Mycobacterial Virulence. Virulent Strains of *Mycobacteria tuberculosis* Have Faster In Vivo Doubling Times and Are Better Equipped to Resist Growth-inhibiting Functions of Macrophages in the Presence and Absence of Specific Immunity

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Summary

The kinetics of growth of two virulent strains of mycobacteria (*M. tuberculosis* Erdman and *M. tuberculosis* H37Rv) and two attenuated strains (*M. tuberculosis* H37Ra and *M. bovis* Bacillus Calmette-Guerin [BCG]) were studied in the lungs, livers, spleens, and kidneys of severe combined immunodeficient (SCID) mice and of their coisogenic CB-17 immunocompetent counterparts. It was found, in keeping with the findings of earlier investigators (Pierce, C. H., R. J. Dubos, and W. B. Schaefer. 1953. *J. Exp. Med.* 97:189.), that in immunocompetent mice, virulent organisms grew progressively only in the lungs, whereas the growth of attenuated organisms was controlled in all organs. In SCID mice, in contrast, virulent mycobacteria grew rapidly and progressively in all organs, as did BCG, although at a slower rate. However, H37Ra failed to grow progressively in any organs of SCID mice, unless the mice were treated with hydrocortisone. In fact, hydrocortisone treatment enabled virulent, as well as attenuated, organisms to grow strikingly more rapidly in all organs of SCID mice and in all organs of CB-17 mice. A histological study showed that in SCID mice, multiplication of mycobacteria in the liver occurs in the cytoplasm of macrophages in granulomas and presumably in macrophages in other organs. It is suggested, therefore, that the macrophages of SCID mice possess a glucocorticoid-sensitive mycobacterial mechanism that prevents virulent and avirulent mycobacteria from expressing their true minimal doubling times. In the absence of this mechanism in the lungs of hydrocortisone-treated SCID mice, the doubling times of Erdman, H37Rv, BCG, and H37Ra were 17.7, 17.4, 44.6, and 98.6 h, respectively. The possible importance of a rapid multiplication rate for mycobacterial virulence is discussed.

Strains of *Mycobacteria tuberculosis* are considered virulent if they have been isolated from persons with active disease and are capable of causing progressive infection in experimental animals. Attenuated strains are derived from virulent ones and have lost the ability to grow progressively in animals. The suitability of mice for studying host resistance to virulent and attenuated strains of mycobacteria was demonstrated some time ago by Pierce et al. (1) who showed that standard strains considered virulent for humans are also virulent for mice, and that virulence is associated with the ability of these strains to grow progressively in the lungs, rather than in other organs, regardless of the route of infection. Attenuated strains, in contrast, were shown to be capable of only limited growth in all organs, presumably because of the growth inhibitory action of acquired immunity.

It is now well established that the control and subsequent resolution of infection with attenuated mycobacteria, such as the attenuated strain of *Mycobacteria bovis*, Bacillus Calmette-Guerin (BCG)[1], is dependent on an acquired mechanism of cellular immunity that is predominantly mediated by $CD4^+$ T cells. Evidence for this conclusion is seen in publications (2, 3) showing that mice selectively depleted of $CD4^+$ T cells, but not $CD8^+$ T cells, are rendered incapable of resolving BCG infection, and that mice in the process of resolving BCG infection possess $CD4^+$ T cells capable of transferring some level of immunity to irradiated recipient mice (4). There is also evidence (5) that CD4 T cells are responsible for the limited antimycobacterial resistance offered by mice before succumbing to infection with virulent mycobacteria. It is apparent, therefore, that attenuation of *M. tuberculosis* and *M. bovis* is associated with loss of ability to grow progressively in mice in the face of an acquired mechanism of T cell–mediated immunity.

Obviously, attenuated mycobacteria are of interest because of their possible usefulness as vaccines. For example, it has been shown (6, 7) that mice infected with BCG or attenu-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/06/1723/11 \$2.00
Volume 177 June 1993 1723–1733

ated *M. tuberculosis* are protected to various degrees against subsequent infection with virulent organisms. However, the ability of BCG to immunize humans is far less certain, in that its effectiveness in a number of vaccine trials has varied enormously, from providing 80% to zero protection (8). This has led some to suggest (8, 9) that more immunogenic BCG vaccines be developed, perhaps with the aid of recombinant DNA technology. However, it might be premature to embark on a search for more immunogenic vaccines until it is first known whether lack of immunogenicity is the reason for the limited effectiveness of BCG as a vaccine. It seems possible, until shown otherwise, that virulence of *M. tuberculosis* is based on an ability to avoid destruction by even high levels of acquired specific immunity. Therefore, a rational approach to the design of an efficacious antituberculosis vaccine will require some understanding of the meaning of mycobacterial virulence.

It was with a view to understanding mycobacterial virulence that the study described here was undertaken. The results show, as an extension of the original findings of Pierce et al. (1), that virulent mycobacteria, in contrast to attenuated ones, have the capacity to grow progressively in the lungs of mice in the face of an acquired mechanism of CD4 T cell–mediated immunity that is capable of controlling mycobacterial growth in other organs. It is shown, in addition, that virulent organisms have a faster doubling time than attenuated organisms in SCID mice, and an even faster doubling time in SCID mice whose immunological-independent defenses have been compromised by treatment with hydrocortisone.

Materials and Methods

Mice. CB-17 *scid/scid* (SCID) and coisogenic CB-17 mice were employed when they were 8 wk of age. They were supplied by the Trudeau Institute Animal Breeding Facility and were known to be free of common viral pathogens according to the results of routine screening performed by the Research Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri. These mice were also free of *Pneumocystis carinii* as a result of having been bred from B and T cell–engrafted parents (10). They were housed in isolator cages supplied with filtered air, and provided with sterilized food and water.

Mycobacteria. All strains of mycobacteria were obtained from the Trudeau Mycobacterial Culture (TMC) Collection. Virulent strains employed were *M. tuberculosis* Erdman (TMC No. 107) and *M. tuberculosis* H37Rv (TMC No. 102). Both were supplied as frozen (−70°C) log-phase, dispersed cultures in Proskauer and Beck (P and B) medium (Difco Laboratories, Detroit, MI) containing 0.01% Tween 80. To ensure virulence, the cultures were thawed and used to seed Tween 80-free P and B medium on which *M. tuberculosis* was allowed to grow as a surface pellicle over several weeks of subculturing. The final culture was homogenized in a glass tube fitted with a teflon pestle to break up bacterial rafts, and the homogenate used to seed a large volume of P and B medium containing 0.01% Tween 80 in which *M. tuberculosis* grew as a submerged, dispersed culture at 37°C. The culture was harvested towards the end of log-phase growth and dispersed in 1-ml volumes that were frozen and stored at −70°C to serve as stock cultures for experiments. For each experiment, a vial was thawed, subjected to 5 s of ultrasound to break up aggregates, diluted in PBS containing 0.01% Tween 80, and 0.2 ml used to inoculate mice via a lateral tail vein. Attenuated strains employed were *M. tuberculosis* H37Ra (TMC No. 201) and *M. bovis* BCG Pasteur (TMC No. 1011). These were also supplied as frozen log-phase cultures in P and B medium containing 0.01% Tween 80. They were prepared for inoculation as described for virulent strains. All strains were enumerated in the lungs, kidneys, livers, and spleens of infected mice by preparing homogenates of these organs in PBS containing 0.05% Tween, and plating 10-fold serial dilutions of the homogenates on enriched agar (Middlebrook 7H11; Difco Laboratories). Colonies were counted after 3–4 wk incubation at 37°C.

