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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.

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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 presentations by authorities in the field on topics related to spirochetal and tick-borne diseases, with an emphasis on Lyme borreliosis.

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

*B. burgdorferi*—Seek and Ye Shall Find

Expanding the Envelope

Kenneth B. Liegner

The borreliae, present on this earth for eons, evolved alongside mammalian life forms in a host-parasite relationship, no doubt long before the appearance of humankind. It should not surprise us, then, that we have much to learn about the range of diseases that the borreliae may undergo and the true scope of infection of the human inhabitants of this planet.

We are at the threshold of a new and exciting era in the understanding and conquest of Lyme borreliosis. Direct antigen detection methods may strike a discordant note with antibody testing's "perfect union of the Spheres," but when clinically validated and honed to optimal sensitivity and specificity, these will be powerful tools in the exploration of the pathogenesis and clinical manifestations of borrelial disease. Researchers as well as rank and file physicians will be able to diagnose with confidence and will have measurable indices of disease activity as these tests begin to become commercially available.

However, with these advances, we will begin to perceive just how daunting an adversary the borreliae really are and how commonly affected the populace of endemic regions. It is my prediction that a much wider range of human disease, now only hinted at, and maybe even totally unexpected, will eventually be linked to borrelial infection in humans. The borreliae are resilient, phoenix-like. Cutting-edge direct antigen detection methods will corroborate the conclusions already quite apparent from the relatively few reports of culture isolation of borreliae from human subjects following antibiotic treatment now in the worldwide peer-reviewed literature (1–5). The rarity of such isolations should not lead to the conclusion that this phenomenon is rare but only that this has been difficult to prove conclusively with methods available until recently. Nocton et al. (6) demonstrated polymerase chain reaction (PCR) positivity in serial synovial fluids of 100% of patients treated with conventional oral antibiotics and in 37% of those treated with longer oral and/or intravenous antibiotic regimens. The authors opined that the presence of Bb-DNA implied viable organisms. Bradley et al. (7) had similar findings and conclusions in their study of serial synovial fluids using Bb-specific DNA detection. Borreliae are, however, tissue tropic and the absence of detection of Bb-specific DNA in body fluids does not exclude their presence in interstitial, intra-cellular, and parenchymal sites. The Rocky Mountain Laboratory antigen capture method, because it depends on the direct detection of the myriad spirochetes by each living borrelial spirochete, promises to have a far higher yield in tissues and fluids than DNA detection relying on genomic or even of multiget probes (8, 9). Systematic application of direct antigen detection methods to suspect populations will reveal the true extent of the disease and define the ratio of seropositive to seronegative cases. Seronegativity may be due to T cell anergy and not only as a result of early application of antibiotic therapy (10). Claims that seronegative Lyme disease is rare or that it is common are currently unverifiable.

A tissue repository should be established for tissues from humans suspected of borrelial disease, including autopsy materials, for in depth study using all currently available classic histologie as well as cutting-edge research methods. The pathologist is, after all is said and done, the final arbiter of truth in clinical matters (11–13).

With recognition of chronic persistent infection, we will begin to look at disease pathogenesis quite differently. A persisting pathogen may induce noxious injury over not just days, weeks, or months, but years and decades, and even the natural life of the host. Slowly simmering infection can induce a wide variety of host responses, both direct and immune-mediated. The treatment approach may need to be very different in this circumstance than for a readily extirpated bacterium such as staphylococcus or streptococcus (14–17). Chronic infection may require chronic treatment. Definitive cure, while theoretically possible, may not be achieved using currently available methods in chronically infected patients. This dilemma should prompt a determined effort to develop definitive means of curing the infection (18, 19).

A focus on problems associated with prolonged antibiotic therapy (20) has diverted attention from the much more ominous and insidious spread of borrelial disease in the general population. This vast de facto and unintended "Tuskegee" experiment of nature has far greater long-term societal impact in terms of personal suffering, economic loss (21), disability, and death (13, 22) than complications of intensive treatment for a serious disease that are, to some degree, unavoidable.

Often, sequelae of borrelial disease are treated as independent disease entities without being traced back to the inciting etiology. For example, Goodman and colleagues found an incidence of seropositivity for *Borrelia burgdorferi* four times higher amongst patients awaiting cardiac transplantation for chronic congestive cardiomyopathy compared to a control population (23). Yet, this very costly and debilitating illness and the fabulously expensive cardiac transplantation and its aftermath is not counted in the economic impact of Lyme disease. Dementing diseases are amongst society's costliest illnesses. What percentage of patients requiring placement in long-term care facilities for organic brain syndromes really represent unrecognized and untreated end-stage chronic neuroborreliosis (24)? This question deserves to be answered.
Lyme disease should be included in the differential diagnosis of a wide range of neurologic syndromes. The National Neurologic Research Bank holding tissues for various neurologic diseases including multiple sclerosis, Alzheimer's disease, and motor neurone disease should begin to be systematically evaluated for evidence of borrelial etiology and pathogenesis using the newer direct antigen detection methods (25). Theoretic conceptualizations already developed for other "slow" infections of the central nervous system (CNS) may find application in borrelial disease of the CNS (26). In depth evaluation of some patients previously thought to have multiple sclerosis has uncovered significant evidence of CNS and/or systemic borrelial infection and good response to intensive antibiotic treatment (27, 28).

The role of borrelial infection also needs to be systematically studied using modern methods in psychiatric syndromes (29) (including derangements leading to domestic violence, suicide, and homicide), attention deficit disorder, various arthritides, "idiopathic" or "autoimmune" diseases, chronic fatigue syndrome, and fibromyalgia. Stunning electron photomicrographs of Haupl and colleagues demonstrate Lyme spirochetes nested parallel to collagen fibers from synovial tissue removed from a patient previously intensively treated for Lyme disease (3) (see Fig. 1). Klampner and colleagues' beautiful confocal photomicrographs conclusively prove intracellular localization of borreliae within human fibroblasts (30) (see Fig. 2). Is it not likely then, that Lyme disease-associated fibromyalgia with pain localized to fibroblast-produced collagen-rich fasciae and entheses may not be due to the persistence of living borreliae? Although their conclusions were otherwise, the data of Dinerman and Steere clearly demonstrated antibiotic responsiveness of symptoms in their series of patients with Lyme disease-associated fibromyalgia (31).

While the reality and extent of chronic persistent infection needs to be more widely recognized, it also must be acknowledged that there may be self-perpetuating immunemediated mechanisms of injury that may coexist with active infection or be operative following eradication of the pathogen. For the latter circumstance, antibiotic treatment of a prolonged nature would be futile. Sorting out which cases are due to chronic persistent infection and which are not will be a major achievement of direct antigen detection methods. Creative immune-modulating interventions may prove more effective in inducing remission and averting ongoing injury than antibiotic treatment for this subset of patients, or these may have a combined role with antibiotic therapy in patients having active infection (32).

We should be humble before this disease. Until there is general agreement on a "gold standard" for diagnosis of active Lyme disease, presently available standard and research assays must be viewed as approximations of the truth only (33). Likewise, limits placed on the geographic range of the infection must be greeted with extreme skepticism, as success in documenting the nearly ubiquitous borreliae in a given natural setting depends largely on the determination and experience of the investigator.

At the present time, there exists no substitute for the clinical judgement of an experienced treating physician knowledgeable about the manifold presentations of the disease, adept in listening to the patient and in observing, as in other natural phenomena, the interaction of host and pathogen. Skillful application of antibiotics continues to be the mainstay of treatment for what is, first and foremost, an infectious disease.

