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Suppression of Babesiosis in BCG-Infected Mice and Its Correlation with Tumor Inhibition

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Infection of mice with Bacillus Calmette-Guérin (BCG) provided good protection against *Babesia* species. The intensity and duration of this protection was similar to that established after natural recovery from babesiosis. It developed too soon after the first exposure to the parasite, and was too radioresistant, to be attributable to specific antibody production. In addition, the parasites degenerated within circulating erythrocytes. This phenomenon is inconsistent with phagocytosis or lysis of parasites or parasitized cells, or prevention of entry of parasites into erythrocytes, causing the observed protection. Hence the phenomenon is best explained by the release of a nonspecific mediator that can limit multiplication of parasites within erythrocytes. These results not only throw light on mechanisms of immunity against hemoprotozoa. There are many points of similarity between the nonspecific protection BCG and *Corynebacterium parvum* provide against *Babesia* species and inhibition of tumor growth by these agents. Therefore, babesiosis in mice may be a convenient experimental model for assessing stimulation of the mononuclear phagocyte system, which appears to be the basis of nonspecific immunity against bacteria, parasites, and tumors.

Infections with *Babesia* species greatly increase cattle production cost in certain areas of the world, particularly the wet tropics. Interest in babesiosis in humans has also increased recently (8, 10). Mice can readily be infected with certain species of both *Babesia* and *Plasmodium*, with considerable cross-protection (7), implying that both these parasites arouse the same defense system. Thus basic information on immunity to babesiosis may also be applicable to malaria.

We have found (4) that infection with *Mycobacterium bovis* strain Bacillus Calmette-Guérin (BCG) in appropriate schedules totally protects mice from infection with *Babesia microti* and *B. rodhaini*, and from the high parasitemia and death that *Plasmodium vinckei* causes. This paper expands our earlier report, extending the argument that neither an increased specific immune response nor phagocytosis, but a nonspecific soluble effector substance, is the best explanation for this protection. This may have important implications for our understanding of the protective effect of BCG and *Corynebacterium parvum* against other infectious agents and tumors, as well as immunity to babesiosis itself.

MATERIALS AND METHODS

Experimental animals. All mice were of CBA/Ca strain, bred in specific pathogen-free conditions at the Clinical Research Centre, Harrow, from Carshalton stock. Most were 5 to 6 weeks old when the experiments were initiated, but sex and age did not appear to affect the outcome. On several occasions a number of mice from the stock were splenectomized to confirm that they were free of *Eperythrozoon* species.

Protozoa. *B. microti* (King strain) and *B. rodhaini* (Antwerp) were stored in liquid nitrogen as a 50% dilution of parasitized blood in 4 M dimethylsulfoxide and passaged once before use. Blood from mice with a rising parasitemia was used to initiate experimental infections. Almost all injections were given intraperitoneally, but intravenous inoculation made no difference to the rate of onset or severity of the parasitemia or to protection by BCG. The course of infection was monitored daily or every second day by examining Giemsa-stained thin blood smears. Parasitemias were expressed as the percentage of erythrocytes infected.

BCG. BCG was obtained directly from Glaxo Ltd., Greenford, Middlesex, England, and, except where stated, had not been freeze-dried. Portions (2 ml), each containing approximately 2×10^8 viable units, were stored at −70°C. It was established that 200 μl of the suspending medium, given intravenously, would not protect mice against *B. microti*. Freeze-dried BCG was reconstituted with distilled

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water to 5 × 10^7 viable units per ml just before use. Unless otherwise specified, BCG was given intravenously.

Thymectomy and splenectomy. Both thymus lobes were removed by aspiration with a water-jet vacuum pump via a midline anterior mediastinal approach. Complete thymectomy was eventually confirmed at autopsy. Splenectomy was performed through a dorsoventral incision high on the left flank, the blood vessels being sealed by electrocautery. Sham operations of both procedures were performed on control mice. In all instances animals were anesthetized with ether and the incisions were closed with a single Michel clip.

Electron microscopy. Several drops of tail blood were fixed in combined glutaraldehyde-osmium tetroxide, postfixed in uranyl acetate, and embedded in agar essentially as described by Hirsch and Fedorko (11). Cut agar blocks were dehydrated in a graded series of ethanol solutions and embedded in Epikote 812 (14). Thin sections were cut on a Sorvall MT-2 ultramicrotome, stained with alcoholic uranyl acetate (34) followed by lead citrate (20), and examined in an AEI EM 6B electron microscope. At least 100 parasites were counted in each mouse when estimating the incidence of abnormal forms.

