INFECTION AND IMMUNITY, Dec. 1994, p. 5698–5701
0019-9567/94/$04.00+0

Vol. 62, No. 12

Antibody Response to a *Babesia bigemina* Rhoptry-Associated Protein 1 Surface-Exposed and Neutralization-Sensitive Epitope in Immune Cattle

TENDAI C. USHE,[1] GUY H. PALMER,[1] LILIANA SOTOMAYOR,[2]† JULIO V. FIGUEROA,[3] GERALD M. BUENING,[3] LANCE E. PERRYMAN,[1]‡ AND TERRY F. MCELWAIN[1]*

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040[1]; Department of Infectious Diseases, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611-0633[2]; and Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri—Columbia, Columbia, Missouri 65211[3]

Received 14 April 1994/Returned for modification 14 July 1994/Accepted 15 September 1994

Protective immunity against *Babesia bigemina* is hypothesized to involve antibodies directed against merozoite surface-exposed epitopes. Levels of antibody against a rhoptry-associated protein 1 (RAP-1) B-lymphocyte epitope, defined by surface-reactive and inhibitory monoclonal antibodies, in immune cattle sera were determined. All cattle produced antibodies to the epitope; however, there was limited correlation between immune protection induced by infection or RAP-1 immunization and the level of antibody to the neutralization-sensitive B-lymphocyte epitope examined.

Bovine babesiosis caused by *Babesia bigemina* is one of several major tick-borne hemoparasitic diseases that are inadequately controlled and cause substantial livestock production losses in tropical and semitropical regions (6, 8). The induction of protective immunity by deliberate infection of naive cattle (premunition) has been the primary basis for control of babesiosis by vaccination in areas of endemicity (1, 6). While effective at inducing protective immunity, premunition has biological and technical drawbacks that limit its utility (1). Efforts towards improved control of babesiosis by vaccination are directed in part at the identification and characterization of molecules for inclusion in recombinant derived or synthetic vaccines (10, 13).

Immunization of cattle with *B. bigemina* rhoptry associated protein 1 (RAP-1) induces partial protection against experimental challenge characterized by a significant reduction in parasitemia (10). In addition to its association with the rhoptry organelles, RAP-1 epitopes are exposed on the surface of extracellular live merozoites (7, 9). Monoclonal antibodies (MAbs) 14.16.1.7 and C2F3G3 bind a surface exposed region of RAP-1 that is conserved among strains and biologic clones of *B. bigemina* isolated from Africa, the Caribbean, North America, and South America (3, 4, 9, 10). In contrast, other merozoite surface polypeptides of these same isolates and clones are antigenically polymorphic (10). Importantly, the addition of MAb C2F3G3 to in vitro cultures of *B. bigemina* inhibits merozoite growth, as indicated by significant decreases in [^{3}H]hypoxanthine incorporation and parasite multiplication (2), suggesting that antibody may be capable of blocking a function of the RAP-1 molecule necessary for parasite invasion or growth.

The mechanisms of immunity induced by babesial infection or RAP-1 immunization have not been defined, but they could be mediated in part by serum neutralizing antibodies. If so, a high concentration and/or affinity of antibodies directed specifically against surface-exposed and neutralization-sensitive epitopes may be necessary for optimum protection. Calves immunized with RAP-1 develop high titers of antibody to whole RAP-1 but, in general, have low levels of antibody against merozoite surface-exposed epitopes as measured by immunofluorescence assay with live merozoites (10). The antibody response to specific surface-exposed B-lymphocyte epitopes, including the neutralization-sensitive epitope bound by MAb C2F3G3, in RAP-1-immunized calves or calves immunized by infection is unknown. Thus, in this study we examined the relationship between immune protection and antibody response to the surface-exposed, neutralization-sensitive epitope(s) bound by MAbs 14.16.1.7 and C2F3G3 in calves immunized with native RAP-1 or live *B. bigemina* merozoites.

