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EFFECTS OF MYCOBACTERIUM BOVIS BCG, BACTERIAL LIPOPOLYSACCHARIDE AND HYDROCORTISONE ON THE DEVELOPMENT OF IMMUNITY TO PLASMODIUM BERGHEI (*)

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SUMMARY

Mycobacterium bovis (BCG) significantly enhanced the development of immunity by CFW, C57BL/6, C57BL/10ScN and BALB/c (Nu+) mice to the erythrocytic stages of **P. berghei.** Mice treated with BCG required fewer cycles of infection and Fansidar (pyrimethamine + sulfadoxine) cure in order to develop solid immunity than untreated, immunized mice. However, those, mice treated with BCG at 30 days before the initiation of immunization showed an earliear loss of immunity to **P. berghei** than those mice which received BCG at 14 days or no BCG. Thus, BCG enhanced the host immune response to **P. berghei** during an initial infection, but shortened the length of immunity so that mice were more susceptible to **P. berghei** during subsequent infections. Treatment of CFW, BALB/c and C57BL/6 mice with bacterial lipopolysaccharide or hydrocortisone acetate (HDC) caused the animals to require more cycles of infections and drug cure in order to attain immunity than controls. Treatment of immunized C57BL/10ScN mice with hydrocortisone completely abolished their ability to survive a P. berghei infection.

KEY WORDS: **Mycobacterium bovis** — BCG — Bacterial Lipopolysaccharide Immunity to **Plasmodium berghei** — Effect of Hydrocortisone Mycobacteriosis.

INTRODUCTION

Bacterial lipopolysaccharide (LPS) has been found to delay the day of death and/or the onset of parasitemia in **P. berghei** and **P. vivax** infections [20,32]. Hydrocortisone has been found to exacerbate **P. berghei** infections [13] or to delay the day of death and the onset of parasitemia [28]. Killed **Corynebacterium parvum** and BCG are each capable of enhancing host resistance to **Plasmodium** and **Babesia** species [8,9,22,24,30]. However, SMRKOVSKI & STRICKLAND [30] reported recently that under the appropriate conditions treatment with BCG interfered with the development of protective immunity to **P. berghei** by mice which had been previously immunized by irradiated sporozoites. In a previous study, we found that mice became solidly immune to **P. berghei** after several cycles of infection and Fansidar cure [12]. In light of the foregoing, we undertook the present study to determine the effects of BCG, HDC, and LPS on the ability of several strains of mice to develop solid immunity to the erythrocytic forms of **P. berghei.**

(*) This study is based on a dissertation submitted by J. J. Ferraroni to Graduate School of the University of Montana in partial fulfillment of the requirements for the Doctor of Philosophy degree.

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MATERIALS AND METHODS

Animals CFW, C57BL/6, and C57BL/10ScN mice were obtained from the National Institute of Allergy and Infectious Diseases Rocky Mountain Laboratory, Hamilton, MT. The BALB/c (Nu/+) mice were obtained from a breeding colony in the Microbiology Departament animal facility at the University of Montana. All mice were male, 6- to 8-weeks-old and weighed 20-25g at the beginning of each experiment. Mice were fed mouse lab chow and water **ad libitum.**

Parasite: The NK65 strain of **Plasmodium berghei** was originally obtained from the University of New Mexico, Albuquerque, NM, and had been maintained by weekly blood passages in Swiss-Webster mice for 5 yr before being used in this study. All mice were each inoculated intraperitoneally (IP) with physiological saline containing 10^7 **P. berghei**-parasitized erythrocytes. The inoculating suspension of infected erythrocytes was kept at 4°C and the interval between bleeding donor and inoculating recipient mice never exceeded 40 min.

Mycobasterium bovis BCG: **Mycobacterium bovis**, Bacillus Calmette-Guérin (BCG, Paris strain) was obtained from a stock culture maintained in the Microbiology Department at the University of Montana. The stock culture was diluted with Dubos Broth to an optical density of 200 (Klett 200) as determined by a Klett-Summerson n.° 42. Klett 200 suspensions of BCG were stored at-70°C. After thawing, the suspension was diluted to a "Klett 100" suspension by adding an equal volume of Dubos Broth. To determine the number of colony-forming units of BCG, five 0.1 ml 10^{-4} to 10^{-8} dilutions were each plated in duplicate on Dubos agar plates, which were then sealed in plastic bags and incubated at 37°C for 28 days. The number of viable BCG organisms was determined by counting visible colonies. BCG was suspended in a saline solution containing 0.5% tween 80. Two tenths ml of this suspension containing 10^8 viable BCG organisms was injected IP or intravenously (IV) into each recipient at 30 or 14 days before the first inoculations of **P. berghei.**