Irradiation. Mice were irradiated by placing them 1 m from a ^{60}Co source installed in an irradiation unit (Pantatron Ltd.), which gave a dose rate of 0.71 rad/s, as calibrated with a chemical dose meter by the National Physical Laboratories.

RESULTS

Time interval between BCG and *B. microti* infections. Six groups of four to six mice were given 10^6 *B. microti* at times ranging from 5 to 180 days after an injection of 2 × 10^7 BCG. A seventh group was given BCG 5 days after they had received 10^6 *B. microti*, 3 days before the parasites were detectable on thin smears. There was no protection when BCG was given after the parasite, even though the infection was not yet patent. The longer the interval between BCG and subsequent infection, the more effectively the parasite was suppressed (Table 1).

TABLE 1. *Effect of increasing the interval between 2 × 10^7 BCG and subsequent infection with 10^6 B. microti on the intensity of protection obtained*

Time between BCG and *B. microti* (days)	Peak parasitemia (%)	Duration of >1% parasitemia (days)
−5	47	9
5	8	4
10	5	3
14	0.5	nil
28	nil	nil
90	nil	nil
180	nil	nil
Controls (no BCG)	48	9

Dose and route of injection of BCG. We investigated the effects of reducing the dose and varying the route of injection of BCG, leaving the time interval before challenge constant. Nine groups of four mice were given 10^5, 10^6, or 10^7 BCG either subcutaneously (s.c.), intraperitoneally (i.p.), or intravenously (i.v.) and challenged with 10^6 *B. microti* 1 month later (Fig. 1). One hundred times the dose had to be given s.c. to achieve the degree of protection produced by 10^5 BCG given i.v. Doses of 10^6 and above,

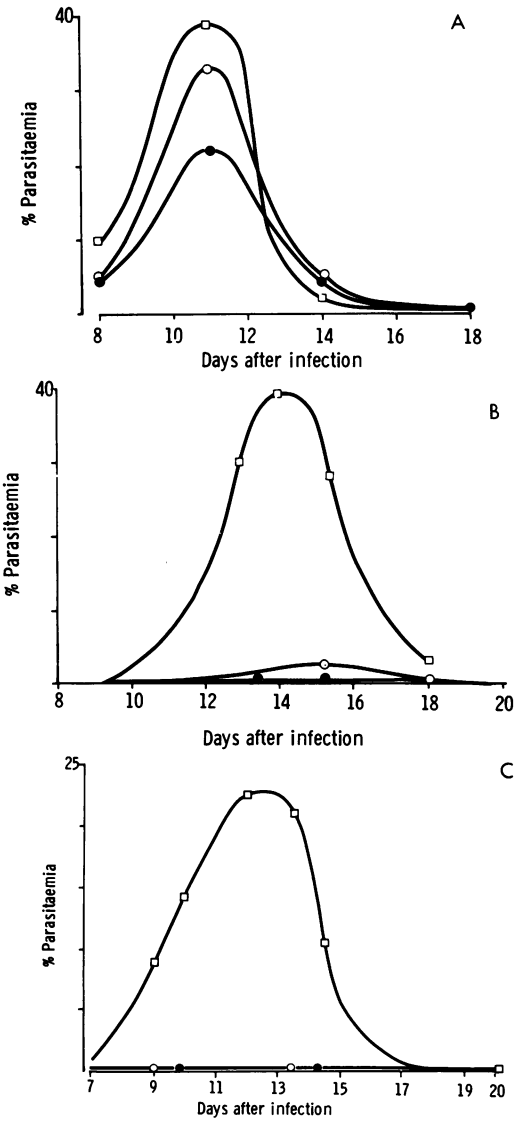


FIG. 1. *Effect of dose and route variations in injection of BCG on subsequent infection with B. microti. (A) 10^5 BCG; (B) 10^6 BCG; (C) 10^7 BCG.* (□) *subcutaneous;* (○) *intraperitoneal;* (●) *intravenous.*

given i.p. or i.v., but not s.c., protected very well—indeed, after 10^6 i.v. or 10^7 i.p. or i.v., the protection was absolute.