T Cell Depletion. Mice were depleted of CD4 T cells by injecting them intravenously at the times of infection indicated with 0.5 mg of anti-CD4 mAb produced by hybridoma GK1.5 (TIB 207; American Type Culture Collection, Rockville, MD). The hybridoma was grown as an ascites in immunodepressed mice, and the ascites was harvested and the mAb purified according to procedures described previously (3).

Cytofluorometry. The SCID mouse colony was sampled routinely to check for B and T cell "leakiness". This involved examining suspensions of LN cells with a FACScan® cytofluorometer (Becton Dickinson & Co., Mountain View, CA) after incubating the cells with FITC-conjugated $F(ab')_2$ fragments of anti-CD4, anti-CD8, or anti-Ig mAbs according to procedures described previously (3).

Histology. Small pieces of livers were fixed overnight in 10% phosphate-buffered formalin, washed for 2 h in tap water, dehydrated in 70 and 100% ethanol, and embedded in glycol methacrylate (JB-4 embedding kit; Polysciences, Inc., Warrington, PA) according to the suppliers instructions. 1–2-μm sections were cut with glass knives using a ultramicrotome (Porter Blum MT-1; Sorvall, Inc., Newtown, CT). The sections were stained with Ziehl-Neelsen stain, or with a phenol-crystal violet stain (11) at 60°C followed by brief decolorization with 95% ethanol. Micrographs were taken with a Nikon Microphot-Fx microscope.

Hydrocortisone Acetate. This was obtained as an emulsion from United Research Laboratories (Philadelphia, PA). It was given subcutaneously in a dose of 2.5 mg at 5-d intervals.

Results

Growth Potential of Virulent Versus Attenuated Mycobacteria in SCID and CB-17 Mice. Because specific immunity would be expected to restrict the growth of virulent and attenuated mycobacteria to different degrees, it was reasoned that a true comparison of the growth rates of these organisms in vivo can only be made by following their growth in mice that are incapable of generating specific immunity. Therefore, experiments were performed that compared the growth of attenuated and virulent mycobacteria in SCID mice, as well as in their immunocompetent CB-17 coisogenic counterparts.

Figs. 1–4 show the growth of *M. tuberculosis* Erdman, *M. tuberculosis* H37Rv (virulent strains), *M. tuberculosis* H37Ra, and BCG (attenuated strains), respectively, in the lungs, kidneys, spleens, and livers of SCID and CB-17 mice over a 60-d period of infection. It can be seen that in CB-17 mice, the main difference between virulent and attennuated mycobacteria was that the former, but not the latter organisms, grew progressively in the lungs. In the other organs of CB-17 mice, none of the strains grew progressively, although it is apparent that Erdman was the more virulent of the two virulent strains,

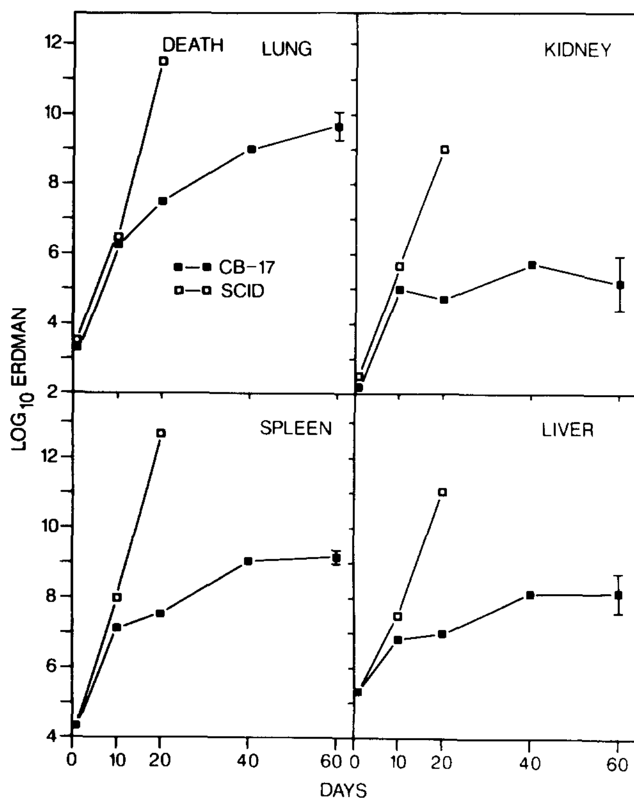


Figure 1. Growth of *M. tuberculosis* Erdman in the lungs, kidneys, spleens, and livers of SCID and CB-17 mice inoculated with 2 × 10^5 bacilli i.v. Means of five mice per time point.

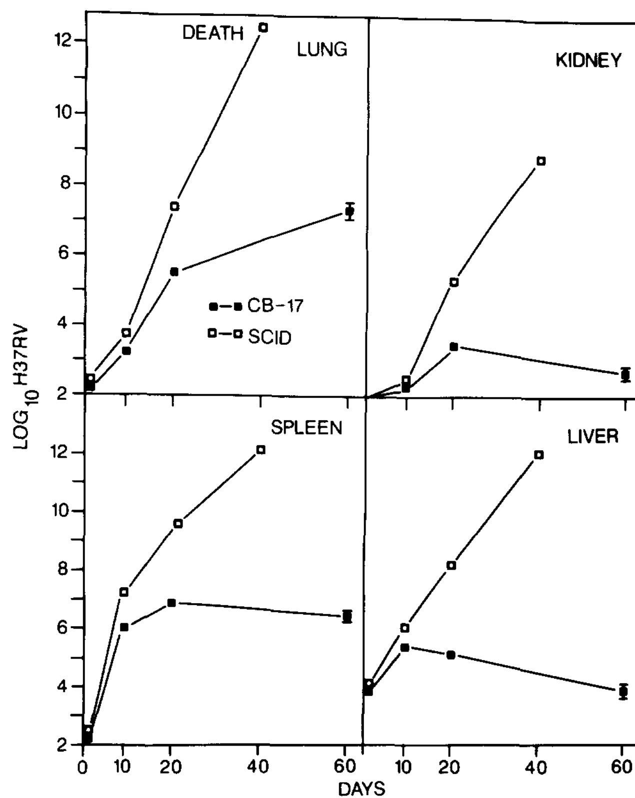


Figure 2. Growth of *M. tuberculosis* H37Rv in the lungs, kidneys, spleens, and livers of SCID and CB-17 mice inoculated with 10^4 bacilli i.v. Means of five mice per time point.

because its numbers plateaued, whereas those of H37Rv decreased. Indeed, on the basis of its growth characteristics in the liver, spleen, and kidney, H37Rv appeared no more virulent than BCG or H37Ra.

