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Protective Vaccination Against Virulent *Babesia bovis* with a Low-Molecular-Weight Antigen

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A *Babesia bovis* low-molecular-weight antigen was purified from crude material by using affinity adsorption techniques first with a mouse monoclonal antibody and then by adsorption with normal bovine sera. The antigen was then further purified by gradient gel electrophoresis. Analysis by Western transfer revealed only one antigen band, with an apparent molecular mass of 29 kilodaltons. A band (12 by 0.3 by 0.6 cm) corresponding to this antigen was excised from the acrylamide gel and injected twice 4 weeks apart, together with 2.5 ml of Freund complete adjuvant, into nine nonsplenectomized adult cattle. The vaccinated cattle and five susceptible control animals were challenged with a virulent homologous strain 4 weeks after the second vaccination. None of the vaccinated animals was clinically affected, whereas three of five controls were severely affected. Control animals had significantly greater declines in packed cell volume and greater rises in temperature and parasitaemias than vaccinated animals.

Protection against virulent *Babesia bovis* infection has been demonstrated in a number of ways. Live organisms attenuated by either rapid passage through splenectomized calves (2) or exposure to γ radiation (15) have been shown to protect cattle against subsequent virulent heterologous challenge. Furthermore, the infection of cattle with a single strain, be it virulent or avirulent, protects the animals against subsequent virulent heterologous challenge for at least 4 years (13). Preparations of dead antigenic material have been shown to produce various degrees of protection against virulent heterologous challenge. Protection as strong as that induced by natural infection with crude and semi-defined preparations of *B. bovis* parasites when the vaccinated cattle were challenged with a virulent heterologous strain has also been demonstrated (12, 14). Poor protection has been demonstrated with supernatant fluid from in vitro-cultured *B. bovis* against virulent heterologous challenge (17), whereas a moderate degree of protection against homologous challenge has also been demonstrated with this material (10). Strong protection against virulent homologous challenge has also been demonstrated with two distinct affinity-purified fractions (20, 21). In this paper, we report on the further characterisation of one of these fractions (21) and its use as an immunogen in nonsplenectomized 18-month-old *Bos taurus* cattle.

MATERIALS AND METHODS

Animals. The 3-month-old calves and 18-month-old cattle of mixed *Bos taurus* breeds were obtained from a tick-free area. These animals were housed under tick-free conditions at our laboratories for the duration of the experiment. All animals were bled before the commencement of experimental procedures, and their sera were examined by indirect immunofluorescence (IFA) (8) and radioimmunoassay (RIA) (21) to confirm their previous nonexposure to *B. bovis*. The calves were splenectomized 2 weeks before infection with a stabilate of *B. bovis* (Samford strain) which had been stored in the vapor phase of liquid nitrogen.

Parasites. The virulent tick-transmitted Samford strain of *B. bovis* was used for antigen preparation, infective challenge, and serological assays. In addition the virulent tick-transmitted Lismore strain was also used for IFA studies.

Monoclonal antibody production. These techniques have been described in a previous paper (21). The clone 15 B1-1-G3-G7-D7 (15B1) and negative NS1 mouse myeloma ascites fluid were used exclusively.

Antigen purification. Soluble parasite antigen was prepared from sonicated whole *B. bovis*-infected erythrocyte preparations (14). An insoluble gel of glutaraldehyde-cross-linked, antibody-containing mouse ascites fluid derived from the 15B1 clone was prepared (16). This antibody is a very labile immunoglobulin M and could not be separated from contaminating ascites proteins without severely affecting its biological properties. For this reason it was used as a stabilized cross-linked gel. The gel obtained from 20 ml of ascites fluid was used as an immunoadsorbant to solid-phase adsorb 10 ml of crude antigen by mixing at 26°C for 2 h. The Immunoadsorbant gel was then washed with 50 ml of 0.15 M phosphate-buffered saline (pH 7.2) and centrifuged 1,000 × *g* for 5 min, and this procedure repeated until the supernatant had no A_{280}. The gel was then washed with 10 ml of phosphate-buffered saline containing 2 M urea, centrifuged, and then washed with 50 ml of phosphate-buffered saline. Antigen was then eluted from the gel with 10 ml of phosphate-buffered saline containing 1% sodium dodecyl sulphate and supernatant eluate obtained by centrifugation. Eluates were monitored for serological activity by RIA. Sodium dodecyl sulfate was removed from the eluate with 2 to 3 drops of 2 M potassium acetate. The eluate was then mixed with an equal volume of a glutaraldehyde-polymerized gel which was derived from a pool of normal bovine serum from 10 animals with sera known to be negative to *B. bovis*. This step was necessary to remove or minimize

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erythrocytic isoantigens and blood group antigens (5). After being mixed at 26°C for 2 h, the mixture was centrifuged and the supernatant containing the antigen retained.