Reconstituted freeze-dried BCG. It was possible that the freeze-drying and reconstitution to which commercial BCG is subjected might alter its ability to protect mice against babesiosis. This was not the case: when 10^5, 10^6, or 10^7 reconstituted BCG were given to three groups of six mice, the results on challenge were the same as when the challenge followed BCG that had never been freeze-dried.

Duration of protection. Various groups of four to six mice, which had been protected against *B. microti* or *B. rodhaini* by prior infection with BCG, were kept for long periods and rechallenged. The timing protocols are set out in Fig. 2. In all cases, 2×10^7 BCG had been given, and each challenge was with 10^6 parasites. Parasitemias were monitored every 2nd day while parasitemias of controls were above 1% and every 4th day for the rest of the experiment. No greater than trace levels of parasites were ever seen in mice that had received BCG, so we are sure that the continued protection was not due to an undetected recrudescence providing natural immunization. Once protected against babesiosis with BCG no mouse has yet reverted to susceptibility for up to 10 months after the BCG injection.

Intra-erythrocytic death of parasites. Since *B. microti* die inside circulating erythrocytes when a mouse overcomes the infection naturally (5; I. A. Clark, J. E. Richmond, E. J. Wills, and A. C. Allison, manuscript submitted), it was important to ascertain whether this also occurs in BCG-protected mice. Thus, to produce a parasitemia high enough for the organisms to be examined from the time of injection, 10^9 *B. microti* were given to groups of nine mice either infected with 2×10^7 BCG 100 days earlier, recently recovered from *B. microti*, or previously unexposed to this parasite. Two mice from each group were killed after 12 h, and another two, after 24 h. Phagocytosis of either parasites or parasitized erythrocytes was not seen on impression smears of spleens or livers.

Parasitemias of the remaining animals were followed for a week. The BCG-treated mice disposed of their parasites as rapidly as did the recovered animals (Fig. 3). Furthermore, erythrocytes on smears from both these resistant groups showed the inclusions that we have demonstrated by electron microscopy studies on recovering mice (5; Clark et al., manuscript submitted) to be degenerate intra-erythrocytic parasites (Fig. 4).

To study this aspect further, samples of blood from five mice given 2×10^7 BCG 34 days earlier, three mice fully recovered from a primary infection with the parasite, and two control mice, all given 10^9 *B. microti*, were examined by electron microscopy. Normal babesias (Fig. 5A) showed the fine structure described by others (1, 22). Parasites had begun to degenerate by 12 h in BCG-protected animals, and the number of abnormal organisms was increased after a further 12 h (Table 2). Large numbers of condensed intra-erythrocytic organisms (Fig.

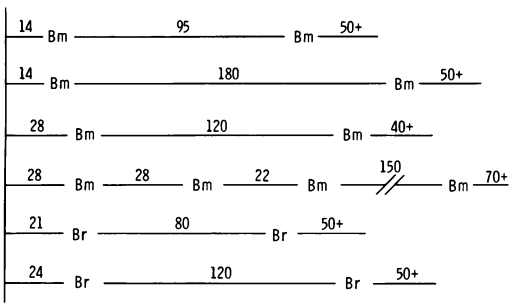


FIG. 2. *Duration of the protection of mice from babesiosis by prior infection with 2×10^7 BCG. BCG is given at left hand side of diagram; B.m., 10^6 B. microti given; and B.r., 10^6 B. rodhaini given. The figures are time intervals in days.*

