JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1098–1103 Vol. 33, No. 5
0095-1137/95/$04.00+0

Serologic Cross-Reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and Human Granulocytic Ehrlichia

J. STEPHEN DUMLER,[1]* KRISTIN M. ASANOVICH,[1] JOHAN S. BAKKEN,[2] PHILIP RICHTER,[3]†
ROBERT KIMSEY,[4] AND JOHN E. MADIGAN[3]

Department of Pathology, University of Maryland Medical Center, Baltimore, Maryland 21201[1]; Section of Infectious Diseases, The Duluth Clinic, Duluth, Minnesota 55805[2]; and Department of Medicine and Epidemiology[3] and Department of Entomology,[4] School of Veterinary Medicine, University of California, Davis, Davis, California 95616

Received 2 December 1994/Returned for modification 5 January 1995/Accepted 9 February 1995

Homology in the 16S rDNAs shows that the agent of human granulocytic ehrlichiosis (HGE) is closely related to the veterinary pathogens *Erlichia equi* and *Erlichia phagocytophila*. After HGE, patients develop antibodies reactive with *E. equi* and *E. phagocytophila*; thus, we hypothesized that these species are closely related and share significant antigenicity. Antisera from humans, horses, dogs, and cattle were tested by indirect fluorescent-antibody assay (IFA) for antibodies reactive with *E. equi* and other ehrlichiae and tested by immunoblot to identify the specific reactions with *E. equi*. All convalescent-phase sera from human patients with HGE and from animals infected or immunized with *E. equi* or *E. phagocytophila* had antibodies reactive with *E. equi* by IFA; no reactions with *Ehrlichia chaffeensis* occurred with these sera, and only one horse naturally infected with *E. equi* had a serologic reaction against *Ehrlichia sennetsu*. Human and animal sera obtained after infection or immunization with other *Ehrlichia*, *Rickettsia*, and *Bartonella* species did not react with *E. equi* by IFA. *E. equi* immunoblots revealed as many as 19 bands with equine anti-*E. equi* serum. All HGE agent, *E. equi*, and *E. phagocytophila* antisera tested reacted with a 44-kDa antigen of *E. equi*, while other anti-*Ehrlichia* spp. sera reacted with this antigen rarely or not at all. HGE agent, *E. equi*, and *E. phagocytophila* antisera but not other sera also reacted occasionally with 25-, 42-, and 100-kDa antigens. Most sera reacted with antigens between approximately 56 and 75 kDa, probably heat shock proteins. The HGE agent, *E. equi*, and *E. phagocytophila* share significant antigenicity by IFA and immunoblot. Coupled with the nearly identical nucleotide sequences of 16S rRNA genes, these data indicate that *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichia are closely related or identical species.

Infections caused by members of the genus *Ehrlichia* have been best recognized in animals, and they have been recognized more recently in humans (2, 8, 19). Currently, three distinct groups (genogroups) of the genus *Ehrlichia* are identified on the basis of similarities in the nucleotide sequences of the 16S rRNA genes (1). These groups may be designated by the historically precedented prototype species: the *Ehrlichia canis* group includes *E. canis*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ewingii*, and perhaps *Cowdria ruminantium*; the *Ehrlichia phagocytophila* group includes *E. phagocytophila*, *Ehrlichia equi*, a newly recognized but unnamed human agent known as the human granulocytic ehrlichia (2, 4), *Ehrlichia platys*, and perhaps some members of the genus *Anaplasma*; and the *Ehrlichia sennetsu* group includes both *E. sennetsu* and *Ehrlichia risticii* and probably *Neorickettsia helminthoeca*. Among all of these species, only those which frequently infect mononuclear phagocytes have been cultivated in vitro. Although some serologic cross-reactivity has been reported to exist among the species of *Ehrlichia*, antigenic cross-reactivity among members of the *E. canis* group and among members of the *E. sennetsu* group is much stronger than that between members of different genogroups.

Ehrlichiae are often described as leukocytic rickettsiae, and each species has a tropism for cells of a specific hematopoietic lineage. *E. canis*, *E. chaffeensis*, *E. muris*, *E. sennetsu*, and *E. risticii* are predominantly associated with infection of mononuclear phagocytes and may be cultivated in vitro. Conversely, all of the members of the *E. phagocytophila* genogroup and *E. ewingii* are detected in peripheral blood granulocytes. Because granulocytic ehrlichiae have not been cultivated in vitro, comparatively little is known about relationships among members of the *E. phagocytophila* group.

E. phagocytophila is the etiologic agent of tick-borne fever, a febrile infection of sheep, goats, and cattle, and is believed to occur predominantly in Europe and perhaps in Africa (19). *E. equi* is a morphologically indistinguishable species which causes a febrile infection in horses and is also believed to be tick transmitted (11). This disease is infrequently diagnosed but is thought to occur widely throughout North and South America and perhaps may affect horses in Europe (16). Experimental transmission of *E. equi* from horses to other species including cattle, goats, sheep, and nonhuman primates is inefficient (13). In spite of these experimental data, *E. equi* has been isolated by primary inoculation of blood from naturally infected dogs into susceptible horses (15), and high seroprevalence rates in dogs have been documented in some regions (22).

Anderson et al. (1) recently proposed that *E. phagocytophila* and *E. equi* probably represent a single species on the basis of near identical sequences of the 16S rRNA genes present in these organisms. We have recently described human infection with a closely related (or identical) species on the basis of amplification of the 16S rRNA gene present in the blood of infected patients who had ehrlichial morulae detected in pe-

* Corresponding author. Phone: (410) 706-1820. Fax: (410) 706-8414. Electronic mail address: jdumler@umabnet.ab.umd.edu.

