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Common and Isolate-Restricted Antigens of *Anaplasma marginale* Detected with Monoclonal Antibodies

TRAVIS C. McGUIRE,[1]* GUY H. PALMER,[1] WILL L. GOFF,[2] MACK I. JOHNSON,[1] AND WILLIAM C. DAVIS[1]

Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University,[1] and the Hemoparasitic Diseases Research Unit, U.S. Department of Agriculture,[2] Pullman, Washington 99164

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***Anaplasma marginale*-infected erythrocytes were examined for the presence of maturation, isolate-restricted, and isolate-common antigens by indirect immunofluorescence with monoclonal antibodies. A panel of 18 monoclonal antibodies was used; none of the antibodies reacted with *Anaplasma ovis*, *Babesia bigemina*, *Babesia bovis*, *Trypanosoma brucei*, *Trypanosoma congolense*, or uninfected bovine erythrocytes. Antigens common to all six *A. marginale* isolates were detected by nine antibodies. Single isolates from Florida, Southern Idado, Northern Texas, and Virginia and two isolates from Washington state had four patterns of reactivity with a second panel of nine antibodies. Antigenically distinct stages were not detected, as sequential smears taken daily during acute infection had the same pattern of reactivity. The results demonstrate antigenic heterogeneity among isolates of *A. marginale* and the presence of common antigens. This information allows grouping of different isolates and, more importantly, provides a method for the indentification and isolation of common antigens for diagnostic tests.**

Anaplasmosis, a hemolytic disease of cattle and other ruminants (18), is caused by *Anaplasma marginale*, a member of the order *Rickettsiales* (18). Transmission is primarily by ticks and, to a lesser extent, biting flies (7, 9). The disease has a worldwide distribution and is one of the major cattle diseases in several areas of the world, including regions of the United States (18, 19). After infection, intraerythrocytic *A. marginale* organisms are seen within 3 to 6 weeks; parasitized erythrocytes increase in number over the next 4 to 9 days (18). At the peak of parasitemia, ca. 10% of the cattle die, whereas survivors gradually recover. The recovered animals remain persistently infected (carriers), with a small number having recurrent clinical disease (18). The recurrence in carriers may be caused by the appearance of antigenically different organisms or by other mechanisms.

There are four major lines of evidence indicating important differences among various isolates of *A. marginale*. (i) The most important difference is the inability of a given isolate to induce protection against heterologous isolate challenge. The ability of each isolate to confer cross-protection against heterologous challenge has not been studied in detail, but evidence for the failure of cross-protection has been reported previously (3, 12). For example, four calves that were carriers of the *A. marginale* Florida isolate were suspectible to challenge with the Oregon isolate (12). In the same study, three calves that were carriers of the Oregon isolate resisted challenge with the homologous isolate, and a single Florida isolate carrier resisted challenge with the Florida isolate. Additional studies by heterologous challenge of premunized animals have also indicated isolate differences (14). (ii) The morphology of the isolates differs, with some having an appendage ("tails"), whereas others, including the Florida isolate, lack an appendage ("tailess") (10, 11). There is no known function for the appendage. (iii) The reactivity of antibody in the complement fixation test is greater with homologous antigen than with antigen prepared from heterologous isolates (K. L. Kuttler and L. D. Winward, Vet. Microbiol., in press). (iv) There are protein differences between the Florida and Washington-Okanogan isolates, as determined by two-dimensional gel electrophoresis (1).

During experiments to produce monoclonal antibodies (MoAbs) to protein antigens of *A. marginale* for identification and isolation of protection-inducing antigens, we found antibodies that could differentiate among the various isolates examined. We report here the reactivity of a panel of MoAbs made to three geographically distinct isolates with these three and an additional three isolates. These antibodies were used to type the various isolates and to help identify common antigens among isolates for use in the development of diagnostic tests.

MATERIALS AND METHODS

***A. marginale* isolates.** The origin of the six isolates used is given in Table 1. The isolates were stored in liquid nitrogen as an infected blood stabilate with dimethyl sulfoxide (16). Ten milliliters of the thawed stabilates was inoculated intramuscularly into splenectomized, 6-month-old Holstein calves. The infected calves were monitored daily by determination of hematocrit, level of parasitemia, and clinical appearance. Blood smears for indirect immunofluorescence assays (IFA) were prepared daily from the day parasitemia reached 1% through the time of peak parasitemia with hemolytic crisis. The blood smears were prepared as follows: the blood was washed three times in phosphate-buffered saline (0.01 M sodium phosphate–0.14 M NaCl [pH 7.4]) at 675 × *g* with aspiration of the buffy coat, packed erythrocytes were suspended 1:1 in equal parts of fetal bovine serum–phosphate-buffered saline, and smears were made and stored at −40°C until use.

