ORIGINAL ARTICLES

Availability and Nature of Commercial Tick Control Services in Established and Emerging Lyme Disease Areas of New Jersey
Terry L. Schulze, PhD; Robert A. Jordan, PhD; and Robert W. Hung, MS

Experimental Infection of Neonatal Calves with Borrelia burgdorferi
Karen A. Molinari Clark, MS; Mazhar I. Khan, DVM, PhD;
Herbert J. VanKruiningen, DVM, PhD, MD; and Sandra L. Bushmich, MS, DVM

Bone Marrow as a Source for Borrelia burgdorferi DNA
Lesley Fein, MD, MPH and Richard C. Tilton, PhD

Lyme Disease and the Clinical Spectrum of Antibiotic Responsive Chronic Meningoencephalomyelitides
Kenneth B. Liegner, MD; Paul Duray, MD; Martha Agricola, FNP;
Carl Rosenkilde, MD, PhD; Lawrence A. Yannuzzi, MD; Martina Ziska, MD;
Richard C. Tilton, PhD; Dagmar Hulinska, PhD; Jeff Hubbard, MD; and Brian A. Fallon, MD

PHOTOGRAPHIC SECTION

Ixodes scapularis: Male, Female, and Nymph Compared to Sesame Seeds

Ixodes scapularis: Mating Adults
James L. Occi
Even when the media coverage stops, our medical coverage continues.

Doctors of the World A non-profit, non-sectarian, humanitarian organization dedicated to providing essential medical care to those in need. • If you haven’t heard of us before, that may be because the people who need the most medical attention aren’t always in the places that get the most media attention. And while what we do may not always make the front page, our doctors and nurses are always on the front lines. • Whether it’s a clinic in their neighborhood or a camp for refugees, our volunteers provide medical and humanitarian aid to those who need it most. • So, whether you can give of your own time, or make a donation to deploy one of our medical professionals, we need you. Call our toll-free number today for more information about Doctors of the World, and join the thousands of others who are helping us to help those who need it most.

CALL US TOLL-FREE FOR MORE INFORMATION 1.888.817.HELP

375 West Broadway • New York • New York • 10012   E-mail address: dow@jgc.apc.org  web site: www.doctorsoftheworld.org
ORIGINAL ARTICLES

Availability and Nature of Commercial Tick Control Services in Established and Emerging Lyme Disease Areas of New Jersey ................................................................. 44
   Terry L. Schulze, PhD; Robert A. Jordan, PhD; and Robert W. Hung, MS

Experimental Infection of Neonatal Calves with Borrelia burgdorferi .................................................. 49
   Karen A. Molinari Clark, MS; Mazhar I. Khan, DVM, PhD;
   Herbert J. VanKruiningen, DVM, PhD, MD; and Sandra L. Bushmich, MS, DVM

Bone Marrow as a Source for Borrelia burgdorferi DNA ................................................................. 58
   Lesley Fein, MD, MPH and Richard C. Tilton, PhD

Lyme Disease and the Clinical Spectrum of Antibiotic Responsive Chronic Meningoencephalomyelitides ................................................................. 61
   Kenneth B. Liegner, MD; Paul Duray, MD; Martha Agricola, FNP;
   Carl Rosenkilde, MD, PhD; Lawrence A. Yannuzzi, MD;
   Martina Ziska, MD; Richard C. Tilton, PhD; Dagmar Hulinska, PhD;
   Jeff Hubbard, MD; and Brian A. Fallon, MD

PHOTOGRAPHIC SECTION

Ixodes scapularis: Male, Female, and Nymph Compared to Sesame Seeds ................................................. 74

Ixodes scapularis: Mating Adults ............................................. 74
   James L. Occi
ILLUSTRATIVE EDITOR

James H. Katz, MD, Medical Center at the University of California, San Francisco

The Journal of Spirochetal and Tick-borne Diseases (ISSN 1060-0201) (GST 123970449) is published quarterly by SLACK Incorporated, 6900 Grove Road, Thorofare, New Jersey 08086. Dates of publication are: March, June, September, and December, on the third week of the publication month.

Copyright 1997 by Lyme Disease Foundation Inc. All rights reserved. No part of this publication may be reproduced or transmitted in any form without written permission by the Executive Director of the Lyme Disease Foundation, One Financial Plaza, Hartford, CT 06103-2610.

The Journal of Spirochetal and Tick-borne Diseases does not hold itself responsible for statements made by any contributors. Statements of opinion expressed in the journal reflect the views of the author(s) and not the official policy of the Lyme Disease Foundation.

Subscription Rates: Physicians: $75.00 yr.; Institution: $95.00 yr.; Single copies: $25.00. Students, fellows, and residents: $45.00 yr.; Foreign: add $20.00 for postage ($10.00 for Canada). To receive student/resident rate, orders must be accompanied by name of affiliated institution, date of term, and signature of program/residency coordinator or institution letterhead. Orders will be billed at single rate until proof of status is received. Back issues can be ordered at a cost of $25.00 per issue. Back issues sold in conjunction with a subscription are on a prorated basis. Requests for orders should be sent to the Journal of Spirochetal and Tick-borne Diseases, SLACK Incorporated, 6900 Grove Road, Thorofare, New Jersey 08086.

Change of address notices, including both the old and new addresses of the subscriber, should be sent at least 1 month in advance of effective date. Include old and new addresses and label from recent issue. The publisher cannot accept responsibility for undelivered copies.

Postmaster: Send change of address to: Journal of Spirochetal and Tick-borne Diseases, SLACK Incorporated, 6900 Grove Road, Thorofare, NJ 08086. Third class postage paid at Thorofare, NJ 08086. Advertising: SLACK Incorporated, 6900 Grove Road, Thorofare, New Jersey 08086. Although all advertising material is expected to conform to ethical standards, acceptance does not imply endorsement by the Journal.

Back issues can be ordered at a cost of $25.00 per issue. Back issues sold in conjunction with a subscription are on a prorated basis. Requests for orders should be sent to the Journal of Spirochetal and Tick-borne Diseases, SLACK Incorporated, 6900 Grove Road, Thorofare, New Jersey 08086.

Editorial content: Topics relating to understanding disease mechanisms and the application of better diagnostic techniques and treatment strategies for all individuals suffering from spirochetal and tick-borne diseases. Letters to the Editor in the form of correspondence related to material published in the Journal or some aspects of spirochetal and tick-borne diseases may be submitted. Such letters, if related to work previously published in the journal, will be referred to the author of the original work for a response.
Availability and Nature of Commercial Tick Control Services in Established and Emerging Lyme Disease Areas of New Jersey

Terry L. Schulze, PhD; Robert A. Jordan, PhD; and Robert W. Hung, MS

ABSTRACT

A preliminary survey of a community where Lyme disease was hyperendemic showed that a significant number of households hired pest control firms to control ticks. However, the availability and nature of commercial tick control services for residential property across New Jersey has not been studied.

Questionnaires pertaining to tick control services were mailed to all registered pest control firms in established (Monmouth County) and emerging (Hunterdon County) Lyme disease areas in New Jersey. Of the 208 registered firms in Monmouth County, 63 (30.4%) responded, 30 of which offered tick control services. In Hunterdon County, 15 of 34 (44.1%) registered firms responded to the survey, with 9 offering tick control programs. All firms in both counties relied on acaricides as the principal control method, although 30.0% and 55.6% in Monmouth and Hunterdon Counties, respectively, claimed to offer non-chemical alternatives. In both counties, Ixodes scapularis Say nymphs were targeted most frequently, although the majority of firms also made applications against other life stages. Regardless of county, woodland edge was the site treated most frequently, while wooded areas received the fewest applications. Most firms treated a combination of sites. Carbaryl, chlorpyrifos, and cyfluthrin were the most commonly used acaricides, and were often used in combination. The majority of firms in both counties charged less than $150 to treat a 1.0 acre (0.4 ha) property.

The frequency of application and the sites targeted for treatment suggest that many firms lack an understanding of basic tick ecology and the role that each life stage plays in disease transmission.

Key words: Ixodes scapularis, acaricides, Lyme disease, control

BACKGROUND

Early epidemiological investigations identified Monmouth County as the principal focus of Lyme disease in New Jersey, accounting for 54.7% (n = 117) of all reported cases between 1978 and 1982.1,2 By the end of the 1980s, Monmouth County alone reported 365 of the state’s 1,633 Lyme disease cases (22.4%) and averaged 30.4 cases annually during that period. Between 1990 and 1995, 614 additional cases were reported in Monmouth County, with a yearly average of 102.3 cases (Schulze, unpublished data). As such, Lyme disease has been established in Monmouth County for well over a decade.

Although the average number of cases each year in Monmouth County during the 1990s has increased over 3-fold compared to the 1980s, Monmouth County accounted for only 9.3% of Lyme disease cases statewide between 1990 and 1995. This disparity is due, in large part, to the emergence of Lyme disease in a number of northern counties, most notably Hunterdon County. By the end of the 1980s, only 33 Lyme disease cases were reported from Hunterdon County, representing only 2.0% of all cases reported in New Jersey during that period. However, Hunterdon County has experienced a significant increase in the number of Lyme disease cases between 1990-1995, with 1,395 cases or 21.2% of the...
state total (n = 6,588) during this period. The average number of cases reported annually has risen from about 2.8 during the 1980s to 232.5 during 1990-1995 (Schulze, unpublished data). This 83-fold increase in case reporting makes Hunterdon County one of the fastest emerging Lyme disease areas in the nation. The black-legged tick, *Ixodes scapularis* Say (L. dammini) Spielman, Clifford, Piesman, and Corwin) is now well established in both Monmouth and Hunterdon Counties.

Despite the fact that Lyme disease is the most important vector-borne disease in the United States, and the majority of transmission is believed to be peridomestic, little is known about the availability and nature of commercial tick control services. As part of a tick control study in a hyperendemic community, 18% of 50 surveyed households reported hiring commercial pest control firms to provide chemical tick control services at least once per year. Stafford conducted the only comprehensive study of tick control services to date. However, that state wide study concentrated as much on the business profile of pest control firms as it did on the nature of actual tick control services, and did not fully probe regional differences in tick control services within the state. This study emphasized collection of information on commercial tick control services available to homeowners and attempted to identify differences in these services between established and emerging Lyme disease areas. The need for expanded professional training and public education programs is discussed.

**MATERIALS AND METHODS**

Lists of registered pest control firms operating in Monmouth and Hunterdon Counties were obtained from the Pesticide Control Program, New Jersey Department of Environmental Protection. Each firm in Monmouth County (n = 208) and Hunterdon County (n = 34), were sent a letter of introduction and questionnaire about tick control services in 1995. The survey instrument was limited to 10 questions designed to elicit information about control techniques against *I. scapularis*, including acaricides and formulations used, sites of application, frequency of application, alternative control methods, and application costs.

**RESULTS**

**Survey response: Monmouth County**

Of the 208 registered pest control firms, 63 (30.3%) returned the questionnaires. There were 12 (5.8%) questionnaires returned with no forwarding address, suggesting that the firms were no longer in business. Of the 63 firms that responded, 30 (47.6%) indicated that they offered tick control services. These 30 firms answered all questions pertaining to tick control services and acaricide use, but 9 (30.0%) declined to provide information about fees charged for applications.

**Survey response: Hunterdon County**

Of the 34 (44.1%) registered pest control firms completed and returned questionnaires. Of the 15 respondents, 9 (60.0%) claimed to offer tick control services. These 9 firms responded to all questions pertaining to tick control services and acaricide use, while 4 (44.4%) failed to respond to questions regarding fees.

**Tick control services: Monmouth County**

Of the 30 firms surveyed, 24 (80.0%) provided tick control services as part of a lawn care-landscaping package. All 30 firms relied on acaricides as the principal method of control, but 9 (30.0%) offered nonchemical alternatives, principally in the form of tick habitat removal and distribution of educational materials. Only 8 of 30 (26.7%) attempted to survey properties for ticks prior to application.