Science is all about measuring things. Once objective measures of disease activity are widely available, rational approaches to treatment will replace those based on convention or blind obedience to authority, and the medical neglect now so frequent in chronic Lyme borreliosis will take its well-deserved place in the history of medicine, and not in modern practice.

REFERENCES

VI INTERNATIONAL CONFERENCE ON LYME BORRELIOSIS

The International Conference on Lyme Borreliosis is organized every two years. Following the 1992 meeting in Arlington, VA, this year’s conference was held in Bologna, Italy on June 19-22. The conference was attended by approximately 500 people, which is the most numerous (and therefore representative) gathering on Lyme borreliosis in the world. The Journal of Spirochetal and Tick-borne Diseases has asked conference attendee Doctor Donta to give us his view on the conference. Sam T. Donta, M.D., is Professor of Medicine, Infectious Diseases, and Molecular Medicine at Boston University and Boston VA Medical Centers.

Question:

Russell C. Johnson opened the Conference with the state-of-the-art lecture "Lyme Disease—Past, Present and Future." How do you personally see the present state of Lyme disease and its current trends?

Answer:

The present state of Lyme disease is beset by many problems in the areas of diagnosis and treatment. The pathogenesis of Lyme disease is also poorly understood and may remain difficult to elucidate because the human disease may not be similar to animal models. Especially difficult will be the elucidation of the pathogenesis of the neurosensory components (e.g., paresthesias, cognitive dysfunction, pain, and fatigue) not easily studied in animal models.

Advances in serologic assays to help in the diagnosis of Lyme disease are needed and should be possible, using recombinant DNA technology to develop a pool of antigens (e.g., 23kD, 31kD, 34kD, 39/41kD) for use in ELISA tests to provide treated specificity than current, whole-organism-based ELISAs. These tests may be of limited value, however, if humoral immunity does not adequately reflect the most important host responses to infection with the Lyme borrelia. For example, some patients with strong IgG responses are asymptomatic, whereas others with similar responses are symptomatic. Then there are those patients who appear to have Lyme disease by clinical criteria who have weak or limited humoral responses ("seronegative Lyme") in whom the diagnosis cannot be confirmed or dismissed.

The treatment of Lyme disease also leaves a lot to be desired. Treatment of the earliest recognizable stage of Lyme disease (i.e., the ECM rash) is associated with a high degree of success, whereas treatment of symptoms that occur later is not uniformly successful. Issues such as which antibiotic regimen and duration of therapy need to be resolved by well-designed, placebo-controlled, clinical trials. An understanding of the pathogenesis of the disease and the nature of any latent infection will likely be key to advances in this area.

The development of vaccines to prevent Lyme disease is a needed area of research. It was gratifying to see the data that support the use of the whole-killed cell vaccine in veterinary medicine. Similar whole-cell vaccines should also be tested in humans, but manufacturers have chosen instead to develop a univalent, outer surface protein A (OspA), vaccine. The results of experiments in animals are encouraging, and similar results in humans would be most welcome.

Question:

The sessions on biology and genetics documented the differing interests of American and European researchers: in the United States, OspA is predominantly researched versus primary interest in outer surface protein C (OspC) in Europe. Please compare the two approaches.

Answer:

Study of the role of each of the outer surface proteins is important in our understanding of the biology of the Lyme spirochete and the pathogenesis of Lyme disease. Work with OspA has led to the development of a potential vaccine, whereas work with OspC suggests that this is the earliest surface protein that is recognized by infected individuals. The available evidence does not support the idea the European strains are OspC rich and American isolates OspC poor, nor is there compelling evidence that European isolates differ from American isolates. A more interesting hypothesis is that there are arthritogenic strains and neurotropic strains, but the evidence for this is not strong at present.

Question:

What was the most important announcement in the area of basic science? Is there a significant clinical application for that research?

Answer:

There were several important developments in the areas of basic science directed toward the understanding of the biology of the organism and pathogenesis of the disease. One of the most notable reports was that by Klimpper et al. who elegantly demonstrated that Borrelia (B.) burgdorferi, which do not possess intrinsic proteolytic enzymes, are capable of binding host plasmin and urokinase enzymes, which resultant proteolytic activities in vitro and enhanced pathogenicity in a mouse model. Although a clinical application has not yet been established, discoveries such as this may allow us to eventually devise means to prevent or abrogate the infectious process.

Question:

The session on immunity deliberated the long stretch from uncertainties about B. burgdorferi biology understanding to human massive vaccination trials. How would you judge developments in this area? In what way(s) was your opinion supported or disputed at the general discussion on vaccine development?
Answer:

With any vaccine designed to prevent infection by a particular microbe, one of the difficult issues is the importance of any antigenic variation by the organism. Influenza A virus, for example, is capable of major antigenic shifts, thus requiring annual changes in the vaccine used to protect susceptible individuals. The major surface proteins (OsP and OsPc) of B. burgdorferi vary antigenically in isolates derived from differing geographic areas, but the extent to which this variation contributes to host immunity and development of a successful vaccine needs further evaluation. With OsPc, there is a highly conserved region among different isolates, and if immunity to this part of the protein is associated with protection from disease, then antigenic variation in a different part of the protein might not be of any significance for vaccine development. Conversely, if antigenic variation is an important issue, then attention will need to be given to the development of multivalent vaccines, similar to that used for the development of pneumococcal polysaccharide vaccines.

One intriguing report in the session on Immunity that is of potential importance, not only in our understanding of the pathogenesis of the disease but in vaccine development as well, describing the binding by IgM antibodies of gangliosides in neural tissues. If this "autoimmune" reaction is associated with the development of certain neurological deficits or dysfunction (e.g., paresthesias, cognitive deficits, pain), further research may lead to means to prevent or interfere with this response. As relates to vaccine development, one potential danger would be the unanticipated development of antibodies to neural tissue, with resultant nerve damage or dysfunction. It will be important to delineate which borrelial antigens are responsible for the development of this antiganglioside antibody.

Question:

Various theories have been suggested for the pathogenic mechanisms employed in Lyme infection. The Lyme disease research community's approach toward this issue seems to be fragmented. In your opinion, is the community approaching this issue properly, or should we be focusing attention differently in this matter?

Answer:

Investigation of any and all areas relating to the pathogenesis of Lyme disease is important. Greater attention should be devoted, however, to an understanding of how host cells other than professional phagocytes, such as neural cells and endothelial cells, that may be the target and reservoir for borrelial infection, deal with the intracellular organisms. Understandably, researchers with an interest in one particular area will be apt to investigate matters that relate to that area; for example, a rheumatologist and immunologist will be more likely to examine immune responses, and a researcher who studies macrophages will examine phagocyte-parasite interactions.

Also, a problem previously mentioned is the limited applicability of animal models to the human disease. Therefore, there are several areas in addition to pathogenesis (e.g., diagnosis, treatment, vaccines) in which greater focus is needed on clinical research.

Question:

P. Abelard's "Four Rules for Argument and Investigation" to evaluate the clinical part of the meeting states, "Use systemic doubt and question everything. Learn the difference between statements of rational proof and those merely of persuasion." How would you apply these rules to the clinical part of the Conference?

