INFECTION AND IMMUNITY, Sept. 1991, p. 3340–3342
0019-9567/91/093340-03$02.00/0

Vol. 59, No. 9

Strain Variation of *Babesia bovis* Merozoite Surface-Exposed Epitopes

GUY H. PALMER,[1]* TERRY F. McELWAIN,[1] LANCE E. PERRYMAN,[1] WILLIAM C. DAVIS,[1] DAVID R. REDUKER,[2]† DOUGLAS P. JASMER,[1] VARDA SHKAP,[3] EUGENE PIPANO,[3] WILL L. GOFF,[2] AND TRAVIS C. McGUIRE[1]

Department of Veterinary Microbiology and Pathology, Washington State University,[1] and Animal Diseases Research Unit, Agricultural Research Service, U.S. Department of Agriculture,[2] Pullman, Washington 99164, and Department of Parasitology, Kimron Veterinary Institute, Beit Dagan, Israel[3]

Received 4 February 1991/Accepted 27 June 1991

***Babesia bovis* merozoites are exposed to antibodies during the extraerythrocytic phase, and surface polypeptides bearing exposed epitopes are possible immunogens. Monoclonal antibodies reactive with the merozoite surface bind either immunodominant epitopes expressed diffusely on the merozoite surface or, alternatively, epitopes expressed in a polar pattern. Epitopes expressed diffusely on the immunodominant 42- and 44-kDa merozoite polypeptides were not conserved among strains from geographically diverse regions. In contrast, epitopes expressed in a polar pattern on the merozoite surface were conserved among nine strains and clones. Identification of variable and conserved epitopes provides a basis for defining antigenic variation and cross-protective immunity.**

Immunization of cattle with merozoite antigens induces protection against challenge with the hemoprotozoan *Babesia bovis* (6, 13). Merozoite surface epitopes are exposed to the immune system during the extraerythrocytic phase prior to invasion of new target erythrocytes. We have identified five polypeptides (of 225, 60, 44, 42, and 16 kDa) that bear epitopes exposed on the surfaces of live merozoites (3, 10) of a Mexico strain (13). Cattle protected against homologous challenge with the Mo7 biological clone of the Mexico strain develop high titers of antibody to the 225-, 60-, 44-, and 42-kDa polypeptides (4), and immunization of cattle (with the recombinantly derived 44-kDa polypeptide) and rabbits (with the recombinantly derived 60-, 44-, and 42-kDa polypeptides) induces antibodies reactive with the surfaces of live Mexico strain merozoites (10, 14). In this study, we tested the conservation of merozoite surface-exposed epitopes among *B. bovis* strains from North America, Israel, and Australia.

The merozoite surface-reactive monoclonal antibodies (MAbs) used were developed against the Mexico strain and included single MAbs reactive with the 225- (MAb 23/8.34), 44- (23/70.174), and 16-kDa (23/28.57) polypeptides (3, 10). Three MAbs identified surface-exposed epitopes on the 42-kDa polypeptide (MAbs BABB35A4, 23/10.36.8, and 23/3.16.45), and four MAbs reacted with exposed epitopes of the 60-kDa polypeptide (BABB75A4, MBOC79B2, 23/38.120.8, and 23/53.156.77) (10). To identify the number of independent epitopes recognized by the multiple MAbs reactive with either the 42- or 60-kDa polypeptides, the ability of unconjugated MAb to inhibit binding of Sepharose 4B-conjugated MAb to radiolabeled *B. bovis* was determined by using a competition radioimmunoassay (9). Briefly, immunoglobulin was purified from ascitic fluid, quantitated, and coupled at 10 mg/ml to Sepharose 4B (9). The reactivity of each Sepharose-MAb conjugate was confirmed by immunoprecipitation of [^{35}S]methionine-labeled *B. bovis* Mexico strain as previously described (3). For the inhibition assay, a range of 0.1 to 30 μg of unconjugated MAb was incubated for 30 min at 4°C with 10^6 cpm of detergent-solubilized [^{35}S]methionine-labeled merozoites. Following the primary incubation, 5 μg of MAb conjugated to Sepharose was added and incubated for 30 min at 4°C. The Sepharose-MAb beads were washed four times, and Sepharose-bound radioactivity was determined following the addition of scintillant (9).

