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Host Defenses in Murine Malaria: Nonspecific Resistance to *Plasmodium berghei* Generated in Response to *Mycobacterium bovis* Infection or *Corynebacterium parvum* Stimulation

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Infection with *Mycobacterium bovis* (BCG) or injection of killed *Corynebacterium parvum* protected some strain B6D2 F1 (C57BL/6×DBA/2) mice but did not protect strain ICR or A mice from lethal challenge with *Plasmodium berghei* strain NYU-2. B6D2 mice were not protected against challenges delivered immediately after intravenous injection of these materials, but rather protection developed by day 7 and persisted through at least day 84. Infections in protected mice progressed to about 10% parasitemia in parallel with infections initiated with the same inoculum in untreated controls. However, infections in most of the protected mice were cleared subsequently, whereas infections in untreated controls were uniformly fatal. A small number of treated mice developed protracted high-level erythrocytic infections, which led to markedly delayed death. BCG-infected mice which survived *P. berghei* infections had a factor in their sera which protected passively immunized recipients from *P. berghei*. BCG-infected mice passively immunized with protective serum controlled *P. berghei* infections better than normal mice given the same amount of the same serum and challenged with the same *P. berghei* inoculum. The capacity of BCG-infected B6D2 mice to resist *P. berghei* infection was not directly related to the pattern of growth of BCG, to the degree of splenomegaly, or to the level of activation of macrophages (measured as microbicidal capacity) caused by BCG infection. Therefore, I concluded that (i) BCG infection or injection of killed *C. parvum* altered the immunological potential of B6D2 mice in such a way as to allow the production of measurable levels of a protective humoral factor in response to infection with *P. berghei*; (ii) BCG infection caused the generation of a capacity which, when expressed in the presence of immune serum, provided an anti-*P. berghei* capacity which was superior to that provided by BCG infection alone or immune serum in the absence of BCG infection; and (iii) not all strains of mice could be protected from *P. berghei* by BCG or *C. parvum* injection.

Previous studies have shown (19, 21, 23) that vaccination with Formalin-killed erythrocytic *Plasmodium berghei* parasites causes the generation in strain ICR mice of an immunity which protects against virulent challenge. At times, the incorporation of immunopotentiating agents, such as *Mycobacterium bovis* (BCG) or *Corynebacterium parvum*, into the anti-*P. berghei* vaccination regimen increases the level of immunity compared with controls (23). However, when these adjuvants are injected in the absence of the erythrocytic plasmodial antigen, they do not protect strain ICR mice from virulent *P. berghei* (23).

Because ICR mice are bred randomly and thus are not suited for some immunological studies, I studied the mechanism of immunity to *P. berghei* after injection of killed erythrocytic antigens in syngeneic F1 hybrid B6D2 (C57BL/6 × DBA/2) mice. Strain B6D2 mice responded to injections of killed erythrocytic parasites like strain ICR mice; i.e., they developed a long-term resistance to subsequent virulent challenge (19). However, unlike ICR mice, some B6D2 mice injected with either BCG or *C. parvum* in the absence of the plasmodial antigen were capable of resisting a virulent challenge of *P. berghei*.

The purpose of this study was to characterize this nonspecific protection further and to search for its mechanism of action.

MATERIALS AND METHODS

Animals. Female mice of the randomly bred strain ICR, the inbred strain A, and the F1 hybrid strain B6D2 (C57BL/6 × DBA/2) were obtained when they were 5 to 6 weeks old from the Trudeau Institute, Saranac Lake, N.Y., or from a colony maintained at the University of Maryland (breeding stock obtained from the Trudeau Institute). These mice were maintained on a standard diet (4RF; Charles River formula; Agway, Waverly, N.Y.) and were provided water ad libitum.

Plasmodia. *P. berghei* strain NYU-2 was maintained as a frozen (−70°C) stock, which was passaged in ICR mice before it was used for challenges. Infections were initiated by injecting *P. berghei*-parasitized erythrocytes (PRBC) into the lateral tail vein.

***M. bovis* (BCG).** The Pasteur strain of BCG was obtained from the Mycobacterial Culture Collection, Trudeau Institute (TMC 1011), and was diluted in a sterile 0.15 M saline solution before use. Quantitation of viable BCG cells was accomplished by plating 10-fold dilutions of a stock seed culture or a tissue homogenate on Middlebrook 7H-10 medium (Difco Laboratories, Detroit, Mich.). An infection was initiated by injecting a selected number of colony-forming units (CFU) of BCG into the lateral tail vein in 0.2 ml.

C. parvum. *C. parvum* was obtained from Burroughs Wellcome Co., Research Triangle Park, N.C., as a formolized suspension. Before use, the bacteria were washed and suspended at a concentration of 2 mg/ml in 0.15 M NaCl. Each mouse was inoculated with 0.2 ml (400 μg) of this suspension in the lateral tail vein.

Listeria monocytogenes. The EGD strain of *L. monocytogenes* was maintained as a frozen (−70°C) seed culture in Trypticase soy broth. For each experiment, an ampoule of this seed culture was thawed and diluted in 0.15 M NaCl. Each *L. monocytogenes* infection was initiated by injecting 10^5 CFU in 0.2 ml into the lateral tail vein. *L. monocytogenes* was quantitated by plating 10-fold dilutions of the seed culture or a tissue homogenate on Trypticase soy agar.

Serum transfer. Blood was collected by cardiac puncture of anesthetized mice and allowed to clot, and the serum was removed. The sera were filtered (pore size, 0.22 μm) and stored at −70°C until they were assayed.

Two assays were used to measure serum anti-*P. berghei* activity. In the first assay, each recipient was inoculated intraperitoneally with 1 ml of serum, and 1 h later it was challenged by intravenous inoculation of 10^4 PRBC. Blood smears were prepared at daily intervals thereafter. The course of infection in the recipients of serum was compared with the course of an infection initiated with the same plasmodial inoculum in normal mice. The data are presented as increases in mean interval to 1% parasitemia. Increase in mean interval to 1% parasitemia was determined by subtracting the mean number of days required for control animals to reach 1% infection from the number of days required for serum recipients to reach 1% infection (e.g., a value of 1 means that serum treatment delayed the onset of 1% infection for 1 day).

In the second assay, each mouse was inoculated intraperitoneally with 1 ml of serum 1 day before *P. berghei* challenge and with 0.5 ml of serum 1 h before *P. berghei* challenge (10^4 PRBC, intravenously); this was followed by 0.5 ml of serum on days 1 and 2 of *P. berghei* infection. The course of parasitemia is presented.

