Immunoprophylaxis with BCG of Experimental *Echinococcus multilocularis* Infections

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Previous studies have demonstrated that prophylactic treatment with BCG protects cotton rats (*Sigmodon hispidus*) against experimental infections with *Echinococcus multilocularis*; this treatment can, however, induce granulomatous reactions. In an attempt to identify a minimum prophylactic dose of BCG which would not induce granulomas, cotton rats were treated intraperitoneally with various doses of BCG (10^1 to 10^7 colony-forming units [CFU]) and then inoculated intraperitoneally with one brood capsule of the parasite. Consistent and complete protection was obtained by the inoculation of as few as 10^3 CFU of BCG. A dose of 10^1 CFU gave no protection whatsoever, and 10^2 CFU gave only partial protection. Doses larger than 10^3 (10^5 , 10^7 CFU) also afforded complete protection but gave rise to granulomatous lesions. At the time of the inoculation of the parasite, protection coincided with a general elevation of leukocytes, especially cells of the monocyte/macrophage series. It is proposed that these results support evidence for the macrophage being the principal potential effector cell in hydatid disease.

Little is known concerning the dose-response relationship in the antitumor activity initiated by BCG. In most instances, reducing the dose of BCG eventually eliminates its stimulating effects on the immunological response (3, 4), although BCG can be effective at doses as low as 800 colony-forming units (CFU) (9). Rau and Tanner (11) have found that intraperitoneal (i.p.) pretreatment of cotton rats (*Sigmodon hispidus*) with 2.64×10^7 CFU of BCG inhibited the growth and metastasis of an inoculum of 200 protoscolices of *Echinococcus multilocularis*. More recently, it has been shown that an i.p. inoculation of BCG completely inhibited the subsequent development of non-metastasizing *E. granulosus* infection in Mongolian gerbils (13). In spite of the immunoprophylactic effect of BCG in hydatid disease, both of these studies reported the development of numerous small granulomatous lesions in the peritoneal cavity as a consequence of the treatment.

Other workers (12) have also reported the formation of chronic granulomatous lesions both at the site of BCG inoculation and within the regional lymph nodes; this response was, however, considered necessary for the elimination of tumor cells. Nevertheless, it would be desirable to develop a treatment schedule that does not induce the formation of tubercular granulomas in the host, since these bacterial infections might deflect some of the protecting responses of that host away from its primary target. Conse-

quently, this study was initiated to determine the minimum effective prophylactic dose of BCG for experimental *E. multilocularis* infections and to relate any protective effects to changes in the leukocyte population induced by this treatment and/or the parasite infection.

MATERIALS AND METHODS

The parasite. Brood capsules of *E. multilocularis* used in the present study were obtained from stock infections maintained at the Institute of Parasitology by vegetative transfer of the metacestode in cotton rats (1). To minimize biological variation, all replicate infections were induced by the implantation of a cephalic brood capsules (approximately 0.25 cm in diameter) obtained from a single infected cotton rat.

Animals. Eight-week-old, laboratory-reared cotton rats of both sexes (85 to 120 g) were used throughout this study. All animals were housed individually in stainless-steel cages and were provided with commercial rat chow and water ad libitum. To reduce variation further, cotton rats were grouped by body weight to constitute blocks and/or replicates. The animals received a single injection of BCG and were inoculated with the parasite 14 days later. All replicates were inoculated with the parasite at the same time; the experiment lasted for 8 weeks.

BCG. The strain of BCG used in this study was a lyophilized vaccine, specifically prepared for use in cancer immunotherapy by the Institute Armand-Frappier, Montreal. The vaccine contained, according to the manufacturer, 8.9×10^6 CFU/mg (lot no. 1750-4); the range of the viable counts was 8×10^6 to 12×10^6 CFU/mg. The lyophilized vaccine was rehydrated

with phosphate-buffered saline (PBS), pH 7.2, according to the instructions of the manufacturer; suspensions containing 10^1 , 10^2 , 10^3 , 10^5 , and 10^7 CFU/ml were prepared in PBS from this stock.

Determination of the level of stimulation by BCG 2 weeks after treatment. To determine the level of stimulation induced by BCG at the time of the inoculation of the parasite, 1-ml doses of 10^1 , 10^2 , 10^3 , 10^5 , and 10^7 CFU of BCG in PBS were each injected i.p. into four cotton rats; four control cotton rats were injected i.p. with 1 ml of PBS. Fourteen days later, all the cotton rats were exsanguinated by cardiac puncture under ether anesthesia. At autopsy, each animal was weighed, total and differential leukocyte counts were made of the peritoneal cells, the spleen was weighed, and, whenever present, the total weight of granuloma within the peritoneal cavity was obtained.

