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ANTIBODIES TO GRANULOCYTIC EHRLICHIAE IN WHITE-FOOTED AND COTTON MICE IN EASTERN UNITED STATES

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ABSTRACT: Serum samples, collected from *Peromyscus leucopus* (white-footed mouse) or *Peromyscus gossypinus* (cotton mouse) during 1987 through 1990 in Florida, Georgia, Maryland, Mississippi, and North Carolina (USA), and in 1997 in southern Connecticut were analyzed by indirect fluorescent antibody (IFA) staining methods or Western blot procedures for antibodies to granulocytic ehrlichiae. Of the 82 sera from white-footed mice in Connecticut tested by IFA methods with either the BDS or NCH-1 strain of the human granulocytic ehrlichiosis (HGE) agent, 45 (55%) and 42 (51%) of the samples contained antibodies to these strains, respectively, at concentrations ranging from 1:80 to 1:2560. One (2%) of 43 sera from *P. leucopus* captured in Assateague Island (Maryland) had a titer of 1:80, while three (20%) of 15 sera from *P. gossypinus*, captured in Sapelo Island (Georgia) and four (40%) of 10 sera from cotton mice caught in Amelia Island (Florida) had antibodies to the NCH-1 strain at titers of 1:160 to 1:1,280. Fifty-five sera from *P. leucopus* in Cape Hatteras (North Carolina) and 30 sera from *P. gossypinus* in Mississippi were negative. Western blot analyses confirmed seropositivity for 19 (95%) of 20 mouse sera positive by IFA staining methods, including samples from both mouse species captured in Connecticut, Maryland, or Florida. There were key banding patterns to proteins having molecular masses of about 44, 80, 105, 110, or 120 kDa. Both serologic assays can be used to determine if mice have been exposed to granulocytic ehrlichiae. These rodents also may be useful in surveillance programs to identify endemic sites for HGE and in performing laboratory studies on immune responses to the etiologic agent.

Key words: Cotton mouse, *Ehrlichia phagocytophila* genogroup, human granulocytic ehrlichiosis, *Peromyscus gossypinus*, *Peromyscus leucopus*, serosurvey, white-footed mouse.

INTRODUCTION

Human granulocytic ehrlichiosis (HGE) occurs in areas of the northern hemisphere where *Ixodes pacificus*, *Ixodes scapularis*, and *Ixodes ricinus* ticks are abundant. In the United States, most human cases have been reported in the northeastern and upper midwestern regions (Walker and Dumler, 1996), areas where there are populations of white-tailed deer (*Odocoileus virginianus*). The causative agent, *Ehrlichia equi* or a closely related agent in the *Ehrlichia phagocytophila* genogroup, has been isolated from humans and horses in localities where infected *I. scapularis* nymphs and females abound (Goodman et al., 1996; Madigan et al., 1996; Heimer et al., 1997). Similar to the ecology of Lyme borreliosis and human babesiosis, white-footed mice (*Peromyscus leucopus*) are probably important reservoirs for this bacterium in nature. *Peromyscus gossypinus* (cotton mouse) is parasitized by ticks in southern states and also may carry granulocytic ehrlichiae. The present study was conducted to determine the prevalence of antibodies to the HGE agent in *P. leucopus* and *P. gossypinus* at widely separated sites in eastern and southern United States, to perform Western blot analyses to identify antigenic protein banding patterns in positive sera, and to compare results of immunoblotting methods with those of indirect fluorescent antibody (IFA) staining procedures.

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MATERIALS AND METHODS

Blood samples, taken from archived collections, were obtained from white-footed mice or cotton mice from areas with known tick activity during 1987 through 1990 in Georgia at Cumberland Island (30°52′N, 81°26′W), Ossabaw Island (31°48′N, 81°5′W), and Sapelo Island (31°30′N, 81°15′W); three counties in Mississippi including Franklin (31°28′N, 90°53′W), Prentiss (34°37′N, 88°33′W), and Sharkey (32°55′N, 90°50′W); Assateague Island in Maryland (38°5′N, 75°12′W); Cape Hatteras in North Carolina (35°13′N, 75°31′W); at Amelia Island in Florida (30°60′N, 81°46′W); and in 1997 in Connecticut where Lyme borreliosis also occurs (Magnarelli et al., 1991, 1992, 1995a; Oliver et al., 1993; Sanders et al., 1995). Animals were captured during spring through fall in Sherman box traps (H. B. Sherman Traps, Inc., Tallahassee, Florida USA) and bled in accordance with protocols approved by an institutional animal care and use committee at the Connecticut Agricultural Experiment Station. In Connecticut, mice were captured in tick-infested forests located in Bridgeport, East Haddam, Lyme, and Old Lyme. East Haddam and Old Lyme are located in southern Connecticut between Bridgeport (41°10′N, 73°12′W) and Lyme (41°23′N, 72°20′W). All sera were stored at −60 C until antibody analyses could be conducted.

