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Authors: Magnarelli, Louis A., Stafford, Kirby C., IJdo, Jacob W., and Fikrig, Erol

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ANTIBODIES TO WHOLE-CELL OR RECOMBINANT ANTIGENS OF *BORRELIA BURGDORFERI*, *ANAPLASMA PHAGOCYTOPHILUM*, AND *BABESIA MICROTI* IN WHITE-FOOTED MICE

Louis A. Magnarelli,[1,4] Kirby C. Stafford III,[1] Jacob W. IJdo,[2,3] and Erol Fikrig[2]

[1] Department of Entomology, The Connecticut Agricultural Experiment Station, PO Box 1106, New Haven, Connecticut 06504, USA
[2] Section of Rheumatology, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, USA
[3] Current address: College of Medicine, Division of Rheumatology, University of Iowa, Iowa City, Iowa 52242, USA
[4] Corresponding author (email: louis.magnarelli@po.state.ct.us)

ABSTRACT: Serum samples were obtained from white-footed mice (*Peromyscus leucopus*) in tick-infested areas of Connecticut during the period 2001 through 2003 and analyzed for antibodies to *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti*. Emphasis was placed on the evaluations of highly specific recombinant VlsE or protein (p) 44 antigens of *B. burgdorferi* and *A. phagocytophilum*, respectively, in a newly developed enzyme-linked immunosorbent assay (ELISA) as well as testing sera with whole-cell antigens by conventional ELISA or indirect fluorescent antibody staining methods. Of the 414 mouse sera analyzed, 310 (75%) had antibodies to whole-cell *B. burgdorferi*, whereas 157 (38%) were positive to the VlsE antigen. The latter nearly equaled the overall antibody prevalence rate (37%) computed when sera were tested separately with the p44 antigen. Mice were exposed to these pathogens and *B. microti* (antibody prevalence = 25%) in extreme northern Connecticut as well as the southern coastal areas of the state, thus indicating further geographic expansion of these infections. Fifty-three (13%) sera from widely separated sites had antibodies to all three pathogens. With expression and immunological recognition of VlsE and p44 antigens in *P. leucopus*, separate incorporation of these fusion proteins in an ELISA was very helpful in confirming past or current infections and in identifying specific foci for *B. burgdorferi* and *A. phagocytophilum*.

Key words: *Anaplasma phagocytophilum*, antibodies, *Babesia microti*, *Borrelia burgdorferi*, ELISA, *Peromyscus leucopus*.

INTRODUCTION

In areas of northeastern United States, where *Ixodes scapularis* ticks are abundant, *Borrelia burgdorferi* sensu stricto, *Anaplasma phagocytophilum*, and *Babesia microti* infect human beings, domesticated animals, or wildlife species. Human cases of Lyme borreliosis are most frequently reported (Walker et al., 1996; Centers for Disease Control and Prevention, 2002). Larvae and nymphs of *I. scapularis* feed on birds and several species of mammals, including white-footed mice (*Peromyscus leucopus*). These rodents are chief reservoirs for *B. burgdorferi* and *B. microti* and, to a lesser extent, also might be important in the epizootiology of *A. phagocytophilum*.

Compared to cats, dogs, and white-tailed deer (*Odocoileus virginianus*), white-footed mice have a more restricted home range. Because serum antibodies to different tick-borne pathogens have been detected in these rodents (Magnarelli et al., 1995, 1997), serologic investigations of field-collected mice can yield important information on the geographic distribution and spread of disease organisms.

Analyses for serum antibodies can provide useful information on antibody prevalence and humoral responses to specific surface and internal antigens of pathogens. In tests of human and deer sera, the use of certain recombinant antigens (i.e., fusion proteins), such as VlsE of *B. burgdorferi* and protein (p) 44 of *A. phagocytophilum*, has improved the specificity of enzyme-linked immunosorbent assay (ELISA) results (Magnarelli, 2002, 2004a, b). The main purpose of the present study was to further investigate the geographic distributions of tick-borne infections in white-footed mice. The

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specific objectives were to: 1) develop a polyvalent ELISA, incorporating highly specific VlsE and p44 antigens; 2) compare these results with those of conventional analyses containing whole-cell antigens; and 3) to determine antibody prevalence rates for *B. burgdorferi, A. phagocytophilum*, and *B. microti* infections in widely separated sites in Connecticut.