Answer:

In the evolution of the understanding of any "new" diseases, it seems unfortunately inevitable that dogmatic statements are made, and premature conclusions reached before the true nature of the disease is appreciated. This has happened and continues to occur with Lyme disease, in which case the definition of the disease, an understanding of the natural course and sequelae of the disease, and treatment of the disease have been subjected to polarized statements and insupportable conclusions. For example, circular reasoning has been used to define the disease; i.e., those with positive ELISA tests have the disease while those without do not; instead, the clinical spectrum of disease should have been and should be delineated, and better criteria for the definition should be developed. As well, no controlled treatment trials have been conducted to examine whether or not different antibiotics and longer duration of therapy are of value in the management of patients with persistent symptoms. It has been understandably difficult to know how to deal with patients who have sensory complaints (e.g., pain, paresthesias, fatigue) without physical findings, including patients who originally met the standard definitions of Lyme disease, but dismissing these complaints or rediagnosing them as having chronic fatigue or fibromyalgia seems illogical and impertinent. Greater efforts are needed by both researchers and clinicians to understand and better deal with these related clinical problems.

Question:

Standardization of Lyme disease serodiagnosis was the most important topic discussed in the session on diagnostic tests. What is your opinion on a standardized two-test (FLA-ELISA-Western blot) approach, proposed by the Centers for Disease Control and Prevention? (Note: Readers not familiar with the proposed criteria are referred to the CDC Lyme Disease Surveillance Summary, Vol. 5 (1).)

Answer:

Until ELISA tests are redesigned and proven to be of value, standardization of existing tests will be a futile and potentially harmful "innovation" in attempting to diagnose Lyme disease. The current ELISAs utilize whole organisms, which de facto are going to be associated with cross-reactions. Therefore, only very highly reactive antibodies will be detected, which may be diagnostic for only some, but not all or most, individuals with Lyme disease. ELISAs based on a pool of recombinant antigens with higher specificity (see answer to the first question) should provide more reliable results but will be reliable only if found to correlate with patients who have Lyme disease based on the clinical picture. Western blots are currently more accurate but are more tedious to perform, are of limited value because the results are more qualitative than quantitative, and are not readily standardizable. And if humoral antibody responses are not well correlated with the course and symptomatology of late Lyme disease, as, for example, might be cellular-immune responses, then the exercise in standardization of seroassays will indeed be futile.
Question:

Russell C. Johnson said at one point in his opening presentation, "... the future looks bright." Is "bright" the way you see the future for Lyme disease?

Answer:

Based on our current state of affairs, it seems difficult to see a bright future for Lyme disease. The diagnosis of Lyme disease is limited by inaccurate seroassays and PCR assays that may not be adequate to detect persistent infection in either certain sites or in intracellular, latent infections. The treatment of Lyme disease, especially in the later stages, is unsatisfactory. Nonetheless, support of research in Lyme disease has led and should continue to lead to a greater understanding of the basic biology of the organism and the pathogenesis of the disease. Vaccine development may hold the promise of an effective means to prevent the disease. A greater open-mindedness and less dogma is needed in the scientific community to help to better direct and design research that will eventually result in better control of Lyme disease.

The questions were prepared by Martina Ziska, M.D.
Fig. 1 A “triangular” shaped erythema migrans on a 28-year-old woman in the third trimester of pregnancy. She was treated with antibiotics with rapid clearing of the rash and with no sequelae of later Lyme disease complications. The baby was born uneventfully and has remained seronegative and completely well. (Courtesy J. Katzel, M.D.)

Fig. 2 A “comma” shaped erythema migrans on a 15-year-old woman in the first trimester of pregnancy. Antibiotics were dispensed early, but the patient was noncompliant and subsequently lost to follow-up. The clinical course is unknown, and the results of the pregnancy are unknown. (Courtesy J. Katzel, M.D.)

Fig. 3 A “double bubble” erythema migrans (EM) on a 60-year-old man with two, side by side, bites from two *Borrelia* infected *Ixodes* ticks. He had no other complaints or systemic illness, presenting only with these two asymptomatic rashes. Antibiotics cleared the EM lesions, and the patient has had no sequelae of later-stage Lyme disease in 4 years of follow-up. (Courtesy J. Katzel, M.D.)
FIG. 4 As opposed to the three cases of solitary erythema migrans lesions representing early localized infection shown on the previous page, this case demonstrates dissemination of the disease, evidenced by multiple erythema migrans and systemic illness. This 35-year-old Asian woman developed erythema migrans on the chest at the site of an infected tick bite. Of note is the shading differences between tanned and untanned skin. The punctum of the tick bite was directly on the tan line. A secondary erythema migrans (not caused by a second bite) is seen on the contralateral upper arm, presumably a result of hematologic spread. The patient was febrile, severely fatigued, had myalgias and arthralgias, cystitis, and malaise. A 4 month course of antibiotics resolved the skin lesions and the systemic symptoms. At 1 year follow-up, she remains seropositive but is back to her normal state of health and all activities.
(Courtesy J. Katzeln, M.D.)
Figs. 5 and 6 Neurologic complications, both peripheral and central, are commonly associated with Lyme disease, in early as well as in disseminated disease and in chronic persistent disease. Bell’s palsy shown here is one of the most common neurologic presentations. This temporary paralysis of the 7th cranial nerve, known as the facial nerve, may follow trauma, viral infections, or infected tick bites. In areas endemic for Lyme disease, antimicrobial antibiotics, rather than steroids, may be the preferred treatment for Bell’s palsy, even when the etiology is obscure or unknown.

(Courtesy J. Katz, M.D.)
Fig. 7. Fibromyalgia or fibrositis is shown here. This 45-year-old man appears older than his age because of his uncomfortable, stiff, hunched over posture, which has been unrelenting. (Courtesy R. Ritter, P.A.)

Fig. 8. Recurrent Lyme disease induced true arthritis and synovitis with effusion of the knee, shown here in this 65-year-old man with Lyme disease antibody titers of 1:65,000. Synovial fluid was PCR positive. Antibiotics seem to hasten the resolution of the swollen knee but have not protected him from recurrent episodes of arthritis. (Courtesy J. Katzel, M.D.)

Fig. 9. This case of Acrodermatitis Chronica Atrophicans (ACA) was provided by Dr. R.J. Scrimanti. Here we see violaceous edematous skin and bilateral fibrous bands in the pretibial skin area of the lower legs. Although infrequently reported in the United States, ACA does occur but must be carefully searched for to be appreciated as a chronic late-stage manifestation of Lyme disease. (Courtesy E. Aberer, M.D.)
Immune Capture and Cultivation of *Borrelia burgdorferi*

David W. Dorward, Ph.D.,* and Claude F. Garon, Ph.D.

Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Microscopy Branch, Hamilton, Montana

The routine difficulty in isolating *Borrelia (B.) burgdorferi* from mammalian hosts complicates the accurate diagnosis of Lyme disease and obscures efforts to monitor the effectiveness of treatments. Factors that contribute to the difficulty include the apparent sparse distribution of the spirochetes in hosts and the extended generation time of primary isolates, both of which favor growth of chance bacterial and fungal contaminants. In order to concentrate *B. burgdorferi* from complex mixtures, and reduce numbers of contaminating bacteria, we developed a system that allows for the capture and subsequent cultivation of the spirochete. The system immobilizes the spirochetes within sterile glass capillary tubes, which have been coated on the interior surface with cell-surface directed polyclonal rabbit IgG antibodies. After thoroughly rinsing tubes containing captured spirochetes, the tubes are placed in culture media for cultivation. *In vitro* results showed that between 10 and 100% of spirochetes in a sample can be captured and propagated, whereas the numbers of contaminating bacteria can be reduced by 100 to 1000 fold. In an experiment involving three infected *Ixodes scapularis* ticks, two mice infected by inoculation with virulent low-passage *B. burgdorferi*, and two mice inoculated with avirulent high passage *B. burgdorferi*, spirochetes were recovered without contamination from one of three tick midgut samples, the spleen of one and the urinary bladders of both mice infected with low passage spirochetes. Without use of the capillary tubes, all three tick samples rapidly became contaminated, and spirochetes were not recovered from the infected mice. No growth of spirochetes or other bacteria occurred in preparations from mice challenged with high passage spirochetes. These results suggest that the system may facilitate primary isolations of *B. burgdorferi* from ticks and mammals.