Electrophoresis. Affinity-purified antigen, together with lysate fractions of *B. bovis*-infected and normal erythrocytes, were electrophoresed on 5 to 15% gradient acrylamide containing 0.1% sodium dodecyl sulfate and 0.5 M urea (19). A series of protein standards (Pharmacia, Uppsala, Sweden) ranging from phosphorylase *b* (94 kilodaltons [kd]) to α-lactalbumin (14 kd) were run on adjacent tracks. In addition 3 ml of affinity-purified antigen was then electrophoresed on preparative slabs (12 cm by 12 cm by 3 mm) under the same conditions. A central longitudinal section of 5 mm in width was cut from the gel and stained with 0.25% Coomassie blue R250 (Bio-Rad Laboratories, Richmond, Calif.) in 7% acetic acid–30% methanol–63% water for 1 h and then destained with the same solvent. The standards were similarly stained. In addition some gels were stained for peroxidase activity to detect hemoglobin and its subunits (3). A section of gel (12 by 0.3 by 0.6 cm) containing the target antigen was tested by RIA with monoclonal 15B1 antibodies and negative ascites fluid as probes. A 6-mm band corresponding to the mobility of the target antigen was cut from each preparative gel and stored at −20°C. Such bands constituted one animal dose, and each band was the equivalent of the amount of antigen contained in 2.5×10^9 whole parasites. Previous experiments with crude parasite erythrocyte preparations had shown that this dose was sufficient to induce strong protective immunity (12).

Western transfer analysis. Proteins in acrylamide gels were transferred to nitrocellulose sheets (Bio-Rad Laboratories) (18). Antigens were detected by either appropriate bovine (both positive and negative) and anti-bovine sera (9) or mouse monoclonal antibody (both positive and negative ascites fluid) and rabbit antisera to mouse immunoglobulins conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories).

Serological analyses. Antibody levels in bovine sera were determined by RIA with both affinity-purified and crude lysate antigens with appropriate labeled secondary antisera as described previously (21). The antigenicity of the various eluates from the immunoadsorption procedure was tested by using mouse 15B1 monoclonal antibody and negative ascites fluid and labeled rabbit anti-mouse immunoglobulin G. IFA with sera from both vaccinated and control cattle was performed (3).

Vaccination regime. Nine acrylamide gel strips (12 by 0.3 by 0.6 cm) were emulsified in a Virtis tissue blender with an equal volume of Freund complete adjuvant (2.5 ml) and used to subcutaneously vaccinate nine cattle. Vaccination at a site different from the first was repeated after 4 weeks.

Five cattle were retained as unvaccinated controls. Freund complete adjuvant and acrylamide was not administered to them, as previous experience in our laboratory has shown that nonspecific immunity to *B. bovis* infections is not produced by this procedure.

All cattle were challenged with 10^4 cells of the virulent S strain of *B. bovis* 4 weeks after the second vaccination.

Jugular blood was obtained daily, and packed cell volumes (PCV) were assessed by using a microhematocrit technique. Thick blood films were examined daily, and parasitemias were calculated as a number of organisms per microliter of blood (11). Rectal temperatures were obtained daily. Citrated plasma was also obtained daily, and assays for conglutinin, cryofribrinogen and fibrinogen were assessed by using techniques previously described (4, 6, 7). Changes in these parameters are reliable indices for the assessment of the severity of the disease (5).