Each of the three merozoite surface-reactive MAbs against the 42-kDa polypeptide bound a different epitope, as demonstrated by a complete lack of cross-inhibition with each different free MAb (Table 1). Of the four MAbs reactive with the surface of the 60-kDa polypeptide, BABB75A4 and MBOC79B2 reciprocally inhibited binding, indicating recognition of the same or an overlapping epitope (Table 1). There was no inhibition between MAbs BABB75A4 or MBOC79B2 when tested against either 23/38.120.8 or 23/53.156.77, indicating a minimum of two independent surface-exposed epitopes on the 60-kDa polypeptide (Table 1). A one-way partial inhibition occurred between MAbs 23/38.120.8 and 23/53.156.77 (Table 1), indicating differences in affinity for an identical epitope or recognition of nonidentical epitopes on the 60-kDa polypeptide. Each conjugated MAb could be 100% inhibited by the same unconjugated MAb, although the concentration of unconjugated antibody required varied with each MAb (Table 1). No inhibition occurred between any of the conjugated MAbs and an unrelated unconjugated MAb (ANA22B1, reactive with *Anaplasma marginale*).

The three independent exposed epitopes on the 42-kDa polypeptide and a single epitope on the 44-kDa polypeptide are diffusely expressed over the surface of live Mexico strain merozoites as previously demonstrated by immunofluorescence assays (10). Conservation of these epitopes was examined for the following nine *B. bovis* strains and clones (location and year of isolation are given): Mexico (northern Mexico, 1979); Mo7 (biological clone derived from the Mexico strain) (11); Texas (southern Texas, 1978); Israel-BbV (Israel, 1963); Isr-B and Isr-C (biological clones derived from the Israel-BbV strain); Israel-BbTD (Upper Galilee, Israel, 1982); Australia-L (New South Wales, 1965) (7); and

* Corresponding author.
† Deceased.

TABLE 1. Epitope mapping by competitive inhibition radioimmunoassay

MAb-Sepharose conjugate[a] (polypeptide bound)	Inhibiting MAb[b,c]	% Inhibition[d]
BABB35A4 (42 kDa)	BABB35A4	100 (3 μg)
	23/3.16.45	0
	23/10.36.8	0
23/3.16.45 (42 kDa)	23/3.16.45	100 (3 μg)
	BABB35A4	0
	23/10.36.8	0
23/10.36.8 (42 kDa)	23/10.36.8	100 (0.3 μg)
	BABB35A4	0
	23/3.16.45	0
BABB75A4 (60 kDa)	BABB75A4	100 (3 μg)
	MBOC79B2	100 (3 μg)
	23/38.120.8	0
	23/53.156.77	0
MBOC79B2 (60 kDa)	MBOC79B2	100 (1 μg)
	BABB75A4	100 (1 μg)
	23/38.120.8	0
	23/53.156.77	0
23/38.120.8 (60 kDa)	23/38.120.8	100 (0.3 μg)
	23/53.156.77	100 (30 μg)
	BABB75A4	0
	MBOC79B2	0
23/53.156.77 (60 kDa)	23/53.156.77	100 (10 μg)
	23/38.120.8	0
	BABB75A4	0
	MBOC79B2	0

[a] Five micrograms of each MAb conjugated to Sepharose-4B was added per tube.
[b] All MAbs were tested in duplicate over a range of 0.1 to 30 μg.
[c] ANA22B1, an unrelated monoclonal antibody reactive with *Anaplasma marginale* MSP-1a, was used as a negative control with all Sepharose-bound MAbs.
[d] The amount at which 100% inhibition was observed is given in parentheses; 0% inhibition was at the highest concentration tested (30 μg).

Australia-S (Queensland, 1969) (7). The biological clones (B and C) of the Israel BbV strain were derived as previously described (11). Merozoites were purified by Percoll gradient centrifugation (12) and standardized to 5×10^7 merozoites, and viability was confirmed by staining with 6-carboxyfluorescein diacetate as previously described (8). All four MAbs (BABB35A4, 23/10.36.8, 23/3.16.45, and 23/70.174) bound 100% of live merozoites of the Mexico and Texas strains and the Mo7 biological clone as determined by immunofluorescence (Table 2). Binding occurred diffusely over the entire merozoite surface of live gradient-purified merozoites and of acetone-fixed intraerythrocytic merozoites in peripheral blood smears. Binding of merozoites sequestered in the cerebral vasculature was determined by using frozen cerebrum sections from calves with acute babesiosis. Prominent sequestration of parasitized erythrocytes within cerebral capillaries was confirmed for each strain by microscopic examination of Giemsa-stained frozen sections. All four MAbs bound 100% of Mexico and Texas strain merozoites within sequestered parasitized erythrocytes. None of the four MAbs reacted with merozoites of the two Australia strains, the two Israel strains, and the two Israel clones when examined by immunofluorescence using acetone-fixed parasitized erythrocytes in peripheral blood smears (Table 2). There was no MAb binding to the live merozoite surface or to merozoites within parasitized erythrocytes sequestered in cerebral capillaries (only Australia strains were examined).