Determination of the minimum effective prophylactic dose of BCG against *E. multilocularis*. Cotton rats were placed in groups of 10 or 12 individuals according to body weight, as described above. The animals in each group were treated i.p. with the appropriate dose of BCG suspended in a 1-ml volume of PBS, pH 7.2. Two weeks later, half of the animals from each treated and control group were anesthetized with Nembutal (Abbott Laboratories, Montreal), and a brood capsule was surgically implanted into the peritoneal cavity of each animal. All other animals were sham-inoculated, as controls.

Forty-two days postinoculation of the parasite, all animals were exsanguinated by cardiac puncture under ether anesthesia and processed as described above, and the weights of the parasite cysts were obtained.

Harvesting and staining of the peritoneal exudate cells. A small incision was made in the abdominal wall of the exsanguinated animals, and a 10- μ l volume of the exudate was drawn into a Unopette (Becton-Dickinson, Rutherford, N.J.); a total cell count was obtained by using a hemocytometer. An additional 5- μ l sample was used to prepare two cover slip smears for a differential cell count according to Ehrlich's two-cover glass method. The remaining peritoneal cells were collected using a modification of the method of Evans and Alexander (5). Briefly, 2 volumes of 5 ml of tissue culture medium 199 (Difco) were injected into the peritoneal cavity of the animal, the peritoneal cavity was massaged, and the cells were aspirated and counted.

The cover slips were stained with both Wright and Giemsa stains. Differential leukocyte counts were obtained by counting 1,000 leukocytes on each stained cover slip. The data were analyzed by the analysis of variance and Duncan's test for multiple ranges.

RESULTS

The results of the total and differential leukocyte counts of the peritoneal exudate of animals that had been treated 2 weeks previously with 10^1 , 10^2 , 10^3 , 10^5 , or 10^7 CFU of BCG or PBS are summarized in Fig. 1.

Only those cotton rats which had been treated 2 weeks previously with an i.p. injection of 10^3 or 10^7 CFU exhibited total leukocyte counts that

FIG. 1. Levels of stimulation of peritoneal leukocytes of cotton rats 2 weeks after inoculation with various doses of BCG or PBS. Vertical bars depict the mean $\pm$ 95% confidence intervals.

were significantly higher ($P \leq 0.05$) than those of control animals treated with PBS. Total leukocyte counts were not significantly elevated in animals that had been treated with 10^1 , 10^2 , or 10^5 CFU. The increase in the number of leukocytes was due principally to a significant elevation in the population of monocytes; neither the polymorphonuclear cells nor the lymphocytes were significantly increased at any of the doses studied. Macroscopic BCG granulomas were found in those animals that had been treated with 10^5 and 10^7 CFU of the vaccine.

The total and the differential peritoneal cell responses of cotton rats treated with BCG, and subsequently inoculated with one brood capsule of the parasite, are illustrated in Fig. 2. The results presented here are of two separate experiments: in the first experiment, cotton rats were

FIG. 2. Levels of stimulation of peritoneal leukocytes of cotton rats inoculated with BCG and *E. multilocularis*. Hatched bars represent animals treated with BCG or PBS and subsequently inoculated with the parasite. Open bars represent sham-inoculated controls previously treated with BCG or PBS. Vertical bars depict the mean $\pm$ 99% confidence intervals. POLY, Polymorphonuclear leukocytes; LYMPH, lymphocytes; MONO, monocytes/macrophages; TOTAL, total leukocytes.

treated with 10^1 , 10^2 , and 10^3 CFU of BCG; in the second experiment, doses of 10^3 , 10^5 , and 10^7 CFU were used (appropriate PBS controls were done in both cases). Since the total leukocyte counts of the peritoneal exudates of animals treated with PBS or 10^3 CFU of BCG in both experiments were not significantly different ($P \leq 0.01$), the results of the two experiments were pooled and are presented together in Fig. 2.

The number of total leukocytes tended to increase with an increase in the dose of BCG, up to 10^3 CFU; at higher doses, a decline in total leukocytes to normal, or nearly normal, values was evident. The concomitant parasite infection enhanced the total peritoneal leukocyte num-

bers in cotton rats treated with 10^2 and 10^3 CFU. Cotton rats treated with 10^3 CFU and subsequently inoculated with the parasite exhibited the greatest elevation in leukocytes of all the groups; this elevation was reflected in a significant increase in the total numbers of monocytes and lymphocytes. None of the other doses produced significant increases in the total or differential cell counts.

