EDITORIALS

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Original research, developments, or clinical observations, which include original research data and are fundamental contributions to the knowledge or understanding of spirochetal and tick-borne diseases, are all eligible for consideration for the prize. The paper must be a first-time publication of the author’s own work. Reference to pertinent earlier work by the author may be included to give perspective.

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The award will be presented at the 1995 Physician Conference. In cases of multiple authorship, the prize will be divided equally between or among the authors.
GUEST EDITORIAL

How Do We Control Tick-Borne Diseases?

Tom G. Schwan, Ph.D.

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Lyme disease, relapsing fever, Rocky Mountain spotted fever, ehrlichiosis, Q fever, tularemia, Colorado tick fever, Powassan encephalitis, babesiosis, and tick paralysis. This is an impressive list of human illnesses presently known in North America that may result from the bite of one of numerous species of hard (Ixodid) and soft (Argasid) ticks. The agents that cause these diseases include a variety of bacteria, viruses, protozoa, and in the last example, a toxin produced in the tick's salivary glands. The new cases occurring each year from all of these infections probably number between 10 and 15 thousand, although cases of Lyme disease outnumber all of the others combined. The clinical spectra of these maladies are diverse and vary from apparent infection or transient rashes to chronic disabilities, acute fulminant infections, and sometimes death. Yet, if I were asked if any of these diseases should be eradicated, I would have to answer no. Controlled? Yes. Eradicated? No. Let me explain. All of these illnesses result from millions of years of evolution and complex biological interactions of microbes, blood-feeding arthropods (including ticks), small and large species of wild mammals, birds, even a few species of reptiles, domestic animals, and of course the human beings that become ill. Supporting these webs of life and death are photosynthetic and saprophytic plants, the physical habitat, and climatic patterns providing suitable amounts of warmth and moisture. The occasional illnesses and less frequent deaths from tick and other vector-borne agents in North America are unfortunate parts of the price that we, as humans, must pay to coexist with the other life-forms on our planet. If it were feasible to eradicate any of these diseases (and it is not), it would require the elimination of at least one but probably many species of animals that together maintain a particular disease-causing agent in nature. It would be absurd to desire the elimination of any species under the auspices of eradicating tick-borne diseases. For like with humans, the negative qualities we perceive in other species are overshadowed by their inherent value and what they contribute to a diverse and natural world. A world without any of the species responsible for perpetuating the microbes transmitted to humans by ticks would be far less interesting. Having proclaimed that I want to keep ticks and tick-borne agents around, I believe that we need to act responsibly and strive to reduce the suffering that can result from tick bites. How do we proceed? Because of the numerous and diverse biological components that are responsible for these zoonoses, which characterize all the tick-borne diseases in North America, the scientists and medical professionals that work on these problems have equally diverse interests and expertise. National and international meetings on Lyme disease typically include sessions on clinical medicine, epidemiology, microbiology, molecular biology, immunology, therapeutics, diagnostics, tick biology and control, ecology and wildlife studies, and, more recently, vaccine development. To reduce time lost to Lyme disease and other tick-borne diseases, all of us who work on any aspect of these infections must communicate, share our ideas and results, and cooperate with each other. Such behavior appears to be difficult for many, probably due in part to the increasingly competitive arena of funding for biomedical research, and possibly exacerbated by a person's need for recognition and the fear that acknowledging what others have done before them will somehow reduce their own efforts or how their efforts are perceived by others (including those holding the purse strings). Lately, I have been reading again the many reviews on ticks and tick-borne diseases written by the late Harry Hoogstraal, wishing he were still alive but thankful for the legacy and spirit he left us. Most of the newcomers to tick-borne diseases who have been drawn together because of Lyme disease probably never met Harry and are not aware of what he meant to us and what he contributed to the study of ticks and tick-borne diseases throughout the world. Harry died on February 24, 1986, on his 69th birthday, ending the life and career of an incredible man whose many accomplishments included authoring over 500 scientific publications on ticks and tick-borne diseases. Harry was also an extremely generous man and supported many younger scientists who were interested in ticks by sending them to meetings, giving them money, sending them books, suggesting research topics, and helping rewrite manuscripts while refusing authorship. Scientific meetings were always the better for his presence and have been the less since his passing. More than any other person, Harry Hoogstraal unified the study of ticks and tick-borne diseases, and he was the catalyst for others in the field to communicate and work together. Today, we badly need more people who can lay their egos and self-interests aside and work together if we are to quickly and effectively reduce the morbidity and mortality resulting from tick-borne diseases. Of course, we need highly skilled diagnosticians to recognize these illnesses when presented before them. We need specific and sensitive laboratory tests to help identify when tick-borne diseases are the problem and when they are not. We need patients who are correctly diagnosed with these diseases to receive prompt and appropriate antibiotic therapy when it is available. We need to reduce the number of tick bites because this is how all of these infections occur. We would benefit from (but not need if everything else in this list was met) effective vaccines used appropriately for people living in truly endemic regions where tick-borne agents have been identified. We need programs that educate the public about these diseases and what they can do to reduce the risk of tick bites and possible illness or death. And of course, we need meetings and journals for scientists to present new
information in open forums with fair, objective, and critical but constructive evaluation. But we also need more people like Harry Hoogstraal with the spirit to help rather than to hinder the work of others. As the human population continues to grow and has increasing contact with the natural world, "new" tick-borne diseases will undoubtedly continue to emerge. Three such diseases have done so in the last 25 years: babesiosis, Lyme disease, and ehrlichiosis. Eradication of these and the other tick-borne diseases is not an option; however, we can reduce their impact on us as we coexist with the animals maintaining them. We must preserve our natural biological communities, enjoy them, learn from them, but be aware of the risks and protect ourselves. To achieve these goals, we must work together.
Analysis of Relapsing Fever Spirochetes from the Western United States

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Tick-borne relapsing fever occurs in scattered foci throughout much of the western United States. *Borrelia hermsii*, *B. turicatae*, and possibly *B. parkeri*, are the etiological agents and these spirochetes are maintained in zoonotic cycles involving rodents and soft ticks of the genus *Ornithodoros*. Five isolates of *B. hermsii* were examined by numerous molecular and genetic techniques and compared to other species of *Borrelia*. Plasmids in the isolates of *B. hermsii* were similar except for one (YOR-1) from northern California. This isolate also differed from the others of *B. hermsii* by not having DNA amplified by the polymerase chain reaction (PCR) when using primers specific for variable major protein (Vmp) genes 7 and 21. These results demonstrate that the repertoire of Vmp genes differs among isolates of *B. hermsii*. DNA probes based on vmp sequences also hybridized to *B. coriacaeae*, the suspected agent of epidemic bovine abortion disease of cattle, suggesting that this spirochete may also cause a relapsing phenomenon.

Key words: *Borrelia hermsii*, tick-borne-relapsing fever, ticks

INTRODUCTION

Tick-borne relapsing fever of humans in the western United States is presently known to be caused by two or three closely related species of *Borrelia (B.)* spirochetes transmitted by Argasid ticks in the genus *Ornithodoros (O.).* (1). The specific status of these bacteria, which includes *B. hermsii*, *B. parkeri*, and *B. turicatae*, is based on their apparent vector specificity for different species of ticks (2). In 1942, Davis identified *B. hermsii* and *B. parkeri* as new species of *Borrelia* based on laboratory studies in which these spirochetes were transmitted only by the ticks *O. hermsii* and *O. parkeri*, respectively (3). Brumpt had named *B. turicatae* 9 years previously based on this spirochete’s specificity to *O. turicata* (4). While some have questioned the validity of using vector specificity as the sole criterion for establishing species of *Borrelia*, very little work has been done since the studies of Davis to address the genetic relatedness of the recognized species of relapsing fever spirochetes in North America (5) and to re-examine the issue of vector specificity and spirochete distribution in nature using newer molecular techniques. Such efforts were no doubt hindered by the lack of an artificial culture medium until 1971, when Kelly described a liquid broth that allowed him to successfully maintain *B. hermsii in vitro* continuously for 8 months while the spirochete retained its infectivity in mice (6). Subsequent improvements of this medium have now allowed for the successful cultivation of many species of *Borrelia*, providing the opportunity for molecular and genetic studies of these spirochetes (7). Over the last 15 years, significant advances have been made toward understanding the structural properties of the relapsing fever spirochete’s genome and defining, in part, the genetic mechanisms that control antigenic variation (8–15). However, nearly all of the recent molecular and genetic studies have been restricted to one strain (HS1) of *B. hermsii* isolated from one adult *O. hermsii* tick collected from eastern Washington.

Other isolates of *B. hermsii* and the other species of relapsing fever spirochetes remain virtually unstudied.

One of our long-term goals is to understand the mechanisms responsible for the apparent specificity of the different species of relapsing fever spirochetes for different species of *Ornithodoros* ticks, as described by Davis many years ago (3). Another goal is to examine the antigenic behavior of *B. hermsii* during infection in its tick vector. As a prerequisite, we need to first develop molecular diagnostic techniques that will rapidly identify these spirochetes to their currently accepted taxon and to examine isolates within each species to identify conserved and variable molecular and genetic determinants. Toward these objectives, we recently developed both a DNA hybridization probe (16) and a monoclonal antibody (17) that rapidly identified *B. hermsii* from the other species of *Borrelia* currently known to exist in North America. In this report, we examine three uncharacterized isolates of *B. hermsii* from California and compare them with other *Borrelia* spp.