To determine whether the 42- and 44-kDa polypeptide epitopes present in the North American strains were highly conformationally dependent, merozoites of all strains and clones were solubilized, reduced, denatured (2% sodium dodecyl sulfate, 1% Nonidet P-40, 100 mM dithiothreitol, 20% methanol) (9), and then reacted with the four MAbs in immunoblots. Antibody reactivity to denatured polypeptides was detected by sequential incubation with rabbit anti-murine immunoglobulin and ^{125}I-labeled protein A followed by autoradiography (3). All four MAbs bound the 42- and 44-kDa polypeptides in the North American strains, whereas no binding was detected with the Australia and Israel strains (data not shown). The resistance to denaturation of these North American strain epitopes is consistent with the existence of non-conformation-dependent linear epitopes (1, 5, 9). Therefore, the antigenic diversity among strains is likely due to variation within the epitope coding sequence rather than to a conformational effect from variation elsewhere in the polypeptide.

In contrast to the 42- and 44-kDa polypeptides, surface-exposed epitopes on the 225-, 60-, and 16-kDa polypeptides

TABLE 2. Conservation of merozoite surface-exposed epitopes among *B. bovis* strains

MAb	Fluorescence pattern[a]	Polypeptide (epitope)	Strain reactivity[b]
BABB35A4	Merozoite surface; diffuse	42kDa (epitope 1)	Mexico and Texas
23/3.16.45	Merozoite surface; diffuse	42kDa (epitope 2)	Mexico and Texas
23/10.36.8	Merozoite surface; diffuse	42kDa (epitope 3)	Mexico and Texas
23/70.174	Merozoite surface; diffuse	44kDa (epitope 1)	Mexico and Texas
23/8.34	Merozoite surface; polar + parasitized erythrocyte membrane	225kDa (epitope 1)	All strains tested
BABB75A4	Merozoite surface; polar	60kDa (epitope 1)	All strains tested
MBOC79B2	Merozoite surface; polar	60kDa (epitope 1)	All strains tested
23/38.120.8	Merozoite surface; polar	60kDa (epitope 2)	All strains tested
23/53.156.77	Merozoite surface; polar	60kDa (epitope[c])	All strains tested
23/28.57	Merozoite surface; polar	16kDa (epitope 1)	All strains tested

[a] Pattern of fluorescence on live merozoites and acetone-fixed intraerythrocytic merozoites in peripheral blood and sequestered in cerebral vasculature. Reactivity patterns were identical for all positive strains.
[b] Strains tested: Mexico, Texas, Israel BbV, Israel BbTD, Australia-L, and Australia-S. The Mo7 clone from the Mexico strain and the B and C clones of the Israel BbV strain reacted identically to the parent strains.
[c] One-way partial inhibition occurred with MAb 23/38.120.8 (Table 1).

are expressed in a polar pattern on live Mexico strain merozoites (3, 10). All six MAbs, binding a minimum of four independent epitopes (one each on the 16- and 225-kDa polypeptides and at least two on the 60-kDa polypeptide), reacted with merozoites from each of the nine strains and clones (Table 2). The polar binding pattern on intraerythrocytic merozoites of all strains was detected by immunofluorescence on peripheral blood smears and in sections of cerebral vasculature. All MAbs bound 100% of the live merozoites of the five strains and clones tested (live merozoites of the Israel strains and clones were not tested). In addition to merozoite binding, MAb 23/8.34 (reactive with the 225-kDa polypeptide) also bound the membranes of erythrocytes parasitized by each strain. Binding of MAb 23/8.34 to both the merozoite and the cytoplasmic side of the erythrocyte membrane has previously been described for the Mexico strain (10) and is hypothesized to be related to the invasion process. The presence of dual binding in all strains examined indicates that the mechanism leading to antigen deposition on the erythrocyte membrane is the result of a conserved function. All six MAbs reacted with the appropriately sized denatured polypeptide for each strain in immunoblots (data not shown), again consistent with linear epitopes that are not highly dependent upon the conformation of the native polypeptide.