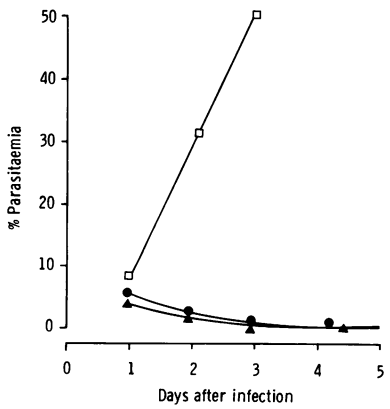
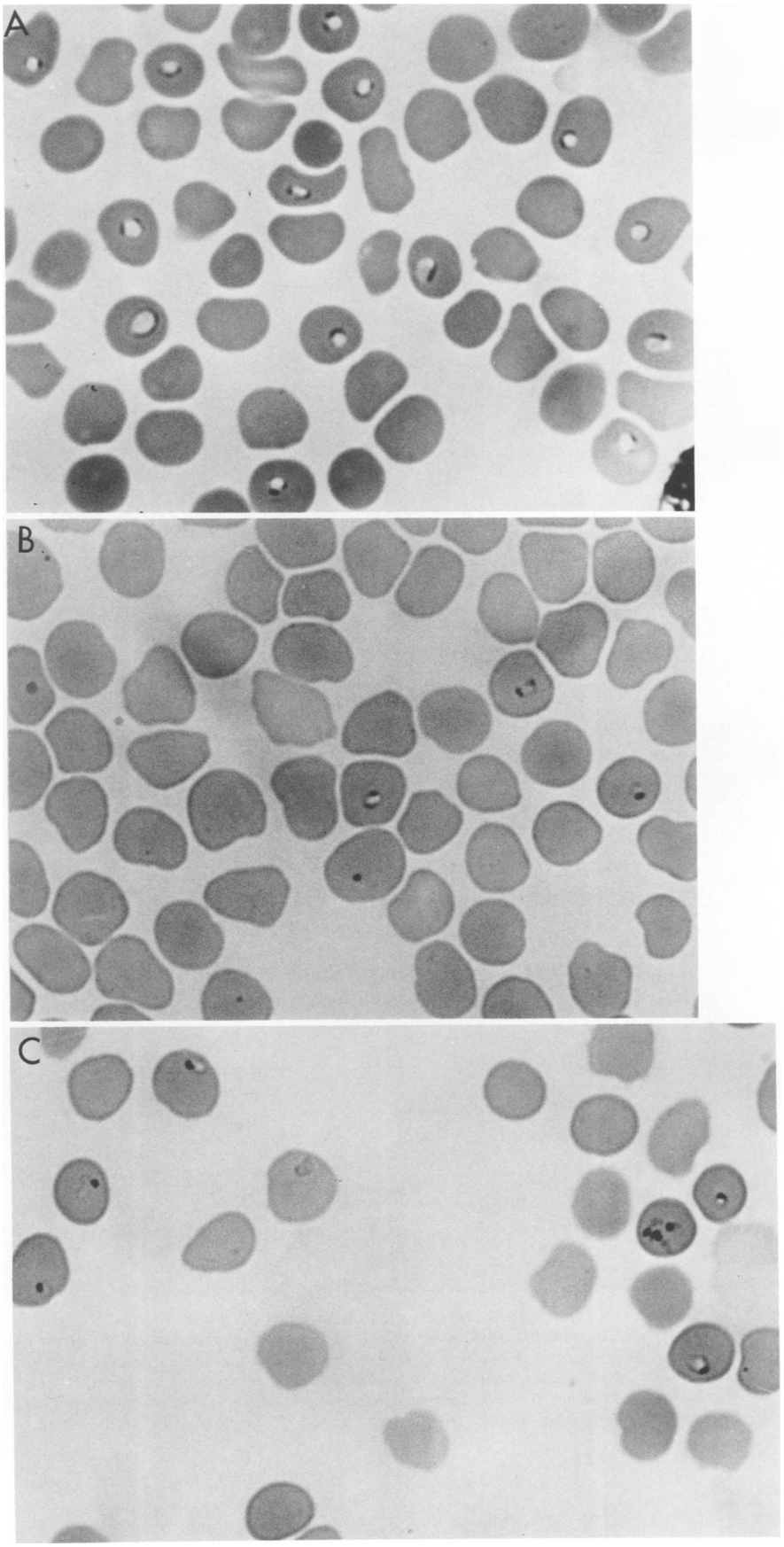


FIG. 3. *Strength of protection conferred by BCG. B. microti (10^9) given to mice infected with 2×10^7 BCG 100 days earlier (●); mice recently recovered from infection with B. microti (▲); and controls (□).*