† Present address: Office of Veterinary Clinical Services, Office of Animal Resources, University of California, San Diego, La Jolla, CA 92093.

ripheral blood neutrophils (2, 4). Despite the significant homology among these species and isolates, few other data exist to support the grouping of the agents within a single species. Classification within this genus has been difficult and in the past was based mostly upon morphology, vertebrate and invertebrate hosts, cell tropism, geographic location, and some serologic cross-reactions (21). In fact, since the relationships among the members of the *E. phagocytophila* genogroup are incompletely defined, infections by members of this genogroup are presumed to be caused by the agent described in that mammalian species and in the geographic region where the animal resides. Objective methods for identification and classification are not readily available.

In order to better define the antigenic relationships and to provide additional data possibly useful for identification and classification of *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichia, we studied the serologic reactions of horses, cattle, dogs, and humans infected with these *E. phagocytophila* genogroup granulocytic ehrlichiae. Each of these agents elicited strong cross-reactive antibodies when tested by indirect fluorescent-antibody assay (IFA) with *E. equi*- and *E. phagocytophila*-infected leukocytes as the antigen, while no antibodies directed against *E. chaffeensis* and rare reactions with *E. sennetsu* were detected. Moreover, sera from these animals and humans had antibodies that reacted with a large number of *E. equi* proteins by immunoblot. The close genetic relationships and strong IFA and immunoblot serologic cross-reactions elicited by these granulocytic ehrlichial agents suggest that *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichia are similar or identical (1, 4) and yet are distinct from other recognized species in the genus *Ehrlichia*. The marked differences in biologic and ecologic associations cannot be accounted for by these studies, and thus an explanation of the observed differences requires more definitive molecular and biologic analysis to establish the true relationships among these human and animal pathogens.

MATERIALS AND METHODS

Serum samples. Equine, canine, bovine, and human infections with granulocytic ehrlichiae were recognized by the presence of a typical clinical presentation and morulae present only in circulating neutrophils. Serum was obtained during the acute phase of the infection when possible and 2 weeks to several months later during convalescence. All sera were stored frozen until serologic testing was performed. Sera were obtained from two convalescent horses experimentally infected with *E. equi* (one serum was obtained from a horse infected with the MRK strain of *E. equi* [11] at the Equine Research Laboratory, School of Veterinary Medicine, University of California, Davis, and the other sample was obtained courtesy of R. Corstvet, Louisiana State University, Baton Rouge), one convalescent horse in Minnesota naturally infected with *E. equi* (courtesy of B. Greig, University of Minnesota, St. Paul), two dogs from Minnesota naturally infected with *E. equi* (courtesy of B. Greig), 10 cattle from Switzerland naturally infected with *E. phagocytophila* (courtesy of J. Liz, University of Neuchâtel, Neuchâtel, Switzerland), and eight human patients with granulocytic ehrlichiosis (2). Control sera were obtained from two normal *E. equi*-seronegative horses, one horse convalescent from experimental *E. risticii* infection, one *E. canis*-seronegative dog, two *E. canis*-seropositive dogs, two dogs convalescent from experimental *E. ewingii* infection, one *E. equi*-seronegative normal bovine, three normal healthy human volunteers with no history of ehrlichiosis, two humans convalescent from Rocky Mountain spotted fever (*Rickettsia rickettsii* infection), one human convalescent from scrub typhus (*Rickettsia tsutsugamushi* infection), and two humans convalescent from *E. chaffeensis* infection. To test the specificity of the *E. equi* immunoblots, we used additional antisera prepared by experimental infection or inoculation with live organisms, including mouse anti-*Rickettsia typhi* (courtesy of Abdu Azad, University of Maryland School of Medicine), mouse anti-*E. sennetsu*, and mouse anti-*Rochalimaea henselae* and anti-*Rochalimaea quintana* (both courtesy of Philippe Brouqui, Marseilles, France). Inactivated antigens suspended in adjuvant (Ribi Immunochem, Hamilton, Mont.) were used to immunize rabbits for the preparation of anti-*E. sennetsu* serum, anti-*E. risticii* serum, and anti-*E. chaffeensis* serum (5). Normal rabbit serum and normal mouse serum were obtained without any ehrlichial immunization. Sera were stored frozen until used.

Experimental *E. equi* infections for antigen production. Horses were inoculated intravenously with fresh or thawed blood stabilates from horses previously infected with *E. equi* MRK as described previously (16). A preinoculation serum was obtained, and convalescent-phase serum was then obtained 3 to 4 weeks after the infection to verify by serologic testing that the resulting illness and typical ehrlichial morulae that were obtained resulted from *E. equi* infection. For the preparation of *E. equi*-infected leukocytes, EDTA-anticoagulated blood was obtained daily after the onset of fever and the percentage of neutrophils which contained morulae was calculated by assessing Wright-stained peripheral blood smears. Blood (2 to 3 liters) was collected in acid-citrate-dextrose anticoagulant for preparation of the antigen when 40 to 70% of the peripheral blood neutrophils contained morulae. Bovine *E. phagocytophila*-infected leukocytes for antigen preparation were provided on acetone-fixed glass multiwell slides (courtesy of J. Liz) and were prepared as previously described (14).