Lipopolysaccharide (LPS): LPS was obtained from Dr. K. B. Von Eschen, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, MT. The LPS was extracted from whole cell extractions of **Salmonella minnesota** ET, wild type, strain 1114,Ø-H20. Ten micrograms LPS in 0.2 ml distilled water was injected IP into each recipient on the same day of the first inoculation of **P. berghei.**

Hydrocortisone (HDC): HDC acetate (Sigma Chemical Co., Saint Louis, MO) was prepared as follows. A 0.85% saline solution was prepared which also contained 0.4% tween 80, 0.9% benzyl alcohol, 0.5% carboxy-methyl cellulose, and 50 mg HDC acetate/ml. On tenth ml of this solution which contained 5 mg of HDC was injected subcutaneously (SC) into each recipient on the same day of the first inoculation of **P. berghei.**

Chemotherapy: All animals received 6 or 7 cycles of **P. berghei** infection and Fansidar cure (CIC). Fansidar (tablet form) was dissolved in distilled water and administered by gavage in a single dose (1 mg pyrimethamine + 20 mg sulfadoxine/kg body weight) 5-6 days after parasite inoculation when the parasitemia was 40-50%. Parasite clearance usually occured 2-3 days after drug treatment. At 7 days after drug treatment, mice were inoculated IP with 10^7 **P. berghei** infected erythrocytes. We had determined previously that Fansidar had no effect on **P. berghei** if mice were inoculated with the parasite 4 or more days after a single oral dose of Fansidar.

Immunity — Survival time was used to assess the degree of immunity to **P. berghei** by mice treated with BCG, LPS or HDC. The study consisted of six experiments (A-F), each of which contained 3,4 or 5 groups of 10 mice. Experiment A was composed of 5 groups of CFW mice. Groups 1 and 2 received BCG IP or IV, respectively, 30 days before inoculation of **P. berghei.**

Groups 3 and 4 received BCG IP or IV, respectively, 14 days before **P. berghei** inoculation. Group 5 served as a control. Experiment B was the same as experiment A, except that C57BL/10 ScN mice were used. Experiment C had four groups of C57BL/6 mice. Group 1 received BCG IP 14 days before **P. berghei** inoculation, groups 2 and 3 received LPS and HDC, respectively, at same day of parasite inoculation and group 4 served as control. Experiment D was the same

as experiment C, except that (BALB/c (Nu/+) mice were used. Experiment E consisted of 4 groups of CFW mice. Group 1 received BCG IP 14 days before **P. berghei** inoculation, and group 4 was a control. Experiment F was performed as experiment E except that C57BL/10ScN mice were used.

Fading of immunity — Those CFW and C57BL/10ScN mice which had been treated with BCG 14 or 30 days before immunization by CIC and which survived **P. berghei** challenge, were each inoculated with 10^7 P. berghei — infected erythrocytes at 120 days after the last infection. Untreated, immunized mice served as a control. The number of deaths for each group of mice was recorded for 60 days after inoculation of each mouse with **P. berghei.**

Significant differences among various experimental groups were determined by the student's t-test at **P.** values of ≤ 0.05.

RESULTS

Effects of BCG, LPS and HDC on the ability of Fansidar-cured mice to develop immunity to P. berghei — Significantly better protection against **P. berghei** was obtained in all four strains of mice treated with BCG than control mice, whereas LPS and HDC had a significant deleterious effect (Figs. 1-4,8). Each strain of mice treated with BCG required fewer cycles of infection and cure in order to become immune and significantly more mice survived the **P. berghei** infection than did controls (Figs. 1-4). In BCG-treated mice, 100% of the CFW and C57BL/6 mice survived a normally lethal inoculation of **P. berghei** after 5 CIC (Figs. 2,4); 90% of the BALB/c and 70% of the C57BL/10ScN mice survived after 6 or 7 CIC (Figs. 1, 3). In all four strains of mice, HDC had a significantly greater deleterious effect than that of LPS (Figs. 1-4). In each strain of mice, treatment with LPS or HDC delayed the onset of immunity and caused an increase in the number of CIC necessary to obtain immune animals (Figs. 1-4,8). In C57BL/10ScN mice, treatment with HDC completely abolished the ability of immunized animals to survive a **P. berghei** infection (Figs. 1).