Key words: Lyme disease, Ticks, Spirochetes, Isolation, Pure culture

INTRODUCTION

Since the demonstration of *Borrelia (B.) burgdorferi* as the infectious agent of Lyme borreliosis (1), numerous studies cited in two comprehensive reviews have documented the difficulty of culturing the spirochetes from or observing spirochetes in infected mammalian hosts (2, 3). Factors such as the reportedly sparse distribution of *B. burgdorferi* in mammals, the fastidious growth requirements, and the relatively slow growth rate of this spirochete compared to potential bacterial contaminants compound the problems associated with aseptic primary isolations (1–4). These problems have prompted several attempts to develop and define media, which optimize maintenance and growth of *B. burgdorferi*, while minimizing chance contamination with antibiotics and filtration (5–8).

A recent study demonstrated that intact spirochetes, which occur at an apparently low density in tissues and fluids of infected hosts, can be captured and retained on surfaces containing adsorbed antibodies directed toward cell-surface extracellular *B. burgdorferi* antigens (9). That study also found relatively few contaminating organisms or host cells adhering to antibody-activated surfaces, suggesting that the antibodies effectively blocked nonspecific adherence to the surfaces.

In order to determine whether such selective immobilization of *B. burgdorferi* cells could be exploited for aseptic capture and cultivation of this spirochete, we designed and developed an antibody-activated, capillary tube-based selective culture system. In this system, IgG antibodies, generated against extracellular membrane vesicle concentrates, or against an extracellular 83 kDa multiprotein complex, which had previously been shown to recognize the OspA and OspB proteins and had proven specific for *B. burgdorferi* (9, 10), were adsorbed onto the inner surface of Caraway capillary tubes. Suspensions of spirochetes containing or lacking contaminating bacteria or specimens from infected hosts were drawn within the tube and incubated. After expelling the contents of the tubes, the tubes were washed with sterile buffer and placed in culture medium containing or lacking antibiotics. This paper describes results that show that *B. burgdorferi* can be selectively and aseptically cultured from *in vitro* and *in vivo* samples using this system.

MATERIALS AND METHODS

**Bacteria**

*Borrelia burgdorferi* strain Sh-2-82 was maintained in BSK II media as previously described (4). The infection studies described were performed using organisms from *in vitro* passage 7 (low passage) and &gt;250 (high passage). *Escherichia (E.) coli* and *Staphylococcus (S.) aureus* strains 11775 and 12600 (American Type Culture Collection, Rockville, MD) were grown at 37°C in Luria broth. All cultures were harvested at mid- to late log phase.

**Experimental infections**

Three adult *Ixodes scapularis* ticks and four RML white mice were infected with *B. burgdorferi*, as previously described (11). Tick infections were verified by dark field microscopy prior to primary isolation experiments. After 6 weeks, the mice were sacrificed and the urinary bladders and spleens were recovered and triturated, as previously described (11). Midguts from experimentally infected ticks were also excised and prepared for culture, according to previously published procedures (1, 4). One-half of each sample was inoculated into BSK II, and the remainder was

*Corresponding author
utilized for capillary tube isolation procedures described below.

**Capillary tube preparation, immune capture, and cultivation procedure**

An apparatus capable of both manipulating Caraway capillary tubes (Fisher Scientific, Pittsburgh, PA) and repeatedly filling and evacuating the tubes was designed and constructed for this study (Fig. 1). The figure shows a micropipettor equipped with a 1 to 200-μL polypropylene tip, which has been clipped off to allow insertion of a capillary pipet sleeve (Baxter Healthcare Corporation, McGaw Park, IL). Figure 2 depicts a schematic diagram of the immune capture and cultivation procedures. Caraway capillary tubes were cleaned and etched by immersion in 1 N NaOH for 1 hour at room temperature. After thoroughly rinsing in distilled water, the tubes were sterilized by autoclaving and dried. Once dried, the tubes were "activated" with antibodies as follows. Purified IgG antibodies derived from rabbit serum generated against extracellular membrane vesicle concentrates or against a gel-purified, 83-kDa extracellular protein band (9, 10) were dissolved at 1 mg per mL of 0.1 M ammonium bicarbonate and filter-sterilized through 0.2-μm membranes (Anotoe Separations Ltd., Banbury Oxon, UK). A 200-μL volume of each antibody solution, or of 0.1 M ammonium bicarbonate, was drawn aseptically into the capillary tubes and expelled. Each tube was then placed horizontally into a sterile container, and the tubes were dried under vacuum at room temperature. Once dried, the tubes were either used immediately or stored at −20°C. For some experiments, ammonium bicarbonate was replaced by Dulbecco’s phosphate-buffered saline pH 7.2. However, tubes coated with phosphate-buffered saline were used immediately because drying and storage left salt deposits in the tubes.

Following such preparations, antibody-activated or nonactivated capillary tubes, treated with buffer only, were used for isolating and culturing *B. burgdorferi* from in vitro cultures and in vivo sources. For in vitro experiments, pure cultures of *B. burgdorferi*, or mixed cultures of *B. burgdorferi* and either one or both *E. coli* and *S. aureus*, were titrated by 10-fold dilutions into sterile 12 × 100 mm culture tubes (Becton Dickinson Co., Oxnard, CA). The total number of bacteria used in each experiment was estimated by endpoint dilution. The final volume in each culture tube was 2 mL. Following dilution, activated or nonactivated capillary pipets were placed in the tubes and incubated at room temperature. After 1 hour, the tubes were removed and washed by drawing 200 μL of sterile 0.1 M ammonium bicarbonate into the tube and expelling the buffer. Five such washes were performed. The tubes were then placed into 12 × 100 mm culture tubes containing 3 mL of BSK II medium and incubated at 35°C for up to 3 weeks or until growth was evident in the tubes. The presence of spirochetes in such cultures was verified by dark-field microscopy. The percentage recovery of spirochetes from such experiments is given as a range of values indicative of the most dilute tube from which spirochetes were captured and cultivated compared to the endpoint dilution tube containing viable spirochetes (i.e., if the endpoint of a dilution series occurred at 10^−2 and spirochetes were successfully captured and cultivated from the same dilution, the efficiency was 10 to 100%).

For capture and cultivation of spirochetes from in vivo sources, 100 μL of triturated mouse tissue, or tick midgut preparation was drawn into an activated capillary tube. The tube was then placed horizontally into a sterile beaker and incubated for 1 hour at room temperature. Following incubation, the tubes were repeatedly washed in ammonium bicarbonate and placed in tubes containing culture medium.
as described above. An equal volume of tritrate was also placed directly into culture tubes containing 3 mL of medium.