FIG. 4. *Appearance of B. microti 24 h after 10^9 organisms had been given to (A) previously unexposed mice, (B) mice given 2×10^7 BCG 100 days earlier, and (C) mice recently recovered from infection with B. microti. ×1,000.*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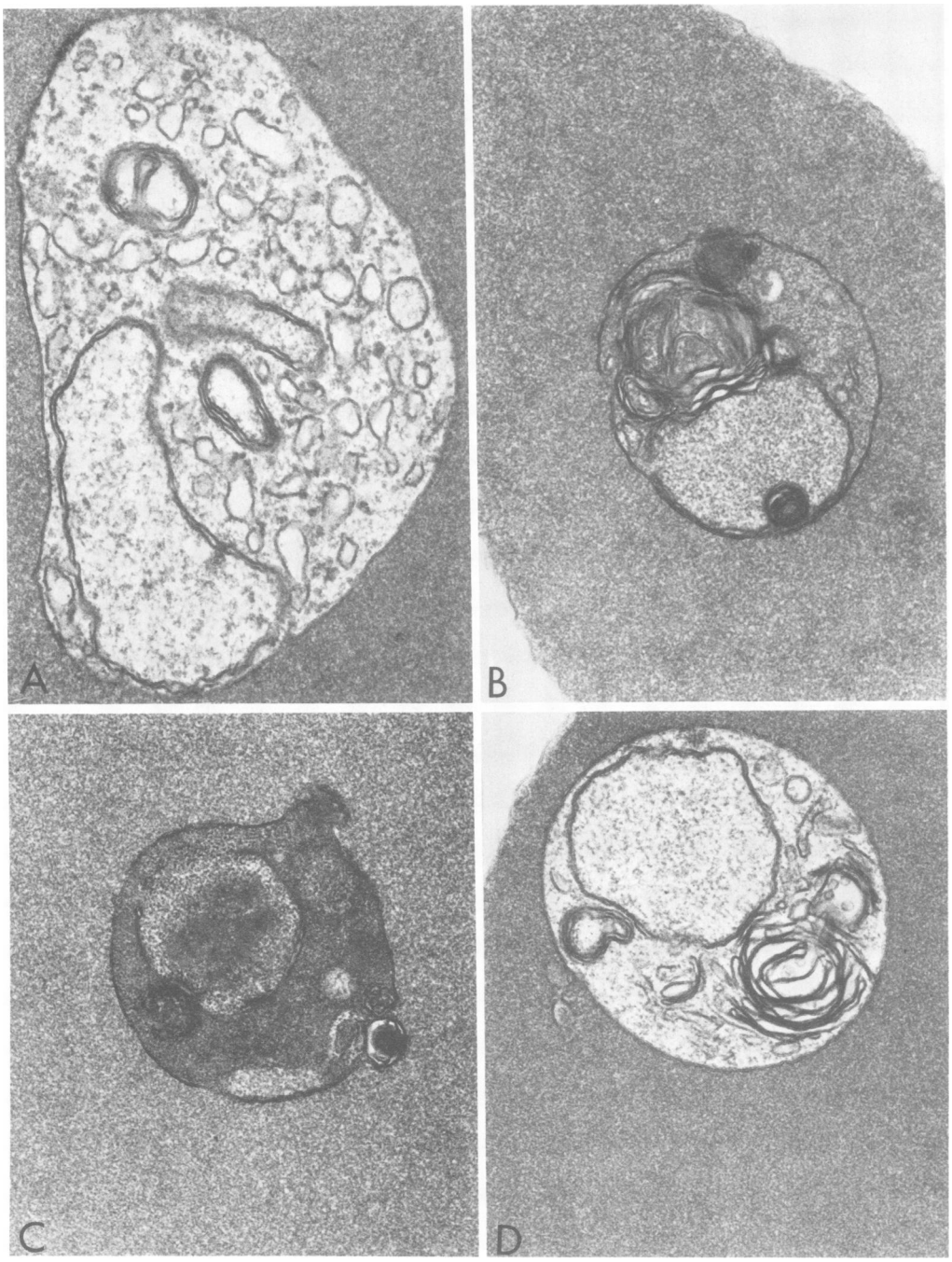

FIG. 5. *Intra-erythrocytic B. microti 24 h after injection into BCG-treated mice. Electron micrographs ×45,000. (A) Normal trophozoite. (B) B. microti with myelin degeneration of cytoplasmic membranes, loss of cytoplasmic organelles, and increased density of nucleoplasm and cytoplasmic matrix. (C) Condensed B. microti. The cytoplasmic organelles are no longer recognizable and there is partial dissolution of the nuclear envelope. (D) Degenerating form that has developed large numbers of cytoplasmic membranes.*

5B, C), identical to those found when mice recover naturally from a primary infection (5; I. A. Clark et al., manuscript submitted), were observed (Table 2). In addition, many babesiae developed multiple cytoplasmic membranes without undergoing condensation of the nucleus or cytoplasmic matrix (Fig. 5D; Table 2).