Effect of time and route of BCG administration on the development of immunity by CFW and C57BL/10ScN mice — In each strain of mice, IV administration of BCG provided

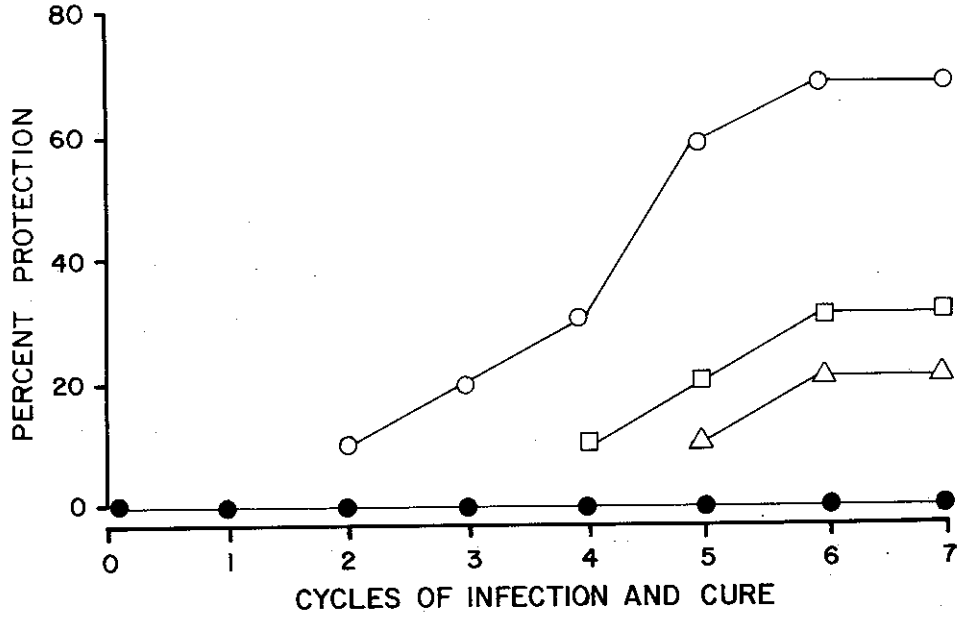

Fig. 1 — Effects of BCG, LPS and HDC on the ability of C57BL/10ScN mice, which were subsequently immunized by several cycles of infection and Fansidar cure, to survive a lethal inoculation of **P. berghei.**
○, BCG; △, LPS; ●, HDC; □, control.

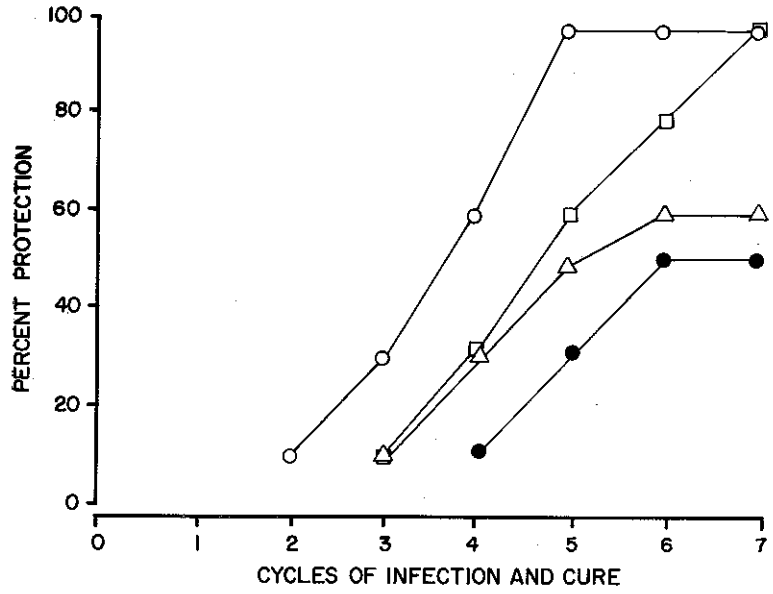

Fig. 2 — Effects of BCG, LPS and HDC on the ability of C57BL/6 mice, which were subsequently immunized by several cycles of infection and Fansidar cure, to survive a lethal inoculation of **P. berghei**.
○, BCG; △, LPS; ●, HDC; □, control.