Assay of systemic macrophage bactericidal activity. Changes in macrophage-mediated bactericidal activity were measured by comparing the 24-h growth of an intravenous challenge of *L. monocytogenes* in experimentally manipulated mice with the growth of bacteria from the same inoculum in control mice. The recovery of fewer bacteria from treated mice than from control mice was evidence of activation of macrophage microbicidal capacity, whereas the recovery of greater numbers of bacteria from treated animals was evidence of a loss of macrophage bactericidal capacity. This is an established procedure for determining levels of macrophage microbicidal activity and is discussed elsewhere (22, 24, 27).

The data on the microbicidal capacity of macrophages are expressed as a resistance index, which was obtained by subtracting the $\log_{10}$ geometric mean number of bacteria recovered from experimentally treated mice from the $\log_{10}$ geometric mean number of bacteria recovered from control animals challenged with the same bacterial inoculum. In all experiments in which the resistance index was used, mice were sacrified for enumeration of *L. monocytogenes* 24 h after *L. monocytogenes* infection. For example, resistance indexes of +1 and −1 mean that the bacteria grew 10-fold less and 10-fold more, respectively, in treated animals than in controls. This convention causes groups with enhanced bactericidal capacities to have values greater than the control value, which is normalized to 0. The meaning of the resistance index in terms of macrophage activation has been discussed by North (25).

Vaccination. A Formalin-killed *P. berghei* antigen prepared by NH_4Cl lysis of infected erythrocytes from strain ICR mice was used as a vaccine. The preparation of this antigen and some of its characteristics as a vaccine have been described previously (19, 21, 23). The vaccination regimen consisted of five intravenous injections (10^8 plasmodial particles per injection) on days 0, 3, 7, 10, and 14.

Statistics. The statistical significance of differences between treated and control groups was determined either by the Student *t* test or, when more than two groups were tested, by analysis of variance and the Tukey test.

RESULTS

Course of *P. berghei* infection. Figure 1 shows the courses of *P. berghei* infections initiated by the intravenous injection of 10^4 PRBC into normal and BCG-infected strain B6D2, A, and ICR mice. Some B6D2 mice infected with BCG 28 days before *P. berghei* challenge survived, whereas all B6D2 mice not previously infected with BCG died. All strain A and ICR mice died of *P. berghei* infection, whether they were infected with BCG or not.

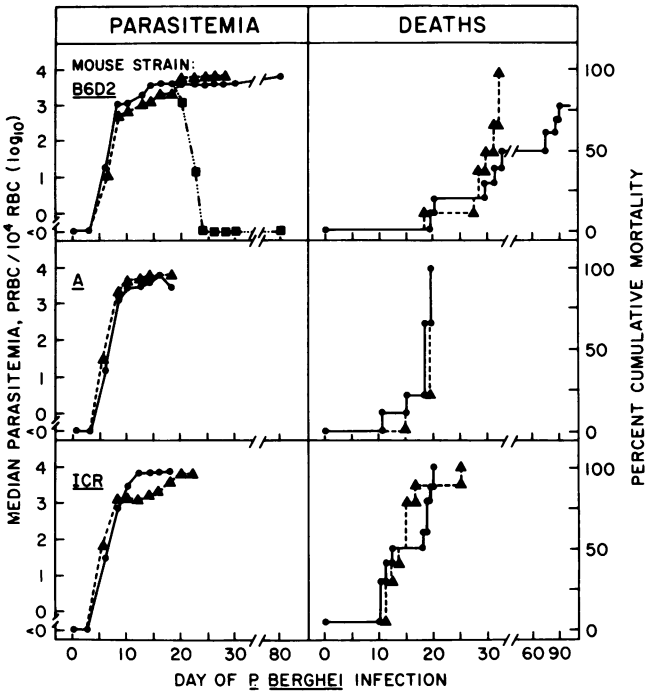

FIG. 1. *Course of parasitemia and patterns of death after intravenous inoculation of 10^4 PRBC into strain B6D2, A, and ICR mice which were (●) or were not (▲) infected previously with BCG. The pattern of parasitemia for those BCG-infected B6D2 mice which rapidly cleared patent blood infections (two mice; see text) is also shown (■). There were 10 mice per group. RBC, Erythrocytes.*

A total of 50% (5 of 10) of the BCG-infected B6D2 mice survived more than 30 days after *P. berghei* challenge (the time required for all non-BCG-infected B6D2 mice to die). However, only two of these five animals cleared patent erythrocytic infection. These two animals cleared overt parasitemia by day 24. The three mice which survived for an extended time after *P. berghei* challenge but ultimately died on days 75, 85, and 89 had persistent high levels of parasitemia and were wasted. Of the four mice alive on day 80, two contained no visible parasites, weighed 26.9 g (standard deviation, 0.14 g) and had 6.70×10^9 (standard deviation, 0.72×10^9) erythrocytes per ml of blood (samples obtained from snipped tails). The other two had parasitemias of 9,630 and 9,821 PRBC per 10^4 erythrocytes, weighed 17.2 g (standard deviation, 0.07 g), and had 3.95×10^8 (standard deviation, 2.75×10^8) erythrocytes per ml of blood.

In the experiment shown in Fig. 1, BCG infection provided 50% of the B6D2 mice with enhanced resistance to *P. berghei* (measured as prolonged survival). However, less than one-half of the mice which showed lengthened survival times showed a capacity to clear parasites. In subsequent experiments, few BCG-infected animals showed this pattern of extended parasitemia followed by death. They either (i) died at about the same time as the controls infected with the same *P. berghei* inoculum or (ii) cleared patent parasitemia and survived. Thus, in subsequent experiments, survival values were based on counts of mice which were robust in appearance on day 49 after *P. berghei* challenge.

Figure 1 also shows that the length of survival after infection of the normal control mice with *P. berghei* varied markedly among strains (mean survival, 28.5 days for strain B6D2, 19.0 days for strain A, and 13.5 days for strain ICR). This difference in length of survival of different strains of mice afer virulent challenge has been reported previously (12, 23, 31). The reason that different strains of mice show differences in the duration of survival after *P. berghei* challenge is not known.

Patent infections did not develop after a second *P. berghei* challenge in B6D2 mice which had, as the result of BCG infection, recovered from a previous *P. berghei* infection (Fig. 2).