There were also considerably more condensed organisms in animals receiving *B. microti* after complete recovery from an initial infection than in controls given the same large dose of parasite (Table 2).

Irradiation or cyclophosphamide treatment of BCG-infected mice. In two experiments four mice that had received 2×10^7 BCG 30 days earlier and four untreated mice were irradiated with 650 or 900 rad. On the following day these mice, together with four that had received BCG only and four controls, were given 10^9 *B. microti*. Neither dose of irradiation (Fig. 6) nor an injection i.p. with cyclophosphamide at a dose of 300 mg/kg (Fig. 7) made BCG-infected mice susceptible to babesiosis (Fig. 7). If, on the other hand, a dose of 650 rad was given 2 days after the BCG, the protection against 10^8 *B. microti* injected 12 days later was abrogated (Fig. 8). There were five mice in each group in this experiment. The route of injection of BCG and the short interval before giving this number of parasites account for the relatively high parasitemia in the protected group.

Sensitivity to hydrocortisone. Two groups of four mice received 2×10^7 BCG, and 85 days later these animals and controls were given 10^6 *B. microti*. The following day and every 4th day for the duration of the experiment the mice in one of the BCG-infected groups were injected i.p. with hydrocortisone acetate at a dose of 100 mg/kg. This resulted in recrudescence of the parasite (Fig. 9).

Thymus independence of protection. To ascertain whether T cells are necessary for BCG to protect mice from *B. microti*, seven congenitally athymic "nude" mice, four mice thymectomized as adults (ATx) 4 months previously, and four intact mice were given 2×10^7 BCG, and 14 days later the mice were challenged with 10^6 *B. microti*. All groups were protected compared with controls that had not received BCG, though the protection was not as good in the nude mice as in the intact or ATx mice (Fig. 10), indicating a requirement for long-lived T cells.

Effect of splenectomy. Twelve mice, four of

TABLE 2. *Electron microscopy-determined morphology of intra-erythrocytic B. microti in BCG-treated, recovered, and control mice*

Mice	Time (h)	Normal babesias (%)	Condensed babesias (%)	Babesias with excess membranes (%)
BCG mice + 10^9 *B. microti*	12	84	7	9
	24	64	26	13
Recovered mice + 10^9 *B. microti*	12	90	10	0
	24	80	19	1
Control mice + 10^9 *B. microti*	24	96	3	1

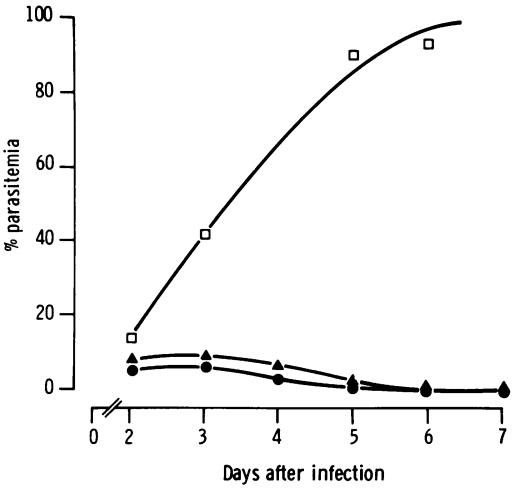

FIG. 6. *Inability of 540 or 900 rad to remove inhibition of 10^9 B. microti in BCG-infected mice. (●) Mice given 2×10^7 BCG 4 weeks earlier and 900 or 650 rads on day −1. (▲) BCG alone; (□) controls.*

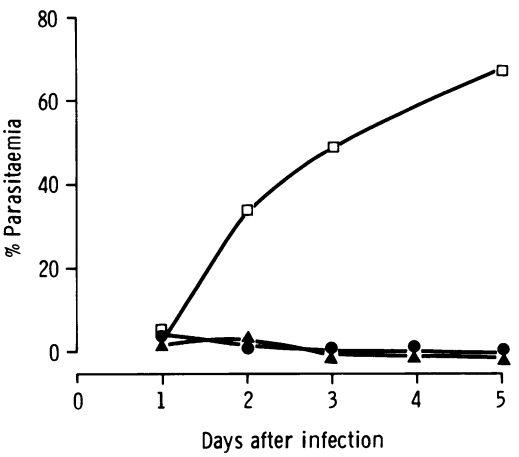

FIG. 7. *Inability of cyclophosphamide (CY) to remove inhibition of 10^9 B. microti in BCG-infected mice. (●) Mice given 2×10^7 BCG 6 weeks earlier, and CY (300 mg/kg) on day −1. (▲) Same BCG, but no CY; (□) controls.*