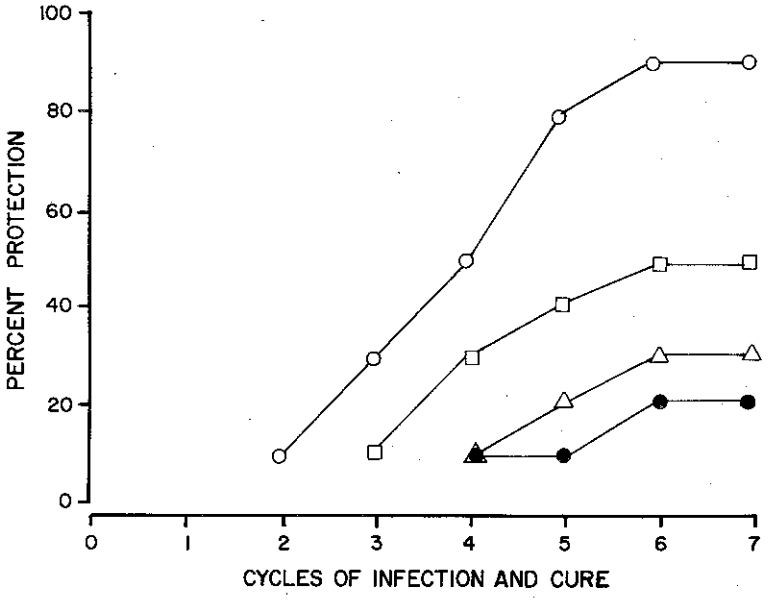

Fig. 3 — Effects of BCG, LPS and HDC on the ability of BALB/c (Nu/+) mice, which were subsequently immunized by several cycles of infection and Fansidar cure, to survive a lethal inoculation of **P. berghei**.
○, BCG; △, LPS; ●, HDC; □, control.

slightly more significant protection than did IP administration (Figs. 5,6). Administration of BCG at 30 days before the initiation of immunization by infection and drug cure provided significantly greater protection than did BCG administration at 14 days (Figs. 5, 6). After 4 or 5 cycles of infection and cure, nearly 100% of the mice survived that had received BCG IV or IP at 14 or 30 days. In about 90% of the C57BL/10ScN mice treated with BCG IV or IP at 30 days became immune to **P. berghei.** The least amount of protection (about 70%) was observed in those C57BL/10ScN mice which had been treated with BCG at 14 days (Fig. 6).

Fading of immunity in immunized CFW and C57BL/10ScN mice treated with BCG. CFW and C57BL/10ScN mice that had received BCG at 14 or 30 days before CIC and had survived were inoculated with **P. berghei** at 120 days after the last **P. berghei** infection. Fifty to 80%

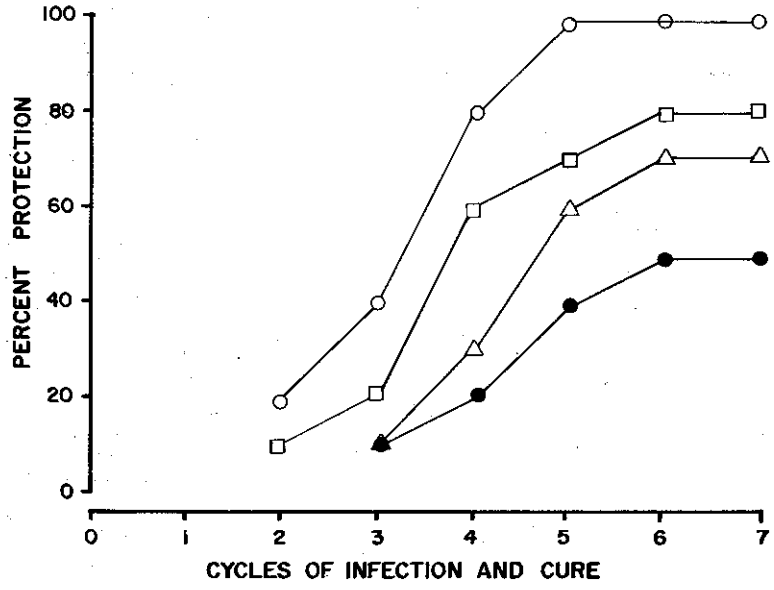

Fig. 4 — Effects of BCG, LPS and HDC on the ability of CFW mice, which were subsequently immunized by several cycles of infection and Fansidar cure, to survive a lethal inoculation of **P. berghei**. ○, BCG; △, LPS; ●, HDC; □, control.