Indirect fluorescent antibody staining methods with the BDS or NCH-1 strain and Western blot analyses with the NCH-1 strain were used to detect total immunoglobulins to granulocytic ehrlichiae. The BDS strain is a human isolate from Minnesota that had been inoculated into and maintained in a horse. The NCH-1 strain was isolated from an ill person in Nantucket (Massachusetts, USA; Telford et al., 1996) and grown in human promyelocytic leukemia cell (HL-60, American Type Culture Collection CCL-240) cultures at Yale University. Infected horse neutrophils or HL-60 cells were fixed to glass microscope slides by acetone treatment. Details on the source and use of fluorescein-conjugated antibodies, negative and positive control sera, the specificity of antibody tests, and on the conservative grading of fluorescence of morulae have been reported (Magnarelli et al., 1997a). Sera from seven white-footed mice born in the laboratory and unexposed to ticks or tick-borne agents were used as negative controls. An additional positive control serum from an inoculated C3H/HeN mouse (Sun et al., 1997) also was included. Distinct reactivity of ehrlichial morulae at or above a serum dilution of 1:80 was considered positive for antibodies. Procedures for Western blot analyses of mouse sera closely follow those described earlier (Sun et al., 1997; Magnarelli et al., 1997b). Twenty mouse sera with antibodies to the NCH-1 strain of the HGE agent were retested at a dilution of 1:100 with this antigen and a 1:1,000 dilution of commercially prepared alkaline phosphatase-labeled anti-*P. leucopus* immunoglobulins (Sigma Chemical Company, St. Louis, Missouri, USA). The substrate reagent (BCIP/NBT), used to visualize banding patterns, also was purchased (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA). A distinct band to the 44 kDa protein was considered most important in verifying evidence of infection by the HGE agent. Uninfected lysates of HL-60 cells were included as controls in parallel tests of all sera tested with preparations of infected HL-60 cells.

RESULTS

Mice had serologic evidence of exposure to granulocytic ehrlichiae in Connecticut, Georgia, Maryland, and Florida. During 1997, 45 (55%) of 82 white-footed mouse sera from Connecticut were positive by IFA staining methods when the BDS strain was tested, while 42 (51%) sera were reactive with the NCH-1 strain (Table 1). The positive specimens were obtained in the period of April through September, a time during which all mice were captured. Aside from one *P. leucopus* serum sample from Assateague Island which had a low concentration of antibodies (titer = 1:80), no other sera from this sampling site, Cumberland and Ossabaw Islands (n = 29), Cape Hatteras (n = 55), or Mississippi (n = 30) had evidence of exposure to granulocytic ehrlichiae. However, four of 10 *P. gossypinus* sera, obtained from Amelia Island in 1989, contained antibodies to the BDS and NCH-1 strains, while three of 15 cotton mouse sera obtained from Sapelo Island in 1990 were positive.

Antibodies in sera from four tick-infested areas in Connecticut frequently reacted with both the BDS and NCH-1 strains. Seropositivity results for assays with the former strain ranged from 33% for samples collected in Lyme to 67% for specimens obtained in Old Lyme (Table 2). Similar results were recorded when the

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TABLE 1. Detection of serum antibodies to the BDS or NCH-1 strains of granulocytic ehrlichiae in *Peromyscus leucopus* and *Peromyscus gossypinus* captured in the eastern and southern United States during 1987 through 1990 and in 1997.

Sampling areas	Year	Number of serum samples tested	Number (%) with antibodies[a] to strains	
			BDS	NCH-1
Connecticut	1997	82[b]	45 (55)	42 (51)
Georgia	1988–1990	44[c]	NT	3 (7)
Florida	1989	10[c]	4 (40)	4 (40)
Maryland	1987	15[b]	0	1 (7)
Maryland	1988	21[b]	0	0
Maryland	1989	7[b]	0	0
Mississippi	1990	30[c]	0	0
North Carolina	1987	10[b]	0	0
North Carolina	1988	22[b]	0	0
North Carolina	1989	23[b]	0	0

[a] A titer of ≥1:80 was considered positive by indirect fluorescent antibody staining methods; NT = Not tested (limited antigen supply).
[b] *Peromyscus leucopus.*
[c] *Peromyscus gossypinus.*

NCH-1 strain was included in IFA staining methods. Moreover, maximal antibody titers were comparable, but geometric means for antibody concentrations ranged between 14 and 64. In general, assay results were highly concordant when findings for both strains were compared; 79 (96%) of 82 specimens were positive (n = 42) or negative (n = 37) by IFA staining methods, while the remaining three sera were positive to the BDS strain and negative to the NCH-1 strain.