Effect of dose of BCG. It has been established that the degree of lymphoid cell proliferation (17) and the dynamics of the immunological response to BCG infection (16) are affected

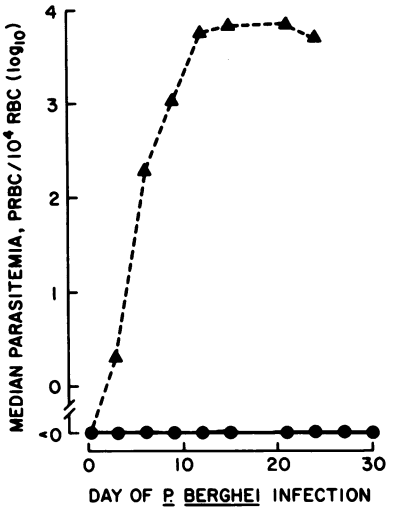

FIG. 2. *Course of parasitemia after intravenous inoculation of 10^6 PRBC into normal mice (▲) and mice which, as the result of an established BCG infection, had survived a previous P. berghei infection (●). The first P. berghei infection was on day 28 after BCG infection. The second P. berghei challenge was on day 42 after the first challenge (i.e., day 70 after BCG infection). There were 10 mice per group. RBC, Erythrocytes.*

by the number of mycobacteria used to initiate the infection. Furthermore, it has been established that BCG infection causes activation of macrophages (4, 26). The following experiment was conducted to determine whether I could establish a relationship between dose-related responses to BCG infection and the capacity of mycobacteria to protect B6D2 mice from *P. berghei*. The data in Fig. 3 through 6 were obtained from one large group of mice infected with the same BCG inoculum.

Figure 3 shows the percentage of mice which survived *P. berghei* challenges delivered 14, 28, or 56 days after intravenous infection with either $10^{8.5}$, 10^7, or 10^5 CFU of BCG. Figure 4 shows the courses of BCG infections in spleens and livers. Resistance to *P. berghei* was related most directly to the number of BCG CFU inoculated as the challenge and not to the subsequent course of the BCG infection.

Figures 5 and 6 show the changes in spleen weights and macrophage-dependent antiheterologous bacterial capacities which occurred in response to injections of increasing doses of BCG. The magnitude and duration of each were related directly to the number of BCG used for challenge. However, the mice showing the greatest increases in spleen weight and the greatest increases in macrophage activity were not the best-equipped to resist *P. berghei* (Fig. 3).

Effect of dose of PRBC. It has been established that the number of PRBC used for challenge affects the level of protection in mice vaccinated against *P. berghei* (23, 36). To determine the effect of challenge size on BCG-associated nonspecific protection, I infected mice with BCG (10^7 CFU) and then challenged them at intervals through day 56 with between 10^2 and 10^6 PRBC. There were survivors at each dose of PRBC tested (Fig. 7).

Effect of source of PRBC. In the experiments described above, the *P. berghei* PRBC used for challenge were obtained from infected strain ICR mice. To determine whether the presentation of *P. berghei* in allogeneic cells was important to the expression of nonspecific resistance, the parasites were passaged four times in strain B6D2 mice. The parasites from this

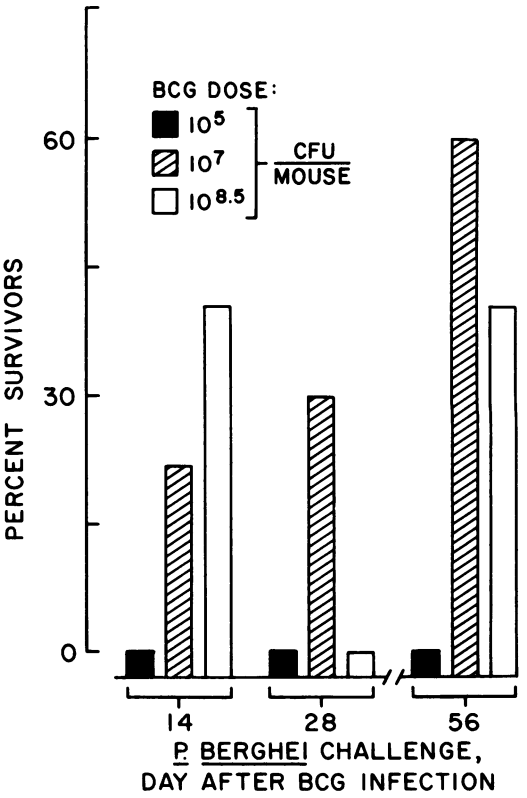

FIG. 3. *Evidence that BCG-associated protection from P. berghei is linked to the dose of BCG used to initiate the mycobacterial infection. Mice were infected by intravenous injection of the indicated numbers of BCG CFU, and they were challenged at different times thereafter by intravenous injection of 10^4 PRBC. Survivors were observed only in groups that received $\geq 10^7$ BCG CFU. There were 10 mice per group. All normal control mice challenged with P. berghei at each of the indicated times died (data not shown).*

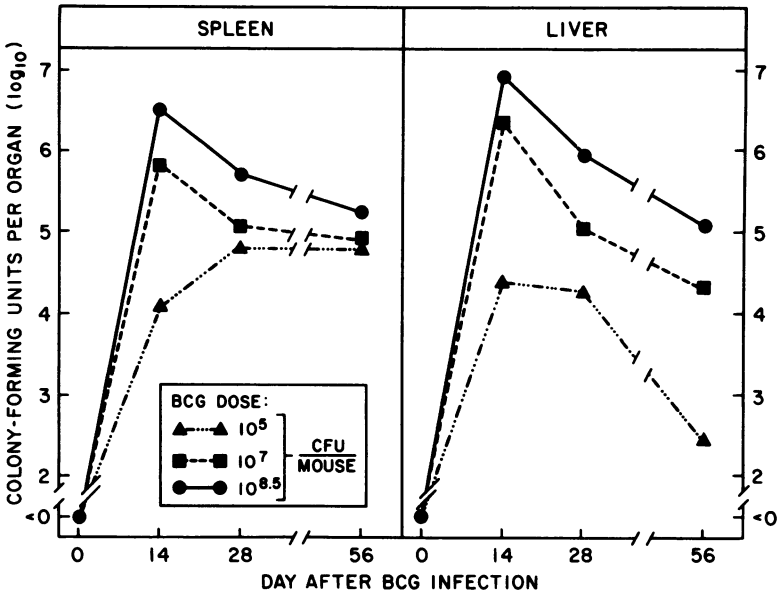

FIG. 4. *Patterns of growth of BCG after intravenous inoculation of different numbers of CFU. Spleens and livers were collected immediately before (day 0) and on the indicated days after BCG inoculation. There were five mice per group.*