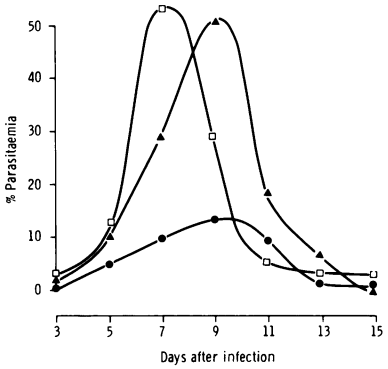

FIG. 8. *Removal of inhibition of B. microti in BCG-infected mice by 650 rad 2 days after 2 × 10^7 BCG i.p. B. microti (10^8) given 12 days after the irradiation.* (▲) *BCG and irradiation;* (●) *BCG alone;* (□) *controls.*

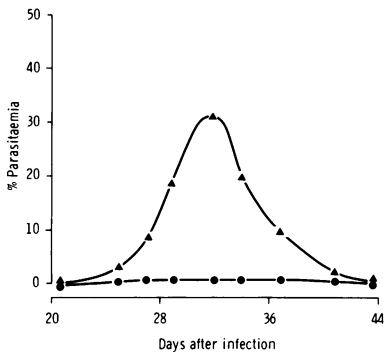

FIG. 9. *Removal of inhibition of B. microti by hydrocortisone acetate.* (▲) *Mice given 10^6 B. microti 85 days after 2 × 10^7 BCG and then 100 mg of hydrocortisone acetate per kg every 4th day.* (●) *Same BCG and babesia but no hydrocortisone acetate.*

which had been splenectomized 28 days earlier, were given 2 × 10^7 BCG. Fourteen days later all 12 and 4 controls were given 10^6 *B. microti*. Four of the eight intact BCG-infected mice were splenectomized 9 days after infection with *B. microti*. Splenectomy before BCG did not remove subsequent protection, but splenectomy after protection had been achieved caused a temporary recrudescence of the parasites (Fig. 11), as it does in mice protected with *C. parvum* (unpublished data) and in animals previously recovered from babesiosis (21). The time of onset of recrudescence in this group spanned 8 days, but, in order to demonstrate the mean peak recrudescence, all four were regarded as having risen synchronously with the first infection to become patent.

DISCUSSION

Prior infection of mice with BCG confers protection against subsequent babesiosis apparently as strong (Fig. 3) and durable (Fig. 2) as that after natural recovery. Resistance of BCG-infected animals to a variety of infectious agents has often been attributed to enhanced specific immune responses or increased phagocytic capacity, but our results indicate that neither plays a major role against *Babesia* species.

Clearly, this protection cannot be due to the adjuvant effect (9) of BCG on antibody production, since many *B. microti* die within 24 h of injection into BCG-infected mice (Table 2), too soon for an appreciable specific response to have been mounted (33). This occurs even when

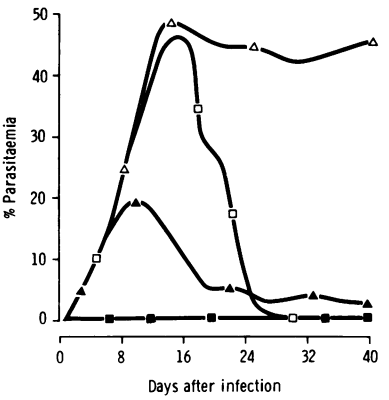

FIG. 10. *Protection of T cell-deprived mice from B. microti with BCG. B. microti (10^6) were given to nude mice, ATx mice, and intact controls. All were given either 2 × 10^7 BCG or saline 14 days earlier. Nude mice with* (▲) *and without* (△) *BCG; intact or ATx mice with* (■) *and without* (□) *BCG.*

