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Coexistence of Antibodies to Tick-Borne Pathogens of Babesiosis, Ehrlichiosis, and Lyme Borreliosis in Human Sera

LOUIS A. MAGNARELLI,[1]* J. STEPHEN DUMLER,[2] JOHN F. ANDERSON,[1] RUSSELL C. JOHNSON,[3] AND EROL FIKRIG[4]

Department of Entomology, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504[1]; Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland 21201[2]; Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota[3]; and Section of Rheumatology, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06520[4]

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Serum specimens from persons with or without Lyme borreliosis were analyzed by indirect fluorescent antibody staining methods for total immunoglobulins to *Babesia microti*, *Ehrlichia chaffeensis* (Arkansas strain), and *Ehrlichia equi* (MRK strain). There was serologic evidence of human exposure to multiple tick-borne agents in 15 (6.6%) of 227 serum samples obtained in Connecticut and Minnesota. Of these, 10 serum samples were from Connecticut patients who had erythema migrans and antibodies to *Borrelia burgdorferi* (range, 1:160 to 1:40,960). A maximal antibody titer of 1:640 was noted for a *B. microti* infection, whereas titration end points of 1:640 and 1:1,280 were recorded for *E. chaffeensis* and *E. equi* seropositives, respectively. In specificity tests, there was no cross-reactivity among the antisera and antigens tested for the four tick-borne pathogens. On the basis of serologic testing, a small group of persons who had Lyme borreliosis had been exposed to one or more other tick-borne agents, but there was no clinical diagnosis of babesiosis or ehrlichiosis. Therefore, if the clinical picture is unclear or multiple tick-associated illnesses are suspected, more extensive laboratory testing is suggested.

Ticks are abundant in or near forests where white-footed mice (*Peromyscus leucopus*), white-tailed deer (*Odocoileus virginianus*), and other mammals abound. Human residences are sometimes located in foci for Lyme borreliosis or babesiosis, illnesses associated with populations of *Ixodes scapularis* (black-legged tick). These ticks transmit *Borrelia burgdorferi* sensu stricto and *Babesia microti* in inland areas of the Northeast as well as on nearby islands off the coast (26, 27, 30). Human cases of both diseases are well documented there (4, 7, 17, 30). Serologic analyses of human sera have confirmed clinical diagnoses by detecting antibodies to these etiologic agents. Moreover, both pathogens have been isolated from white-footed mice and meadow voles (*Microtus pennsylvanicus*), and as in *I. scapularis*, coinfection was demonstrated (3, 26).

Human ehrlichiosis is another emerging problem in tick-infested areas. *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis, occurs in southern states (1, 10). Human granulocytic ehrlichiosis, which is caused by *Ehrlichia equi* or a closely related agent (5, 8), is present in Minnesota and Wisconsin and may occur elsewhere. *Amblyomma americanum* (Lone star tick) is believed to be a vector of *E. chaffeensis* (2, 14), while *I. scapularis* and *Dermacentor variabilis* (American dog tick) are suspected vectors of *E. equi* or a closely related agent (5, 8). Unidentified rickettsia-like organisms which share antigens with ehrlichiae have been observed in the hemocytes (blood cells) of *I. scapularis* and *D. variabilis* (20) with or without the presence of *B. burgdorferi* in tick midguts.

With the continued reversion of agricultural land to forests and the building of human residences in and near tick-infested areas, there has been increased exposure of persons to hard-bodied ticks. The prevalence of tick bites is particularly high during the warmer months, a period when ticks are more active and when persons visit recreational areas or are more active outdoors. Consequently, there is greater potential for human exposure to multiple tick-borne pathogens during the summer. This retrospective study was conducted to determine if persons who have been diagnosed with Lyme borreliosis also carry antibodies to *B. microti*, *E. chaffeensis*, or *E. equi*.