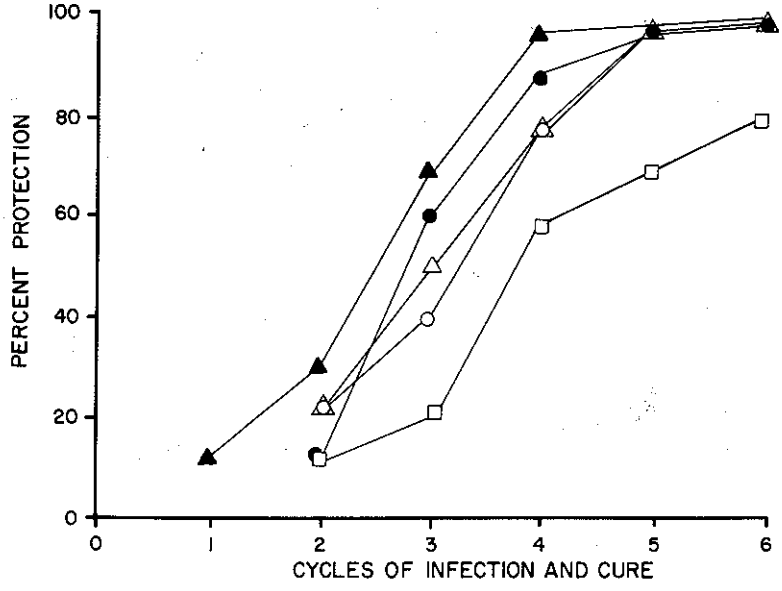

Fig. 5 — Effects of BCG, administered at 14 or 30 days before 1 6 cycles of infection and Fansidar cure, on the ability of CFW mice to survive a lethal inoculation of **P. berghei**. ○ IP and △ IV administration of BCG at 14 days; ● IP and ▲ IV administration of BCG at 30 days; □ control.

of the control mice and mice which had received BCG at 14 days before CIC survived, whereas all of the C57BL/10ScN and CFW mice that had received BCG at 30 days before CIC had died by 30 and 50 days, respectively, after **P. berghei** inoculation (Fig. 7).

DISCUSSION

It has been known for some time that killed **Mycobacterium tuberculosis** can potentiate the development of resistance to malarial parasites when administred in conjunction with specific antigen. FREUND et al.[15] demonstrated that killed **M. tuberculosis** and blood forms of **Plasmodium knowlesi** emulsified in paraffin oil and lanolin extract conferred significant protection to rhesus monkeys against a normally lethal inoculations of erythrocytic forms of **P. knowlesi**. Treatment of monkeys with incomplete Freund's adjuvant or antigen alone before inoculation of erythrocytic forms of **P. know-**

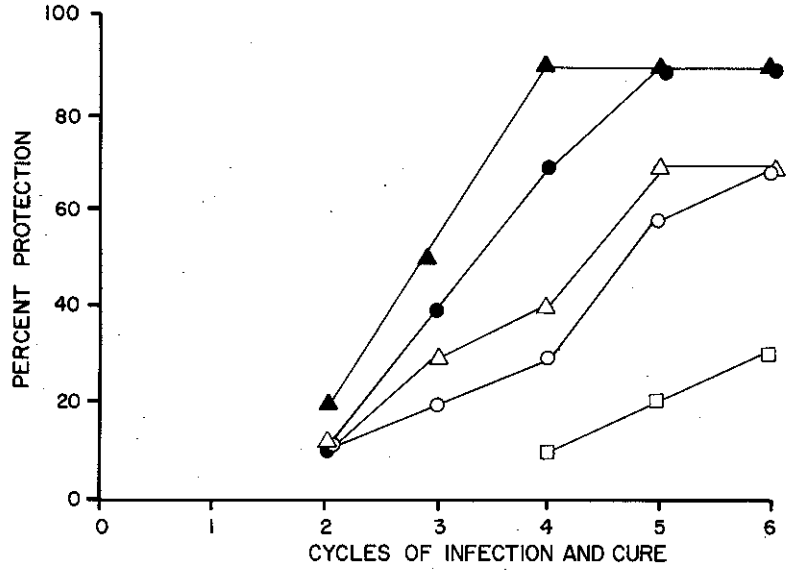

Fig. 6 — Effects of BCG, administered at 14 or 30 days before 1-6 cycles of infection and Fansidar cure, on the ability of C57BL/10ScN mice to survive a lethal inoculation of **P. berghei**. ○ IP and △ IV administration of BCG at 14 days; ● IP and ▲ IV administration of BCG at 30 days; □ control.