RESULTS

Antigen purity. Electrophoresis and Coomassie blue staining of the affinity-purified eluate showed only two protein bands, one being 29 kd and the other being 14 kd. However, both positive and negative lysate fractions contained numerous protein bands (Fig. 1). Both bands in the affinity-purified eluate could also be stained for peroxidase (data not shown). Analysis of nitrocellulose blots of affinity-purified material indicated several bands, and all but one were detected by normal bovine sera or by secondary marker antibody. A single 29-kd band was detected with bovine antisera from vaccination. In addition, nitrocellulose blots of crude antigen, when developed with monoclonal 15B1 antibody, showed an identically sized band. This band could not be demonstrated with negative ascites fluid. Figure 2 shows nitrocellulose blots of crude antigen developed with bovine *B. bovis* antisera (lane A) and monoclonal antibody to 15B1 (lane B). RIA analysis of the gel band by using 15B1 antibody also showed strong activity, which again could not be demonstrated with negative ascites fluid.

Serology. Sera from vaccinated animals remained negative 4 weeks after the primary vaccination by the IFA test. The sera of the animals then became progressively positive until 8 weeks after the primary vaccination, when the average titer was 1/200 to 1/400. Sera reacted with the heterologous L strain antigen by the IFA test and gave titers similar to those of the homologous system. Sera specifically stained the parasite, with no erythrocyte staining. Hybridoma fluid gave similar staining.

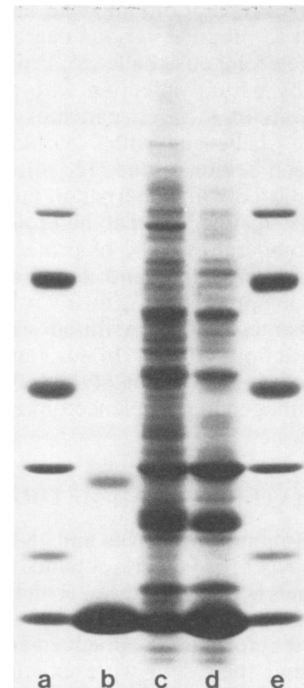

FIG. 1. Gradient acrylamide electrophoresis of marker proteins (lanes a and e), 15B1-affinity adsorbed crude soluble fraction of *B. bovis*-infected erythrocytes (lane b), crude soluble fraction of *B. bovis*-infected erythrocytes (lane c), and crude soluble fraction of uninfected erythrocytes (lane d).

Vaccination challenge. All animals survived the challenge. However, whereas none of the vaccinated cattle appeared clinically affected, three of the five controls were severely affected.

Clinical signs in control animals included jaundice, labored respiration, ataxia, anorexia, and muscle weakness. Significantly higher temperatures were observed in controls on days 2 through 6 ($P < 0.05$), 7 ($P < 0.001$), 8 ($P < 0.01$), 9 through 12 ($P < 0.05$), 13 ($P < 0.01$), and 14 ($P < 0.05$). These signs, which are indicative of severe disease, were not observed in vaccinated animals or in two of the control animals. The variable response by control animals was expected due to the small number of outbred animals used.

The PCV was significantly lower in control as opposed to vaccinated animals on days 12 through 14 ($P < 0.05$). The maximum drop in PCV in controls was 42.9 ± 7.0% below preinfection levels on day 14, and in vaccinates it was 28.9 ± 3.4% on the same day. The parasitemia was significantly higher in controls on day 12 ($P < 0.025$) and days 13 to 14 ($P < 0.05$).

Data for temperature, PCV, and parasitemia are detailed in Fig. 3.

Changes in plasma fibrinogen levels fluctuated between 12.4% below and 16.6% above preinfection levels in control animals, but the levels fell consistently in vaccinated animals to 27.3% below preinfection levels (Fig. 4).

Plasma conglutinin levels fell slowly in both groups from day 1 but rose briefly to twice normal levels in the vaccinated group on day 8 before falling again.

Cryofibrinogen was detected qualitatively in the sera of two controls 12 DPI.

DISCUSSION

The data presented in this paper show that a polypeptide seemingly of babesial origin can induce a protective response in cattle after challenge with the virulent organism from which it was derived. The polypeptide, which is presumably a derivative of a native protein used in a previous experiment (21), was as protective under the conditions of this experiment as was crude extract derived from 5×10^9 organisms. However, an exact analogy cannot be drawn between the two vaccine trials, for although both were in nonsplenectomized adult cattle, the challenge organisms for crude and semicrude vaccines were heterologous, whereas homologous organisms were used in this current study (12, 14). A direct comparison between the 15B1-purified antigen in native form (21) and crude material (7) can be made, however, for both experiments were conducted with homologous challenge in splenectomized calves. In this instance only one calf died in each of the vaccinated groups, 4 days after the death of the last control (all of which died), and significant differences in the drop in PCV, rise in temperature, and parasitemia were observed. It seems, therefore, that under homologous challenge conditions at least this small polypeptide is as protective as crude material.