Most of the serum samples included in the present study were obtained in Connecticut. Some of these specimens had been tested previously for antibodies to *B. burgdorferi* in other investigations (4, 18, 19, 21). Five study groups were established. Persons diagnosed with Lyme borreliosis were divided into two groups: those who had erythema migrans (n = 40) with subsequent cardiac, neurologic, or arthritic disorders, as documented by physicians, and those who lacked or did not recall having expanding skin lesions but who had arthritis (n = 15). All serum samples had antibodies to *B. burgdorferi* (titers of 1:320 to 1:20,480) as determined by a polyvalent, standard enzyme-linked immunosorbent assay (ELISA) with whole-cell antigen coated to polystyrene plates (21). In addition, these persons lived in or had entered tick-infested sites in Connecticut. Other serum samples were from 106 persons who were being tested for syphilis (premarital screening program). These subjects were apparently healthy and had no histories of spirochetal infections. The fourth study group included five serum samples from persons who had secondary or latent syphilis and contained antibodies to *Treponema pallidum* with titers of 1:64 or greater in standard fluorescent antibody adsorption tests. The last group consisted of six serum samples from laboratory personnel who had no histories of tick-borne infections. These samples were used as normal controls in all analyses. Most serum samples were obtained from physicians or the Connect-

* Corresponding author. Mailing address: Department of Entomology, The Connecticut Agricultural Experiment Station, P. O. Box 1106, New Haven, CT 06504-1106. Phone: (203) 789-7241. Fax: (203) 789-7232.

TABLE 1. Reactivity of human serum samples to *B. burgdorferi*, *B. microti*, *E. chaffeensis*, and *E. equi* by IFA staining methods or ELISA

Study group[a]	No. of serum samples tested[b]	No. (%) with antibodies to:			
		B. burgdorferi[c]	*B. microti*	*E. chaffeensis*	*E. equi*
Lyme borreliosis (erythema migrans)[d]	40	40 (100)	3 (7.5)	4 (10)	3 (7.5)
Lyme arthritis[e]	15	15 (100)	0	0	0
Premarital screening[f]	106	0	0	2 (1.9)	1 (0.9)
Syphilis[g]	5	1 (20)	0	0	0
Normal controls[h]	6	0	0	0	0

[a] Serum samples were obtained from patients in Connecticut during 1985 to 1987.
[b] Serum samples were tested by an ELISA for antibodies to *B. burgdorferi* and by IFA staining methods for the remaining organisms.
[c] Results were published earlier (18, 19) and are listed here for comparison.
[d] Erythema migrans with supportive serologic test results.
[e] No erythema migrans, but arthritis with antibodies to *B. burgdorferi*.
[f] Serum samples were collected from apparently healthy subjects who had no histories of spirochetal infections and who were tested for antibodies to *T. pallidum*.
[g] Antibodies to *T. pallidum*, detected by the Venereal Disease Research Laboratory and fluorescent antibody adsorption tests, cross-reacted with *B. burgdorferi* (19), but there was no clinical evidence of Lyme borreliosis.
[h] Negative controls (no evidence of spirochetosis).

icut Department of Health during the period of 1985 through 1990 and had been stored at −60°C at the Connecticut Agricultural Experiment Station. For comparative analyses, an additional 25 serum specimens were obtained from persons who were treated for *B. burgdorferi* infections during 1994 at the Yale University Lyme Disease Clinic. In addition to having erythema migrans, these persons had one or more of the later manifestations of Lyme borreliosis and had produced antibodies to *B. burgdorferi* (titers of 1:640 to 1:40,960 in a polyvalent ELISA).

In view of recent reports of human granulocytic ehrlichiosis in Minnesota and Wisconsin (5, 8), it was appropriate to test sera from persons who lived in or near tick-infested areas of the upper midwestern United States. *I. scapularis* populations are abundant in sections of both states. Fifty-five serum samples were obtained from the University of Minnesota. Of these, 30 specimens were from persons who were suspected of having Lyme borreliosis and had class-specific immunoglobulin (Ig) M or IgG antibodies to *B. burgdorferi* (strain 297), as determined by ELISAs or Western blot (immunoblot) analyses (13) performed at the University of Minnesota. In the latter, banding patterns that included reactivity to key proteins of *B. burgdorferi*, such as those with molecular masses of 24, 31, 34, 39, 41, and 88 kDa, were considered evidence of exposure to this spirochete. Twenty-five serum samples were used as negative controls, since there were no histories of tick-borne diseases.