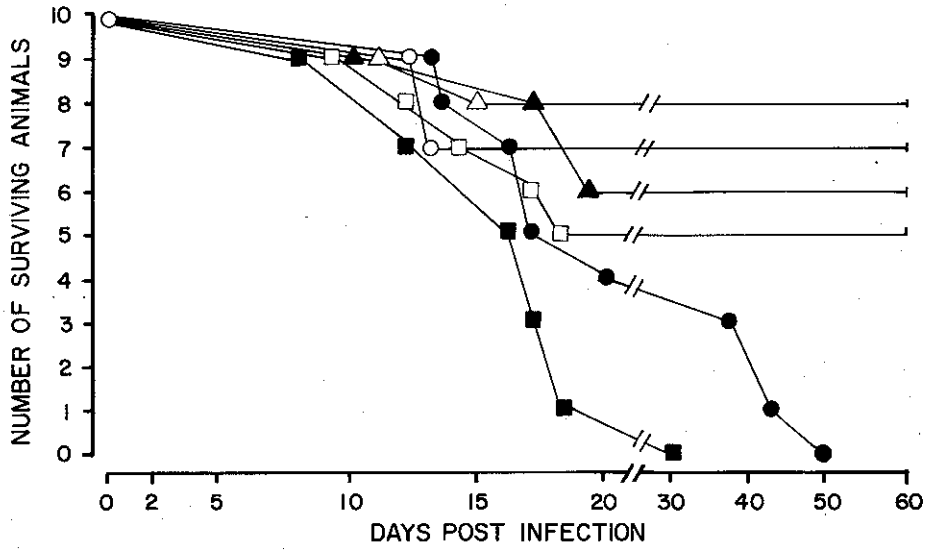

Fig. 7 — Fading of immunity in CFW and C57BL/10ScN mice that had survived a lethal inoculation of **P. berghei** after being treated with BCG 14 or 30 days before immunization by 6-7 cycles of infection and Fansidar cure. Mice were inoculated with **P. berghei** at 120 days after the last **P. berghei** infection. CFW control, △ ; BCG given to CFW mice at 14 ○ or 30 ● days before **P. berghei** inoculation; C57BL/10ScN control, ▲ ; BCG given to C57BL/10ScN mice at 14 □ or 30 ■ days before **P. berghei** inoculation.

lesi or **P. falciparum** does not provide as much protection as parasite antigen combined with complete Freund's adjuvant (CFA) [4,5,21]. Likewise, BGC parasite antigen isolated, emulsified in adjuvant 65 does not protect rhesus monkeys from **P. knowlesi**, whereas BCG and parasite antigen emulsified in adjuvant 65 does provide protection [27]. Live **M. tuberculosis** given to the Indian brown monkey in the absence of antigen or adjuvant lengthens the prepatent period of **P. knowlesi** infection and protects some monkeys against lethal **P. cynomolgi** infection [1].

Administration of Mycobacteria to mice before inoculation of plasmodial blood forms