Regarding the frequency of application, 8 (26.7%) firms treated residential properties only once. Of these, 6 firms directed their applications against nymphs, while 1 farm each treated for adults or larvae only. Four of 30 (13.3%) firms made applications against *I. scapularis* nymphs and larvae. There were 14 (46.7%) firms that made applications against each life stage and would make applications at the request of the homeowner. Two additional firms admitted that they would perform acaricide applications only at the request of the homeowner. The remaining 2 firms reported treating on a monthly basis from March through November. Of the 30 firms, 26 (86.7%) directed control efforts against nymphs, 20 (66.7%) at larvae, and 17 (56.7%) targeted adults (Fig 1).

Of the 30 firms polled, 27 (90.0%) reported making applications to the woodland edge, 23 (76.7%) to lawns, 22 (73.3%) to landscaping, and 4 (13.3%) to woodland (Fig 2). However, most of the applications were made to various combinations of sites: 4 (13.3%) firms treated all possible sites, while the largest percentage of firms (43.3%) treated a combination of woodland edge, lawn, and landscaping; 4 (13.3%) firms treated the woodland edge and lawn; 3 (10.0%) the woodland edge and landscaping; 2 (6.7%) the woodland edge, landscaping, and woodland; and 1 (3.3%) lawn and landscaping. Of the remaining 3 firms, 2 (6.7%) treated the woodland edge exclusively, while 1 (3.3%) treated only lawns.

**Tick control services: Hunterdon County**

Of the 9 firms performing tick control in Hunterdon County, 6 (66.7%) offered these services as part of a lawn care-landscaping package. All 9 firms relied on
acaricides as the principal method of control, while 5 (55.6%) provided nonchemical alternatives, including habitat modification, education, and rodent control. Only 1 firm (11.1%) attempted to survey properties for ticks prior to acaricide application.

Regarding the frequency of application, 1 firm (11.1%) treated residential properties once; 6 firms (66.7%) made applications against all active stages of *I. scapularis*; and 4 of 9 (44.4%) indicated that they also treated at the request of the homeowner. Two (22.2%) firms treated against nymphs and adults, but 1 also indicated it would make applications at the request of the property owner. Overall, of the 9 firms, 8 (88.9%) directed control efforts against nymphs, 6 (66.7%) at larvae, and 8 (88.9%) treated for adults (Fig 1).

All 9 firms reported making applications to the woodland edge and landscaping: 5 (55.6%) to lawns, and 3 (33.3%) to woodland (Fig 2). However, most of the applications were made to various combinations of sites. All possible sites were treated by 3 (33.3%) firms, while the largest percentage of firms (44.4%) treated a combination of woodland edge and landscaping. Two firms (22.2%) treated the woodland edge, landscaping, and lawn.

**Acaricide usage: Monmouth County**

The most commonly applied acaricide was carbaryl, being used by 19 of 30 (63.3%) firms. Chlorpyrifos was used by 16 (53.3%) firms, while cyfluthrin was used by 5 (16.7%). Permethrin, cyhalothrin, bendiocarb, trichlorfon, and fluvalinate were each used by 1 firm. There were 16 of 30 (53.31) firms that indicated they used a combination of up to 3 acaricides, the most common combination being carbaryl and chlorpyrifos (n = 9). The remaining firms used a single acaricide, with carbaryl (n = 6) and chlorpyrifos (n = 6) being the most popular choices. Host-targeted permethrin (Damminix, EcoHealth, Boston, MA) was used by 3 (10.0%) firms.

With respect to formulation, 22 of 30 (73.3%) firms used liquid formulations (wetable powder, flowable, emulsifiable concentrate) exclusively or in combination with granular formulations. Granular formulations alone or in combination with liquids were used by 21 of 30 (70.0%) firms. There were 13 of 30 (43.3%) firms that used a combination of granular and liquid, while 9 of 30 (30.0%) firms used liquid formulations only, and 8 (26.7%) used granular materials exclusively.

**Acaricide usage: Hunterdon County**

The most commonly used acaricide by Hunterdon County respondents was carbaryl, which was used exclusively or in combination by 5 (55.6%) firms. Chlorpyrifos, cyfluthrin, and permethrin were each used by 2 (22.2%) firms. Cyhalothrin, cypermethrin, bendiocarb, and pyrethrum were each used by 1 firm. There were 5 (55.6%) firms that used a combination of up to 4 acaricides, with no 2 firms using the same combination. Host-targeted permethrin was used by 2 (22.2%) firms.

With respect to formulation, all 9 firms used liquid formulations exclusively or in combination with granular formulations. Three of 9 (33.3%) firms used granular formulations alone or in combination with liquids. Three of 9 (33.3%) used a combination of granular and liquid, while 6 (66.7%) firms used liquid formulations only and 3 (33.3%) used granular formulations exclusively.
Application costs: Monmouth County

Of the 30 firms offering tick control services, 9 (30.0%) declined to provide information on fees charged to the homeowners for acaricide applications. The 21 firms that responded provided 13 different treatment charges ranging from $80 to $250 per acre (0.4 ha). The mean charge was $159.05 per acre; 13 (61.9%) of the 21 firms charged less than $150 per acre.

Application costs: Hunterdon County

Of the 9 firms offering tick control, 4 (44.4%) did not provide information on fees charged to the homeowners for acaricide applications. The 5 firms that responded reported fees ranging from $90 to $180 per acre. The mean charge was $113 per acre. Only 1 firm charged more than $150 per acre.

DISCUSSION

Survey response

Although the Connecticut survey\(^{11}\) and this study approached data collection in a different fashion, both received a similar level of response. Stafford opted to survey all licensed applicators statewide and received a response rate of 38.8%\(^{11}\). In New Jersey, individual firms were polled in an effort to avoid certain biases. It was felt that surveying individual applicators rather than firms, would skew the resulting data in favor of larger firms with more applicators and, presumably, more extensive training programs. Stafford showed that larger firms tended to be more likely to offer tick control services than smaller firms.\(^{11}\) Nevertheless, the level of response was similar in Monmouth (30.3%) and Hunterdon (44.1%) Counties. Surprisingly, only 16.4% of the 348 respondents in the Connecticut study offered tick control services, compared to 47.6% of the 30 responding firms in Monmouth County and 60.0% of the 15 firms from Hunterdon County. The seemingly greater availability of commercial tick control services in New Jersey may reflect the fact that both Monmouth and Hunterdon Counties are highly endemic for Lyme disease, whereas the Connecticut survey may have combined data from endemic and nonendemic areas. Since Monmouth County has 5 times the population of Hunterdon County, the difference in the number of registered firms between the counties was expected.

Tick control services

All firms offering tick control services in both Monmouth and Hunterdon Counties relied on acaricides, a finding similar to that reported by Stafford in Connecticut.\(^{11}\) In Monmouth and Hunterdon Counties, 70.0% and 55.6%, respectively, relied on acaricides exclusively. The majority of Monmouth County (80.0%) and Hunterdon County (66.7%) firms offered tick control as part of a more comprehensive pest control package.

The frequency of application varied dramatically within and between counties. In Monmouth County, the number of applications ranged between once per year to monthly for 8 months. Nearly one-half of the firms made applications against all life stages and over one-half would treat at the owner's request, regardless of the seasonal activity of a particular life stage. Nymphs were targeted most frequently (86.7%), but two-thirds of the firms made applications against larvae and over one-half treated properties to control adult ticks. In Hunterdon County, application frequency ranged between 1 and 3 applications per year, with two-thirds of the firms making applications against each life stage and 55.6% of firms treating at the owner's request. Nymphs and adults were targeted by 88.9% of the firms.

Stafford reported that 61.4% of Connecticut applicators treated areas upon the owner's request.\(^{11}\) Significantly fewer Connecticut applicators targeted each life stage, compared to New Jersey, but admitted to treating properties more than 2 times during the summer months. Nearly twice as many applicators in Connecticut surveyed for ticks before making applications compared to Monmouth County firms, and 4 times more often than those offering tick control services in Hunterdon County.

Regarding the sites of application, 90% of the Monmouth County firms treated the lawn-woodland edge interface. About 75% of firms treated lawns and landscaping, while only 13.3% treated woodland. In Hunterdon County, all firms treated woodland edge and landscaping, with lawns and forests being included as target areas by 55.6% and 33.3% of firms, respectively. In both counties, the majority of firms treated a combination of sites. Connecticut applicators treated the lawn-woodland border (60-62%) and lawn (greater than 25%) less frequently compared to New Jersey firms.

Surveys of Connecticut applicators and New Jersey firms revealed that a variety of insecticides were used. However, the same compounds (carbaryl, chlorpyrifos, cyfluthrin) were used most frequently in both studies, but the ranking of use was different. Monmouth county firms used liquid (73.3%) and granular (70.0%) formulations with similar frequency, while Hunterdon County firms seemed to prefer liquid formulations. Connecticut applicators also preferred liquid over granular formulations. Only a small percentage of applicators or firms used host-targeted permethrin.

The per acre costs for tick control were similar in both studies, although the range of fees was broader and the mean fee was higher in Connecticut. It is unclear whether the wide disparity in costs reflect a tick only application (high) or general pest control package application (low).
The majority of applicators or firms charged less than $150 per acre.

Assuming that property owners are interested primarily in disease prevention, the frequency of application and the sites targeted for treatment seem to suggest that many firms lack an understanding of basic tick ecology and the role that each life stage plays in the transmission of tick-borne pathogens. For example, two-thirds of the firms made applications to control *Ixodes scapularis* larvae, a stage that has little or no role in disease transmission and is considered the most difficult stage to control. Over 75% of Monmouth County firms and more than 50% of Hunterdon County firms routinely treated lawns (Fig 2), despite the fact that large expanses of lawn generally are not recommended for treatment. Further, 80% of Monmouth County firms and 66.7% of Hunterdon County firms offered tick control as part of a broader pest control package. As such, tick control in Monmouth County may have become more of an ancillary activity achieved largely through the control of other pests, compared to Hunterdon County, where Lyme disease is considered emergent. Although the apparent excessive treatment may be due, in part, to the respective firms' lack of knowledge of tick ecology and management techniques, it is interesting to note that the majority of respondents admitted that they would treat at the request of the property owner, regardless of tick activity. This suggests that sound tick control practices may be in conflict with existing business practices. As a partial solution, pest control operators engaged in tick control services should receive training regarding tick ecology, assessment, and management techniques as part of the pesticide applicator re-certification process. In addition, government agencies charged with Lyme disease education should expand their mandate to include the preparation and distribution of materials on proper frequency and appropriate sites of application to the general public. Such an approach will enhance the efficacy of tick control, while limiting the amount of acaricides applied to the environment.

ACKNOWLEDGMENTS

This study was partially supported by a Cooperative Agreement (US0/CCU/206567-06) between the New Jersey Department of Health and Senior Services and the Centers for Disease Control and Prevention.

The authors thank R. Chadwick Taylor (Freehold Area Health Department); John W. Beckley (Hunterdon County Health Department); and their respective staffs for assistance in distributing and compiling the questionnaires.

REFERENCES


Experimental Infection of Neonatal Calves with *Borrelia burgdorferi*

Karen A. Molinari Clark, MS; Mazhar I. Khan, DVM, PhD; Herbert J. VanKruiningen, DVM, PhD, MD; and Sandra L. Bushmich, MS, DVM

**ABSTRACT**

Four neonatal calves were inoculated intraperitoneally, intramuscularly, subcutaneously, and intradermally with a total of $2 \times 10^4$ organisms from a low passage culture of *Borrelia burgdorferi*. Two control calves were inoculated with sterile BSK media in a similar manner. The six experimental calves were maintained for 7 to 8 weeks. The *B. burgdorferi* infected calves demonstrated an erythematous skin lesion (from which *B. burgdorferi* could be cultured) at the site of inoculation 2 to 3 weeks following infection. Rashes resolved spontaneously within 1 to 2 weeks. Unilateral lymphadenopathy of the left prefemoral lymph node occurred concurrent with the development of skin lesions and persisted throughout the study in all four infected calves.