Western blot analyses were conducted to identify antigenic protein banding patterns and to confirm results of IFA staining methods. Immunoblotting of 20 samples, positive by IFA staining methods and including sera from both mouse species, revealed antibody reactivity to the following proteins of the NCH-1 strain: 44, 80, 105, 110, and 120 kDa in 19 (95%) sera. The positive sera were from Bridgeport (n = 10) and Old Lyme (n = 4), Assateague Island (n = 1), and Amelia Island (n = 4). All positives showed distinct reactivity with the 44 kDa protein (Fig. 1, 2). Less frequent reactions were noted for the 80 kDa, 105 kDa, 110 kDa, and 120 kDa, peptides. The remaining serum sample was positive by IFA staining methods at a titer of 1:160, but in two attempts by Western blot analyses, results were negative. In analyses of *P. gossypinus* sera from Amelia Island, the four samples positive by IFA staining methods were also positive by immunoblotting methods (Fig. 2) and

TABLE 2. Reactivity of white-footed mouse sera obtained in southern Connecticut during 1997 to the BDS and NCH-1 strains of granulocytic ehrlichiae.

Sites	Number of serum samples tested	BDS strain			NCH-1 strain		
		Number (%) positive	Antibody titers		Number (%) positive	Antibody titers	
			Range	Mean[a]		Range	Mean[a]
Bridgeport	41	23 (56)	80–2,560	44	22 (54)	80–1,280	51
East Haddam	11	6 (55)	80–640	36	6 (55)	80–2,560	47
Lyme	12	4 (33)	80–320	14	4 (33)	80–1,280	17
Old Lyme	18	12 (67)	80–1,280	64	10 (56)	80–1,280	47

[a] Geometric means; the average titer for a negative serum sample was 1:4 (n = 25 samples tested).

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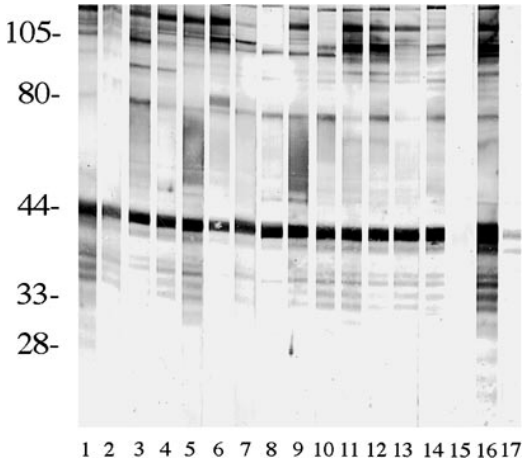

FIGURE 1. Representative immunoblots of polyvalent antibody responses to lysates of the NCH-1 strain of the human granulocytic ehrlichiosis organism in infected HL-60 cells. Molecular masses indicated in kilodaltons (kDa). Lanes 1-14 show reactivity patterns of seropositive *P. leucopus* from Connecticut; lane 15 indicates no reactivity of serum from a healthy laboratory-born white-footed mouse (negative control); lane 16 shows reactivity of a C3H/HeN mouse inoculated with the NCH-1 strain (positive control); and lane 17 indicates weak reactivity of a white-footed mouse from Assateague Island, Maryland to the 44 kDa protein.

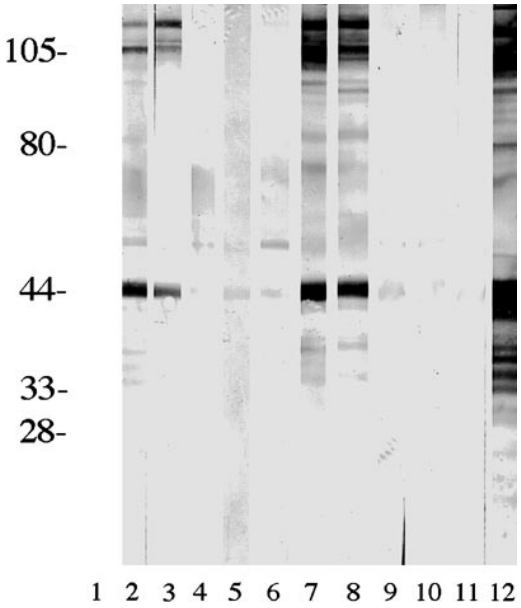

FIGURE 2. Representative immunoblots of polyvalent antibody responses to lysates of the NCH-1 strain of the human granulocytic ehrlichiosis organism in infected HL-60 cells. Molecular masses indicated in kilodaltons. Lanes 2, 3, 7 and 8 show reactivity patterns of seropositive *P. gossypinus* from Amelia Island, Florida; lane 11 contained a negative serum control from a laboratory-born *P. leucopus*; Lane 12 shows reactivity of a C3H/HeN mouse inoculated with the NCH-1 strain (positive control); and lanes 1, 4-6, 9 and 10 indicate no or very weak (graded as negative) reactivity of field-collected *P. gossypinus* sera from Amelia Island, Florida.

showed marked reactivity to the 44 kDa, 105 kDa, and 110 kDa proteins. Sera from laboratory-born mice were negative in all tests, and there was no reactivity of positive mouse sera to uninfected lysates of HL-60 cells.