RESULTS

Previous work had shown that surfaces activated with F(abs) fragments or IgG antibodies, directed against B. burgdorferi extracellular membrane vesicle concentrates, could capture B. burgdorferi cells and antigens and concurrently reduce adsorption of an excess of biological material from other sources to these activated surfaces (9). In order to determine whether such activated surfaces could be adapted to the capture and culture of B. burgdorferi, we designed and constructed an apparatus for manipulating capillary tubes, which could be activated and utilized for these purposes (Figs. 1 and 2). We found that use of this instrument facilitated repeated aseptic filling and expelling of precise volumes of the solutions and suspensions used in these experiments.

In order to determine whether antibody-activated capillary tubes could be used to capture and cultivate B. burgdorferi cells, we incubated capillary tubes activated with either anti-vesicle or anti-83 kDa IgG antibodies, or non-activated capillary tubes, in culture tubes containing serial 10-fold dilutions of log-phase cultures, and then we rinsed and transferred the capillary tubes into fresh medium. We also examined whether use of the tubes allowed recovery of spirochetes from medium intentionally contaminated with E. coli. In these experiments, cultures were supplemented with 50 µg of rifampin per milliliter of medium in order to mimic conditions used for primary isolations from ticks and mammals (4). The results are shown in Table 1.

Table 1 shows the inverse log values of dilutions from which growth of either B. burgdorferi or E. coli occurred after transfer into fresh culture medium. Each value is the average of two dilution experiments. At least 10³ spirochetes per milliliter of medium were present in the original culture. Without antibody activation, 2 to 3 logs fewer spirochetes were recovered in cultures incubated by capillary tube transfers. Transfers using capillary tubes coated with either antibody dissolved in phosphate-buffered saline resulted in 10 to 100% recovery of B. burgdorferi. Recovery of 1 to 10% of spirochetes was observed using antibodies dissolved in ammonium bicarbonate. When approximately equal numbers of E. coli cells were added to B. burgdorferi cultures and the mixtures were serially diluted, growth of E. coli was evident overnight. No attempt was made to quantify spirochetes in such mixtures. As we found with pure B. burgdorferi cultures, the numbers of E. coli recovered after transfer via nonactivated capillary tubes were reduced by 2 to 3 logs. Approximately the same reduction in numbers of E. coli occurred using antibody-activated tubes. In several cases, using either antibody dissolved in either buffer, pure cultures of B. burgdorferi were recovered from the mixtures at dilutions greater than those resulting in growth of E. coli. Because of these results showing purification of B. burgdorferi in both buffer systems, and because of the preparative advantages cited above, we used ammonium bicarbonate systems for all further experimentation.

We also examined whether deletion of rifampin from BSK II medium affected the recovery of B. burgdorferi from mixed cultures (Table 2). In these experiments, we added S. aureus to mixtures of E. coli and B. burgdorferi. Preliminary experiments had shown that S. aureus, a common contaminant of skin biopsies, was inhibited by BSK II containing rifampin (data not shown). Like Table 1, Table 2 lists the average inverse log value of endpoint dilutions containing viable bacteria from four dilution experiments. As described above, capillary tubes prepared without antibody reduced the transfer efficiency of B. burgdorferi by 2 to 3 logs, and the recovery of B. burgdorferi from diluted cultures using antibody-activated capillary tubes ranged from 10 to 100%. Adsorbed antibodies also reduced the numbers of E. coli and S. aureus present in capillary tube transfers from mixed cultures by more than two logs. Furthermore, pure cultures of B. burgdorferi were recovered from two of four and three of four bacterial mixtures, respectively, using capillary tubes activated with antisnecile and anti-83 kDa antibodies.

For an in vivo analysis, we examined whether antibody-activated capillary tubes could assist in the recovery of B. burgdorferi from laboratory-infected mice and ticks. For analysis of ticks, the midguts of three infected ticks were removed, examined by dark-field microscopy for spirochetes and cultured both by direct inoculation of medium and by the capillary tube methods. After 24 hours, all three samples cultured directly were visibly contaminated with bacteria, whereas no contamination was evident in the capillary tube cultures. After nearly 2 weeks, two of the three capillary tube cultures showed fungal contamination. We recovered B. burgdorferi from the remaining culture. In a mammalian experiment, bladders and spleens from two mice

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<th>Table 1</th>
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<td>**Inverse Log of Endpoint Dilution Resulting in Growth of **( \text{Borrelia burgdorferi} ) or <strong>Escherichia coli</strong></td>
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<tr>
<td><strong>Sample</strong></td>
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<tr>
<td><strong>B. burgdorferi</strong></td>
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<tr>
<td>Original culture</td>
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<tr>
<td>Tubes-buffer</td>
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<td>Tubes/anti-vesicle</td>
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<td>Tubes/anti-83</td>
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<tr>
<td><strong>B. burgdorferi and E. coli</strong></td>
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<td>Tubes-buffer/anti antibody</td>
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<td>Tubes/anti-83</td>
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ND = not determined (contaminated).

* B. burgdorferi recovered from two of two mixed cultures.

† B. burgdorferi recovered from one of two mixed cultures.
infected with virulent spirochetes and two mice inoculated with high avirulent spirochetes were supplied as "blind" samples by Dr. Tom Schwan (Rocky Mountain Laboratories). These samples were also cultured with and without use of antibody-activated capillary tubes. Pure cultures of *B. burgdorferi* were recovered from both bladders and one spleen from animals infected with low passage organisms, whereas no isolates were obtained from direct inoculations into culture medium. Spirochetes were not cultured from animals inoculated with high passage spirochetes by either method.

### DISCUSSION

The results of this study showed that antibodies directed against certain surface components of a fastidious pathogen can be used to capture the microorganism from potentially contaminated source material onto surfaces that can then be used to transfer the captured spirochetes into sterile medium for aseptic cultivation.

Initially, we found that surfaces activated with antibodies dissolved in phosphate-buffered saline could be used to capture spirochetes and successfully inoculate fresh growth medium. However, such tubes had to be used immediately or stored wet, due to extensive salt deposits that remained after drying. Therefore, we examined whether the volatile buffer, ammonium bicarbonate, was suitable for these experiments. We found that ammonium bicarbonate could readily be removed under vacuum, presumably leaving an antibody film on the inner surface of the glass Caryawan pipes. We also found that pretreating the glass with 1 N NaOH allowed for retention of an even layer of antibody solution within the tubes after expulsion of the majority of the solution. Without this treatment, or after acid treatment, the antibody solution formed beads on the glass surfaces (data not shown).

Using procedures described in the text and diagrammed in Figs. 1 and 2, we found that *B. burgdorferi* could be captured and propagated from pure and mixed cultures using antibody-activated capillary tubes. Comparisons between buffers used to coat the tubes suggested that tubes coated with antibody dissolved in phosphate-buffered saline were slightly more efficient than tubes coated with antibodies in ammonium bicarbonate. This was true for both retention of *B. burgdorferi* and exclusion of contaminating *E. coli*. Whether ammonium bicarbonate reduced the adherence of antibody to the glass surfaces, reduced the activity of the antibodies, or killed a proportion of attached spirochetes is unclear. However, because tubes produced using ammonium bicarbonate allowed recovery of *B. burgdorferi* from a mixture of *B. burgdorferi* and *E. coli* and provided superior storage capabilities, ammonium bicarbonate was used as a buffer in subsequent experiments.

