Journal of Spirochetal and Tick-borne Diseases

Volume 1 June 1994 Number 2

First Announcement

VIII ANNUAL SCIENTIFIC CONFERENCE ON LYME BORRELIOSIS and other Spirochetal and Tick-borne Diseases

Vancouver, British Columbia, Canada April 27 & 28 1995

Site and Date

The VIII Annual Scientific Conference on Lyme Borreliosis and other diseases will be held in Vancouver, British Columbia, April 27 and 28, 1995. The exact location will be announced later. Activities of the Conference will include scientific sessions, exhibits and social events.

Conference Host: Diane Kindree, B.S. N. B.C. Lyme Borreliosis Society
Conference Chair: Martina Ziska, M.D. Lyme Disease Foundation
Conference Co-Chairs: S. N. Banerjee, Ph.D. The B.C. Centre for Disease Control
Willy Burgdorfer, Ph.D., M.D. (Hon.) NIH/RML
Bettina Wilske, M.D. Max von Pettenkofer Institute
Poster Session Chair: Craig Cleveland, M.D. East Hyde Park Internal Medicine

Conference Theme

The VIII Annual Scientific Conference on Lyme Borreliosis and other diseases is an interdisciplinary scientific forum designated to stimulate the exchange of new information among those interested in these diseases of increasing worldwide significance. The special emphasis will be given to mechanisms of Lyme borreliosis persistency.

Scientific Program

The scientific program will include both oral and poster scientific presentations. The main topics will be designated at the Call For Presentations.

Publications

The program and abstract of the papers and posters will be published and distributed to scientific registrants at the Conference.

Exhibits

The Conference invites commercial companies to exhibit their products.

IMPORTANT DATES:

November 30, 1994 Deadline for receipt of abstracts of papers and posters

February 28, 1994 Notification of acceptance of papers and posters

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Dedicated to:
"Science and Art in Spirochetal and Tick-Borne Diseases"

Information for Authors and Editorial Policy

The following guidelines are in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" and 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 upon 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 four weeks. Rejected manuscripts are usually returned to authors within six weeks. Decisions about potentially acceptable manuscripts may take somewhat longer.

The Journal will publish material defined within the categories described below.

Reviews

Each issue includes a series of articles on the state of the art 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.

Each manuscript should present a comprehensive state-of-the-art analysis and should be accompanied by an abstract of 300 words or less summarizing the 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 three-week periods with subsequent notification to the authors within five weeks of submission. Submitted articles may relate to any area of spirochetal and tick-borne diseases.

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 all should sign a letter. 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 of research articles 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 usually 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 Editor-in-Chief.

Submission of Manuscript

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

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Lyme Disease Foundation
1 Financial Plaza
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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 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 acknowledgement 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 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.

Tables

Submit tables typed double spaced and provide a heading for all columns with a comprehensive title on separate sheets.

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 used.

References

References should be numbered in order of citation in the text. The standard form of INDEX MEDICUS should be followed. Numbered references to personal communications, unpublished data, and manuscripts either ''in preparation'' or ''submitted for publication'' are unacceptable.

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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.

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Journal of Spirochetal and Tick-Borne Diseases

CALL FOR PAPERS

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wants to encourage potential authors
to submit manuscripts
to all sections of the Journal.
Manuscripts without requirements, designated in the ''Information to Contributors,'' will be automatically rejected.

CALL FOR PHOTOGRAPHS

The *Journal of Spirochetal and Tick-Borne Diseases*
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for publication in the Photographic Section of the Journal.
The Editor-in-Chief should be contacted for further details.

Journal of Spirochetal and Tick-Borne Diseases

Topic review for upcoming issues:

September 1994

Aspects of Lyme Borreliosis Pathogenesis
Guest Editors: *Claude F. Garon, Ph.D.*

December 1994

Clinical Manifestations of Lyme Borreliosis: An Enlarging Spectrum?
Guest Editors: *Kenneth B. Liegner, M.D.*

March 1995

Focusing on the Tick-Borne Disease in North America:
Relapsing Fever, Ehrlichiosis and Babesiosis
Guest Editor: *Tom G. Schwan, Ph.D.*

June 1995

Treponemal Biology and Pathogenesis at the Cellular and Molecular Level
Guest Editor: *James N. Miller, Ph.D.*

Journal of
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Lyme Disease Foundation Prize

To be awarded for an original paper, contributing significantly to the understanding of spirochetal and tick-borne diseases, published in *JSTD* the first year of publication.

The Lyme Disease Foundation Prize will be awarded to the author of an outstanding original research, development, or clinical observation, published in *JSTD*. **The value of the first prize is $5000, the value of the second prize is $2500**; the winners will also receive a certificate. The current competition period begins with the June 1994 issue and ends with the June 1995 issue. Funding for this award is a gift from an anonymous donor.

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

Throughout the competition period, readers are invited to nominate papers appearing in *JSTD*. Nominations must be typed, and the following information provided: the title of the paper, issue in which it was published, author's name, and a brief statement of justification for nomination. Nominations should be submitted to the Editor-in-Chief, JSTD, 1 Financial Plaza, Hartford, CT 06103-2610, and **must be received on or before July 31, 1995.** Final selection will rest with a panel of distinguished scientists appointed by the Editor-in-Chief of *JSTD*.

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

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EDITORIAL
The Cradle of Lyme Borreliosis

K. Weber and W. Burgdorfer

Many scientific articles on Lyme borreliosis emphasize that the story of this disease began with the description of certain observations made in the United States in the middle of the 1970s. The first papers of the Yale investigators recognized that a skin manifestation of the illness, erythema migrans, had been described in Europe, but since they found no evidence for a previous characterization of joint and cardiac manifestations observed in their patients (1), they thought they were dealing with a hitherto undescribed illness and named it Lyme arthritis and later Lyme disease. Was the cradle of Lyme borreliosis in Europe or the United States? To arrive at a correct answer, one must go back to see what was known in Europe about this disease by 1975. As recently outlined (2), the history of Lyme borreliosis can be divided into several periods.

Period one lasted from 1883 until 1945. During this time, the three classical dermatological manifestations, acrodermatitis chronica atrophicans (ACA), erythema migrans, and, to a certain extent, borrelial lymphocytoma, have become known since 1883, 1909, and 1911, respectively (2). Hellerström was the first to recognize the relationship between erythema migrans and meningitis in a single case (3), whereas Garin and Bujadoux observed tick bite, erythema (not recognized as erythema migrans), and meningoradiculoneuritis in another case (4). In 1941 and 1944, Bannwarth published his possibly prospective observations on 15 patients of the early neurological manifestations of "chronic meningitis"; unfortunately, he missed the relationship to tick bite and erythema migrans in his otherwise very careful presentation (2).

Period two lasted from 1946 until 1975. When the antibiotics appeared on the market, European dermatologists soon realized that the three cutaneous disorders mentioned earlier showed rapid improvement or disappeared completely after antibiotic therapy. This induced a new wave of enthusiasm, led to a search for the causative organism, and stimulated clinical work anew (2). Spirochetes were discussed as a possible causative agent. Hauser wrote a lengthy Handbuch article linking the three cutaneous disorders together and emphasized the role of ticks (5). In 1966, Hopf presented a thorough description of the peripheral neuropathy associated with ACA (6). Hopf and Stroux thought to have found evidence for clustering of ACA in a certain region of Lower Frankonia, Germany (7). The relationship of tick bite, erythema migrans, and neurological manifestations had been acknowledged by 1975 (2). In 1974, one of the present authors (KW) emphasized in a case report that erythema migrans and the accompanying meningitis were due to one and the same bacterium, mentioned borreliae as a possible causative agent, and described the successful treatment of "erythema chronicum migrans meningitis" with high-dosed penicillin G (8). Before that report, the prevailing view among neurologists was that the neurological manifestations in association with the erythema migrans were due to a virus.

Joint and cardiac involvement were mentioned in several case reports but not to an extent as to acknowledge it as belonging to a disease. European authors had not given a name to this disease besides the attempt to put cutaneous and neurological manifestations together (8). An arthropathy associated with ACA had been described repeatedly, but Hauser doubted its significance as a special manifestation (5).

Period three started in 1976 when Allen C. Steere and coworkers began to describe, in a series of papers, observations that they designated Lyme disease. Many new aspects and thus the full extent of the disease became known due to this and other work in the United States and Europe in the following years. The etiologic agent remained elusive until 1981 when one of the present authors (WB) and coworkers showed it to be a spirochete associated with the tick vector *Ixodes dammini* from Shelter Island, New York (9), and *I. ricinus* in Europe (10). This discovery led to the fourth and still ongoing period that is characterized by worldwide research on clinical, epidemiological, bacteriological, and ecological aspects of Lyme disease and its causative agent, *Borrelia burgdorferi*.

Where now stood the cradle of Lyme borreliosis? Prior to 1976, the extent of the illness was known only to a few European specialists in the fields of dermatology and neurology. The full extent of the European work published by 1975, not seldom written in German, was and is not appropriately recognized in the present literature. The cradle of Lyme borreliosis stood in the former German town, Breslau, where Buchwald published a case report on ACA in 1883 (11). However, the Lyme story and the discovery of the causative agent were the beginning of new periods in the long history of the disease, and these periods yielded the decisive impetus.

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Bacteriophages and Ultrastructural Alterations of *Borrelia Burgdorferi* Induced by Ciprofloxacin

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In a former study, a lysogenic isolate of *Borrelia burgdorferi* harboring two different types of tailed A-1 and B-1 bacteriophages inducible by subinhibitory ciprofloxacin concentrations was described. In the present study, two further *Borrelia burgdorferi* isolates obtained by culture from a nymphal *Ixodes ricinus* tick and from human skin were exposed to increasing concentrations (0.125 to 8 μg/mL) of ciprofloxacin.
The *in vitro* minimal inhibitory concentration (MIC) was determined to be 1 μg/mL by a broth dilution method. In both isolates, belonging to the genospecies *Borrelia burgdorferi, sensu stricto* A-1 bacteriophages were discovered exclusively at subinhibitory concentrations of ciprofloxacin (0.125 to 0.5 μg/mL). After exposure of the isolates to ciprofloxacin concentrations coinciding with or exceeding the MIC, the following alterations of the borrelial ultrastructure became visible: (1) at a ciprofloxacin concentration of 1 μg/mL electron-lucent swollen areas within the protoplasmic cylinder complex of otherwise intact cells as well as very short borrelial cell fragments, (2) at a ciprofloxacin concentration of 2 μg/mL numerous small-membrane defects of the peptidoglycan layer, (3) at ciprofloxacin concentrations of 4 and 8 μg/mL disruption of the protoplasmic cylinder complex into many small particles. These ultrastructural alterations caused by high ciprofloxacin concentrations proved to be clearly different from the features of phage-induced cell lysis found at subinhibitory ciprofloxacin concentrations.

Key words: Borrelia burgdorferi, Ciprofloxacin, Ultrastructure, Bacteriophages, Electronmicroscopy

INTRODUCTION

Recently, we reported on the discovery of two different types of bacteriophages induced by subinhibitory concentrations of ciprofloxacin in a *Borrelia burgdorferi* skin isolate and described the typical phage-induced alterations of the borrelial morphology (1).

Ciprofloxacin is a fluorinated, piperazin-substituted quinolone related to nalidixic acid. By inhibiting the bacterial DNA-gyrase, this drug has a high *in vitro* activity against many gram-positive and gram-negative bacteria (2). In several reports, the ultrastructural alterations of ciprofloxacin treated gram-negative and gram-positive bacteria were described comprehensively (2–4). To our knowledge, there are only two studies dealing with the *in vitro* susceptibility of *Borrelia burgdorferi* to ciprofloxacin. Preac-Mursic et al. reported in 1987 that ciprofloxacin showed only low activity against *Borrelia burgdorferi* (5). Similar results were reported by Levin et al. (6) in 1993. So far, we are not aware of reports concerning ultrastructural alterations of spirochetes caused by ciprofloxacin.

In the present study, we determined the *in vitro* minimum inhibitory concentration (MIC) of ciprofloxacin for two *Borrelia burgdorferi* isolates and examined the morphological alterations of the borrelial cells after treatment with ciprofloxacin concentrations ranging from the MIC of 1 to 8 μg/mL.

Moreover, we examined borrelial cells exposed to subinhibitory concentrations of ciprofloxacin in order to look for the presence of further lysogenic isolates.

MATERIALS AND METHODS

Borrelia burgdorferi isolates

The *Borrelia burgdorferi* skin isolate was obtained by biopsy from an erythema migrans lesion located at the left mamma of a 63-year-old woman. The tick isolate was cultivated from a nymphal tick removed from a patient visiting our out-patient clinic.

Isolation and subcultivation of the borreliae were accomplished in BSK II-medium (7) modified by adding 0.15% agarose (Serva, Fine Biochemicals Inc., Paramus, New Jersey, No. 11397) (8). The two isolates were classified by nondenaturing polyacrylamide gel electrophoresis of RNA complementary to amplified *Borrelia burgdorferi*-specific gene segments (9, 10). Both isolates were found to belong to the genospecies *Borrelia burgdorferi* sensu stricto, according to the *Borrelia burgdorferi* subspecies classification delineated by Baranton et al. (11).

Evaluation of MICs of ciprofloxacin

In vitro susceptibility to ciprofloxacin (Bayer, Leverkusen, No. 521532) was determined via the broth dilution method (5). Here, 100 μL of an actively growing culture (log-phase) containing 10^7 cells/mL were added to tubes with 9.9 mL BSK II-medium, resulting in a final concentration of 10^5 cells/mL. Ciprofloxacin concentrations ranged from 0.125 to 8 μg/mL. Control tubes without antibiotics were inoculated with 100 μL of the log-phase culture. Each concentration was prepared in triplicate. Cultures were examined for the presence of spirochetes by dark-field microscopy after 5 days of incubation at 33°C. The MIC was defined as the lowest concentration of ciprofloxacin completely inhibiting growth, i.e., at which the spirochete count was 10^5 cells/mL or less.

The number of spirochetes was determined by using a Petroff Hauser counting chamber.

Preparation for electron microscopy

Each tube was centrifuged at 4000 × *g* for 20 minutes at 33°C. The resulting pellets were suspended in SMC [0.03% sucrose in redestilled water with 0.01 M $CaCl_2$ and 0.01 M $MgCl_2$ added (12)]. Two drops of each suspension were

*Corresponding author.

