Relative Immunogenicity of Streptomycin-Sensitive and -Resistant Strains of BCG

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Received for publication 21 March 1973

Normal, specific pathogen-free mice were vaccinated intravenously with increasing amounts of a streptomycin-resistant variety of BCG Tice (BCG SM^R). The behavior of BCG SM^R in the lungs, liver, and spleen was followed quantitatively for up to 50 days. One or two intravenous doses of 10⁶ viable organisms were steadily eliminated from the tissues without producing detectable tuberculin sensitivity or raising resistance to a subsequent challenge with *Mycobacterium tuberculosis* strain Erdman. But mice receiving six weekly injections of 10⁶ viable BCG SM^R or a single injection of 10⁸ BCG SM^R by the intravenous route did develop effective levels of antituberculous resistance. Heat inactivation of the BCG SM^R inoculum removed the organism's protective activity which could, however, be restored by incorporation of the organisms into Freund adjuvant. The ability of living BCG SM^R to induce an effective antituberculous resistance when introduced into the tissues in an appropriate manner is discussed in terms of the mechanism of antituberculous immunity.

The immunogenicity of live mycobacterial vaccines seems to depend upon the ability of the infecting organism to survive, although not necessarily to multiply in vivo (5). Cross-protection studies carried out in mice infected with several strains of BCG, *Mycobacterium kansasii*, or *M. intracellulare* indicate that protection against a subsequent tuberculous challenge is achieved only when the primary infecting strain is able to survive within normal mouse phagocytes for an extended period of time; closely related strains of *Mycobacteria* which were unable to survive in vivo were nonprotective, despite the presence of cross-reacting antigens (4). The absence of immunogenicity in living mycobacterial vaccines was first observed for a streptomycin-resistant strain of BCG Tice; the corresponding isoniazid-resistant variant was as immunogenic as the drug-sensitive parent strain, however (11). The absence of immunogenicity in BCG SM^R was not due to a lack of sensitizing antigens because a suspension of the organism in Freund adjuvant induced excellent levels of tuberculin sensitivity in both mice and guinea pigs (5a). The present investigation extends these earlier findings by showing that BCG SM^R is immunogenic for CD-1 mice if

viable organisms are introduced into the tissues in such a way that substantial numbers of bacteria are present continuously for several weeks.

MATERIALS AND METHODS

Animals. Four-week-old specific pathogen-free CD-1 mice (Charles River Farms, Mass.) were maintained under isocaps on sterile bedding and fed sterile food and water ad lib. (4).

Organisms. *Mycobacterium bovis* BCG Tice (TMC no. 1028) was obtained from the Trudeau Mycobacterial Culture Bank, Saranac Lake, N.Y. A streptomycin-resistant mutant able to multiply in the presence of 125 µg of streptomycin sulfate per ml was isolated from this strain (11). *M. tuberculosis* Erdman (TMC no. 107) was also obtained from the Trudeau Culture Collection. All cultures were grown in Proskauer and Beck liquid medium; the surface pellicle was removed after 10 to 14 days of incubation at 37 C, homogenized in buffered 1% gelatin (pH 6.8) as described elsewhere (10) and stored at -70 C (13). The viability of the frozen suspensions remained high for at least 2 years. When an inoculum was required, a frozen ampoule was thawed rapidly at 37 C, homogenized in 10 ml of buffered gelatin, and diluted in sterile saline; the suspension was immediately injected into the mice. The viability of the inoculum

was checked by plating serial 10-fold dilutions of the final suspension on Middlebrook 7H10 agar (Difco, Detroit). Plates were sealed in plastic bags and incubated at 37 C for 3 to 4 weeks before counting.

Killed vaccines were heated at 80 C for 10 min and checked exhaustively for sterility. The suspension was lyophilized without further washing and stored at room temperature in vacuo. Vaccines containing killed mycobacteria suspended in Freund adjuvant were prepared as described elsewhere (5a, 8).

Enumeration of in vivo populations. Groups of five randomly selected mice were killed and the lungs, livers, and spleens were removed aseptically and homogenized separately in sterile saline (4). The homogenates were diluted suitably in saline and plated onto 7H10 agar. Where appropriate, bovine and human strains of *Mycobacteria* were distinguished by double plating the suspension on 7H10 agar, with and without 2 μ g of thiophene carboxylic acid hydrazide per ml (7).

Challenge infection. Mice were infected intravenously with approximately 10^5 *M. tuberculosis* Erdman, and the number of residual vaccine and challenge organisms in the lung, liver, and spleen were determined over a 20-day period.

Tuberculin sensitivity to 2.5 μ g of purified protein derivative (PPD) (Parke Davis, Mich.) injected into a hind footpad was measured after 3 and 24 h by using dial gauge calipers (4).