MATERIALS AND METHODS

*Borrelia Strains and Cultivation. Borrelia hermsii* HS1 (ATCC 35209) serotype C originated from *O. hermsii* collected near Spokane, WA (18). *Borrelia hermsii* FG was isolated at Rocky Mountain Laboratories in April 1987 from the blood of a human with relapsing fever in Washington. *Borrelia hermsii* CON-1, MAN-1, and YOR-1 were isolated from the blood of humans with relapsing fever in California (19). *Borrelia coriacaeae* CO53 (ATCC 43381) was isolated from *O. coriacaeae* from California (20, 21). *Borrelia parkeri*, *B. turicatae*, and *B. anserina* were isolated from *O. parkeri*, *O. turicatae*, and a domestic chicken, respectively, and were in the Rocky Mountain Laboratories bacterial pathogen collection. *Borrelia burgdorferi* B31 (ATCC 35210) was isolated from *Ixodes scapularis (= dammini)* from Shelter Island, NY (22). Live borrelial cultures were maintained in BSK-II medium (23) at 34°C and passaged twice a week.

*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis. Whole-cell
lysates of *Borrelia* were prepared as described (24). The Laemmli buffer system (25) was used with a Vertical Gel Electrophoresis System (Bethesda Research Laboratories-Gibco, Gaithersburg, MD) following the instructions of the manufacturer. After electrophoresis, lysates were transferred to nitrocellulose in a Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Richmond, CA) using the Towbin buffer system. The *Borrelia* genus-specific monoclonal antibody H9724 (26) was used at a dilution of 1:50. Membranes were prepared and examined by 125I-protein A radiography, as described previously (24).

**DNA Purification.** Total DNA was purified from 500-mL stationary phase cultures of the various borreliae, as described previously (27). A technique that enriches for plasmid DNA was also used for some of the DNA preparations (28). All samples were precipitated in cold 95% ethanol, washed twice in 70% ethanol, suspended in TE (10 mM Tris [pH 7.6], 1 mM EDTA), and quantified by UV A260.

**DNA Analysis.** Profiles of plasmid DNA were examined by agarose gel electrophoresis using a Mini-Sub DNA Cell (Bio-Rad Laboratories), as described previously (29). Briefly, undigested samples of DNA were electrophoresed in 0.3% agarose gels with TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA) to separate plasmids. This was accomplished by electrophoresing for 5 minutes at 50 V and continuing for 16 hours at 12 V and staining with ethidium bromide.

**DNA Probes.** Three DNA probes were used to examine DNA samples of borreliae in either dot blot or Southern blot hybridizations. Probe E4 was described previously (16) and is a cloned 570-bp HindIII fragment that contains the 5' end of the variable major protein 7 (vmp7) gene and the adjacent upstream sequence of the expression locus of *B. hermsii* HS1. Probes V-7 and V-21 were 890-bp amplification products of the polymerase chain reaction (PCR) described below, using primers unique to the vmp7 and vmp21 genes, respectively. Probes were nick translated by using a commercial kit (Boehringer Mannheim Biochemical, Indianapolis, IN) and labeled with [α-32P]dCTP following the instructions of the manufacturer. Unincorporated isotope was separated from the labeled DNA by centrifugation in a Mini Spin Column (Worthington Diagnostics, Freehold, NJ). Probes were denatured by heating to 95°C for 5 minutes and cooled on ice until use.

**DNA Hybridization Procedures.** Serial twofold dilutions of borrelial DNA were applied directly to membranes using a 96-well dot blot manifold (Bio-Rad Laboratories), as described previously (16). Borrelial plasmids separated by low percentage agarose gel electrophoresis were transferred to membranes by the method of Southern (30), as described previously (16). Dot blot hybridizations were done on GeneScreen Plus membranes and Southern blots were done on GeneScreen membranes (Dupont, NEN Research Products, Boston, MA) following the instructions of the manufacturer. Hybridization conditions, buffer, and membrane washes were described previously (16). DNA hybridization patterns were determined by exposing Kodak X-Omat film to the membranes at -70°C with an intensifying screen and development with a Kodak X-OMAT M20 processor.

**Polymerase Chain Reaction (PCR).** Nucleotide sequences for primers were chosen from sequences published for the vmp7 and vmp21 genes (11) and are as follows: vmp7, 5'-TGT-GAG-ATG-TGT-TGG-GAT and 5'-ACT-GGC-TCT-TGT-GAA-CCT; and vmp21, 5'-TTT-CAG-ATA-CAT-TAG-GCT and 5'-CCT-GCT-GGT-TTT-GGA-TCT. The predicted size of both amplification products is 890 bp. Oligonucleotide primers were synthesized with a

**RESULTS**

Spirochete cultures designated MAN-1, CON-1, and YOR-1 were each isolated from the blood of human patients clinically ill with relapsing fever in California. These isolates were provided to us by Jane Wong, Microbial Diseases Laboratory, California Department of Health Services, Berkeley, CA. These spirochetes were compared to the prototype strain HS1 of *B. hermsii* and to other species of *Borrelia* known to occur in North America.

Protein profiles of the three new isolates were consistent for relapsing fever spirochetes and grouped together with *B. hermsii* (Fig. 1A). The new isolates also bound monoclonal antibody H9724 (Fig. 1B), which is specific to flagellin of all known members of the genus *Borrelia* tested to date. Reactivity with this antibody identified the new isolates as *Borrelia* spp. Reactivity with monoclonal antibody H9826 identified the spirochetes MAN-1, CON-1, and YOR-1 as *B. hermsii* (17).

Samples of total DNA from the cultured spirochetes were examined by several techniques and demonstrated that the YOR-1 isolate varied from the other isolates of *B. hermsii*. Agarose gel electrophoreses of total, undigested DNA showed that the plasmid profile of the YOR-1 isolate was distinctly different from four other isolates of *B. hermsii* (Fig. 2). The PCR amplification of a chromosomal target resulted in
Fig. 2. Agarose gel electrophoresis of *Borrelia* DNA demonstrating the unique plasmid profile of the YOR-1 isolate. Size standards are shown on the right in kilobase pairs (kb).

all *B. hermsii* isolates yielding the predicted 276 base pair product while DNA of *B. burgdorferi* was not amplified (Fig. 3). However, dot blot hybridization using the E4 probe derived from *B. hermsii* HS1 with DNA from 10 borreliae demonstrated again that YOR-1 varied from the other isolates of *B. hermsii* (Fig. 4). This probe bound significantly less under high stringency to YOR-1 than to the other isolates of *B. hermsii*. This same probe was also used following the separation of plasmid DNA in an agarose gel and transfer to a membrane for hybridization (Fig. 5). Of the four typical isolates of *B. hermsii*, this probe hybridized to two plasmids (Fig. 5B). This pattern resulted from the *vmp7* portion of the probe hybridizing to the linear plasmid containing the silent loci of *vmp* genes and from the other part of the probe hybridizing to a different linear plasmid containing the expression locus. Note that for an exposure of 18 hours, very little hybridization was detected with YOR-1 (Fig. 5B). With a longer exposure of 72 hours, hybridization with two distinctly different-sized plasmids in the YOR-1 isolate was seen (Fig. 5C). This is probably due to the presence of sequences homologous to both the expression site and the silent copies of *vmp* genes on these different-sized plasmids in YOR-1.

Next, we attempted to amplify by PCR an 890 base pair region of either the *vmp7* or *vmp21* genes based on sequences obtained from the prototype strain HS1. While four of the isolates of *B. hermsii* yielded the predicted amplification product using primers specific for *vmp7* (Fig. 6A), no amplification was detected from YOR-1 or the other species of *Borrelia* tested. Attempts to amplify DNA using

Fig. 3. PCR amplification products of the predicted 276 base pairs from relapsing fever spirochetes but not the Lyme disease spirochete, *B. burgdorferi*. Size standards are shown on the left in base pairs.

Fig. 4. *Borrelia hermsii* probe E4 hybridized with six twofold serial dilutions of standardized concentrations of total DNA from 10 *Borrelia* isolates.
Fig. 5. (A) Plasmid profile and Southern hybridization using *B. hermsii* probe E4 with film exposure of (B) 18 hours and (C) 72 hours. Size standards are shown on the left in kilobase pairs (kb). Note the reduced and unique pattern of hybridization of this probe with DNA from the YOR-1 isolate of *B. hermsii*.

primes specific for *vmp21* resulted in only two isolates of *B. hermsii* yielding a product (Fig. 6B). These results suggest that there was most likely sequence variation in the *vmp* genes among the *B. hermsii* isolates.

