Resistance to Babesia spp. and Plasmodium sp. in Mice Pretreated with an Extract of Coxiella burnetii

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Mice injected intravenously with a commercially available extract of Coxiella burnetii prepared for use as the antigen in the complement fixation diagnostic test for Q fever were subsequently resistant to infection with Babesia microti, Babesia rodhaini, and Plasmodium vinckei petteri. The parasites appeared to die inside circulating erythrocytes. Protection was unaffected by exposing the pretreated mice to 900 rads on the day before they were infected. To explain these findings, it is postulated that pretreatment with Coxiella extract protects by potentiating the interferon-inducing capacity of the challenge dose of protozoa, which perhaps leads to enhanced activity of natural killer cells. Tumor necrosis factor also warrants investigation.

We have previously presented evidence that BCG (5) and killed Corynebacterium parvum (6) protect mice from subsequent infection with Babesia spp. and certain Plasmodium spp. Brucella abortus S19 also protects mice against both Babesia microti (19) and Plasmodium vinckei (J. Harvey, A. Kaplun, and I. A. Clark, unpublished data). As well as causing splenomegaly and inducing granulomatous reactions, these agents suppress certain experimental tumors (17, 33, 54) and protect against apparently unrelated bacteria (2, 11, 34). Arguments have been presented that the protection which these agents afford against hemoprotozoa is not due to shared antigens, enhanced production of specific antibody, or phagocytosis, but has its basis in the nonspecific immunity expressed during infection with these bacteria (6, 8).

Because Coxiella burnetii also shares these characteristics (26, 27, 32, 43), I decided to test whether the similarity extended to protection against blood parasites as well. The results demonstrated that a commercially available extract of C. burnetii protects mice against B. microti, Babesia rodhaini, and P. vinckei petteri.

MATERIALS AND METHODS

Animals. Female CBA/Ca H mice, 6 to 8 weeks old, were used in these experiments. Mice from the same stock had recently been found, after splenectomy, to be free of Eperythrozoon sp.

Protozoa. B. microti (King's strain), B. rodhaini (Antwerp), and P. vinckei petteri (from D. Walliker) were stored at −70°C and maintained by serial passage before use in these experiments. The course of infection was monitored regularly by Giemsa-stained thin blood smears, and the degree of infection was expressed as the percentage of erythrocytes infected.

Coxiella extract. A commercially available extract of C. burnetii (phase 1; Nine-Mile strain), obtained from Commonwealth Serum Laboratories, Melbourne, Australia, was used in these studies. It had been prepared by the method described by Stoker (51) for use as the antigen in the complement fixation diagnostic test for Q fever in humans. The batch employed (batch 081-1) contained 350 μg more protein and 38.3 μg more carbohydrate per ml than did control antigen extracted commercially by the same procedure from uninfected eggs (T. Higgins, unpublished data). In all cases, the extract was injected intravenously, and the parasites were given intraperitoneally as parasitized erythrocytes suspended in saline.

Endotoxin. Escherichia coli serotype O128:B12 lipopolysaccharide B (trichloroacetic acid extracted; Difco Laboratories, Detroit, Mich.) was used in these studies. It was stored in saline at −20°C at a concentration of 1 mg/ml and subsequently diluted so that the required dose was contained in 200 μl.

RESULTS

B. microti. (i) Protective effect of different doses of Coxiella extract. In the initial experiment five mice were injected with 200 μl of Coxiella extract, and another five were injected with the same volume of control extract. Both of these groups and a third which was previously untreated were infected 3 weeks later with 106 B. microti. Protection was absolute in the mice given Coxiella extract, with no parasites seen in their smears then or during the 3-week period after a second dose of 106 B. microti was given 26 weeks later. The other two groups underwent a usual primary infection (Fig. 1 and
2, control groups). Neither extract visibly affected the mice, although on histology the group injected with Coxiella extract showed granulomas which were the same as those previously reported during Q fever (43). These lesions were particularly evident in the liver and splenic red pulp.

To establish the minimum effective dose of Coxiella extract, a range of doses between 5 and 100 μl was given to additional groups of five mice each. After 3 weeks these also received 10⁶ B. microti. As Fig. 1 shows, protection was appreciable even in the mice given 5 μl, and it was greater with higher doses. Very few parasites were detected in smears from those given 20 μl of extract, and only a few isolated sightings were made in smears from mice given 50 or 100 μl. No parasites were seen after the 50-μl group was again challenged after 24 weeks.