DISCUSSION

White-footed mice or cotton mice had evidence of exposure to granulocytic ehrlichiae in Connecticut, Georgia, Maryland, and Florida. Human cases of granulocytic ehrlichiosis, Lyme borreliosis, and babesiosis occur in Connecticut (Hardalo et al., 1995; Magnarelli et al., 1995b), and mice carry antibodies to multiple tick-borne pathogens (Magnarelli et al. 1997a). Antibodies to granulocytic ehrlichiae were detected in sera from mice captured during the period 1983 through 1993 (Magnarelli et al., 1997a), but seropositivity rates were lower (≤36%) than in 1997. Our documentation of mice seropositive to granulocytic ehrlichiae in Maryland is consistent with earlier reports on infected rodents (Bunnell et al., 1998) and deer (Massung et al., 1998) there. Antibodies to granulocytic ehrlichiae have not been previously reported in *P. gossypinus* on Amelia Island and Sapelo Island. Although there was no serologic evidence of granulocytic ehrlichiosis in mice from Cumberland and Ossabaw Islands, Mississippi, or North Carolina, more extensive studies including isolation attempts and the application of polymerase chain reaction (PCR) methods are needed to delineate the geographic distribution of infected ticks and forest-dwelling mammals. Different species of rodents probably harbor granulocytic ehrlichiae in widely separated sites in the United States.

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Comparative serologic testing with different strains of granulocytic ehrlichiae revealed little or no differences in results for white-footed mice. Variable antibody test results have been reported in analyses of dog sera (Magnarelli et al., 1997b) and human sera (Asanovich et al., 1997) when different strains of the HGE agent were used in IFA staining methods. However, we conclude that either the BDS or NCH-1 strain can be used as antigen for antibody detection in *P. leucopus* or *P. gossypinus.* Moreover, there was close agreement in results of IFA staining methods and Western blot analyses for both mouse species. Since immunoblotting methods were particularly useful in confirming and interpreting results of IFA staining methods, we suggest that the former be relied on to further assess the performance of IFA assays.

Key banding patterns were observed in immunoblots of white-footed mouse and cotton mouse sera. Most importantly, the frequent and strong reactivity of antibodies to the 44 kDa peptide parallels findings for infected C3H/HeN mice (Sun et al., 1997), humans (IJdo et al., 1997; Ravyn et al., 1998), and dogs (Magnarelli et al., 1997b). These results reaffirm that this protein is a key serological marker for infection with the HGE agent or *E. equi.* The apparent high sensitivity and specificity of assays with this peptide (IJdo et al., 1997) indicate that it is an excellent candidate for use as a recombinant antigen in automated enzyme-linked immunosorbent assays and specialized Western blot analyses. The gene encoding the 44 kDa antigen has been cloned and expressed (IJdo et al., 1998a; Zhi et al., 1998), and purified antigens are available for further evaluation. The less frequent reactivity of antibodies to the 105kDa, 110kDa, and 120kDa proteins also is noted in human and domestic animal infections caused by the HGE agent or *E. equi* (IJdo et al., 1997; Magnarelli et al., 1997b), but these peptides appear to be less important markers in laboratory analyses for granulocytic ehrlichial infections in mice. Reactivity to the 80 kDa peptide is least specific. Studies indicated that this antigen is a heat shock protein homolog similar to a *Borrelia burgdorferi* heat shock protein and that antibodies produced to this spirochete bind to the 80 kDa ehrlichial protein (IJdo et al., 1998b).

As in Lyme borreliosis and human babesiosis, white-footed mice are probably epizootiologically important in the occurrence of granulocytic ehrlichiae. These rodents can maintain the HGE agent and infect immature *I. scapularis* (Telford et al., 1996). Further, PCR methods and serologic tests have detected the DNA of the HGE agent or antibodies to this bacterium in white-footed mice captured in Minnesota (Walls et al., 1997) and Rhode Island (Yeh et al., 1997). Therefore, these rodents should be included in surveillance studies to determine if the HGE agent is endemic in tick-infested sites and can be used to conduct additional studies on immune response. With the advances made in culturing the HGE agent from human and horse blood in HL-60 cell cultures (Goodman et al., 1996; Heimer et al., 1997), it should be feasible to isolate and characterize granulocytic ehrlichiae from white-footed mice and cotton mice.

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