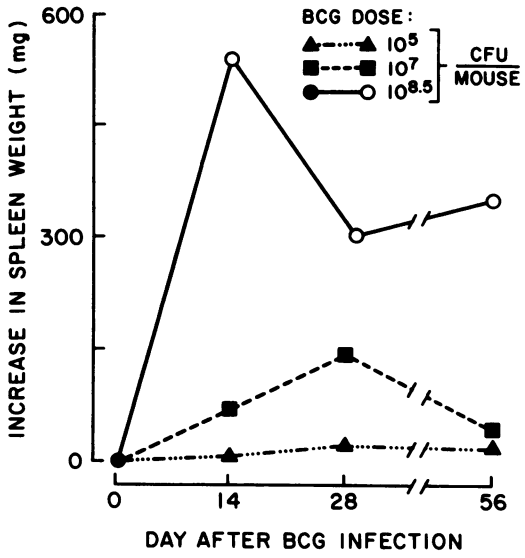

FIG. 5. *Demonstration that the magnitude and duration of splenomegaly are directly related to the dose of BCG. Mice were inoculated intravenously with the indicated numbers of BCG CFU; at intervals thereafter mice from each of these groups and normal mice were sacrificed, and their spleens were weighed. The differences in mean weight between the spleens from the BCG-infected mice and the spleens from the control mice are shown. Those points where the BCG-infected spleens were significantly ($P < 0.01$) heavier than the control spleens are indicated by open symbols. There were 10 mice per group per time point.*

fourth passage and the parasites from an ICR donor were then used separately to challenge mice infected 28 days previously with BCG and control mice. Figure 8 shows that some BCG-infected B6D2 mice resisted *P. berghei* presented in either syngeneic or allogeneic erythrocytes.

Response of strain B6D2, A and ICR mice to BCG infection. It was clear that BCG-infected B6D2 mice responded differently to *P. berghei* challenge than either BCG-infected strain A or BCG-infected strain ICR animals. Therefore, these strains of mice (i) may show differing courses of BCG infection or (ii) may respond differently to BCG infection, and, in turn, these differences may correlate with the capacity to resist *P. berghei*. To test these hypotheses, strain B6D2, ICR, and A mice were infected with BCG, and the courses of the mycobacterial infections and the selected effects of BCG infection on the different hosts were determined. The data in Fig. 9 through 12 can be compared directly since they were generated from one large group of BCG-infected mice.

Some BCG-infected B6D2 mice challenged with *P. berghei* between 7 and 84 days after BCG infection survived *P. berghei* infection, but none of the B6D2 mice challenged with *P. berghei* 1 h after BCG infection survived (Fig. 9). In contrast, normal B6D2 mice and all strain A and ICR mice uniformly succumbed to the same *P. berghei* challenges.

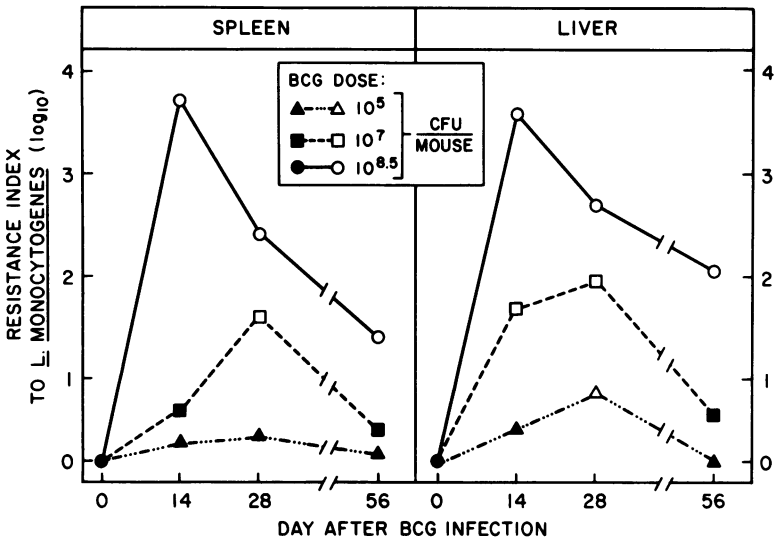

FIG. 6. *Demonstration that the level and duration of systemic nonspecific macrophage microbicidal activity generated in response to BCG infection are directly related to the number of BCG CFU used to initiate the mycobacterial infection. BCG-infected and normal mice were challenged at the indicated times with 10^5 CFU of L. monocytogenes intravenously. The difference in the 24-h growth of L. monocytogenes between control mice and BCG-infected mice is shown as the resistance index. Those groups of BCG-infected mice which differed significantly ($P < 0.01$) from the controls are indicated by open symbols. There were five mice per group per point.*

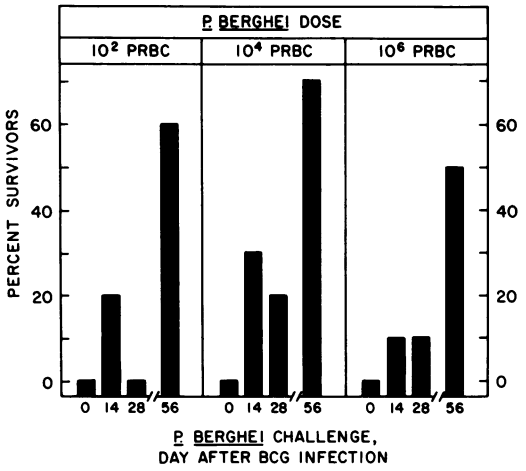

FIG. 7. *Effect of the number of PRBC in the challenge inoculum on the level of protection. Mice were infected with 10^7 BCG intravenously, and then BCG-infected and normal mice were challenged with the indicated numbers of PRBC at intervals from 1 h (day 0) through day 56 after the initiation of the BCG infection. All normal mice used as infection controls at each of the indicated times died and are not represented on the figure. There were 10 mice per group.*

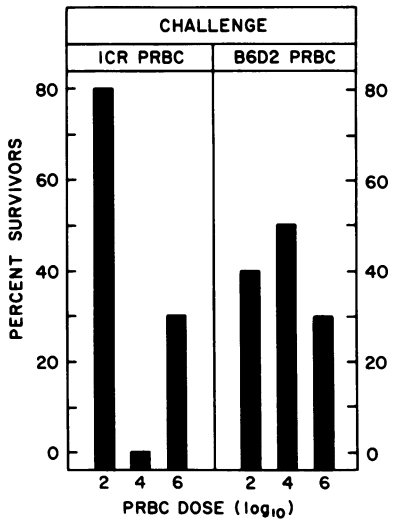

FIG. 8. *Evidence that BCG infection-associated resistance to P. berghei is expressed against parasites delivered in syngeneic (B6D2) and allogeneic (ICR) erythrocytes. Mice were infected by the intravenous injection of 10^7 BCG CFU and then challenged 28 days later by the intravenous injection of the indicated numbers of PRBC. There were 10 mice per group.*

Figure 10 shows the growth of BCG in the spleens and the livers of the tested mouse strains. There were no marked differences in BCG growth between groups or strains of mice which were resistant to *P. berghei* and those which were not.