(ii) Time interval between Coxiella extract and B. microti. Groups of five mice each were infected with 10⁶ B. microti at various times after they had received 50 μl of Coxiella extract. Figure 2 shows the results of the shorter-term experiments, when the interval was 0, 3, or 7 days. When either 8 or 16 weeks had elapsed before the mice were infected, no parasites were subsequently detected. Thus, the longer the interval between injection of Coxiella extract and subsequent infection, the more effectively the parasite was suppressed. Also, when the extract was given 7 days after the infection had been initiated but was not yet patent, it did not diminish the ensuing parasitemia (Fig. 2).

(iii) Intra-erythrocytic death of parasites. It was established that mice pretreated with 200 μl of Coxiella extract were protected against doses of B. microti as high as 10⁹ (Fig. 3). Inocula of this size ensure that parasites are immediately apparent in the blood in sufficient numbers for their structure and fate to be studied. As in mice protected against B. microti with BCG (5), C.

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**Fig. 1.** Minimum effective dose of Coxiella extract against subsequent infection with B. microti. Symbols: ●, 5 μl; □, 10, 20, 50, or 100 μl; ○, controls. Ranges of 2 standard errors of the mean are indicated.

**Fig. 2.** Effect of altering the interval between 50 μl of Coxiella extract and infection with B. microti. Symbols: Δ, extract given 7 days after parasite; extract given 0 (■), 3 (□), or 7 (○) days before parasite; ○, controls. Ranges of 2 standard errors of the mean are indicated.

**Fig. 3.** Radiation resistance of protection against B. microti by pretreatment with Coxiella extract. Symbols: ▽, 200 μl of extract given 4 weeks earlier and 900 rads given on day -1; ▽, extract alone; ■, 900 rads alone; ○, controls. Ranges of 2 standard errors of the mean are indicated.
parvum (6), or B. abortus S19 (19), the inclusion bodies shown to be degenerate intra-erythrocytic parasites (7) were present in erythrocytes of the protected mice in these experiments. The dead parasites had the same appearance and were as common as those previously found by light microscopy (5-7, 19) and electron microscopy (5, 7). Again, parasites could not be detected in macrophages on impression smears from spleens and livers of protected mice.

(iv) Protection after irradiation. Protection that is achieved by the death of the infectious agent within circulating erythrocytes is presumably mediated by a soluble factor. The possibility that this factor was antibody specific for parasite antigens was therefore investigated. In two experiments 10 mice injected with 200 μl of Coxiella extract 4 weeks earlier and 10 controls were infected with 10⁶ B. microti. One-half of each group of 10 had been exposed to 900 rads 24 h previously. As Fig. 3 shows, this dose of irradiation, given when it could be expected to affect profoundly the production of antibody specific for B. microti (53), did not affect the course of protection. In addition, parasitemias in the pretreated groups fell behind those of the controls within 24 h of infection, too soon for appreciable production of specific antibody.

B. rodhaini. Because one B. rodhaini is invariably fatal to unprotected mice, this parasite was used as a more rigorous test of the efficacy of Coxiella extract against Babesia spp. in this host. Six mice were injected with 200 μl of Coxiella extract. After 2 weeks, these mice and six controls received 10⁶ B. rodhaini. As Fig. 4 shows, the controls died on day 8 or 9, whereas the pretreated mice all survived, having experienced only low parasitemias. In view of the effect of a longer time interval between pretreatment and infection on the degree of protection against B. microti (Fig. 2), the suppression of B. rodhaini may have been even more marked if it had been given later.

P. vinckei petteri. Of 15 mice injected with 200 μl of Coxiella extract, 5 were infected with 10⁶ P. vinckei petteri 3 weeks later, and the remaining 10 were infected the following week. All 15 were protected compared with controls (Fig. 5). Protection against this parasite was also radiation resistant; 15 mice pretreated with 200 μl of Coxiella extract 4 weeks earlier retained their resistance to infection with P. vinckei petteri (10⁷ cells) even though they had been exposed to 900 rads 24 h previously (Fig. 6). As in mice protected with C. parvum (6), dead parasites were present in circulating erythrocytes as the parasitemias decreased in protected mice.

Hyperreactivity to endotoxin. Guinea pigs infected with C. burnetii are hyperreactive to injection with bacterial endotoxin (44). To ascertain whether this extends to mice and whether it could be induced with the Coxiella extract used in these experiments, mice pretreated with extract were injected with several doses of E. coli endotoxin. The pretreated mice were much more sensitive to this material than were normal mice (Table 1).