MATERIALS AND METHODS

White-footed mice were caught during the period 2001 through 2003 in Sherman box traps (H. B. Sherman Traps, Inc., Tallahassee, Florida, USA) placed in or near forests at residential sites during late spring and summer in Fairfield (Westport and Weston), New London (Groton and Old Lyme), and Litchfield (Canaan, Cornwall, and Salisbury) Counties in Connecticut. *Ixodes scapularis* ticks are present at all locations. Sites in southern Connecticut are located between Weston (41°12′N, 73°22′W) and Groton (41°21′N, 72°04′W), whereas Canaan (42°01′N, 73°19′W), Cornwall (41°50′N, 73°19′W), and Salisbury (41°59′N, 73°25′W) are in the northwestern part of the state. Mice were bled following protocols approved by an institutional animal care and use committee at The Connecticut Agricultural Experiment Station, ear tagged, and released into the environment. Following centrifugation, sera were obtained and promptly stored at −60 C until antibody analyses could be conducted.

Enzyme-linked immunosorbent assays (ELISA) or indirect fluorescent antibody (IFA) staining methods were used to detect total antibodies to whole-cell or recombinant antigens of *B. burgdorferi, A. phagocytophilum*, and *B. microti*. Details on the use of an ELISA with whole-cell *B. burgdorferi* (strain 2591) and on IFA methods with the NCH-1 strain of *A. phagocytophilum* or whole-cell *B. microti* have been described (Magnarelli et al., 1995, 1997, 1999). Recombinant VlsE (VlsE1-HIS), a His_6-tagged version of the full-length VlsE protein of *B. burgdorferi*, and p44 of *A. phagocytophilum* are surface-exposed antigens previously used in an ELISA to detect antibodies in white-tailed deer (Magnarelli et al., 2004a), cattle (Magnarelli et al., 2004b), and human beings (Magnarelli et al., 2002). The full-length VlsE antigen was produced at the University of Texas Medical School Science Center in Houston, Texas, as described (Lawrenz et al., 1999), whereas the p44 antigen was made at Yale University (IJdo et al., 1999). All antigens were coated to flat-bottomed polystyrene plates (Nunc A/S, Roskilde, Denmark) at concentrations of 1 μg/ml for VlsE, 2.5 μg/ml for p44, and 5 μg/ml for whole-cell *B. burgdorferi* to achieve optimal reactivity. Commercially prepared (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA), affinity-purified horseradish peroxidase-labeled goat anti-*P. leucopus* immunoglobulins were diluted to 1:4000 in phosphate-buffered saline solution (PBSS) for use in an ELISA with VlsE or p44 antigens. Sera were diluted in PBSS (pH 7.2) to 1:160, 1:320, and 1:640 for testing with whole-cell or recombinant antigens, and if positive, were retested at higher dilutions to determine titration endpoints and to check reproducibility of results. All plates contained positive and negative control sera for *P. leucopus*, as done in earlier studies (Magnarelli et al., 1997), plus controls for PBSS and conjugated antibodies. The reactivity of all antigens was further verified by testing positive human and deer sera (Magnarelli et al., 2002, 2004a).

To determine cut-off values for positive results, 18 negative *P. leucopus* sera, used in earlier studies (Magnarelli et al., 1997, 1999), were analyzed by ELISA. Net absorbance values, which represent differences in optical density (OD) readings for reactions with or without antigen, were calculated for serum dilutions of 1:160, 1:320, and ≥ 1:640. Critical regions for positive results were established by performing statistical analyses (three standard deviations plus the mean) of net OD values. In an ELISA with VlsE antigen, net OD values of 0.06 and 0.04 were considered positive for serum dilutions of 1:160 and ≥1:320. These OD values were much lower than those established for an ELISA with whole-cell antigen (0.18, 0.15, and 0.11). Cut-off values of 0.10, 0.07, and 0.06 were used in analyses with the p44 antigen of *A. phagocytophilum*.