In SCID mice, the situation was different in that virulent strains grew rapidly and progressively in the liver, spleen, and kidneys, as well as in the lungs, although fastest in the lungs. BCG also grew progressively in all organs of SCID mice, but at a substantially slower rate than virulent strains. It is obvious moreover, that BCG also grew fastest in the lungs, meaning that its growth, like that of virulent strains, was more restricted in other organs in the absence of a specific immune response. H37Ra, on the other hand, failed to grow progressively in any organ of SCID mice, indicating the possibility that in the absence of an immune response the growth of this organism was self-limiting.

Control of Growth of H37Rv in the Livers, Spleens, and Kidneys of CB-17 Mice Is Dependent on CD4 T Cells. It is shown by the foregoing results that, whereas growth of virulent mycobacteria in immunocompetent mice is progressive in the lungs, it is controlled in other organs after 10–20 d of infection. This indicates that a mechanism of immunity is acquired in response to infection with virulent mycobacteria which is efficiently expressed in all organs except the lungs. Because resolution of BCG infection has been shown to depend predominantly on the function of CD4 T cells (2, 3), it was anticipated that this same subset of T cells would prove to be necessary for control of infection with virulent organisms. That this is the case is shown in Fig. 5, where it can be seen that H37Rv-infected CB-17 mice injected intravenously with 0.25 mg of anti-CD4 mAb on days 5, 10, 20, 30, and 40 of infection were greatly deficient in their capacity to inhibit the growth of this organism in their livers, spleens, and kidneys. Moreover, treatment with anti-CD4 mAb caused a substantial increase in the rate of growth of this organism in the lungs. These results do not exclude the participation of $CD8^+$ T cells and other T cells in the immune response.

Hydrocortisone Treatment Enhances Growth Rates of All Strains of Mycobacteria in SCID Mice. The foregoing results show that virulent mycobacteria grow rapidly and progressively in all organs of SCID mice, indicating that there is little resistance offered to the growth of these organisms in the absence of acquired specific immunity. BCG also grew progressively in SCID mice, but more slowly, indicating that in the absence of specific immunity it has a slower doubling time. Growth of H37Ra, however, was inhibited in SCID mice at an early stage of infection, making it likely that an immunologically independent mechanism of resistance is acquired that is capable of restricting the growth of this highly attenuated strain. If such a mechanism does develop, it is likely

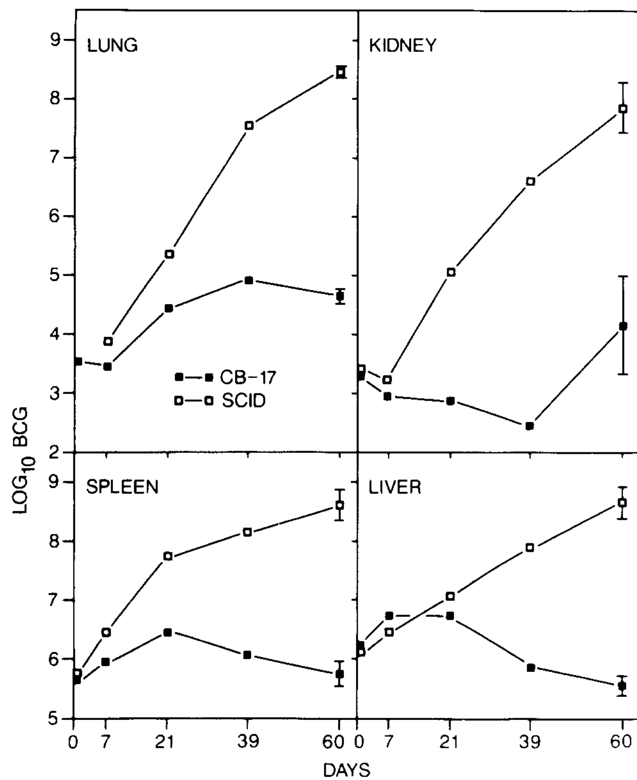


Figure 3. Growth of *M. bovis* BCG in the lungs, kidneys, spleens, and livers of mice inoculated with 2 × 10^6 bacilli i.v. Means of five mice per time point.

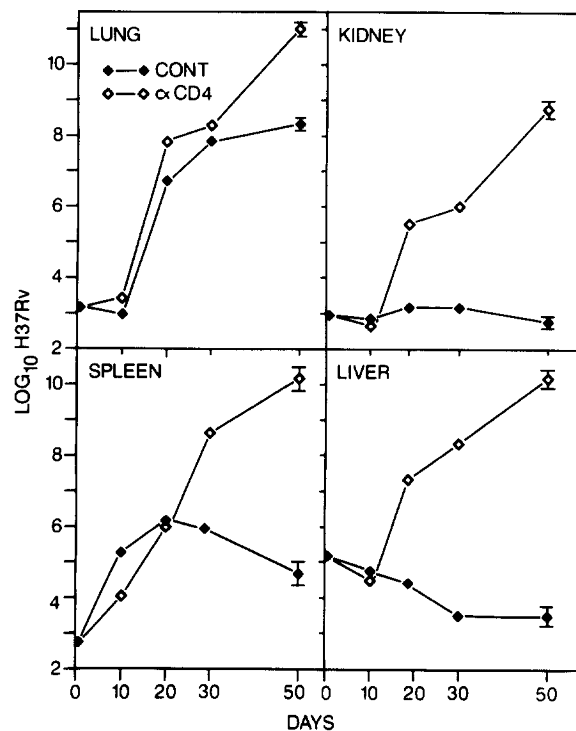


Figure 5. Exacerbation of *M. tuberculosis* H37Rv infection in CB-17 mice by intravenous injection of 0.5 mg of anti-CD4 mAb on days 5, 10, 20, 30, and 45 of infection. Control mice received equivalent amounts of rat IgG. Infection was initiated with 2 × 10^6 H37Rv i.v. Means of five mice per group per time interval.

that it also serves to restrict the growth of virulent mycobacteria in SCID mice. To investigate this possibility, the growth of virulent and avirulent organisms was followed in SCID mice treated with hydrocortisone. It was reasoned that, because glucocorticoids have been shown to inhibit the antimicrobial functions of macrophages in vitro (12, 13), these compounds should also be capable of inhibiting this function of macrophages in vivo.