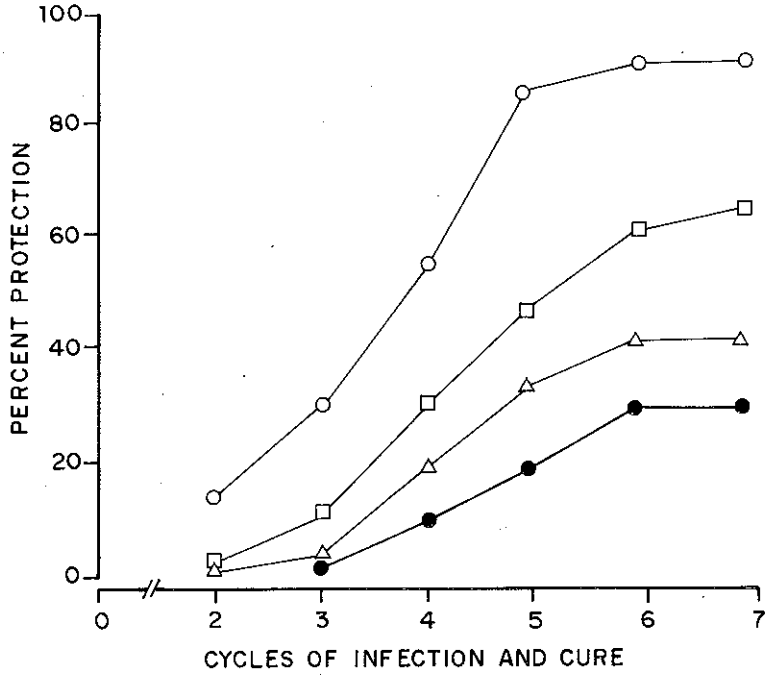

Fig. 8 — Effects of BCG, LPS and HDC on all four strain of mice combined which were immunized by infection and Fansidar cure before **P. berghei** inoculation; BCG; ○ ; LPS, △ ; HDC, ● ; control, □ .

usually confers partial or complete resistance to the parasite. Immunization of mice with parasite antigen in CFA does not protect mice against **P. berghei** [26], whereas BCG has been found to nonspecifically protect murine hosts against rodent malaria [7]. Erythrocytic stages of **P. berghei** plus BCG has been found to provide significant protection of mice against **P. berghei** but the degree of protection varied with the antigen lot [26]. Usually, BCG alone does not confer resistance to **P. berghei** [22] but it does protect mice from **P. vinckei** and reduces or ablates the temporal peripheral parasitemia of a **P. yoelii** infection [7].

Mice successfully immunized by a single or several CIC without BCG treatment [10,11,12]; usually retain the ability to survive subsequent **P. berghei** challenge for at least 120 days. In the present study, we found that BCG treatment of mice before immunization by several CIC decreased the number of CIC required to elicit solid immunity in CFW, C57BL/10ScN, C57BL/6 and BALB/c strains of mice. Murphy & Lefford [23] also found that BCG treatment followed by two vaccinations with erythrocytic stages of **P. berghei** significantly increased the number of mice surviving parasite challenge, but that there was a more rapid loss of malarial immunity.

SMRKOVSKI & STRICKLAND [30] found that BCG interfered with the development and maintenance of immunity to sporozoite challenge in mice immunized by irradiated sporozoites of **P. berghei**. Recently, SMRKOVSKI [29] reported that IV administration of BCG more immunosupressive than BCG given IP, SC or intramuscularly. Intravenous administration of BCG only has been reported to confer nonspecific resistance to **Plasmodium** infections in mice [7]. In each strain of mice the curve of protection with intravenous administration seems to afford a slightly higher protection than I. P. (Figs. 5, 6), although this may not be significant. Administration of BCG at 30 days before the initiation period of immunization by CIC apparently provided greaten protection than did 14 days.

Our findings are similar to those of SMRKOVSKI & STRICKLAND [30] in that BCG appears to enhance the development of immunity but interferes with the maintenance of resistance to **P. berghei**. Because irradiated sporozoites were rendered inefficatious in recipient mice by either pre-or post-treatment with BCG, SMRKOVSKI & STRICKLAND [30] proposed that

BCG interfered with both the inductive phase of immunity and memory

Thus because only one sporozoite needs to survive to initiate this infection, an alternative hypothesis for the deleterious effect of BCG upon memory might be the possibility of recruitment of effector T-cells to sites of BCG-initiated granulomas not permitting the action of these cells to contribute to the host defense against this new infection. This would be an alternative to the simulation of supressor T-cells, as proposed by SMRKOVSKI [29].