All infected calves developed a serological response to *B. burgdorferi*, as determined by immune fluorescent antibody (IFA) test and Western blot (WB) analysis. Dissemination of *B. burgdorferi* occurred in all infected calves. *B. burgdorferi* were detected, by culture and/or PCR, in urine, blood, synovial fluid, and tissue samples (kidney, bladder, spleen, and tarsal synovium). Minimal histopathological changes occurred in the infected calves. The most common lesion was a lymphocytic infiltrate noted in skin; scattered lymphocytic foci also occurred in renal cortex, heart, synovial tissue, and liver. This study has demonstrated that neonatal calves experimentally inoculated with *B. burgdorferi* develop disseminated infection, shed *B. burgdorferi* in the urine and produce a specific serological response to the organism.

**Key words:** *Borrelia burgdorferi*, cattle

**INTRODUCTION**

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and transmitted by *Ixodes spp* ticks.1 The disease has been recognized in humans and in a number of domestic animal species including cattle, horses, sheep, dogs, and cats.2-12 In livestock the pathogenesis and transmission of this disease remain poorly defined. Disease expression among cattle, as well as other domestic animal species, varies. Many animals remain clinically normal while demonstrating antibodies to *B. burgdorferi*.5,7,13

Clinical signs of Lyme disease reported in cattle include lameness, stiffness, swollen joints, laminitis, arthritis, fever, weight loss, decreased milk production, erythematous skin rash and abortion.5,9

Diagnosis of Lyme disease is often difficult and is based on clinical signs, exclusion of other diseases, serological response to *B. burgdorferi* antigens and response to antibiotic therapy.6-9,13 Positive serology is helpful in making a diagnosis, but it indicates previous exposure only, not necessarily active or clinical infection.5,6 In cattle it has been suggested that antibody testing may not be as dependable as in other domestic animals due to cross-reacting antibodies to other *Borrelia spp* (*Borrelia coriaceae* and *Borrelia theileri*) or perhaps rumen spirochetal organisms.5,6 It has been reported that cattle with confirmed infection (ie, *B. burgdorferi* organisms demonstrated) may develop only low antibody titers.5,5 Definitive diagnosis depends on the demonstration of *B. burgdorferi* in body fluids or tissues of infected animals. This is often difficult due to: sparse numbers of organisms in affected fluids or tissues, difficulty in recovering the organisms because of special media requirements and low replication time, and

From the Department of Pathobiology, The University of Connecticut, Storrs, CT.

The authors wish to recognize the support of The University of Connecticut Research Foundation, Storrs, and the technical support of Jenny Lomastro, BS, and Jennifer Schneider, BS, at The University of Connecticut.

Address reprint requests to Sandra L. Bushmich, MS, DVM, Department of Pathobiology, U-89, The University of Connecticut, 61 North Eagleville Road, Storrs, CT 06269-3089.
the need for darkfield microscopy or special stains to demonstrate the organisms.6,7,9,13-15

The objective of this study was to further characterize bovine serological and pathological responses to *B. burgdorferi*, as well as determine patterns of organism dissemination in tissues and body fluids by utilizing an experimental calf model.

**MATERIALS AND METHODS**

**Animals**

Neonatal Holstein bull calves, weighing approximately 40 kg, were obtained from the University of Connecticut dairy herd, Storrs. Each calf was removed from the dam at birth and not allowed to nurse. Serologic testing was not conducted on the dams. The calves were given oral bovine rotavirus and coronavirus vaccine ("Calf-Guard", Norden Laboratories) and one oral dose of synthetic colostrum diluted to one liter. Synthetic colostrum (Colostrix (454 g), Fisons Animal Health, Minneapolis, MN or First Milk Formula (272 g), Land O’ Lakes, Ft. Dodge, IA) was tested by Western blot analysis to insure the absence of antibodies to *B. burgdorferi*. Calves were transported to an isolation facility, where they were individually housed in wire pens on wood shavings. They were fed milk replacer (Blue Seal Lawrence, MA) for 2 weeks, then grain and hay were added to their diet *ad libitum*.

**Inoculum**

A second or third passage Connecticut *B. burgdorferi* tick isolate (Provided by Dr. John Anderson, Connecticut Agricultural Experimental Station, New Haven, CT) was used for animal inoculations. Experimental injections contained approximately $2 \times 10^6$ log phase organisms suspended in Barbour-Stoenner-Kelly (BSK) medium. Cell counts were determined using a bacterial counting chamber (Petroff Hauser bacterial counting chamber, Hauser Scientific, Blue Bell, PA).

**Experimental plan**

Prior to inoculation all calves were determined to be free of *B. burgdorferi* antibodies (IgG) by immune fluorescent antibody (IFA) and Western blot analyses; urine culture for *B. burgdorferi* was negative. Four calves (calves 1-4) were inoculated with *B. burgdorferi* at 6 days of age in the left flank as follows: following surgical skin preparation (hair clipped, site scrubbed with tamed iodine followed by alcohol wipes 3 times, then allowed to dry prior to procedure), 0.5 mL of the inoculum was administered intraperitoneally, intramuscularly, subcutaneously, and intradermally for a total inoculum volume of 2 mL. Two control calves (calves 5 and 6) were inoculated in a similar manner with an equal volume of sterile BSK medium.

Daily physical examination of all calves included: rectal temperature, pulse and respiration rate, evaluation of attitude, appetite and gait, visual examination of feces, palpation of peripheral lymph nodes, examination of joints for swelling, and of skin for erythematous skin lesions. Circumferences of both carpi and tarsi were measured on a weekly basis. Blood was collected 3 times weekly aseptically from the jugular vein. Blood analyses included packed cell volume (PCV), total protein (TP), dark field examination and culture (3 times per week), PCR for *B. burgdorferi* (once a week) and chemistry screen (weeks 0, 3, and 7). Midstream urine was collected 3 times a week and analyzed by darkfield microscopy, culture, and fluorescent antibody (FA) testing; weekly samples were analyzed by PCR/slot blot for *B. burgdorferi*. Four millimeter skin biopsies of dermis and subcutis were taken from aseptically prepared erythematous skin lesions of infected calves 3 and 4. A carpal synovial fluid sample was taken at the time of the skin biopsy. Control calves 5 and 6 received similar skin biopsy and arthrocentesis 2 weeks post-inoculation. Calves were maintained for 40-60 days then killed by IV injection of euthanasia solution (Beuthanasia D, Schering-Plough Animal Health, Kenilworth, NJ). Necropsy and histopathological examination with culture and PCR of selected tissues was done on each calf.

**Serology**

Weekly serological responses to *B. burgdorferi* were evaluated by immune fluorescent antibody test and Western blot analysis.

**Immune fluorescent antibody**

A previously described IFA technique was used.16 Multi-well acetone resistant slides were coated with a single layer of whole *B. burgdorferi* suspension. *B. burgdorferi* used were a cocktail of the isolate used for inoculation plus several other low passage isolates. Calf sera were prepared in two-fold serial dilutions (beginning with 1:32 and ending with 1:1024) and incubated in wells for 30 minutes at 37°C. Slides were rinsed twice in phosphate buffered saline (PBS) and once in distilled water. Fluorescein isothiocyanate (FITC) labeled goat anti-bovine heavy and light chain IgG (Kirkegaard and Perry, Gaithersburg, MD) (diluted 1:400), was applied to each well, incubated and rinsed as before. Slides were examined at $400 \times$ using a microscope with a UV light source. A titer of $\geq 1: 64$ was considered positive.

**Western Blot analysis**

Sodium dodecyl sulfate polyacrylamide gel elec-
trophoresis was performed on cultured *B. burgdorferi* using a modification of the method described by Laemmli. Spirochetes were prepared by washing 150 mL of whole cells from log phase cultures of *B. burgdorferi* three times by centrifugation at 10,000 g for 15 minutes in a 0.1% solution of mercaptoethanol and phosphate buffered saline (PBS). Protein concentration was estimated by the Bradford method. Approximately 300 μg of *B. burgdorferi* protein was mixed with 17 μL of sample buffer (0.25 mol/L, 40% glycerol, 2% sodium dodecyl SDS, 20% 2- mercaptoethanol, 0.025% bromophenol blue) and denatured by boiling for 5 minutes. *B. burgdorferi* protein and molecular weight standards (prepared according to manufacturer) (Beuthanasia D, Schering-Plough Animal Health) were subjected to electrophoresis using 10% resolving gels and 4% stacking gels. Electrophoresis was performed at a constant current of 100 mA until dye-front reached 1 cm from the bottom of the gel. Proteins were pre-equilibrated in transfer buffer (25 mM Tris base, 38 mM Glycine, 20% methanol, pH 8.3) for 30 minutes, then transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad, Richmond, CA) at 70 volts for 2.5 hours using a Trans-blot Electrophoretic Transfer Cell (Bio-Rad) as previously described by Towbin et al. Following transfer, nitrocellulose membranes were stained in Ponceau’s stain (Sigma Chemical Co., St. Louis, MO), for approximately 10 minutes to determine if proteins were properly transferred. The membranes were cut into strips, placed in a small tray and blocked with 2% BSA and 1% horse serum in Tris buffered saline for one hour at room temperature with rocking. Strips were rinsed (three 10 minute washes) in wash buffer (0.85% NaCl, 12% Tris, 0.05% Tween 20), overlaid with experimental calf sera (diluted 1:100) and incubated as before for two hours. Serum was removed, strips were washed as described and goat anti-bovine heavy and light chain IgG phosphatase labeled conjugate (Kirkegaard and Perry, Gaithersburg, MD) (diluted 1:2500) was added and incubated for one hour. The strips were rinsed as before and reacted with BCIP/NBT phosphatase substrate (Kirkegaard and Perry) until optimal protein band development was achieved (1-10 minutes). The reaction was stopped by the removal of substrate and rinsing the strips several times with distilled water.

**ANTIGEN TESTING**

**Culture**

All urine, blood, synovial fluid, skin biopsies and post-mortem tissues and fluids (kidney, bladder, testis, spleen, heart (myocardium and septum), brain (cerebrum, cerebellum), prefemoral lymph nodes, synovial tissue (carpi and tarsi), pericardial fluid, cardiac blood, urine, aqueous humor, synovial fluid, and CSF were cultured for *B. burgdorferi* in Barbour-Stoenner-Kelly medium as previously described. Tubes were inoculated in tandem, using one tube of media without antibiotics and one with ciprofloxacin (40 mg/mL) and rifampicin (20 mg/mL) added to retard the growth of contaminants. Approximately 0.5 mL of fluid or 15-20 mg of minced tissue sample was inoculated into 7 mL of sterile BSK media. Samples were incubated at 32°C and examined by darkfield microscopy after one week incubation, then bi-weekly for one month. Identity of spirochete positive cultures was confirmed by PCR analysis.

**Darkfield examination**

Darkfield microscopy was used to examine blood, urine, and synovial fluid. One drop of fluid was examined at 200× and 400× magnification. Before examining fresh urine, approximately 20-50 mL was centrifuged at 11,000 × g for 20 minutes, all but 3 mL of the supernatant was poured off and discarded. The remaining fluid was mixed with the sediment then examined microscopically.

**Fluorescent antibody staining**

Direct fluorescent antibody staining was used to test all fluids excluding premortem blood samples. Two separate drops of fluid were placed on a glass microscope slide, air dried, then fixed in acetone for 10 minutes. Urine was prepared as described above before testing. Approximately 2 drops of fluorescein conjugated polyclonal goat anti-Borrelia antibody (Kirkegaard and Perry) (1:20 dilution) was applied to each droplet. Slides were incubated at room temperature in a moisture chamber for 30 minutes, then placed in PBS for 10 minutes and rinsed in distilled water three times. Slides were examined at 200× and 400× using a microscope with a UV light source for the presence of stained spirochetes.

