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Serotyping Isolates of *Anaplasma phagocytophilum* by Using Monoclonal Antibodies

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Ten mouse monoclonal antibodies (MAbs) that react with *Anaplasma phagocytophilum* (the human granulocytic ehrlichiosis agent) Webster isolates were developed. Seven different isolates of *A. phagocytophilum* were subtyped with these MAbs. Western blot analysis revealed that these MAbs reacted mainly with 41- to 46-kDa Msp2 proteins. Six MAbs reacted with all isolates. Four other MAbs reacted with human isolates from Wisconsin, but not with human isolates from New York or with animal isolates. Three different serotypes were identified. These features may lead to the development of other specific MAbs in order to provide tools for antigenic characterization of human isolates of *A. phagocytophilum*.

Anaplasma phagocytophilum (comb. nov. of the human granulocytic ehrlichiosis [HGE] agent, *Ehrlichia phagocytophila*, and *Ehrlichia equi*) is included among emerging ehrlichial infections of humans and animals. The agent infects granulocytes and causes mild to severe clinical manifestations, including fever, headache, leukopenia, thrombocytopenia, and anemia among others. Most clinical cases of human *A. phagocytophilum* infection have been reported from the United States, but some were from European countries, and several isolates have been cultivated in vitro. Whereas infection in humans is most often self-limited, a persistent subclinical infection in deer and mice may allow for long-term survival in these reservoir hosts.

The sequences of 16S rRNA and the *groE* gene of *A. phagocytophilum* isolates from different origins are nearly identical. However, *ankA* gene analysis distinguishes isolates from different geographic locations (3, 8, 10) and separates *A. phagocytophilum* into three clades: two in North America and one in Europe. Although, Western blot antigen profiles of isolates from the same geographic regions show little diversity, marked antigenic variation is noted among isolates from different geographic regions. The chief source of antigenic variation is due to differences in the major immunodominant outer surface proteins of *A. phagocytophilum*, which are encoded by a multicopy gene family called *msp2* (or *p44*). This gene possesses two conserved regions flanking a unique hypervariable core with three extremely hypervariable central domains that are likely to provide surface phenotype diversity (7).

Monoclonal antibodies (MAbs) are potentially useful for identification and subclassification of ehrlichial agents because of their highly specific affinity for unique epitopes, potentially including those encoded in *msp2* hypervariable regions. Many MAbs specific for other rickettsial agents have been developed and proven useful for taxonomic purposes (6, 11–14). In this study, we assessed MAbs specific for the *A. phagocytophilum* Webster strain (isolated in a human in Wisconsin) and examined their reactivity against isolates from different hosts and geographic locations.

Seven isolates of *A phagocytophilum* from diverse geographic locations were used in this study (Table 1). All isolates were cultivated in the human promyelocytic leukemia cell line HL-60 (American Type Culture Collection, Manassas, Va.) in RPMI 1640 medium with 10% fetal bovine serum for <10 passages, with the exception of the BDS strain, which was initially isolated by inoculation of human blood into a horse and then cultivated in HL-60 cells for <3 passages. *A. phagocytophilum* isolates were purified by a method described previously (4). Briefly, infected HL-60 cells were mechanically disrupted, and cell debris was removed by centrifugation. Bacteria in the supernatants were harvested and purified by Renografin density gradient purification.

Six-week-old female BALB/c mice were immunized with cell-free *A. phagocytophilum* Webster strain. The bacteria were harvested after mechanical disruption of infected HL-60 cells and separated from cell debris by centrifugation at 150 × *g* for 10 min. Mice were immunized with 500 μl of cell-free bacteria mixed with Freund's complete adjuvant (vol/vol) by intraperitoneal injection three times at 1-week intervals. One and 2 weeks after the third injection, the mice were boosted twice by injection of 500 μl of cell-free bacteria into the tail vein. Five days after the last boost, the mice were euthanized, and spleen cells were fused with SP2/0-Ag14 myeloma cells by using 50% polyethylene glycol (molecular weight, 1,300 to 1,600; Sigma Chemical Co., St. Louis, Mo.). Fused cells were grown in hybridoma medium (Seromed, Berlin, Germany) with 17% fetal bovine serum (Gibco BRL) and hypoxanthine-aminopterin-thymidine selective medium (Sigma Chemical Co.) at 37°C in a humidified atmosphere supplemented with 5% CO_2. The culture supernatants were screened for antibodies to the *A. phagocytophilum* Webster strain by immunofluorescence assay (IFA). Hybridomas producing antibodies were subcloned twice by limiting dilution. The isotype of the MAbs was determined with a mouse MAb isotyping kit (RPN29; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). The

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TABLE 1. Isolates of *A. phagocytophilum* used in this study and their reactivity to MAbs by IFA

Isolate	Origin	Antibody reactivity by IFA[a]			Location
		Group A	Group B	Rabbit PAbs	
96HE27 (Webster)	Human	+	+	+	Wisconsin
96HE54 (Spooner)	Human	+	+	+	Wisconsin
97HE97	Human	+	+	+	Wisconsin
E. equi MRK	Horse	+	+	+	California
96HE158 (NY8)	Human	−	+	+	New York
MD-HGE	Human	−	+	+	New York
97E13	Dog	−	+	+	Minnesota
BDS	Human[b]	−	−	+	Wisconsin

[a] Group A includes 1D1B12, 1D1D9, 3H5H7 and 3H5H5. Group B includes 5B3B11, 5B3C10, 5B3D8, 5B3G6, 5B3H4, and 5B3H5. PAbs were raised in a rabbit exposed to the *A. phagocytophilum* Webster strain in our laboratory.