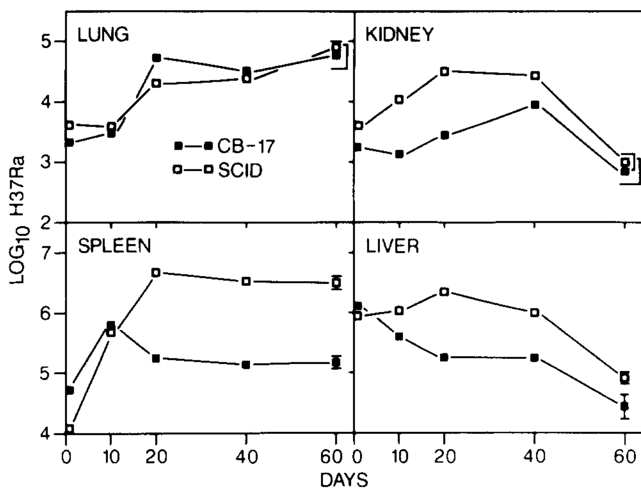


Figure 4. Growth of *M. tuberculosis* H37Ra in the lungs, kidneys, spleens, and livers of mice inoculated with 2 × 10^6 bacilli i.v. Means of five mice per time point.

The results in Table 1 show quite clearly with all mycobacteria investigated that 2.5 mg of hydrocortisone given subcutaneously on days 5, 10, and 20 of infection caused a large increase in bacterial load in all organs of SCID and CB-17 mice by day 25 of infection, at which time some cortisone-treated SCID mice infected with virulent strains began to die. As an example of the striking infection-enhancing action of hydrocortisone, it can be seen that hydrocortisone-treated SCID mice inoculated with 10^5 Erdman or 10^5 H37Rv contained $10^{13.24}$ and $10^{12.60}$ organisms, respectively, in their lungs on day 25 of infection, compared with $10^{11.75}$ Erdman and $10^{7.93}$ H37Rv in the lungs of control SCID mice. Hydrocortisone caused a similar increase in the numbers of virulent organisms in the lungs and other organs of CB-17 mice, but the absolute numbers reached by day 25 were considerably lower than those reached in the organs of SCID mice. The growth of BCG was also substantially increased in the organs of SCID and CB-17 mice given hydrocortisone. Of particular interest, however, is the finding in Table 1 showing that hydrocortisone caused an increase in the numbers of H37Ra in the organs of SCID and CB-17 mice. As shown in Fig. 4, this organism failed to grow progressively in any organ of control SCID mice. Therefore, the results with hydrocortisone show that failure of H37Ra to grow

Table 1. *Enhancing Effect of Hydrocortisone on Mycobacteria Infection in SCID and CB-17 Mice (Log$_{10}$ Bacteria/Organ on Day 25)*

Mice	Cortisone treatment	Strain	Lung	Kidney	Spleen	Liver
SCID	–*	Erdman	11.75 ± 0.29	7.55 ± 0.11	12.51 ± 0.15	10.06 ± 0.14
SCID	+		13.24 ± 0.08	10.59 ± 0.21	14.01 ± 0.14	14.00 ± 0.10
CB17	–		9.41 ± 0.14	4.67 ± 0.18	7.93 ± 0.24	6.58 ± 0.18
CB17	+		10.57 ± 0.09	5.33 ± 0.24	10.09 ± 0.22	7.63 ± 0.44
SCID	–	H37Rv	7.93 ± 0.11	6.70 ± 0.32	9.80 ± 0.13	8.58 ± 0.11
SCID	+		12.60 ± 0.23	9.45 ± 0.38	12.84 ± 0.12	12.30 ± 0.29
CB17	–		5.10 ± 0.15	2.43 ± 0.10	7.24 ± 0.14	4.88 ± 0.07
CB17	+		8.64 ± 0.14	3.41 ± 0.25	10.21 ± 0.15	6.01 ± 0.07
SCID	–	H37Ra	4.48 ± 0.20	4.01 ± 0.10	7.19 ± 0.16	6.04 ± 0.14
SCID	+		5.36 ± 0.20	4.48 ± 0.16	9.10 ± 0.15	9.01 ± 0.10
CB17	–		3.84 ± 0.08	1.81 ± 0.26	4.66 ± 0.09	4.34 ± 0.08
CB17	+		4.24 ± 0.15	2.56 ± 0.17	5.63 ± 0.18	5.41 ± 0.22
SCID	–	BCG	5.90 ± 0.13	5.70 ± 0.21	7.00 ± 0.09	6.90 ± 0.13
SCID	+		8.00 ± 0.19	6.32 ± 0.37	7.72 ± 0.10	7.80 ± 0.35
CB17	–		5.40 ± 0.08	4.45 ± 0.27	6.10 ± 0.14	6.11 ± 0.23
CB17	+		6.20 ± 0.31	4.80 ± 0.28	7.30 ± 0.30	7.50 ± 0.02

Mice were infected intravenously with 10^5 Erdman, 10^5 H37Rv, 10^6 H37Ra, or 10^6 BCG.
* 2.5 mg were given subcutaneously on days 10, 15, and 20.

progressively in SCID mice must be due to active growth inhibition by a cortisone-sensitive mechanism of resistance.

The influence of this growth-restrictive mechanism on the growth of virulent and avirulent mycobacteria is best illustrated in Table 2 which lists the doubling time of each strain in different organs of SCID and CB-17 mice treated or untreated with hydrocortisone. With a knowledge of the numbers of organisms on day 1 of infection, the doubling times were obtained by calculating the number of times that each strain needed to divide (as a population) in order to reach the numbers present on day 25. If it is assumed that SCID mice treated with hydrocortisone offered no resistance to mycobacterial replication, then, it can be concluded that virulent organisms have a much shorter intrinsic doubling time in vivo than attenuated ones, and that BCG has a shorter doubling time than H37Ra. Since the same ranking applies to doubling times measured in SCID control mice, it follows that virulent organisms are equipped to grow faster in the presence, as well as in the absence of this hydrocortisone-sensitive defense mechanism.