**Polymerase chain reaction**

Polymerase chain reaction (PCR) was used to detect *B. burgdorferi* in weekly urine and blood samples as well as the following postmortem samples: kidney, bladder, spleen, prefemoral lymph nodes, heart (septum and myocardium), cerebrum, synovial fluid, and CSF. Chromosomal DNA was extracted from body fluids and tissues as previously described. The primer pair used amplifies a specific 309 base pair segment of *B. burgdorferi* outer surface protein A (Ospa) and have been previously described by Malloy et al. This primer set will not amplify Osp A of other *Borrelia* spp, including *B. coreaeae* a pathogen of cattle. Primers were synthesized by the Biotechnical Services at the University of Connecticut, Storrs. A GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, CT) was used to amplify gene products as previously described. Triplicate posi-
tive and negative controls were included in each PCR run; PCR sample preparation and conduction were conducted in a laboratory dedicated to this purpose.

**Slot blot hybridization**

PCR amplified DNA product of *B. burgdorferi* (200 ng) was used as DNA probe for slot blot hybridization. *B. burgdorferi* DNA was digested using 10 units of EcoRI in 20 µL of reaction buffer. The digested DNA was heat denatured (95°C for 5 minutes) and end labeled with [gamma-32P]-dATP using a Gibco BRL 5' DNA terminus (Gibco BRL, Life Technologies, Gaithersburg, MD). Amplified DNA products (1 µg) were digested using EcoRI in reaction buffer. Samples were incubated with the enzyme for one hour at 37°C, followed by the addition of 6 X SSC (0.9 M NaCl, 0.09 M sodium citrate pH 7.0) was added and the digested DNA was heat denatured at 95°C, 5 minutes. The samples were applied to a Zeta Probe membrane (Bio-Rad) using a slot blot manifold (Bethesda Labs, Bethesda, MD) under vacuum pressure. The membrane was air-dried, baked for 2 hours at 80°C and sealed inside a plastic bag with prehybridization buffer (10× denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA), 6 X SSC, 20 mM sodium phosphate, 4% SDS, salmon sperm DNA (80 µg/mL)) and incubated for 60 minutes at 59°C.

The labeled DNA probe, heat denatured at 95°C for 5 minutes, was added with fresh prehybridization buffer to the Zeta-Probe membranes and allowed to hybridize on a rocker overnight at 59°C. The membranes were removed from the bag and washed once in a solution made up of 3 X SSC, 1% SDS and distilled water at room temperature for 30 minutes. This was followed by a second wash in a solution made up 1 X SSC, 1% SDS and distilled water at room temperature for 30 minutes. The membranes were washed for a final time in a solution made up of 0.1 X SSC, 1% SDS and distilled water at 60°C for 45 minutes. Membranes were air dried and exposed to x-ray film for approximately 24 hours using an intensifying screen at -70°C.

**Pathology/histopathology**

Necropsy was conducted immediately following euthanasia. Selected tissues (listed previously) were collected aseptically for *B. burgdorferi* culture. Tissues from all major organ systems, excluding endocrine, were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 micrometers and stained with H & E. Selected tissues with lesions were stained with the Modified Steiner method and by immunocytochemistry (ICC) (Ying Liu, MS thesis, Storrs, CT) for the presence of spirochetes. In the latter, polyclonal rabbit anti *B. burgdorferi* serum (ViroStat, [code No. 0301], Portland, ME) at a dilution of 1:20,000 was applied, followed by the addition of the secondary antibody, biotinylated goat anti-rabbit (dilution 1:500), and then a streptavidin-horse radish peroxidase conjugate. Amino-ethylcarbazole (AEC) served as the chromogen. The tissue sections were then counterstained with Lerner-2 hematoxylin, coated with crystal mount, and the slides were coveredslipped.

**Statistical analysis**

The paired *t* test was used to compare the prefemoral lymph node sizes of the infected and control groups.

**RESULTS**

**Clinical and laboratory findings**

Calves remained bright and alert with good appetites throughout the study. Rectal temperature, pulse and respiration rates remained within normal ranges in both infected and control group calves. Three of the four infected calves (1, 3 & 4) and both control calves (5 & 6) had mild diarrhea, which developed between weeks 1 and 3 of infection and resolved following oral electrolyte treatment. Other clinical disorders which occurred in the calves include: a cough from week 1-7 in calf 1, an episode of mild bloat in calf 3 (week 7) which resolved following stomach intubation, and a soft tissue abscess in the buccal mucosa of calf 4 (days 23-41), which drained and healed spontaneously; abscess material was not cultured.

Erythematous skin lesions appeared at the site of inoculation (left flank) of all four *B. burgdorferi* infected calves 2 to 3 weeks following infection. The skin lesions first appeared as small red macules, which expanded to 5
<table>
<thead>
<tr>
<th>Week Following Inoculation</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf 1</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td>41,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
</tr>
<tr>
<td>WB</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
</tr>
<tr>
<td>Calf 2</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td>41,34</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
</tr>
<tr>
<td>WB</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41,34</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
</tr>
<tr>
<td>Calf 3</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td>41,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
</tr>
<tr>
<td>WB</td>
<td></td>
<td></td>
<td></td>
<td>41,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
<td>41,39,34,31</td>
</tr>
<tr>
<td>Calf 4</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
</tr>
<tr>
<td>WB</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
</tr>
<tr>
<td>Calf 5</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
</tr>
<tr>
<td>WB</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td></td>
</tr>
<tr>
<td>Calf 6</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
</tr>
<tr>
<td>WB</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td>41,39</td>
<td></td>
</tr>
</tbody>
</table>

Table 1

Immune fluorescent antibody (IFA) test and Western blot (WB) values for B. burgdorferi infected calves (calves 1-4) and noninfected control calves (calves 5 & 6). IFA results are shown as inverse titers, 1:64 is considered a low positive. WB results are shown as antibodies to 41, 39, 34 and 31 kDa proteins of B. burgdorferi.

Serology

Table 1 summarizes the antibody response to B. burgdorferi in all calves. All calves were seronegative by IFA and Western blot analysis prior to experimental inoculation. The four infected calves developed a positive IFA antibody titer (≥ 1:64) by the fourth week of infection, which increased throughout the study in calves 1 thru 3. Calf 4 maintained a 1:64 titer from weeks 4 to 7. Control calf 5 remained IFA negative (≤ 1:64) throughout the trial. Control calf 6 developed a positive antibody titer (1:64) 7 weeks following experimental inoculation; however Western blot analysis of its sera did not reveal antibodies specific to B. burgdorferi.

Western blot analysis of sera from all 4 infected calves demonstrated a sequential development of antibodies to B. burgdorferi specific outer surface proteins A and B (OspA, 31 kDa and OspB, 34 kDa) (Table 1 and Figs 2A). In calves 1 thru 3 these antibodies were detectable 3 to 4 weeks following experimental infection. The serological response to these proteins was delayed and weak in calf 4. In this calf antibody to the 34 kDa protein was not detectable until the fifth week postinoculation and an antibody response to the 31 kDa protein was detectable at week seven postinoculation (Table 1). Antibodies directed against the nonspecific 41 kDa flagellar protein and the reportedly B. burgdorferi specific 39 kDa protein were apparent in both infected and control calves (Table 1 and Figs 2A and 2B). Antibody response to the flagellar (41
Table 2.
Urinary culture and PCR results for B. burgdorferi infected calves (calves 1-4) and noninfected control calves (calves 5 & 6).

<table>
<thead>
<tr>
<th>Week of Inoculation</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf 1</td>
<td>-</td>
<td>+P</td>
<td>+P</td>
<td>+P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calf 2</td>
<td>-</td>
<td>+P</td>
<td>+P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+C,P,FA</td>
<td>+C,P</td>
</tr>
<tr>
<td>Calf 3 (NS)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calf 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+P</td>
<td>+P</td>
<td>+P</td>
</tr>
<tr>
<td>Calf 5 (control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calf 6 (control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

NS=No Sample (There was no sample available for PCR/slot blot; the sample was examined by FA and darkfield microscopy and was negative for Borrelia burgdorferi).
C= culture; P= PCR; FA= Fluorescent antibody staining; *Skin biopsy (+) for Bb (culture and PCR); **Synovial fluid sample for PCR (+); ***Blood sample PCR (+); Note: Identity of all spirochete positive cultures was confirmed by PCR/slot blot hybridization.

kDa) protein remained weak throughout the course of infection in all calves. Response to the 39 kDa protein became stronger with time in all of the infected calves, but remained weak in the control calves (Figs 2A and 2B). Immunoblots of the synthetic colostrum fed to all calves revealed antibody to the nonspecific 41 kDa flagellar protein only.

**Antigen testing**

*B. burgdorferi* was detected in urine of all 4 experimentally infected calves. Results of urine culture and PCR (demonstrated by slot blot hybridization) are presented in Table 2. *B. burgdorferi* was also detected by PCR in occasional blood and synovial fluid samples taken from infected calves, and by culture and PCR in all skin biopsy samples taken from infected calves (Table 2). All control calf samples were PCR and culture negative. Direct darkfield microscopy of urine, blood, or synovial fluid samples did not reveal spirochetes in either infected or control calves. Direct fluorescent antibody (FA) staining revealed spirochetes in the urine of calf 2 during week 6 of infection; FA staining of all other samples was negative (Table 2).

At necropsy, *B. burgdorferi* was isolated by culture from kidney and bladder of calf 2, and spleen and left hock synovial tissue of calf 4. All positive cultures were confirmed as *B. burgdorferi* by PCR/slot blot hybridization. PCR performed directly on tissues and body fluids collected post-mortem (listed previously) did not detect *B. burgdorferi*.

**Necropsy and histopathologic results**

At necropsy the sites of inoculation were well healed and without erythema or thickening. In all 4 *B. burgdorferi* infected calves the left (side of inoculation) prefemoral lymph node was significantly (*p* ≤ 0.01) larger than the right prefemoral lymph node (Fig 3). One of the four *B. burgdorferi* inoculated calves had a buccal ulcer that extended from the mucosa of the left cheek through to the epidermis, consisting primarily of a granulocytic infiltrate. Histologically, the 4 calves inoculated with *B. burgdorferi* had lymphocytic, plasmacytic infiltration at the site of inoculation in the left flank; control calves did not. This appeared predominately in-between hair follicles and around blood vessels. The enlarged left prefemoral lymph node of one infected calf had cortical hyperplasia and that of another infected animal had sinus histiocytosis. The other 2 infected calves’ prefemoral lymph nodes were no different histologically than those of controls.

Three of the 4 infected calves had focal tonsillitis, characterized by granulocytes and foreign material in a few crypts; so did both controls. Three of the four infected subjects had focal patchy interstitial pneumonitis, a change not seen in the two controls. One infected calf had few foci of lymphocytic nephritis bilaterally; one had a few foci of lymphocytic synovitis in one hock joint; and another had few focal lymphocytic aggregates in portal triads of the liver and subendocardially in the interventricular septum of the heart.

These changes were not observed in the control calves. Spirochetes were not demonstrable in the discussed foci when stained by Steinzer’s silver method or immunocytochemistry.

**DISCUSSION**

In this study we have utilized an experimental calf model to further define the pathogenesis of *B. burgdorferi* infection in cattle. Neonatal calves were susceptible to infection as demonstrated by the development of an ery-
Figs 2A and 2B: Western Blot analysis of antibody to B. burgdorferi in a B. burgdorferi inoculated calf (calf 2) (A) and for a BSK inoculated control calf (calf 6) (B). Serum was tested weekly from day 0 to post-inoculation week 7. The results in each immunoblot lane are as follows; lane 1: positive control experimentally B. burgdorferi infected calf serum; lane 2: negative control noninfected calf serum; lane 3: conjugate control goat anti-bovine H&L chain IgG (diluted 1:2500); lane 4: pre-inoculation; lane 5: week 1; lane 6: week 2; lane 7: week 3; lane 8: week 4; lane 9: week 5; lane 10: week 6; lane 11: week 7. Molecular weights are in kilodaltons (kDa).

erythematous rash at the site of B. burgdorferi inoculation, a serological response to B. burgdorferi and by detection of B. burgdorferi in body fluids and tissues.