In the current experiment three of five control animals became clinically ill, but none died. In contrast, none of the nine vaccinated animals was clinically affected. Significant differences in the drop in PCV, rise in temperature, and parasitemia were observed between the two groups, but

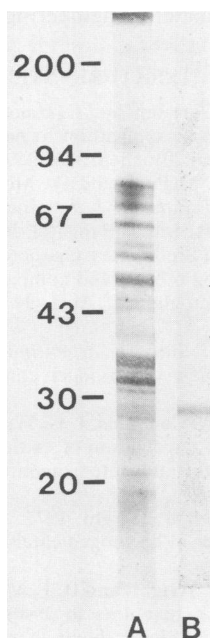

FIG. 2. Western transfer analysis of crude soluble fraction of *B. bovis*-infected erythrocytes. Lane A, Stained with bovine antisera from naturally infected cattle; lane B, 15B1 mouse monoclonal antibody. Relevant protein standards are shown on the left-hand side.

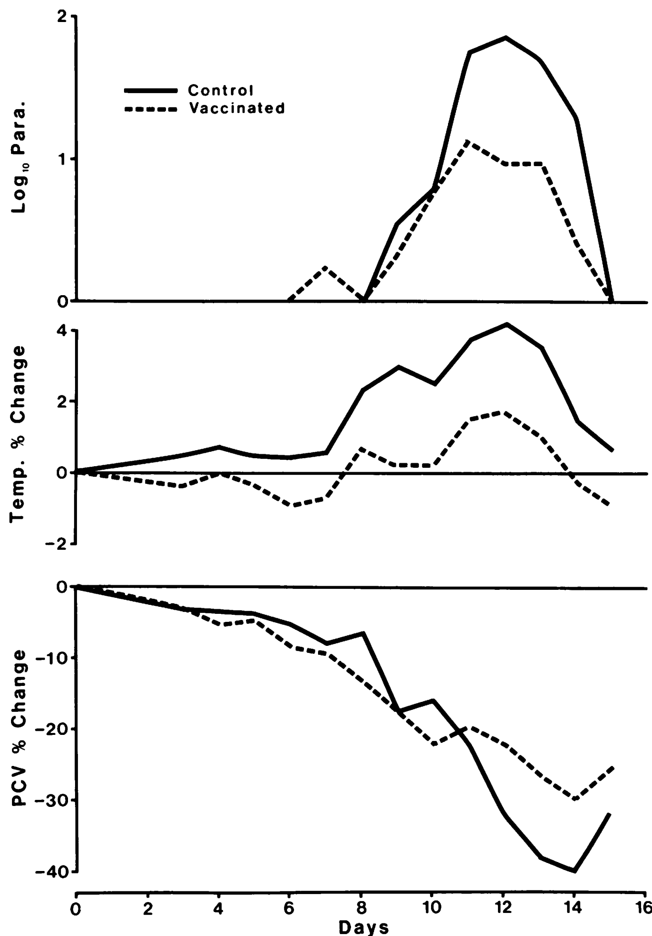

FIG. 3. The percent change in PCV and temperature (°C) and the log parasitemia (para) per microliter of blood for control and vaccinated cattle.