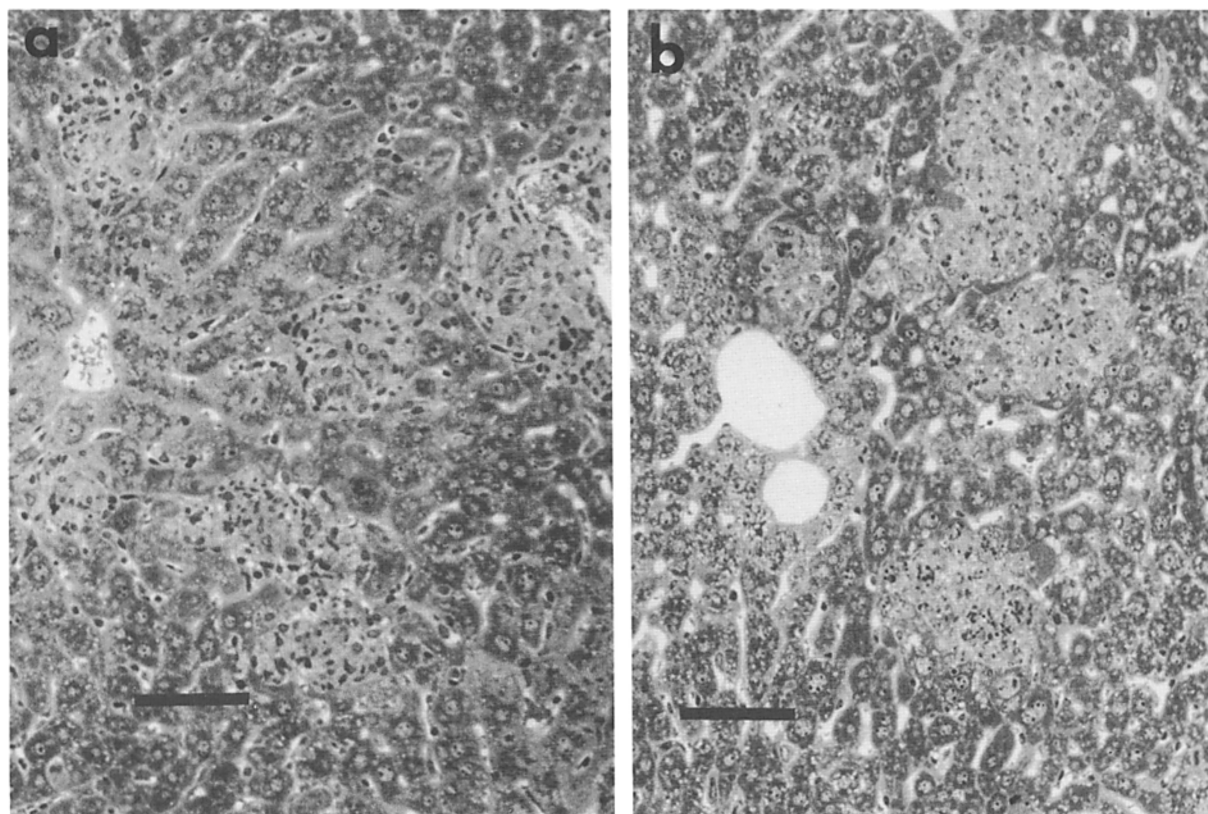
Growth Kinetics of BCG in the Organs of Hydrocortisone-treated Mice. The foregoing results clearly show that SCID mice possess a glucocorticoid-sensitive mechanism that enables them to restrict the growth of virulent, as well as attenuated strains of mycobacteria, as evidenced by a faster rate of growth of all strains in all organs of SCID mice treated with hydrocortisone. However, because these results were obtained on a single day of infection, it was considered important to determine the effect of giving hydrocortisone on the kinetics of mycobacterial growth over a relatively long period of infection. This was done by following the growth of BCG in untreated and hydrocortisone-treated SCID and CB-17 mice inoculated intravenously with 2 × 10^6 BCG. In this experiment hydrocortisone was given every 5 d of infection beginning on day 15. Day 15 was chosen to start hydrocortisone treatment in this experiment, and day 10 in the preceding experiments, because an ongoing histological study (North, R. J., and A. Izzo, manuscript in preparation) shows that by these times of infection, BCG is already in macrophages in developing granulomas in the livers and spleens of SCID and CB-17 mice. As can be seen in Figs. 6 and 7, hydrocortisone treatment caused a substantial increase in the rate of BCG growth in all organs of SCID and CB-17 mice between days 15 and 40 of infection, with the largest increase occurring in the lungs. It can also be seen that the rate of BCG growth in hydrocortisone-treated SCID mice was faster and more extensive than in hydrocortisone-treated CB-17 mice.

The purpose of the micrographs depicted in Figs. 8–10 is to show the consequences of hydrocortisone treatment on the cellular events at foci of infection in the livers of SCID mice. The results of a more extensive ongoing histological study of the consequences of hydrocortisone treatment on

Table 2. *Doubling Times of Mycobacteria Populations in Different Organs of SCID and CB-17 Mice Untreated or Treated with Hydrocortisone*

Mice	Cortisone treatment	Strain	Lung	Kidney	Spleen	Liver
				h		
SCID	–*	Erdman	21.0	34.4	21.4	36.4
SCID	+		17.7	21.4	18.0	19.9
CB17	–		28.3	70.2	48.0	135.5
CB17	+		24.0	55.9	30.0	77.8
SCID	–	H37Rv	32.0	32.1	23.8	38.9
SCID	+		17.4	21.3	16.7	21.1
CB17	–		64.2	168.4	35.7	197.2
CB17	+		27.8	86.3	22.2	86.3
SCID	–	H37Ra	197.2	423.5	58.1	NC
SCID	+		98.6	198.6	35.5	55.9
CB17	–		321.7	NC‡	NC	NC
CB17	+		184.6	NC	187.0	NC
SCID	–	BCG	96.3	91.2	133.6	157.8
SCID	+		44.6	68.8	85.8	86.7
CB17	–		102.1	231.3	248.2	NC
CB17	+		69.3	157.8	91.4	123.8

Doubling times determined from a knowledge of bacteria present in each organ on day 1 of intravenous infection and on day 25 as shown in Table 1.
* Cortisone given as for Table 1.
‡ Not calculated (NC) because the number of bacteria on day 25 was no greater than on day 1.



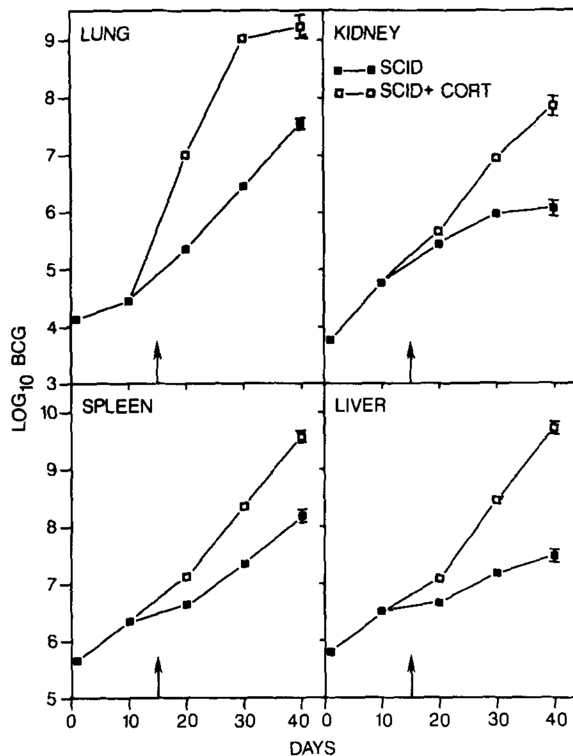


Figure 6. Growth of BCG in the lungs, kidneys, spleens, and livers of SCID mice and SCID mice treated with 2.5 mg of hydrocortisone acetate (SCID + CORT) at 5-d intervals beginning on day 15 of infection initiated with 5 × 10^5 BCG i.v. Means of five mice per group per time interval.