[b] Originally from a human patient, but the agent was purified from horse neutrophils after experimental infection.

specificity of the MAbs was tested by IFA and with *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Neorickettsia helminthoeca*, *Neorickettsia risticii*, *Neorickettsia sennetsu*, *Rickettsia rickettsii*, *Rickettsia prowazekii*, *Rickettsia montanensis*, *Rickettsia conorii*, *Coxiella burnetii*, *Bartonella henselae*, and *Bartonella quintana* as antigens.

Purified, cell-free *A. phagocytophilum* isolates and HL-60 control cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) under reducing conditions. Prestained SDS-PAGE standards were used as a reference. The separated antigens were transferred to nitrocellulose membranes. After protein transfer, the nitrocellulose membranes were incubated overnight in phosphate-buffered saline (PBS) with 3% skim milk to block nonspecific binding. After a 15-min wash in PBS, the membranes were incubated with supernatant containing MAb diluted 1:10 in 3% skim milk–PBS at room temperature for 1 h and washed three times with PBS for 5 min. After incubation at room temperature for 1 h with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) diluted 1:1,000 in 3% skim milk–PBS and three washes in PBS, color was developed with a Bio-Rad ALP coloring kit.

Screening of MAbs by IFA using the Webster strain of *A phagocytophilum* shows that 6.2% of hybridoma clones (26 of 418) produced reactive antibodies. Subsequently, 10 antibody-producing hybridomas (1D1B12, 1D1D9, 3H5H7, 3H5H5, 5B3B11, 5B3C10, 5B3D8, 5B3G6, 5B3H4, and 5B3H5) with titers in culture supernatant that ranged from 8 to 256 were selected. MAbs from all 10 clones recognized the *A. phagocytophilum* Webster strain, but not *E. chaffeensis*, *E. canis*, *N. risticii*, *N. sennetsu*, *N. helminthoeca*, *R. rickettsii*, *R. prowazekii*, *R. montanensis*, *R. conorii*, *C. burnetii*, *B. henselae*, *B. quintana*, or HL-60 cells. All MAbs were of the IgG1 isotype.

IFA and Western blot analysis of the selected MAbs on seven isolates of *A. phagocytophilum* revealed three different phenotypes (Tables 1 and 2). All isolates reacted with *A. phagocytophilum* Webster strain rabbit polyclonal antibody (PAb) raised in our laboratory. Four MAbs (group A) produced by hybridomas 1D1B12, 1D1D9, 3H5H7, and 3H5H5 reacted with the human isolates from Wisconsin (Webster, Spooner, and 97HE97) and with the equine MRK strain. Six MAbs (group B) produced by hybridomas 5B3B11, 5B3C10, 5B3D8, 5B3G6, 5B3H4, and 5B3H5 derived from the same master plate reacted with all isolates except the BDS strain.

Western blot analysis revealed that MAbs reacted mainly with the 41- to 46-kDa Msp2 proteins of *A. phagocytophilum*. The Western blot patterns of MAbs were almost identical in each group (Table 2 and Fig. 1). The MRK strain (California) reacted as a single band with all MAbs, while the BDS strain (Wisconsin) did not react with any.

Msp2, p44, and 44-kDa antigen are names for the major immunodominant surface protein of *A. phagocytophilum*. Encoded by multiple gene paralogs in a single gene family dispersed throughout the genome, Msp2 is characterized by amino and carboxy termini that are highly conserved among paralogs and by a central hypervariable domain (2, 15, 16). Models predict that the conserved regions are likely to be less antigenic and are probably membrane associated (2). MAbs specific for the 42- to 45-kDa Msp2 of *A. phagocytophilum* have been characterized previously (9) and generally react between a narrow range of molecular masses from 36 to 46 kDa. Although the 78- and 180-kDa bands revealed by the MAbs in isolates from humans in the upper Midwest may represent reactivity with epitopes of other ehrlichial proteins, they may also represent immunoreaction with epitopes in multimers of individual Msp2 proteins.

Among the MAbs examined, some recognized Msp2 epitopes in isolates derived from humans in the upper Midwest only. Analysis of sequences and loci of *msp2* paralogs transcribed by *A. phagocytophilum* suggest that variations in their sequences are not random but are restricted by geography and host (2, 7). Thus, the most likely explanation for these observations is that there are only a limited number of genomic clones and *msp2* within a specific geographic region (2) and that the predominant *msp2* paralogs expressed by these related strains share common epitopes (17).