To determine whether MAbs 14.16.1.7 and C2F3G3 bound identical or distinct RAP-1 epitopes, a competitive inhibition enzyme-linked immunoabsorption assay (CI-ELISA) based on recombinant Mexico strain RAP-1 antigen was developed (11, 12). The ability of each MAb to inhibit the binding of the other MAb was determined by using isotype-specific detection of MAb binding. Briefly, *Escherichia coli* XL1-Blue cells (Stratagene Inc., La Jolla, Calif.) transformed with plasmid pBbg58 encoding recombinant RAP-1 (11, 12) were grown overnight at 37°C in 500 ml of Luria-Bertani broth containing 60 mg of ampicillin per ml (5). Bacteria pelleted by centrifugation at 2,500 × *g* were resuspended in an equal volume of pH 7.2 phosphate-buffered saline (PBS) and freeze-thawed once. Recombinant bacteria were solubilized in 1% deoxycholic acid–0.1% Tris (pH 8.8), diluted in citrate coating buffer (pH 5.5), and used to coat Immulon 2 microtiter plates. Following overnight incubation, wells were washed with PBS and blocked for 1 h at 37°C with 350 μl of PBS containing 0.5% gelatin and 1% bovine serum albumin (blocking buffer). The wells were then washed twice with PBS containing 0.2% Tween-20. To measure inhibition of MAb 14.16.1.7 (immunoglobulin G1 [IgG1] isotype) binding by MAb C2F3G3 (IgG2b isotype), MAb C2F3G3 was added at concentrations ranging from 0.002

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-9696. Fax: (509) 335-7424.

† Present address: 213 Yakima Valley Highway, Sunnyside, WA 98944.

‡ Present address: College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606.

to 20 μg/ml and incubated for 30 min at 37°C, and wells were washed five times with PBS–0.2% Tween 20. After incubation for 30 min at 37°C with MAb 14.16.1.7 at 0.06 μg/ml and washing, bound MAb 14.16.1.7 was detected with peroxidase-labeled rat MAb directed against mouse IgG1 (Zymed, South San Francisco, Calif.). Inhibition of MAb C2F3G3 binding by MAb 14.16.1.7 was similarly measured by using a MAb C2F3G3 concentration of 0.04 μg/ml and MAb 14.16.1.7 concentrations from 0.012 to 12 μg/ml. Bound MAb C2F3G3 was detected with peroxidase-labeled rat MAb directed against IgG2b (Zymed). The color reaction which was developed with ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] peroxidase substrate was quantified by determination of the optical density at 405 nm, and an inhibition curve for each MAb was constructed.

In this assay, MAb 14.16.1.7 was able to efficiently inhibit the binding of MAb C2F3G3 to recombinant RAP-1 antigen (50% inhibition of 0.04 μg of C3F3G3 per ml required 0.12 μg of 14.16.1.7 per ml). By extrapolation from the reciprocal inhibition curve, the concentration of MAb C2F3G3 required for 50% inhibition of 0.04 μg of MAb 14.16.1.7 per ml was determined to be nearly equal (0.16 μg/ml), indicating that the two MAbs bound the same epitope or very closely spaced epitopes and that the binding affinities of the MAbs were similar.

To analyze the temporal development of antibodies against the epitope bound by MAbs 14.16.1.7 and C2F3G3, two groups of four 6-month-old Holstein calves were experimentally infected with 5×10^7 *B. bigemina*-parasitized erythrocytes. Group 1 was inoculated with a Mexico strain, and group 2 was inoculated with a Puerto Rico strain (the origin of each strain has been previously reported (2, 9). Packed-cell volume, level of parasitemia, and rectal temperature were determined daily for 30 days postinfection or until packed-cell volume had stabilized to preinfection levels. Sera were collected from the calves throughout acute infection, and the development of antibody against the epitope was assessed by using inhibition of MAb C2F3G3 and 14.16.1.7 binding in CI-ELISA. The rRAP-1 CI-ELISA procedure described above was used with the modification that bound MAb 14.16.1.7 was detected with peroxidase-labeled goat antibodies against murine IgG (Sigma, St. Louis, Mo.). Five known negative bovine serum samples and one known positive control serum sample were tested in each assay. Results for the experimental wells were expressed as the percent inhibition determined by the following formula: [(mean of negative control sera-mean of duplicate wells of test serum)/mean of negative control sera] × 100. By this procedure, 25 bovine serum samples from a *B. bigemina*-free region (northwest United States), randomly selected from samples submitted to the Washington Animal Disease Diagnostic Laboratory for unrelated serologic assays, were tested and had a mean inhibition of 6.2% with a standard deviation of 7.2%. All eight *B. bigemina*-infected calves developed detectable antibody to the neutralization-sensitive RAP-1 epitope between 5 and 15 days postinfection (Fig. 1). The antibody level was maintained throughout the course of acute disease (3 weeks) and persisted after clinical remission. At 6 weeks postinfection, there was no significant difference between the RAP-1 epitope-specific antibody responses of Mexico strain-infected and Puerto Rico strain-infected calves. Thus, serum antibodies to Puerto Rico strain *B. bigemina* recognized the recombinant Mexico strain RAP-1 epitope and were present at the same level as serum antibodies in calves infected with the homologous Mexico strain of *B. bigemina*.