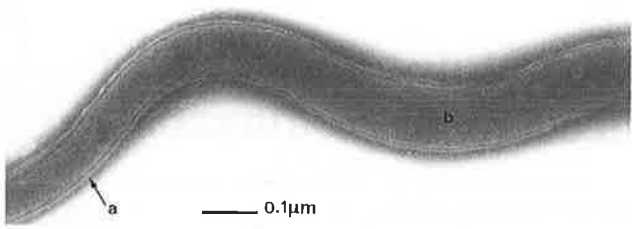

FIG. 1. Untreated Borrelia burgdorferi *with a (a) smooth-structured outer cell membrane without blebs and (b) unchanged peptidoglycan layer. Phosphotungstate stain, × 68,000, fixed. Bar = 0.1 µm.*

placed on grids for electron microscopy. In some experiments, the samples were negatively stained with 2% phosphotungstic acid for 30 seconds. In other experiments, the samples were first fixed with 1.5% glutaraldehyde (pH 7.2, in 0.1 M PO_4-buffer) and then negatively stained with 1% phosphotungstic acid for 30 seconds.

We decided to examine fixed and unfixed borrelial cells of each ciprofloxacin concentration, as the specific ciprofloxacin-induced cell alterations were better visible in the fixed samples; the bacteriophages, however, were better visible in the unfixed samples.

RESULTS

Ciprofloxacin susceptibility

The mean MIC of both isolates was 1 µmL.

Ultrastructure of untreated Borrelia burgdorferi cells

The untreated spirochete in Fig. 1 confirms the often-described structural characteristics of borrelial cells (12–15).

No phages were observed in borreliae grown in the untreated control cultures.

A-1 bacteriophages induced by subinhibitory ciprofloxacin concentrations

While the majority of the cells presented a regular shape, approximately 20% of the borrelial cells of both isolates showed severe abnormalities of ultrastructure when exposed to subinhibitory ciprofloxacin concentrations, ranging from 0.125 to 0.5 µg/mL. In the phage-carrying and morphological-altered borreliae, the outer envelope appeared to be undamaged, while the protoplasmic cylinder showed at least three different stages of destruction (1):

(1) numerous irregular constrictions of the normally smooth-structured peptidoglycan layer,
(2) disruption of the protoplasmic cylinder into several segments within a largely intact outer envelope, and
(3) small plasmolyzed protoplasma cylinder debris particles within an enlarged and irregularly shaped outer envelope.

In both isolates, plasmolyzed cells were filled with clusters of numerous unassembled heads and tails of bacteriophages (Fig. 2) showing an A-1 morphology (1, 16–18). According to the classification of Ackermann (17), this type consists of an isometric head (30 nm), a thin collar, and a long contractile tail (length 50 to 64 nm, width 13 to 19 nm) with a baseplate. In contrast to our former study (1), only unassembled heads and tails of A-1 bacteriophages could

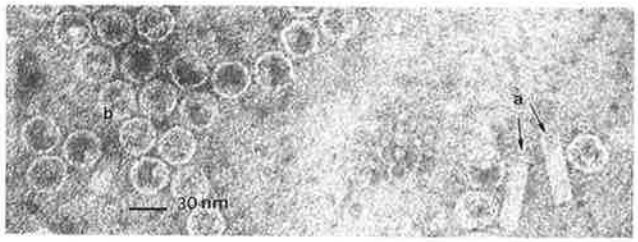

FIG. 2. (a) Unassembled tails and (b) heads of A-1 bacteriophages inside of a Borrelia burgdorferi *cell. Phosphotungstate stain, × 330,000, unfixed. Bar = 30 nm.*

be observed within the borrelial cells. We detected no phages in borrelial cells exposed to ciprofloxacin concentrations equal to or higher than the MIC or in the untreated controls.

ULTRASTRUCTURE OF BORRELIA BURGDORFERI EXPOSED TO CIPROFLOXACIN CONCENTRATIONS ≥ 1 µg/mL

As previously described, nearly 20% of the cells showed severe *phage-induced* morphological alterations at subinhibitory ciprofloxacin concentrations. In the remaining borreliae, which presumably were not infected by temperate phages, no ultrastructural changes were seen when exposed to ciprofloxacin concentrations from 0.125 to 0.5 µg/mL.

The majority of borrelial cells exposed to a ciprofloxacin concentration of 1 µg/mL showed irregular constrictions of the peptidoglycan layer, which were located near the end of the cell (Fig. 3a). Obviously, as a result of these irregularly located constrictions, abnormally short distinct fragments of borrelial cells (Fig. 3b) became visible. The size of these fragments ranged from 0.6 to 0.8 µm. Moreover, the protoplasmic cylinder complex of *Borrelia burgdorferi* cells exposed to 1.0 µg/mL of ciprofloxacin showed electron-lucent swellings (Fig. 4a).

At a ciprofloxacin concentration of 2 µg/mL, approximately 75% of the treated cells revealed numerous defects of the peptidoglycan layer (Fig. 5a). The diameters of the protoplasmic cylinder complex varied from 0.09 to 0.20 µm (Fig. 5 arrows). Finally, at 4 and 8 µg/mL (Fig. 6a), in almost all cells the protoplasmic cylinder complex and the outer envelope were disrupted into many small plasmolyzed particles.

At ciprofloxacin concentrations from 0.125 to 2 µg/mL, spherical structures were seen (Fig. 7). Coiled up spirochetes were lying within these spheres.

No bacteriophages became visible in borrelial cells treated

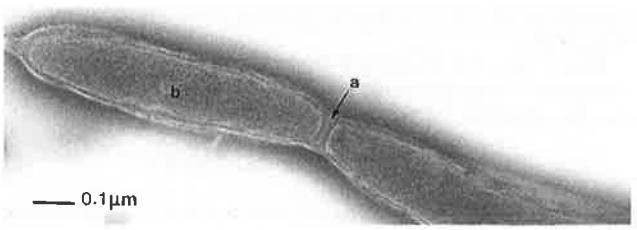

FIG. 3. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 1 µg/mL. (a) Irregular constriction of the peptidoglycan layer at the cell periphery. (b) Abnormal short fragments of borrelial cells showing a length of 0.8 µm. Phosphotungstate stain, × 97.000, fixed. Bar = 0.1 µm.*

FIG. 4. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 1 µg/mL. (a) Electron-lucent swelling of the protoplasmic cylinder complex. Phosphotungstate stain, × 97,000, fixed. Bar = 0.1 µm.*

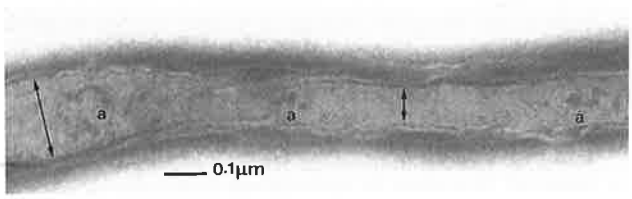

FIG. 5. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 2 µg/mL. (a) Numerous defects of the peptidoglycan layer. Note the different diameters of the protoplasmic cylinder complex (arrows). Phosphotungstate stain, × 97,000, fixed. Bar = 0.1 µm.*

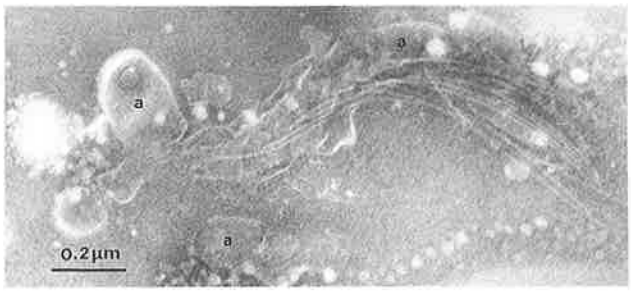

FIG. 6. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 8 µg/mL. (a) Debris of the disrupted protoplasmic cylinder complex partially enclosed by fragments of the outer envelope. Phosphotungstate stain, ×90,000, unfixed. Bar = 0.2 µm.*

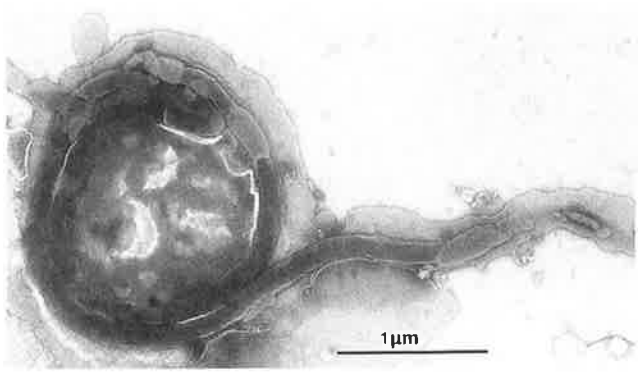

FIG. 7. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 0.125 µg/mL. Coiled-up spirochete forming a spherical structure (spheroplast). Phosphotungstate stain, × 37,000, unfixed. Bar = 1 µm.*

with ciprofloxacin concentrations of 1 µg/mL (MIC) and more.

DISCUSSION

The MIC of 1 µg/mL for our *Borrelia burgdorferi* strains was comparable to that found by investigators of other studies (5, 6). Our data confirm that *Borrelia burgdorferi* shows only moderate susceptibility to ciprofloxacin.

The ultrastructural morphology of our untreated *Borrelia burgdorferi* isolates (Fig. 1) corresponded with former morphological descriptions by Barbour and Hayes (13), Hovind-Hougen and coworkers (12, 14), and Hayes and Burgdorfer (15). Also, the measurements for length and diameter as well as the numbers of flagella were characteristic for Borrelia species (12–15).

Both *Borrelia burgdorferi* isolates examined in this study contained temperate bacteriophages showing an A-1 morphology that were inducible exclusively by subinhibitory ciprofloxacin concentrations (Fig. 2). These phage-carrying *Borrelia burgdorferi* cells showed severe ultrastructural alterations of their morphology (1), which completely differed from the ciprofloxacin effects on borreliae observed at concentrations of 1 to 8 µg/mL. Induction of prophages occurred only at subinhibitory ciprofloxacin concentrations, presumably as production and release of bacteriophages depend on an undisturbed metabolism of the host organism. Including our former study (1), we examined two erythema migrans isolates and one tick isolate for the presence of bacteriophages. All lysogenic borreliae contained A-1 bacteriophages, the first skin isolate in addition a B-1 bacteriophage (1).

Besides these phage-induced morphological alterations of borrelial cells, other severe ciprofloxacin-induced ultrastructural changes could be observed at concentrations of 1 µg/mL (MIC) and more. The normal cell division was considerably disturbed at a ciprofloxacin concentration of 1 µg/mL (MIC). Multiplication of *Borrelia burgdorferi* occurs by binary transverse fission (13). Usually, cell division is started by constriction of the peptidoglycan layer in the middle of a long cell (13). Obviously, as a result of the irregular constriction of the peptidoglycan layer in the periphery of abnormal elongated borrelial cells, very short cell fragments became visible (Fig. 3). The damaging effect of ciprofloxacin first led to swellings (Fig. 4a), after that to membrane defects (Fig. 5a), and finally to the disruption of the protoplasmic cylinder complex (Fig. 6).

At ciprofloxacin concentrations ranging from 0.125 to 2 µg/mL, large spherical forms filled with remnants of the protoplasmic cylinder complex, as described before (Fig. 7), were observed (13, 15), but the significance and function of such structures are still unknown. In comparison with the results of Voigt and Zeiler (2), Elliott et al. (3), and Rodgers et al. (4), who demonstrated that ciprofloxacin primarily affected areas located in the cell wall of gram-negative and gram-positive bacteria, we found severe morphological alterations concerning mainly the protoplasmic cylinder complex of *Borrelia burgdorferi*.

In contrast to penicillin-treated borreliae, which showed morphological alterations even at subinhibitory concentrations (Schaller M, Neubert U. Morphology of *Borrelia burgdorferi* exposed to benzylpenicillin. Infection, in press), in nonlysogenic borreliae, no ciprofloxacin-induced changes were visible at concentrations below the MIC.

This may be a further explanation why ciprofloxacin does not show the same *in vivo* efficacy (Meisel C. personal

communication) in comparison to the β-lactam antibiotics preferentially used in treatment of early Lyme-borreliosis (5, 6).

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Prevalence of *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* in Southern Germany

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In the years 1985 and 1986, we investigated 2802 *Ixodes (I.) ricinus*—1212 adults, 1157 nymphs, and 433 larvae—collected in eight different regions in the southern part of Germany for the presence of *Borrelia burgdorferi*. We determined stage-, season- and region-dependent differences of the infection rates: 352 (12.6%) of all ticks [239 adults (19.7%), 108 nymphs (9.3%), and 5 larvae (1.2%)] were found to be positive by direct immunofluorescence. In all locations examined, *I. ricinus* was infected with *Borrelia burgdorferi* with always the highest infection rate in adult ticks. The percentage of infected ticks (calculated without larvae) varied between 6.9 and 30.5%. Comparison of geoecological data, vegetation, and tick density with the infection rates of ticks revealed no clear correlation. Remarkably, infection rates were particularly high in the region with the highest year-isotherme.
In four regions, ticks were collected in the spring (April/May) and late summer (August/September) of 1985. In two regions, infection rates increased significantly from spring to late summer, whereas in the other two regions, no difference was observed. Overall, we found a seasonal increase from 14.3 to 20.1%, which was especially pronounced in nymphs (from 9.3 to 16.1%).

Key words: Borrelia burgdorferi, Ixodes ricinus, Tick infection rate, Lyme borreliosis

INTRODUCTION

The multisystem disorder Lyme borreliosis is the most frequent arthropod-borne disease in Europe. The hard tick *Ixodes (I.) ricinus* is the primary vector of the Lyme disease agent, *Borrelia (B.) burgdorferi* sensu lato (in this paper, the term *B. burgdorferi* for the three genospecies of *B. burgdorferi* sensu lato present in Europe will be used). This tick species is widely distributed in central Europe, but only few data from Germany are available about infection rates of *I. ricinus* with this spirochete. Little is known about the factors influencing infection rates or about the risk of infection for humans (1–8).

The present study was conducted during 1985 and 1986 in the southern part of Germany. Preliminary results of this work have been reported in 1987 (4). The purpose of this study was to determine the prevalence of *B. burgdorferi* in immature and adult populations of *I. ricinus* in different regions and seasons and to evaluate the influence of geoecological conditions on infection rates.