Our failure to amplify DNA above could have resulted from only minor differences in DNA sequence between the prototype strain and the other isolates. Therefore, we next used the entire 890 base pair fragments of the *vmp7* and *vmp21* genes amplified from the prototype strain HS1 as probes in dot blot hybridizations (Figs. 7 and 8). The *vmp7* probe (V7) hybridized as strongly to the same four isolates that were also amplified by PCR using *vmp7*-specific sequences (Fig. 7). Weaker hybridization was also detected with YOR-1, *B. parkeri*, *B. turicatae*, and *B. coriaceae*. The *vmp21* probe (V21) hybridized strongest with the two isolates that were also amplified by PCR using *vmp21*-specific primers (HS1 and FG) (Fig. 8). Again, weaker hy-
Fig. B. Borrelia hermsii probe V21 hybridized with six twofold serial dilutions of standardized concentrations of total DNA from 10 B. hermsii HS1 DNA using primers specific for the vmp21 gene and has 890 base pairs.

hybridization was detected with all of the samples except B. anserina and B. burgdorferi. These results demonstrated that, although YOR-1 was not amplified by PCR using primers specific for either vmp7 or vmp21, homologous sequences to these two variable membrane protein genes are present in its genome. These probes also identified homologous sequences to the variable major protein genes in B. parkeri, B. turicatae, and B. coriaceae (Figs. 7 and 8). The former two species are also relapsing fever spirochetes and the presence of genes involved with antigenic variation was not surprising. To date, only a single isolate of B. coriaceae (CO53 used in the present study) exists, and nothing is known concerning its behavior during infection in mammals. Our data suggest that B. coriaceae has genes homologous to vmp genes of B. hermsii, and therefore, this spirochete might be a relapsing fever spirochete and have the ability to alter its cell surface to avoid the host’s immune response.

DISCUSSION

We have begun to identify molecular and genetic differences among a small group of relapsing fever spirochetes. All five isolates examined were identified as B. hermsii by protein profiles, PCR amplification of a conserved chromosomal target, and their reactivity with genus and species-specific monoclonal antibodies. Yet, among these five isolates, some obvious differences were apparent. Each varied slightly in plasmid profile. The YOR-1 isolate, however, displayed a plasmid pattern unique and strikingly different from the other B. hermsii ssp. examined. In addition, dot blot or Southern blot hybridizations with three DNA probes originating from the prototype strain HS1 demonstrated that there were differences in two vmp genes between YOR-1 and the other four isolates of B. hermsii. The PCR amplification using primers based on sequences specific to the vmp7 gene also separated YOR-1 from the other isolates of B. hermsii. Picken also identified slight sequence variation in the flagellin gene of YOR-1 compared with the prototype strain HS1 of B. hermsii (32). The PCR amplification based on sequences specific to the vmp21 gene also demonstrated differences among the other four isolates, with only strains HS1 and FG being amplified. This was the first attempt, and admittedly only a start, to compare genes responsible for antigenic variation among isolates of B. hermsii. A much larger analysis is currently underway in our laboratory using additional isolates recently acquired from human patients residing in other localities. All identified sequences of vmp genes of B. hermsii to date have originated from a single strain, HS1. Now we have begun to see a pattern of variation among a small sample of five isolates. The vmp7 gene was highly conserved in four of the five isolates while vmp21 was contained in only two of the five isolates. Although YOR-1 genes are not identical to vmp7 nor vmp21 genes of other isolates, it does have homologous vmp genes including a sequence that is homologous to the expression site. The identification of YOR-1 as B. hermsii was based on reactivity with monoclonal antibody H9826 (17) but has been confirmed by sequencing its 16S rRNA and the fact that in our laboratory only O. hermsii, but not O. parkeri or O. turicata was capable of transmitting this spirochete (Gage, Marconi, and Schwan, unpublished observations).

SUMMARY

Relapsing fever spirochetes and their tick vectors are found in numerous foci throughout much of the western United States (33). The mechanisms for restricted maintenance and transmission of each of the currently recognized species of Borrelia by their respective species of Ornithodoros tick are unknown. In this investigation, we have begun to identify and analyze differences among isolates B. hermsii. Despite considerable variation in genetic and molecular determinants among isolates, unpublished studies in our laboratory have shown that these spirochetes are transmitted only by O. hermsii. Thus, they share the determinants responsible for vector specificity described by Davis so many years ago (3). Our studies will continue in an attempt to identify the mechanisms that restrict these spirochetes to specific species of ticks.

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REFERENCES


Senile Plaques, Neurofibrillary Tangles, and Neurupil Threads Contain DNA?

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It has recently been reported that microorganisms—spirochetes—may play a role in the etiology of Alzheimer’s disease (AD). Further ultrastructural analysis revealed that the helically shaped microorganisms isolated or cultured from the Alzheimer’s brain possess axial filament that taxonomically distinguishes spirochetes from other bacteria. The 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes. DNA staining that employs DAPI is the most popular method for the detection of cell culture mycoplasmas. We expected that DAPI would also bind to the DNA of other bacteria, namely, to the DNA of spirochetes. If senile plaques, neurofibrillary tangles, and neurupil threads are indeed formed by spirochetes, they would consequently also contain DNA and would, therefore, be visualized with DAPI. Histological brain sections of AD cases, control cases, and smears of reference spirochetes were stained with DAPI without and with DNase pretreatment and were examined with a fluorescent microscope. When using this sensitive fluorescent DNA stain, not only reference spirochetes may be visualized, but also senile plaques, neurofibrillary tangles as well as neurupil threads. DNase pretreatment abolish the DAPI staining of these structures, indicating that they contain DNA. Based on observations reported here we suggest that the DNA found in the Alzheimer’s type changes may be of bacterial origin and therefore would be in agreement with our earlier observations that spirochetes may well contribute to the pathogenesis of AD.

Key words: Alzheimer’s disease, DNA, Microorganisms, Spirochetes

INTRODUCTION

The etiology of Alzheimer’s disease (AD) (1), which is characterized by a slow, progressive appearance of memory disturbances and dementia, is unknown and the treatment unresolved.

In AD, the most consistent histopathological changes are the senile plaques, named also neuritic or argyrophilic plaques, and the neurofibrillary tangles localized in the cerebral cortex. Senile plaques are complex, mostly spherical structures, usually 100 to 150 μm of diameter containing fine argyrophilic filamentous structures, made of both neuronal and non-neuronal elements. Different components are usually described in senile plaques: degenerating neurites, neurupil threads, extracellular amyloid fibrils, and reactive cells including microglia and astrocytes. Neurofibrillary tangles are formed by pathological argyrophilic filamentous structures, often extending into the apical dendrite (2).

In addition to tangles and plaques, argyrophilic filamentous structures, named “curly fibers” or “neurupil threads,” disseminated in the neurupil were also reported in AD. Following the description of Braak et al. (3), the slender silver-stained structures follow tortuous courses. They could be traced, even in 100-μm-thick preparations for only short distances. They were independent from each other and did not form continuous networks. Using morphometric image analysis, the mean length of neurupil threads in AD was found to be 21.9 ± 11.01 μm (4).

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It has recently been reported that in 14 autopsy cases with histopathologically confirmed AD, spirochetes were found using dark-field microscopy in blood and cerebrospinal fluid and were isolated from brain tissue (5). We used the method described for the isolation of the Treponema pallidum from tissue by Delacrétaz (6). Samples of the isolation fluid were examined with dark-field microscopy. Spirochetes were cultured from the blood of four out of five AD cases investigated in a modified Noguchi medium (5, 7). Moreover, spirochetes were cultured from the brain tissue in three of four AD cases tested in Barbour-Stonner-Kelly (BSK) medium. These observations have suggested that Alzheimer’s disease may correspond to the late stage of neurospirochetosis (5). Further ultrastructural analysis demonstrated that the helically shaped microorganisms isolated and cultured from the Alzheimer’s brain possess axial filaments, indicating that they taxonomically belong to the order Spirochaetales (8).

The 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) is a fluorescent dye that binds selectively to DNA (9). This fluorescent dye by its specificity and sensitivity is frequently used for the detection of mycoplasma infection in cell cultures. We expected that DAPI would also bind to the DNA of other bacteria, namely, to the DNA of spirochetes. If senile plaques, neurofibrillary tangles, and neurupil threads are indeed formed by spirochetes, they would consequently also contain DNA and would, therefore, be visualized with DAPI.

MATERIAL AND METHODS

Part of the material used in this study was the same as described in a previous work (5). From 10 out of the 14
AD cases investigated earlier (5), and from 10 new, neuropathologically confirmed AD cases (including two familial AD cases), where spirochetes were isolated from the brain tissue, 7-µm-thick frozen sections from the unfixed parietal cortex were cut on a cryostat, postfixed with methanol for 2 minutes, and stained with 3 µg/mL of DAPI (Boehringer, 236 276) in methanol, for 30 minutes at 37°C. The sections were rinsed in distilled water for 5 minutes; mounted with gum arabic; coverslipped; and examined with a fluorescence microscope either in UV light, using G 365/11 excitation and LP 397 barrier filters as well as Bp 485/20 excitation and LP 520 barrier filters.