Intravenous injection of 10^7 CFU of BCG caused marked splenomegaly in all strains of

mice tested (Fig. 11). The capacity of B6D2 mice to survive *P. berghei* infection did not correlate with more rapid development, longer persistence, or greater level of splenomegaly.

Figure 12 shows that strain B6D2, A, and ICR mice developed enhanced levels of macrophage bactericidal activity in response to BCG infections. The dynamics of the development and the waning of this capacity differed slightly among strains, but there was no reproducible pattern that was predictive of groups capable of withstanding *P. berghei* challenge.

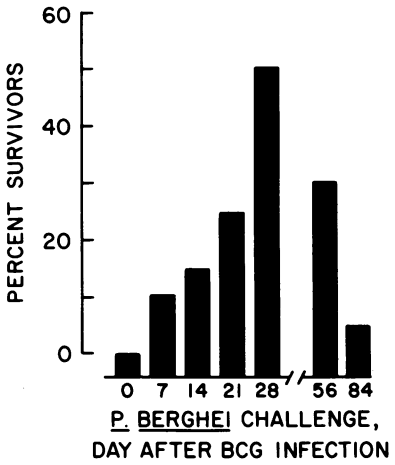

FIG. 9. *Survival after challenge with P. berghei of mice previously infected with BCG. Strain B6D2, A, and ICR mice were inoculated with 10^7 BCG CFU intravenously, and at intervals from 1 h (day 0) through day 84 thereafter groups of these mice and normal mice of each strain were inoculated intravenously with 10^4 PRBC obtained from an ICR donor. All normal mice and all BCG-infected strain A and ICR mice died of the P. berghei infection (data not shown). The histograms show the levels of survival of BCG-infected B6D2 mice. There were 10 mice per group per point.*

C. *parvum* protects B6D2 mice from *P. berghei*. Like BCG infection, stimulation with killed *C. parvum* causes splenomegaly (1, 13) and activation of macrophage bactericidal capacities (33) and acts as an adjuvant (3, 14, 34). Because of these similarities, *C. parvum* was tested to determine whether, like BCG, it could protect mice from *P. berghei*. Strain B6D2, A, and ICR mice were injected intravenously with *C. parvum* (400 μg) and then challenged at intervals with *P. berghei*. Splenomegaly and macrophage bactericidal activity were also measured. The data in Fig. 13 through 15 were obtained from one large group of mice treated with the same inoculum of *C. parvum*.

Figure 13 shows that by day 14 after *C. parvum* injection some *C. parvum*-treated B6D2 mice developed a capacity to resist virulent *P. berghei*, which persisted through at least day 56. As with BCG treatment, no *C. parvum*-treated strain A or ICR mice survived *P. berghei* infection, and all strain B6D2, A, and ICR mice which were not treated with *C. parvum* died.

Figures 14 and 25 show that the expected splenomegaly and activation of macrophage microbicidal activity occurred after injection of *C. parvum* into strain B6D2, A, and ICR mice. Again as with BCG infections, the dynamics and magnitudes of these responses did not allow identification of those mice which would survive *P. berghei* infection.

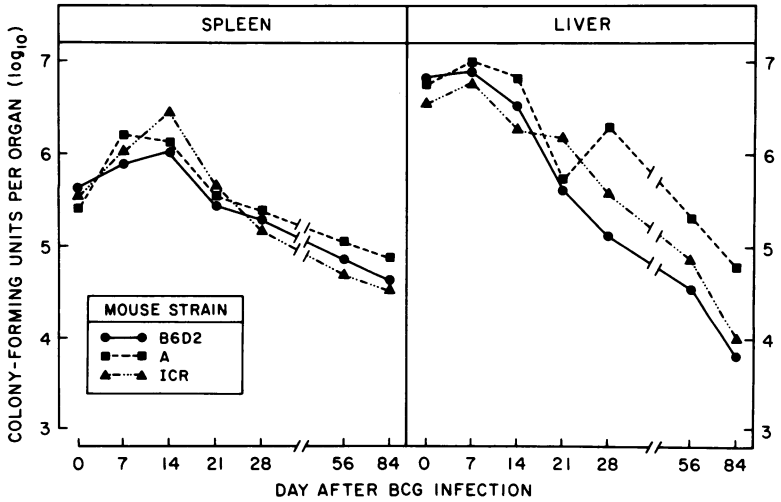

FIG. 10. *Patterns of growth of BCG in spleens and livers after intravenous injection of 10^7 CFU into strain B6D2, A, and ICR mice. Spleens and livers were collected at intervals from 1 h (day 0) through day 84 after BCG infection. There were five mice per group per point.*

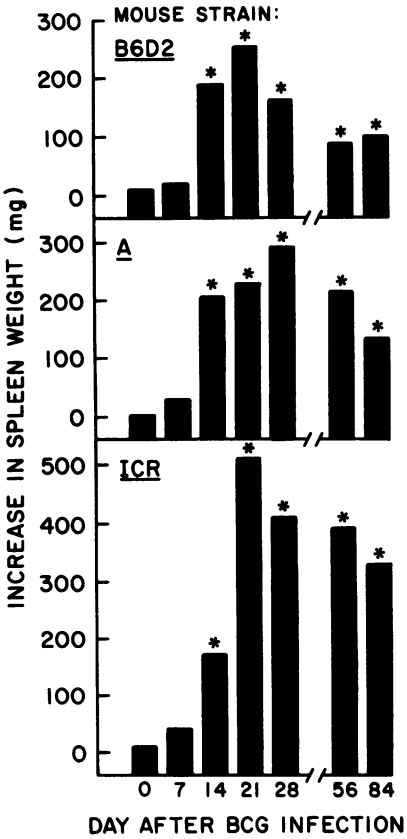

FIG. 11. *Development of splenomegaly after intravenous injection of 10^7 BCG CFU into strain B6D2, A, and ICR mice. The differences in mean spleen weight between BCG-infected and normal age-matched mice of the same strain are shown, as determined at intervals from 1 h (day 0) through day 84 after inoculation of BCG. There were 10 mice per group per point, and those groups of BCG-infected mice which had spleens significantly ($P < 0.01$) heavier than control spleens are indicated with an asterisk.*

BCG infection allows the development of a humoral factor with anti-*P. berghei* activity. Figure 2 shows that B6D2 mice which recovered from *P. berghei* infections as the result of BCG infection had the capacity to restrict a second larger *P. berghei* challenge with such efficiency that patent parasitemia did not develop. Immunity of this quality has been observed previously with both *P. berghei* (19, 21) and *Plasmodium yoelii* (22), although in each instance the response was generated by means other than BCG or *C. parvum* treatment. In these previous studies, sera from highly immune animals protected naive recipients from the homologous parasite. Thus, because of the pattern of response to second challenges shown in Fig. 2, it was possible that BCG altered the immunoresponsiveness of treated mice in such a way as to allow the development of a humoral factor with anti-*P. berghei* capacity.