Preparation of *Ehrlichia* species antigens. *E. equi*-infected leukocytes were harvested from acid-citrate-dextrose-anticoagulated blood by sedimentation at 1 *g* at 4°C overnight. Erythrocytes were removed from the leukocyte-rich fraction by osmotic lysis, and infected leukocytes were harvested by centrifugation. The pellet was suspended to approximately 10^5 leukocytes per ml in phosphate-buffered saline (PBS) with 2% fetal bovine serum and 0.05% sodium azide. Samples (10 μl each) were applied to each well of 12-well Teflon-coated glass slides, which were air dried, fixed in acetone for 10 min, and stored in airtight containers at −70°C until used. *E. chaffeensis* and *E. sennetsu* IFA antigens were prepared by in vitro propagation in DH82 cells and $P388D_1$ cells, respectively, as described previously (7). For the preparation of the immunoblot antigen, infected leukocytes were lysed by sonication with a Branson sonicator for 1 to 2 min at a low setting, and complete lysis was determined by microscopic evaluation of the lysate by Diff-Quik staining. The lysates were then incubated with DNase (50 μg/ml) and RNase A (50 μg/ml) for 45 min at 37°C and layered onto 30% diatrizoate meglumine (Hypaque-76; Sanofi Winthrop Pharmaceuticals, New York, N.Y.) gradients. The ehrlichiae were purified from host cell material by ultracentrifugation at 87,000 × *g* for 75 min at 4°C. The ehrlichial pellet was resuspended in 2 ml of 200 mM sucrose, 50 mM potassium phosphate, and 1 mM glutamine buffer, pH 7.4, and washed twice, and the protein concentration was determined by the microBCA method (Pierce, Rockford, Ill.). The ehrlichial stocks were adjusted to a protein concentration of 2 mg/ml, and 1-ml aliquots were frozen at −70°C. Uninfected horse leukocytes were obtained from an *E. equi*-, *E. chaffeensis*-, and *E. sennetsu*-seronegative healthy horse and purified as described above. The whole cells were sonicated and treated with DNase and RNase as described above, and the sonicate was used as the immunoblot antigen since pilot studies showed that no pellet was present after ultracentrifugation was performed as for *E. equi*-infected leukocytes.

SDS-PAGE and immunoblot preparation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (12). The density gradient-purified *E. equi* and normal horse leukocyte sonicates were suspended in final sample buffer (8% 2-mercaptoethanol, 40% glycerol, and 0.4% bromphenol blue in 60 mM Tris-HCl, 1 mM EDTA, and 2% SDS buffer, pH 6.8) at a protein concentration of 2 mg/ml. Initially, 20 μl was loaded onto lanes of 5% polyacrylamide stacking–12.5% polyacrylamide separating gels. Subsequently, the quantity of protein was calculated per lane surface area for use in preparative gel electrophoresis. Each gel included a set of seven prestained molecular size standards (BRL-GIBCO, Bethesda, Md.). Gels were electrophoresed at 15 mA until the dye front eluted from the gel. The electrotransfer of proteins was performed by the method of Towbin et al. (25), with modifications, as described previously (3). Briefly, SDS-PAGE gels were overlaid with nitrocellulose sheets, and proteins were transferred at a constant 24 V in phosphate buffer for 2 h at 4°C. The transfer was assessed by the presence of prestained molecular size markers on the nitrocellulose sheet. The nitrocellulose was air dried and cut into 4-mm-wide strips for use in immunoblotting. The strips were stored at −20°C until used.

IFA. The immunofluorescent assay for antibodies was performed by a modification of the procedure of Madigan et al. (16). Because preliminary studies indicated that nonspecific fluorescence occurred with dilutions of 1:40 or less, all sera were initially diluted and screened at a 1:80 dilution. Thawed sera were diluted in PBS with 0.5% nonfat dry milk, and 10 μl of the diluted sera was applied to wells of the *E. equi*-infected horse leukocyte multiwell antigen slides. The slides were incubated in a moist chamber for 1 h at ambient temperature, and unbound antibody was removed with two changes and a 5-min soak in PBS. The slides were then rinsed with deionized water and air dried. The appropriate fluorescein isothiocyanate-conjugated secondary antibody (anti-horse immunoglobulin [Ig] G, anti-dog IgG, anti-bovine IgG, anti-human IgG plus IgA plus IgM, anti-mouse IgG, or anti-rabbit IgG [KPL, Gaithersburg, Md.]) diluted in PBS with 0.5% nonfat dry milk was applied to each well, and the slides were again incubated in a moist chamber for 1 h at ambient temperature. The slides were rinsed in two changes of PBS, soaked for 5 min in PBS supplemented with 0.005% Evans blue, and rinsed again in PBS to remove residual stain. Slides were then mounted with PBS-glycerol (1:9) and examined with a fluorescent microscope. Each run included an appropriate positive and negative control serum. A reactive pattern was considered present when definite fluorescent ehrlichia and morula morphology were observed within the cytoplasm of cells. Equivocal morphology and fluorescent intensity were resolved by repeating the test. Sera reactive when diluted 1:80 were then serially titrated to determine the end point titer. Sera were tested for IFA antibodies to *E. phagocytophila* at a 1:80 dilution,

but because of the limited quantities of antigen available, titration was performed only on selected reactive sera. *E. phagocytophila* IFA titers for Swiss cattle convalescent from tick-borne fever were provided courtesy of Jorge Liz (University of Neuchâtel, Neuchâtel, Switzerland) and are included for comparison.