Four antigens were used in serologic analyses to quantitate concentrations of serum antibodies. Washed whole cells of *B. burgdorferi* (strain 2591) were used in an ELISA as described earlier (21). The *B. microti* antigen consisted of infected hamster erythrocytes recovered from animals inoculated with whole blood from an infected person who lived in Stonington, Conn., and who was diagnosed with babesiosis (4). Methods of preparing this antigen for indirect fluorescent antibody (IFA) staining and criteria for determining antibody-positive results have been reported elsewhere (4, 9). Briefly, serial twofold dilutions of human sera were screened for total Igs with a 1:80 dilution of polyvalent fluorescein isothiocyanate-labeled goat anti-human Ig (Organon Teknika Corp., Durham, N.C.) mixed with 1.0% Evan's Blue. When six normal serum samples were tested at dilutions of 1:80 or greater, there were no false-positive reactions. Therefore, distinct fluorescence of *Babesia* antigen at or above the 1:80 dilution for test sera was considered positive. In addition to negative control sera, a positive human serum with homologous antibody to *B. microti* (titer of 1:1,280) was included in the analyses. Human sera also were tested with *E. chaffeensis* or *E. equi* antigens by IFA staining methods. The former was cultured in DH 82 cells and was kindly provided along with human sera containing antibodies to the Arkansas strain by J. E. Dawson and J. G. Olson of the Centers for Disease Control and Prevention (Atlanta, Ga.). The *E. equi* antigen (MRK strain) was kindly supplied by J. Madigan, University of California (Davis, Calif.). Human sera with antibodies to *E. equi* or a closely related agent were from two patients diagnosed with granulocytic ehrlichiosis (5, 8); titration end points were 1:10,240 or greater. The fluorescein isothiocyanate-conjugated antiserum included in analyses for antibodies to *B. microti* was used at a dilution of 1:40 without Evan's Blue stain in phosphate-buffered saline (PBS) solution (pH = 7.2) as the second antibody in tests with ehrlichial antigens. Since screening of the six normal human serum samples at dilutions of 1:80 or greater revealed no false-positive reactions, distinct fluorescence of ehrlichial antigens at or above this dilution for test sera was considered evidence of past or current infection. This cutoff titer is the same as or close to those established by other investigators (5, 8, 11, 12). The grading of the intensity of fluorescence to determine titration end points was done conservatively. All analyses contained controls for conjugated antibodies and positive and negative sera. In addition, a 1:80 dilution of serum antibodies raised in a horse experimentally infected with *E. equi* (MRK strain), provided by J. Madigan, was used for IFA staining methods, along with a 1:40 dilution of fluorescein isothiocyanate-labeled goat anti-horse IgG antibodies (specific for heavy and light chains; Cooper Biomedical, Melvern, Pa.) in a PBS solution to verify the reactivity of the *E. equi* antigen.

Homologous and heterologous tests of antisera and antigens were conducted to assess specificity. Included were six serum samples from persons who were diagnosed with babesiosis (4), four serum samples from patients who had ehrlichiosis (two each for *E. chaffeensis* infections and granulocytic ehrlichiosis), and three serum specimens from individuals who had *B. burgdorferi* infections (18). Homologous antibody titers for the *Babesia* infections ranged from 1:1,280 to 1:5,120, while those for *B. burgdorferi* and ehrlichial infections varied from 1:320 to 1:2,560 and 1:5,120 to 1:40,960, respectively. An additional five serum samples from persons who had syphilis and antibodies to *T. pallidum* (19) were included for comparison. Serum dilutions of 1:80 to 1:320 of each sample were screened against *B. burgdorferi*, *B. microti*, *E. chaffeensis*, and *E. equi* by IFA staining methods.

Serum samples with or without Igs to *B. burgdorferi* also contained antibodies to *B. microti*, *E. chaffeensis*, or *E. equi*. Of the 40 serum samples obtained during 1985 to 1987 from Connecticut patients who had erythema migrans and antibody concentrations of 1:640 to 1:20,480 to *B. burgdorferi*, 8 (20%) had serologic evidence of exposure to other tick-borne pathogens (Table 1). In one sample, there were antibodies to both ehrlichial agents (titers of 1:160 to 1:320) and to *B. burgdorferi* (titer of 1:640). Of the three serum samples containing antibodies to *B. microti* (range, 1:160 to 1:640) and *B. burgdorferi* (range, 1:640 to 1:1,280), one specimen had evidence of *E. equi* infection (titer of 1:1,280). Similarly, 3 of 106 serum samples from apparently healthy persons who were being tested for syphilis in a premarital screening program and who were lack-