Figure 16 shows the results of an experiment which tested this hypothesis. Mice infected with BCG and normal mice were challenged with *P. berghei*, and subsets of each group were bled at intervals. Sera collected 42 and 64 days after *P. berghei* challenge of BCG-infected mice provided naive recipients with significant ($P < 0.05$) levels of protection against *P. berghei*, but no serum from any of the other groups provided protection.

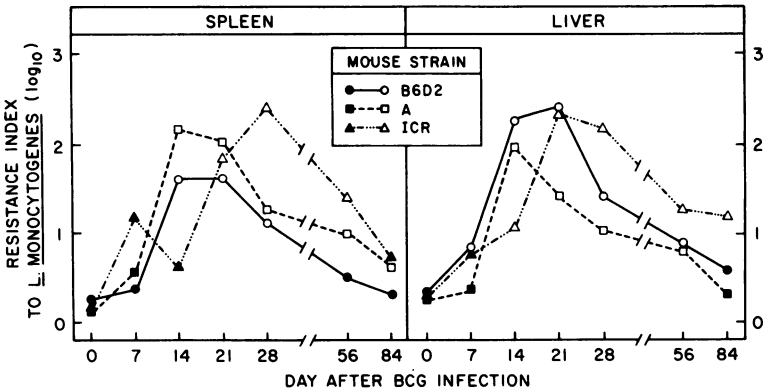

FIG. 12. *Levels of macrophage bactericidal capacity generated in response to BCG infection of strain B6D2, A, and ICR mice. Mice were inoculated intravenously with 10^7 BCG CFU and at the indicated times thereafter were challenged by intravenous injection of 10^5 L. monocytogenes CFU. Significantly fewer viable L. monocytogenes ($P < 0.01$, indicated by the open symbols) were recovered at 24 h after L. monocytogenes challenge from some groups of BCG-infected mice than from control mice challenged with the same L. monocytogenes inocula. There were five mice per group per point.*

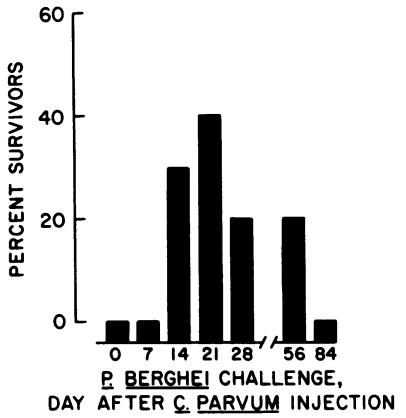

FIG. 13. *Evidence that killed C. parvum can protect B6D2 mice from P. berghei. Strain B6D2, A, and ICR mice were inoculated intravenously with 400 μg of C. parvum. At intervals from 1 h (day 0) through day 84 thereafter, groups of these mice and normal mice of each strain were challenged by intravenous injection of 10^4 PRBC obtained from an ICR donor. All normal mice and all C. parvum-treated strain A and ICR mice died of the P. berghei infection and are not represented on the figure. The histograms show the levels of survival of C. parvum-treated B6D2 mice. There were 10 mice per group.*

BCG infection enhances passive immunity. Although Fig. 16 shows that BCG infection allowed some B6D2 mice to develop humoral anti-*P. berghei* activity, it was not clear whether BCG acted solely as an adjuvant in the production of the humoral factor or, alternatively, whether BCG infection stimulated an ancillary defense which functioned in conjuction with or in addition to the passively transferrable humoral defense. I reasoned that if the latter were the case, then the injection of immune sera into BCG-infected recipients might result in a higher level of protection against *P. berghei* than the injection of the same sera into normal mice. This hypothesis was tested in the following experiment. Because only a fraction of B6D2 mice survived *P. berghei* infection and took 64 days to generate peak anti-*P. berghei* sera (Fig. 16), a serum pool with anti-*P. berghei* activity was made from mice vaccinated against *P. berghei* by injection of Formalin-killed erythrocytic parasites and subsequently infected with virulent organisms. The serum was collected on day 14 after the *P. berghei* infection (19).

Figure 17 shows that normal mice inoculated with the immune serum showed delays in the onset of *P. berghei* infection compared with normal and BCG-infected mice challenged with the same *P. berghei* inoculum but not inoculated with the immune serum. Furthermore, BCG-infected recipients of immune serum were better equipped to combat *P. berghei* than recipients of the same immune serum which were not infected with BCG. This was demonstrated by (i) delay of approximately 4 days in the appearance of 1% parasitemia in BCG-infected mice compared with normal recipients of immune serum and (ii) the survival of three of four BCG-infected mice, compared with none of four normal mice inoculated with the same immune serum.

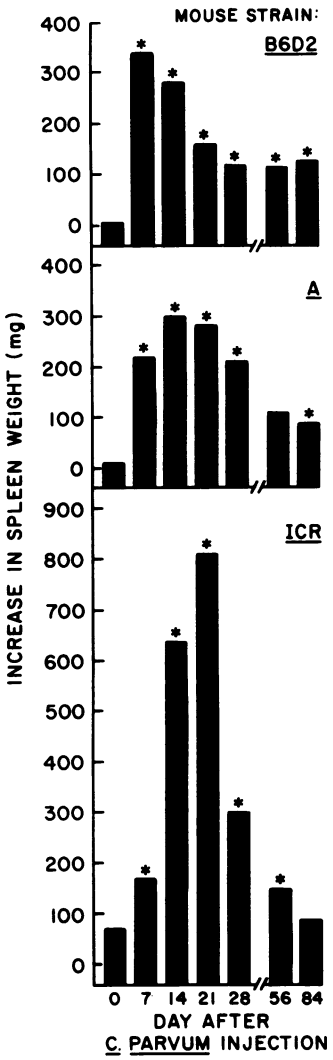

FIG. 14. *Development of splenomegaly after intravenous injection of 400 μg of killed C. parvum into strain B6D2, A, and ICR mice. The differences in mean spleen weight between BCG-infected and age-matched control mice of the same strain are shown, as determined at intervals from 1 h (day 0) through day 84 after inoculation of BCG. There were 10 mice per group, and those C. parvum-treated groups with spleens significantly ($P < 0.01$) heavier than control spleens are indicated with an asterisk.*