MATERIALS AND METHODS

Study areas and tick collection. An amount of 2802 *I. ricinus* (1212 adults, 1157 nymphs, and 433 larvae) were collected in eight different regions of the southern part of Germany in the spring (April/May) and late summer (August/September) of 1985 and in the spring (April/May) of 1986 by flagging the low vegetation. Seven areas are located within a 60-km range around Ulm, and one area is situated in the north of München (Fig. 1). Collecting seasons for the different regions were: spring 1985 for Illertissen, Stoffenried, and Streitheim; spring and late summer 1985 for München, Stafflangen, and Bernstadt; spring 1986

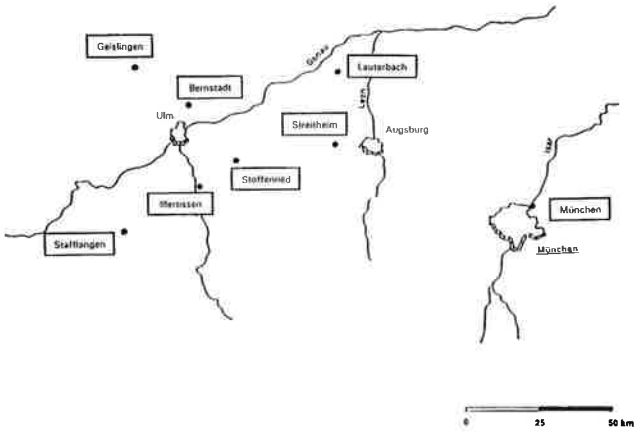

FIG. 1. Collecting regions in southern Germany.

for Geislingen; and all three seasons for Lauterbach. The size of the regions varied between 1.5 and 2 km^2. Six of the eight areas—Stafflangen, Stoffenried, Streitheim, Geislingen, Illertissen, and Bernstadt—were further subdivided in either three or four sectors, each about 0.5 km^2. To obtain comparable results, we collected between 126 and 223 nymphs and adults (average: 182) per region and season. The mean tick density of each region was estimated from the time necessary to collect an appropriate number of specimen. A collecting time of more than 2 days was considered as low, of about 2 days as medium, and of 1 day or less as high tick density. Additional geoecological data for the different regions are listed in Table 1.

Detection of borreliae in ticks. After washing the ticks for 2 to 3 minutes in distilled water, 96% ethanol, and phosphate-buffered saline (PBS) pH 7.4, smears of dissected midgut (adults) or whole ticks (immatures) were prepared on slides in a drop of PBS. After air-drying, the slides were fixed with methanol for 15 minutes and then incu-

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TABLE 1
***Prevalence of* B. burgdorferi-*Infected* Ixodes ricinus**

	Number	Positive by IFA	
		Number	(%)
Adults	1212	239	19.7
Females	616	117	19.0
Males	596	122	20.5
Nymphs	1157	108	9.3
Larvae	433	5	1.2
Total	2802	352	12.6

Ticks were collected in spring 1985, late summer 1985, and spring 1986 at München, Illertissen, Stafflangen, Lauterbach, Streitheim, Stoffenried, and Geislingen in the southern part of Germany.

bated for 30 minutes with fluorescein isothiocyanate-conjugated rabbit immune serum against *B. burgdorferi* (rabbits were immunized nine times in 4 months by injection of whole cells of *B. burgdorferi* strain B31, kindly provided by Willy Burgdorfer, Hamilton, MT). For detection of borreliae, we used an immunofluorescence microscope (Leitz Laborlux 12) and evaluated 40 visual fields at a magnification of 400 times. A smear was considered positive if at least three borreliae could be identified. In addition, we estimated the number of borreliae in each smear: <2 borreliae per visual field was regarded as low, 2 to 4 as medium, and >4 borreliae as high positive.

RESULTS

During 1985 and 1986, a total of 2802 *I. ricinus* (1212 adults, 1157 nymphs, and 433 larvae) were collected by flagging in eight different regions of southern Germany.

A total of 352 (12.6%) ticks were positive by direct immunofluorescence assay (IFA) (Table 1). Infection rate was 1.2% for larvae, 9.3% for nymphs, and 19.7% for adults (males 20.5%, females 19%) (Table 1). Only nymphs and adults were included in the geographic and seasonal analyses (Tables 2 through 5) because larvae showed only sporadic occurence and low infection rates.

To ensure a correct comparison of infection rates between the different regions, we evaluate only results obtained from ticks collected within the same season. In spring 1985, seven regions were investigated: infection rates varied between 3.6% in Bernstadt and 30.3% in München (6% versus 34.9% for adults and 0.9% versus 25% for nymphs, respectively) (Tables 2 and 3). In late summer 1985, infection rates varied between 14.1% in Stafflangen and 30.7% in München (15.9% versus 32.7% for adults and 11.7% versus 26.8% for nymphs, respectively) (Table 3). In each region, the infection rate of adults was higher than that of nymphs.

To evaluate differences among infection rates between closely located areas, we split six regions into three or four sectors of about 0.5 km^2 (Table 4). In spring 1985, no obvious differences could be found among different sectors in Illertissen, Stafflangen, or Bernstadt, respectively. In Stoffenried, positivity rates ranged from 4.2 to 20.7%; in Streitheim, from 7.3 to 19.2%; and in Geislingen (spring 1986), from 3.6 to 13%. It is remarkable that infected ticks were detected in all sectors examined.

To evaluate seasonal differences, ticks were collected in four regions (München, Stafflangen, Lauterbach, and Bernstadt) in spring as well as in late summer of the year 1985 and, in addition, in Lauterbach again in spring 1986

TABLE 2
***Infection Rates of* I. ricinus B. burgdorferi**

			Positive	
		Number	Number	(%)
München**	Adults	216	73	33.8
	Nymphs	148	38	25.7
	Total	364	111	30.5
Stafflangen**	Adults	145	28	19.3
	Nymphs	228	22	9.6
	Total	373	50	13.4
Bernstadt**	Adults	228	26	11.4
	Nymphs	218	14	6.4
	Total	446	40	9.0
Lauterbach***	Adults	217	39	18.0
	Nymphs	193	17	8.8
	Total	410	56	13.7
Illertissen*	Adults	95	23	24.2
	Nymphs	96	5	5.2
	Total	191	28	14.7
Stoffenried*	Adults	118	18	15.3
	Nymphs	64	2	3.1
	Total	182	20	11.0
Streitheim*	Adults	108	21	19.4
	Nymphs	106	8	7.5
	Total	214	29	13.6
Geislingen****	Adults	85	11	12.9
	Nymphs	104	2	1.9
	Total	189	13	6.9

*Collecting time was spring 1985.
**Collecting time was spring and late summer 1985.
***Collecting time was spring and late summer 1985 and spring 1986.
****Collecting time was spring 1986.

(Table 3). We found a rise of the infection rates from 14.3% in spring to 20.1% in late summer. With respect to individual regions, a considerable increase was observed in Bernstadt from 3.6 to 14.3% and in Lauterbach from 10.7 to 24.6%, whereas in the other two areas, there was no obvious difference between spring and late summer. In Lauterbach, where we conducted an additional survey in spring 1986, we observed a renewed decrease in infection rates (from 24.6 to 6.9%). With all areas together, about the same prevalence could be found for adults in spring (19.5%) and late summer (23.8%) but, in contrast, an increase for nymphs from spring (9.3%) to late summer (16.1%) (Table 4).

In addition, we estimated the quantity of borreliae in each individual tick (Table 6). In all regions (with the exception of Lauterbach), the majority of the infected ticks carried only small numbers of borreliae (<2 per visual field), whereas in Lauterbach, the majority of the positive *I. ricinus* were classified as high positive (>4 per visual field).

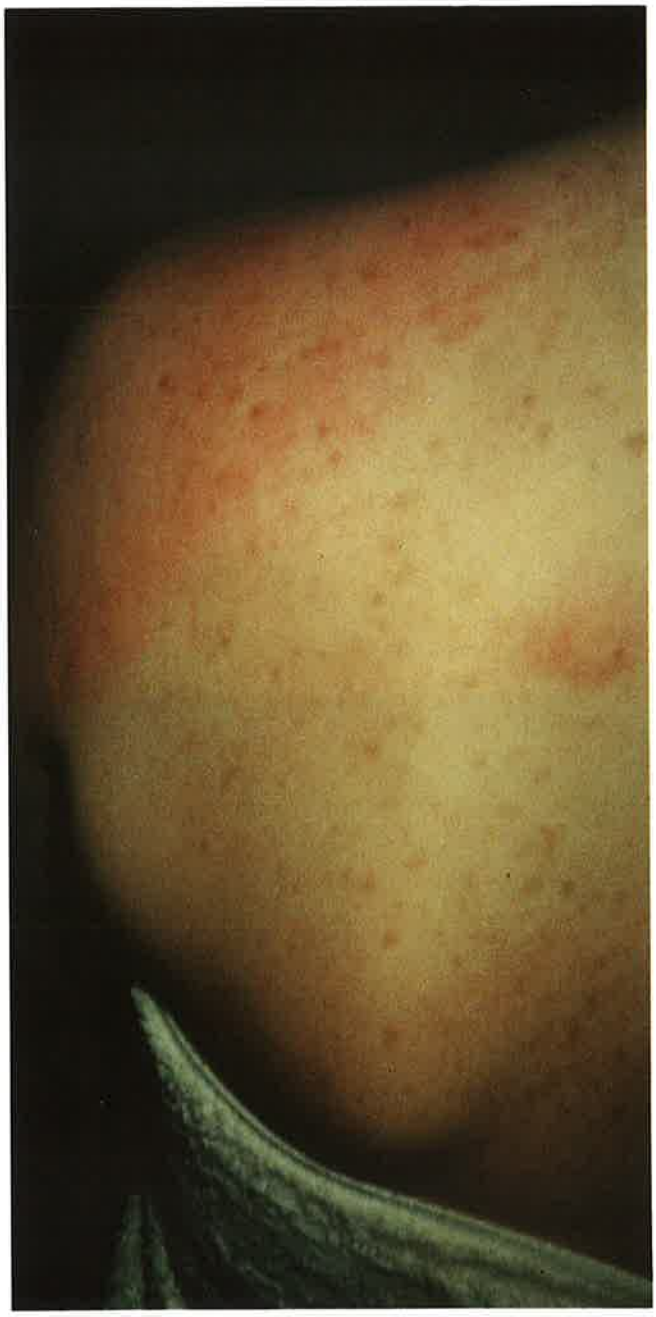
DISCUSSION

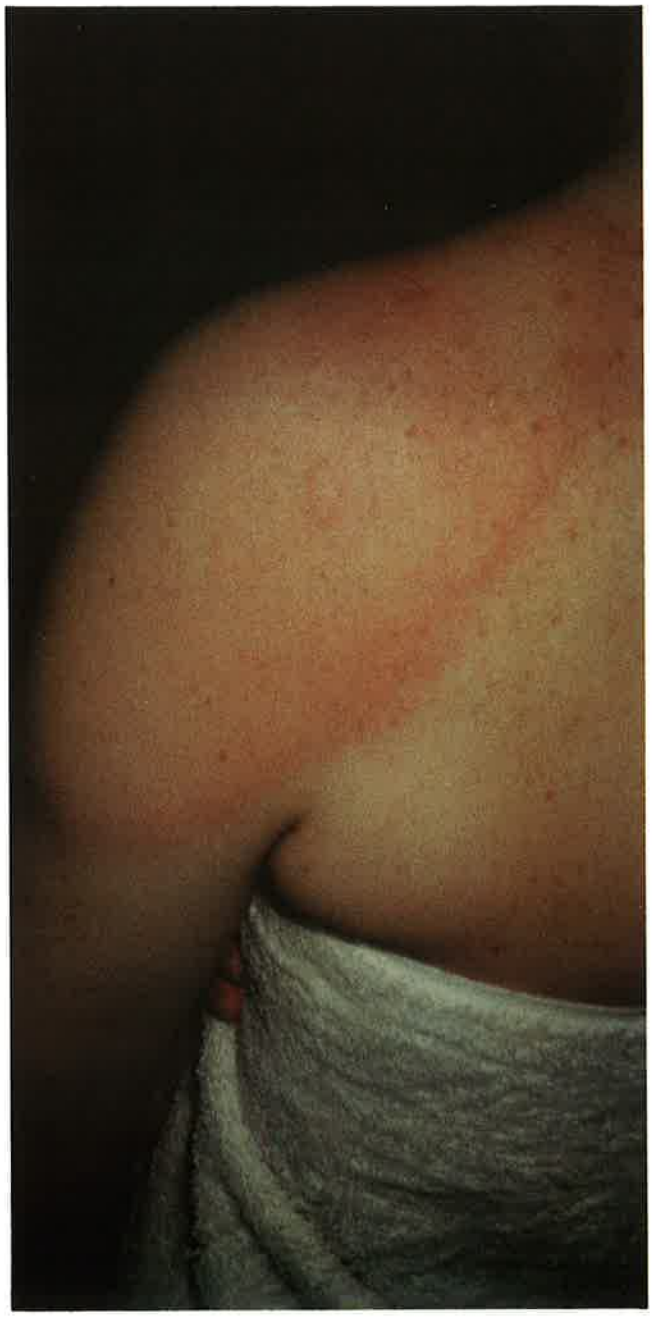
Our results suggest that in southern Germany, infection of *I. ricinus* with *B. burgdorferi* is frequent and widespread.

In this study, 352 (12.6%) of 2802 *I. ricinus* were found to be infected with *B. burgdorferi*. According to the literature, infection rates of *I. ricinus* (mainly reported for adults and nymphs) varied between 0 and 30% (1–9).

Regarding the different stages, we found an obvious increase in infection rates from larvae to adults. The low in-

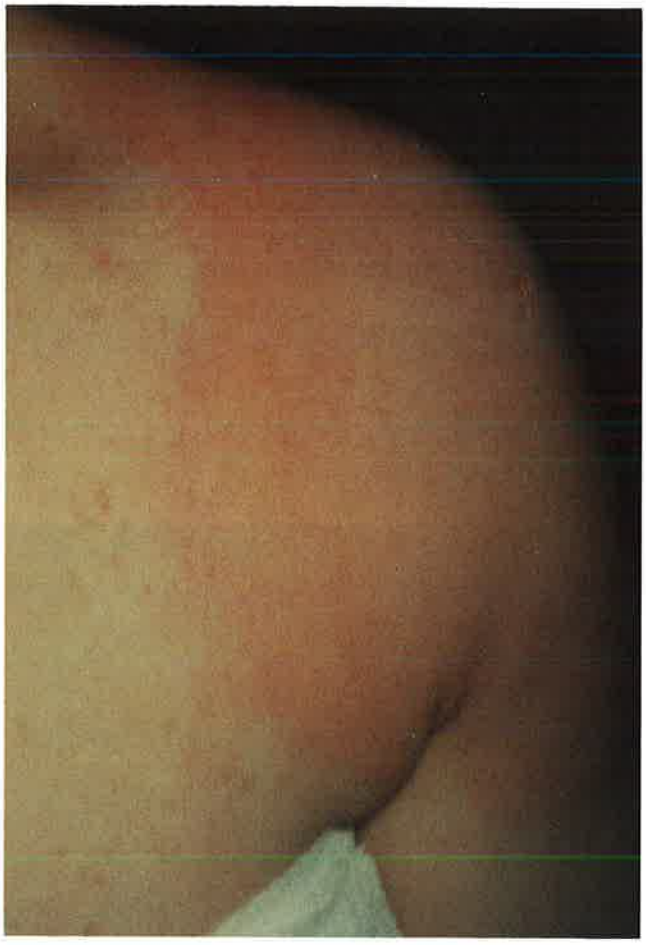
DOES LYME DISEASE EXIST IN AUSTRALIA?