RESULTS

Vaccination of mice with live streptomycin-resistant BCG Tice (BCG SM^R). When normal mice were infected intravenously with approximately 10^6 BCG SM^R, the number of viable organisms found in the lungs, liver, and spleen decreased steadily until no viable bacteria could be detected in the lungs on day 21. The liver and spleen populations also de-

clined sharply at first, although the rate of inactivation in the spleen slowed somewhat after 14 days, and this fact, together with the larger initial populations of bacteria in these organs, resulted in the persistence of small numbers of BCG SM^R in both organs for as long as 50 days. However, this residual viable population represented only about 0.1% of the initial inoculum.

The BCG SM^R-vaccinated mice were reinfected with the same organism on day 28. The behavior of the reinfected BCG SM^R population closely resembled that of the primary infection (Fig. 1) so that regression curves prepared for the lung, liver, and spleen populations over the first 14 days of the primary and secondary infections showed few differences (Table 1). The doubly vaccinated mice were then superinfected intravenously with 3×10^5 viable *M. tuberculosis* strain Erdman. A substantial increase in the numbers of virulent tubercle bacilli found in the lungs, liver, and spleen over the next 14 days was recorded (Fig. 1). The BCG SM^R-vaccinated mice were tuberculin negative at the time of challenge. However, after the Erdman challenge, the level of footpad reactivity increased sharply to a peak at about day 14, after which it again declined. This pattern of footpad reactivity in the BCG SM^R-vaccinated mice after the Erdman challenge resembled that reported earlier for nonsensitized animals (9).

A second group of mice were infected intravenously with approximately 10^6 viable BCG SM^R at weekly intervals for 6 weeks; the lung, liver, and spleen populations are recorded in Fig. 2. Comparison of the slopes of the regression

FIG. 1. Survival curves for BCG SM^R injected intravenously into normal mice on days 0 and 28. Lr, liver; Sp, spleen; Lg, lung. The mice were challenged intravenously on day 50 with 3×10^5 *M. tuberculosis* strain Erdman (broken lines). The arrows represent the inoculum sizes. The vertical lines represent the increase in foot thickness measured 24 h after injection of 2.5 μ g of PPD. An average increase of 1.8 or more units (0.18 mm) in foot thickness was significant at the 1% level.

TABLE 1. Slopes and intercepts of regression lines prepared from BCG Tice and BCG SM^R growth curves obtained for the spleen, liver, and lungs of CD-1 mice after intravenous inoculation

Vaccine	Spleen			Liver			Lung		
	Intercept	Slope	r ⁺ ^a	Intercept	Slope	r ⁺	Intercept	Slope	r ⁺
BCG SM ^R (10 ⁶)									
Dose 1	4.63	-0.088	-0.9165	5.71	-0.104	-0.9866	3.50	-0.167	-0.9855
2	5.19	-0.090	-0.9737	6.22	-0.090	-0.9904	4.42	-0.150	-0.8652
3	4.99	-0.144	-0.9963	5.59	-0.090	-0.9954	3.76	-0.190	-0.9620
4	5.09	-0.171		6.62	-0.231		4.46	-0.169	
5	5.39	-0.265		5.44	-0.192		3.51	-0.170	
BCG SM ^R (10 ⁸)									
Dose 1	6.73	-0.137	-0.9492	7.55	-0.100	-0.9737	5.77	-0.105	-0.8976

^a r⁺, Coefficient of correlation.

FIG. 2. Viable bacteria in the lung (Lg), liver (Lr), and spleen (Sp) after intravenous infection with approximately 10⁶ BCG SM^R at weekly intervals. On day 50 the mice were superinfected with 10⁵ Erdman (broken lines).

curves obtained after each sequential dose of BCG SM^R over the 5-week period indicated that the rate of inactivation of the in vivo population generally increased after the third and subsequent injections (Table 1). There was little change in the rate of decay of BCG SM^R in the lung, however. The multiply immunized mice showed an enhanced antituberculous reactivity (revealed by the reduced growth of *M. tuberculosis* Erdman in the three test organs). The latter curves should be compared with the typical control Erdman infection shown in Fig. 5. The vaccinated mice were tuberculin negative at the time of challenge, but they subsequently developed an accelerated tuberculin response to the Erdman challenge.

To put the foregoing observations into a better perspective, a group of control mice were infected intravenously with 10⁶ of the parental strain, BCG Tice (Fig. 3). Viable counts for the vaccinating organism increased substantially in the lung and to a smaller extent in the spleen over the first 14 days. There was then a slow

decline in viability in all three test organs over the remainder of the vaccination period. The vaccinated mice developed significant tuberculin sensitivity and showed immediate, substantial resistance against the Erdman challenge (Fig. 3).