A *z*-test was applied to determine significant differences in test results. The statistical software program (SigmaStat, SPSS Inc., Chicago, Illinois, USA) included the Yate's correction. Values of $P<0.05$ were considered significant. Cohen's kappa statistic was used to determine concordance of results for assays with whole-cell or recombinant antigens of *B. burgdorferi* and *A. phagocytophilum* (Systat, SPSS Inc., Chicago, Illinois, USA).

RESULTS

Antibodies to one or more tick-borne pathogens were detected in white-footed mice at all sampling sites. Antibody

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TABLE 1. Presence of serum antibodies to whole-cell or recombinant antigens of *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, or *Babesia microti* in white-footed mice in Connecticut, as detected by using polyvalent enzyme-linked immunosorbent assays (ELISAs) or indirect fluorescent antibody (IFA) staining methods.

Sampling		Total sera tested	*B. burgdorferi* No.(%)[a] with antibodies to		*A. phagocytophilum* No.(%)[a] with antibodies to		*B. microti*
County	Year		WC[b]	VlsE[b]	WC[b]	p44[b]	No.(%) positive[a,b]
Fairfield	2001	26	22(85)	13(50)	6(23)	5(19)	2(8)
	2002	45	41(91)	23(51)	15(33)	19(42)	4(9)
	2003	28	14(50)	2(7)	7(25)	9(32)	1(4)
Litchfield	2002	58	44(76)	27(47)	16(28)	19(32)	6(10)
	2003	128	89(70)	35(27)	34(27)	44(34)	8(6)
New London	2001	36	25(69)	15(42)	13(36)	18(50)	22(61)
	2002	73	59(81)	38(52)	34(47)	34(47)	48(66)
	2003	20	16(80)	4(20)	4(20)	3(15)	9(45)
Totals		414	310(75)	157(38)	129(31)	151(37)	100(24)

[a] Percent positive equals the number of positives divided by total sera tested in the respective category by year and county.

[b] Recombinant VlsE and p44 antigens were tested by ELISA methods. Whole-cell *B. burgdorferi* was used in an ELISA, whereas *A. phagocytophilum* and *B. microti* whole-cell antigens were tested by IFA staining methods.

prevalence rates were highly variable, regardless of whether whole-cell or recombinant antigens were used (Table 1). Moreover, antibodies to *B. burgdorferi* whole-cell or recombinant VlsE antigens were detected more frequently than antibodies to *A. phagocytophilum* or *B. microti* antigens. There were differences, however, in how sera reacted to the test antigens. Of the 414 total sera analyzed, 310 (75%) had antibodies to whole-cell *B. burgdorferi*, whereas 157 (38%) samples were positive to VlsE. These results were statistically significant ($z=10.668$, $P<0.001$). Percentages of positive sera to whole-cell antigens were statistically greater than those calculated for an ELISA with VlsE in each year at sites in all three counties. A kappa value of 0.314 indicated discordant results for all sera tested by an ELISA with whole-cell or VlsE antigens, regardless of year and county (kappa range = 0.118–0.410). In laboratory analyses for *A. phagocytophilum* antibodies, the antibody prevalence rate was higher for samples tested by ELISA with the p44 antigen (37%) as compared to IFA staining methods with whole-cell antigens (31%); however, these results were not statistically significant ($z=1.749$, $P=0.080$). Similarly, a kappa value of 0.785 for all sera tested shows concordance of results for both assays and in each separate field for each year and county (kappa range = 0.478–0.890). There also was evidence of *B. microti* infections in mice at widely separated sites, but 81 (79%) of the total 102 positive sera were from animals captured in Groton and Old Lyme.