Immunoblot staining. Immunoblot staining was performed by a standard method, with modifications (3). In brief, blotted strips were blocked for 10 min in blocking buffer (PBS with 0.05% Tween 20, 0.5% nonfat dry milk, and 1% normal goat serum [Sigma, St. Louis, Mo.]). Sera were diluted 1:80 in blocking buffer and incubated with the antigen strips for 3 h at ambient temperature with rocking. Unbound antibody was removed by three PBS-Tween 20 washes, and the secondary antibodies (all diluted 1:200 in blocking buffer) were incubated with the strips for 1 h at ambient temperature with rocking. Biotinylated secondary antibodies included goat anti-horse IgG, goat anti-bovine IgG, goat anti-dog IgG, goat anti-human Ig (IgG plus IgA plus IgM), horse anti-mouse IgG, and goat anti-rabbit IgG (all from KPL). The strips were washed and reacted with streptavidin-alkaline phosphatase (diluted 1:1,000; Dako, Carpinteria, Calif.) for 1 h at ambient temperature with rocking. Bound antibody complex was detected with fast red-naphthol phosphate or 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium substrates. The migration of *E. equi* antigens was determined by comparison with a standard curve generated with the molecular size standards for each immunoblot strip preparation.

RESULTS

IFA. Serologic reactions and titers are shown in Table 1. Sera obtained from horses convalescent from experimental or natural *E. equi* infection (sera 1 to 3) uniformly had titers in excess of 640, and each serum sample was reactive when tested for *E. phagocytophila* antibodies by IFA. Several healthy normal horses with no history of *E. equi* or other ehrlichial infection had no evidence of serologic reactivity by IFA for either *E. equi* or *E. phagocytophila* (sera 5 and 6). Similarly, the sera obtained from 10 Swiss cattle with tick-borne fever (*E. phagocytophila* infection, sera 12 to 21) were reactive (titers, $\geq$160) with the *E. equi* antigen, while sera from two healthy California cattle (sera 22 and 23) were nonreactive when assayed for antibodies to *E. equi* and *E. phagocytophila*. Sera from two Minnesota dogs with granulocytic ehrlichiosis (sera 7 and 8) had high titers ($\geq$2,560) of *E. equi* antibodies and antibodies reactive with *E. phagocytophila*, while control *E. canis*-seronegative dog serum (serum 11) lacked any reactivity with either *E. equi* or *E. phagocytophila*. Sera obtained from eight human patients with granulocytic ehrlichiosis (sera 24 to 31) reacted strongly with *E. equi* and *E. phagocytophila*, and sera from normal healthy human volunteers with no history of ehrlichiosis (sera 37 to 39) were nonreactive. All sera were tested for antibodies against both *E. chaffeensis* (also used as a surrogate for *E. canis* and *E. ewingii*) (7, 20) and *E. sennetsu*, and only one serum, from a horse with naturally acquired *E. equi* infection (serum 3), demonstrated a cross-reaction, with an *E. sennetsu* antibody titer of 640. The sera from the *E. risticii*-infected horse (serum 4) and rabbit immunized with *E. risticii* (serum 46) both produced high titer antibodies reactive with *E. sennetsu*, in keeping with the close phylogenetic relationship of these two ehrlichiae.

To establish the specificity of the IFA reactions, multiple sera from various mammalian sources known to contain antibodies directed against *Ehrlichia* species, including *E. chaffeensis* (sera 32 and 33), *E. canis* (serum 9), *E. ewingii* (serum 10), *E. risticii* (sera 4 and 46), and *E. sennetsu* (sera 40 and 45), were tested for antibodies reactive with *E. equi*. Of these sera, none had antibodies reactive with *E. equi* by IFA.

Immunoblots. Results of immunoblots with *E. equi* as the antigen are shown in Fig. 1 and 2. Sera obtained from experimental and natural *E. equi* infections of horses (sera 1 to 3) were used initially to establish the profile of *E. equi* immunoreactive proteins by immunoblot analysis. With these antisera, it was expected that the profile of antigens reactive with nonhyperimmune antiserum could be established to serve as a framework for further studies. Immunoblots performed with three different horse *E. equi* antisera yielded a maximum of 19 discernible antigens of different molecular sizes, ranging from 14 to 156 kDa. Most immunoblots demonstrated five or fewer bands. Only one, a 44-kDa antigen, was consistently present when tested with each antiserum on several different occasions. However, bands of 14, 25, 35, 40, 42, 60, 65, 70, and 100 kDa were often detected. Identical antigen preparations were tested for nonspecificity by immunoblot analysis with serum obtained from *E. equi*-seronegative, normal horses (sera 5 and 6). A 75-kDa antigen was most frequently detected with these antisera, and antigens of 14, 16, 20, 35, 40, 56, 60, 65, and 70 kDa were detected less frequently. Thus, the most specific antigen markers for *E. equi* infection include the 100-, 44-, 42-, and 25-kDa antigens. Since the 44-kDa antigen was uniformly present, a tentative defining pattern of *E. equi* immunoreactivity includes the 44-kDa antigen and at least one other band of 100, 42, or 25 kDa. By this criterion, all but one immunoblot reaction of the *E. equi* antisera tested on several different occasions would be considered reactive, and no serum from a normal horse would be considered reactive.

To confirm the specificity of the immunoblot, multiple antisera obtained from naturally infected, experimentally infected, and immunized animals were tested, and representative results are shown in Fig. 2. Many sera, including sera from uninfected humans and animals, reacted with the group of antigens between 56 and 75 kDa, bands that probably represent heat shock proteins (6). In addition, some sera also reacted with the 156-, 140-, and 100-kDa antigens, suggesting conserved epitopes among ehrlichiae and some rickettsiae. Occasional reactions were noted with both the 40- and 44-kDa antigens, limited only to serum reactive with other members of the genus *Ehrlichia*, including canine anti-*E. canis*, rabbit anti-*E. chaffeensis*, and human anti-*E. chaffeensis*. These cross-reactions with the 44-kDa antigen were usually faint, and no other serum contained antibodies reactive with the 25-, 42-, or 100-kDa group of *E. equi* antigens. Thus, an immunoblot profile which includes the 44-kDa antigen and at least one additional antigen of 25, 42, or 100 kDa strongly suggests infection with *E. equi* or a closely related organism.