We also found that antibody-activated capillary tubes were capable of isolating *B. burgdorferi* from mixed cultures, without the use of supplemental rifampin (4). In these experiments, we included *S. aureus* along with *E. coli* in mixed cultures. Although overnight growth of the serial dilutions suggested nearly a 10-fold excess of contaminating bacteria in the original mixture, *B. burgdorferi* was successfully recovered from most samples using activated capillary tubes. Thus, both in systems supplemented with or lacking rifampin, the capillary tubes coated with antibodies enabled purification of *B. burgdorferi* from mixed cultures at nearly the same rate as from a diluted pure culture of *B. burgdorferi*. Using tubes lacking antibodies, recovery rates from pure cultures were reduced by 100 to 1000 fold, and we were unsuccessful in recovering *B. burgdorferi* from mixed cultures.

An earlier study had shown that both anti-vesicle and anti-83 kDa antibodies bind to *B. burgdorferi* whole cell and extracellular antigens (9). However, the anti-vesicle antibodies also bound to *B. hermsii* and mammalian antigens, whereas the anti-83 kDa antibodies bound only to antigens specific to geographically diverse isolates of *B. burgdorferi* (*sensu lato*) (9). Therefore, we used anti-83 kDa antibodies exclusively for experiments involving recovery of spirochetes from tick and marine samples. No isolates were obtained by direct inoculations of media with preparations from these sources. It is unknown whether such *in vitro* results with log-phase cultures correlates well with spirochetal cell surface interactions *in vivo*. However, *B. burgdorferi* was isolated from one of three ticks and three of four samples from infected mouse organs, using the activated capillary tubes. Whether the recovery from ticks could be improved by adding an antimycotic to primary culture medium remains to be determined.

Taken together, these results show that *B. burgdorferi* can be captured onto antibody-activated surfaces and cultivated by transferring such surfaces into culture medium. Furthermore, the results suggest that systems based on such capture, including the capillary tube methods described here, may facilitate aseptic primary isolation of *B. burgdorferi* from ticks and mammalian samples.

As stated above, this study did not include experiments...
designed to maximize the efficiency of recovery of *B. burgdorferi* from diverse samples. We presume that experimental variables could be defined that increase the probability for successful isolation of this organism or that target other organisms for cultivation. For example, development of systems utilizing substrates such as polystyrene, with enhanced antibody-binding affinities might increase the capacity for capturing organisms (12). Similarly, repeated contact of comparatively large volumes of source material to activated surfaces might allow retention of additional targeted microorganisms, providing an increased chance of effective isolation. Furthermore, since rinsing apparently reduces the level of contaminants from immobilized spirochetes, it is likely that such immune capture methods would facilitate amplification of *B. burgdorferi* DNA target sequences by polymerase chain reaction. Improvements in these areas may have significant impact on the ability to detect and obtain isolates of pathogenic or free-living microorganisms for genetic, pathogenic, therapeutic, or other experimental purposes.

We thank Dr. Willy Burgdorfer and Dr. Tom Schwan for providing tick and mouse material, respectively. We also thank Dr. Burgdorfer and Dr. Kenneth Gage for critical review of this manuscript, Mr. Robert Evans and Mr. Gary Hettrick for graphic support, and Ms. Betty Kester for manuscript preparation.

Reprint requests: David W. Dorward, Department of Health and Human Services, Rocky Mountain Laboratories, Microscopy Branch, Hamilton, MT 59840.

REFERENCES

Epidemiology of Lyme Borreliosis in Southern Germany during the Years 1987 through 1990

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The purpose of this study was to further complete epidemiological data about Lyme borreliosis reported in a former study in the period from 1983 until 1986. Investigated characteristics were age and sex distribution, incubation period, seasonal cumulation, and prevalence of IgM and IgG antibodies among seropositive patients.

From 1987 until 1990, 74,517 samples were investigated for antibodies to *Borrelia (B.) burgdorferi* by immunofluorescence assay. Of these, 1200 seropositive patients were included as clinically defined cases of Lyme borreliosis: 37% showed erythema migrans (EM); 1.1% showed lymphocytoma cutis benigna; 1.3% showed carditis; 33% showed neurolymeborreliosis stage II (NLB II); 15% showed arthritis; and 12% showed acrodermatitis chronica atrophicans (ACA). Seventeen percent of the patients had more than one clinical manifestation. Among adults, more women than men had EM and ACA, whereas more men had NLB II and arthritis. As to EM and NLB II, a bimodal distribution was observed with one group of patients over 40-years-old and another one of children under 15-years-old. The incubation period for 38% of EM cases was less than 1 week, and for 45% of NLB II patients, it was between 2 and 5 weeks. Seasonal onset of EM was between May and August and of NLB II between June and August. In EM patients, IgM antibodies prevailed IgG antibodies during the first 6 weeks; NLB II patients, however, more frequently showed IgG antibodies than IgM antibodies from the beginning of the disease. Sera from children with NLB II only were more often IgM positive than IgG positive during the first 6 weeks.

Our results are in good accordance with other European investigations and supplement our results from 1983 until 1986. Apparently, the investigated clinical and epidemiological features of Lyme borreliosis are uniform in Europe and have not changed significantly during the past 8 years.

**INTRODUCTION**

Lyme borreliosis is the most common human tick-borne disease in Europe and the United States (1). The etiologic agent of the disease was detected by Burgdorfer et al. (2). Since recent genetic analysis revealed that borreliae causing Lyme disease belong to three different species, the official name for the Lyme disease spirochetes is *Borrelia burgdorferi sensu lato* (3). However, for simplicity, the traditional name *B. burgdorferi* will be used.

In contrast to the United States, cases of Lyme borreliosis are not registered by the national health services in Europe. However, epidemiological studies have been conducted in several European countries (4–10) including our own study on 375 cases from Southern Germany registered between 1983 and 1986 (11). Subsequently, an enlarged number of 1200 clinically defined cases could be collected in our institute during the period 1987 through 1990. The purpose of the present study was to re-assess epidemiological features as age and sex distribution, geographical occurrence, incubation periods, and seasonal prevalence. The epidemiological data were compared with data from our previous study and those from other regions in Europe. The increase of registered cases is apparently due to more widespread knowledge of the disease.

**MATERIAL AND METHODS**

**Patients.** During the years 1987 through 1990, questionnaires were sent out to the clinician about all patients with positive serology (see below). Information was requested about age, sex, date and place of tick bite, onset and duration of the disease, as well as clinical symptoms. Only patients with clinically defined Lyme borreliosis were included; these were patients with erythema migrans (EM), lymphocytoma cutis benigna (LCB), carditis, neurolymeborreliosis stage II (NLB II), arthritis, and acrodermatitis chronica atrophicans (ACA); chronic neurolymeborreliosis (CNLB) was only assumed when the CSF/serum index (CSI) was elevated (see below). In addition, seronegative patients with clinically defined EM from a dermatological practice were included.

**Geographical Distribution.** As far as arthropod bites were reported, their geographical locations were plotted on a map according to the postal code of the area; in other cases, residences of patients were indicated.