Frozen sections of the parietal cortex from four patients where the neuropathological examination did not reveal AD-type changes, and we did not find spirochetes, were also stained with DAPI. With respect to the four control cases, two patients were hospitalized with ischemic heart failure due to myocardial infarct. The neuropathological examination revealed hypertensive encephalopathy and no cerebral lesion, respectively. One patient had Wernicke's aphasia associated with agraphia, without deterioration of the other cognitive functions. In this case, the clinical and the neuropathological diagnosis was that of glioblastoma localized in the left temporal lobe. The neuropathological diagnosis of the fourth patient with chronic alcoholism was that of Wernicke's encephalopathy.

In order to remove DNA, another set of sections before staining with DAPI was treated with 1 mg/mL of DNase I (Boehringer, 1284 932) diluted in PBS containing 5 mmol/mL of Mg++, at pH 7.8 at 37°C for 3 hours. The same procedure was also carried out using RNase free DNase I (Boehringer, 776 785).

To eliminate the possibility of an unspecific binding of the DAPI to amyloid, a set of DNase I treated sections were post-stained with Thioflavin S, a fluorochrome, widely used for the demonstration of amyloid in AD (10).

Smears of spirochetes from the B31 strain of Borrelia burgdorferi were treated and examined in the same manner.

RESULTS

The examination of the DAPI-stained brain sections of the 20 neuropathologically confirmed AD cases in UV light showed not only a strong silver-white fluorescence of nuclei of resident cells of the central nervous system (CNS) but also senile plaques, neuropil threads, and neurofibrillary tangles, which all exhibited a similar fluorescence. A strong yellow fluorescence against a dark-green background of all of these structures were obtained when the sections were examined with the Bp 485/20 excitation and LP 520 barrier filters, generally used for the detection of the fluorescein isothiocyanate (FITC) fluorescence (Figs. 1A through 1C). DNase I or RNase free DNase I pretreatment abolished not only the DAPI staining of the CNS cell nuclei but also that of the senile plaques, neurofibrillary tangles, and neuropil threads (Fig. 1F). Sometimes a slight signal persisted in the core of the plaques.

When the sections after DNase treatment were post-stained with Thioflavin S, the senile plaques showed a strong yellow fluorescence indicating that the DNase does not abolish the visualization of the amyloid (Fig. 1G).

In four control cases, without AD-type changes, for those without spirochetes, on frozen sections stained by DAPI, only nuclei of the CNS cells showed strong fluorescence (Fig. 1D).

Reference Borrelia burgdorferi spirochetes (B31 strain) when stained with DAPI showed a similar yellow-green fluorescence to those of CNS cell nuclei (Fig. 1E). We observed a striking similarity between the morphology of the DAPI-stained reference spirochetes and those of the neuropil threads. Both are helically shaped, elongated, slightly coiled structures.

DISCUSSION

The DAPI is a fluorescent dye that binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity, yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence (9, 11). Its specificity was found to be similar to that of the fluorescent DNA-binding benzimidole derivative Hoechst 33258 (9, 11). Mitochondrial DNA does bind the fluorochrome but at levels imperceptible by routine fluorescent microscopy (12). Mycoplasma, however, which have approximately 10 times the DNA content of mitochondria, are readily detected as bright foci against the dark background (9, 12). Bacteria being prokaryotic cells contain DNA, but they differ from the eukaryotic cells in that the nuclear material is not surrounded by a limiting nuclear membrane. As we demonstrated here, reference spirochetes by their DNA content may also be visualized by DAPI. The observations that senile plaques, neurofibrillary tangles, and neuropil threads all bind to DAPI and the fact that DNase pretreatment abolishes the DAPI staining of these structures indicate that they contain DNA.

It is difficult to explain the presence of DNA, particularly in senile plaques and in the neuropil threads. In neurons, due to neuronal cell death, one may expect to find free DNA secondary to karyorrhexis in AD. However, in other pathologies (cerebral hypoxia, infarct, or glioblastoma) also associated with neuronal or other cell destruction, we never found the same distribution of the free DNA in the form of the neuropil threads, as in AD. In biopsy specimens, we can sometimes observe elongation of cell nuclei due to artifacts of electrocoagulation but quite different from the form of the neuropil threads.

The helically shaped morphology of the distribution of the DNA in the neuropil threads is similar to the distribution of the DNA in the reference spirochetes. The term “curly fibers,” used as a synonym for neuropil threads (2, 3), also describes well the morphology of the spirochetes. In addition, the length of the neuropil threads is reported to be 21.9 ± 11.01 µm (4), which may also correspond to the length of the spirochetes. These observations suggest that the DNA found in the fine filamentous structures of the AD-type changes may be bacterial, namely, spirochetal DNA. In the core of some senile plaques, because of the dense deposition of amyloid, the DNA may not be as easily accessible for the action of the DNase. This may explain that a slight DAPI signal remained in the core of some plaques after DNase treatment.

A similar strong fluorescence to that obtained with DAPI of the senile plaques, neurofibrillary tangles, and neuropil threads in the AD cortex may be obtained with Thioflavin S, known to detect amyloid (10). The fact that DNase treatment does not abolish the Thioflavin S fluorescence of the senile plaques but does abolish the DAPI staining indicates, in agreement with Russel et al. (9), the specificity of DAPI as a sensitive DNA fluorescent stain.

It is important to explain that the DAPI-stained structures, except the CNS cell nuclei, are the same as those also positive for Thioflavin S a fluorochrome that is used
Fig. 1. Photomicrographs illustrating that senile plaques, neurofibrillary tangles, and neuropil threads in Alzheimer's disease contain DNA when stained with the 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), a sensitive and selective DNA fluorochrome. We show here that DAPI is also useful for the visualization of the bacterial DNA of spirochetes. The histological sections were taken from the parietal cortex of two out of the 20 neuropathologically confirmed AD cases and from the parietal cortex of one of the four control cases investigated in this study. Photomicrographs A, C, F, and G were taken from the parietal cortex of a familial AD case, and B was taken from a sporadic AD case. (A) Senile plaques and neuropil threads (arrows) showing fluorescence when stained with DAPI and, here, examined with Bp 485/20 excitation and LP 397 barrier filters. (B) Senile plaques exhibiting a similar fluorescence to those of cell nuclei (arrows) when stained with DAPI. (C) Arrows point to the DAPI-stained neurofibrillary tangles of a neuron. (D) Photomicrograph of a DAPI-stained section derived from the parietal cortex of a control case, without AD-type changes. Only CNS cell nuclei exhibit fluorescence. (E) Smear of *Borrelia burgdorferi* spirochetes (B 31 strain) cultured in a synthetic medium stained with DAPI, showing a yellow-green fluorescence. (F) In order to remove DNA, before staining with DAPI, an adjacent section to that illustrated in A and C was treated with DNase I. The fluorescence of the senile plaques and neuropil threads disappeared. In rare senile plaques, a slight signal in the core of the plaque remained, as shown here. The DAPI-staining of this section was performed at the same time and with the same DAPI solution as those sections shown in A and C. (G) DNase I pretreatment does not abolish the fluorescence of amyloid due to Thioflavin S. Bar in A corresponds to 10 μm and is the same for C and D. Bar in B corresponds to 30 μm and is the same for F and G. Bar in E represents 10 μm.
worldwide for the demonstration of the amyloid deposition in senile plaques, neurofibrillary tangles, and neuropil threads in AD. We recently observed that cultured reference spirochetes of the B 31 strain of *Borrelia burgdorferi*, isolated spirochetes of the Nichols strain of *Treponema pallidum*, as well as oral spirochetes when stained with Thioflavin S exhibit a similar yellow-green fluorescence to those of senile plaques, neurofibrillary tangles, and neuropil threads (unpublished data). This observation is in agreement with our previous finding (5) that spirochetes (B 31 strain of *Borrelia burgdorferi* and Nichols strain of *Treponema pallidum*) showed a positive immunoreaction with a monoclonal antibody against the N-terminal part of the amyloid precursor protein (APP), suggesting that the APP or at least an APP-like protein (APLP) may be an integral part of this infectious agent (5). Jarrett and Lansbury (13) reported that the amino acid sequence of OsmB (28–44), a periplasmic outer membrane-associated lipoprotein of the *Escherichia coli*, is similar to the C-terminal region of the β-amyloid protein of AD. This peptide was shown to form amyloid fibrils in vitro that bond Congo red. These data suggest that these gram-negative bacteria (spirochetes and *Escherichia coli*) contain an amyloidogenic protein that resembles the β-amyloid protein and may explain that they are stained with Thioflavin S. These notions seem to reinforce the possibility that the structures stained for both Thioflavin S and DAPI may well be of bacterial origin. Because of the morphology of the distribution of the DNA in helically shaped structures in AD, different, e.g., from that of *Escherichia coli* and similar to the distribution of the DNA found in reference *Borrelia* spirochetes, we suggest that it may correspond to spirochetal DNA.