MoAbs. The production and initial screening of MoAbs to *A. marginale* have been previously described (4). The immunogens used were (i) fusion A, mixed Virginia and Washington-Okanogan *A. marginale* bodies purified on a Renografin gradient (5); (ii) fusion R, Virginia isolate purified on a Renografin gradient (5); (iii) fusion O, Washington-

* Corresponding author.

TABLE 1. Summary of *A. marginale* isolates used in this study

Designation	Yr isolated	Place of isolation (reference no.)
Florida	1955	Florida (19)
Virginia	1972	Southern Virginia (Kuttler and Winward, in press)
Washington O[a]	1981	Okanogan, Wash. (1)
Washington C[b]	1982	Clarkston, Wash. (Kuttler and Winward, in press)
Southern Idaho	1983	Caldwell, Idaho (this study)
Northern Texas	1977	Throckmorton, Tex. (Kuttler and Winward, in press)

[a] Washington-Okanogan.
[b] Washington-Clarkston.

Okanogan isolate purified on a sucrose gradient (20); (iv) fusion F, Florida isolate *A. marginale* bodies purified by differential centrifugation (G. H. Palmer and T. C. McGuire, J. Immunol., in press). All MoAbs described above were double cloned (4) before evaluation by IFA. The culture supernatants from the double-cloned cells were reacted in double immunodiffusion versus separate commercial antisera to mouse isotypes immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, IgM, and IgA (Bionetics Laboratory Products Division, Charleston, S.C.). Supernatants negative in immunodiffusion were concentrated and retested. Of the 18 MoAbs, 17 reacted with only one of the isotype antisera, whereas 1 MoAb gave a consistently negative reaction. The concentration of immunoglobulin in the supernatants was determined by single radial immunodiffusion (8) with the appropriate antiserum and the appropriate commercial purified immunoglobulin (Bionetics) as a standard. The 17 MoAbs of known isotype were concentrated to 0.1 mg/ml, and the other MoAb of unknown isotype was concentrated by an amount representing the mean required to reach 0.1 mg/ml by the MoAbs of known isotype. All subsequent testing was done with the concentrated antibodies.

IFA. The thawed blood smears were fixed in acetone for 5 min, and small squares were delineated with fingernail polish. Hybridoma supernatants were added and incubated for 30 min at room temperature. The slides were washed three times in phosphate-buffered saline, and then fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin was added for 30 min. The slides were washed three times and examined by fluorescent microscopy. Estimation of the percentage of infected erythrocytes reacting with each antibody was done by counting the number of IFA-positive organisms per field and comparing this with the number of organisms per field on an adjacent part of the slide that was stained with Wrights stain and examined under bright-field illumination.

Reactivity with other hemoparasites. The specificity of the MoAbs for *A. marginale* was tested by IFA on blood films from cattle infected with *Babesia bigemina* (13) and *Babesia bovis* from Mexico (15), sheep infected with *Anaplasma ovis* from Idaho (17), and rats infected with *Trypanosoma brucei* (2) and *Trypanosoma congolense* from Uganda (6).

RESULTS

Reactivity of initial hybridoma supernatants with homologous and heterologous isolates. The results of the IFA with the initial hybridoma supernatants from the four fusions are presented in Table 2. Analysis of 2,534 supernatants by IFA yielded 229 positive for the homologous organisms, with 220 supernatants binding *A. marginale* determinants common to the three primary isolates and 9 supernatants binding none or only some of the heterologous isolates. Cell lines producing the nine isolate-restricted antibodies and another nine cell lines producing isolate-common antibodies were double cloned, and the supernatants were concentrated as described above.

Differentiation of isolates by MoAbs. The concentrated MoAbs were tested by IFA with six *A. marginale* isolates obtained from separate areas in the United States (Table 3). MoAbs A-15^{D2}, A-22^{B1}, F-34^{C1}, and F-35^{A1} bound to 100% of the *A. marginale* bodies present in infected erythrocytes of all isolates. MoAbs F-19^{A6}, O-50^{A2}, O-58^{A2}, O-66^{A2}, and O-70^{A2} reacted with *A. marginale* bodies from all isolates; however, organisms in only ca. 70% of the infected erythrocytes on any slide were stained. Four MoAbs (R-17^{A6}, R-19^{A6}, R-83^{B3}, R-94^{C1}) made to the Virginia isolate were specific for the Virginia isolate; these MoAbs bound to ca. 45% of the organisms present. Two MoAbs made to the Washington-Okanogan isolate bound only to that isolate and the Washington-Clarkston isolate, with each MoAb binding ca. 45% of the *A. marginale* bodies. A single MoAb (F-22^{A4}) to the Florida isolate was specific to that isolate, with recognition of 50% of the organisms present. With the isolate-restricted MoAbs used as a panel (Table 3), the six *A. marginale* isolates could be divided into four groups based on reactivity patterns: (i) the Virginia isolate, (ii) the Florida isolate, (iii) the two Washington isolates, and (iv) the northern Texas and southern Idaho isolates.

