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Salmonellosis in Mice Infected with *Mycobacterium bovis* BCG

II. Resistance to Infection

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Mycobacterium bovis BCG-infected mice were found to be consistently more resistant than normal mice to superinfection with *Salmonella typhimurium*. This resistance was manifested by a decreased mortality and by a decrease in the number of viable *Salmonella* in the BCG mice 3 to 4 days after challenge. Antibody production, as determined in the serum by the complement-dependent bactericidal system or in the spleen by the Jerne plaque technique, was either equivalent to or less than that of normal mice. Therefore, the immunity to *S. typhimurium* possessed by BCG-infected mice cannot be the expression of a greater or more rapid antibody response. By exclusion, these findings appear to support the concept of "cellular immunity."

It has been observed that animals infected with *Mycobacterium bovis* BCG acquire resistance not only to tuberculous infection but to infections by other apparently unrelated bacteria, such as *Salmonella* (1). The nonspecificity of this resistance is inconsistent with an antibody-mediated immunity but is quite compatible with the concept of a cell-mediated immunity as described by Mackaness (4). However, since it has been shown that mice respond to salmonella infection with a very rapid antibody synthesis (3), it is difficult, in the in vivo setting, to divorce the increased resistance of the tuberculous mice from antibody production. In fact, one could postulate that the induced immunity is effected via an adjuvant-like activity of the BCG, leading to a greater or a more rapid antibody response, or both. In this study, we investigated this possibility by assaying the antibody response to salmonella infection in normal and in BCG-infected mice.

MATERIALS AND METHODS

Animals. Strain CD-1, female, pathogen-free mice weighing 18 to 22 g were obtained from Charles River Laboratories, North Wilmington, Mass. The mice were housed in air-conditioned quarters and were given water and chow ad libitum.

Bacteria. *S. typhimurium* strain 7, obtained from M. Herzberg (Department of Bacteriology, University of Hawaii), was grown at 37 C in Brain Heart Infusion Broth (Difco) on a shaker for 4 hr. At that time the cultures contained approximately 10^9 bacteria/ml. *M. bovis* BCG was maintained by weekly transfers in Dubos Liquid Medium (BBL) containing 0.5% albumin.

Experimental procedure. BCG infection was established in mice by intravenous injection via tail vein of 0.2 ml of a 10- to 14-day culture, or approximately 10^8 *Mycobacteria* per mouse. Salmonella infections were established in mice 10 days after BCG infection by intraperitoneal inoculation of *Salmonella* in 0.5 ml of 0.15 M saline. Mortality was tabulated until 14 days after the last death (usually more than 21 days after salmonella infection). Total counts of *Salmonella* in the tissues of mice were obtained by homogenizing the carcasses in a Waring Blendor as previously described (10).

Antibody titers. Bactericidal antibody titers were determined by the microtiter method described by Kenny and Herzberg (3). The mice were bled by cardiac puncture, and the sera were stored at −40 C until use. Serial twofold dilutions of serum in saline were made by using a Micro-Titer Kit (Cooke Engineering Co., Alexandria, Va.). To 0.025 ml of each dilution, 0.05 ml of fresh precolostral calf serum and 0.025 ml of a saline suspension of *S. typhimurium* strain 7 containing 10^5 bacteria/ml were added. After incubation at 37 C for 1 hr, 0.025 ml of the mixture was removed and diluted in 1.0 ml of saline, of which 0.2 ml was then plated in duplicate on Trypticase Soy Agar plates (Difco) to enumerate viable bacteria. The antibody titers were expressed as the reciprocal of the serum dilution required to kill 50% of the *Salmonella*.

Plaque-forming cell assay. The number of spleen cells from normal and from BCG mice capable of producing hemolytic plaques with erythrocytes sensitized with lipopolysaccharide from *S. typhimurium* was determined during salmonella infection by a modified

Jerne technique (2). Since the spleens of both normal and BCG mice contained plaque-forming cells when plated with sheep erythrocytes, sets of duplicate plates containing unsensitized sheep erythrocytes served as controls.