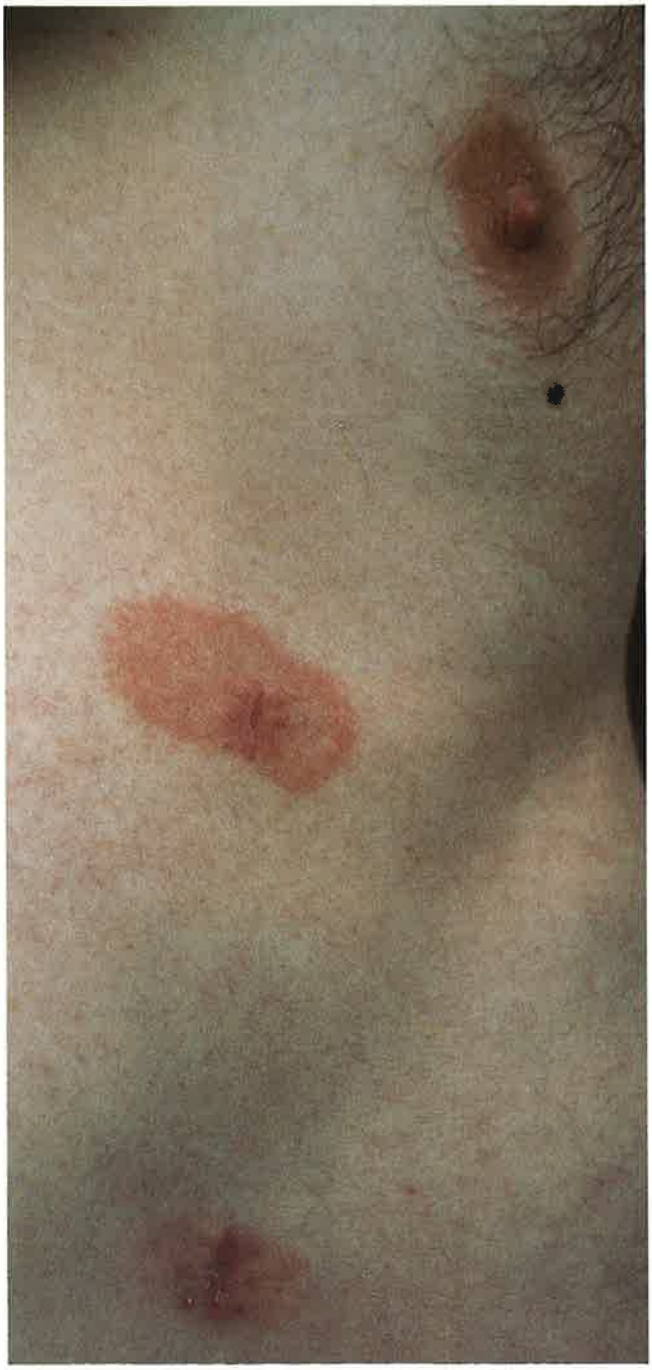
Case 6

Developing Erythema Migrans lesion in left shoulder region (posterior view)



Case 6

Developing Erythema Migrans lesion in left shoulder region (anterior view)



Skin lesions on chest wall, case 2 (see text). Lesions continued to enlarge after skin biopsies were taken.

Fully engorged adult female *I. holocyclus*, ventral surface

Partially engorged adult female *I. holocyclus,* dorsal surface

Haemaphysalis species, dorsal surface

TABLE 3
Regional Prevalence of B. burgdorferi *in* I. ricinus*—Seasonal Comparison*

	Spring 1985			Late Summer 1985		
		Positive			Positive	
	Number	Number	(%)	Number	Number	(%)
München						
Adults	106	37	34.9	110	36	32.7
Nymphs	92	23	25.0	56	15	26.8
Total	198	60	30.3	166	51	30.7
Stafflangen						
Adults	76	17	22.4	69	11	15.9
Nymphs	120	8	6.7	108	14	13.0
Total	196	25	12.8	177	25	14.1
Bernstadt						
Adults	116	7	6.0	112	19	17.0
Nymphs	107	1	0.9	111	13	11.7
Total	223	8	3.6	223	32	14.3
Lauterbach						
Adults	71	11	15.5	71	20	28.2
Nymphs	69	4	5.8	55	11	20.0
Total	140	15	10.7	126	31	24.6
All regions						
Adults	369	72	19.5	362	86	23.8
Nymphs	388	36	9.3	330	53	16.1
Total	757	108	14.3	692	139	20.1
	Spring 1986					
Lauterbach						
Adults	75	8	10.7			
Nymphs	69	2	2.9			
Total	144	10	6.9			

Ticks were collected in München, Stafflangen, Lauterbach, and Bernstadt.

Collecting time was spring and late summer 1985 (all regions); Lauterbach, additional collection in spring 1986.

TABLE 4
Infection Rates of Adults and Nymphs in Closely Located Sectors

Region	Sector Number	Infection Rate (%)
Illertissen	I	15.5
	II	10.2
	III	17.9
Stafflangen	I	13.7
	II	10.3
	III	13.3
Bernstadt	I	3.6
	II	4.0
	III	2.6
Stoffenried	I	9.4
	II	20.7
	III	4.2
Streitheim	I	7.3
	II	12.5
	III	19.2
	IV	15.7
Geislingen	I	3.6
	II	13.0
	III	5.0

Collecting time was spring 1986 in Geislingen and spring 1985 for the other regions.

fection rate of larvae (1.2%) indicates that transovarial transmission may occur but appears to be a rare event that plays only a minor role for infection of *I. ricinus*. Nymphs and adults, therefore, attain their infection as larvae or nymph while feeding on competent reservoir animals.

In Germany, higher infection rates in adults compared to nymphs were also reported from Pelz et al. (6) and Kahl et al. (5), whereas Matuschka et al. (9) described that infection rates in nymphs ". . . may even marginally exceed those for adults!". In our study, the percentage of borrelia-positive adults was in all regions and all seasons higher than that of nymphs. Little is known about the factors influencing the stage-dependent infection rates. Probably, the competence of hosts to carry and transmit borreliae to the vector plays a major role. Matuschka et al. (8) have shown that different kinds of deer in Germany—highly frequented hosts for all development stages of *I. ricinus*—are incapable to infect ticks and even eliminate pre-existing spirochetal infection in attached *I. ricinus*. Assuming that transstadial transmission is an effective mechanism for the spread of *B. burgdorferi* in *I. ricinus* (10), infection-suppressing host factors could explain the lower infection rates in adults compared to nymphs.

To elucidate decisive factors influencing the infection rate of *I. ricinus* with *B. burgdorferi*, we compared geoecological data, structure of vegetation, waters, and tick density with infection rates in each region. Kurtenbach et al. (7) found that increasing tick density leads to an overproportional rise in infection rate. Aeschlimann et al. (11) showed the influence of altitude on tick populations: Up to 1000 m above sea level, *I. ricinus* was abundant, between 1000 and 1500 m it was rare, and over 1500 m it was missing. In addition, they could demonstrate that infection rates decrease as altitude increases (study areas between 400 and 700 m). In our study, these findings could not be confirmed. Neither tick density nor altitude had a clear influence on the infection rates. Five of the eight regions are located at about the same altitude (510 to 540 m); however, the infection rates in these areas varied between 3.6 and 30.3%. In addition, there was no difference in infection rates between Lauterbach (410 m) and Stafflangen (600 m). In Geislingen, the altitude varied between 420 m (sector 1) and 690 m (sectors 2 and 3). In this area, lowest infection rate correlates with lowest altitude. We therefore conclude that in our study region, altitude seems to have no crucial influence on the infection rate of *I. ricinus* with *B. burgdorferi*; however, this might be the case in other locations.

An interesting finding of the present study was that the highest infection rate was observed in the region with the highest year-isotherme (München).

Seasonal comparison revealed a tendentious increase in total infection rate toward late summer. Especially in nymphs, we found a high increase (between about 2-fold and 10-fold) from spring to late summer in three of four regions. The study in Lauterbach, which showed again a decrease in infection from late summer 1985 to spring 1986, suggests that we have to expect a higher incidence of infected ticks in late summer. To our knowledge, only one study investigated seasonal influences on the dynamics of spirochetal infection rates of *I. ricinus*: Mejlon and Thomas (12) did not find a distinct seasonal pattern with respect to spirochetal infection prevalence in *I. ricinus* nymphs.

In our study, most of the infected *I. ricinus* contained only a small number of borreliae. However, a notable exception was observed in Lauterbach, where about 40% of the infected ticks carried high numbers of borreliae: in 1984, two persons acquired a tick bite in this region (forest in the

TABLE 5
Geoecological Data

	Altitude (Meters above Sea Level)	Mean Rainfall (mm)*	Year-Isotherm (°C)*	Short Description of the Study Regions (Main Vegetation, Waters)	Tick Density	Infection Rate** (%)
München	530	925	8.0	Leafy forest, a lot of underbrush, brooks, around a river	High	30.3
Illertissen	510	800	7.5	Leafy and marshy birch wood, a lot of underbrush, around a river	Low	14.7
Stafflangen	600	850	7.5	Old conifer wood, plantations, few underbrush, no waters	Medium	12.8
Lauterbach	410	700	7.5	Conifer wood, a lot of underbrush, two brooks, marshy sectors	High	10.7
Bernstadt	540	700	7.5	Mixed, leafy, and conifer wood, underbrush, one small river	Low	3.6
Stoffenried	510	750	7.5	Conifer and mixed forest, few underbrush, no waters	Medium	11.0
Streitheim	520	800	7.5	Conifer forest, few underbrush, no waters	Medium	13.6
Geislingen	420 to 690	900	7.0	Mixed, leafy, and conifer wood, sloap, few underbrush, no waters	Medium	6.9

*Long-term follow-up.
**Collecting time was spring 1986 in Geislingen and spring 1985 for the other regions.

TABLE 6
Quantity of Borreliae in the Infected Ticks*

	Number of Positive Ticks	Degree of positivity					
		Low		Medium		High	
		Number	(%)	Number	(%)	Number	(%)
München	111	56	50.5	36	32.4	19	17.1
Stafflangen	53	39	73.6	12	22.6	2	3.8
Lauferbach	56	20	35.7	14	25.0	22	39.3
Bernstadt	41	27	65.9	9	22.0	5	12.2
Illertissen	28	18	64.3	8	28.6	2	7.1
Stoffenried	20	17	85.0	3	15.0	0	0.0
Streitheim	29	16	55.2	6	20.7	7	24.1
Geislingen	14	9	64.3	3	21.4	2	14.3
Total	352	202	57.4	91	25.9	59	16.8

*All regions, seasons, and stages.
Degree of positivity: low: <2 borreliae per visual field; medium: 2 to 4 borreliae per visual field; and high: >4 borreliae per visual field.

neighborhood of a sports ground) followed by serologically and clinically proven Lyme borreliosis (one case of Lyme arthritis and one case of neuroborreliosis). It has been postulated that *B. burgdorferi* may be transmitted by either regurgitation of gut contents or via salivary glands (13, 14). In both instances, a high number of borreliae in infected ticks may result in a more rapid transmission after tick attachment. There is no doubt that the risk of transmission is higher from heavily infected ticks.

The degree of infection in *I. ricinus* seems to be variable: similar findings were reported by Burgdorfer et al. (15) who found that in *I. dammini*, "some ticks contained only a few spirochetes, others contained large numbers." Pelz et al. (6) reported between 5000 and 485,000 borreliae per tick.

Although we report a high prevalence of borrelia-infected *I. ricinus* in the investigated regions, it is not possible to give a clear statement about the risk of infection for humans. Too little is known about the conditions for successful transmission from vectors to humans and, in addition, about differences in pathogenicity of borreliae detected by IFA in ticks. Further investigations are necessary to clarify the role and interaction of the numerous influencing factors.

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Does Lyme Borreliosis Exist in Australia?

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The existence of an indigenous form of Lyme borreliosis (LB) in Australia has not yet been confirmed as isolation of the causative organism from clinical specimens collected from candidate patients has not yet been achieved. Candidate spirochetes, resembling *Borrelia burgdorferi* have been isolated from *Ixodes holocyclus* ticks but growth cannot be sustained in standard media. Erythema migrans, arthritis and radiculopathy have been described in candidate LB cases in Australia. Immunoblotting of sera from such cases indicates that antibodies to flagellin and OspA antigens of European isolates, *Borrelia garinii* and *Borrelia afzelii*, are commonly detected, while such seroreactivity to *B. burgdorferi* sensu stricto is uncommon. Based on clinical and immunoblot data on candidate Australian LB cases, we postulate that an indigenous form of LB occurs in Australia, possibly caused by spirochetes more closely related to *B. garinii* and *B. afzelii* than *B. burgdorferi sensu stricto*.

Key words: Lyme, Indigenous, *Ixodes holocyclus*, Immunoblotting

LYME BORRELIOSIS IN AUSTRALIA

With few published clinical case reports of possible Lyme borreliosis acquired in Australia (1–3), the existence of a genuine, indigenous form of Lyme borreliosis (LB) in Australia has not yet been confirmed. Efforts to isolate *Borrelia (B.) burgdorferi* from candidate tick vectors have so far proved inconclusive (6). Despite this, we believe that there is an indigenous form of LB based on clinical and immunoblot data that we have collected since 1991. From the northern hemisphere experience, there is characteristically a delay between recognition of indigenous LB cases and isolation and identification of causative spirochetes. The Australian experience is likely to be similar. Furthermore, on an island continent with much unique native fauna, it would not be unexpected to find significant differences in LB epidemiology, clinical manifestations, and even etiological spirochetes when Australian and northern hemisphere LB are compared. Review articles always reference the first human case of indigenously acquired LB in Australia as a male who developed rash and subsequent arthritis following a bite from an "unidentified insect" in Hunter Valley, New South Wales (NSW) (1). While he appeared to have erythema migrans (EM), it is doubtful whether LB was the cause of arthritis. The next clinical case was reported from the NSW Central Coast, just north of Sydney in 1986 (2). The only other indigenous cases reported in refereed journals, both in the same letter, were from a coastal area south of Sydney in 1986 (3). All four indigenous cases had a rash consistent with EM.