We reported earlier that in a case with concurrent AD and Lyme disease, using specific monoclonal antibody against *Borrelia burgdorferi* (5), spirochetes were found in senile plaques and in neurons. In addition, immunostained individual spirochetes were also found in the neuropil with a morphology that may correspond to that described for “curly fibers” in AD. The same structures were now visualized with DAPI, indicating that they contain DNA. Taken together, these observations suggest that the DNA distributed in the fine filamentous structures of the senile plaques, the neurofibrillary tangles, and the neuropil threads, stained also with Thioflavin S, may well correspond to spirochetal DNA.

Despite these arguments for a final conclusion, investigations using specific nucleic acid probes were undertaken and will be the subject of further communication.

We would like to strengthen that we do not interpret the present observations and those published earlier (5, 8) in that all AD cases are the late manifestation of Lyme borreliosis. It was reported that more than 25% of hospitalized patients, without a clinically active form of Lyme borreliosis, from three different regions of Switzerland shows a positive serology for Lyme disease (14, 15). One may expect that in a no hospitalized healthy population, this percentage would be even lower. The positive serology of these patients indicates that they once had an infection with *Borrelia burgdorferi*, and probably, a large part of them will never develop any manifestation of the disease. We expect that only a part of this population with positive serology for Lyme disease may be considered in a latent stage and may later—even decades later—develop tertiary neuroborreliosis in the form of AD. The incidence of AD (16), which affects a large proportion of the aged population, is higher than the population at risk for developing AD due to neuroborreliosis. Spirochetes, in general, have a high meningo- and neuropitropism; therefore, other spirochetes, e.g., oral spirochetes, intestinal spirochetes, and those so-called commensal spirochetes, may all be considered as possible candidates to develop tertiary neurospirochitoses in the form of AD. Therefore, the characterization of the spirochetes is needed for a final conclusion concerning this matter.

CONCLUSIONS

Using a specific fluorochrome for the detection of DNA (DAPI), we found that senile plaques, neurofibrillary tangles, and neuropil threads all contain DNA and show a similar yellow-green fluorescence to those of the CNS cell nuclei and to those of the reference spirochetes. These observations, taken together with our previous findings (5, 8), suggest that the DNA in the plaques, tangles, and neuropil threads may be of bacterial origin and seem to reinforce the hypothesis (5, 8) that spirochetes may well contribute to the pathogenesis of AD.

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REFERENCES


Lyme Disease in the Elderly

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Lyme disease (LD) is now the most common tick-borne illness in the United States, affecting individuals of all ages. Little, however, is published about clinical patterns of the disease in the elderly population. Twenty-five cases in elderly patients (≥ 60 years) were reviewed. The mean age was 69.24 +/− 1.29 years and the median age was 68 years (range, 60 to 83 years). Clinical patterns, serology, and response to intravenous (IV) or oral antibiotic therapy were noted. Fourteen patients (56%) were initially treated for early Lyme disease. Of these fourteen, three had positive LD serology, two of whom had markedly high titers, and were among five patients who presented with acute LD superimposed on chronic LD through reinfection. The remaining 11 patients (44%) were initially treated for late LD. Ten of these patients were seropositive, three of whom had markedly high titers. In the entire group, markedly positive LD serologies were found in five patients (20%; mean age = 69.4 +/− 2.88 years [range, 65 to 72 years] and median age = 71 years); all five were highly symptomatic. Eleven of the 16 patients treated for late LD received IV antibiotics, and the remaining five took oral antibiotics. The findings suggest that LD behaves similarly in the elderly as in the general population. Nonetheless, certain complaints commonly associated with the so-called normal aging process, especially rheumatic complaints, may be symptomatic of active LD and thus must be carefully evaluated in patients exposed to endemic areas.

Key words: Lyme disease, elderly, ticks, polynynalgia rheumatica

INTRODUCTION

Since the first case of Lyme disease (LD) was identified in the United States in 1975, this complex multisystem illness caused by the spirochete Borrelia burgdorferi has become the nation’s most prevalent tick-borne illness (1). Although incidence is highest for children under 15 (3), the disease affects individuals of all ages. A review of the medical literature on LD reveals numerous studies on adults and children but none that focus specifically on the elderly (≥60 years) (2). Thus, while special considerations for diagnosing and treating the infection in children have been described, no similar descriptions exist for older patients.

In this paper, we review the clinical and laboratory findings in a group of patients aged 60 or older treated for LD. Our purpose is to elucidate any correlations among age, presentation, and therapeutic outcome with specific attention paid to range of symptoms, antibody levels, and antibiotic response.

PATIENTS AND METHODS

We reviewed records of all patients >60 years old (n = 25) treated for LD from 1988 to 1993. Twenty-four lived along the coast of southern New Jersey (known to be endemic for LD) and one patient was a visitor to the area. One of the authors (PWP) cared for all patients at the Lyme Disease Center for South Jersey, Asbury, New Jersey. Data were collected on age, sex, tick bite history, symptoms and signs of Borrelia (B.) infection, serology for B. burgdorferi, diagnosis (early or late LD), antibiotic therapy, and outcome.

Each case met the Centers for Disease Control’s (CDC’s) definition for LD: erythema migrans or at least one late manifestation indicative of musculoskeletal, nervous, or cardiovascular system involvement, or laboratory confirmation of infection (2). Laboratory confirmation requires a positive IgM or IgG antibody found by indirect immunofluorescence assay, enzyme-linked immunosorbent assay, or immunoblot assay.

We measured outcome by response to antibiotic therapy, defining a positive response as the complete or marked resolution of symptoms on clinical evaluation. Oral antibiotics included amoxicillin (plus probenecid), azithromycin, cefuroxime, and doxycycline; IV antibiotics included cefotaxime, ceftriaxone, cefuroxime, and penicillin. (Table 2)

RESULTS

The mean age of the patients was 69.24 +/− 1.29 years, and the median age was 68 (range 60 to 83 years). Thirteen were males with a mean age of 67.46 +/− 1.33 and a median age of 67 (range 61 to 79). The 12 females had a mean age of 71.17 +/− 2.20 and a median age of 72.5 (range, 60 to 83).

Early Lyme Disease

We diagnosed early LD in 14 (56%) of the 25 patients. Symptoms included musculoskeletal discomfort in 10 patients, erythema migrans alone in three, and erythema migrans accompanied by fatigue and atioventricular block in one. Three of the patients with early LD had a positive serology at the time of diagnosis. Among the 14, five had early LD imposed on late LD through reinfection (Table 1: patients 2, 16, 18, 19, and 22), and all but one of these gave a history of recent tick bite.

Late Lyme Disease

Late-stage Lyme disease was initially found and treated in 11 patients (44%), of whom 10 had positive LD serologies. Three had markedly high titers (Table 1). Ten of the 11 patients had musculoskeletal symptoms, including two with neurologic deficits involving cognition, memory, and motor function. The remaining patient presented with depression alone.
**TABLE 1**

Clinical Findings and Serology in 25 Elderly Patients with Lyme Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years) and Sex</th>
<th>Tick Bite History</th>
<th>Symptoms/Signs</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60/F</td>
<td>No</td>
<td>Ataxia, ankle pain, arthralgias, neurologic deficits (cognitive, memory, functional), headache, visual disturbance, ringing in ears, vertigo</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>5</td>
<td>63/F</td>
<td>Yes(^a)</td>
<td>Arthritis, aseptic meningitis, arthralgias, Bell’s palsy</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>8(^a)</td>
<td>65/M</td>
<td>Yes(^a)</td>
<td>Headache, fatigue, Bell’s palsy, facial paresthesia, arthralgias, neurologic deficits (cognitive, memory, functional)</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>10</td>
<td>66/M</td>
<td>No</td>
<td>Neurologic depression</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>12(^a)</td>
<td>68/M</td>
<td>No</td>
<td>Arthralgias, headache, stiff neck, fatigue, multiple joint swelling</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>14(^a)</td>
<td>71/M</td>
<td>Yes</td>
<td>Arthritis, swollen knee (tender and warm) with severe degenerative disease, persistent arthralgias</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>16(^a)</td>
<td>71/F</td>
<td>No</td>
<td>Arthralgias, myalgias, headache, stiff neck, numbness in hand, memory problem (remembers having rash similar to bull’s-eye lesion); fatigue</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>17</td>
<td>71/M</td>
<td>No</td>
<td>Arthralgias, swollen knee, fatigue, stiff neck, headache</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>18(^a)</td>
<td>72/F</td>
<td>Yes</td>
<td>Erythema migrans, headache, dizziness, stiff neck, fatigue, arthralgias, numbness in calf</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>19</td>
<td>73/F</td>
<td>Yes</td>
<td>Erythema migrans, flulike syndrome, stiff neck, headache, knee pain, arthralgias, fatigue</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>23</td>
<td>79/M</td>
<td>Yes</td>
<td>Headache, arthralgias, myalgias, hip pain, stiff neck, blurred vision, numbness in hands, mild ankle edema</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>24</td>
<td>81/F</td>
<td>No</td>
<td>Bull’s-eye lesion (1 year prior), headache, stiff neck, arthralgias in legs</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>25</td>
<td>83/F</td>
<td>Yes</td>
<td>Arthralgias, myalgias, stiff neck, headache</td>
<td>Late Lyme</td>
</tr>
</tbody>
</table>