**Serological Methods.** Antibodies to *B. burgdorferi* were detected using an indirect immunofluorescence assay after absorption of sera with *Treponema phagedenis* (IFA-Abs.) and rheumatoid factor (RF) absorption for the IgM test, as described in reference 12. Antibody titers ≥ 1:64 were regarded as positive, which corresponds to a cut-off in blood donors of 99% for IgG and 100% for IgM (13). For neurological patients with elevated IgG IFA-Abs. titers (≥1:2) in the cerebrospinal fluid (CSF), the CSF/serum index (CSI) was calculated as the quotient of quantitative arbitrary ELISA units measured in paired samples of CSF and serum which previously had been diluted to equal IgG concentrations;

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*Corresponding author

**Results presented in this publication are part of the thesis of Klara Rieth, Medical Faculty at Ludwig-Maximilians-Universität, München, Germany; in preparation.
Table 1

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<td>19,104</td>
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<td>21,368</td>
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<td>349</td>
<td>300</td>
<td>256</td>
<td>224</td>
<td>1,129</td>
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Of the 1705 returned questionnaires, 1200 contained relevant and sufficient data (see Table 2). The study comprised 1132 patients with positive B. burgdorferi serology and 68 patients without positive serology but with clinically defined EM.

Clinical Manifestations. Table 3 shows the number of patients with one, two, or three chronologically distinct manifestations and the frequency of clinical symptoms.

Chronic neurolymeborreliosis was only assumed—because of the clinically variable picture—when a significantly elevated CSF/serum index (CSI) (14) could be demonstrated. This was the case in 4 patients.

Age and Sex Distribution. We evaluated 619 male and 581 female patients. Figure 1 shows the sex and age distribution of the main manifestations.

For EM, the male/female ratio was 1:1.3; for neurolymeborreliosis (NLB II), the male/female ratio was 1:0.7, and for arthritis, it was 1:0.6. Among ACA patients, we found a significant majority of women (1:2). Differences in sex distribution were highly significant (p < 0.001) for all manifestations.

There was no significant difference in sex distribution for each of the manifestations (n = 243) in children below 15-years-old. Neurolymeborreliosis stage II was the most common disease (104 children, 43%), whereas ACA was very rare (4 children, 2%), as compared to 163 ACA patients (11.9%) among a total of 184 distinct manifestations in adults.

Figure 1 shows a bimodal age distribution for EM and NLB II: 13% and 19% of patients were below 11-years-old and 57% and 50% of patients between 41 and 70-years-old respectively. As to arthritis, numbers were too small to obtain a significant result about age distribution. The majority of ACA patients was older than 40-years-old (80%).

Geographical Distribution. Figure 2 shows the topographical data from a total of 967 patients from Southern Germany. 450 patients could recall an arthropod bite: 393 of them had been bitten by a tick and 57 by another arthropod.

Of the total patients from Southern Germany, 331 patients could recall the geographical location where they had been bitten (shown with full dots); these cases were concentrated in urban areas in contrast to rural regions such as the north-eastern or the southwestern part of Southern Germany. The other 636 patients did not recall the location of a bite and were plotted with open circles representing their residences.

Incubation Periods. For 552 patients who remembered a tick bite, the incubation period was calculated from the date of the tick bite to the onset of symptoms (see Figure 3). The percentage of patients recalling a tick bite is highest for EM (58%) and lowest for the late manifestations (18%).

Erythema migrans occurred in 38% during the first week after the tick bite; 45% of NLB II cases occurred between 2 and 5 weeks, 21% for more than 10 weeks, and 6% for 5 months or more after the tick bite. Late manifestations
Fig. 1. Sex and age distribution.

Fig. 2. Geographical distribution of locations of tick bites (●) and residences (○) in Southern Germany.
Erythema migrans (n=295)

Neurolyme borreliosis Stage II (n=183)

Fig. 3. Incubation periods for early manifestations.

(arthritis or ACA) showed no clearly defined incubation period: only 62 (29%) and 31 (19%) patients, respectively, could recall a tick bite; in 79% of 62 patients and 94% of 31 patients, incubation took more than 10 weeks, and in 50% and 71%, it took more than 1 year.

Seasonal Distribution. In early manifestations of Lyme borreliosis, a characteristic seasonal distribution was observed corresponding to the activity of ticks and to the different incubation periods (Figure 4).

Most cases of EM (58%) and NLB II (61%) occurred between June and August. For late stages, no characteristic seasonal distribution could be stated.

Figure 5 demonstrates that in 1988, EM reached its maximum between June and July, which is earlier than in other years (e.g., 1987). In 1988, the onset of symptoms for NLB II patients was also earlier than in other years. In 1990, a widespread occurrence of EM cases between April and August was observed in contrast to the clear-cut maximum in the other years (data not shown).

Sero logical Results. Results of the first serological examination only were evaluated. At the time of presentation, 26% of the patients with EM did not show any serological response. Sixty-four percent of the patients with EM who were seropositive at the first examination showed an IgM antibody response; 47% of them showed IgG antibodies. At least one test was positive in 74%. Of the neurological patients (NLB II), 77% were seropositive at the first examination, 79% of these being positive for IgG and 32% for IgM antibodies. In stage III of Lyme disease, 98 and 99% of patients with arthritis and ACA, respectively, were positive. Only 5% of these patients had IgM antibodies, but 97% and 98% had IgG antibodies.

Figure 6 shows the relation of IgM and IgG antibody response in seropositive patients for EM and NLB II in correlation with time elapsed between the onset of symptoms and the first serological examination. Among seropositive EM patients, IgM reactivity in the second week was higher than in the first week. In contrast, IgG reactivity in the second week was less than in the first week, but it increased again in the following period.

EPIEMIOLOGY OF LYME BORRELIOSIS

In seropositive children (<15-years-old) with EM, 92% showed IgM reactivity during the first 2 weeks (n = 12), whereas IgG antibodies were rarely seen (25% in the first and 10% in the second week). After the second week, the part of IgG antibody response was increased again and reached 80% after more than 6 weeks (n = 15). Adults with EM, however, showed to an equal extent IgG antibodies (50%) and IgM antibodies (56%) in the first week; IgG antibodies decreased to 33% in the second week and consecutively increased again to 88% after more than 6 weeks.

Children with NLB II showed slightly more IgM antibodies (65%) than IgG antibodies (57%) in the first week (n = 23); IgG antibodies consecutively decreased to 50% during the second week (n = 10), and they increased again to 100% after more than 6 weeks (n = 9). Both IgM and IgG antibody reactivity was more frequent at the first examination in children than in adults.

In 205 of 323 patients with NLB II as the first manifestation, CSF/serum pairs were examined as the first sample; 104 patients (51%) had elevated CSI. During the course of the disease, a total of 215 patients (66%) developed a significantly elevated CSI. At the first examination, 32 patients of 101 without B. burgdorferi—specific intrathecal antibody formation had elevated IgM antibodies in serum, and 16 had elevated IgM antibodies in CSF.

TPHA was performed for 514 patients out of 1132 with positive B. burgdorferi serology; 504 of them were negative (≤1:10), whereas 10 were reactive (five of these only borderline up to 1:40). All these patients had clear clinical symptoms of EM, NLB II (together with elevated CSI), or ACA; all sera were negative in VDRL.

DISCUSSION

Patients. Our study provides a representative overview of the epidemiological situation in Southern Germany. The increased number of cases as compared to our previous study (11, 16) is probably not due to a real increase of cases but reflects increased awareness of the disease. Although this evaluation supplies more reliable data about the epidemiology of Lyme borreliosis, some limitations must be considered: clinically unequivocal cases—especially EM—are often not serologically examined. In addition, serologically negative cases—in particular, EM—have not been included in this study, except for 68 patients of a Munich dermatological practice.