None of the Ixodes species that transmit LB in the northern hemisphere are found in Australia, but another Ixodid tick, *Ixodes (I.) holocyclus*, commonly bites man, transmitting *Rickettsia australis*, the cause of spotted fever in Australia (Queensland tick typhus) and also causes a toxin-mediated paralysis in children and domestic dogs (4). Its distribution is along the eastern seaboard in Queensland, NSW, and Victoria, where it extends into the Great Dividing Range of mountains in some areas (5). Spirochetes that have both the morphology and some structural characteristics of Borreliae have been recovered from *I. holocyclus* but cannot currently be sustained in culture (see below) (6, 7).

In 1991, Wills et al. reported detection of spirochetes in cultures from engorged adult and nymphal *I. holocyclus* ticks (7). Isolation generally took 8 weeks, but contamination of cultures has remained a persistent problem, despite use of culture methods recommended by North American and European researchers (8, 9). Numbers of spirochetes from each culture are low and, when isolated, demonstrate extremely fastidious growth. Further study of the spirochetes is thus difficult. Despite this, 70/167 (42%) of ticks processed yielded spirochetes (7). The ticks were collected mostly in the Manning River district of NSW, a region in which we are now finding clinical cases (10). Purification and analysis of 4 isolates demonstrates that all have borrelia-like polyacrylamide gel electrophoresis (PAGE) profiles and react (albeit) weakly with a monoclonal antibody to OspA (H5332) (11). Polymerase chain reaction (PCR) products were obtained using primer sets for the flagellin (*fla*) and rRNA genes; one strain additionally produced a specific OspA product (Shafren et al., unpublished). Morphological resemblance to *B. burgdorferi* is shown by immunofluorescence with flagellin-specific monoclonal antibody (H9724) demonstrating spirochete morphology resembling that of *B. burgdorferi* (data obtained by Dr. V. Bundoc, University of Texas) (12). We therefore believe that there are Borreliae in Australian ticks that are structurally similar to the agents of LB as described in the northern hemisphere. Whether they are pathogenic for humans or animals requires correlation with clinical isolates when they are eventually made.

When investigating *likely* new endemic areas, persistently negative results from initial research cast doubts on whether local transmission of LB occurs. For example, Russell et al. obtained 78 spirochete isolates from 35 separate locations along the eastern seaboard of Australia (6). They processed over 10,000 ticks, comprising 12 species, but, following analysis of the spirochete isolates, they *queried* the existence of so-called *Lyme disease* in Australia (6). *Ixodes holocyclus* was shown in laboratory studies not to be a competent vector for B31 strain (13). These negative results have not deterred our research as one of us

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(BJH) regularly sees clinical cases of LB acquired in Australia, examples of which are illustrated in Table 1. Failure to respond to standard treatment regimens has also not deterred our investigations, as every treatment regimen has a measurable failure rate, and currently, the optimal treatment for LB is not known (14). European researchers, in particular, have isolated spirochetes from skin and cerebrospinal fluid (CSF) in clinical relapses despite standard and even prolonged courses of intravenous antibiotics (15).

Despite increasing anecdotal reports of tick-bite-associated Lyme-like illness in eastern coastal Australia, and increasing referrals of sera to clinical diagnostic laboratories for Lyme serology, the only published body of data on which to base an Australian case definition are the three published cases of EM. Our initial case definition is based upon that developed by the United States Centers for Disease Control and Prevention (CDC), which, in itself, is problematic (16). It is acknowledged that this definition was developed for epidemiological purposes and, so, will exclude many clinical cases. Its use may also be inappropriate to another endemic area like Australia, especially since clinical manifestations of LB appear to vary with different genospecies of Borreliae from different parts of the world (17). If EM is either not common or not recognized, many LB cases will be missed. Definition of an endemic area before an isolate of the causative spirochete has been made in that area may have to be based on detection of cases with EM as in Steere's original investigation (18). We have seen a number of such cases and are confident that such clinical cases can define an endemic area in the initial stages of the investigation. The case definition can always be modified as knowledge improves. For serological confirmation of cases with manifestations other than EM, we chose the Western Blot method due to lack of specificity of screening tests like immunofluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA), and hemagglutination tests (19). In immunoblots, we have tested for antibodies to the OspA (31kD) and flagellin (41kD) antigens. OspA appears to show little (if any) cross reactivity with organisms other than Borreliae that cause LB (20). OspA thus represents the most likely unique antigenic protein of Lyme borreliae to which antibodies are made. Even though they are not very specific, showing considerable cross reactivity, antibodies to flagellin may be the only ones detected in early LB. As yet, we have not tested for IgM antibodies or for antibodies to low molecular weight proteins like the 21kD OspC, especially since OspC may not be expressed by some LB strains. Although the presence of antibodies to OspA may be specific to LB, early in the course of LB antibodies to OspA are not commonly seen; indeed, some persons never make antibodies to OspA (21). Accordingly, we acknowledge that sensitivity of immunoblots based upon the presence of antibodies to OspA will be low, but specificity was considered more important to assess whether indigenous cases of LB are occurring in Australia.

Table 1 displays clinical summaries for six patients seen by one of us (BJH) together with immunoblot data (Fig. 1–3). The patients live in known areas of tick infestation, along the eastern seaboard of NSW, although case 6 sustained the tick bite in Central Australia, outside the known area of distribution of *I. holocyclus*, the postulated tick vector of LB in Australia. Patients 5 and 6 had the typical EM rash, followed by systemic illness. Skin biopsies in cases 2, 5, and 6 showed lymphohistiocytic infiltrates, predom-

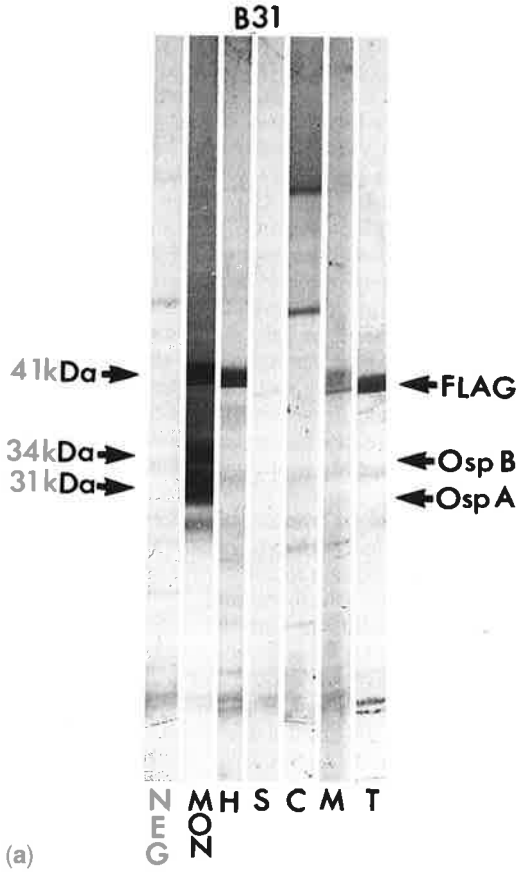
TABLE 1
Clinical Summaries and Immunoblot Data of Six Patients who Acquired Lyme Borreliosis in Australia (Adapted from Reference 12 with Permission)

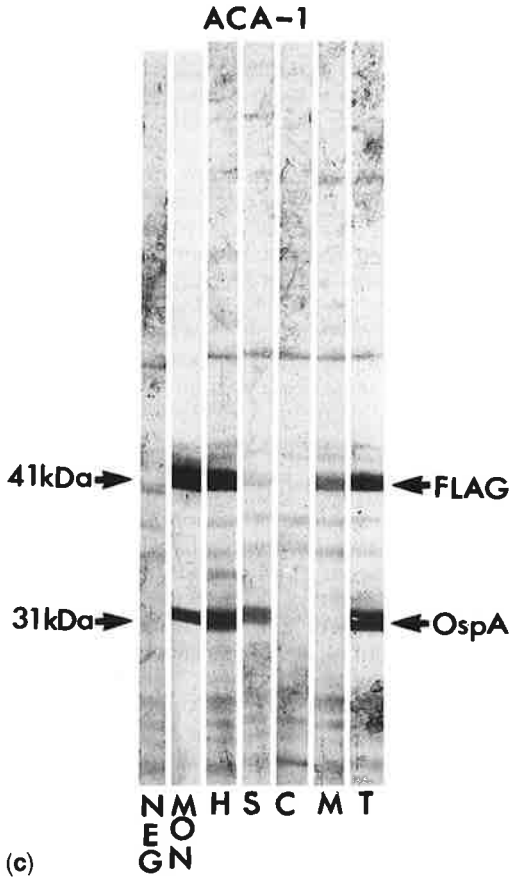
Clinical Summary: Six Australian LB Cases[a]				
Case	Age/Sex	Illness (Duration)	Immunoblot	Therapy (Days)
1	71 female	Rash, radiculitis (4 months)	NBS16:F(+)A(+) ACA1:F(+)A(+) B31:F(+)A(−)	 D(28)
2	47 male	Rash, CFS (12 years)	NBS16:F(+)A(+) ACA1:F(+)A(−) B31:F(+)A(+)	 D(28) R + C (28)
3	9 male	Arthritis, fevers (4 months)	NBS16:F(+)A(−) ACA1:F(+)A(+) B31:F(+)A(−)	E(28) Cf(28) H
4	60 male	Rash, arthritis, fibromyalgia (3 years)	NBS16:F(+)A(−) ACA1:F(+)A(+) B31:F(+)A(−)	D(56) R + C (28) Cf(28) H
5	52 female	EM (recurrent) fibromyalgia (2 years)	NBS16:F(+)A(−) ACA1:F(+)A(−) B31:F(+)A(−)	 D(28) Cf(14)
6	41 female	EM (recurrent) arthritis (14 months)	NBS16:F(+)A(+) ACA1:F(+)A(−) B31:F(−)A(−)	D(56) Cf(56) H

[a]Infections were all acquired in coastal NSW except case 6. EM = erythema migrans; CFS = chronic fatigue syndrome (by criteria of Holmes et al. (22); (fibromyalgia by criteria of Wolfe et al. (23)). Criteria for EM and arthritis followed CDC case definition (16).

ACA1, NBS16, B31 used for immunoblots: F = flagellin; A = OspA; (+) = positive; and (−) = negative.

Therapy: D = doxycycline 200 mg/day; R = roxithromycin 600 mg/day; C = cotrimoxazole 320/1600 mg daily; Cf = ceftriaxone 2 g/day; H = Herxheimer reaction with treatment. Days of treatment = total over 1 or more courses of therapy.





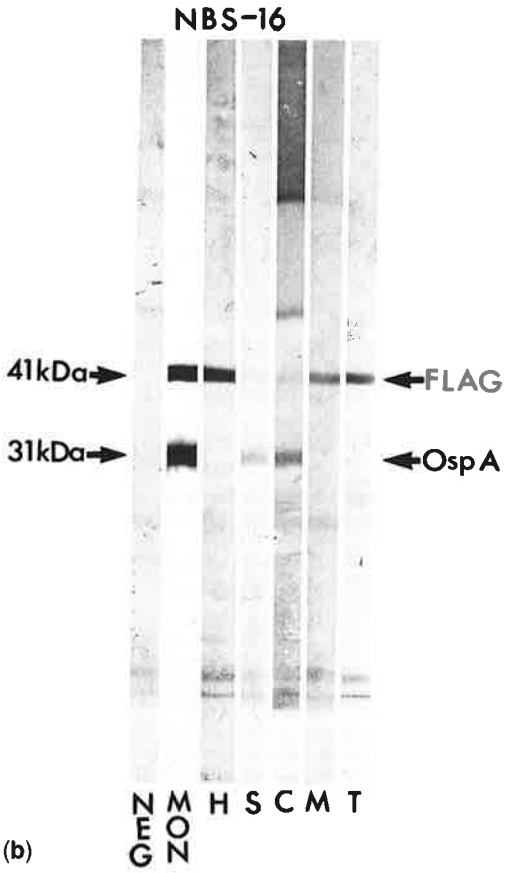


FIG. 1. Immunoblots on sera from Australian Lyme borreliosis cases using different species of B. burgdorferi *sensu lato. NEG = negative control; MON = monoclonal antibodies bound to 41kDa, OspB, and OspA proteins; H,C,M,T = cases 4, 2, 5, and 3, respectively, in Table 1. Immunoblots for cases 1 and 6 not shown (see text). (a) B31* (B. burgdorferi *sensu stricto); (b) NBS-16* (B. garinii)*; (c) ACA-1* (B. afzelii)*.*

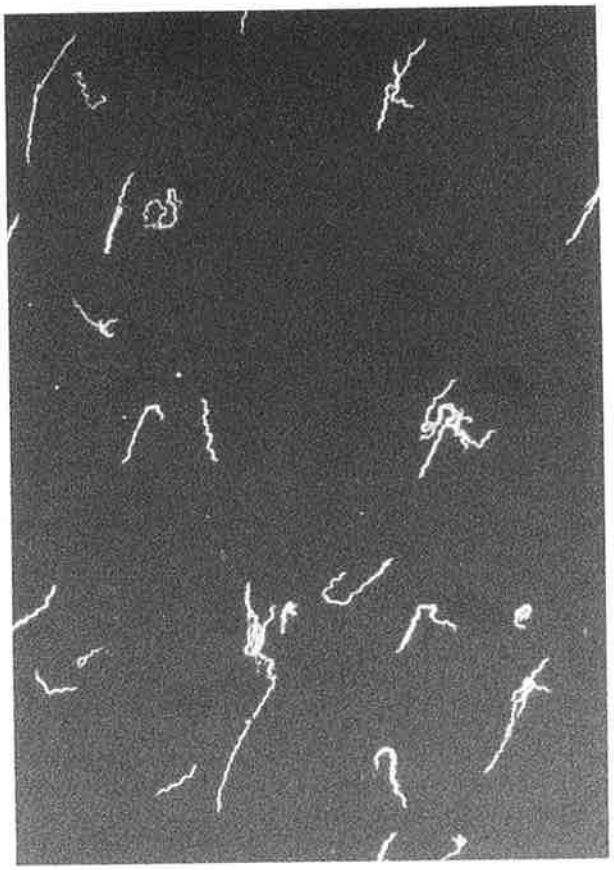


FIG. 2 B. burgdorferi *B31: immunofluorescence stain with flagellin monoclonal antibody H9724 (courtesy of Dr. V. Bundoc and Professor A. Barbour).*

FIG. 3 Borrelia *isolated from engorged* I. holocyclus *ticks in eastern Australia: immunofluoresence stain with flagellin monoclonal antibody H9724 (courtesy of Dr. V. Bundoc and Professor A. Barbour).*

inantly perivascular and in dermis, except case 2 had significant infiltrate around a pilosebaceous follicle. Warthin-Starry silver stains for spirochetes were negative in all cases. Physician-observed joint swelling, for which no other cause was found, was required for the diagnosis of arthritis. Recurrent EM rashes were seen in cases 5 and 6 despite antibiotic treatment. Herxheimer reaction was not uncommon. Antibodies to OspA of North American strain B31 (*B. burgdorferi* sensu stricto) were not commonly detected, but antibodies to OspA of two European strains were. These belonged to the two other species groups of *B. burgdorferi* sensu lato: *B. garinii* (NBS-16) and *B. afzelii* (ACA-1), isolates from a tick in Sweden and an acrodermatitis chronica atrophicans skin lesion, respectively (provided by Professor A. G. Barbour, Texas).