The two groups of calves were hyperimmunized by a second inoculum of 5×10^7 parasitized erythrocytes of the homologous strain (group 1 received the Mexico strain; group 2 received the Puerto Rico strain). To assess the level of homologous protection induced by infection, the calves were challenged at 312 days postinfection with 5×10^7 parasitized erythrocytes from the homologous strain. Seronegative uninfected control calves were also challenged with each strain. Acute disease was monitored as described above. All eight immunized calves were protected from severe anemia, the hallmark of acute *B. bigemina* infection, after homologous challenge, as demonstrated by the significant difference between the decreases in packed-cell volume in infected and naive control animals (Table 1). No parasites were detectable in Puerto Rico-infected calves after homologous challenge, and the duration of fever was significantly shorter than in nonimmunized controls. The calves were then challenged (374 days after the initial infection) with 5×10^7 parasitized erythrocytes of the heterologous strain. Seronegative nonimmunized calves were again identically challenged. Compared with nonimmunized calves, all eight hyperimmunized calves were protected from anemia and had a significantly shorter duration of fever (Table 1). In addition, calves immunized with the Puerto Rico strain had a significantly shorter duration of parasitemia following Mexico strain challenge than nonimmunized control calves (Table 1).

The levels of antibody against the RAP-1 epitope bound by MAbs 14.16.1.7 and C2F3G3 in three groups of calves were compared: group 1 calves were immunized by multiple infection with Mexico strain *B. bigemina*, group 2 calves were immunized by multiple infection with Puerto Rico strain *B. bigemina*, and group 3 calves were immunized with whole RAP-1. The immunization of calves with purified whole RAP-1 and response to virulent *B. bigemina* challenge (group 3 animals) have been previously described (10). Briefly, five seronegative calves immunized at 2-week intervals with 50 μg of RAP-1 initially in complete and subsequently in incomplete Freund's adjuvant were challenged with 3×10^9 *B. bigemina*-infected erythrocytes. RAP-1-immunized calves were protected against challenge, as determined by a significant decrease in peak levels of parasitemia compared with levels in adjuvant-immunized controls.

RAP-1-monospecific sera collected from calves in the previous immunization trial (10) prior to challenge and immune sera from the two groups of experimentally infected calves obtained prior to homologous and heterologous challenge were tested at reciprocal dilutions of 10^1, 10^2, and 10^3 in MAb-based CI-ELISA. The results were expressed as percent inhibition determined by comparison with the five negative serum samples used in all assays, and the group mean percents inhibition of MAb 14.16.1.7 and MAb C2F3G3 binding were compared among all groups at each serum dilution (Table 2). When inhibition of binding of each respective MAb was compared within a group of calves, there was no significant difference noted at any serum dilution. Serum from RAP-1-immunized calves had a statistically higher mean percent inhibition of MAb binding at all three dilutions than the Mexico strain- and Puerto Rico strain-infected groups at the time of homologous or heterologous challenge (Table 2). There was no significant difference in mean percent inhibition between Puerto Rico and Mexico strain-infected calves (Table 2). Calves immunized with RAP-1 and partially protected against challenge developed a high titer of serum antibodies against the RAP-1 neutralization-sensitive epitope (titer range of 10^3 to 10^4 [data not shown]). In contrast, two infected calves (one with Mexico strain and one with Puerto Rico strain *B. bigemina*) had no detectable inhibition of binding of either MAb at a 1:10 serum dilution at the time of homologous or heterologous challenge. A minimal level of detectable serum