Lipopolysaccharide, boiled for 1 hr, was added to 1% suspension of thrice-washed erythrocytes to a final concentration of 0.4 mg/ml in 0.15 M saline. After 1 hr of incubation at 37 C, the red cells were washed three times and resuspended in 0.15 M saline.

Four normal and four BCG mice were killed by cervical fracture before salmonella infection and at daily intervals thereafter. The spleens were removed, minced with scissors, and pressed through a stainless steel mesh (no. 120) into 10.0 ml of cold Hanks basal salt solution. Clumps of cells were dispersed by repeated pipetting.

Tubes containing 2.0 ml of 0.8% Agar (Difco) in Eagle Medium with 1,000 units of penicillin per ml were prepared daily and maintained at 45 C. Approximately 1 hr before use, 0.2 ml of diethylaminoethyl (DEAE) dextran (10 mg/ml) was added and mixed. To these tubes, either erythrocytes or erythrocytes coated with lipopolysaccharide were added (0.2 ml of a 30% cell suspension). Spleen cells (0.2 ml) were added, mixed, and poured quickly over a base layer of 1% agar (Agar with DEAE dextran) in 15 by 100 mm petri dishes. After 1 hr of incubation at 37 C, 3.0 ml of a 1:10 dilution of fresh guinea pig serum in barbital-buffered saline (*p*H 7.6) was added. After a 1 hr additional incubation, all plaques clearly visible under a stereoscopic microscope were counted.

RESULTS

The mortality of the BCG-infected mice was consistently less than that of normal mice after intraperitoneal challenge with *S. typhimurium*. The differences in mortality in 26 paired groups (data combined in Table 1) were highly significant ($P < 0.001$) when tested with the Student *t* test. By probit analysis, the difference in the susceptibility of the two groups of mice to salmonella infection appeared to be 5- to 10-fold.

This resistance was also evident in the numbers of viable *Salmonella* cells recovered from the carcasses of normal and of BCG mice when enumerated at intervals after salmonella infection. After an intraperitoneal challenge of 3×10^5 bacteria in normal mice, there was a tendency toward an increase in the number of bacteria with time, culminating in a high mortality (22/24) with a mean time till death of 7.2 days (Fig. 1). In contrast, BCG mice given the same challenge showed a decrease in bacterial numbers

TABLE 1. *Mortality after salmonella infection in normal and BCG-infected mice*

Inoculum	Mortality (deaths/total)[a]	
	Normal	BCG
4×10^1	19/50 (38%)	4/50 (8%)
7×10^2	16/40 (40%)	5/40 (13%)
7×10^3	9/40 (23%)	2/41 (5%)
5×10^4	59/86 (69%)	35/93 (38%)
4×10^5	103/112 (92%)	75/124 (60%)
2×10^6	15/15 (100%)	14/15 (93%)

[a] Total mortality in normal mice was 221/343 (63%); total mortality in BCG mice was 135/363 (37%).

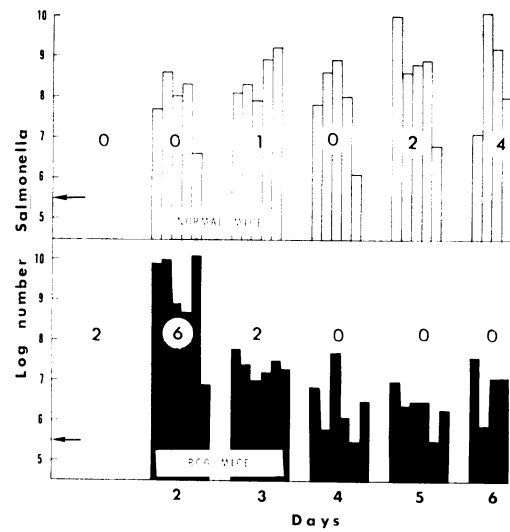

FIG. 1. *Number of viable Salmonella cells in BCG-infected and normal mice after salmonella infection. Each bar represents counts from one mouse. The arrow indicates the inoculum. Mortality and mean times till death were 22/24 and 7.2 days in normal mice, and 11/20 and 2.0 days in the BCG mice. The circled numbers indicate the number of deaths prior to sampling each day.*