We compared immunoblot results for patients judged likely to have LB based on clinical assessment by one of us (BJH) with healthy controls from the Newcastle area and for patients with connective tissue diseases. The clinical assessment was done independent of any knowledge of the immunoblot testing and vice versa. All patients had acquired their illness in Australia. For LB cases, 21/23 (>90%) had detectable Flagellin antibody by immunoblot while 13/23 (55%) had antibody to OspA. This contrasted with the low levels of antibodies to OspA seen in the other groups (Table 2). Differences were statistically significant.

We have found European strains of Borrelia most useful for immunoblotting in candidate Australian LB patients. Antibodies are detected to OspA of these strains in such patients rather than to OspA of the North American *B. burgdorferi* B31 strain (Table 3). When a local Australian clinical isolate is made, and can be grown in amounts adequate for immunoblotting, obviously we will study immunoblots with such isolates. Until then, northern hemisphere isolates will be used.

For individual cases, there was also variation in seroreactivity as the pattern of OspA positivity varied. Results were compared for 13 patients who produced antibodies to OspA. Of the 13 patients, 11 had antibody to NBS-16 OspA and 7 to ACA-1 OspA, but only 4 had detectable antibodies

TABLE 2
Immunoblot Reactivity LB Cases/Controls[a,b]

Category	Persons Tested	Number Positive	(%)
A. Healthy volunteers	92	2	(2.2)
B. Rheumatic illness— not LB	56	2	(3.3)
C. LB cases	23	13	(55.0)

[a]Seroreactivity to OspA proteins of European strains of *B. burgdorferi* of candidate Lyme borreliosis cases, compared with healthy controls and patients with connective tissue diseases. All patients had negative syphilis serology by TPHA (*Treponema pallidum* hemagglutination test) (adapted from Reference 12 with permission).

[b]European isolates NBS-16, ACA-1 used (see text)

A,B: not significant (Chi squared); A,C: significant $p < 0.01$; C,B: significant $p < 0.05$; and AB,C: significant $p < 0.01$.

TABLE 3
Immunoblot Reactivity of 686 Referred Sera[a,b]

Reactivity Pattern	Number	(%)
OspA + flagellin		
NBS-16 only	44	(6.4)
ACA-1 only	18	(2.6)
B31 only	4	(0.9)
NBS-16 & ACA-1 (both)	4	(0.6)
NBS-16 & B31 (both)	3	(0.4)
ACA-1 & B31 (both)	3	(0.4)
Subtotal	78	(11.3)
Trace OspA + flagellin	32	(4.6)
Strong flagellin (all strains)	50	(7.4)
Total	160	(23.3)

[a]Sera referred for Lyme serology 1/1/93–9/20/93.
[b]Three strains used: B31, NBS-16, ACA-1.

to OspA of both NBS-16 and ACA-1. This may indicate antigenic heterogeneity in causative spirochetes in Australia.

Because we have detected Borrelia-specific antibodies in the serum of candidate clinical cases of LB acquired in Australia, we hypothesise that an indigenous form of LB exists in Australia. The acquisition of at least one case outside the area of distribution of *I. holocyclus* indicates that ticks other than this species can transmit LB in Australia. The findings of Piesman and Stone should be reinterpreted in view of this observation (13). One could argue that the presence of antibodies to OspA, with or without antibodies to flagellin, is weak serological data for the existence of LB in Australia. Should not antibodies to antigens like OspC, the 94kD protein, and others also be sought? Should immunoblots use recombinant antigens (e.g., OspA protein or other immunogenic proteins)? We considered these approaches initially but discounted them because of increasing evidence of genetic diversity of spirochetes associated with LB worldwide. Heterogeneity of European isolates for OspA compared with North American isolates is well known. When comparing European to North American isolates, Barbour et al. demonstrated variable binding of monoclonal antibodies to OspA proteins as well as different arrangements of OspA-associated DNA sequences (24). In a newly described endemic area in Japan, four of eight clinical *B. burgdorferi* sensu lato isolates failed to react with H5332 but still possessed OspA-like protein bands on SDS-PAGE analysis, indicating different epitopes compared with European and North American strains (25). Using restriction fragment length polymorphism (RFLP) analysis, virtually all clinical isolates in Japan were dissimilar to representative isolates from Europe and North America (25).

We feel that our approach is also supported by the work of Wilske et al. (26) who, using a panel of monoclonal antibodies for OspA, identified at least seven different OspA serotypes with one other OspA serotype that did not bind any of the monoclonal antibodies. Of 128 OspA-expressing strains, serotype 1 corresponded to *B. burgdorferi* sensu stricto, serotype 2 to *B. afzelii*, and serotypes 3 through 7 to *B. garinii*; serotype X was the label given to three strains from *I. dentatus* ticks that had varying molecular mass of OspA proteins and varying binding patterns for monoclonal antibodies. Only one monoclonal antibody bound to all seven OspA serotypes. Analysis of OspA partial amino acid sequences showed highly conserved regions but a significant variable region. To detect local cases of LB in Australia, recombinant OspA and other proteins for immunoblots may lack essential epitopes and may well be inappropriate. The demonstrated geographic genetic heterogeneity of spirochetes and OspA serotypes is likely to be of relevance to LB in Australia and to the identity of putative local spirochetes. Since *B. burgdorferi* has been isolated from migratory birds and their ticks (27, 28), and migratory birds travel annually from LB endemic areas in northern Japan and eastern Asia to the whole Australian east coast, one would suspect that some (possibly most) Australian isolates are likely to more resemble isolates from these areas than areas in North America or Europe. Recombinant proteins from well-characterized North American strains may be of little use for immunoblotting in Australian LB cases.

More appropriate is our approach to perform immunoblots using strains from the 3 different genospecies of *B. burgdorferi* sensu lato. We also feel that recent work by Assous et al. further supports this approach (29). They performed immunoblots on sera from known LB patients with EM, meningoradiculitis, arthritis or acrodermatitis chronica atrophicans (ACA) using strains from the three different species groups of *B. burgdorferi* sensu lato. For patients with EM or meningoradiculitis, against *B. afzelii* (two strains), no antibodies to OspC were detected, and in few cases were antibodies to the 94kD protein detected; against *B. garinii* (three strains), antibodies were detected to OspC in most cases but not to 94kD in every case; against *B. burgdorferi* sensu stricto (three strains), no antibodies were detected to OspC in many cases. For the same strains with sera from patients with ACA or arthritis, antibodies to OspC were only variably detected. We feel that these serological reactions to different genospecies resemble those we report for OspA and flagellin. Lack of detection of antibodies to OspC and the 94kD protein may be relevant to Australian patients, a point that we are currently addressing.

The clinical manifestations of LB in Australia appear to resemble those seen in the northern hemisphere. The spectrum of illness and frequency of clinical manifestations remain to be determined however. For the moment, treatment recommendations follow northern hemisphere guidelines recognizing that optimal therapy is yet to be established (14). Treatment of chronic cases of LB is problematic as persistent symptoms and relapses are seen often in the northern hemisphere (14). Similar problems have been observed in Australian cases, as illustrated by 4 of the 6 cases presented in Table 1, where repeat courses of oral and intravenous antibiotics have been given. This need was particularly obvious in cases 5 and 6 where recurrent EM occurred. Case 1 has just commenced ceftriaxone therapy. The only case of complete resolution of symptoms and signs and apparent cure was case 3, following ceftriaxone therapy. Incorrect diagnosis and/or persisting coexistent illness to explain poor response to therapy is possible, but physician-observed recurrences of the original EM rash in cases 5 and 6 are best explained by failure of antibiotic therapy.

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Late Complaints after Erythema Migrans

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A lot of treatment studies have been carried out, but no antibiotic has been proved to avoid late manifestations of Lyme disease. Our interest focused on late manifestations following uncomplicated erythema migrans (UEM) and complicated erythema migrans (CEM) after a median observation period of 30 months. To compare the therapeutic, serological, and clinical outcome, 161 patients were re-examined prospectively.

Late complaints could be observed in 31/161 (19%) of patients, more often in CEM than in UEM (36% versus 12%). Patients with late sequelae were more often seropositive than the total collective (77% versus 67%), at least once during the observation period, as against 12 of 13 patients who needed several therapy cycles (92%). Seven seropositive patients did not respond to oral antibiotic treatment even after several cycles. Amoxicillin/clavulanic acid treated patients had late complaints in 8% in contrast to penicillin V (15%) and doxycycline (17%) treated persons.

Seropositivity before treatment has a negative influence on the course of erythema migrans (EM) disease. Immunogenetic disposition might be responsible for repeated infections and for treatment failures in a certain patient group.

Key words: Erythema migrans, Complicated and uncomplicated, Late complaints, B. burgdorferi-antibodies

INTRODUCTION

First reports on the relation of erythema migrans (EM) to tick bites date back to the beginning of this century (1). In the early fifties, it was reported that antibiotics efficiently act on EM (2). With the detection and cultivation of a previously unknown spirochete from *Ixodes (I.) dammini* ticks by Burgdorfer et al. in 1982 (3), the door was opened to study its antimicrobial susceptibility *in vitro* and *in vivo* (4–7). Several therapeutic studies have been carried out by different groups of researchers (4, 6, 8–13) to ascertain the best antibiotic treatment in early Lyme borreliosis in order to prevent late manifestations of the disease, as well as serological studies, to investigate the role and titers of specific antibodies in EM and associated complaints (14–18). For oral treatment following intensive use of penicillin, tetracyclines were found to be superior (4). Erythromycin, highly effective *in vitro*, resulted in more treatment failures than penicillin or tetracycline (4). A well-documented report describes the effectiveness of amoxicillin combined with probenecid or doxycycline for 21 days (11), and a randomized study supports the use of ceftriaxone intramuscularly (13).

The purpose of this study was to evaluate the course of EM in a group of well-documented cases. The frequency of complications was correlated with the duration of EM, type of therapy, serological results, and status of reinfection.

MATERIALS AND METHODS

Patients. One hundred sixty-one patients (116 female, 45 male) with EM were reinvestigated after therapy in the following 6, 12, and 24 months and up to 5 years (median 30 months) a minimum of four times. Every patient's history was evaluated with respect to arthralgia, polyarthritis, headache, fatigue, fever, and cardiac and autoimmune diseases. Patients with a history of these symptoms before the onset of EM were excluded.

Erythema migrans was classified as uncomplicated erythema migrans (UEM), when no accompanying symptoms were noted, and as complicated erythema migrans (CEM), when the occurrence of EM was accompanied by systemic symptoms (e.g., fatigue, fever, chills, headache, migratory musculoskeletal pains, and arthralgia).

Any symptoms that occurred during the observation period were recorded. These were viewed as late manifestations of the spirochetal infection when a concomitant infectious disease, trauma, or other underlying degenerative disease could be excluded.

In patients with an elevated IgG antibody level and negative IgM antibodies less than 4 weeks after the tick or insect bite or beginning of EM, a second episode of Lyme disease (reinfection?) was considered (14, 19, 20).

Treatment. Ninety-four patients with EM received oral phenoxymethylpenicillin 1.5 million IU tid for 10 to 14 days, 35 were treated with doxycycline 100 mg bid orally for 10 to 14 days, and 12 patients received amoxicillin 500 mg plus clavulanic acid 125 mg tid orally for 20 days. Because of persistent concomitant symptoms like arthralgia, cephalea, fatigue, polyneuritis, myalgia, fever, and lymphadenitis in CEM, or arising complaints in UEM during or up to 1 month after therapy, six UEM and seven CEM patients required retreatment. The sequence of antibiotic therapies was varied, but in all regimens, penicillin or doxycycline were primarily used, and all patients received both antibiotics. Seven patients refused antibiotic therapy (Table 1).

Serological methods. Antibodies against *Borrrelia (B.) burgdorferi* were evaluated by enzyme-linked immunosorbent assay (ELISA) (21) before, 3 to 5 weeks after, 6 months after, and 2 to 5 years after treatment. An ELISA unit of 4.7 was calculated as the threshold level. A supernatant of an ultrasonicated whole cell preparation of *B. burgdorferi*