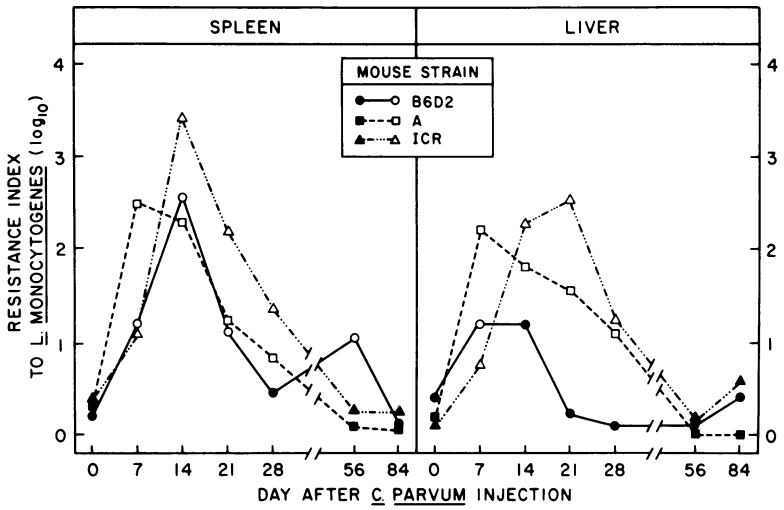

FIG. 15. *Levels of macrophage bactericidal capacity generated in response to C. parvum injection into strain B6D2, A, and ICR mice. Mice were inoculated intravenously with 400 μg of C. parvum and at the indicated times thereafter were challenged by intravenous injection of 10^5 L. monocytogenes CFU. Significantly fewer viable L. monocytogenes ($P < 0.01$, indicated by open symbols) were recovered at 24 h after L. monocytogenes challenge from some groups of C. parvum-treated mice than from controls challenged with the same L. monocytogenes inocula. There were five mice per group per point.*

DISCUSSION

My results show that BCG infection or *C. parvum* stimulation of strain B6D2 mice but not strain A or ICR mice can protect a variable proportion of the treated mice from otherwise lethal *P. berghei* infections and that protected mice have the capacity to produce a humoral factor with anti-*P. berghei* activity. Therefore, my results are consistent with the suggestion that the efficacy of these nonspecific stimulants is linked in some way, to the production of a protective humoral factor, possibly antibody. However, this study did not provide a full explanation of the mechanism of this nonspecific protection.

Three groups of possible mechanisms which might act singly or together can be considered. First, others have argued, mostly through studies of nonspecific stimulant-attenuated babesial or *Plasmodium vinckei* (5–7) infections, that stimulants cause the production of non-antibody-soluble mediators capable of adversely affecting intra-erythrocytic parasites. If such mediators were generated directly in response to BCG or *C. parvum*, it might be expected that (i) they would act against parasites immediately upon challenge, thereby causing the early course of parasitemia in protected mice to differ from that in controls and (ii) sera collected after BCG infection might protect recipients from *P. berghei*. My findings argue against these possibilities since infections in protected BCG-infected or *C. parvum*-treated mice developed at the same rate as in controls and serum collected after BCG infection did not protect recipients against *P. berghei*. However, my findings do not preclude the possibilities that mediators with anti-*P. berghei* activity were short lived, were generated via an indirect or delayed pathway, or required the simultaneous presence of soluble and fixed effectors for their expression.

Second, it could be argued that BCG or *C. parvum* acts as an adjuvant, allowing stimulated mice to generate a different class or a greater quantity of immunological effector. Ample evidence in support of the immunomodulatory capacities of BCG (4, 16–18, 26) and *C. parvum* (1, 3, 13, 14, 33, 34) has been presented by others. However, in my study it was not clear whether the development of the humoral anti-*P. berghei* activity resulted from adjuvant actions of the stimulants. It is possible that, via a mechanism different from any mechanism which would be considered a conventional adjuvant action, the stimulants simply allowed mice to survive long enough to generate demonstrable amounts of protective humoral factors.

Third, it could be argued that the stimulants may cause the generation of an as-yet-unidentified defense which could function in addition to or in conjunction with humoral factors in controlling parasites. Direct evidence in support of this hypothesis was obtained in one experiment in which BCG-infected mice infused with immune serum could control *P. berghei* better than

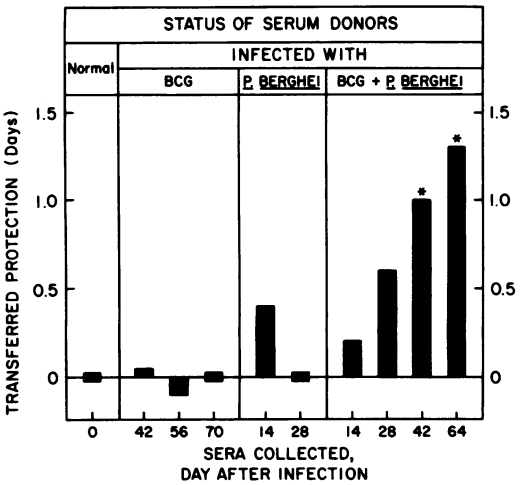

FIG. 16. *Demonstration of anti-P. berghei activity in sera collected after P. berghei infection of B6D2 mice previously infected with BCG. A group of mice was inoculated intravenously with 10^7 BCG CFU, and some of these mice were challenged 28 days later by the intravenous injection of 10^4 PRBC. At the indicated times, sera were collected from (i) mice infected with BCG and subsequently infected with P. berghei, (ii) mice infected with P. berghei but not with BCG (iii) mice infected with BCG but not with P. berghei, and (iv) normal mice. This figure shows that only sera collected 42 or 64 days after P. berghei infection of mice previously infected with BCG delayed the onset of infection in passively immunized recipients for a statistically significant interval (denoted by the asterisks). Note that days 14, 28, and 42 after P. berghei infection of BCG-infected mice were days 42, 56, and 70 after BCG infection. There were three to five recipients per group.*

normal mice which received the same amount of the same serum. However, the mechanism through which BCG infection provided this enhanced protection was not identified.

This and other studies have established clearly that manipulations which may be independent of the presentation of plasmodial antigens may markedly affect the capacity of rodents to resist sporozoite (15, 28) or erythrocytic malaria (5–7). However, in no instance has the mechanism(s) of this nonspecific resistance been defined. It is not clear whether nonspecific resistance to malaria is solely a laboratory phenomenon or whether it represents the expression of a defense mechanism which is also a part of the immunospecific defense against malaria.