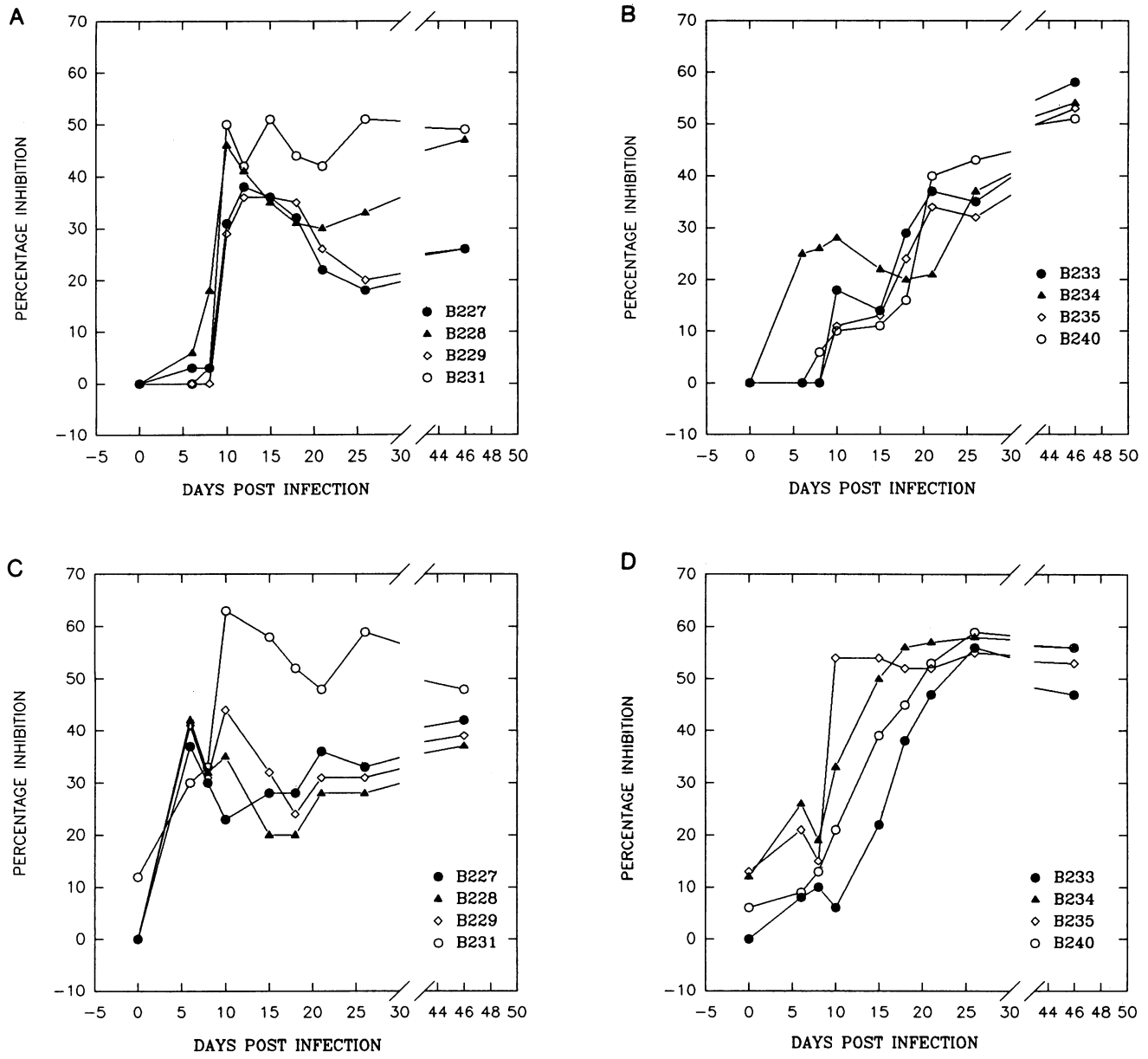

FIG. 1. Temporal antibody response to a RAP-1 neutralization-sensitive epitope as measured by inhibition of binding of MAb 14.16.1.7 (A and B) and MAb C2F3G3 (C and D) during acute *B. bigemina* infection in calves experimentally inoculated with either the Mexico strain (A and C) or the Puerto Rico strain (B and D).

antibody was present in these calves at a 1:2 serum dilution, despite an overall titer of serum antibody against whole merozoites, as determined in indirect immunofluorescence assay (9), or whole native RAP-1, as determined in direct ELISA (10), that either was not different from or was higher than those of other calves within their respective groups (data not shown).

We expected that calves immune to subsequent *B. bigemina* challenge would have high levels of antibody against the specific RAP-1 surface-exposed and neutralization-sensitive B-lymphocyte epitope bound by MAbs 14.16.1.7 and C2F3G3. In this study we showed that calves in the acute phase of babesiosis develop antibodies against this epitope and that epitope-specific antibodies persisted after clinical remission. However, the serum antibody level was low, and in two infected calves that were protected against challenge, antibody was detectable only at a 1:2 serum dilution.