The development of an erythematous rash at the site of inoculation was the only significant clinical sign observed and was consistent in all infected calves. In humans, erythema chronicum migrans (ECM) is a hallmark of B. burgdorferi infection and ECM dermal tissue is a good source for recovering B. burgdorferi, as was also shown in this study.26,27 Erythematous skin lesions have been reported in naturally infected cattle.8 They are rarely seen during spontaneous infection of other animal species, possibly because of hair covering; however spirochetes have been isolated from the skin of infected animals lacking a rash.28,29 Lymphocytes, the major cellular component of human ECM,30 were observed as the main cellular infiltrate at the site of B. burgdorferi inoculation in all four infected calves.

Use of a tick mode of infection, as well as a greater number of experimental animals, may have produced additional clinical signs. Distinct differences in clinical and serological response have been observed in dogs experimentally inoculated with cultured B. burgdorferi compared to those exposed experimentally via B. burgdorferi infected ticks.29

Disseminated B. burgdorferi infection occurred in all infected calves. B. burgdorferi was demonstrated in the urine, blood, premortem synovial fluid, and in several tissues taken postmortem (blood, kidney, bladder, spleen, and synovial tissue). Pathological changes observed in skin, lung, lymph node, heart, liver, and synovium of the infected calves may also represent a response to disseminated B. burgdorferi infection; control calves did not have similar lesions at these sites. In this study, B. burgdorferi was more readily detected in pre-mortem urine and blood samples by PCR than by culture or FA staining; however, culture was more successful than direct PCR on tissue samples collected at necropsy. Factors involved may include the concentration of urine by high speed centrifugation prior to PCR, the sparse numbers of organisms in tissues, and the ability of PCR to detect nonviable organisms.31

Spirochetes were detected in the urine of all infected calves but in the blood of only one. Early or intermittent spirochetemia may have contributed to the difficulty in demonstrating B. burgdorferi in the blood of the calves.32,33 The pattern of urine shedding of B. burgdorferi in infected calves was variable and persisted over a period of weeks in some calves. Positive urine cultures obtained demonstrate that live organisms are present in the urine of infected calves. More frequent analysis of urine and blood
may provide a clearer pattern of urine shedding and spirochetemia. The demonstration of *B. burgdorferi* in the urine of infected cattle has lead investigators to speculate that perhaps, like leptospirosis, *B. burgdorferi* may be transmitted among cattle by contact with urine.4,7,9,34 *B. burgdorferi* is more labile than *Leptospira* spp and, therefore this type of transmission would likely require direct contact between voided urine and mucous membranes.6

The 4 infected calves developed a specific serological response to *B. burgdorferi* as determined by IFA and western blot analysis. Antibody titers increased throughout the course of infection in all infected calves with the exception of calf 4. Calf 4 produced a low antibody titer and its response to Osp A and B was both weak and delayed compared to the other infected calves. This calf also demonstrated the most widely disseminated infection; *B. burgdorferi* was detected in its blood, ante-mortem urine, and several tissues post-mortem. The phenomenon of *B. burgdorferi* infection with weak serological response has also been observed in natural disease.4,5,35

Antibodies to the nonspecific flagellar protein (41 kDa) of *B. burgdorferi* were detected by Western blot analysis in all of the calves beginning on day 0, as well as in the synthetic colostrum fed to the calves. Antibodies to this protein are common in adult cattle and probably represent antibodies formed against other flagellated flora in ruminants.5,6 The antibodies appear to have been passively transferred from the synthetic colostrum. The positive IFA titer seen in control calf 6 is also a response to the flagellar protein, as supported by western blot analysis. An antibody response to the reportedly *B. burgdorferi* specific 39 kDa protein26 was also common to both *B. burgdorferi* infected calves and noninfected control calves. This response in the infected calves became more distinct throughout the course of infection but remained weak in the control calves. Antibody to the 39 kDa has been commonly found in sera of cattle from nonendemic regions and therefore, may not be a specific marker of *B. burgdorferi* infection in cattle (Bushmich, unpublished data). In human disease, the presence of antibodies to the 39 kDa protein is commonly seen in patients with late manifestations of disease but antibodies against this protein have also been found on occasion, in patients with syphilis suggesting that there may be cross-reactive epitopes within this protein.37

The serum chemistry panels in both infected and control calves did not reveal any major organ dysfunction, and hematocrit levels were within normal range. Depressed serum globulin and total protein levels may reflect lower levels of immunoglobulins present in synthetic compared to natural colostrum. Alkaline phosphatase elevations may be due to elevated bone isoenzyme commonly observed in young animals (W Hoffman, personal communication).38 The diarrhea observed in the infected calves is not thought to be related to, or the consequence of, the *B. burgdorferi* inoculation; control calves also experienced diarrhea. The miscellaneous clinical signs observed in the infected calves (cough, bluet, buccal ulcer) may or may not be related to the introduction of *B. burgdorferi*.

This study utilized neonatal calves as an experimental model for studying the pathogenesis of Lyme borreliosis in cattle. We have demonstrated that following experimental inoculation with *B. burgdorferi* there is a sequential development of antibodies to specific *B. burgdorferi* proteins in these calves. Experimentally infected calves developed an erythematous rash from which *B. burgdorferi* was cultured. They also shed *B. burgdorferi* in urine for several weeks duration and *B. burgdorferi* was disseminated to other organs/tissues. It was demonstrated that calves can be spirocheticemic and spirocheturic without the development of clinical disease. Further investigations are needed to determine the pattern and duration of spirochetemia and spirocheturia in cattle as well as clarify the clinical and pathophysiological manifestations associated with *B. burgdorferi* infection in naturally infected cattle.

REFERENCES


Bone Marrow as a Source for *Borrelia burgdorferi* DNA

Lesley Fein, MD, MPH and Richard C. Tilton, PhD

**ABSTRACT**

The diagnosis of persistent Lyme disease has depended on the detection of serum antibody to *Borrelia burgdorferi*. Patients may lose their immune response over time or it may be abrogated by antimicrobial therapy. These case reports describe patients with chronic Lyme disease and a reactive bone marrow polymerase chain reaction (PCR). After appropriate and aggressive treatment, specific DNA may persist in sequestered sites such as bone marrow.

**Key words:** polymerase chain reaction, outer surface protein A gene, *B. burgdorferi* DNA

**INTRODUCTION**

Diagnosis of Lyme disease by laboratory methods continues to be difficult. Most tests rely on the production of IgM and/or IgG antibody to *Borrelia burgdorferi*. Antibody production may be abrogated by antimicrobial agents or may not occur at all. IgM and IgG antibody responses may fluctuate over time. Complicating these immune responses is the lack of reliability of many of the commercially available serological tests. Only recently has standardization of confirmatory Western blot testing been discussed in an organized forum and recommendations published for the use of proficiency panels.

There is a need for methods to directly detect *B. burgdorferi* in body fluids. Gene amplification, notably polymerase chain reaction (PCR), satisfies the requirements for both sensitivity and specificity. Several reports document the advantages of PCR for detection of DNA in synovial fluid and cerebrospinal fluid as well as other body fluids such as blood and urine. Relatively few, if any, reports have cited bone marrow as a diagnostic specimen.

The following case reports describe patients who had a reactive PCR for *B. burgdorferi* DNA in the bone marrow and positive *B. burgdorferi* antibody tests.

**Case #1**

This 51-year-old female was seen for the first time in April 1989 with a Lupus-like illness. There were insufficient clinical and laboratory data to fulfill the American Rheumatology Association criteria for Systemic Lupus Erythematosus (SLE). She was treated with nonsteroidal anti-inflammatory agents. From October 1989 to January 1991, she reported frequent respiratory infections, sinusitis, increasing joint pains, headaches, and fatigue. She was started on Plaquinil (hydroxychloroquine sulfate) for autoimmune arthritis in March 1991, but was symptomatic through June of 1991 with frequent upper respiratory infections on intermittent antimicrobial agents. By September 1991, she complained of severe headaches, fatigue, nausea, joint swelling, and urinary symptoms. She was again placed on various regimens of oral antimicrobials in addition to Plaquinil and tapering doses of steroids.

In June 1992 she was admitted to the hospital with chest pain (left hilar) and aorticopulmonary adenopathy. All of her laboratory tests were within normal limits, except antinuclear antibodies (ANA), which were present at a titer of 1:40. The gallium scan was positive in the left hilum and mediastinum. An abdominal CT scan was unremarkable. A biopsy of a mediastinal lymph node revealed noncaseating granulomas, which were atypical for sarcoidosis. Progressive improvement was seen through January of 1993 when she developed an acute influenza-like illness associated with severe arthritis and neck stiffness. The patient was started on oral Biaxin (clarithromycin) and Suprax (cefixime) for presumptive...
seronegative Lyme disease. Initiation of treatment was followed by a flare of an acute necrologic syndrome including vertigo, headaches, and profound fatigue. She became progressively worse until March 1993 when she began intravenous antimicrobial (IV) therapy with Claforan (ceftoxime). By that time her ANA was repeatedly negative but she experienced multiple Herxheimer reactions on Claforan requiring adjustment of dosage.

After 2 months of IV antimicrobial agents, her condition improved. She had no additional antimicrobials until March 1994 when she developed gradually recurring symptoms of headaches, joint pains, fevers, paresthesia, profound fatigue, and difficulty with word-finding. The ANA’s remained either negative or weakly positive. An ELISA antibody test for Lyme disease was performed with a commercial kit and was reactive on June 2, 1994 but was not confirmed by Western blotting. She was maintained on Zithromax (azithromycin) and Plaquenil. She continued to have severe fatigue, requiring total bed rest. Immunological studies were normal except for IgG and lambda monoclonal gammopathy. In September 1994, a bone marrow biopsy was performed. The PCR for *B. burgdorferi* DNA was reactive. She was again started on IM 1.2 mg BiCillin (penicillin) on October 3, 1994 and oral Zithromax 250 mg BID. The patient was maintained on the above regimen until clinical remission with progressive improvement of all her symptoms. Repeat immunological studies continue to be within normal limits.

**Case #2**

This 59-year-old female was seen for the first time on April 8, 1994 after being treated by another physician for Lyme disease. She had multiple influenza-like illnesses in 1992 through 1993 and was treated with short courses of oral antibiotics. She went to Spain in 1993. This trip was followed by the onset of severe pain and weakness. The patient had been a professional singer and could no longer sing. When tested for Lyme disease, the IgM Western blot was reactive. She was started on treatment for Lyme disease in August 1993 consisting of IV Claforan (Cefotaxime) for 8 weeks. She initially felt better but then relapsed. Intravenous ampicillin was administered for 1 month followed by Claforan (9 g/d) for 2 weeks which was reduced to 6 g/d. By May of 1994, there was lack of response and she was started on IV vancomycin. The patient responded well but relapsed when vancomycin was discontinued. Vancomycin was restarted in June of 1994 and the patient responded well.

Therapy was continued through July followed by pulse vancomycin and BiCillin LA (penicillin). By August 1994, she was very symptomatic with fevers and arthritis. The IV vancomycin was stopped and Plaquenil started in addition to intramuscular (IM) BiCillin. The patient was noted to have hypogammaglobulinemia and anemia and treated with IV gammaglobulin and transfusions. The patient did well until October 1994 when both symptoms and anemia recurred. The consulting hematologist performed a bone marrow biopsy that was unremarkable except for a positive PCR (*B. burgdorferi*). By November 1994 she had recurrent fevers and arthritis. Her anemia is stable on Epogen (epoetin alpha) and her strength is improving on Plaquenil. She has had two gammaglobulin transfusions for recurrent hypogammaglobulinemia. Throughout this time, both her IgG and IgM Western blots have remained consistently positive. The patient now reports that she is feeling better.