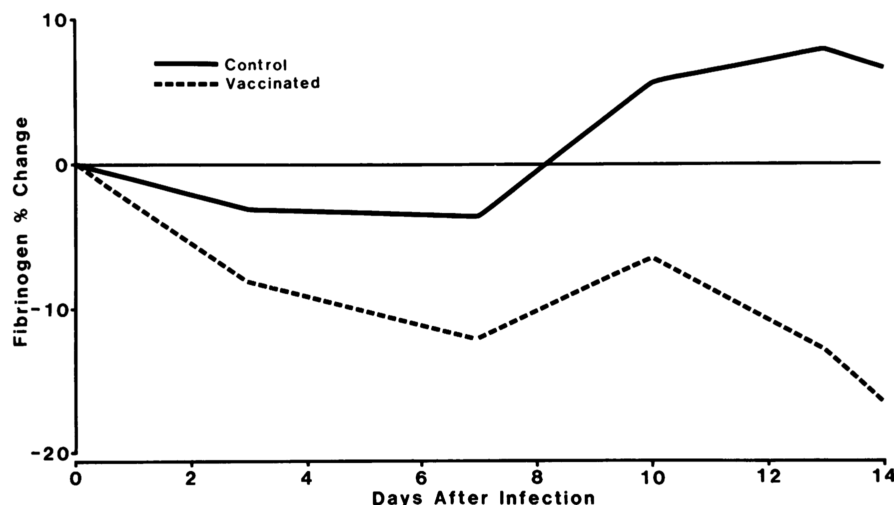

FIG. 4. The mean percent change in plasma fibrinogen levels for control and vaccinated cattle.

plasma conglutinin and fibrinogen levels were relatively similar in both groups. However, the severity of infection in control animals was similar to that observed in nonsplenectomized susceptible cattle in previous experiments in which the Samford challenge strain was used (19). Variability of reaction occurs in these experiments due to the small number of outbred animals used.

Staining of polyacrylamide gels of affinity-purified material for peroxidase revealed two bands, one with a molecular mass of 29 kd, and the other with a molecular mass of 14 kd. As this reaction is specific for haem compounds, it seems likely that the larger-molecular-mass material is a dimer of the smaller hemoglobin subunit derivative. It appears, therefore, that the *B. bovis*-derived antigen comigrates with the larger hemoglobin subunit. This is substantiated by fluorescent antibody staining, as antisera only stain parasites and not uninfected erythrocytes. Moreover, a protective response in cattle is induced, whereas hemoglobin compounds do not induce such a response. In addition, monoclonal antibodies stain only the 29-kd band upon Western transfer, and then only weakly.

The desorption of hemoglobin subunits together with the parasite antigen after affinity adsorption of crude material is a major problem in the purification of this polypeptide. The amount of hemoglobin derivatives can be reduced by prior adsorption with CM-Sephadex (20). However, due to the high avidity of the 15B1 antibody for the 29-kd polypeptide, relatively high concentrations of detergent are necessary to elute the parasite polypeptide. As the glutaraldehyde gel matrix also contains haptoglobin, which strongly binds hemoglobin, this use of detergent results in coelution of hemoglobin subunits from haptoglobin and babesial antigen from antibody. The subsequent removal of sodium dodecyl sulfate from the eluate and further adsorption of the latter with normal bovine serum resulted in the further reduction of nonspecifically bound material that would otherwise have reacted with negative bovine sera in Western transfers (B. V. Goodger, unpublished data; reference 5). The phenomenon of nonspecific binding of proteins, including hemoglobin, to affinity chromatography columns has been reported by other authors (1). But by using appropriate controls, especially the use of Western blotting with normal as well as infected erythrocyte preparations, and by using negative as well as positive sera and by selective adsorption procedures, most nonspecifically bound proteins can be identified.

Previous work on native material indicated that the 15B1 antigen was also complexed to large host molecules (21). The latter may act as carriers, thus enhancing immunogenicity. Therefore, it is the intention of our group to pursue further trials with 15B1 antigen by modifying its presentation to the host in a number of ways to determine whether its immunogenicity can be enhanced. Such procedures would include polymerization and immobilization on inert carriers. A number of other target antigens have now been isolated, and screening experiments with these have commenced. It may be that a cocktail of antigens will eventually be required in an effective vaccine, although based on the information to date, the 29-kd polypeptide is a highly effective immunogen in its own right, is required in only small quantities, and serologically at least, is cross-reactive with a series of strains (21). Because of its small size, it is also an ideal candidate for in vitro synthesis by genetic engineering.