**Seronegative**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years) and Sex</th>
<th>Tick Bite History</th>
<th>Symptoms/Signs</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>61/M</td>
<td>Yes</td>
<td>Arthralgias, stiff neck, headache, visual discomfort</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>3</td>
<td>61/F</td>
<td>Yes</td>
<td>Erythema migrans, fatigue, atrioventricular block</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>4</td>
<td>62/M</td>
<td>Yes</td>
<td>Erythema migrans</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>6</td>
<td>63/M</td>
<td>Yes</td>
<td>Erythema migrans</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>7</td>
<td>64/F</td>
<td>Yes(^a)</td>
<td>Erythema migrans</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>9</td>
<td>65/M</td>
<td>Yes</td>
<td>Erythema migrans, headache, mild fever, knee pain, hand paresthesia, stiff neck and shoulder discomfort, backache, edema in legs</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>11</td>
<td>67/M</td>
<td>No</td>
<td>Arthralgias, stiff neck, ankle edema</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>13</td>
<td>68/M</td>
<td>Yes</td>
<td>Erythema migrans, mild stiff neck, shoulder discomfort</td>
<td>Late Lyme</td>
</tr>
<tr>
<td>15</td>
<td>71/M</td>
<td>Possible</td>
<td>Erythema migrans, mild knee pain, fatigue, headache, stiff neck, arthralgias</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>20</td>
<td>75/F</td>
<td>Yes</td>
<td>Erythema migrans, mild fatigue, arthralgias</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>21</td>
<td>75/F</td>
<td>No</td>
<td>Erythema migrans, arthralgias, myalgias, headache, stiff neck, paresthesia in arms, fatigue</td>
<td>Early Lyme</td>
</tr>
<tr>
<td>22</td>
<td>76/F</td>
<td>Yes(^a)</td>
<td>Erythema migrans, mild fatigue, stiff neck, paresthesia in arms, arthralgias, myalgias, knee pain, ankle discomfort, headache, fatigue, edema, swollen knee</td>
<td>Early Lyme</td>
</tr>
</tbody>
</table>

\(^a\)Mean age = 69.24 ± 1.29 year; median age = 68 year (range, 60 to 83 years).

\(^a\)Multiple tick bites.

\(^a\)Markedly positive Lyme disease serology.

**TREATMENT**

Oral antibiotics produced a positive therapeutic response within a month in nine of the patients with early LD (64%) (Table 2: patients 3, 4, 6, 7, 9, 13, 15, 20, and 21), all of whom were seronegative when diagnosed.

A total of 16 patients were eventually treated for late-stage LD, including five with markedly positive LD serologies (Table 1: patients 8, 12, 14, 16, and 18). These five had a mean age of 69.4+/−2.88 years (range, 65 to 72) and a median age of 71 years and showed more intense symptoms and signs of late LD (multiple arthralgias and myalgias, headache, fatigue, and paresthesias). Eleven of the 16 patients received IV antibiotics, while five took oral antibiotic therapy alone because either limited resources or inadequate health insurance benefits prevented hospitalization.

**DISCUSSION**

In a large retrospective study by Steere et al. (6), the median age of patients (n = 314) with LD was 26 (range, 2 to 88 years). It is clear, however, from reviewing the cases in our practice over a 5-year period that LD is not rare among older patients and that they present with a full range of signs and symptoms. There are several reasons to believe that more attention should be focused specifically on this group.

We are likely to see more cases of LD among the elderly purely as a result of a projected increase in the number of individuals in this age range. Over the next 50 years, people 85 years and older are expected to be the fastest-growing segment of the United States population; by the end of this century, the number of persons between the ages of 65 and 85 will increase from 26 to more than 30 million (7). Moreover, because older Americans are not only living longer
but are probably engaging in more outdoor recreational activity as well, those who live in or travel to endemic LD areas may face an increased risk of infection.

There are suggestions that older adults are relatively more susceptible to LD. This may reflect a generally observed increase in vulnerability to infection with aging, variously ascribed to senescence of the immune system, underlying health problems, alterations in nonspecific or local host defenses, and protein-calorie malnutrition (8). Senescence of the immune system may be particularly important in LD, as human and animal studies indicate that the host’s immune response partially determines pathologic changes in the disease (9). Parallels between LD and babesiosis, an illness in which advanced age may be the single most important risk factor, suggest the need for further study of the role of immune defenses (10, 11). Both are tick-borne diseases with the same vector and reservoir animals, although babesiosis is confined almost entirely to the elderly and immune-suppressed individuals (27).

A study by Siee et al. (22) also implies that the risk of acquiring LD increases with age. In a longitudinal assessment of the clinical and epidemiological features of LD in 162 long-term residents of a small island close to the south shore of Cape Cod, 26 (16%), including children and adults,
experienced symptoms of LD. Subclinical infection, however, seemed confined to adults. Of 121 asymptomatic residents who gave blood samples, 10 adults (8%)—aged 20 or older—had high titers of IgG antibodies to *B. burgdorferi*, with the highest percentage of cases appearing in adults older than 60 years. This contrasts with no elevated antibody titers in 13 asymptomatic children despite the fact that 82% gave at least one blood sample. The authors caution that although both symptomatic and subclinical infection do appear to increase with age, these may be artificial observations because older residents had more person-years of tick exposure than children and because the proportion of those giving blood rose with age.

A number of factors may obscure symptomatic LD in the elderly. Musculoskeletal pains caused by *Borrelia* infection may be mistaken for the aches and pains common in old age, thus delaying a request for medical attention. Neurologic deficits such as cognitive problems and memory loss may limit a patient's ability to provide accurate history. Fever, a common early symptom of LD (59%) (6), was part of the presentation in only 1 of 25 patients we saw. This may reflect a physiologic blunting of the fever response in the elderly, but it carries a warning that the slight or absent fever in this population does not reliably exclude LD.

Published cases of LD in older patients also suggest that other unrelated medical conditions, such as hypothyroidism (12), chronic renal failure (13), and diabetes mellitus (14) may make the diagnosis more difficult. Our review (Table 1) points out the likelihood of encountering cases of acute LD imposed on chronic LD with pronounced rheumatic symptoms.

Such symptoms can be confused with those of polymyalgia rheumatica (PR) in particular (23), leading to invalid assumptions (24, 25). Both LD and PMR often present with nonspecific signs and symptoms of inflammation, including marked rises in erythrocyte sedimentation rates (ESR)—greater than 100 mm/h in PMR and a similar level of 85 mm/h in the case of patient 23 shown in Table 1. Positive LD serology has also been noted in patients with PMR exposed to endemic areas.

Rare presentations of LD in the elderly have also been described, including neuroborreliosis in patients with stroke (16) and with cerebellar symptoms (17) and LD associated with acute respiratory distress syndrome (15), acrodermatitis atrophicans (18), acute purulent meningitis (19), and cutaneous B-cell lymphoma (20, 21).

Plocker et al. (26) failed, however, to find consistent correlation between age and symptoms. In an Austrian study of 82 patients treated for cutaneous symptoms of LD, the authors attributed most of the neurologic signs observed in 34 (41%) patients to underlying disorders, with old age (defined as ≥80 years of age) the most likely cause in five patients with encephalopathy and four with polyneuropathy. Similarly, they blamed age, not LD, for atrioventricular block in two seropositive patients aged 78 and 82. No correlation was found between patient age and LD serology by indirect immunofluorescence assay, enzyme-linked immunosorbent assay, or immunoblot assay.

As in other age groups, successful treatment of LD in the elderly depends on prompt diagnosis and proper antibiotic therapy, although physiologic changes that occur with aging can alter the pharmacokinetic and pharmacodynamic properties of medications. Among these are reduced gastric acidity, decreased gastric emptying rate, and diminished small bowel motility (8). Tetracyclines, for instance, are known to be catabolic in the elderly. It is also necessary to consider the possibility of age-related adverse drug reactions, such as anaphylaxis from penicillin (more common among older patients) and adverse drug-drug interactions, such as seizures produced by erythromycin plus theophylline (8).

Another concern in LD treatment in the elderly is compliance. Patients with mental and physical deficits caused by old age or LD may not comply fully with an antibiotic regimen and thus may be at risk for possible recurrences that require extended therapy. More serious complications of noncompliance in early LD are the development of severe late-stage symptoms and, in rare cases, death (3–5). Compliance is less of an issue with IV antibiotics, the preferred route of administration for late-stage LD, but, as was seen with five of the cases reviewed here, uncontrollable social and economic factors sometimes necessitate oral therapy as a second choice.

CONCLUSIONS

In terms of severity of symptoms, degree of positivity found by current serological assays, and response to antibiotic therapy, whether oral or parenteral, LD appears to behave similarly in the elderly as in the general population. We found no predictable pattern of symptoms and signs in the “young” geriatric population, but questions about its presentation in the “oldest old” (>85 years) remain. Review of the 25 cases described here indicates that, in areas known to be endemic for LD, the clinician should consider *Borrelia* infection in the differential diagnosis when evaluating elderly patients with rheumatic complaints, fatigue, or neurologic deficits.