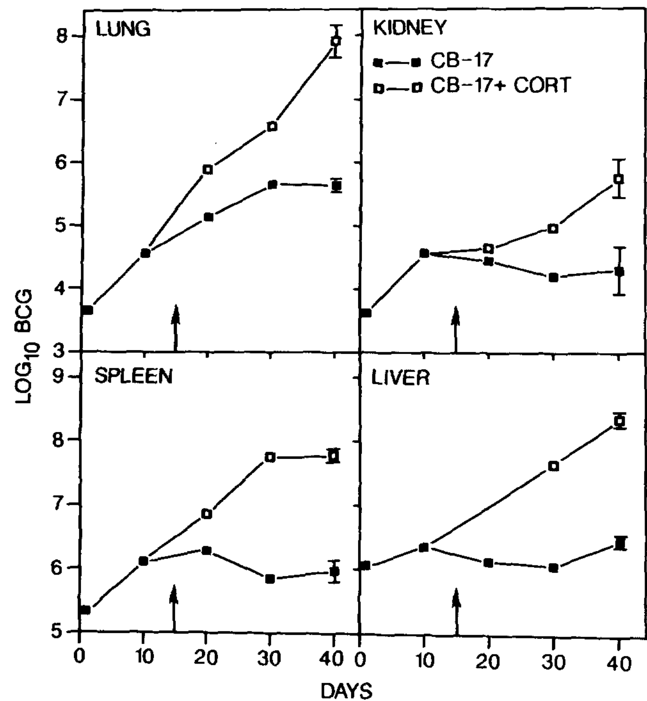


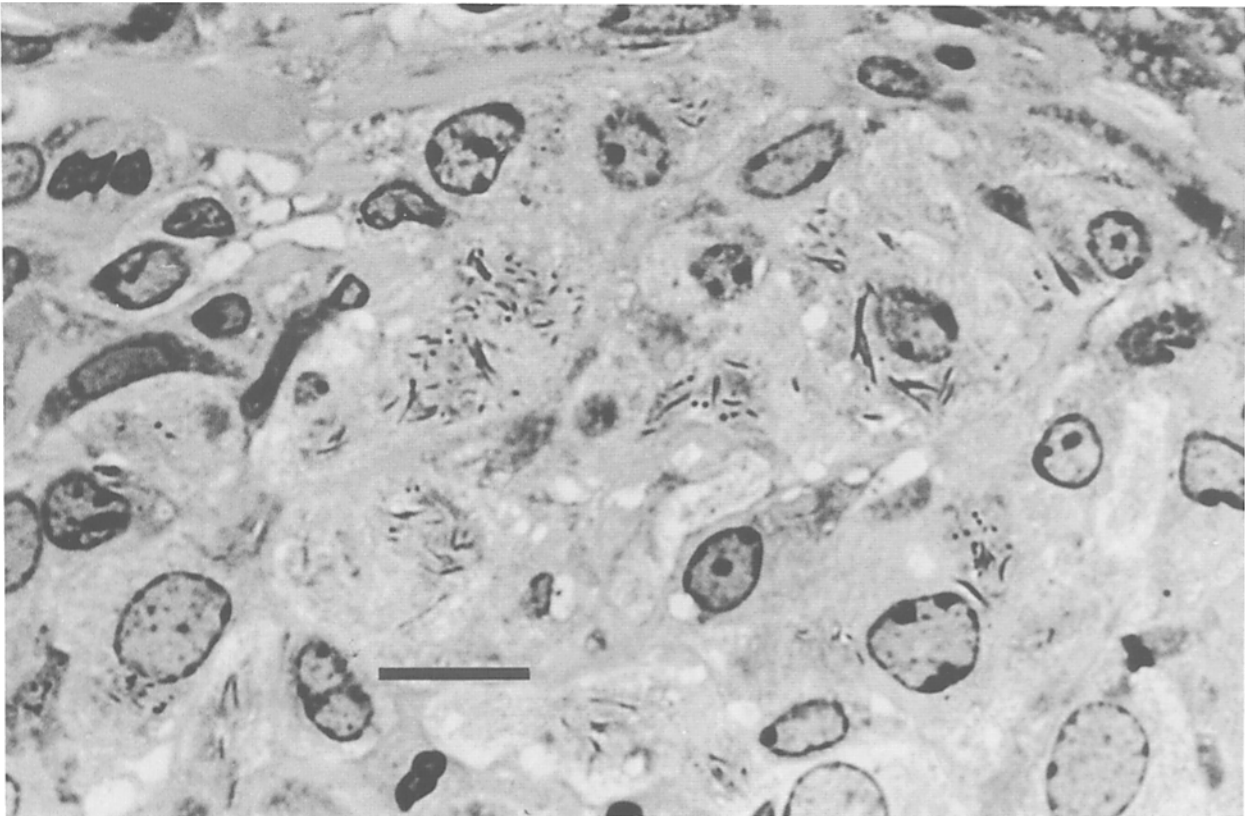
Figure 7. Growth of BCG in the lungs, kidneys, spleens, and livers of CB-17 mice and CB-17 mice treated with hydrocortisone as described for Fig. 6. The experiment was done concurrently with that shown by Fig. 6. Means of five mice per group per time interval.