Titration endpoints and geometric means varied. Antibody titers ranged from 160 to 20,480 in an ELISA with whole-cell, VlsE, or p44 antigens. A maximum titer of 40,980 was recorded for seven sera, obtained from animals captured in southern Connecticut (Groton and Westport/Weston areas), when samples were tested with whole-cell *B. burgdorferi* antigens by an ELISA. In general, there were high concentrations of antibodies, as evidenced by the majority of the titers (63%) being 2,560 or greater and by the corresponding elevated geometric means. The geometric means for an ELISA with whole-cell or VlsE antigens were 3,588 and 1,326, respectively. The geometric mean (3,235) for seropositivity to the p44 antigen of *A.*

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phagocytophilum nearly equaled that calculated for results of an ELISA with whole-cell *B. burgdorferi* antigen.

There was serologic evidence of mouse exposure to multiple tick-borne agents. Of the 337 sera that tested positive to one or more antigens, 93 (28%) samples had antibodies to *B. burgdorferi* and *A. phagocytophilum*, 39 (12%) tested positive to *B. burgdorferi* and *B. microti*, four (1%) were positive to *A. phagocytophilum* and *B. microti*, and 53 (16%) sera had antibodies to all three pathogens. However, the number of positive sera with antibodies to *B. burgdorferi* only ($n=129$) greatly exceeded those for single reactions to *A. phagocytophilum* ($n=11$) or *B. microti* ($n=8$).

Positive sera reacted differently to whole-cell or recombinant antigens of *B. burgdorferi* and *A. phagocytophilum*. The number of antibody positive sera to whole-cell and VlsE antigens ($n=154$) nearly equaled the total number of positives to whole-cell antigens only ($n=156$). The remaining three positive sera reacted only to the VlsE antigen. In tests for *A. phagocytophilum* antibodies, results were confirmed when 120 positives reacted to whole-cell and recombinant p44 antigens by IFA staining methods and an ELISA, respectively. The remaining positives were single reactions to either whole-cell ($n=9$) or p44 antigens ($n=31$).

DISCUSSION

There was serologic evidence of *B. burgdorferi, A. phagocytophilum*, and *B. microti* infections in *P. leucopus* at study sites in all three counties. These results and published findings on isolations of these pathogens or on the detection of the DNA of these disease agents in ticks, white-footed mice, or white-tailed deer from northeastern United States (Anderson et al., 1979; Anderson et al., 1991; Schwartz et al., 1997; Stafford et al., 1999; Courtney et al., 2003; Magnarelli et al., 2004a) clearly demonstrate widespread geographic distribution of numerous foci. Our serologic findings from Litchfield County, however, indicate a more northern distribution of these disease agents in Connecticut. Presence of *B. microti* at these sites is particularly noteworthy because this pathogen was previously thought to be restricted mainly to the coastal areas of northeastern United States (Anderson and Magnarelli, 2004; Kogut et al., 2005). The presence of *B. microti*-infected ticks in the Lower Hudson Valley Region of New York State extending from Westchester County northward into Columbia County (Kogut et al., 2005) parallels our findings. Because concurrent tick and human infections of *B. burgdorferi* and *A. phagocytophilum* also have been demonstrated (Nadelman et al., 1997; Schwartz et al., 1997), both should be considered in differential diagnoses in cases of suspected tick-associated diseases.

Coinfections have been documented for *P. leucopus* (Anderson et al., 1991; Stafford et al., 1999). This is not surprising because these rodents are often heavily parasitized by larvae and nymphs of *I. scapularis* during the warmer months, and once infected with these pathogens, these animals serve as reservoirs. Simultaneous infections of *B. burgdorferi* and *A. phagocytophilum* in laboratory mice resulted in increased concentrations of both pathogens and more severe Lyme arthritis, compared to mice that were only infected with *B. burgdorferi* (Thomas et al., 2001). Therefore, the combined effects of pathogenic organisms on the immune systems of certain hosts, such as *P. leucopus*, might be epidemiologically significant by enhancing reservoir competency and, possibly, by allowing for further amplification of infectious agents in nature.