**DISCUSSION**

*B. burgdorferi* DNA was detected from bone marrow samples of these patients. The DNA was amplified with specific primers for the highly conserved Ospa (outer surface protein A) gene sequence of *B. burgdorferi*. The amplification and detection system used detected 10 copies of the Ospa gene in the sample. The unique Ospa primers are highly specific for *B. burgdorferi* and do not cross-react with closely related organisms such as *B. hermsii*. Known positive serum and urine samples, as well as a standard curve of *B. burgdorferi* DNA are included in each assay to confirm assay sensitivity and reproducibility throughout the sample processing, amplification and detection steps. Since PCR can be adversely affected by inhibitors of Taq polymerase commonly found in clinical specimens, all samples are required to pass a strict inhibition test that consists of the amplification, and detection of a small amount of a known DNA sequence added to an aliquot of the test sample. Samples that fail the inhibition test are repurified and retested. Negative controls included a series of buffer controls, extracts from normal human cells, and negative patient material. All negative controls must test negative before the PCR process can be validated. Amplification products were detected using a proprietary microtiter-based capture hybridization assay for the Ospa gene. This system generates a strong signal with greater sensitivity than conventional hybridization formats. Southern blot hybridization with nonradioactive alkaline phosphatase probes specific for the Ospa sequence, was used as a confirmatory method for ampligen detection.

To assure the validity of the sensitive PCR system, stringent quality control practices were observed. Sample processing, preparation, and reagent formulation functions were carried out in separate areas within the laboratory dedicated exclusively for PCR. Reagents were pretested and pre aliquoted for one time usage. Further precautions included the use of uracil-N-glycosylase to minimize ampligen cross-contamination. In cases such as
these, the presence of B. burgdorferi DNA can be detected in patients with active infection. Body fluids have been shown to contain small levels of Borrelia, which may be difficult to detect by culture, but may be detectable with the use of the PCR assay that is capable of detecting as little as the equivalent of less than 10 organisms per mL of fluid. This laboratory performs more than 2500 PCR tests per month for a variety of infectious agents and has never had a contamination incident. Manak et al suggested that PCR reactivity could be cyclical. That is, B. burgdorferi DNA may be released into the circulation in small amounts at unpredictable time intervals but apparently associated with antibiotic therapy. They also reported that reactive PCR tests may be observed in patients with no detectable antibody to B. burgdorferi and who fulfilled the Centers for Disease Control’s criteria for Lyme disease. Of 16 patients with chronic Lyme disease and a reactive PCR, only 4 were seropositive.

The significance of B. burgdorferi DNA in the bone marrow is unclear. Some investigators believe that B. burgdorferi is an intracellular bacterium. It is possible that the bone marrow serves as a reservoir for the Lyme disease agent and that sporadic appearance of nucleic acid in the circulation occurs after a DNA shower from the bone marrow, possibly stimulated by antimicrobial induced cell lysis. Thus, the bone marrow biopsy, albeit invasive, may prove to be a clinically significant procedure in a patient whose signs and symptoms are not sufficiently focused to rule out other diseases.

Unlike many suspected Lyme disease patients, these 2 individuals had a serologic diagnosis of Lyme disease and received extensive and seemingly adequate antibiotic treatment over a several-year period. They developed hematologic abnormalities, which resulted in a bone marrow biopsy. Symptoms of Lyme disease persisted in spite of aggressive treatment.

This report demonstrates several phenomena:
1. Despite aggressive IV and oral antimicrobial treatment, B. burgdorferi may persist in sequestered areas of the body such as bone marrow.
2. The yield on PCR testing for Lyme disease may be enhanced by testing bone marrow specimens or, by extrapolation, testing leukocytes, ie, buffy coat, where the organism may reside intracellularly protected from immune defenses. The bone marrow provides a specimen resource rich in nucleated cells, which may harbor the spirochete in dormant or active form. We clearly do not advocate this invasive testing for all patients but rather those who are refractory to standard therapy and have concomitant immunologic or hematologic abnormalities.
3. There does not appear to be a clear correlation between reactive Lyme serology and PCR positivity, although one patient had a persistently positive IgG and IgM Western blot and the other a positive ELISA test.

In conclusion, these case reports suggest that bone marrow may be an important site for detection of B. burgdorferi DNA.

REFERENCES
Lyme Disease and the Clinical Spectrum of Antibiotic Responsive Chronic Meningoencephalomyelitides

Kenneth B. Liegner, MD; Paul Duray, MD; Martha Agricola, FNP; Carl Rosenkilde, MD, PhD; Lawrence A. Yannuzzi, MD; Martina Ziska, MD; Richard C. Tilton, PhD; Dagmar Hulinska, PhD; Jeff Hubbard, MD; and Brian A. Fallon, MD

ABSTRACT

Intensive study of four patients with chronic meningoencephalomyelitis believed due to Lyme disease revealed seronegativity and/or variable seroreactivity and chronic persistent infection as common threads. Evaluation of these complex cases required determined study over time using all known methods (i.e., culture isolation, histologic, immuno-

hista
dmicrographic, direct antigen detection as well as standard serologic methods) on tissues as well as serial study of blood, cerebrospinal fluid (CSF) and urine. Prolonged intravenous antibiotic therapy conferred clinical benefit in each case and withholding of treatment resulted in clinical deterioration.

Key words: Lyme disease, meningoencephalomyelitis, persisting infection, seronegativity, B. burgdorferi, syphilis, multiple sclerosis, systemic lupus erythematosus

INTRODUCTION

It is commonly held that patients with late Lyme disease are almost invariably seropositive and antibiotic treatment of limited duration is generally curative. However, the phenomenology of chronic neuroborreliosis has not been fully elucidated. We have encountered a significant number of patients who have been seronegative for months to years despite serious neurologic illness of long standing thought due to Lyme disease. Antibiotic treatment in these cases, while conferring benefit, has seemed unable to eradicate the infection regardless of route of administration or duration of therapy. Four such cases of chronic meningoencephalomyelitis have been extensively studied and their response to treatment carefully documented. Detailed presentation of these cases may serve to illumine this cryptic disorder, which can be as difficult to treat as it is to diagnose.

CASE REPORTS

Case #1

A 39-year-old woman with a two-year history of progressing spastic quadraparesis, cranial nerve palsies, and persistent unexplained CSF pleocytosis was evaluated beginning in 1989. She had been diagnosed with idiopathic thrombocytopenic purpura (ITP) in 1975 and underwent splenectomy in 1976. She had lived in northern Westchester county, New York and northern California but gave no history of tick attachments or of erythema migrans.

No diagnosis was established after a year of observation and testing, and serologic studies for Lyme disease in serum and CSF were repeatedly negative. CSF examination in 1990 showed lymphocytic pleocytosis, elevated IgG, and absence of oligoclonal bands or myelin basic protein. Anticardiolipin and antinuclear antibodies were present and Raji cell assay and C1Q immune complexes were elevated. HIV and HTLV-I antibodies were negative.

An empiric trial of intravenous antibiotic treatment with cefotaxime (CFOTX) for 21 days in April 1990 resulted in no clinical improvement and no change in CSF pleocytosis. Thereafter she was treated with 4 months of minocycline with no clinical benefit. The patient remained wheelchair-bound.
B. burgdorferi grew from CSF in December 1991 at which time the patient first became seropositive despite at least 4 years of clinical illness. She was treated with CFOTX (4 g IV Q 8 hrs once weekly) with complete resolution of pleocytosis after 13 weeks and constitutional symptoms improved. Despite continuation of one weekly IV therapy for 10 months, there was gradual neurologic deterioration. Intravenous antibiotics were discontinued December 1992.

Methylprednisolone sodium succinate was given intravenously, 1 g daily for 5 days, followed by prednisone over a six-week period for the possibility of systemic lupus erythematosus. Pleural effusions developed within one week of starting steroids along with severe encephalopathy and debilitation. She could not remember conversations held minutes earlier and was unable to hold a cup, roll over in bed, or transfer from bed to wheelchair. Computed axial tomography of the chest revealed pleuropericardial effusions (Fig 1).

A pleuropericardial window was created for diagnostic and therapeutic purposes. Fibrinous pericarditis was present with infiltration of plasma cells and macrophages and spirochete-compatible structures were seen with modified Steiner silver and phycoerythrin stains, as well as a touch preparation (Figs 2-5).

Intravenous CFOTX 6 g daily was administered for the next 3½ months with dramatic improvement of her encephalopathy. The pleuropéricardial effusions improved (Fig 6). The patient was able to walk 500 feet
with a rolling walker and was able to go home. A further 3 months of daily CQOTX was administered but the patient’s health insurer refused authorization for any subsequent intravenous antibiotic therapy.

The patient became increasingly encephalopathic over the next 6 months. Daily intravenous CQOTX was reinstituted in June 1994 and mental status improved as confirmed by serial neuropsychological testing before and after 4 months of treatment.

Several specimens of plasma and urine between February and July of 1995 were found to be PCR positive for B. burgdorferi-specific DNA. From July 1995 through April 1996 the patient was treated with intramuscular benzathine penicillin. On this treatment she felt poorly, encephalopathy worsened, and she lost the ability to ambulate. Plasma PCR for B burgdorferi-specific DNA was again positive February 1996. CSF analysis March 1996 showed 14 lymphocytes/mm³, elevated protein (57 mg %) and slight elevation of IgG. Oligoclonal bands were present in both CSF and serum. Myelin basic protein was absent. CSF Lyme PCR and OspA antigen were negative as were Lyme-specific immune complexes in serum and CSF. Authorization for additional intravenous antibiotic therapy was refused by the insurer. Encephalopathy and debilitation worsened (Table 1).

**Case #2**

In the fall of 1985 a 61-year-old outdoorsman residing in the Catskill region of New York State developed a large round rash on one thigh. A physician was consulted but no treatment was given. The following winter unremitting headache, low grade fever, paresthesias and truncal instability developed. Lumbar puncture demonstrated lymphocytic pleocytosis. Lyme ELISA was negative. Dysphasia and a progressive stroke syndrome developed. A diagnosis of “vasculitis” was given and the patient was treated with steroids and cyclophosphamide for a number of months with progressive deterioration to a level of functioning slightly above a persistent vegetative state. Lyme ELISA was positive in 1988. Treatment with intramuscular ceftriaxone (CFTRX) for 14 days resulted in slight improvement.

In 1992, computed axial tomography of the brain
Table 1 (Case I)

<table>
<thead>
<tr>
<th>Clonazepam</th>
<th>Diagnoses</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/92 CSF WBC 3,000-7,000 lymph 11% monos Cell 23,30,41,42,55,50</td>
<td>ANA 1:20000</td>
<td>12/9250-1/2103 CFTX 2 g/day</td>
</tr>
<tr>
<td>12/93 CSF WBC 3,000-7,000 lymph 7% monos</td>
<td>12/9350-15250 Methyldopa Hydrocortisone Sodium Saccharide 1 g/day</td>
<td></td>
</tr>
<tr>
<td>2/25/93 prednisone 50 mg/5</td>
<td>3/25/93-5/13/93 prednisone 90 mg/day</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 (Case I)

<table>
<thead>
<tr>
<th>Clonazepam</th>
<th>Diagnoses</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/30/93</td>
<td>350 cc thoracentesis</td>
<td>3/30/93 pleural fluid complement WBC Bx HgPC or in one lab, negative in another</td>
</tr>
<tr>
<td>450 cc thoracentesis</td>
<td>350 cc thoracentesis</td>
<td>450 cc thoracentesis</td>
</tr>
<tr>
<td>350 cc thoracentesis</td>
<td>350 cc thoracentesis</td>
<td>350 cc thoracentesis</td>
</tr>
<tr>
<td>350 cc thoracentesis</td>
<td>350 cc thoracentesis</td>
<td>350 cc thoracentesis</td>
</tr>
</tbody>
</table>

Note: Please see abbreviations and reference ranges for Tables I-IV following Table IV.
Fig 8. Case 2: Coronal section of brain at level of the temporal horns showing massive hydrocephalus at autopsy, July 1993.

Fig 9. Case 2: Floor of the fourth ventricle and brainstem viewed following removal of cerebellum, at autopsy, July 1993. Prominent ependymitis is evident.

Fig 10. Case 2: Section through cerebellar cortex overlying floor of the fourth ventricle at the foramen of Luschka, showing florid granulomatous meningoencephalitis and ependymitis [original magnification 40 X].

Fig 11. Case 2: Floor of the IVth ventricle showing mixed granulomatous and acute inflammation [original magnification 40 X].