DISCUSSION

Previous reports have referred to the activity of C. burnetii against Brucella suis in vivo (32) and to the in vitro suppression of Listeria monocytogenes by supernatants from macrophages
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Symbols: \( \Delta \), 200 \( \mu l \) of extract given 4 weeks earlier and 900 rads given on day \(-1\); \( \square \), extract alone; \( \& \), 900 rads alone; \( \circ \), controls. Ranges of 2 standard errors of the mean are indicated.

![Graph showing radiation resistance of protection against P. vinckei petteri by pretreatment with Coxiella extract.](image)

**TABLE 1. Hyperreactivity of mice pretreated with Coxiella extract to endotoxin**

<table>
<thead>
<tr>
<th>Intraperitoneal endotoxin dose (( \mu g ))</th>
<th>No. of deaths at 24 h/no. in group</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5/5/5</td>
<td>0/5/5</td>
</tr>
<tr>
<td>25</td>
<td>5/5/5</td>
<td>0/5/5</td>
</tr>
<tr>
<td>100</td>
<td>5/5/5</td>
<td>0/5/5</td>
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<tr>
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<td>ND(^a)</td>
<td>0/5/5</td>
</tr>
<tr>
<td>800</td>
<td>ND(^a)</td>
<td>4/5/5</td>
</tr>
</tbody>
</table>

\(^a\) ND, Not done.

previously infected in vivo with *C. burnetii* (26). Intraleisional injection of killed *C. burnetii* also causes tumor regression (27).

This appears to be the first example of *C. burnetii* or an extract derived from this organism protecting against a protozoan parasite. The characteristics of this protection seem very much to parallel those we have previously described in mice infected with BCG (5, 8) or pretreated with killed *C. parvum* (6). In particular, the effect is radioresistant (Fig. 3 and 6); therefore, it is evidently not caused by enhanced production of specific antibody. This type of antiprotozoal activity is possessed not only by the rickettsial extract described in this paper, but also by three genera of bacteria (5, 6, 19), the yeast extracts zymosan and glucan, and chlorite-oxidized oxamyllose, a synthetic polycarboxylate (Clark, manuscript submitted for publication). The likelihood that this range of agents shares a protective antigen with these hemoprotozoa seems remote. In addition, no antibodies were detected by an indirect fluorescence test in mice pretreated with BCG (5) or *C. parvum* (6), which produce protection with the same characteristics as that afforded by *Coxiella* extract.

Nevertheless, this protection results in intrerythrocytic death of the parasites, implicating some type of soluble mediator. Interferon warrants consideration, because *C. burnetii* induces interferon in mice (25) and other interferon inducers have some activity against hemoprotozoa (3, 24). It is unlikely that the mechanism of the protection induced by *Coxiella* extract is this simple, however, because its kinetics are much different (weeks) than those of the appearance of interferon in mice injected with *C. burnetii* (hours). The protective effect of BCG (5), *C. parvum* (6), and *B. abortus* S19 (19) against *Babesia* spp. also outlasts the ability of these bacteria or their extracts to induce detectable interferon (30, 33, 58).

Nevertheless, it is still conceivable that an interferon could be mediating the observed protection. As well as protecting mice against these parasites, BCG (52), *C. parvum* (4), *B. abortus* S19 (1), *Eperythrozoon coccoides* (15), and *C. burnetii* (Table 1 and reference 44) all enhance the responsiveness of mice to the lethal effects of bacterial endotoxin. With at least BCG (59) and *E. coccoides* (14) this hyperreactivity includes an increased efficiency of interferon induction by endotoxin. *Plasmodium* sp. can induce interferon (21) and has been reported to contain material functionally similar to an endotoxin (39). Likewise, a lipopolysaccharide has recently been isolated from *Trypanosoma cruzi* (28), a recognized interferon inducer (46) which BCG also protects against (40). Thus, if the increased efficiency of interferon induction by endotoxin which occurs in mice pretreated with BCG (59) and *E. coccoides* (14) proves to extend to *C. parvum*, *B. abortus*, and *C. burnetii* (as does the hyperreactivity to other effects of endotoxin), pretreatment with these agents may protect by potentiating the interferon-inducing capacity of the challenge dose of protozoa. The maintenance of protection in irradiated mice (Fig. 3 and 6) is consistent with the radioreactivity of induction of interferon by endotoxin (9).