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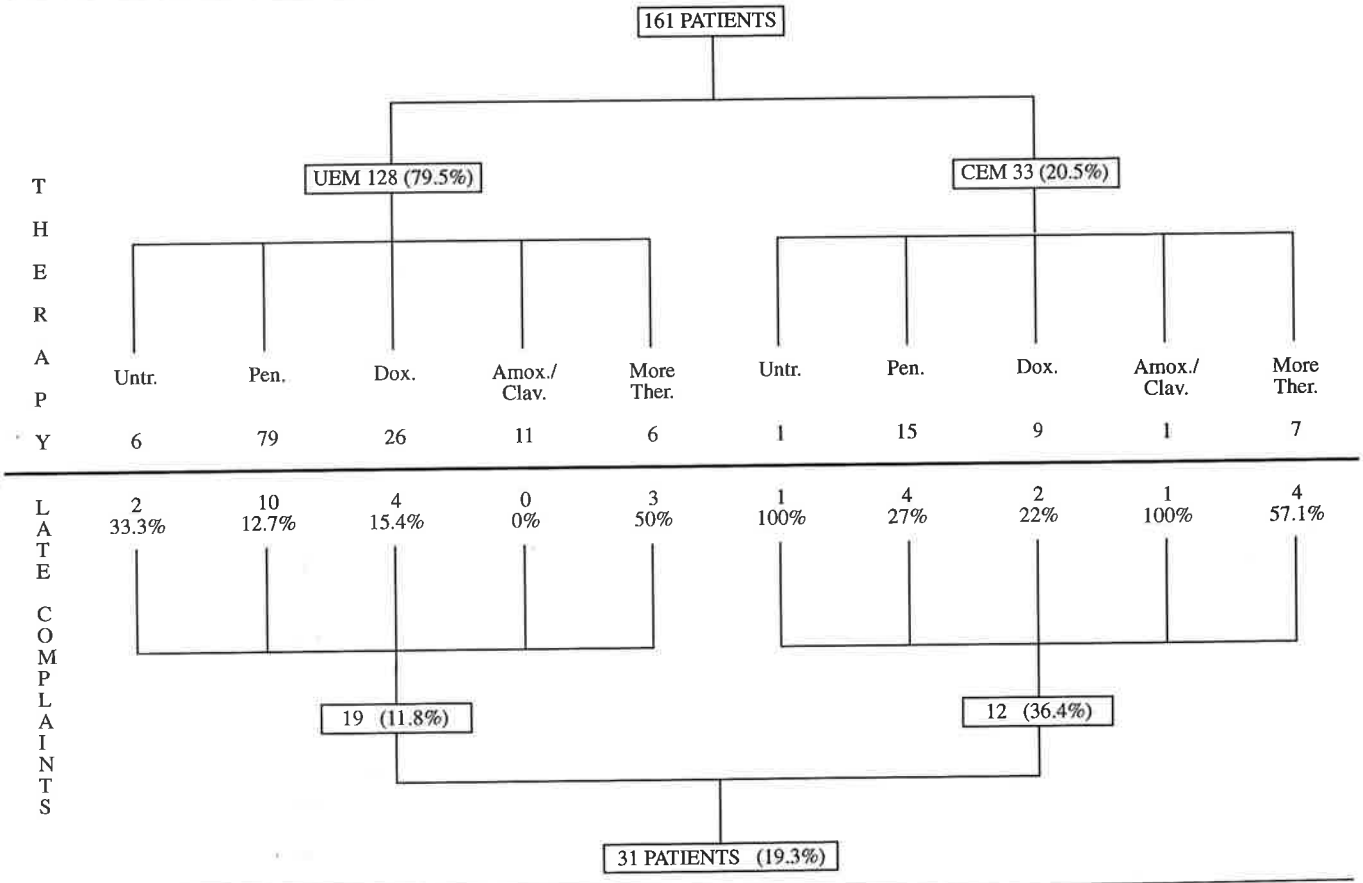
TABLE 1
Frequency of Late Complaints in Patients with UEM and CEM at the Last Follow-Up After a Mean Period of 3 Years



Untr. = untreated; Pen. = phenoxymethylpenicillin; Dox. = doxycycline; Amox./Clav. = amoxicillin/clavulanic acid; More Ther. = several antibiotic cycles.

There was a high significant difference in the occurence of late complaints between UEM and CEM ($p < 0.01$) and a significant difference between the treatment groups in relation to late complaints in UEM ($p = 0.046$) in contrast to CEM ($p = 0.185$).

B31 strain, 1 μg per well, was used as an antigen. Photometrically determined optical density (OD) values were quantified in ELISA units, defined as the differential value of the base 3 logarithmic dilution at OD 0.2 from the comparison of a known positive serum with the test serum.

Statistical analyses. The analyses were calculated by a chi-square test; values <0.05 were interpreted as significant.

RESULTS

1) Clinical course

- The median observation period was 30 months (22 months to 5 years).
- Tick bites were reported in 53 patients (32.9%), insect bites from mosquitos or horseflies in 46 patients (28.5%), an unidentifiable spider bite in 1 patient, and a thorn injury in 1 patient. No specific data were given for 60 patients (37.3%).
- A total of 128 patients had UEM (79.5%), and 33 patients had complicated CEM (20.5%) (Table 1).
- Median duration of EM before treatment was 26 (3 to 270) days (UEM 21 days and CEM 38 days); median duration of EM with subsequent late complications was 46 days (UEM 56 days and CEM 21 days).
- The most frequently recurring late complaints (Table 2)

TABLE 2
Late Complaints

	UEM ($n = 19$)	CEM ($n = 12$)
Monosymptomatic	13	3
Polysymptomatic	5	9
Monoarthralgia	4	2
Oligoarthralgia	8	9
Arthritis	—	1
Dysaesthesia	3	2
Cephalea	2	4
Myalgia	2	3
Fatigue	—	2
Dactylitis	4	—
Acrodermatitis chronica atrophicans	—	1
Morphea	—	1
Juxta-articular nodules	1	—
Neuralgia	2	—
Carpaltunnel-syndrome	1	1

TABLE 3
Late Complaints of 161 EM Patients Depending on Treatment

EM	Number of Patients Total	Late Complaints (n)	Late Complaints (%)
Penicillin	94	14	14.9
Doxycycline	35	6	17.1
Amocycillin/Clavulanic acid	12	1	8.3
More therapy cycles	13	7	53.8
Untreated	7	3	42.8

were mild to moderate severe mono- and oligoarthralgia, starting between 2 and 36 months after EM and lasting from 3 to 42 months. The predominant localization was that part of the body with previous EM. Arthritis occurred only in one patient with primary CEM. Dactylitis with painful swelling of the fingers could only be observed after UEM and fatigue only after CEM. In addition, in the CEM group, one patient developed acrodermatitis chronica atrophicans (ACA) of the legs three years after EM and one circumscribed scleroderma at the site of EM after half a year.

- Recurring complaints after more than 2 years were seen in 19 patients with UEM (11.8%) and in 12 patients with CEM (36.4%) (Table 1).

Treatment

Erythema migrans cleared in all patients after several days. Penicillin and doxycycline proved equally effective in preventing late complaints (85/87% in UEM, 73/78% in CEM) (Tables 1 and 3). Amoxicillin/clavulanic acid was ineffective in one patient with CEM but had no treatment failure in UEM. Patients who needed more than one therapeutic cycle did not recover in up to 53.8% of the cases. Jarisch Herxheimer-like reactions at the beginning of treatment and lasting up to 1 week occurred in 3/128 patients with UEM and in 4/33 patients with CEM. Manifestations included flulike symptoms, fever, chills, and fatigue.

Serological findings

- At least once during the observation period, 108 patients (67%) were seropositive (Table 4); this includes 24 out of 31 patients (77.4%) with late complaints. In this latter group of patients, seropositivity before therapy and at the time of the last control was roughly 50% (Table 6).

  Doxycycline-treated patients developed antibodies to *B. burgdorferi* more often than penicillin-treated patients, but without statistical significance. After treatment with amoxicillin/clavulanic acid, the number of seropositives was lowest. Patients who received more than one therapy cycle were seropositive in a higher percentage than untreated patients (92.3% versus 71.4%).
- IgM antibodies were found positive in 15.6% of the cases before therapy and decreased to 11.9% after therapy (data not shown). In UEM patients, IgG seropositivity before therapy was seen in 36.7%, as against 42.5% after therapy. In contrast, in CEM patients, 72.7% were seropositive before and 62.9% after therapy.
- At the last control, 62 of all patients were seropositive (38.5%): 45 of the UEM group (35.2%), and 17 of the CEM group (51.5%) (Table 5). Patients with UEM and late symptoms were more often seropositive (47.7%) than patients without late symptoms (33.0%); however, no statistical relevance could be evaluated. In contrast, at the last control, patients with CEM and late complaints were only seropositive in 41.7% versus 57% of patients without persistent complaints. In the doxycycline group, 50% of the patients with late symptoms were seropositive, whereas only 35.7% of the penicillin-treated patients were seropositive.

Reinfection

According to the definition, a *B. burgdorferi* reinfection was suspected in 28 of the 161 patients (17.4%) due to IgG seropositivity before treatment at a median duration of EM of 12 days; 10 of these 28 patients developed late complaints (35.7%; UEM 47.1% versus CEM 18.9%; $p = 0.000$).

DISCUSSION

Erythema migrans can be diagnosed clinically except in atypical cases, but it is still difficult to identify complaints that appear weeks or months after clearing of EM by antibiotic treatment as being late manifestations of Lyme disease (22–24).

According to reports in the literature, late complications such as arthralgias, headache, and fatigue have been associated with chronic Lyme borreliosis in seropositive and seronegative patients (25). In our patients, arthralgia and dactylitis caused by swelling of the juxta-articular connective tissue appeared clinically as tenderness on pressure. Joint alterations demonstrable by X-ray were never observed. Dactylitis was seen in association with UEM, whereas fatigue was only observed in patients of the CEM group (26). Migratory musculoskeletal pains either persisted from several months to more than 3 years or recurred after symptom-free intervals. Dysaesthesias could be confirmed by clinical neurological examination. Headache and fatigue in our patients were of subjective nature. Because of the prospective follow-up and the personal knowledge of patients, statements of patients could be evaluated objectively. For the patients, the complaints were disturbing life quality for a certain time, but they have not been seen so severe as to cause encephalopathy or disabling "rheumatic" or cardiac

TABLE 4
IGM and/or IGG-Antibodies against* B. burgdorferi *at Least Once during Observation Period

	UEM	CEM	ALL EM
Penicillin	48/79 (60.7%)	12/15 (60.0%)	60/94 (63.8%)
Doxycycline	28/26 (69.2%)	8/9 (88.9%)	26/35 (74.3%)
Amoxicillin Clavulanic acid	4/11 (36.4%)	1/1 (100%)	5/12 (41.7%)
More therapy cycles	5/6 (83.3%)	7/7 (100%)	12/13 (92.3%)
Untreated	4/6 (66.7%)	1/1 (100%)	5/7 (71.4%)
Total	79/128 (71.6%)	29/33 (87.9%)	108/161 (67.0%)

TABLE 5
IGG-Seropositive Patients at the Last Control

		No Late Complaints (n = 130)			Late Complaints (n = 31)		
		UEM	CEM	Total	UEM	CEM	Total
Penicillin	34/94 (36.2%)	22/69	7/11	29/80 (36.3%)	3/10	2/4	5/14 (35.7%)
Doxycycline	13/35 (37.1%)	7/12	3/7	10.29 (34.5%)	3/4	0/2	3/6 (50.0%)
Amoxicillin/Clavulanic acid	2/12 (16.7%)	2/11	0/0	2/11 (18.2%)	0/0	0/1	0/1
More therapy cycles	8/13 (61.5%)	2/3	2/3	4/6 (66.7%)	2/3	2/4	4/7 (57.1%)
Untreated	5/7 (71.4%)	3/4	0/0	3/4 (75.0%)	1/2	1/1	2/3 (66.7%)
Total	62/161 (38.5%)	36/109 (33.0%)	12/21 (57.0%)	48/130 (36.9%)	9/19 (47.7%)	5/12 (41.7%)	14/31 (45.2%)
		UEM 45/128 (35.2%)		CEM 17/33 (51.5%)		all EM 62/161 (38.5%)	

TABLE 6
Comparison of IGG-Antibodies against B-burgdorferi *in Patients with Late Complaints before Therapy and at Their Latest Control*

	Before Therapy	Latest Control
UEM	9/19 (47.4%)	9/19 (47.4%)
CEM	7/12 (58.3%)	5/12 (41.7%)
TOTAL	16/31 (51.6%)	14/31 (45.2%)

disease. All these symptoms improved gradually during the observation period, as also reported by Steere et al. (27).

The importance of critical view of complaints is addressed in a recent retrospective study of 82 patients, who were treated adequately or nonadequately for cutaneous manifestations of Lyme borreliosis (28). Several systemic complaints were described in these patients. Although symptoms like atrioventricular blocks caused by hypertensive cardiomyopathy, polyneuropathy after acrodermatitis chronica atrophicans, or carpal tunnel syndrome have been associated with Lyme borreliosis, these were attributed to old age or chronic alcoholism in this study.

One of the factors influencing the course of Lyme disease has been seen in the duration of EM, as it was twice as long in patients with CEM than in UEM. In most of the treatment studies of early Lyme disease, antibiotics were given within the first 4 weeks of illness (9, 10, 12). Asbrink and Olsson, however, reported on general symptoms in 53% in patients with a disease duration longer than 3 weeks, compared to 23% of EM lasting less than 3 weeks (12). A significant correlation between the duration of therapy and clinical outcome was not found by others (13).

The evaluation of the efficacy of the various antibiotics used in this study is restricted because of the heterogeneity of our patient groups with regard to the type of antibiotic and duration of treatment. Unresponsiveness to the first treatment schedule and ongoing systemic symptoms despite clearing of EM was observed in 8% of patients. Despite several antibiotic treatment cycles, the complaints did not improve after oral therapy in 7 of 13 cases. Persisting complaints seen in these patients were present even more often than in patients who refused therapy (Table 3). This is in accordance with reports by Steere et al. (23) and Sigal and Patella (24) who noticed that musculoskeletal symptoms will not improve in a certain percentage of patients even after repeated administration of antibiotics. It was further shown that the response to several different antibiotics may be determined by genetic variations of the host immune response; especially, the presence of HLA-DR4 was significantly associated with the failure of treatment, (29) although this association could not be observed in Europe, as reported by Herzer (22).

A significant anti-*B. burgdorferi* antibody titer was always concomitant with worse prognosis, in that patients unresponsive to treatment were seropositive in 92% and that CEM patients were seropositive in a higher percentage than UEM patients. The epidemologic range of seropositivity to *B. burgdorferi* in healthy blood donors has been evaluated as 8% for the area of Vienna where this study was also done (30). As reported, IgM antibodies appear within 1 to 3 weeks after disease onset, and IgG antibodies require 4 to 6 weeks to develop (14, 19, 20). We assumed that IgG antibodies detectable earlier than 4 weeks after tick or insect bite might point to previous contact with *B. burgdorferi*. The possibility of reinfection was repeatedly mentioned in the literature (31). Seropositivity before therapy, due to a long duration of EM or due to reinfection, predict a more severe course of disease by developing CEM or causing late symptoms.

The pathogenesis of late complaints must remain unanswered. In case of the detection of *B. burgdorferi* DNA in body fluids or affected tissues, persistent infection can be suggested (34). Viable borreliae can escape eradication by antibiotics due to intracellular location (35), can withdraw into immuno-privileged sites or can persist in the host through antigenic variation similar to *B. hermsii* (19, 32, 33). On the other hand, nonviable remnants of borreliae such as surface blebs, borrelia antigens, or tissue components acting as antigens by molecular mimicry can maintain inflammatory processes (23, 36–38).

Considering all these possible hypotheses, further studies are needed for optimizing the choice of antibiotics and the duration of therapy.

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Cutaneous Manifestations of Lyme Borreliosis in Europe

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Lyme borreliosis is accompanied by several cutaneous manifestations. Three manifestations are pathognomonic for this infectious disease, if they are present in their typical appearance: erythema migrans, borrelial lymphocytoma, and acrodermatitis chronica atrophicans (ACA). In addition, Lyme borreliosis can occasionally be associated with a borrelial dermatomyositis-like syndrome and localized scleroderma (Table 1).