ing serologic evidence of Lyme borreliosis had antibodies to *E. chaffeensis* or *E. equi*; titers ranged from 1:320 to 1:640. Maximal antibody titers of 1:640 and 1:1,280 were recorded for the remaining serum samples reactive to *E. chaffeensis* and *E. equi*, respectively. The other tested sera from Connecticut patients, including the specimens obtained from the Yale Lyme Disease Clinic, were negative when *B. microti* and the ehrlichial antigens were included in the analyses. There were 2 of 30 serum samples from the Minnesota study group that each had antibodies to *E. chaffeensis* at a titer of 1:320. Both persons had erythema migrans, and clinical diagnoses of Lyme borreliosis were serologically confirmed by an ELISA and Western blot analyses. The remaining 25 serum samples from Minnesota were negative in all trials.

In tests of specificity, there was little or no cross-reactivity noted. There were no false-positive reactions when 1:80 serum dilutions of four homologous antiserum samples to *E. equi* or *E. chaffeensis* were analyzed with heterologous antigens of these bacteria or with *B. microti* and *B. burgdorferi*. Similarly, the six serum samples from patients who had babesiosis and homologous antibody titers to *B. microti* ranging from 1:1,280 to 1:5,120 were negative when tested against the three other antigens. In the analyses of three other serum specimens with antibodies to *B. burgdorferi* (homologous titer range, 1:320 to 1:2,560) or five serum samples from syphilitic patients with antibodies to *T. pallidum* (range, 1:320 to 1:5,120), the results were likewise negative in tests for antibodies to *B. microti* and ehrlichiae.

Persons diagnosed with Lyme borreliosis and having erythema migrans and Igs to *B. burgdorferi* also carried antibodies to *B. microti*, *E. chaffeensis*, or *E. equi*. Babesiosis and ehrlichiosis were undiagnosed, and it is possible that these infections were mild or asymptomatic. It also is unclear whether or not these infections were concurrent. Nonetheless, these patients had a high probability of tick exposure and may have had multiple tick bites.

Endemicity of Lyme borreliosis in or near tick-infested forests of southern New England is well recognized (4, 7), and the occurrence of simultaneous infections with *B. burgdorferi* and *B. microti* in humans has been reported (6, 23, 24). Our serologic findings add further evidence that ehrlichial infections occur in tick-infested areas. PCR and DNA detection procedures have verified the presence of *E. equi* in *I. scapularis* collected in Connecticut (22). Therefore, patients who present with unexplained febrile illnesses marked by severe headache, arthralgias, and myalgias and who have leukopenia, anemia, and/or thrombocytopenia following tick bites may have had exposure to multiple tick-borne pathogens, including ehrlichiae. Paired sera from patients should be tested against *B. burgdorferi*, *B. microti*, *E. chaffeensis*, and *E. equi*. When the clinical picture is unclear or if multiple infections are suspected, laboratory diagnosis also should include complete blood cell counts, blood chemistry analyses, PCR and DNA detection procedures whenever possible (5, 8, 25), and microscopic examinations of blood smears to help identify *B. microti* and ehrlichiae. *B. microti* infects erythrocytes (9, 28), whereas *E. chaffeensis* and *E. equi* primarily target mononuclear phagocytes and polymorphonuclear leukocytes, respectively (5, 10, 15). That there was no cross-reactivity in antibody tests with antisera and antigens of *B. microti*, *E. chaffeensis*, and *E. equi* in the present study should help to differentiate these infections serologically. We recognize, however, that variations in antibody responses exist among infected hosts and that minor serologic cross-reactivity can occur among certain ehrlichial infections.

Travel histories of persons who had antibodies to *E. chaffeensis* or *E. equi* in Connecticut and Minnesota were unavailable. It is possible that some of these individuals may have been bitten by ticks outside of these states. Although most human infections of *E. chaffeensis* are diagnosed and documented in south central and south Atlantic states (15, 16), our serologic test results indicate a possible distribution in northern states. A human case from Cape Cod, Mass., has been reported (29). There was no travel outside Cape Cod for 1 to 3 weeks before the onset of the illness, and there was exposure to ticks. The authors concluded that the ehrlichial infection had been acquired on Cape Cod. Isolation of ehrlichiae from or detection of ehrlichiae in ticks, rodents, deer, or other possible mammalian reservoirs or identification of these bacteria by PCR methods and DNA detection techniques is needed to further document and confirm the presence of *E. chaffeensis* and *E. equi* in southern New England and Minnesota.

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