Sera obtained from humans convalescent from granulocytic ehrlichiosis, dogs convalescent from *E. equi* infection, and a cow convalescent from *E. phagocytophila* infection were also tested by *E. equi* immunoblot (Fig. 1). The results were similar in that sera from all well-defined cases of ehrlichiosis presumed to be caused by a member of the *E. phagocytophila-E. equi* genogroup (proven by demonstration of granulocytic ehrlichiae on peripheral blood smears and IFA serologic reactions with *E. equi*) had antibodies reactive with the 44-kDa antigen and the 25-, 42-, or 100-kDa antigen. Of sera tested from eight human patients convalescent from granulocytic ehrlichiosis, all had the characteristic 44-kDa band, seven had additional bands at 42 kDa, and six had weak bands at 25 kDa (data not shown for all sera). Paired acute- and convalescent-phase sera were tested for four of these eight patients, each with acute-phase *E. equi* IFA titers of $<$80. By immunoblot, two of these acute-phase sera had weak 44- and 42-kDa bands, which increased in intensity when the convalescent-phase sera were tested (serum pair from one patient shown in Fig. 1, lanes 9 and 10). Acute-phase sera from the other two patients had no detectable *E. equi* antibodies by immunoblot, but 44- and 42-kDa bands were evident when convalescent-phase sera were tested (Fig. 1, lanes 11 to 14).

Sera obtained from dogs with *E. equi* infection demonstrated

TABLE 1. Host species and IFA titers of sera

Serum	Host species	Agent	Serologic reaction	IFA antibody titer			
				E. equi	*E. phagocytophila*	*E. chaffeensis*	*E. sennetsu*
1	Horse	*E. equi*	Infection (experimental)	≥20,480	≥80	<80	<80
2		*E. equi*	Infection (experimental)	≥1,280	≥80	<80	<80
3		*E. equi*	Infection (natural)	5,120	≥80	<80	640
4		*E. risticii*	Infection (experimental)	<80	ND[a]	<80	≥1,280
5		None	None	<80	<80	<80	<80
6		None	None	<80	ND	<80	<80
7	Dog	*E. equi*	Infection (natural)	2,560	≥80	<80	<80
8		*E. equi*	Infection (natural)	5,120	≥80	<80	<80
9		*E. canis*	Infection (experimental)	<80	ND	≥1,280	<80
10		*E. ewingii*	Infection (experimental)	<80	ND	≥1,280	<80
11		None	None	<80	ND	<80	<80
12	Bovine	*E. phagocytophila*	Infection (natural)	≥1,280	≥80	<80	<80
13		*E. phagocytophila*	Infection (natural)	≥320	2,560[b]	<80	<80
14		*E. phagocytophila*	Infection (natural)	≥320	2,560[b]	<80	<80
15		*E. phagocytophila*	Infection (natural)	≥160	5,120[b]	<80	<80
16		*E. phagocytophila*	Infection (natural)	≥320	320[b]	<80	<80
17		*E. phagocytophila*	Infection (natural)	≥160	640[b]	<80	<80
18		*E. phagocytophila*	Infection (natural)	≥320	320[b]	<80	<80
19		*E. phagocytophila*	Infection (natural)	≥320	1,280[b]	<80	<80
20		*E. phagocytophila*	Infection (natural)	≥320	640[b]	<80	<80
21		*E. phagocytophila*	Infection (natural)	≥320	320[b]	<80	<80
22		None	None	<80	<80	<80	<80
23		None	None	<80	<80	<80	<80
24	Human	HGE[c]	Infection (natural)	320	≥80	<80	<80
25		HGE	Infection (natural)	160	<80	<80	<80
26		HGE	Infection (natural)	2,560	320	<80	<80
27		HGE	Infection (natural)	80	80	<80	<80
28		HGE	Infection (natural)	20,480	ND	<80	<80
29		HGE	Infection (natural)	1,280	ND	<80	<80
30		HGE	Infection (natural)	1,280	ND	<80	<80
31		HGE	Infection (natural)	2,560	80	<80	<80
32		*E. chaffeensis*	Infection (natural)	<80	<80	1,280	<80
33		*E. chaffeensis*	Infection (natural)	<80	ND	320	<80
34		*Rickettsia rickettsii*	Infection (natural)	<80	ND	<80	<80
35		*Rickettsia rickettsii*	Infection (natural)	<80	ND	<80	<80
36		*Rickettsia tsutsugamushi*	Infection (natural)	<80	ND	<80	<80
37		None	None	<80	<80	<80	<80
38		None	None	<80	<80	<80	<80
39		None	None	<80	<80	<80	<80
40	Mouse	*E. sennetsu*	Infection (experimental)	<80	ND	<80	640
41		*Rickettsia typhi*	Immunization	<80	ND	<80	<80
42		*Bartonella quintana*	Immunization	<80	ND	<80	<80
43		*Bartonella henselae*	Immunization	<80	ND	<80	<80
44		None	None	<80	ND	<80	<80
45	Rabbit	*E. sennetsu*	Immunization	<80	ND	<80	5,120
46		*E. risticii*	Immunization	<80	ND	<80	≥1,280
47		*E. chaffeensis*	Immunization	<80	ND	≥1,280	<80
48		None	None	<80	ND	<80	<80

[a] ND, not done.
[b] Results courtesy of J. Liz, University of Neuchâtel, Neuchâtel, Switzerland.
[c] HGE, human granulocytic ehrlichiosis.

similar antibody reactivities by immunoblot, with each of the two dogs tested having strong 44- and 42-kDa antigens and weaker 25-kDa antigen bands.