Vaccination with large doses of BCG. Mice were infected intravenously with approximately 10⁸ viable BCG SM^R (or with 10⁸ BCG Tice as a control). The BCG SM^R population in the lung, liver, and spleen declined steadily throughout the experiment (Fig. 4). However, the initial size of the vaccinating inoculum insured the persistence of a substantial population of viable drug-resistant organisms in the liver and spleen throughout the 50-day period of the experiment. None of the vaccinated mice developed detectable footpad reactivity to PPD but were able to express an effective antimycobacterial response against the Erdman challenge (Fig. 4). Control mice immunized with 10⁸ live BCG Tice gave growth curves similar to those already published (5, 7). As a result, more than 10⁷ viable

FIG. 3. Growth curve for BCG Tice injected intravenously into normal mice. The Erdman challenge of 8×10^5 viable organisms was injected intravenously on day 50.

FIG. 4. Survival curves after an intravenous injection of 1.05×10^8 viable BCG SM^R in normal mice. Lr, liver; Sp, spleen; Lg, lung. The mice were challenged with 10^5 Erdman on day 50 (broken lines).

BCG Tice were still present by day 50. These heavily infected mice also remained tuberculin negative until a late stage in the Erdman challenge infection. The behaviour of the Erdman challenge in both groups of anergic mice indicated that they were capable of expressing highly effective antituberculous responses despite the absence of peripheral tuberculin hypersensitivity (7).

Heat inactivation of the 10^8 BCG inoculum destroyed its protective value whether the organisms had been drug sensitive or resistant. Typical growth curves for the Erdman challenge in mice vaccinated intravenously with a single dose of 10^8 heat-killed BCG SM^R suspended in saline are shown in Fig. 5. The mice failed to develop detectable levels of acquired antituberculous resistance so that the growth curves resembled those obtained in normal unvac-

cinated controls. The behaviour of the Erdman challenge in these vaccinated mice was essentially the same whether the antigenic stimulus was introduced by the intravenous or the subcutaneous route or whether the organisms had been streptomycin sensitive or resistant. For this reason, Erdman growth curves obtained in mice pretreated with the BCG Tice preparations have been omitted from Fig. 5.

The heat-inactivated BCG SM^R were also suspended in Freund adjuvant and injected subcutaneously into a group of normal mice. Challenge of these animals 2 weeks later with about 10^8 Erdman was associated with considerable growth by the virulent organisms in vivo (Fig. 6); comparison of these curves with those for the unimmunized controls recorded in Fig. 5 indicates that the single dose of adjuvanted vaccine did result in some protection. However,

mice receiving a second dose of 10^8 BCG SM^R in adjuvant showed increased immunity to the subsequent Erdman challenge (Fig. 6). These mice also showed an accelerated tuberculin response after infection.

DISCUSSION

Earlier protection studies using streptomycin-resistant BCG vaccines (5, 11) indicated that the observed lack of immunogenicity was not due to the absence of sensitizing antigens from these organisms but was somehow linked to their inability to establish stable, ongoing infections in the vaccinated host. This lack of immunogenicity could be overcome by suspending the organism in Freund adjuvant (5a). The present study shows that suspensions of the living organism could also sensitize normal mice when a sufficiently large immunizing inoculum was used (Fig. 4). Protection studies using increasing numbers of living BCG SM^R indicate that this initial population must be large enough to result in a persisting population of 10^5 or more viable organisms over a 3- or 4-week period. This appears to be the minimal

FIG. 5. Bottom, Growth of *M. tuberculosis* Erdman in mice vaccinated intravenously with 10^8 heat-killed BCG SM^R suspended in saline. The histograms represent the tuberculin sensitivity. Top, Growth of *M. tuberculosis* Erdman injected intravenously into normal mice.

FIG. 6. Left, Growth of *M. tuberculosis* Erdman in mice vaccinated 14 days previously with 10^8 heat-killed BCG SM^R suspended in Freund adjuvant. Right, Growth of *M. tuberculosis* Erdman in mice vaccinated with two doses of BCG SM^R in Freund adjuvant at 14-day intervals.

stimulus required to induce detectable levels of antituberculous immunity. Although the BCG SM^R cells are unable to multiply in vivo, sufficient numbers of them remain in the tissues in a metabolically active state to sensitize the mouse to some critical antigenic component. Heat inactivation of the sensitizing inoculum effectively prevents the development of such a state of cellular hypersensitivity unless the organisms are suspended in a Freund type of adjuvant (Fig. 6). Thus the inability of the dead, saline-suspended organisms to protect the mice was not due to the destruction of critical "protective" antigens by the heat treatment. Clearly, the nonmultiplying BCG SM^R population within the liver and spleen releases sensitizing antigens to the surrounding cells in a manner quite different from that occurring when the same amount of unadjuvanted antigenic material is introduced into the tissue by the intravenous inoculation route. Despite the ability of the adjuvanted vaccine to induce an antituberculous response in mice, quantitative differences exist between the living and killed vaccines since the immune response after two doses of 10⁸ killed BCG SM^R in adjuvant was always inferior to that seen in mice receiving a single dose of 10⁸ viable bacilli (cf. Fig. 4 and 6).