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REFERENCES


Human Ehrlichiosis, A Potentially Fatal Tick-Borne Disease

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Key words: Human ehrlichiosis, Human granulocytic ehrlichiosis, Ehrlichiae, Ehrlichia chaffeensis

Ehrlichia (E.) canis, the type species of the genus, was described in Africa in 1935 and subsequently was recognized as the cause of illness in dogs on several continents (1, 2). A particularly virulent form of the disease occurred in U.S. Military working dogs during the Vietnam War, and that outbreak underscored the pathogenic potential of this tick-borne agent. This severe and often fatal form of ehrlichiosis is known as tropical canine pancytopenia (3). Since discovery of the type species in 1935, numerous E. spp. of veterinary medical and human significance have been reported. Veterinary medical pathogens, in addition to E. canis, include E. phagocytophila (tick-borne fever of ruminants), E. equi (equine ehrlichiosis), E. ewingii (canine granulocytic ehrlichiosis), and E. risticii (equine monocytic ehrlichiosis, Potomac horse fever).

Of the three human pathogens assigned to this genus, this review focuses on two. The third, sennetsu ehrlichiosis, has been recognized in southwestern Japan since the late 19th century (4); but it was not until 1953 that the etiologic agent, E. sennetsu, was isolated from a person (5). In the United States, two different ehrlichial agents have been recognized to cause very similar disease syndromes in human beings. The first case of human ehrlichiosis in the United States (acquired in Arkansas) was recognized in 1986 (6). In 1990, an etiologic agent was isolated (7) from a patient, also infected in Arkansas, and shown to be a distinct species, E. chaffeensis (8). More recently, another disease, designated human granulocytic ehrlichiosis (HGE), has been reported from 12 individuals who were exposed in the Upper Midwest (Wisconsin and Minnesota) (9). The etiologic agent appears to be closely related to the domestic animal pathogens, E. phagocytophila and E. equi but has not yet been fully characterized (10).

TAXONOMY

Ehrlichia chaffeensis is one of several species in a genus that was erected in 1945 to accommodate obligately intracellular parasites that have predilection for leukocytes. The type species, E. canis, was originally assigned to the genus Rickettsia, as were several other species now recognized to belong to the tribe Ehrlichiae. The current classification of these obligately intracellular bacteria is as follows:

Order: Rickettsiales
Family: Rickettsiaceae
Tribe: Ehrlichiae
Genus: Ehrlichia

*Corresponding author

Of the several species currently assigned to the genus, E. chaffeensis is apparently most closely related to E. canis and E. ewingii, which are both pathogens of dogs. Likewise, the human granulocytic ehrlichial agent appears to be very closely related to E. phagocytophila and E. equi, which are both parasites of domestic herbivores.

BASIC BIOLOGY OF THE EHRLICHIA SPECIES

Most members of the tribe Ehrlichiae have a narrow tropism for granulocytes or agranulocytes. For example, E. canis, E. risticii, and E. sennetsu parasitize agranulocytes while E. equi, E. ewingii, and the human granulocytic agent are observed exclusively in granulocytes. Both E. phagocytophila and E. chaffeensis appear to be exceptions, at least occasionally parasitizing both agranulocytes and granulocytes.

The Ehrlichiae are nonmotile, gram-negative cocci found in membrane-lined cytoplasmic vacuoles; typically, they occur in aggregates, forming inclusions called morulac. A typical morula observed by light microscopy in stained blood films (or tissue culture material) contains from 10 to 100 organisms (Fig. 1). However, when infected host cells are observed by electron microscopy, single microorganisms can be observed in the cytoplasm (7). The individual organisms are often extremely pleomorphic (Fig. 2); the size of individual organisms is hard to determine, but most are less than 0.5 μm. Some aggregates are extremely dense.

Fig. 1. Photomicrograph of Diff-Quik-stained DH82 cells containing E. chaffeensis morulac (magnification times 1000).

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antibody technique. White-tailed deer are susceptible to experimental infection with *E. chaffeensis* (20). Dogs can also be infected experimentally (21), but there is no strong evidence implicating dogs as epidemiologically important hosts. On the contrary, Harkess et al. (22) and Rohrbach et al. (23) found no increased risk among patients who had contact with dogs. Prazu et al. (24) also found that contact with dogs, even animals with *E. canis* infection, did not constitute a risk for human beings, and Eng and Giles (25) stated that ehrlichiosis was not transmitted from dogs to human beings.

**Human Granulocytic Ehrlichiosis**

Granulocytic human ehrlichiosis is so recently discovered that little is known about geographic distribution or reservoir host(s). All 12 cases reported to date (including two fatalities) occurred in Minnesota and Wisconsin (9, 10). Bakken et al. (9) reported that 11 of 12 (92%) patients had a history of arthropod bite within 10 days of onset of illness and stated that either *Ixodes scapularis* or *Dermacentor variabilis* had been attached to 8 (67%) patients prior to onset of fever.

One human case from New England is confusing because it is unclear whether it was ehrlichiosis due to *E. chaffeensis* or granulocytic ehrlichiosis (26–28). Even in the face of strong serologic evidence of *E. chaffeensis* infection, the diagnosis was questioned. This confusion is understandable and not surprising given that *E. chaffeensis* has also been reported to occur occasionally in granulocytes (6). As stated previously, granulocytic ehrlichiosis has been reported from only 12 people, but neutrophils in every case, to the exclusion of other cells, were parasitized (9, 10). Many more cases of *E. chaffeensis* infection have been reported, but the majority were serologic diagnoses.

**CLINICAL SYNDROME**

Most cases occur between May and October, suggestive of arthropod transmission. Patients present with a flu-like syndrome, often with a history of arthropod exposure. Ehrlichial diseases are characterized by sudden onset of fever, headache, and malaise. Other common symptoms include confusion, myalgia, rigor, sweats, and nausea/vomiting (9, 29). Despite the nonspecific signs and symptoms of this disease, laboratory findings are more constant and frequently include thrombocytopenia, leukopenia, elevated liver enzymes, and, occasionally, anemia.

One early case report of human ehrlichiosis ascribed to *E. chaffeensis* documented bone marrow hypoplasia and suggested diminished production of leukocytes, erythrocytes, and platelets (30). Other bone marrow findings have included myeloid hyperplasia, megakaryocyte, granulomas, marrow histiocytosis, myeloid hypoplasia, pancellular hypoplasia, and normocellular marrow (31). Three postmortem examinations revealed gastrointestinal hemorrhage, mild interstitial pneumonitis, perivascular lymphohistiocytic infiltrates in lung, liver, kidneys, heart, and bone marrow hyperplasia. Focal hepatocyte necrosis, hepatocyte dropout, Kupffer cell hyperplasia, and erythrophagocytosis were also observed (32). Immunostaining with biotinylated globulin from a patient convalescing from ehrlichiosis demonstrated the presence of clusters of ehrlichial organisms (morulae) in splenic cords and sinuses, splenic periarteriolar lymphoid sheaths, hepatic sinusoids, lymph nodes, lung microvasculature, bone marrow, kidney, and epicardium (33).
Less is known about the two fatalities that have been attributed to infection with the human granulocytic agent. Both patients died of multiorgan failure after presenting with cough and pulmonary infiltrates that were obvious upon radiography (9). Diagnosis of the first patient was based upon postmortem findings. The second patient's illness was compounded by several factors, including chronic lymphocytic leukemia with Richter's syndrome, high-dose steroid treatment, and previous splenectomy (9).

Dumler et al. (36) have reported persistent infection in a patient with ehrlichiosis. Although it may be premature to speculate on the extent of persistent infections in human beings, it is known that E. canis infections may persist in dogs for years (37) and that after prolonged asymptomatic periods recrudescence sometimes occurs. Dogs with such chronic (relapsed) conditions are typically more profoundly ill than in initial (acute) infections.

**DIAGNOSIS**

*Ehrlichia* spp. appear as round, dark-purple-stained dots or clusters of dots (morulae) in the cytoplasm of leukocytes upon direct microscopic examination of peripheral blood smears or buffy coat preparations stained by Romanowsky-type techniques (e.g., Giemsa, Wright, or Diff-Quik). Direct examination of such smears is probably useful only during the acute, febrile phase of infection. *Ehrlichia chaffeensis* has been reported occasionally in lymphocytes, atypical lymphocytes, band neutrophils, and segmented neutrophils, but most organisms are observed in monocytes or macrophages. The human granulocytic agent has been found only in neutrophils. Direct examination of buffy coats and peripheral blood smears is a lengthy process, as evidenced by Bakken et al.'s (9) need to inspect at least 800 polymorphonuclear granulocytes per smear for evidence of infection with the human granulocytic agent.

The search for *E. chaffeensis* via immunohistologic techniques has been performed not only on peripheral blood smears but also on formalin-fixed, paraffin-embedded bone marrow biopsy specimens or aspirated clotted marrow. Dumler et al. (31) described use of the biotinylated human anti-*E. chaffeensis*, avidin-alkaline phosphatase or avidin-horseradish peroxidase system. Organisms were detected primarily within histiocytes, but morulae were occasionally present within lymphocytes (31).