Serum samples contained varying concentrations of antibodies to the VlsE and p44 antigens of *B. burgdorferi* and *A. phagocytophilum*, respectively. These results verify that these antigens were expressed when the pathogens infected *P. leucopus*. The antibody-positive preva-

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lence rate derived from the VlsE antigen in ELISA, however, was lower than that for whole-cell antigens, whereas the reverse was noted for recombinant p44 and whole-cell *A. phagocytophilum*. Differences in assay sensitivities are probably caused by one or more of the following: technical factors, including variation in binding of serum antibodies to antigenic epitopes; differences in host immune responses to the disease agent; or differential expression of immunodominant antigens when the pathogen infects hosts. In studies of Erp (outer surface proteins E and F) proteins (Stevenson et al., 2002) of *B. burgdorferi*, extensive variations in Erp sequences occurred among different isolates. Several surface and internal antigens of *B. burgdorferi* are immunologically recognized by mammals (Dressler et al., 1993; Magnarelli et al., 2004b). High concentrations of antibodies to a multitude of nonspecific proteins present in whole-cell preparations can sometimes block or interfere with specific antibody reactivity to key immunodominant proteins, such as VlsE in an ELISA. One would expect stronger and more frequent positive results in tests using multiple antigens of *B. burgdorferi* than to a single antigen. Moreover, *B. burgdorferi* and *A. phagocytophilum* change their surface antigenic components in response to cell-mediated or humoral immune responses in mice (Liang et al., 2004) and horses (Wang et al., 2004), respectively. Therefore, we suspect that the surface-exposed VlsE lipoprotein might not always be expressed when the pathogen infects *P. leucopus*. In addition, high antibody titers, such as those recorded when we tested *P. leucopus* sera with the immunodominant p44 antigen, tend to greatly enhance overall assay performance. Extensive evaluations of the full-length recombinant VlsE and a peptide corresponding to the invariant IR6 region of this antigen with human sera in an ELISA (Lawrenz et al., 1999; Liang et al., 1999, 2001; Magnarelli et al., 2002; Bacon et al., 2003; Schulte-Spechtel et al., 2003) revealed that these antigens are suitable for laboratory diagnosis. Similar results were recorded when deer sera were tested with the VlsE antigen (Magnarelli et al., 2004a). Despite varying test results, separate incorporation of highly specific VlsE or p44 antigens in an ELISA (as an adjunct to using whole-cell antigens) was useful in confirming past or current infections of *B. burgdorferi* or *A. phagocytophilum* in *P. leucopus*.

Although there was evidence of mouse exposure to multiple tick-borne agents, reactions to *B. burgdorferi* alone were most prevalent. Single reactions of sera to *A. phagocytophilum* and *B. microti* also were recorded. In analyses of human (Magnarelli et al., 2002), deer (Magnarelli et al., 2004a), and cow sera (Magnarelli et al., 2004b), high specificity was noted when recombinant VlsE and p44 antigens were tested. Moreover, in challenge studies of *P. leucopus* and C3H/HeJ mice, infected by *A. phagocytophilum*, rodents did not induce antibodies to diagnostically significant *B. burgdorferi* antigens (Bunnell et al., 1999). Because both of these bacteria and the protozoan, *B. microti*, are unrelated organisms and there were numerous antibody reactions to a single pathogen in our analyses, we conclude that serologic cross-reactivity is not a problem when the VlsE or p44 antigens are used in an ELISA.

Serologic investigations of *P. leucopus* can yield important information on the geographic distributions and seasonal changes in prevalence of tick-borne infections. Compared to larger mammalian hosts, such as deer, horses, and humans, mice have smaller home ranges and live for much shorter periods. Serologic studies of *P. leucopus*, therefore, can help identify specific foci and also might be useful in monitoring changes in tick infection rates following management efforts to reduce tick populations at selected sites.

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