Fig 12. Case 2: Anterior quadrant of thoracic spinal cord showing meningitis and focal myelitis [original magnification 40 X].

Fig 13. Case 2: Higher power view of the foramen of Luschka granulomatous inflammation showing giant cells, mononuclear cells, and polymorphonuclear leukocytes [original magnification 400 X].
showed massive hydrocephalus (Fig 7). Electroencephalogram revealed status epilepticus and phenobarbital was prescribed. Lyme serology was negative in one laboratory, yet positive in another. Western blot was nondiagnostic, showing only a 41 kiloDalton band. CSF examination revealed the presence of oligoclonal bands without myelin basic protein and very elevated CSF IgG. Serum showed elevated C1Q immune complexes. OspA antigen capture assay in CSF was strongly positive.

The patient was given daily intravenous CFTRX for one month, then weekly CFOTX (4 g IV Q 8 hr x 3 doses) for one year, with modest improvement in his neurologic status. The patient succumbed to his disease July 1993.
Autopsy revealed severe hydrocephalus (Figs 8,9) and florid meningoencephalomyelitis and ependymitis (Figs 10-13). The CSF was positive for OspA antigen and Lyme-specific immune complexes. Spirochetes were not visualized on histopathologic and immunohistochemical study by light microscopy but borrelia-compatible structures were visualized in formalin-fixed tissues studied by electron microscopy (Figs 14-16) and brain tissue and dura mater were PCR positive for detection of *B. burgdorferi*-specific oligonucleotides (Figs 17A,B)³ (Table II).

Case 3
A 37-year-old woman removed a tiny tick from her left shin in the spring of 1982 while visiting Dutchess County, New York and developed an eruption about the site that persisted for several years. Biopsy of the lesion was read as granuloma annulare. Multisystem symptomatology developed within months of the attachment including polyarthralgia and synovitis, fatigue, headache, paresthesias, cognitive problems, and ocular disorders incuding pars planitis, anterior and posterior granulomatous uveitis, and retinal vasculitis.

Several Lyme ELISAs between 1982 and 1990 were negative. A short course of doxycycline in 1990 conferred some benefit. In June 1990 intravenous CFTRX 2 g/day was given for 42 days with symptomatic improvement. She was then given minocycline, 300 mg/day, for the next 2fi years with progressive improvement.

In the spring of 1993 the patient used minocycline sporadically, and in the summer of 1993 she developed neurologic symptoms. MRI of the cervical spinal cord showed high intensity lesions (Fig 18). Cerebrospinal fluid examination revealed 70 cells (mostly lymphocytes) and markedly elevated protein and IgG. Oligoclonal bands were present in CSF but myelin basic protein was within normal limits. Lyme serologic tests were negative.
in serum and CSF as was CSF culture for *B. burgdorferi*, and OspA antigen in CSF, and PCR and Lyme-specific immune complexes in CSF and serum.

The patient was treated with daily intravenous CFOTX for the next 8 months during which time serial CSF examinations revealed progressive decrease in pleocytosis, protein, IgG and IgG synthesis rates. The last 3 of 5 CSF samplings showed Lyme-specific immune complexes in CSF and Lyme Western blots now demonstrated key *B. burgdorferi*-specific bands (IgG: 28, 30, 39, 93 from one lab and 23, 29, 39 from a second lab). Intravenous antibiotics were discontinued July 1994.

The patient has been maintained on weekly intramuscular benzathine penicillin (4.8 million units IM), combined with azithromycin (750 mg/day) since that time, remaining clinically well. Western blots from February 1996 showed the 39 kDa band on IgM blot and 28, 30, 39, 41 kDa bands on IgG blot and July 1996 showed 20 and 34 kDa bands on IgM and 28, 34, 39, and 58 kDa bands on IgG. Lyme ELISA has been negative throughout (Table III).

**Case 4**

In October 1989 a 40-year-old fire captain developed optic neuritis, constitutional symptoms, and progressive neurologic symptomatology believed on clinical grounds to be multiple sclerosis. MRI of the neuraxis showed hyperintense lesions at a variety of levels. CSF examination was not performed. His condition progressively deteriorated to a wheelchair-bound status despite treatment for multiple sclerosis, including beta interferon. There was no history of deer tick attachments nor of erythema migrans but he had had a large-type tick attachment occurring in the 1970s on Parris Island, South Carolina, followed by some joint symptoms. He had spent a great deal of time in shore areas of Rhode Island, Connecticut, and Massachusetts. All Lyme serologic tests and research assays in blood and CSF were negative in September 1994 and CSF showed a pattern thought pathognomonic for multiple sclerosis.

In view of the patient’s lack of response to treatment
### Table III (Case 2)

<table>
<thead>
<tr>
<th>Q1/21</th>
<th>Diagnostics</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/04</td>
<td>89y r/o child of woman presents an umbilicated</td>
<td>INF 29.2 mg/kg, IgG synthesis rate 101 mg/dL</td>
</tr>
<tr>
<td>4/04</td>
<td>Blood/normal, feet and hands normal</td>
<td>INF 9.6 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>5/04</td>
<td>All leg numbness resolved following 9 months</td>
<td>INF 14.3 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>6/04</td>
<td>No leg numbness, no new fevers,</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>7/04</td>
<td>Essentially normal neurologic exam and status except for</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>8/04</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
</tr>
</tbody>
</table>

### Table III (Case 3)

<table>
<thead>
<tr>
<th>Q1/21</th>
<th>Diagnostics</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/04</td>
<td>59y r/o child of woman presents an umbilicated</td>
<td>INF 29.2 mg/kg, IgG synthesis rate 101 mg/dL</td>
</tr>
<tr>
<td>4/04</td>
<td>Blood/normal, feet and hands normal</td>
<td>INF 9.6 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>5/04</td>
<td>All leg numbness resolved following 9 months</td>
<td>INF 14.3 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>6/04</td>
<td>No leg numbness, no new fevers,</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>7/04</td>
<td>Essentially normal neurologic exam and status except for</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
</tr>
<tr>
<td>8/04</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
<td>INF 17.0 mg/kg, 100 mg/dy</td>
</tr>
</tbody>
</table>
aimed at multiple sclerosis, a five-month empirical treatment trial of daily CFOTX was given between the fall of 1994 and spring of 1995. The patient's neurologic status which had been progressively deteriorating, reversed. Wheelchair bound to start, the patient was able to ambulate at least 100 feet with a walker. Speech became clearer, and movements somewhat more fluid. Synovitis of ankles and knuckle joints was noted during the early phases of treatment.

MRI of neuraxis at completion of therapy showed improvement compared to a pretreatment study with a diminution in the number and size of lesions. CSF examination repeated at completion of 4 months of therapy showed disappearance of oligoclonal bands and myelin basic protein, normalization of IgG synthesis rate and CSF IgG only slightly elevated. Lyme Western blot although not fully diagnostic, showed evolution of key B. burgdorferi-specific bands (IgM 30, 45, 58, 93 kDa, March 1995; and IgG 39, 45, 58, April 1995) and Lyme-specific immune complexes were seen in serum for the first time (optical density 0.634; positive cut-off, greater than 0.215). OsPA antigen in CSF remained negative.

While being treated with intramuscular benzathine penicillin combined with azithromycin, the patient deteriorated with loss of ability to ambulate and development of urinary and fecal incontinence.

Lumbar puncture repeated in September 1995 five months after discontinuing intravenous antibiotic therapy again showed markedly abnormal parameters indicative of multiple sclerosis including oligoclonal bands and elevated myelin basic protein, markedly elevated CSF IgG and IgG synthesis rate as well as lymphocytic meningitis.

Intravenous antibiotics were resumed. The patient regained his ability to ambulate with a walker and to control bowel and bladder. Repeat lumbar puncture March 1996 showed significant improvement in CSF parameters with disappearance of myelin basic protein, clearance of CSF pleocytosis, and decreased CSF IgG and IgG synthesis rate. Lyme-specific IgM immune complexes were detected in CSF (Table IV).
DISCUSSION

Seronegativity implies failure of detection of infection by the patient’s immune defense and also masks the infection from recognition by the patient’s physician. This may set the stage for the development of more serious neurologic or other systemic illness. Dattwyler et al showed that early application of antibiotic therapy may blunt the development of an antibody response. Schutzer has shown that free antibodies may not be demonstrated unless methods to dissociate circulating immune complexes are used. T-cell anergy may be another mechanism to explain both seronegativity and chronic persistent infection. Recently, it has been found in vitro experiments that B. burgdorferi may target, invade, and destroy human B- and T-lymphocytes and may even steal lymphocyte cell membrane.

Analogous to the situation in leprosy, there may be two clinical subsets of patients with Lyme disease defined by the host immune response. Both T- and B-cell deficiencies occurring in the seronegative subset may predispose to expression of more serious illness, as with lepromatous leprosy, and chronic persistent infection. The seropositive subset may resemble tuberculoid leprosy where the severity of illness is limited by the more effective host immune response. Other mechanisms, which may explain survival of borreliae despite antibiotic treatment include intracellularity and the adoption of spheroplast-L forms to evade the lethal effect of cell wall-acting antibiotics.

Case 1 showed clinical illness for at least four years prior to proof of diagnosis. Compelling laboratory evidence for chronic persistent infection was developed numerous times throughout her course including culture, demonstration of spirochetal-compatible forms in pericardial biopsy, and repeated PCR positivity all despite prior and sometimes very prolonged intravenous antibiotics. Weekly treatment with CFOTX was apparently not adequate to control the infection in her case. Maximal improvement was seen only after 6 months of daily intravenous antibiotic therapy, but the patient had suffered a degree of irreversible neurologic injury, and had relapses whenever treatment was discontinued.

This patient showed many markers suggesting lupus including positive ANAs, antinuclearin antibodies, circulating immune complexes, and at one point even the presence of anti-double stranded DNA antibodies. Whether she suffered from a pre-existing connective tissue disease or the autoimmunity she evidenced was merely an epiphenomenon associated with chronic borreliosis is unclear. However, treatment for systemic lupus erythematosus with corticosteroids resulted in severe clinical deterioration, whereas intensive antibiotic treatment reversed this situation and achieved dramatic improvement.

That her borreliosis isolate showed resemblance to strains from the West Coast of the United States on pulse-gel electrophoresis, and that she had lived in California for a few years prior to the development of her ITP makes one wonder whether borreliosis infection may have caused her ITP. One attempt to identify B. burgdorferi-specific DNA in tissue in paraffin blocks from the patient’s splenectomy was negative, however. Her case prompts speculation on the possible role of borreliosis infection in other autoimmune disorders previously thought to be idiopathic.

Case 2 demonstrates the devastating potential of unrec-
ognized and untreated borrelioid infection in genetically susceptible hosts and the disastrous consequences of applying immunosuppressive therapy in cases of unrecognized borrelioid infection. CNS damage was very far advanced by the time the diagnosis was finally made; however, there was still some antibiotic responsiveness. The false teaching that patients with late Lyme disease are almost invariably seronegative led the physicians caring for this patient away from the correct (and treatable) diagnosis.

Case 3 had a clear clinical history indicating Lyme disease. Despite intensive study laboratory corroborations for the diagnosis could not be obtained for some 13 years. A prior six-week course of intravenous CфТRХ did not prevent the development of meningoencephalomyelitis. An eight-month course of intravenous CFOTX was required to resolve disturbed CSF parameters. Lyme-specific immune complexes were demonstrable in the final three of five cerebrospinal fluid examinations and key Lyme disease-compatible bands finally developed on Western blot in serum thereafter. She has been seronegative by ELISA throughout, calling into serious question the validity of using this assay alone as a screening test.