The nine isolate-restricted MoAbs were assayed by IFA on blood smears of Virginia, Florida, and Washington-Okanogan isolates from different calves by a different laboratory. The results were scored by a different observer, and the outcome was the same as that shown in Table 3.

Morphological appearance of initial bodies recognized by MoAbs. The MoAbs described so far yielded a staining pattern characterized by a round, uniformly stained *A. marginale* body within the unstained erythrocytes. In contrast, MoAbs O-23^{A5} and O-24^{D5} reacted with *A. marginale* bodies of all isolates except the Florida isolate (Table 3) and gave a different pattern of staining. In most affected erythrocytes, the staining pattern resembled that described for tails (10, 11). Wedge-shaped structures were present in some infected cells, with circles and crescents present in others. These two MoAbs bound to all of the organisms present on the smears of recognized isolates.

Reactivity of MoAb in different stages of acute infection. The pattern and percentage of *A. marginale* bodies bound by each MoAb was examined on blood smears prepared daily, starting from the day parasitemia reached 1% through the

TABLE 2. IFA of hybridoma supernatants on *A. marginale*-infected erythrocytes

Immunizing isolate	Fusion type	No. tested	No. positive with homologous isolate	No. negative with at least one heterologous isolate
Washington O[a] and Virginia	A	480	26	0
Virginia	R	790	75	4
Washington O[a]	O	970	77	4
Florida	F	294	42	1

[a] Washington-Okanogan.

TABLE 3. Reactivity of monoclonal antibodies with different *A. marginale* isolates

Mo Ab (immunoglobulin isotype)	Reactivity for the following isolates:					
	Virginia	Florida	Washington O[a]	Washington C[b]	Northern Texas	Southern Idaho
A-15^{D2} (G3), A-22^{B1} (63) F-34^{C1} (?), F-35^{A1} (G3)	+	+	+	+	+	+
F19^{A6} (G2a), O-50^{A2} (G1), O-58^{A2} (G2a), O-66^{A2} (G1), O-70^{A2} (G1)	+	+	+	+	+	+
R-17^{A6} (G1), R-19^{A6} (G1), R-83^{B3} (G3), R-94^{C1} (G2b)	+	−	−	−	−	−
O-11^{C2} (G3), O-12^{B5} (G1)	−	−	+	+	−	−
F-22^{A4} (G2b)	−	+	−	−	−	−
O-23^{A5} (G1), O-24^{D5} (G1)	+	−	+	+	+	+

[a] Washington-Okanogan.
[b] Washington-Clarkston.

time of peak parasitemia for each isolate. No changes in the binding pattern were observed. There was no change in the percentage of organisms bound by each antibody; those binding 100, 70, 50, or 45% of the organisms present bound similar percentages, regardless of the level of parasitemia.

Reactivity with other hemoparasites. All 18 MoAbs were reacted with blood smears from animals infected with two *Babesia* spp., two *Trypanosoma* spp., and *A. ovis*. None of the MoAbs bound to the organisms when tested by IFA.

DISCUSSION

We identified two collections of MoAbs which identify determinants common to all six isolates of *A. marginale* tested. MoAbs A-15^{D2}, A-22^{B1}, F-34^{C1}, and F-35^{A1} bound to all organisms from every isolate, regardless of the extent of parasitemia. The second collection, F-19^{A6}, O-50^{A2}, O-58^{A2}, O-66^{A2}, and O-70^{A2}, bound to the same organisms as the first collection, except that only 70% of the organisms were recognized. This 70% binding was uniform among the different isolates and at all stages of parasitemia. The explanation for the failure of MoAbs to bind to a constant number (30%) of *A. marginale* bodies in a blood smear is unknown. The MoAbs could recognize a common maturation antigen present on a constant percentage of organisms in all stages of acute infection.

In addition to the MoAbs that recognized common determinants, nine MoAbs that did not react with all isolates were made. These isolate-restricted MoAbs could be used as a panel to classify the isolates into four groups. The panel reacted identically with the two Washington isolates, which comprised one group. The northern Texas and southern Idaho isolates formed a second group, whereas the Virginia isolate and the Florida isolates had unique reactivities. Seven of the nine MoAbs reacted with only 45 to 50% of the *A. marginale* bodies present in the blood smears. The remaining two antibodies reacted with 100% of the organisms in all isolates except the Florida isolate. The Florida *A. marginale* isolate is tailess, and these two MoAbs may have recognized appendage antigens present in the other organisms, which are all tailed (11).

This panel of MoAbs will be used as follows: those MoAbs recognizing common determinants will be used to identify *A. marginale* bodies in vertebrate and invertebrate host tissues, including cell culture. The MoAbs that were reactive with common determinants will be evaluated as the basis for improved diagnostic tests. The isolate-restricted MoAbs will be used as a panel to classify *A. marginale* organisms; this will allow the correlation of geographical distribution with antigenic differences and the identification of organisms in cross-immunity experiments as being a primary persistent infection or a secondary, heterologous challenge.

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