However, this hypothesis is contradicted by the observation that two isolates of *A. phagocytophilum* from the upper Midwest—strain 97E13, which was isolated from a dog in Minnesota; and BDS, which was isolated from a human in Wisconsin but was first grown by in vivo infection of horse neutrophils—were not recognized by these MAbs. It is now established that transcription of *A phagocytophilum msp2* paralogs is differen-

TABLE 2. Western blot patterns of MAbs raised against the *A. phagocytophilum* Webster strain on seven other *A. phagocytophilum* isolates

Isolate	Origin	Mol mass (kDa) of antigen reacting to MAb		Location
		Group A	Group B	
96HE27 (Webster)	Human	42, **43,** 45, 78, 180	41, **44, 46**	Wisconsin
96HE54 (Spooner)	Human	44, 45	**44,** 46	Wisconsin
97HE97	Human	36, 38, **44, 45**	**43,** 45	Wisconsin
E. equi MRK	Horse	**44**	**44**	California
96HE158 (New York 8)	Human		42, **44,** 46	New York
MD-HGE	Human		**44**	New York
97E13	Dog		**41, 42**	Minnesota
BDS	Human[b]			Wisconsin

[a] IgG1 was the isotype for groups A and B. Major antigens are shown in boldface.

[b] Originally human, but the agent was purified from horse neutrophils after experimental infection.

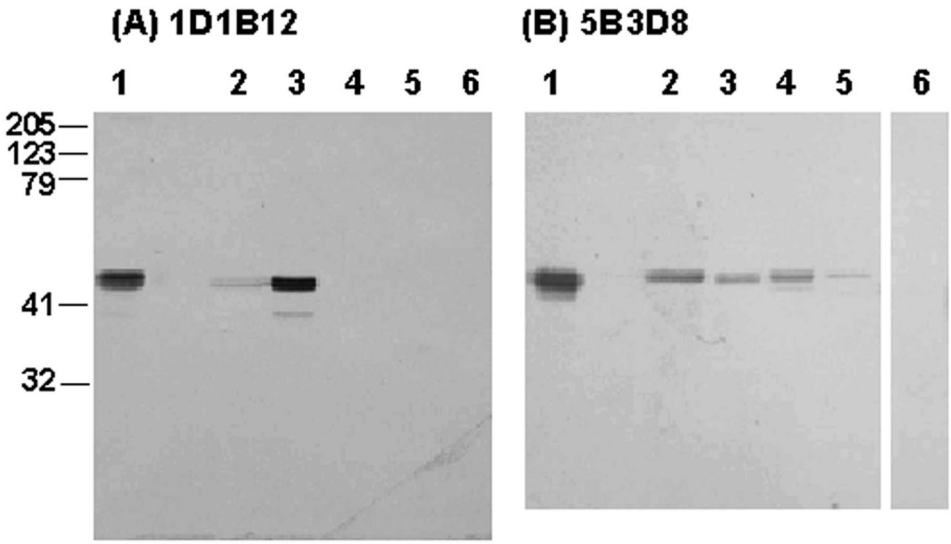

FIG. 1. Western immunoblot analysis of the five *A. phagocytophilum* isolates 96HE27 (Webster; lane 1), 96HE54 (Spooner; lane 2), 97HE97 (lane 3), 96HE158 (New York 8; lane 4), and MD-HGE (lane 5) and uninfected HL-60 cells (lane 6) with MAbs 1D1B12 (A) and 5B3D8 (B). The blot shown in panel A recognized the isolates from Wisconsin only.

tially regulated depending upon the host environment, whether in cell culture, ticks, mice, horses, or humans (5, 7, 17). Thus, these limited data raise the possibility that human infection may influence or select which Msp2-expressing *A. phagocytophilum* clones survive among a population limited by a fixed number of *msp2* genes.

As for the related species *Anaplasma marginale*, it has been suggested that *A. phagocytophilum* antigenic variability results from the transcription of one or a few of the existing paralogs of *msp2*, complemented by gene conversion and recombination of hypervariable sequences (1, 2). Since *A. phagocytophilum* Webster strain rabbit PAb reacted with the BDS strain, while group A and group B MAbs did not, it is possible that the BDS strain expresses a paralog of Msp2 that is usually not expressed by other strains and that PAb may react with epitopes present in the conserved, probably membrane-associated, domains of Msp2.

These MAbs distinguish three serotypes. Serotype 1 strains comprise human isolates from Wisconsin and a horse isolate from California and react with MAbs from group A and B and with the PAb. Serotype 2 strains comprise two human isolates from New York and a dog isolate from Minnesota that are reactive with group B and with the PAb but not with group A MAbs. Finally, serotype 3 includes the BDS strain, which reacted with the PAb but not with any MAbs. Although these antibodies may effectively distinguish isolates at a single time point, it is unlikely that the MAb serotype is a fixed antigenic attribute of the bacterium. Whether the MAb serotype could be useful to distinguish strains by geography or by pathogenicity to humans will require further investigation of a large number of geographically diverse strains under various conditions in vivo, in vitro, and in ticks. These data provide further evidence that specific serotypes exist among isolates from geographically constrained area. These features encourage the development of other specific MAbs to provide tools for antigenic characterization of human isolates of *A. phagocytophilum*.

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