The inability of a single dose of 10⁶ viable BCG SM^R to protect mice against a tuberculous challenge was ascribed to the rapid decline in the numbers of viable mycobacteria within the liver and spleen; this population presumably fell below the critical threshold necessary to induce a detectable antituberculous resistance. As a result, BCG SM^R was reported earlier to be quite nonimmunogenic for CD-1 mice (5, 5a). Even an inoculum of 10⁵ viable BCG Tice was immunogenic for mice because the growth of the organisms in vivo maintained the bacterial population above the minimal sensitizing level for a period of several weeks. Immunization of mice with various substrains of BCG had earlier established the fact that an inoculum of 10⁶ viable bacilli resulted in the development of an acquired antituberculous resistance, regardless of whether the vaccine strain multiplied extensively in vivo or not (11, 7). Variations in the immunogenic qualities of different substrains of BCG are thus best demonstrated with relatively small doses of viable organisms (12). Nonmultiplying strains will then be unable to induce effective levels of tuberculin sensitivity and acquired resistance (5). The present data seem to be consistent with the earlier findings of both Bloch (2) and Pierce et al. (14), who indicated that multiplication of the vaccinating orga-

nisms in vivo was not essential for effective immunization, but that the inoculum must be able to persist in a metabolically active state within the liver and spleen for a period of at least 3 weeks (4).

The ability of regularly repeated doses of BCG SM^R to induce significant levels of acquired resistance appears to be at variance with earlier observations in *Salmonella pullorum*-vaccinated mice (6). This study showed that even repeated doses of viable *S. pullorum* failed to induce significant levels of acquired resistance to an *S. enteritidis* challenge, despite the presence of common somatic antigens in the two organisms (9). The similarities in the survival patterns for *S. pullorum* and BCG SM^R in normal mice has been remarked upon elsewhere (5), so that the demonstration of antituberculous resistance in mice repeatedly exposed to BCG SM^R infection was somewhat unexpected. The difference in the immunogenicity of the two organisms is probably due to variations in their relative rates of inactivation in vivo. *S. pullorum* shows a 10- to 50-fold decrease in viability in vivo over a 24-h period (6), whereas it requires 7 to 10 days to achieve a similar drop in viability in BCG SM^R (Fig. 1). Thus, 10⁷ viable *S. pullorum* reach nonimmunogenic levels within a day or so and even daily reinoculations will be unable to maintain sufficient viable cells in vivo to induce detectable levels of acquired antisalmonella immunity. On the other hand, weekly injections of 10⁶ viable BCG SM^R sustained a population of 10⁵ or more viable mycobacteria within the liver (Fig. 2), and this was apparently sufficient to induce an active antituberculous resistance.

The reason for the inability of BCG SM^R to multiply in vivo is still unknown. Streptomycin-resistant strains of *M. tuberculosis* H₃₇R_v tend to retain the parental strain's mouse virulence (15), although isoniazid-resistant variants may or may not show a substantial decline in mouse pathogenicity (1, 3). Apparently, the development of streptomycin resistance is frequently associated with some degree of attenuation (5), an effect that is exacerbated in the already attenuated BCG organism. As a result, the drug-resistant BCG strain is highly susceptible to inactivation even by unstimulated host phagocytes. Under these circumstances, the organism loses all ability to sensitize the host. Interestingly, there was no corresponding loss in immunogenicity or in the ability of isoniazid-resistant strains of BCG to grow in normal mice (5a, 11). None of several streptomycin-resistant strains of BCG tested had lost their catalase

activity nor was there any significant change in growth rates in liquid cultures. The conclusion must be drawn that mutation to streptomycin resistance in BCG is linked in some way with a concomitant change in the structure of the cell wall which makes the organism more susceptible to intracellular inactivation. It would be interesting to know whether corresponding changes occur in the growth patterns and immunogenicity of streptomycin-resistant variants of attenuated strains of *M. tuberculosis* such as H₃₇R_a or R₁R_v. However, at present there does not seem to be an obvious explanation for the observed loss in the ability of BCG SM^R to survive and multiply in normal mice, and this problem clearly deserves further examination.

ACKNOWLEDGMENT

This study was supported by Public Health Service Grant AI-07809 from the United States-Japan Cooperative Medical Sciences Program administered by the National Institute of Allergy and Infectious Diseases.

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