TABLE 2. *Bacterial numbers and reciprocal antibody titers in BCG and normal mice*

BCG mice[a]		Normal mice[b]	
Log no. of *Salmonella*	Antibody titer	Log no. of *Salmonella*	Antibody titer
8.8	100	9.9	200
8.2	130	9.6	110
7.4	37	9.3	99
6.7	40	9.2	300
6.4	48	9.2	140
		8.3	82
		7.0	60

[a] Mean titer and standard error of the mean was 70 ± 17.

[b] Mean titer and standard error of the mean was 140 ± 29.

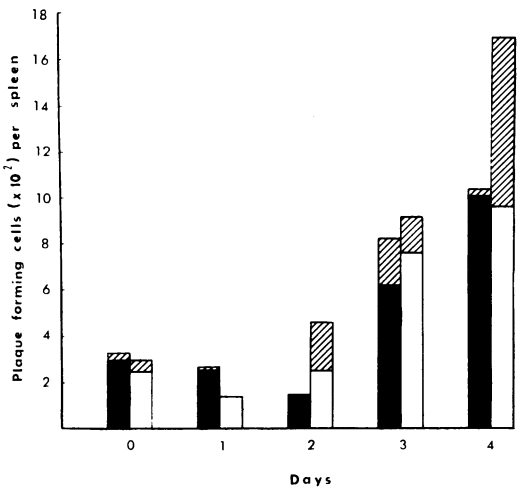

FIG. 2. *Antibody response of spleen cells from normal (white bar) and BCG (black bar) mice after intraperitoneal challenge with 6 × 10⁴ viable S. typhimurium cells. Solid areas represent the number of plaques formed in unsensitized cells. Hatched bar indicates the number of plaques specific for sensitized cells. Each bar represents the mean of four mice. Differences between BCG and normal mice were significant only on day 2. Mortality in the control groups was 60% in normal mice and 10% in BCG mice.*

after an initial rapid increase. Some of the decline in the number of bacteria was undoubtedly due to a selective process, i.e., death of mice containing large numbers of *Salmonella* cells prior to sampling, particularly because most deaths in BCG mice occurred on the second day. This, however, cannot explain the decline entirely, since there was a significant decrease ($P < 0.02$) in bacterial numbers between the third and fourth days, although no deaths occurred during this period.

The bactericidal antibody titers of normal and of BCG mice at 5 days after salmonella infection are listed in Table 2. Also shown are the numbers of *Salmonella* cells recovered from the carcass of each mouse. It is evident that BCG mice contained fewer *Salmonella* at 5 days after challenge and that their bactericidal antibody titers were the same or lower than those of normal mice, but certainly no greater.

