungs on day 20 is a reflection of a larger number per lesion, immunity in this organ was just as efficient at restricting multiplication of *M. tuberculosis* R1Rv as multiplication of *M. tuberculosis* Erdman, even though 1 log more of the latter strain was present at the onset of expression of immunity.

It is obvious that virulence factors provide mycobacteria with a capacity to survive in the lung and cause progressive disease, in spite of a state of host immunity capable of inactivating the organisms in other organs. However, the properties that enable the organisms to persist in the lungs of mice in a metabolically active form and induce destructive pathology have yet to be elucidated. Because acquired immunity is capable of restricting the growth of different strains of *M. tuberculosis* and *M. bovis* to the same extent, even though their potential doubling times differ considerably, it is suggested that their virulence is best gauged by the rate at which they cause lung pathology and death of the host. This would be in keeping with the proposal of Mitchison et al. (17) that virulence of a given strain of *M. tuberculosis* is best measured in terms of the extent to which the strain causes organ pathology during a given time of infection. The alternative suggestion by others (4, 16), that the rate of growth of one strain with respect to another during the first few weeks of infection is a better measure of its virulence, may be misleading, except when large enough numbers of bacilli of either strain are inoculated to ensure rapid death from early-type lung pathology. Under these conditions, it is possible that *M. tuberculosis* Erdman is more virulent than *M. tuberculosis* H37Rv (13).

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