Complete protection of cotton rats from experimental *E. multilocularis* infections was achieved with a prophylactic dose of BCG as small as 10^3 CFU, without the induction of any apparent granulomatous reaction. Two of the five animals treated with 10^2 CFU also did not

develop a hydatid infection. Protection against *E. multilocularis* was also achieved at the higher doses of 10^5 and 10^7 CFU, but this was accompanied by formation of distinct granulomas. Animals treated with 10^1 CFU were not protected at all against the parasite, since *E. multilocularis* infections in these cotton rats reached sizes statistically indistinguishable from those in the controls (Table 1).

There was no splenomegaly (Fig. 3) in any of the treatment groups. There was, however, a tendency for those animals treated with 10^5 and 10^7 CFU of BCG to gain less body weight from the time they were treated to necropsy than those animals treated with lower doses of BCG or PBS (Fig. 3).

TABLE 1. Weight of *E. multilocularis* cysts in control (saline-treated) cotton rats and in animals treated i.p. with 10^1 and 10^2 CFU of BCG

Treatment	Mean cyst wt (g)	± Standard error
Saline	0.85	0.38
10^1 BCG	0.60	0.27
10^2 BCG	0.27	0.26

DISCUSSION

The purpose of this study was to determine the minimum dose of BCG which can protect cotton rats from experimental infections of *E. multilocularis*, without the induction of granulomatous lesions. Animals could not be protected against the parasite by treatment with 10^1 CFU, but partial protection was achieved with 10^2 CFU. Complete protection against hydatid infection was achieved with BCG doses of 10^3 CFU and greater, but protection with 10^5 and 10^7 CFU was accompanied by the formation of BCG granulomas.

A previous report (10) suggests that the environment into which the parasite is inoculated may determine the success or failure of *E. multilocularis* to establish infection. The assessment of the cellular response of animals 2 weeks after treatment with BCG showed that the significant elevation in total leukocytes after treatment with 10^3 CFU was, not unexpectedly, almost exclusively attributable to a significant increase in the numbers of peritoneal monocytes. Furthermore, this dose not only elicited the highest monocyte response, but also completely suppressed the growth of the parasite without

FIG. 3. Hatched bars represent the spleen and total body weights at necropsy of cotton rats treated with BCG or PBS and subsequently inoculated with *E. multilocularis*. Open bars represent sham-inoculated controls previously treated with BCG or PBS. Vertical bars depict the mean weight $\pm$ 95% confidence intervals. *, Animals with granulomas of ≤ 0.1 g; **, animals with granulomas of ≥ 0.1 g.

the induction of granulomas. This observation supports the hypothesis of Baron and Tanner (2) that monocytes are the primary effector cells in this host-parasite system.

The level of cell proliferation 2 weeks after treatment with 10^5 and 10^7 CFU of BCG was, apparently, somewhat lower when compared with the response obtained with 10^3 CFU. This result is consistent with the delayed hypersensitivity reaction induced by BCG which develops, peaks, and diminishes more rapidly with higher than with lower doses (6, 7). Thus, the cellular responses of animals inoculated with 10^5 and 10^7 CFU may have been in the process of returning to normal values, whereas the response of animals treated with 10^3 CFU may have represented a maximum stimulation at 2 weeks.

A dose of 10^4 CFU of BCG was determined to be optimal for stimulating cell proliferation and is also an effective dose for suppressing the growth of the parasite. Doses of 10^1 and 10^2 CFU produced an inadequate level of stimulation of the leukocyte population. At doses of 10^5 and 10^7 CFU, however, the bacilli provoked a host response which ultimately resulted in the formation of granulomas; these granulomas were first observed 2 weeks after treatment and were considerably larger after 8 weeks.

Forty-two days after inoculation of the parasite, leukocyte levels in animals treated with 10^5 and 10^7 CFU of BCG were lower than after treatment with 10^3 CFU. Since tubercular granulomas have been shown to deplete circulating lymphocytes and monocytes (8), the lower levels of leukocytes in animals given 10^5 and 10^7 CFU may have been due to a sequestration of cells for the formation of the granulomas. Furthermore, the reactions to the tubercle bacilli could, conceivably, compete for cells with the hydatid cyst; such reactions surrounding the bacteria would inhibit a maximum antiparasite effect by the cellular response.

It is well documented (8) that an excessive number of BCG can have a disorganizing effect on lymphoid tissues, such as the spleen, so as to interfere with its immunological capabilities. Splenomegaly has been demonstrated in mice treated with BCG and inoculated with *E. granulosus* (13). Although we were unable to detect any splenomegaly, there was less gain in body weight in animals treated with 10^5 and 10^7 CFU (Fig. 3), indicating that high doses of the vaccine may be detrimental to the host.

The results of these experiments indicate that

relatively low prophylactic doses of BCG are effective in controlling experimental *E. multilocularis* infections without the concomitant formation of macroscopic granulomatous lesions.

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