In a previous study, we found that Swiss-Webster and C57BL/6 mice became immune to **P. berghei** after 5 CIC which lasted for 502 days [12]. In the present study, similarly treated mice were immune at 120 days (the longest interval tested) after the last **P. berghei** infection, whereas those animals treated with BCG 30 days before CIC became immune sooner than controls but quickly lost their immunity. BCG infection in the mouse peaks at about 12 days after inoculation and is usually long-lasting [2]. Therefore, BCG probably effects the development of immunity to **P. berghei** during repeated CIC. However, an on going stimulation of the immune system by BCG cannot account for the loss of memory to **P. berghei** by BCG-treated mice. There may be qualitative differences in the development of immunity by mice treated with BCG and then immunized by CIC as compared to untreated, immunized mice. Such differences may account for the fading of immunity (loss of memory) to **P. berghei** by BCG-treated mice and the retention of immunity by untreated, immunized mice. Thus, these findings indicate that nonspecific immunopotentiators such as BCG may enhance the host immune response to malaria parasites during an initial infection, but that they may also shorten the interval of time that the host is immune to the parasite as well as have a deleterious effect upon host resistance to subsequent infections.

In contrast to the persistence of BCG in infected animals, mice are capable of rapidly eliminating LPS and HDC [3,6]. When LPS is given before exposure to antigen, there is an increase in antibody production and a depression in delayed type hypersensitivity (DTH) to sheep red blood cells (SRBC) [17]. Since antibody is known to inhibit T-cell responses to common antigen [19], the adjuvant effect of LPS on antibody production [14,16] may be partially responsible for the decrease in DTH to SRBC. Administration of LPS before the first CIC may have caused a depression of cell mediated immune responses (CMI) necessary for the eventual development of solid immunity to **P. berghei**. Conversely, augmentation of the humoral response might be responsible for the delay in day of death of mice treated with LPS prior to the inoculation of the erythrocytic forms of **P. berghei**. Paradoxically, MACGREGOR et al. [18] could detect no specific antibody by immunofluorescence (IFA) in normal mice nor in LPS-treated mice infected with **P. berghei**, whereas WAKI & SUZUKI [33] observed relatively high IFA in mice infected with **P. berghei**. However, LPS is known to augment reticuloendothelial clearance [18] as well as to mediate the production of an unidentified substance that non-specifically interferes with the development of erythrocytic stages of **P. berghei** [31].

Administration of LPS prior to repeated CIC delayed the development of solid immunity by about one CIC. Twenty-six of 40 control mice became immune, whereas only eighteen of forty LPS-treated mice developed a solid, sterile immunity by the seventh CIC. Thus, the primary effect of LPS appears to be short-lived and limited to the first CIC. If LPS suppressed the development of CMI then CMI would appear to be responsible for the eventual development of immunity to **P. berghei**. Mice that did not develop immunity after 7 CIC usually did not become immune even with additional CIC. Therefore, LPS may have a long-lasting suppressive effect upon the immune response to **P. berghei** by some of the mice.

Hydrocortisone acetate depressed the development of immunity to **P. berghei** to a greater extent than did LPS. In mice HDC causes a short-lived thymic aplasia due to a cytocidal effect on immature, short-lived T-cells [6]. The immune systems of HDC-treated mice evidently lack the capacity to recruit these cells in response to **P. berghei** infection during the first CIC which may impair their ability to develop immunity during subsequent infections.

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RESUMO

Efeito do Mycobacterium bovis BCG, lipopolissacarídeo bacteriano e hidrocortisona no desenvolvimento de imunidade ao Plasmodium berghei em camundongos.

Mycobacterium bovis (BCG) aumenta significantemente o desenvolvimento da imunidade nos camundongos CFW, C57BL/6, C57BL/10ScN e BALB/c (Nu/+) para os estágios eritrocíticos do **Plasmodium berghei**. Camundongos tratados com BCG requerem menos ciclos de infecção com *P. berghei* e cura pelo Fansidar (pirimetamina + sulfadoxina) para desenvolverem imunidade sólida a este parasita do que os controles. Contudo, os animais que receberam BCG 30 dias antes do início da imunização evidenciaram uma perda precoce da imunidade adquirida para o **P. berghei**, quando comparado com os animais que receberam BCG 14 dias antes ou que não receberam BCG. Assim, sendo, o BCG aumentada a indução na resposta imune do hospedeiro ao **P. berghei** no curso de infecções subseqüentes. O tratamento de camundongos CFW, BALB/c e C57BL/6 com lipopolissacarídeo bacteriano ou hidrocortisona faz com que os animais requeiram um número maior de ciclos de infecção e cura para tornarem-se imunes ao **P. berghei** que os controles. O tratamento dos camundongos C57BL/10ScN com hidrocortisona aboliu completamente a sua habilidade de sobrevida subseqüentes a ciclos de infecção com **P. berghei** e cura pelo Fansidar.

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