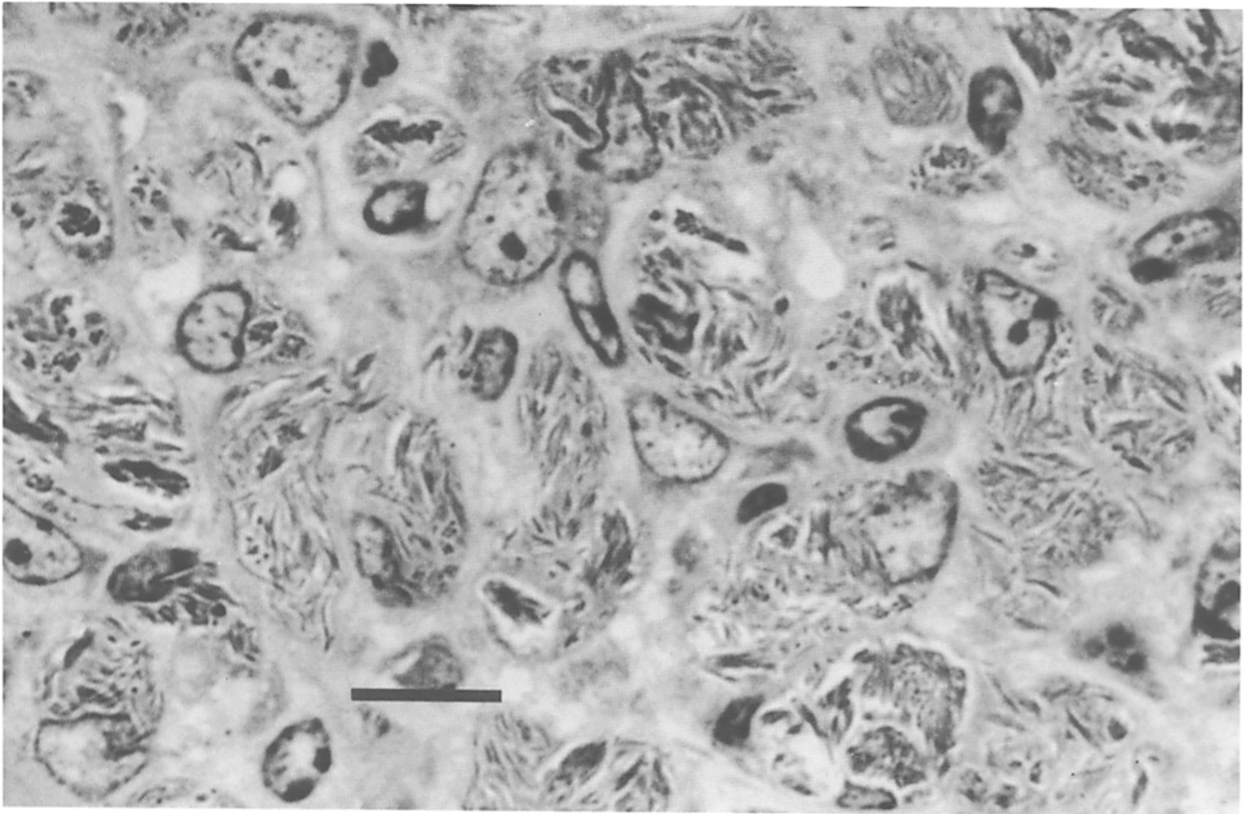
the appearance of infectious foci in different organs of SCID and CB-17 mice will be the subject of a forthcoming publication (North, R. J., and A. Izzo, manuscript in preparation). It can be seen in Figs. 8–10 that in the livers of control SCID mice, BCG was confined to discrete foci of infection that were occupied predominantly by epithelioid macrophages some of which contained BCG. The effect of giving hydrocortisone over a 20-d period was to permit a great deal more BCG multiplication to occur in epithelioid macrophages, to the extent that the cytoplasm of these cells became replete with bundles of bacilli.

Discussion

Tuberculosis is a major world disease that no longer is confined to developing countries, but is now a public health threat in major U.S. cities primarily because of its high incidence in HIV-infected persons (14, 15) who can offer little resistance to its progression. Consequently, AIDS patients are serving as a reservoir for an infectious agent that is not opportunistic, but capable of infecting and causing progressive disease in a considerable proportion of normal people. The problem is being made worse by the increasing incidence of the disease caused by drug-resistant strains of *M. tuberculosis* (16). This situation has served to create a sense of urgency towards developing drugs to treat the disease and designing vaccines to protect against contracting it. Currently, the only available antituberculosis vaccine is BCG, which, according to a number of trials (8), is of limited value in protecting adults against pulmonary tuberculosis. This has led to the suggestion (8, 9) that more immunogenic vaccines be developed, possibly with the aid of recombinant DNA technology. This might allow BCG to be provided with the capacity to express more immunogens, including those that are peculiar to virulent *M. tuberculosis* itself (8). However, it would seem premature at this stage to begin the task of designing a better BCG vaccine without knowing whether lack of immunogenicity is the reason for its limited efficacy. It remains possible, until shown otherwise, that the pathogenicity of virulent mycobacteria is based on a capacity to resist destruction by relatively high levels of immunity generated in response to BCG or *M. tuberculosis* itself. It is reasonable to suggest that the rational design of a better antituberculosis vaccine will require, among other things, an understanding of mycobacterial virulence.

Figure 8. Low-power micrographs of a BCG-infected liver of a SCID mouse (*a*) and a SCID mouse treated at 5-d intervals with 2.5 mg of hydrocortisone (*b*). Infection was initiated 35 d earlier as described for Figs. 6 and 7. Granulomas are obvious, and are bigger in hydrocortisone-treated mice. Section was stained with phenol-crystal violet. Bar, 100 μm.





According to the results presented here, the pathogenicity of two standard virulent strains of *M. tuberculosis* (Erdman and H37Rv) for mice is based on the ability of these strains to grow progressively in the lungs in the face of a systemic mechanism of acquired CD4 T cell–dependent immunity capable of inhibiting mycobacterial growth in the liver, spleen, and kidneys. Attenuated strains (BCG and H37Ra), in contrast, are shown to be incapable of causing progressive infection in any organ in immunocompetent mice. Instead, infection with these organisms is slowly resolved after 2–3 wk of bacterial growth. It has been shown, previously, in this connection (2, 3), that resolution of BCG infection also is predominantly dependent on $CD4^+$ T cells. It was not surprising to find, therefore, that BCG can grow progressively in the lungs and other organs of SCID mice, although fastest in the lungs. Thus, the attenuated organism is opportunistic in the absence of specific immunity, and it is known from a previous study (3) that it is capable of eventually killing SCID mice.

In view of these results, it was not unexpected to find that virulent strains of *M. tuberculosis* also grew rapidly and progressively in all organs of SCID mice, compared to only in the lungs of CB-17 mice. Moreover growth of these strains was fastest in the lungs of SCID mice, meaning that the lungs offer less resistance than other organs to the growth of virulent mycobacteria both in the absence and presence of specific immunity. Again, virulent strains grew much faster than BCG in all organs of SCID mice. Thus, since SCID mice are known to be devoid of immunologically dependent mycobacteria growth-restrictive mechanisms, it may be concluded that the major difference between virulent and attenuated mycobacteria is that the former have much faster doubling times in vivo in the absence of acquired specific immunity. It could be suggested on the basis of the results presented, moreover, that growth of H37Ra is eventually self-limiting because its numbers in all organs of SCID mice is controlled after 2–3 wk of infection.