Serum from one cow recovered from natural tick-borne fever (*E. phagocytophila* infection) was tested. The IFA titer to *E. equi* was ≥1,280, and by immunoblot, a prominent 44- and weaker 80-kDa antigen were detected.

Each serum tested by immunoblot for *E. equi* was simultaneously tested for antibodies directed against normal horse leukocyte antigens. These antisera produced occasional bands in immunoblots of normal horse leukocyte sonicate; however, usually two or fewer bands that were inconsistently present were seen, and the molecular sizes of the antigens detected by these antisera (120, 30, 17, and 10 kDa) were distinct from those identified simultaneously in the *E. equi* antigen (data not shown).

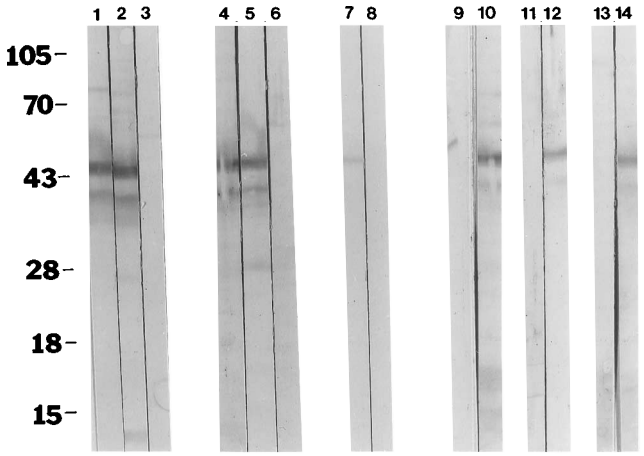

FIG. 1. Immunoblot analysis of electrophoretically separated, density gradient-purified *E. equi* antigens reacted with *E. phagocytophila* genogroup antisera. Sera used included equine anti-*E. equi* from an experimental infection (lane 1, serum 1), equine anti-*E. equi* from a natural infection in Minnesota (lane 2, serum 3), normal horse serum (lane 3, serum 5), canine anti-*E. equi* from two naturally infected dogs in Minnesota (lanes 4 and 5, sera 7 and 8), normal dog serum (lane 6, serum 11), bovine anti-*E. phagocytophila* from a natural infection in Switzerland (lane 7, serum 12), normal bovine serum from California (lane 8, serum 22), and acute-phase (lanes 9, 11, and 13, each with *E. equi* IFA titers of <80; no corresponding sera listed in Table 1) and convalescent-phase (lanes 10, 12, and 14, sera 27, 24, and 25, respectively) paired sera from three human patients with granulocytic ehrlichiosis in the upper midwest United States. Note the consistent presence of a band at approximately 44 kDa and the frequent presence of a 42-kDa antigen in sera from *E. equi*, *E. phagocytophila*, and human granulocytic ehrlichia infections. The approximate molecular sizes are indicated on the left in kilodaltons.

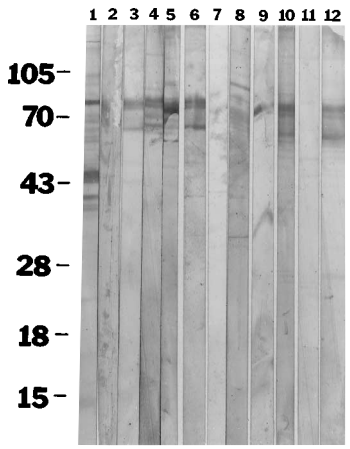

FIG. 2. Immunoblot analysis of electrophoretically separated, density gradient-purified *E. equi* antigens reacted with homologous equine anti-*E. equi* (lane 1, serum 1), normal serum, or antiserum against non-*E. phagocytophila* genogroup *Ehrlichia* species or *Rickettsia* species. Heterologous sera include equine anti-*E. risticii* (lane 2, serum 4), normal horse serum (lane 3, serum 5), human anti-*R. rickettsii* (lane 4, serum 34), human anti-*E. chaffeensis* (lane 5, serum 32), normal human serum (lane 6, serum 39), mouse anti-*R. typhi* (lane 7, serum 41), mouse anti-*E. sennetsu* (lane 8, serum 40), nonimmune mouse serum (lane 9, serum 44), canine anti-*E. canis* (lane 10, serum 9), canine anti-*E. ewingii* (lane 11, serum 10), and normal dog serum (lane 12, serum 11). Note (i) the absence of a 44-kDa antigen for all sera except the control equine anti-*E. equi* and (ii) antigens between approximately 56 and 75 kDa for most sera. Similar results were obtained with additional sera, including a human anti-*R. rickettsii* serum, a human anti-*R. tsutsugamushi* serum, two normal human sera, mouse anti-*Bartonella henselae* and mouse anti-*Bartonella quintana* sera, two dog anti-*E. canis* sera, and rabbit antisera against *E. sennetsu*, *E. risticii*, and *E. chaffeensis* (data not shown). The approximate molecular sizes are indicated on the left in kilodaltons.

DISCUSSION

Anderson et al. were the first to document objective, nonmorphologic similarities between the granulocytic ehrlichiae *E. phagocytophila* and *E. equi* when they demonstrated a 99.9% homology between the 16S rDNAs of these species (1). Recently, this finding was extended to include a granulocytic ehrlichia pathogenic for humans which shares 99.9% and 99.8% homologies with *E. phagocytophila* and *E. equi*, respectively (4). Aside from the similar morphologic appearance and host cell parasitism, no other objective data which corroborate these suggested close relationships are available. The differing host mammalian susceptibilities and geographic localizations of these agents and the diseases with which they are associated are arguments to maintain the separate species designations (16, 17, 19).