Previous studies have established that resistance to *P. berghei* can be conferred upon strain ICR (21, 23) and B6D2 (19) mice through injections of Formalin-killed mixed erythrocyte-stage parasites. Injection of a diluent (0.15 M NaCl) or a mock antigen of formalinized normal erythrocytes failed to protect mice. When vaccinated mice were challenged with virulent *P. berghei*, infection developed through about 10% parasitemia at the same rate as in controls. However, thereafter infections in controls progressed to death, whereas infections in vaccinated mice were cleared (19, 21). A second challenge delivered to mice 28 days after vaccine-mediated recovery from a primary *P. berghei* infection was neutralized immediately, and overt parasitemia was not observed (21). Injection of the vaccine did not directly cause the generation of a protective serum, but the vaccine primed mice in such a way that they produced protective serum after challenge with live *P. berghei* (19). Thus, the patterns of response to *P. berghei* challenge in strain ICR and B6D2 mice immunized with a specific antigen were similar to the pattern of response in B6D2 mice injected with BCG or *C. parvum*. Therefore, it is reasonable to question whether the mechanism of the defense raised by the vaccine differs from the

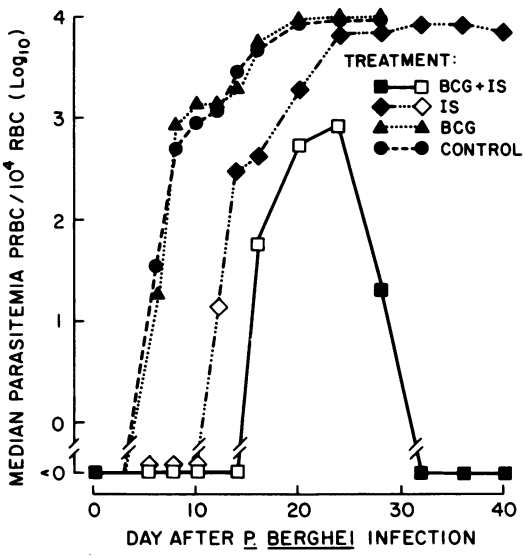

FIG. 17. *Demonstration that BCG infection enhances the expression of passive immunity. Mice were infected with 10^7 BCG CFU intavenously, and 40 days later these mice and normal mice were challenged with 10^4 PRBC intravenously. Some mice were immunized passively by the intraperitoneal injection of 1 ml of immune serum (IS) on the day before P. berghei infection and 0.5 ml of immune serum on days 0, 1, and 2 after P. berghei infection (i.e., a total of 2.5 ml/recipient). This figure shows the courses of parasitemia in the differently treated groups. There were four or five recipients per group. Those groups of treated mice which showed levels of parasitemia which differed significantly ($P < 0.05$) from the levels in the controls on the same day are indicated by open symbols. RBC, Erythrocytes.*

mechanism of defense raised by nonspecific stimulants.

A full answer to this question is not currently available. However, there are significant differences between specific antigen-raised resistance and the resistance raised by injections of stimulants. First, erythrocytic forms of *P. berghei* must be incorporated in sufficient quantities into the immunizing regimen if strain ICR (23) or A (Murphy, unpublished data) mice are to be protected from virulent challenges. Neither mock antigens, BCG, nor *C. parvum* protects these mice. Second, the resistance to *P. berghei* observed in BCG- or *C. parvum*-treated B6D2 mice did not often protect more than 50% of the mice, and the overall pattern of these experiments suggests that the resistance generated in response to these stimulants peaked between 21 and 56 days after stimulant injection and waned markedly by day 84. In contrast, the immunity generated in response to intravenous injection of formolized erythrocytic antigen often protects the majority of B6D2 mice (19). This vaccine-induced resistance also appears to be longer lived than the resistance induced by BCG or *C. parvum*. It has been shown that immunity persists for at least 168 days after intravenous immunization of ICR mice (21) and for at least 400 days after intravenous vaccination of B6D2 mice (Murphy, manuscript in preparation). Furthermore, the pattern of resistance observed after BCG or *C. parvum* treatment is strikingly similar to that exhibited by mice at extended intervals (e.g., months) after recovery from *P. yoelii* infection (2, 20), differing only in that the immunity generated in response to the attenuated infection appeared to be longer lived (at least 418 days long) (20).

It is clear that additional studies designed to probe host defenses against *P. berghei* with a greater degree of resolution will be required to discover the mechanism through which nonspecific stimulants protect animals from *P. berghei* and to differentiate these stimulants from the mechanisms of specific acquired immunity. These studies will be difficult if the model system used for this study cannot be improved because in this model (i) only a fraction of the BCG- or *C. parvum*-treated mice are protected and (ii) the dynamics of the protective response are somewhat unpredictable.

It is clear that antibodies contribute significantly to immunity against malaria (8–11, 35). However, as shown by this and other studies (5–7, 12, 15, 28–30), the known actions of antibodies cannot explain fully the murine defense against plasmodia. Hence, it can be argued that nonantibody defenses may exist. The identification of these putative other defenses and the definition of their relative roles in immunity against malaria are pressing problems. In this study, attention was directed to these problems through attempts to determine whether protection from *P. berghei* could be correlated with (i) the systemic activation of macrophages (a predominantly T-cell-dependent defense mechanism [24], which was measured as microbicidal capacity) or (ii) the degree of increase in spleen size. To test these hypotheses, I took advantage of the knowledge that the magnitude of these responses is determined by the dose of BCG inoculated and the demonstration that resistance to *P. berghei* can be raised in response to BCG or *C. parvum* treatment in strain B6D2 mice but not in strain A or ICR mice. I reasoned that, if either systemic T-cell activity or spleen size was critical to the successful expression of immunity against *P. berghei,* then there would be a direct relationship in B6D2 mice among the number of BCG inoculated, the magnitude of the measured responses, and the resistance to *P. berghei.* Similarly, it might be expected that protected B6D2 mice might show greater bactericidal activity or increased spleen size compared with nonprotected strain A or ICR mice. However, my results demonstrated that BCG-infected B6D2 mice which were protected from *P. berghei* showed increased spleen size and bactericidal capacities compared with nonprotected B6D2 controls but also showed that, above a threshold, the magnitude of splenomegaly or bactericidal activity did not correspond to increased levels of resistance to malaria. When viewed in the perspective of the degrees of splenomegaly and bactericidal activity generated in non-protected strain A and ICR mice in response to BCG or *C. parvum,* it is clear that neither splenomegaly nor the bactericidal capacity of macrophages directly relates to the capacity to resist malaria.

Although this and other studies have shown clearly that protection against malaria can result from the injection of heterologous materials, it is also true that some of these materials may compromise the host defense against malaria. This has been shown most clearly by the recent work of Smrkovski and Strickland (32), who demonstrated that BCG infections which nonspecifically protect against sporozoite *P. berghei* infections in strain A mice simultaneously may render these mice incapable of generating specific immunity in response to a sporozoite vaccination regimen which is successful in normal mice. Again, the mechanism of this BCG infection-associated interference with vaccination is not known.

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