TABLE 1
Cutaneous Manifestations of Lyme Borreliosis

Classical Manifestations:
Erythema migrans
Borrelial lymphocytoma
Multiple erythema migrans-like lesions
Acrodermatitis chronica atrophicans
Other Manifestations:
Borrelial dermatomyositis-like syndrome
Localized scleroderma (morphea)

ERYTHEMA MIGRANS

An "erythema migrans" was first mentioned by Afzelius in 1909. The first cases were described by Balban in 1910, Lipschütz in 1913, and Riehl in 1913 (1).

Erythema migrans is the hallmark of Lyme borreliosis and the typical lesion of stage 1 of this disease.

Erythema migrans is a usually expanding cutaneous erythema starting around the inoculation of *Borrelia (B.) burgdorferi*. The typical lesion consists first of a homogeneous erythema that spreads peripherally. The center tends to clear or to become bluish red as the lesion expands so that a peripheral ring-shaped erythema remains. The ring-shaped peripheral erythema is bright red and about 0.5 to 4 cm in width. The erythema migrans is mostly oval shaped or round and occasionally elongated or rather bizzare. The size of the erythema migrans varies from 3 cm to more than 85 cm and is 20 cm in average (2). The duration of the erythema migrans ranges from 3 days to 2 years. Most lesions lasted longer than 4 weeks in a previous study (3), but patients seem to seek medical advice earlier in recent years. If left untreated, the erythema migrans eventually disappears spontaneously, usually within a few months, sometimes within a week or so. Patients with an erythema migrans of short duration may be prone to have a poor prognosis, but this is not invariably the case (3). If the lesion lasts longer than 6 months, the former designation erythema chronicum migrans appears to be justified, but for all other instances, the designation erythema migrans is preferred (2).

The erythema migrans occurs most frequently on the legs and, in children, on the head, sites where ticks have the easiest access. Almost all other parts of the skin with the exception of the palms and soles can be affected.

Atypical lesions can sometimes be seen. For instance, they can be small and nonmigrating with a size of 2 to 4 cm or the ring-shaped erythema may be interrupted. Our group has repeatedly isolated *B. burgdorferi* from the small lesions mentioned (2).

The erythema migrans can reappear at the original site, e.g., after unsuccessful therapy. If an erythema migrans appears at a different site, especially if more than a year has elapsed since appearance of the first erythema migrans, this is usually due to reinfection. Superinfection must be assumed if an erythema migrans appears in a patient with chronic Lyme borreliosis such as ACA (a few cases have been described).

The erythema migrans lesion may be asymptomic, pruritic, burning, or painful. Pain and burning have been associated with more severe initial disease (3).

Borrelia burgdorferi is usually inoculated by ticks of the Ixodid group. Flying insects apparently can also transmit *B. burgdorferi*. The tick bite remains often unnoticed, especially that of the rather tiny Ixodid nymph. The seeming lack of a tick bite does not, therefore, rule out transmission of *B. burgdorferi* or other infectious agents which might be transmitted by ticks.

Due to the activity of the ticks, the erythema migrans begins predominantly during the period between May through October. However, earlier or later beginnings are possible in regions with warm climate, and a later onset can occur because a long-lasting lesion might go on unnoticed for months.

A variety of symptoms are associated with the erythema migrans (2,3). A comparison with an American study (4) reveals that the occurrence of the symptoms is remarkably similar but that the symptoms are less frequently seen in Germany compared to the United States. This is supported to a certain extent by other more recent investigations (5,6).

Extracutaneous findings in patients with erythema migrans are rather sparse, with the exception of regional lymphadenopathy and low-grade fever (2).

The histopathological changes of the erythema migrans consist of a mild to moderate lymphohistiocytic infiltrate located predominantly around blood vessels. Sometimes, plasma cells can be seen (2). T cells predominate among lymphocytes. Helper T lymphocytes are more common than suppressor T lymphocytes. Langerhans cells are numerous (7). Complement C3 and C4d, fibrin, fibrinogen, IgM, and IgG can be found in the blood vessels (2).

Serological tests are not always positive in patients with erythema migrans. IgM and IgG antibody titers against *B. burgdorferi* can be elevated (8). A negative serological test does not rule out early infection. On the other side, a positive serological test does not always prove the diagnosis of an active infection because of the possibility of a pre-

vious infection with *B. burgdorferi*. In patients with eythema migrans, however, a serological test should always be performed before and after therapy. Serological tests should become negative after therapy, but this may take time (6, 9). Persistently elevated antibody titers may indicate ongoing infection (9).

Borrelia burgdorferi can mainly be identified by isolation (8) or by PCR (10,11). Isolation procedures are not always successful. It remains to be seen whether the PCR yields more reliable results. The diagnosis of a typical erythema migrans rests on clinical grounds alone and does not require a positive laboratory test. This description raises the question how different the erythema migrans is in Europe and the United States. It may be assumed that the three subtypes of *B. burgdorferi* seen in Europe would induce a slightly different appearance and behavior of the European compared to the American erythema migrans. One must be conscious of a possible bias facing this question (12). However, the tendency to a longer duration of the European erythema migrans, the lower frequency of concomitant or subsequent multiple erythema migran lesions and the lower percentage of associated symptoms occurring in Europe might be hints for a true difference between the erythema migrans seen in Europe and in the United States. Of special interest is the rather high occurrence in European erythema migrans patients of *B. afzelii* (group VS 461), which is exclusively seen in patients with ACA (11). The latter affection has only rarely, if at all, been described in native American citizens.

BORRELIAL LYMPHOCYTOMA

The term borrelial lymphocytoma (BL) has only been introduced recently (13, 14); BL is a benign lymphoreticular hyperplasia of the skin caused by *B. burgdorferi*. The BL must be separated from other nonborrelial lymphoreticular proliferations. The BL is not exactly identical with lymphadenosis benigna cutis which may include nonborrelial cases of lymphoreticular hyperplasia. However, many patients with solitary lymphadenosis benigna cutis, or solitary lymphocytoma described previously might indeed have been borrelial lymphocytomas, especially if the lesions were located on the ear lobe or the nipple.

Lymphocytoma is a designation first mentioned by Biberstein in 1923. Lymphadenosis benigna cutis was a term first used by Bäfverstedt in 1943 to separate solitary and multiple benign lymphoreticular lesions from malignant ones.

Clinically, BL is a bluish-red infiltration of the skin, usually about 1 to 5 cm in diameter. It is most commonly a solitary lesion. Multiple lesions have been described, but the true significance of multiple lesions has probably not been evaluated (15).

The BL is most frequently a sign of early Lyme borreliosis, usually belonging to the second rather than to the first stage. In some patients, a BL of the nipple followed within weeks the development of an erythema migrans of the chest (15). In other cases, the LB developed on the ear lobe subsequently to a tick bite at this site (13, 15). The BL can also rarely be seen during the third stage of Lyme borreliosis in connection with ACA.

The BL can be accompanied by regional lymphadenopathy, arthritis, choroiditis, and meningoradiculoneuritis including facial palsy. Untreated BL tends to disappear within months (15).

Borrelial lymphocytoma is histologically characterized by lymphoreticular hyperplasia with or without germinal center formation. Other inflammatory cells such as plasma cells, mast cells, and eosinophis can also be present. The histological picture is that of a pseudolymphoma, which has to be differentiated from true lymphoma.

Most patients with BL have an elevated IgG and/or IgM antibody titer against *B. burgdorferi* (13–15). This helps to establish the diagnosis in unclear cases. The isolation of *B. burgdorferi* is difficult (15). The value of the PCR needs to be shown.

MULTIPLE ERYTHEMA MIGRANS-LIKE LESIONS

If *B. burgdorferi* spreads hematogeneously from its original inoculation site, multiple erythema migran lesions can arise at distant sites. This sign is thought to be a second-stage lesion of Lyme borreliosis. Although originally observed several times in Europe (Lipschütz 1923, Sonck 1956, Hauser 1965, cited in Reference 2), patients in the United States appear to have multiple lesions more frequently and in higher numbers than European patients (2).

Multiple erythema migran lesions can occur at any site of the skin. They are often between 5 and 15 cm in diameter but can be larger. They are either homogeneous or ringshaped. Multiple erythema migran lesions have been observed without and with other symptoms and signs of Lyme borreliosis (2–4, 6); *B. burgdorferi* has been isolated from such lesions (2).

ACRODERMATITIS CHRONICA ATROPHICANS (ACA)

Acrodermatitis chronica atrophicans was first described by Buchwald in 1883. Several case reports followed until 1902 when Herxheimer and Hartmann added 12 of their own cases and introduced the term ACA. They used previous experiences and their own experience to distinguish between an early inflammatory phase and a late atrophic phase of this skin disorder. Not only was ACA the first sign of Lyme borreliosis that has ever been described, its history is fascinating in other respects too. Already in 1910, Finger and Oppenheim included 134 cases of ACA in their textbook on atrophic skin diseases. In 1925, Ehrmann and Falkenstein found certain histologic features of ACA to resemble syphilis. Grüneberg postulated in 1952 that ACA was caused by a spirochete related to Treponea pallidum. In 1954, Götz and his three colleagues proved in a self-trial that ACA is contagious. In 1965, Hauser emphasized the relationship between erythema migrans, BL, and ACA and noted the role of ticks. Three years later, Hopf and Stroux noticed clustering of ACA in Lower Franconia, Germany, and discussed the relationship to ticks (1, 16).

The ACA is a typical sign of late, third-stage Lyme borreliosis (13, 17). The ACA usually starts on the extensor surface of an extremity. The dorsum of the hand or foot, the knee, and the elbow are particularly often affected. The ACA typically starts on the same extremity that has been affected by a previous erythema migrans or meningoradiculoneuritis (Bannwarth's syndrome), but in many cases, patients do not remember such past events. The initial lesion consists of a more or less diffuse bluish-red erythema. Swelling is not infrequently a conspicuous sign of early ACA. Swelling can occasionally be so pronounced and erythema may be so faint that the condition strongly resembles lymphedema.

As time goes by, the other extremities, buttocks, and rarely, the trunk and face may become affected. Regional and general lymphadenopathy often develops. The skin it-

self becomes more or less atrophic as the disease goes on. The atrophy finally resembles a scrambled cigarette paper and the underlying blood vessels become easily visible. Large areas of the skin can become affected in this way, but the extent of involvement is more often limited even if all extremities show signs of the disease (17).

Secondary sclerotic changes of the skin, in the form of either bands or plaques, lichen sclerosus and atrophicans-like changes, and fibroid nodules are other signs seen in association with ACA. Sclerotic plaques are most often found on the lower extremities, and linear sclerotic indurations are sometimes found on the lower arm, as so-called ulnar bands. These sclerotic changes may resemble localized scleroderma, but they differ definitely by being located within an area affected by the ACA. In rare instances, localized scleroderma can be observed at distant sites outside the ACA. Lichen sclerosus and atrophicans-like lesions may also develop within regions affected by the ACA.

Fibroid nodules clinically resemble rheumatoid nodules, but histologically, they are different. They show a homogeneous eosinophilic center surrounded by fascicles of collagen fibers and perivascular infiltrates of lymphocytes and plasma cells. The fibroid nodule is about 1 to 3 cm in diameter. Fibroid nodules sometimes occur in groups. They are mostly located in the vicinity of joints, e.g., beneath the patella, near the olecranon, and adjacent to one of the metacarpophalangeal joints. The nodules are firm and often fixed to the underlying tissue. Fibroid nodules respond surprisingly well to effective antibiotic therapy.

Patients with ACA often do not exert special complaints. However, ACA is rather frequently associated with a peripheral neuropathy (18, 19). The peripheral neuropathy is present in the areas affected by the ACA and also at distant sites.

A peculiar type of articular involvement in the form of subluxations and luxations of small joints of the hands and feet can be present (20). In 1931, Hövelborn postulated that this type of joint involvement might develop in analogy to the ACA, first as inflammatory process and later as atrophy of the affected synovia (21). Some patients have preceding or concomitant arthritis in joints of the affected extremity.

Histologically, ACA starts as a dense inflammatory dermal infiltrate consisting of lymphocytes, histiocytes, and often, plasma cells and more or less pronounced edema. As the disorder progresses, the epidermis more and more flattens, collagen and especially elastic fibers degenerate, the blood vessels are found to be dilated, and the inflammatory infiltrate becomes less pronounced. Besides the dermis, hair follicles, sweat and sebaceous glands also show atrophy. The bone marrow of patients with ACA shows reactive hyperplasia (22).

Patients with ACA have an elevated IgG antibody titer against *B. burgdorferi* (13, 17, 23). The IgM antibody titer was found to be usually negative (13, 23).

The diagnosis of ACA rests primarily on the clinical picture, which is pathognomonic in its typical appearance. The positive serological test confirms the diagnosis and helps to establish the diagnosis in unclear cases.

BORRELIAL DERMATOMYOSITIS-LIKE SYNDROME

Only two patients with borrelial dermatomyositis-like syndrome (BDM) have been described in Europe so far (24). Other patients observed previously might have had some features of this syndrome (24). The BDM in these two patients was characterized by a dermatomyositis-like clinical picture and evidence of an active *B. burgdorferi* infection. The clinical features consisted of suddenly arising, rather diffuse, bluish-red erythemas; edematous swelling of the skin; and muscle weakness. There were more or less pronounced laboratory changes indicative of an inflammatory process. The IgG antibody titer against *B. burgdorferi* was strongly elevated and the IgM antibody negative or borderline. In one patient, *B. burgdorferi* could be recovered from two different skin sites, and spirochetes were detected by silver stain in the other patient.

The histological picture of the skin resembled that of ACA. The examined muscular tissue revealed a slight to moderate lymphocytic infiltrate.

Antibiotic therapy with penicillin G, cefotaxime, and oral tetracycline cleared the condition. Knowledge of this syndrome is important, as it resembles dermatomyositis to a certain extent and is curable within a short period of time.

LOCALIZED SCLERODERMA (MORPHEA)

The sclerotic changes seen in patients with ACA and the annular or round erythematous skin lesions seen in some patients with Lyme borreliosis resemble morphea lesions to a certain degree. This resemblance led several investigators to examine whether or not morphea is due to a *B. burgdorferi* infection. The evidence for that is still controversial.

Several groups believe to have found serological hints for a borrelial origin, at least of some cases of morphea. Other groups failed to confirm these results (25). In a few instances, spirochetes were isolated from morphea lesions (25). In one solitary, longstanding lesion on the thigh of a young woman, *B. burgdorferi* was recovered (V. Preac-Mursic and K. Weber, unpublished). There is a case report of a localized scleroderma developing in the area of a previous erythema migrans (26). In conclusion, one might state that localized scleroderma seems to be induced by spirochetes, possibly *B. burgdorferi*, in a few instances. There is not enough evidence presently to link the majority of morphea cases to the causative agent of Lyme borreliosis. A similar statement can be made in regard to lichen sclerosus and atrophicans.

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