The 42- and 44-kDa polypeptides are major components of the merozoite surfaces of the Mexico and Texas strains and are immunodominant in cattle that have recovered from Mexico strain infection and are protected against further challenge (4). The loss of four independent merozoite surface epitopes on these polypeptides in the Australia and Israel strains and clones suggests that dominant antibody responses are directed against antigenically variable molecules. Changes in virulence have been shown to be generated by sequential in vivo passage of parasitized erythrocytes (2). The variation in the immunodominant 42- and 44-kDa polypeptides cannot be attributed solely to in vivo passage following isolation, since epitopes are conserved on both the second-passage Texas strain and the multiple-passage Mexico strain and have been lost from both the one-passage Israel-BbTD and the multiple-passage Israel-BbV strains. In addition, passage-induced changes are attributed to selection of parasite subpopulations, and the identical reactivities of clones and uncloned parents indicates that selection of these antigenic variants did not occur in passaging. Identity between clones and parents for the examined epitopes was maintained both in strains bearing the 42- and 44-kDa polypeptide epitopes (Mexico strain and the Mo7 clone) and in strains which lost all four epitopes on these polypeptides (Israel BbV strain and clones B and C). Consequently, it appears that epitope variation reflects the presence of antigenic variants in different geographical regions. The rapidity with which these variants arise and their distribution among cattle and tick populations is unknown. Examination of additional strains from the Americas is needed to determine whether variation of 42- and 44-kDa polypeptide epitopes occurs among regional strains. In contrast to the variation observed in the diffusely expressed epitopes, the conservation of the polar-expressed epitopes in all strains examined suggests that these molecules have an obligate role in merozoite growth or replication. The polar distribution is consistent with localization to the apical-complex organelles demonstrated to be involved in *Plasmodium* merozoite invasion of erythrocytes (15). The marked conservation of these *B. bovis* epitopes, combined with their merozoite surface exposure, targets the polypeptides bearing these epitopes (225-, 60-, and 16 kDa) as candidates for experimental immunization and challenge.

We thank Mary Jo Hamilton, Bev Hunter, Pat Mason, and Kay Morris for technical assistance.

This work was supported by the United States-Israel Binational Agricultural Research and Development Fund Projects US-1080-86 and US-1561-88 and the United States Agency for International Development Cooperative Agreement DAN-4178-A-00-7056-00.

REFERENCES

1. **Allred, D. R., T. C. McGuire, G. H. Palmer, S. R. Leib, T. M. Harkins, T. F. McElwain, and A. F. Barbet.** 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc. Natl. Acad. Sci. USA **87**:3220–3224.
2. **Callow, L. L., L. T. Melors, and W. McGregor.** 1979. Reduction in virulence of *Babesia bovis* due to rapid syringe passage in splenectomized cattle. Int. J. Parasitol. **9**:333–338.
3. **Goff, W. L., W. C. Davis, G. H. Palmer, T. F. McElwain, W. C. Johnson, J. F. Bailey, and T. C. McGuire.** 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. Infect. Immun. **56**: 2363–2368.
4. **Hines, S. A., T. F. McElwain, G. M. Buening, and G. H. Palmer.** 1989. Molecular characterization of *Babesia bovis* merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol. Biochem. Parasitol. **37**:1–10.
5. **Keegan, K., and M. S. Collett.** 1986. Use of bacterial expression cloning to define amino acid sequences of antigenic determinants on the G2 glycoprotein of Rift Valley fever virus. J. Virol. **58**:263–270.
6. **Mahoney, D. F.** 1967. Bovine babesiosis: the immunisation of cattle with killed *Babesia argentina*. Exp. Parasitol. **20**:125–129.
7. **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.
8. **McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. J. Immunol. **138**:2298–2304.
9. **Palmer, G. H., S. D. Waghela, A. F. Barbet, W. C. Davis, and T. C. McGuire.** 1987. Characterization of a neutralization-sensitive epitope on the Am105 surface protein of *Anaplasma marginale*. Int. J. Parasitol. **17**:1279–1285.
10. **Reduker, D. W., D. P. Jasmer, W. L. Goff, L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1989. A recombinant surface protein of *Babesia bovis* elicits bovine antibodies that react with live merozoites. Mol. Biochem. Parasitol. **35**:239–248.
11. **Rodriquez, S. D., G. M. Buening, T. J. Green, and C. A. Carson.** 1986. Cloning of *Babesia bovis* by in vitro cultivation. Infect. Immun. **42**:15–18.
12. **Rodriquez, S. D., G. M. Buening, C. A. Vega, and C. A. Carson.** 1986. *Babesia bovis*: purification and concentration of merozoites and infected bovine erythrocytes. Exp. Parasitol. **61**:236–246.
13. **Smith, R. D., M. A. James, M. Ristic, M. Aikawa, and C. A. Vega.** 1981. Bovine babesiosis: protection of cattle with culture derived soluble *Babesia bovis* antigen. Science **212**:335–338.
14. **Suarez, C. E., G. H. Palmer, D. P. Jasmer, S. A. Hines, L. E. Perryman, and T. F. McElwain.** 1991. Characterization of the gene encoding a 60 kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. Mol. Biochem. Parasitol. **46**:45–52.
15. **Thomas, A. W., L. H. Bannister, and A. P. Waters.** 1990. Sixty-six kilodalton-related antigens of *Plasmodium knowlesi* are merozoite surface antigens associated with the apical prominence. Parasitol. Immunol. **12**:105–113.