However, the results obtained with hydrocortisone clearly show that growth of H37Ra is not self-limiting in SCID mice, in that this organism grew to much larger numbers (1–3 log, depending on the organ) over a 25-d period of SCID mice treated with glucocorticoid. Therefore, growth of H37Ra in SCID mice is controlled by a glucocorticoid-sensitive mechanism of antimycobacterial resistance that almost certainly is based on macrophage function. This can be suggested on the basis of results of a histological study still in progress (North, R. J., and A. Izzo, manuscript in preparation) showing that it is in macrophages that H37Ra (and other strains under study here) resides during the course of infection both in SCID and CB-17 mice. Indeed, it is shown here with BCG that this organism resides in macrophages of granulomas in the livers of SCID mice, and that the effect of giving hydrocortisone was to greatly increase the bacillary load in individual macrophages. It has been demonstrated (12, 13), in keeping with this interpretation, that glucocorticoids can inhibit the ability of macrophages to control the growth of certain microbial pathogens in vitro. Regardless of its identity, this mycobacteria growth-restrictive mechanism also functions to retard the growth of virulent mycobacteria in SCID mice, as evidenced by a striking increase in the growth of these organisms in all organs of SCID mice treated with hydrocortisone. Given that it is likely that in the absence of this hydrocortisone-sensitive mechanism SCID mice can exert little, if any, active growth-restrictive influence on either attenuated or virulent mycobacteria, the growth rates of virulent and attenuated mycobacteria in hydrocortisone-treated SCID mice is probably a reflection of their true, intrinsic doubling times in vivo. Thus, the minimum doubling times in the lung (the organ in which all mycobacteria under study grow fastest) for Erdman, H37Rv, BCG, and H37Ra (Table 2) are 17.7, 17.4, 44.6, and 98.6 h, respectively. The same ranking can be given to the doubling times of these strains in other organs of SCID mice treated with hydrocortisone.

In view of these results, it seems reasonable to conclude that the ability to multiply faster in macrophages in vivo is a key characteristic that enables virulent mycobacteria to cause progressive infection in the lungs of immunocompetent mice in the face of a systemic mechanism of specific antimycobacterial immunity capable of controlling the growth of these organisms in other organs. It is apparent that the antimycobacterial function of macrophages in the liver, spleen, and kidneys, but not in the lungs, is sufficiently upregulated by specific immunity to control infection caused by virulent organisms. On the other hand, the antimycobacterial function of lung macrophages is sufficiently upregulated by specific immunity to control the growth of BCG in the lungs. It may well be asked, therefore, whether BCG could overcome the expression of specific immunity in the lungs if it had a faster intrinsic doubling time? Obviously, an answer to this question cannot be given at this time. However, an examination of the BCG growth curves presented here, and other BCG curves recently obtained in this laboratory (North, R. J., and A. Izzo, manuscript in preparation) leads one to believe that this might be the case. For example, the extent of growth of BCG in the lungs of immunocompetent mice is greater than its growth in other organs, because it takes longer for

Figure 9. Light micrograph of infectious focus in the liver of a control SCID mouse infected intravenously 35 d earlier with 2×10^6 BCG. The infectious focus is occupied by a granuloma made up predominantly of epithelioid macrophages, some of which contain BCG. Section was stained with phenol crystal violet. Bar, 10 μm.

Figure 10. Light micrograph of infectious foci in the liver of a SCID mouse infected 35 d earlier with 2×10^6 BCG, and treated at 5-d intervals with 2.5 mg of hydrocortisone beginning on day 15. The epithelioid cells that populate the granuloma have permitted enormously more BCG growth than the epithelioid cells shown in Fig. 8 for control SCID mice. The bundles of bacilli shown in the infectious focus are confined to areas representing the cytoplasm of epithelioid macrophages, some of which have been sectioned to include a nucleus. Section was stained with phenol-crystal violet. Bar, 10 μm.

specific immunity to control the growth of BCG in the lungs. In addition, BCG grows faster in the lungs than in other organs of SCID mice, and it grows progressively in the lungs, but not in other organs, of athymic nude mice and euthymic mice depleted of $CD4^+$ and $CD8^+$ T cells (3).

Not that mycobacterial virulence will be easy to explain mechanistically purely on the basis of a faster intrinsic doubling time. It is difficult to believe, for example, that a faster doubling time enables an organism to escape immune destruction simply by enabling it to outgrow a developing immune response in the lungs. Rather, it seems more likely that the reason for escape from immunity will prove more complicated. For instance, a faster doubling time could result in some of the macrophages in which virulent mycobacteria implant becoming rapidly overloaded with bacilli to the extent of being rendered incapable of processing and presenting antigen. This would serve to make mycobacteria in these macrophages invisible to mycobacteria-specific T cells that need to see processed mycobacterial antigens at the surface of macrophages in the context of class II MHC in order to be stimulated to secrete macrophage-activating lymphokines. Needless to say, this implies that lung macrophages are heterogeneous with respect to origin and functional maturity. It also seems likely that the requirements for the expression of antimycobacterial immunity by macrophages may involve more than lymphokine-mediated upregulation of a single intramacrophage mycobacteriacidal mechanism. The finding presented here which shows that hydrocortisone treatment failed to allow mycobacteria to grow as fast in CB-17 mice as in SCID mice points to the possibility that macrophages in the former mice acquire a microbicidal mechanism in association with T cell–mediated immunity which is resistant to glucocorticoid treatment. According to this line of reasoning, macrophages of immunocompetent mice would come to possess two separate antimycobacterial mechanisms: a preexisting cortisone-sensitive mechanism, as possessed by SCID mice, and a cortisone-resistant mechanism that is brought into play by specific immunity.

Published evidence in keeping with this idea is seen in the demonstration (13) that the baseline ability of human blood monocyte–derived macrophages to inactivate ingested *Listeria monocytogenes* in vitro can be abolished by exposing the macrophages to glucocorticoids. In contrast, the elevated listeriacidal function acquired by these macrophages in response to treatment with IFN-γ is resistant to treatment with glucocorticoids. Again, it has been shown (12) that the ability of mouse macrophages to inhibit the growth of ingested *Toxoplasma gondii* trophozoites is severely suppressed by glucocorticoids, but not if the macrophages are first activated by lymphokines. Presumably a similar cortisone-resistant, macrophage-based microbicidal or microbistatic mechanism needs to be acquired in order for mice to control the growth of virulent *M. tuberculosis*. Needless to say, this mechanism is not acquired by all macrophages in the lung during the course of infection with virulent *M. tuberculosis*. For this reason, the efficacy of an antimycobacterial vaccine needs to be judged on its ability to protect against the growth of virulent mycobacteria in the lungs. According to published studies (6, 7), BCG is highly efficient at immunizing the lungs of mice against subsequent challenge with virulent organisms, provided the BCG is inoculated intravenously, or given by aerosol. However, the results of an ongoing study in this laboratory (North, R. J., and A. Izzo, manuscript in preparation) suggests that the protection afforded by BCG vaccination is not absolute, in that initial destruction of most of the virulent challenge organisms in the lungs is followed by regrowth of surviving organisms after a protracted period of time. It seems possible, therefore, that regrowth of the challenge organism occurs is a small number of lung macrophages that is refractory to the activating signals of lymphokines secreted by specific T cells.

The authors wish to thank Linda Schaefer, Debbie Duso, Lynn Ryan, and Ron LaCourse for expert technical assistance; and Mary Durett for typing.

This work was supported by grant IHP-24 from the Trudeau Institute.

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Received for publication 18 February 1993.

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