The serologic data presented herein demonstrate broad cross-reactivity among members of the *E. phagocytophila-E. equi*-human granulocytic ehrlichia genogroup. Each of these *Ehrlichia* species elicits strong serologic cross-reactivities when tested by IFA, while strong IFA cross-reactions are infrequently encountered when other *Ehrlichia* species outside of this genogroup are used as an antigen. Similarly, IFA cross-reactions are rarely detected when nonhyperimmune antisera produced against *E. chaffeensis*, *E. canis*, *E. ewingii*, *E. sennetsu*, and *E. risticii* are reacted with the *E. equi* antigen. These results partly contradict published reports of broad IFA serologic cross-reactions between out-of-group species, including reactions between *E. sennetsu* and *E. canis*, *E. phagocytophila* and *Cowdria ruminantium*, and *E. chaffeensis* and *E. equi*. Usually, these reactions occur at very low titers and may represent nonspecific reactions or specific reactions to highly conserved antigens among the members of *Ehrlichia* and other genera.

Immunoblot analyses provide further support of the close antigenic relationships within this genogroup, in that all infections and immunizations of members of the group elicited antibodies reactive with a 44-kDa antigen and an antigen of 25, 42, or 100 kDa. Conversely, with the exception of one serum from a dog convalescent from experimental *E. canis* infection, no other antiserum produced by infection or immunization with ehrlichiae outside of this genogroup or directed against *Rickettsia* spp., *Bartonella* (*Rochalimaea*) spp., or normal serum produced this pattern of reactivity. These immunoblot results differ from those described by Nyindo et al., who showed significant immunoblot serologic cross-reactivity among *E. canis*, *E. sennetsu*, *E. risticii*, and *E. equi* (18). This discrepancy could be explained by their use of hyperimmune sera to detect cross-reactive antigens among these *Ehrlichia* species. In spite of these findings, low-level serologic cross-reactions between the members of different *Ehrlichia* genogroups are well described and usually involve only a few specific antigens (3, 5, 18, 20, 23). The reason that these cross-reactions exist is not established, but they could result from a common genetic ancestor for the genus, convergent evolution of species, or simply the presence of antigens that are highly conserved among many eubacterial species, such as homologs of the *E. coli* GroEL or GroES chaperonins shown to exist in *E. chaffeensis* (24).

These results, coupled with the phylogenetic data from 16S rRNA gene sequences, suggest that the bacteria within the *E. phagocytophila* genogroup may constitute a single species of the genus *Ehrlichia*. However, little is known about the biologic basis of the clinical, ecologic, host affinity, and geographic differences which were initially used as the rationale to assign separate species. No *E. equi*-specific band was identified only in sera from *E. equi*-infected horses and dogs when compared with bands in sera obtained from humans convalescent from granulocytic ehrlichiosis and serum obtained from a bovine

with tick-borne fever. Thus, it is likely that *E. equi* immunoreactive proteins are similar to those of *E. phagocytophila* and the granulocytic ehrlichia of humans. However, without similar immunoblot analysis of the immunoreactive proteins of these latter two granulocytic ehrlichiae, no definite conclusions about relationships at the protein level may be drawn.

Aside from our previous description of serologic reactions in human granulocytic ehrlichiosis (2), no previous reports document the serologic cross-reactivities in this genogroup. Only a single case report of *E. equi* infection of a Welsh gelding addresses the possibility that *E. equi* and *E. phagocytophila* may be serologically similar or identical, with inconclusive results (17). In fact, sporadic case reports of *E. equi* infection of horses in Europe seem to reinforce the concept of disparity between these species (16, 17). The nonspecific clinical findings of fever, depression, and decreased activity, among others, and the laboratory abnormalities which are shared between *E. equi* and *E. phagocytophila* infections are of limited value in assessing any relationship (10, 11, 16). However, the poor transmissibility of *E. phagocytophila* from goats, sheep, and cattle to other mammals and the similar poor transmissibility of *E. equi* from horses to cattle, goats, sheep, nonhuman primates, and many other mammals seem to support the concept that these agents have unique biologic properties and should be separately designated, perhaps at the species level. Alternatively, sufficient differences must exist even among isolates of a single species, such as with *E. phagocytophila*, since sheep which are convalescent from infection with an isolate from one geographic region are protected from reinfection with the homologous strain but are not always protected from infection by an isolate from a different geographic region (9).

In spite of the apparent close antigenic and genetic similarities of the ehrlichiae within the *E. phagocytophila* genogroup, it is clear that important biological differences that remain undefined exist among these agents. Since we recently transfused blood from a patient with human granulocytic ehrlichiosis to a horse that developed infection typical for *E. equi*, we must presume that these two agents have not only a close genetic and antigenic relationship but also similar biological capabilities and may represent strains of a single species (unpublished data). It is still premature to reclassify all of these agents into a single species, although given the considerable genetic and antigenic differences among the *Ehrlichia* genogroups, some consideration should be given to assigning the *E. phagocytophila* and *E. sennetsu* genogroups to separate genera. Studies to elucidate the function of ehrlichial proteins and genes which are not highly conserved among bacteria and are unique to this genogroup will be required to define the basis for the existing biologic and ecologic differences. In turn, such studies may also shed light on the mechanisms by which these granulocytic ehrlichiae injure the host and alter host defenses, both of which are key objectives for research on these organisms. If these objectives are met, a clearer understanding of classification within this genus will surface and strategies to diminish human and veterinary ehrlichial morbidity and mortality may be devised.