Previously it was shown that calves immunized with RAP-1 had significantly lower peak levels of parasitemia upon challenge than calves immunized with adjuvant alone (10). The ELISA titer of serum antibody against whole RAP-1 in these calves was 5×10^4, while the titer of antibody against merozoite surface proteins was substantially lower (10^2), as measured by immunofluorescence against live merozoites (10). We have now shown that a high level of serum antibodies directed against the specific neutralization-sensitive and surface-exposed epitope reactive with MAbs 14.16.1.7 and C2F3G3, as measured in CI-ELISA, was present in the same RAP-1-

TABLE 1. Response to homologous and heterologous *B. bigemina* challenge

Infection strain	Challenge strain	n^a	Duration (days)[b] of:		% Decrease in PCV[c]
			Fever[d]	Parasitemia[e]	
Mexico	Mexico	4	0.5 ± 0.5	0.5 ± 0.5	2.3 ± 1.9[f]
None	Mexico	2	1.5 ± 0.5	3.0 ± 2.0	10.5 ± 2.5
Mexico	P.R.[g]	4	1.0 ± 0.7[f]	1.3 ± 0.8	5.0 ± 1.9[f]
None	P.R.	3	2.7 ± 0.5	1.7 ± 0.5	13.7 ± 2.9
P.R.	P.R.	4	0.3 ± 0.4[f]	0.0 ± 0.0[f]	3.0 ± 2.3[f]
None	P.R.	2	1.0 ± 0.0	3.5 ± 1.5	12.0 ± 3.0
P.R.	Mexico	4	0.3 ± 0.4[f]	1.5 ± 1.1[f]	8.8 ± 2.5[f]
None	Mexico	3	3.7 ± 0.5	4.7 ± 0.5	21.3 ± 3.1

[a] Number of calves per group.
[b] Values are means ± standard deviations.
[c] PCV, packed-cell volume. Values are means ± standard deviations.
[d] Defined as a rectal temperature of ≥103°F (ca. 39.4°C).
[e] Defined as ≥0.01% parasitemia.
[f] Significantly different from control values ($P \leq 0.05$, Student's t test).
[g] P.R., Puerto Rico.

immunized calves. Moreover, the titer of antibody against this epitope in RAP-1-immunized animals was significantly higher than in infected calves. Although they were not compared directly in the same immunization trial, the degree of protection against challenge in RAP-1-immunized animals was certainly no better than that in animals immunized by infection. Thus, there was poor correlation between titer of antibody to the C2F3G3 and 14.16.1.7 RAP-1 epitope and protective immunity.

In conclusion, this study has demonstrated that MAbs 14.16.1.7 and C2F3G3 bind the same epitope or very closely related epitopes. The RAP-1 merozoite surface-exposed and in vitro neutralization-sensitive epitope recognized by these MAbs is poorly immunogenic during infection but is significantly more immunogenic in animals immunized with isolated RAP-1. It is clear that immunization of cattle with purified RAP-1 in Freund's adjuvant targets increased levels of high-affinity antibodies against this neutralization-sensitive epitope. However, immune protection induced by infection or RAP-1 immunization does not appear to correlate with the level of epitope-specific antibody. A relatively high concentration of MAb C2F3G3 is required for in vitro inhibition (2). While it is possible that a minimal level of C2F3G3 B-lymphocyte epitope-specific high-affinity antibodies may be sufficient for neutralization in vivo, it seems more likely that B-lymphocyte mechanisms of immunity against RAP-1, if present, may be directed toward other, as-yet-undefined B-lymphocyte epitopes. Alternatively, the contribution of T-cell epitopes in the direct induction of protective immunity may be significant. Experiments are under way to better define these mechanisms of immunity, as well as the domains important for their induction.