Immunohistology has also been performed on paraffin-embedded tissue sections from a patient infected with the human granulocytic agent. The etiologic agent was detected by using a modified immunohistologic method with equine anti-*E. equi* and bovine anti-*E. phagocytophilum* sera (9). Numerous small (1-3-μm diameter) intracytoplasmic morulae were observed in postmortem spleen and peripheral blood neutrophils that were not present in control tissues (9).

The polymerase chain reaction (PCR) has been used to detect both human ehrlichiosis agents known to occur in the United States. The PCR primers based on sequence from variable regions of the 16S rRNA gene have been used to amplify DNA from *E. chaffeensis* (8) and the human granulocytic agent (10). In both instances, sensitivity has been increased by using a nested PCR reaction (10, 20). The outside amplification is performed using either primers based on sequences from a highly conserved region of the 16S rRNA gene or universal primers. The specificity of the assay is dependent upon the internal primers that bind to the 16S gene of the species in question. Since the granulocytic agent has only been amplified from a few patients, the inside primers were designed to amplify a wider group that includes *E. phagocytophilum*, *E. equi*, and the human granulocytic agent. As in the case of successful culture, EDTA blood samples for the PCR technique are optimally drawn during the febrile phase of the disease, prior to antibiotic treatment.

Serologic diagnosis of ehrlichial infections is accomplished by the indirect fluorescent antibody (IFA) test. Continuously infected macrophage cultures (DH82 cells of canine origin) are used as the antigen for *E. chaffeensis* IFA. At CDC, human ehrlichiosis due to *E. chaffeensis* is defined by a fourfold change in immunoglobulin G antibody levels. Until 1990, when *E. chaffeensis* was first isolated, *E. canis* was used as the diagnostic antigen. Comparative tests have shown that *E. chaffeensis* antigen is more sensitive in detecting the homologous antibody during the early stages of the disease (CDC, unpublished data). Serologically, HGE is detected by testing patients' sera with *E. phagocytophilum*-infected neutrophils or *E. equi*-infected leukocytes (9). The majority of patients had a higher antibody titer when *E. equi*-infected cells were used as antigen. However, 2 patients had a 1:80 titer with *E. phagocytophilum* antigen and were <1:80 on *E. equi* (9).

**PREVENTION AND TREATMENT**

Epidemiologic evidence suggests that the agents of human ehrlichiosis are transmitted by ticks. Therefore, precautions against tick exposure must be taken. A study of military personnel documented that permethrin-impregnated clothing is an effective prevention measure (34). However, the availability of this acaricide may be limited in the civilian population. Vaccines are not available for either form of this disease. Recently, Fishbein et al. (29) reported that there was a lower rate of hospitalization, shorter duration of illness, and more rapid defervescence in patients treated with tetracycline (compared with those treated with other antibiotics). This study also showed that chloramphenicol frequently corrected the pyrexia and other clinical signs within 24 to 48 hours. These results contrast with in vitro studies on *E. chaffeensis*, which showed that chloramphenicol was not effective (35).

Apparently, all acute bacterial tick-borne diseases found in the United States are susceptible to tetracycline (29). Fishbein et al. (29) state that standard doses of tetracycline or doxycycline can be used for routine treatment of ehrlichiosis and that chloramphenicol should be used when tetracycline is contraindicated.

**CONCLUSIONS**

Ehrlichiosis has emerged within the last decade as a zoonotic disease. *Ehrlichia chaffeensis* was identified in 1991 as one of the etiologic agents, but the reservoir and vector hosts remain to be determined. The geographical distribution and mode(s) of transmission are also unknown. Recent discovery of a second leukocyte-dwelling pathogen capable of causing disease in people suggests a complex of diseases that may prove difficult to differentiate clinically from Rocky Mountain spotted fever and other rickettsioses. Discovery of multiple ehrlichiae in human beings echoes earlier experience with dogs and horses. Veterinary medical science recognizes disparate ehrlichial disease in both dogs and horses with those pathogens that show predilection for agranulocytes usually producing more severe disease than do those
that parasitize granulocytes. Fortunately, antibiotic therapy is curative when administered in a timely fashion. Identifying reservoir hosts and arthropod vectors presents major challenges; alternate methods of transmission may exist and attempts should be made to determine them. The newly recognized ehrlichial pathogens from human beings present many challenges to the medical community and other biologists.

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REFERENCES


Isolation of Borrelia burgdorferi, the Lyme Disease Spirochete from Rabbit Ticks, Haemaphysalis leporispalustris from Alberta

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Live Borrelia (B.) burgdorferi spirochetes were isolated from one engorged Haemaphysalis (H.) leporispalustris female tick retrieved from an injured snowshoe hare (Lepus americanus) outside the Grande Prairie city limits in Alberta. Dead spirochetes were seen in cultures of 3 other H. leporispalustris female ticks and 1 nymphal tick taken from the same hare. All spirochetes were immunostained by four monoclonal antibodies of B. burgdorferi, viz., OSPA (31 kD), OSPB (34kD), flagellin (41 kD), and P39 (39kD), and were found positive. Polymerase chain reaction for OSPA was positive for one culture tested. Antisera to B. burgdorferi were tested against the live isolate and found to be positive. This represents the first isolation of a Lyme disease spirochete in Alberta. Borrelia burgdorferi isolation from rabbit ticks is also the first such discovery in Canada. Rabbit ticks rarely bite humans, and this may be one of the reasons why Alberta has not reported any human Lyme disease to date.

Key words: Spirochete, Lyme disease, Borrelia burgdorferi, polymerase chain reaction

A snowshoe hare (Lepus americanus) was hit by an automobile on a road through a wooded area just outside Grande Prairie, Alberta on June 21, 1994. The injured hare was brought to the Grande Prairie Animal Hospital by the driver for treatment. The veterinarian, Dr. David Cook, judged the injuries to be fatal and euthanized the animal.

During the examination, six ticks of “varying sizes” were found on the head, face, and neck area of the hare. The ticks were placed in a plastic pill box along with a piece of moistened cotton and sent to the Vector-borne Diseases Laboratory at the Provincial Laboratory in Vancouver for identification and tests for the presence of Lyme disease spirochetes.

There were 4 engorged female and 2 engorged nymphal Haemaphysalis (H.) leporispalustris ticks. Each tick was sterilized, mid gut tissues from each tick were cultured separately in Barbour-Stoner-Kelly medium (1) (BSK) with antibiotics (2) on June 24, 1994. The tubes were examined 5 days later, and motile spirochetes were found in one of the adult female tick cultures. Dead spirochetes were seen in three other adult female and one nymphal tick culture.

All isolates reacted when immunostained with four monoclonal antibodies for Borrelia (B.) burgdorferi, viz., outer surface protein A (OSPA) (31kD), OSPB (34kD), flagellin (41 kD), and P39 (39kD) (OSPA-H5332, OSPB-H6831, flagellin-H9724, and P39-H107-11F3 from Dr. Barbour’s laboratory, Texas). The motile spirochetes were further tested for OSPA gene by a polymerase chain reaction technique (Banerjee et al., unpublished) and found to be positive. Antisera to B. burgdorferi was reacted with this isolate and found to be positive by an immunofluorescent assay (IFA) technique (Banerjee, unpublished). This culture was subsequently rendered axenic by passage through a 0.2-μm filter and antibiotic treatment (2). Further studies to ascertain the SDS-PAGE protein profile of these spirochetes and the DNA sequence of 16S r RNA gene are in progress.

However, based on tests already conducted, it appears that the spirochete isolated from the rabbit tick H. leporispalustris in Alberta is indeed B. burgdorferi, the etiologic agent of Lyme disease, and is identical to the spirochetes isolated in British Columbia (3, 4). This is the first isolation of B. burgdorferi from rabbit ticks in Canada.

The rabbit is well known as a host for Lyme disease spirochetes in the United States (2, 5, 6). Ixodes dentatus larvae, nymphs, and adults retrieved from trapped cottontail rabbits in the New York Botanical Garden yielded B. burgdorferi spirochetes (2). Burgdorfer (7) and Lane (8) found 2 of 174 H. leporispalustris ticks infected with spirochetes indistinguishable from B. burgdorferi. Borrelia burgdorferi was also isolated from rabbit ticks in Texas (9).

Rabbit ticks rarely bite humans (10). However, Brown (11) cites one instance of a rabbit tick as an ectoparasite on man in Canada. This may be one of the reasons why Alberta has not reported any cases of human Lyme disease.

The rabbit tick is widely distributed in Canada. This species is also known to parasitize varieties of mammals and birds. Gregson (12) records the presence of H. leporispalustris in all parts of Canada except in the N.W. Territory and Quebec. The snowshoe hare is also commonly distributed throughout the forests of Canada from the Atlantic to the Pacific (13). More field work will be required to evaluate the roles of hare and rabbit ticks in the enzootiology of B. burgdorferi. Surveillance of domestic animals in Alberta for signs of Lyme disease is also warranted. Further studies of wild rabbit and rodent populations and their ticks need to be conducted as they may be important in the dissemination of Lyme disease.

REFERENCES

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