ACKNOWLEDGMENTS

This work was supported in part by a SRIS award from the University of Maryland School of Medicine and by a grant from the Duluth Clinic, Duluth, Minn.

We acknowledge the generosity of Jorge Liz of the University of Neuchâtel, Neuchâtel, Switzerland, for providing some bovine sera, antigens, and serologic results; Barb Greig of the College of Veterinary Medicine, University of Minnesota, St. Paul, for providing some equine and canine sera; and Ellen Trigiani, University of Maryland School of Medicine, Baltimore, for help with serologic tests.

REFERENCES

1. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson.** 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29:**2838–2842.
2. **Bakken, J. S., J. S. Dumler, S.-M. Chen, M. R. Eckman, L. L. Van Etta, and D. H. Walker.** 1994. Human granulocytic ehrlichiosis in the upper Midwest. A new species emerging? JAMA **272:**212–218.
3. **Brouqui, P., J. S. Dumler, D. Raoult, and D. H. Walker.** 1992. Antigenic characterization of ehrlichiae: protein immunoblotting of *Ehrlichia canis*, *Ehrlichia sennetsu*, and *Ehrlichia risticii*. J. Clin. Microbiol. **30:**1062–1066.
4. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
5. **Chen, S.-M., J. S. Dumler, H. M. Feng, and D. H. Walker.** 1994. Identification of the antigenic constituents of *Ehrlichia chaffeensis*. Am. J. Trop. Med. Hyg. **50:**52–58.
6. **Dasch, G. A., E. Weiss, and J. C. Williams.** 1990. Antigenic properties of the Ehrlichiae and other Rickettsiaceae, p. 32–58. *In* J. C. Williams and I. Kakoma (ed.), Ehrlichiosis, a vector-borne disease of animals and humans. Kluwer Academic Publishers, Boston.
7. **Dawson, J. E., Y. Rikihisa, S. A. Ewing, and D. B. Fishbein.** 1991. Serologic diagnosis of human ehrlichiosis using two *Ehrlichia canis* isolates. J. Infect. Dis. **163:**564–567.
8. **Fishbein, D. B., J. E. Dawson, and L. E. Robinson.** 1994. Human ehrlichiosis in the United States, 1985 to 1990. Ann. Intern. Med. **120:**736–743.
9. **Foggie, A.** 1951. Studies on the infectious agent of tick-borne fever in sheep. J. Pathol. Bacteriol. **63:**1–15.
10. **Gordon, W. S., A. Brownlee, D. R. Wilson, and J. MacLeod.** 1932. "Tick-borne fever." (A hitherto undescribed disease of sheep). J. Comp. Pathol. Ther. **65:**301–307.
11. **Gribble, D. H.** 1969. Equine ehrlichiosis. J. Am. Vet. Med. Assoc. **155:**462–469.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lewis, G. E., D. L. Huxsoll, M. Ristic, and A. J. Johnson.** 1975. Experimentally induced infection of dogs, cats, and nonhuman primates with *Ehrlichia equi*, etiologic agent of equine ehrlichiosis. Am. J. Vet. Res. **36:**85–88.
14. **Liz, J., A. Aeschlimann, and K. Pfister.** 1991. Tick-borne fever (*Ehrlichia phagocytophila*) as a significant disease of cattle in Switzerland, p. 740–750. *In* J. Kazár and D. Raoult (ed.), Rickettsiae and rickettsial diseases. Publishing House of the Slovak Academy of Sciences, Bratislava, Czechoslovakia.
15. **Madewell, B. R., and D. H. Gribble.** 1982. Infection in two dogs with an agent resembling *Ehrlichia equi*. J. Am. Vet. Med. Assoc. **180:**512–514.
16. **Madigan, J. E., S. Hietala, S. Chalmers, and E. DeRock.** 1990. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. J. Am. Vet. Med. Assoc. **196:**1962–1964.
17. **McNamee, P. T., A. P. Cule, and J. Donnelly.** 1989. Suspected ehrlichiosis in a gelding in Wales. Vet. Rec. **124:**634–635.
18. **Nyindo, M., I. Kakoma, and R. Hansen.** 1991. Antigenic analysis of four species of the genus *Ehrlichia* by use of protein immunoblot. Am. J. Vet. Res. **52:**1225–1230.
19. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. **4:**286–308.
20. **Rikihisa, Y., S. A. Ewing, J. C. Fox, A. G. Siregar, F. H. Pasaribu, and M. B. Malole.** 1992. Analyses of *Ehrlichia canis* and a canine granulocytic *Ehrlichia* infection. J. Clin. Microbiol. **30:**143–148.
21. **Ristic, M., and D. L. Huxsoll.** 1984. Ehrlichieae, p. 704–709. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore.
22. **Rodgers, S. J., R. J. Morton, and C. A. Baldwin.** 1989. A serological survey of *Ehrlichia canis*, *Ehrlichia equi*, *Rickettsia rickettsii*, and *Borrelia burgdorferi* in dogs in Oklahoma. J. Vet. Diagn. Invest. **1:**154–159.
23. **Shankarappa, B., S. K. Dutta, and B. L. Mattingly-Napier.** 1992. Antigenic and genomic relatedness among *Ehrlichia risticii*, *Ehrlichia sennetsu*, and *Ehrlichia canis*. Int. J. Syst. Bacteriol. **42:**127–132.
24. **Sumner, J. W., K. G. Sims, D. C. Jones, and B. E. Anderson.** 1993. *Ehrlichia chaffeensis* expresses an immunoreactive protein homologous to the *Escherichia coli* GroEL protein. Infect. Immun. **61:**3536–3539.
25. **Towbin, H., T. Saehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.