TABLE 2. Levels of antibody to 14.16.1.7 and C2F3G3 epitopes

MAb	*B. bigemina* immunogen	n^a	% Inhibition of MAb binding at dilution[b]		
			10^1	10^2	10^3
14.16.1.7	RAP-1	3	67.3 ± 0.6	66.0 ± 14.9	42.0 ± 14.5
	Mexico strain	4[c]	32.5 ± 13.9	10.5 ± 8.1	6.5 ± 13.0
		4[d]	30.0 ± 21.9	13.5 ± 10.0	6.7 ± 13.5
	Puerto Rico strain	4[c]	26.8 ± 13.0	9.3 ± 8.3	5.7 ± 6.7
		4[d]	25.3 ± 14.1	5.0 ± 9.3	4.3 ± 8.5
C2F3G3	RAP-1	3	55.7 ± 7.5	66.0 ± 6.4	46.0 ± 16.5
	Mexico strain	4[c]	22.5 ± 15.5	5.3 ± 6.7	0.0 ± 0.0
		4[d]	29.3 ± 14.1	6.7 ± 10.2	0.5 ± 1.0
	Puerto Rico strain	4[c]	24.8 ± 16.6	3.3 ± 6.5	0.0 ± 0.0
		4[d]	28.0 ± 16.9	4.2 ± 5.0	0.0 ± 0.0

[a] Number of calves per group.
[b] Dilutions are reciprocal serum dilutions. Values are means ± standard deviations. All values obtained with RAP-1 are significantly different from values for *B. bigemina*-infected groups ($P \leq 0.05$, analysis of variance with Fisher's least-squares determination).
[c] Sera were collected at the time of homologous challenge.
[d] Sera were collected at the time of heterologous challenge.

This work was supported by Zimbabwe Ministry of Lands, Agriculture and Rural Resettlement, the African American Institute, U.S. Agency for International Development grants DHR 5600-G-00-1035-00 and DAN 4178-1-00, and USDA NRICGP 92-37204-8180.

We thank Beverly Hunter, Carla Robertson, and Kay Morris for technical assistance.

REFERENCES

1. **Callow, L. L.** 1977. Vaccination against bovine babesiosis, p. 121–149. *In* L. M. Miller, J. A. Pino, and J. J. McKelvy (ed.), Immunity to blood parasites of animals and man. Plenum Press, New York.
2. **Figueroa, J. V., and G. M. Buening.** 1991. In vitro inhibition of multiplication of *Babesia bigemina* by monoclonal antibodies. J. Clin. Microbiol. **29:**997–1003.
3. **Figueroa, J. V., G. M. Buening, D. A. Kinden, and T. J. Green.** 1990. Identification of common surface antigens among *Babesia bigemina* isolates using monoclonal antibodies. Parasitology **100:**161–175.
4. **Figueroa, J. V., G. M. Buening, V. S. Mishra, and T. F. McElwain.** 1992. Screening of a *Babesia bigemina* cDNA library with monoclonal antibodies directed to surface antigens. Ann. N. Y. Acad. Sci. **653:**122–130.
5. **Hoey, T.** 1988. Expression and purification of lacZ and trpE fusion proteins, p. 16.5.1–16.5.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Greene Publishing Associates and Wiley Interscience, New York.
6. **Irvin, A. D.** 1987. Control of tick-borne diseases. Int. J. Parasitol. **17:**649–657.
7. **Machado, R. Z., T. F. McElwain, C. E. Suarez, S. A. Hines, and G. H. Palmer.** 1993. *Babesia bigemina*: isolation and characterization of merozoite rhoptries. Exp. Parasitol. **77:**315–325.
8. **McCosker, D. J.** 1981. The global importance of babesiosis, p. 1–24. *In* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, New York.
9. **McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1987. Antibodies define multiple proteins with epitopes exposed on surface of live *Babesia bigemina* merozoites. J. Immunol. **138:**2298–2304.
10. **McElwain, T. F., L. E. Perryman, A. J. Musoke, and T. C. McGuire.** 1991. Molecular characterization and immunogenicity of neutralization sensitive *Babesia bigemina* merozoite surface proteins. Mol. Biochem. Parasitol. **53:**149–158.
11. **Mishra, V. S., T. F. McElwain, J. B. Dame, and E. B. Stephens.** 1992. Isolation, sequence and differential expression of the p58 gene family of *Babesia bigemina*. Mol. Biochem. Parasitol. **53:**149–158.
12. **Mishra, V. S., E. B. Stephens, J. B. Dame, L. E. Perryman, T. C. McGuire, and T. F. McElwain.** 1991. Immunogenicity and sequence analysis of recombinant p58: a neutralization sensitive, antigenically conserved *Babesia bigemina* merozoite surface protein. Mol. Biochem. Parasitol. **47:**207–212.
13. **Suarez, C. E., G. H. Palmer, S. A. Hines, and T. F. McElwain.** 1993. Immunogenic B-cell epitopes of *Babesia bovis* rhoptry-associated protein 1 are distinct from sequences conserved between species. Infect. Immun. **61:**3511–3517.