LITERATURE CITED

1. **Bergenheim, N., U. Carlsson, and C. Hansson.** 1983. Preparative affinity electrophoresis: application to human erythrocyte carbonic anhydrase. Ann. Biochem. **134:**259–263.
2. **Callow, L. L., L. T. Mellors, and W. McGregor.** 1979. Reduction in virulence of *Babesia bovis* due to rapid passage in splenectomized cattle. Int. J. Parasitol. **9:**333–338.
3. **Goodger, B. V.** 1972. Preliminary characterization of the bovine polymeric Hb binding protein and comparison of some properties with human haptoglobins. Aust. J. Exp. Biol. Med. Sci. **50:**11–20.
4. **Goodger, B. V.** 1977. *Babesia argentina*: observations on the immunogenicity of the cryofibrinogen complex. Z. Parasitenkd. **53:**47–52.
5. **Goodger, B. V., M. A. Commins, I. G. Wright, and G. B. Mirre.** 1984. *Babesia bovis*: vaccination of cattle against heterologous challenge with fractions of lysate from infected erythrocytes. Z. Parasitenkd. **70:**321–329.
6. **Goodger, B. V., and I. G. Wright.** 1977. Acute *Babesia bigemina* infection: changes in fibrinogen catabolism. Z. Parasitenkd. **53:**53–61.
7. **Goodger, B. V., I. G. Wright, and D. F. Mahoney.** 1981. The use of pathophysiological reactions to assess the efficacy of the immune response to *Babesia bovis* in cattle. Z. Parasitenkd. **66:**41–48.
8. **Goodger, B. V., I. G. Wright, D. F. Mahoney, and R. V. McKenna.** 1978. *Babesia bovis* (Argentina): components of the cryofibrinogen complex and their contribution to pathophysiology of infection in splenectomised calves. Z. Parasitenkd.

58:3–13.

9. **Goodger, B. V., I. G. Wright, and D. H. Waltisbuhl.** 1983. The lysate from bovine erythrocytes infected with *Babesia bovis*. Analysis of antigens and a report on their immunogenicity when polymerized with glutaraldehyde. Z. Parasitenkd. **69**:473–482.
10. **Kuttler, K. L., M. G. Levy, M. A. James, and M. Ristic.** 1982. Efficacy of a non-viable culture-derived *Babesia bovis* vaccine. Am. J. Vet. Res. **43**:281–284.
11. **Mahoney, D. F., and J. R. Saal.** 1963. Bovine babesiosis: thick blood films for the detection of parasitaemia. Aust. Vet. J. **37**:44–47.
12. **Mahoney, D. F., and I. G. Wright.** 1976. *Babesia argentina*: immunization of cattle with a killed antigen against infection with a heterologous strain. Vet. Parasitol. **2**:273–282.
13. **Mahoney, D. F., I. G. Wright, and B. V. Goodger.** 1979. Immunity in cattle to *Babesia bovis* after single infections with parasites of various origins. Aust. Vet. J. **55**:10–12.
14. **Mahoney, D. F., I. G. Wright, and B. V. Goodger.** 1981. Bovine babesiosis: the immunization of cattle with fractions of erythrocytes infected with *Babesia bovis* (syn. *B. argentina*). Vet. Immunol. Immunopathol. **2**:145–156.
15. **Mahoney, D. F., I. G. Wright, and P. J. Ketterer.** 1973. *Babesia argentina*: the infectivity and immunogenicity of irradiated blood parasites of splenectomized calves. Int. J. Parasitol. **3**:209–217.
16. **Ternynck, T., and S. Avraneas.** 1976. Polymerization and immobilization of proteins using ethylcholoroformate and glutaraldehyde. Can. J. Immunol. Suppl. **3**:29–35.
17. **Timms, P., R. J. Dalgleish, D. N. Barry, C. K. Dimmock, and B. J. Rodwell.** 1983. *Babesia bovis*: a comparison of culture-derived parasites, non-living antigen and conventional vaccine in the protection of cattle against heterologous challenge. Aust. Vet. J. **60**:75–77.
18. **Towbin, H., T. Staekelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
19. **Wright, I. G., B. V. Goodger, K. Rode-Bramanis, J. S. Mattick, D. F. Mahoney, and D. F. Waltisbuhl.** 1983. The characterisation of an esterase derived from *Babesia bovis* and its use as a vaccine. Z. Parasitenkd. **69**:703–704.
20. **Wright, I. G., D. F. Mahoney, G. B. Mirre, B. V. Goodger, and J. D. Kerr.** 1982. The irradiation of *Babesia bovis*. II. The immunogenicity of irradiated blood parasites for intact cattle and splenectomised calves. Vet. Immunol. Immunopathol. **3**:591–601.
21. **Wright, I. G., M. White, P. D. Tracey-Patte, R. A. Donaldson, B. V. Goodger, D. J. Waltisbuhl, and D. F. Mahoney.** 1983. *Babesia bovis*: isolation of a protective antigen by using monoclonal antibodies. Infect. Immun. **41**:244–250.