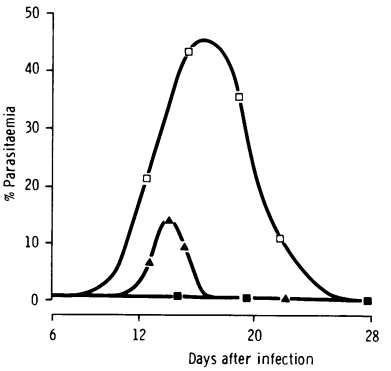

FIG. 11. *Temporary loss of protection in mice splenectomized after BCG and B. microti were given, but protection maintained in mice splenectomized earlier.* (■) *Intact mice and mice splenectomized on day −42 given 2 × 10^7 BCG on day −14 and 10^6 B. microti on day zero.* (▲) *Mice given BCG on day −14 and B. microti on day zero and splenectomized on day 9.* (□) *Mice given B. microti only.*

a dose of 650 or 900 rad, which completely inhibits antibody production (32), has been given 24 h before infection with the parasite (Fig. 6). In contrast, the removal of protection by 650 rad given 2 days after the BCG and 12 days before the parasite (Fig. 8) clearly indicates that cell proliferation with no specificity for *Babesia* species is essential. In addition, BCG given during the prepatent period, when it would have had ample time to exert adjuvanticity, does not protect (Table 1). Moreover, negligible amounts of antibody specific for *B. microti* surface antigens, as detected by an indirect fluorescence test, are present in the serum of mice protected against this parasite by either BCG (4) or *C. parvum* (I. A. Clark, F. E. G. Cox, and A. C. Allison, Parasitology, in press). Our inability to detect cross-reacting antibodies agrees with the findings of workers who have investigated the protective effect of BCG against toxoplasmic retinochoroiditis (31) and of BCG (30) and *C. parvum* (6) against salmonellosis.

Although injection with BCG increases the rate of carbon clearance in mice (2), increased phagocytic capacity is an equally unlikely explanation for the protection observed. Parasites were not detected in monocytes or macrophages, but dead or dying forms were present in circulating erythrocytes in sufficient numbers (Table 2) to account for the observed decrease in parasitemia. In addition, the BCG-induced increase in carbon clearance of mice reaches its maximum 2 weeks after i.v. injection of the bacteria and then declines (12), whereas protection against a first challenge with *Babesia* species is fully maintained for up to 6 months after BCG has been given (Table 1) and for at least 10 months with repeated challenges (Fig. 2). These results are consistent with the lack of correlation between the ability of BCG and other agents to protect mice against Mengo virus and to increase their phagocytic capacity (18).

Since babesias are evidently dying outside macrophages, without the mediation of specific antibodies, one is forced to the conclusion that a nonspecific mediator is involved. It is known that such mediators, elicited from mice infected with BCG (25), can suppress the growth of not only bacteria, a yeast, and bone marrow cells (23) but also a mouse sarcoma (26). We have found that suppression of *B. microti* is much greater when BCG is given i.v. than s.c. (Fig. 1). Hence, the observation (24) that i.v. BCG leads to release of these mediators much more efficiently than when it is given s.c. supports the view that we may be observing another manifestation of the same phenomenon.

Several workers have suggested that BCG and *C. parvum* may suppress tumor growth by the same mechanism (15, 27). Comparison of protection against *Babesia* species induced by BCG and *C. parvum* (Clark et al., Parasitology, in press) and inhibition of certain tumors by these agents reveals some striking similarities: i.v. injection protects more efficiently in vivo (19; Fig. 1), and also endows macrophages with much greater in vitro cytostatic activity against tumors, than does s.c. injection (13); splenectomy after injection, but not beforehand, temporarily interferes with protection (15; Fig. 11); mice deprived of T lymphocytes are protected (28, 35; Fig. 10); and protection is resistant to radiation (16; Fig. 6) and cyclophosphamide (28; Fig. 7) but sensitive to cortisone (29; Fig. 9). Finally, there is now evidence that restriction of tumor growth by BCG may be mediated by soluble factors (3, 17, 26). The intra-erythrocytic death of *B. microti* leads us to the same conclusion for these parasites.

The results presented in this paper indicate that babesias in mice are particularly susceptible to nonspecific immunity, which BCG can induce against a range of infectious agents and tumors. This observation, as well as the large size of the parasite and its location inside erythrocytes, allowing rapid sampling and assessment, gives this model particular advantages for further elucidation of the way in which the widespread protective mechanism induced by BCG exerts its effects.

Moreover, BCG-induced protection against *Babesia* species and specific immunity to this parasite in mice may have the same final mediator, since the protection in each case is equally intense and durable, is effective against the same range of blood protozoa, and leads to intra-erythrocytic death of the parasite with very similar ultrastructural changes.

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