The antibody response to salmonella infection at the cellular level is given in Fig. 2. Normal and BCG mice possessed comparable numbers of plaque-forming cells for unsensitized erythrocytes prior to infection, even though the spleens of the BCG mice were three to four times larger than those of normal mice. Neither group possessed significant numbers of plaque-forming cells for sensitized erythrocytes above the background level. After infection, the number of plaque-forming cells declined and began a steep rise on the second or third day in both groups of animals. Except for the second day, there were no significant differences in the number of plaque-forming cells/spleen between normal and BCG mice. The apparent difference on day four was due only to the extraordinary response of one mouse in the normal group. On the second day after salmonella infection, however, the BCG mice contained significantly fewer ($P < 0.01$) plaque-forming cells than did the normal mice. These data, therefore, indicate that the cellular antibody response of BCG mice was certainly no greater nor more rapid than that of normal mice when each group was challenged with the same number of *Salmonella* cells. The total number of plaque-forming cells in the spleens of the BCG-infected mice was either equivalent to or less than that of normal mice, in spite of the fact that the spleens of the BCG mice were larger. Figure 2 also presents the interesting observation that salmonella infection induced a considerable rise in the number of spleen cells producing hemolysins for unsensitized erythrocytes. In fact, the increase in plaque-forming cells for the unsensitized erythrocytes was similar in magnitude to that for sensitized cells. This phenomenon may be due to cross-reacting antigens shared by *S. typhimurium* and sheep erythrocytes, or to nonspecific stimulation of antibody-producing cells by endotoxin, particularly since it has been observed that immunization of rabbits with *S. typhimurium* leads to production of sheep cell agglutinins (Ivan Čižnár, *personal communication*), and that injection of *Escherichia coli* endotoxin in rats elevates the number of plaque-forming cells for unsensitized sheep erythrocytes (6).

DISCUSSION

These studies show that BCG-infected mice are about 5 to 10 times more resistant than normal mice to salmonella infection as measured by survival. This immunity was also evident in the ability of the BCG mice to suppress the number of viable *Salmonella*. When normal mice were infected, the bacterial population gradually increased over a period of 6 days. In contrast, when the same challenge was given to BCG mice, the population of *Salmonella* was greatest on the second day, indicating a more rapid proliferation of bacteria, as was previously shown (10), but by the third or fourth day it had diminished and was much less than that of normal mice. Therefore, the adaptive response of the BCG mice, which led to their immunity, was evident by the fourth day.

The data on antibody titers and on the number of cells producing antibody lend no support to the possibility that an earlier or greater antibody response played a role in the immunity expressed by the BCG mice. The data even suggest that the antibody response of the BCG mice was diminished. BCG-infected mice are well known to clear foreign particles at an accelerated rate (1) and to contain "activated" macrophages with greater concentrations of lysosomal enzymes (8). It is entirely possible, therefore, that they sequester and degrade antigens in a more efficient manner, and consequently produce less antibody. Also, because BCG mice restrict the multiplication of *Salmonella* after the second or third day of infection, they may be subjected to a smaller immunogenic mass. The relationship of antibody titer to bacterial number (Table 2) lends support to this hypothesis. In both BCG-infected and normal mice, the antibody titer tended to be lower in those mice containing fewer bacteria.

In some respects our results appear to conflict with those of a previous report. Howard et al. (1) reported that immunity to salmonella infection in BCG mice was expressed only by an increased survival time and not by a decreased mortality. In contrast, we found a shortened mean time till death but a significant decrease in mortality. These discrepancies can probably be explained by the differences in the number and virulence of the bacteria used for challenge. Whereas the other authors used a highly virulent strain of *S. enteritidis* and injected small numbers, we used a moderately virulent strain of *S. typhimurium* (intraperitoneal $LD_{50} = 2 \times 10^4$) and injected relatively large numbers. As we previously reported (10), large inocula of *Salmonella* may lead to early death when administered to BCG-infected mice. It appears that the endotoxin content of a large inoculum plus the hyperreactivity of BCG mice to endotoxin permit an early, rapid multiplication of bacteria which may cause an early death. If the BCG mice withstand the initial insult, their increased resistance to infection leads to eradication of most of the infecting microorganisms and ultimate survival of the host.

Studies of immune mechanisms operative in mouse salmonellosis have given rise to conflicting opinions as to the relative importance of humoral versus cellular mechanisms. In some instances protection against salmonella infection has been attributed to antibody (3, 7), and in others a cellular adaptive mechanism was found to be the significant response conferring immunity (5, 9). In the experiments reported in this paper, it was found that a cellular adaptive response induced in mice by BCG infection conferred significant protection against salmonellosis and that this immunity did not correlate with antibody formation. By exclusion, these findings appear to support the concept of cellular immunity.

ACKNOWLEDGMENTS

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