Case 4 demonstrates how closely neuroborreliosis can mimic multiple sclerosis. Given now that seronegativity occurs in Lyme disease, distinguishing the two disorders may be a daunting task. The patient showed resolution of markers thought to be pathognomonic for multiple sclerosis in CSF along with clinical improvement following intensive intravenous antibiotic treatment. Relapse of abnormal CSF findings and of neurologic signs occurred with suspension of intensive treatment. Resolution again followed a second course of intravenous therapy. This case suggests that neuroborreliosis may be misdiagnosed as multiple sclerosis.20,21

On the other hand, a significant body of research had been developed in pre-World War II Germany suggesting a relationship between spirochetal infection and multiple sclerosis.20,21 Steiner averred that the Spirocheta myelophthora he visualized morphologically resembled borreliae rather than treponemes.22 Additional studies using modern direct antigen detection techniques should be conducted to re-examine a potential relationship between borrelioid infection and multiple sclerosis.

That borrelioid infection may result in multiple sclerosis-like illness following months to years of clinical latency and without occurrence of erythema migrans ought to be taken into account in decisions on antibiotic prophylaxis for recognized ixodid tick attachments.23-25

CONCLUSION

Chronic persistent infection and seronegativity are not without precedent in spirochetal disease and are now well accepted phenomena in syphilis. Prolonged and combination antibiotic therapy are being utilized by an increasing number of clinicians to attempt to avert progressive neurosyphilis.26 The hypothesis has been proposed that syphilis may not be a curable infection in the sense of total bacterial eradication with available treatment approaches.27

Relapses following use of potent antibiotics and detection of the Lyme organism or its DNA following treatment likewise demonstrates an inability to completely eradicate the pathogen and permanently halt the pathologic process with current methods of treatment in some patients.28-37 This is a problematic situation because intensive antibiotic treatment is costly, is inconvenient, and carries associated risk for the patient.28 Such antibiotic usage may foster the emergence of strains of other types of bacteria resistant to the antibiotics employed and thus has public health implications. For some patients, however, this may be the only presently available alternative to progressive neurologic deterioration.28,29 In view of this dilemma, the international biomedical research community must give high priority to the development of improved and/or alternate methods of treatment that can definitively cure persisting borrelioid infections responsible for neurologic and other manifestations of chronic Lyme disease.

ACKNOWLEDGMENTS

Patricia Coyle and Zhilian Deng, Department of Neurology, SUNY Stony Brook, performed Ospa antigen and Lyme-specific immune complex testing in CSF and serum in Cases 1-4. The Centers for Disease Control and Prevention, Fort Collins, CO cultured for borreliae and did serologic and PCR testing in Cases 1 and 2. David Perings and Barbara Rutledge, Mayo Clinic Foundation, performed PCR testing and culture for borreliae in Cases 1 and 2 and pulse-gel electrophoresis characterization of the isolate in Case 1. Ben Laft, Department of Medicine, SUNY Stony Brook, provided the PCR primer used in studying autopsy tissues in Case 2. Maria Philipp and Dr. Roberts, Tulane Regional Primate Center, effected immunohistochemical study of CNS tissues in Case 2. Tom Gora provided computer expertise, hardware and software support and assisted in tabulating data.

REFERENCES


*Ixodes scapularis* (black legged tick): female, male, and nymph (left to right) compared to Sesame seeds. (above)

*Ixodes scapularis* mating adults: female below ventral side facing up and male above dorsal side facing up. Background squares = 1mm per side.

James L. Occi  
Biology Department  
Union County College  
Cranford, New Jersey 07016
INFORMATION FOR AUTHORS AND EDITORIAL POLICY

The following guidelines are in accordance with the “Uniform Requirements for Manuscripts Submitted to Biomedical Journals” and the International Committee of Medical Journal Editors (the “Vancouver Group”) statement, agreed at the January 1993 Meeting.

The Journal of Spirochetal and Tick-borne Diseases publishes quarterly reviews and original work studies about any aspect of spirochetal and tick-borne diseases. The primary purpose is to broaden our understanding of spirochetal and tick-borne diseases. Special focus is given to Lyme borreliosis (also known as Lyme disease), as the most prevalent spirochetal and tick-borne disease. The clinical topics may involve all medical disciplines, nursing, and pharmacy, as well as the social, ethical, and biological features of spirochetal and tick-borne diseases.

The Journal is composed of two major sections. One section is devoted to the review of a specific topic that is established by the Associate Editors, and a special guest editor is invited to coordinate the development of up to six manuscripts relating to the specific topic. The second section of original works is composed of unsolicited manuscripts that are subsequently reviewed by the Review Board, as well as external reviewers, depending on the potential for conflict of interest within the editorial panel and the potential interest by the readership.

Expeditious review of all manuscripts is carried out with a projected response time of not more than 6 weeks. Rejected manuscripts usually are returned to authors within 6 weeks. Decisions about potentially acceptable manuscripts may take somewhat longer.

The Journal will publish material defined within the categories described in the following.

Reviews

Each issue includes a series of state-of-the-art articles on a topic related to spirochetal and tick-borne diseases.

The articles represent invited presentation by authorities in the field on topics related to spirochetal and tick-borne diseases, with an emphasis on Lyme borreliosis.

Submissions to this category should present a comprehensive state-of-the-art analysis and should be accompanied by an abstract of 300 words or less summarizing major points.

Peer Review Articles

Original articles of 5000 words or less may be submitted to the editorial office. Each article should be accompanied by an abstract of 300 words or less describing the findings of the original research. All articles will be peer reviewed within a 3-week period with subsequent notification to the authors within 5 weeks of submission.

Case Reports

Specific clinical case reports describing a unique approach to Lyme disease and other related disorders in the area of diagnosis or treatment may be submitted for review. An abstract of 250 words or less should accompany the text.

Correspondence

Letters to the Editor in the form of correspondence related to material published in the Journal or some aspect of Lyme borreliosis and other spirochetal and tick-borne diseases may be submitted. Such letters, if related to work previously published in the Journal, will be referred to the author of the original work for a response. Letters to the Editor should be submitted in duplicate, typewritten and double-spaced, not exceeding 400 words of text and a maximum of five references. Letters should have no more than three authors, and should be signed by all of the authors. Please include a word count.

Receipt of letters is not acknowledged, but correspondents will be notified when a decision regarding publication is made.
Editorials

Editorials may be published, usually at the solicitation of the Associate Editors, but unsolicited submissions that relate to an unusual topic of interest exceeding the usual designation of correspondence, i.e., 1000 words or less, will be considered.

Photographic Section

The topical photographic section will be a regular feature. Photographs pertinent to articles presented in the Journal, as well as other photographs related to any aspect of spirochetal or tick-borne diseases, will be considered for the publication. The guidelines for the submission are designated in Illustrations.

Conflict of Interest

The Journal asks authors to disclose at the time of submission any financial or other arrangements they may have with a company whose product figures in the submitted manuscript or with a company making a competing product. Such information will be held in confidence while the paper is under review and will not influence the editorial decision, but if the article is accepted for publication, the editors will discuss with the authors the manner in which such information is to be communicated to the reader.

Because the essence of reviews and editorials is selection and interpretation of the literature, the Journal expects that authors of such articles will not have any financial or other interest in a company (or its competitor) that makes a product discussed in the article. Potential authors who have questions about these issues should contact the Managing Editor.

Submission of Manuscript

An original and three copies of the manuscript should be submitted to:

Journal of Spirochetal and Tick-borne Diseases
SLACK Incorporated
6900 Grove Road
Thorofare, NJ 08086

Manuscripts containing original material are accepted with the understanding that neither the article nor any part of its essential substance has been or will be published or submitted for publication elsewhere before appearing in the Journal.

All manuscripts should be accompanied by a letter of copyright transmittal. This must be signed and dated by all authors. The letter is required before any manuscript can be considered for publication and should contain the following wording:

"In consideration of The Lyme Disease Foundation taking action in editing my (our) submission, the author(s) undersigned hereby transfers, assigns, or otherwise conveys all copyright ownership to The Lyme Disease Foundation. The copyright so conveyed includes any and all subsidiary forms of publication, such as electronic media. The author(s) declares that the manuscript contains no matter that is, to the best of the author’s knowledge, libelous or unlawful, or that infringes upon any U.S. copyright."

All manuscripts should be submitted with a cover letter indicating the category for which the manuscript should be reviewed. Copies of any closely related manuscripts should be submitted to the Editor along with the manuscript that is to be considered by the journal.

A cover letter, signed by all authors, should identify the person (with the address and telephone number) responsible for negotiations concerning the manuscripts; the letter should make it clear that the final manuscript has been seen and approved by all authors and that they have taken due care to ensure the integrity of the work. Manuscripts should include a title page, abstract, and text, with tables, illustrations, and references below.

For the integrity of the published material, manuscripts describing clinical aspects of Lyme borreliosis must disclose: criteria for patient enrollment into the study and criteria for defining "successful" or "nonsuccessful" Lyme borreliosis treatment.

Manuscripts without these requirements will be automatically rejected.

Titles and Author's Names

With the manuscript, provide a page giving the title of the article; titles should be concise and descriptive (not declarative). Also include a running head of fewer than 40 letter spaces; the name(s) of the author(s), including the first name(s) and academic degree(s); the name of the department and institution in which the work was done; the institutional affiliation of each author; and the name and address of the author to whom reprint requests should be addressed. Any grant support that requires acknowledgment should be mentioned on this page.

Abstract

Provide on a separate page an abstract of not more than 300 words (original and review articles) or 250 words (case report). This abstract should consist of four paragraphs, labeled Background, Methods, Results, and Conclusion. They should briefly describe the problem being addressed in the study, how the study was performed, the results, and what the authors conclude from the results.
Text
All material should be typed and double-spaced. Standard sequence of methods and materials, results, and discussion should be employed with tables and figures numbered in the order in which they are cited in the text. A disk in text format should accompany this.

Tables
Submit tables typed and double-spaced and provide a heading for all columns with a comprehensive title on separate sheets. A disk copy with a separate file for each table should be on the disk containing the text.

Illustrations
Photographs and figures should be submitted as glossy prints 5×7 in., with one copy of each print for each copy of the manuscript. Figure legends should be provided on a separate sheet with identification of the figure. The back of the glossy print should indicate the number of the figure.

References
References should be numbered in order of citation in the text, following the American Medical Association guidelines for references. The standard journal abbreviations from Index Medicus should be followed. Numbered references to personal communications, unpublished data, and manuscripts either “in preparation” or “submitted for publication” are unacceptable.

Drug Names
Generic names generally should be used. When proprietary brands are used in research, include the brand name in parentheses in the Methods section.

Units of Measure
Authors should express all measurements in conventional units, with Systeme International (SI) units given in parentheses throughout the text. Figures and tables should use conventional units, with conversion factors given in legends or footnotes.

Permissions
Materials taken from other sources must be accompanied by a written statement from both author and publisher giving permission to the Journal for reproduction. Obtain permission in writing from at least one author of articles still in press, unpublished data, and personal communication. Contributors are obligated to disclose that submissions constitute their own work unless otherwise specified. Contributors shall indemnify the Journal for any claims or actions instituted against the Journal by individuals claiming that the contribution is not the work of the contributor.

Acknowledgment of Receipt
An acknowledgment is dispatched on receipt.
Our new book, BeginnerNet: A Beginner’s Guide to the Internet and the World Wide Web, was created for all levels of health care professionals to gain a basic understanding of life on-line. The Internet is one of the best places to find the most current information available on a variety of topics, for both work and home. This book describes uses of the Internet and what to do if you are surfing the Web for the first time. The book is 104 pages, 7" X 10", paperback, and the order number is #33225.

The price is only $18.95
(S&H in US is $4.95, outside US $20.00)
We accept credit cards, checks, and money orders.

To easily get your copy today,
call 1-800-257-8290,
1-609-848-1000 or fax 1-609-853-5991
or mail Chris Freeman, SLACK Incorporated,
6900 Grove Road, Thorofare, NJ 08086

You’ll be surfing in no time.

You can ruin a life in one stroke.

Even if you live through a stroke, you might face paralysis, loss of speech, or altered behavior. Things that can make life difficult, to say the least. Strokes occur when the blood supply is cut to part of the brain. And if you currently have high blood pressure or any form of heart disease, your risk of stroke is higher than normal. The American Heart Association urges you to control your blood pressure. By doing so, you’ll reduce your chances for a stroke. And increase the likelihood of a long, happy life.