If interferon is involved in protection against hemoprotozoa by BCG, *C. parvum*, *B. abortus*, *E. coccoides*, and *C. burnetii* extract, a direct action on deoxyribonucleic acid synthesis is an obvious possibility (31). However, in view of recent evidence that interferon inducers or interferon may be active against tumors because they augment natural killer (NK) cell activity (37, 13) and our previous evidence that the pro-
tective effects of BCG and *C. parvum* against *B. microti*, *P. vinckei*, and tumors are remarkably similar (7), NK cells clearly warrant serious consideration in the protection described in this paper. Many observations are consistent with this. Corticosteroids suppress both NK cells (35) and protection against hemoproteozoa (8), and the spleen has a central role in each phenomenon (8, 29). As well as protecting mice against *P. vinckei* and *B. microti*, BCG and *C. parvum* also increase NK cell activity (37, 56). Newcastle disease virus, statolon, and polynosinic acid-polycytidylic acid have been reported to enhance NK activity (13, 37) and protect against *P. berghei* (23), and both mature NK cells (20) and protection against hemoproteozoa by BCG and *Coxiella* extract (8) (Fig. 3 and 6) are radioreistant. Finally, there is some evidence that NK cells secrete a nonspecific soluble inhibitor of deoxyribonucleic acid synthesis (41), which would be consistent with the intra-erythrocytic death of the parasites. Nevertheless, it is conceivable that contact between NK cells and parasitized erythrocytes, to the detriment of the parasite within, could protect in the absence of any detectable mediator. This contact could occur in the red pulp of the spleen.

We have earlier provided indirect evidence (8) that the final mediator may be the same in both BCG-induced protection and natural recovery. Thus, the involvement of interferon in natural recovery from these hemoproteozoa, perhaps acting via NK cells, warrants reappraisal. It provides, for instance, an alternative explanation for the temporary loss of control over protozoal parasites after either splenectomy or injection of corticosteroids. Both of these treatments can impair immunity to protozoa in the presence of stable amounts of specific antibody (12, 49, 55, 57), which is inconsistent with the view that antibody acts alone to mediate protection (10, 42). It may be relevant that mice recently splenectomized (22) or injected with corticosteroid (9) produce much less interferon after injection with endotoxin than do controls.

Similarly, there has been no explanation for reports that cells from the thoracic duct do not adaptively transfer protection against *P. berghei* in rats, whereas spleen cells are excellent for this purpose (45, 50). This is again inconsistent with a mechanism wholly based on antibody, since thoracic duct lymphocytes of primarily immunized rats adaptively transfer the ability to respond to the same antigen with a secondary-type response (16). Conceivably, most of the antigenic stimulation from a parasite restricted to the circulation could occur in the spleen. This should not prevent thoracic duct lymphocyte transfer of memory for antibody production, however, since splenic T cells, and to a lesser extent B cells, readily find their way into the thoracic duct within the collection period employed in the studies with *P. berghei* (48). The explanation of these data (45, 50) may lie in the tissue distribution of NK cells; many are present in the spleen, but low or undetectable numbers are present in the thoracic ducts of rats (36). Finally, the ability of serum from rats infected with *P. berghei* to control this parasite in the homologous host but not in mice (18) is more in keeping with the tendency of interferons to species specificity than with the broad spectrum of species activity shown by antibody.

Another non-antibody-soluble mediator, tumor necrosis factor (TNF), which is apparently different from interferon, has been reported to be released when BCG-, *C. parvum-* or zymosan-treated mice received small doses of endotoxin (4). These workers attribute the nonspecific antitumor effect of these three agents to this mediator. It seems likely that other agents which cause macrophages to proliferate and form granulomas and cause hyperreactivity to endotoxin, accompanied by antitumor activity, may lead to release of TNF in the same circumstances. *C. burnetii* appears to fulfill these criteria (27, 43) (Table 1). Whatever the mediator, a prerequisite for its formation seems, with all protectants, to be the presence of these granulomas in the liver and spleen. As well as TNF (4), endotoxin-induced interferon is reported to originate mainly from macrophages (47).

Thus, both the interferon-NK cell system and TNF are under consideration as possible mediators of the protective effect of *Coxiella* extract against *Babesia* spp. and *P. vinckei petteri*. The species specificity reported for the humoral protective activity against *P. berghei* in rats (18) is absent in TNF (4), which appears to favor an explanation based on interferon, but there seems to be no reason why both mediators could not be involved.

Work to determine the component of the *Coxiella* extract responsible for this protection is in progress. We have so far determined that rigorous treatment of the extract with Pronase does not diminish protection (T. J. Higgins, and I. A. Clark, unpublished data), implying that the active principle is not a protein. The antitumor (27) and antibacterial (26) activities observed after treatment with *C. burnetii* are also attributed to nonprotein components of this organism.

**LITERATURE CITED**


42. Phillips, R. S., and V. E. Jones. 1972. Immunity to Plasmodium berghei in rats: maximum levels of protective antibody activity are associated with eradication of the infection. Parasitology 64:117-127.