Frequency of Clinical Manifestations, Sex, and Age Distribution. The frequency of clinical symptoms were in good accordance with other (mostly serologically based) studies. Erythema migrans was reported to occur in 13% (17) up to 72% (18); neurological cases were described in 7% (18) to 56% (10) and carditis in 1.6% to 8% of patients with Lyme borreliosis (8, 10, 18–20). Arthritis was seen in 15% (10) up to 29% (17), and ACA was reported in 11.5% (6) and 5% (17). In a clinically based study on children, Williams et al. (21) observed relative frequencies of EM, NLB II, and arthritis in 47%, 10%, and 42%, respectively.

Thirty percent of our patients with NLB II had had another manifestation of Lyme borreliosis before, in accordance with Hansen and Lebeck (4) and Pfister and Einfelder (22). Only 8% of our ACA patients recalled another preceding Lyme disorder, in contrast to an average of 43% described in two studies of Ashbrink and coworkers (5, 23).

However, the observed frequencies as well as the age distribution depend on the referring physicians and hospitals, and cases are preselected by seroreactivity; therefore,
Fig. 4. Seasonal distribution of all cases.

Cases of Erythema Migrans of the Years 1987 and 1988

Fig. 5. Seasonal distribution of erythema migrans in 1987 and 1988.
Fig. 6. IgM and IgG in IFA Abs. in seroreactive patients at first examination: A. erythema migrans and B. neurolymeborreliosis stage II.
- IgM IFA Abs. ≥ 1:64
- IgG IFA Abs. ≥ 1:64
- IgM and IgG IFA Abs. ≥ 1:64
the real frequency of clinical manifestations in the population cannot be estimated. Especially the prevalence of EM is probably underestimated in seroepidemiological studies. Sex distribution in our collective was similar to ratios reported by other investigators (4, 6, 10, 19, 23–29). No significant difference in sex distribution was observed in patients below 15-years-old. Some other investigators, however, found that boys were more likely to get NLB II (21, 30) and arthritis (21).

The age distribution followed a bimodal pattern, as described before (11). Benach and Coleman (19) reported 50% and Anda et al. (10) 25% of their Lyme borreliosis patients to be under 20-years-old.

The bimodal distribution was mainly seen in the groups of EM and NLB II patients; for NLB II, this had been described before (4, 10, 11, 18). Hansen and Lebech (4) suggested that elderly people are more frequently registered because NLB II manifestations are more severe and that children probably are more frequently admitted to the hospital than adults because of anxiety of their parents. Furthermore, clinical features in children (facial palsy, meningitis, encephalitis, (30)) differ from those in adults (mainly meningoaradictilitis (22)). This suggests different pathogenetic mechanisms that could also explain the bimodal pattern. The high occurrence of arthritis in children described by Petersen et al. (18) was not found in our study. This is probably due to different patient collectives, but may be also due to the different prevalences of Lyme arthritis in Europe as compared to the U.S. (31–34).

Topography of Lyme borreliosis Cases. As described before (11), cases with known site of infection cumulate in urban areas. No clear concentration of cases along rivers was observed, in contrast to tick-borne encephalitis (15). However, this map is only a registration of the sites of infection of confirmed cases. The cumulation is due to the higher population density, and perhaps also to higher awareness of the disease and a greater number of physicians. Preselection depending on the referring physicians and hospitals also must be considered.

Incubation Periods. Frequency of tick bites reported by our patients is similar to the figures of other studies (6, 19, 21, 22, 35). 20% of our patients with late manifestations could recall a tick bite. However, even a recalled bite may not be the one causing infection.

For early manifestations only, reliable incubation periods could be evaluated. For both EM and NLB II, the incubation periods did not differ from the results of other studies (4, 11, 21, 22, 30, 35).

For late manifestations, incubation periods can rarely be calculated due to frequent lack of recall of a tick bite. As to arthritis, intervals as short as 3 weeks may be due to a misdiagnosed arthralgia at an early stage. Åsbrink and co-workers (5, 23) reported incubation intervals between 6 months and 10 years for ACA referring to a preceeding EM or NLB II.

Seasonal Distribution. Apparently, seasonal distribution depended on the tick activity beginning in early spring and on the incubation periods of the various manifestations. This was also observed in our former study (11) and by other authors (6, 19, 21, 30, 35).

In 1988, the peak for EM cases was about 1 month earlier than in other years; NLB II cases were shifted similarly. In 1990, no significant maximum for EM cases was observed. Both observations might be due to climatic influences.

With respect to arthritis and ACA, the uniform seasonal distribution is an indicator for variable and rather long incubation periods in late manifestations.

Seroreactivity. Due to different inclusion criteria of various studies, the proportion of seropositive patients in the diagnostic groups cannot be compared. The development of IgM antibodies relative to the IgG antibodies in seropositive patients, however, can be determined.

As was expected from former investigations (16), IgM prevaled IgG antibodies during the first 6 weeks of EM. Despite a predominant IgG antibody response after more than 6 weeks, there was still a remarkable group with IgM reactivity.

In contrast to adults, children with NLB II showed high IgM reactivity in the first 2 weeks, as was also found by Christen et al. (30). Possibly, reinfections occur more often in adults than in children.

Similar results (without differentiation between children and adults) were described by Hansen and coworkers (36–38) for patients with EM and NLB II and by Stierstedt et al. (39, 40) for NLB II patients.

Late manifestations showed almost 100% seroreactivity at first examination with only a few cases of IgM reactivity. This is in accordance with our former study and with Staake et al. (6).

Cross-reactions in TPHA. Occasional reactivity of sera from Lyme borreliosis patients in TPHA has been previously observed (12). However, there is no significant difference in the reactivity of reactive TPHA between sera from Lyme borreliosis patients and sera routinely screened for syphilis (unpublished results). Therefore, we do not believe that TPHA reactivity results from infection with B. burgdorferi.

CONCLUSIONS

This study confirms our former report about the period of 1983 through 1986. The considerably increased number of patients makes the data more reliable. Our data are also in good accordance with seroepidemiologic studies from Northern and Southern Europe. Obviously, there are no important differences concerning sex and age distribution, incubation periods, and seasonal cumulation in various regions of Europe. No significant change has been observed over the years.

The authors thank the numerous primary and consultant physicians who supplied information about their patients. We thank especially Peter Herzer, Hans-Walter Pfister, and Klaus Weber for clinical data and Reinhard Boehmer for valuable help with the manuscript.

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Psychiatric Aspects of Lyme Disease in Children and Adolescents: A Community Epidemiologic Study in Westchester, New York

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To date, no community study has examined the psychiatric aspects and or sequelae of Lyme disease (LD) among children. As part of a community epidemiologic study of psychiatric disorders among children ages 9 through 17 in a Lyme endemic county, parents were asked whether their child had ever been diagnosed as having LD, and 10.1% (36/357) responded yes to the LD question. Of the 36, 29 also agreed to take part in a follow-up interview. Sixteen of the 29 children had had physician-diagnosed LD as well as either an erythema migrans rash or a positive serology. Fifteen of these 16 received treatment within 1 month of symptom onset; none of these 15 children were symptomatic longer than 4 months. Only one child had physical symptoms at the time of the interview; she was not treated until 4 months after symptom onset. This child experienced 5 years of intermittent arthritis, cognitive deficits, emotional problems, severe fatigue, and a deterioration in school performance. Courses of oral antibiotics were at first associated with a good response, followed by a resurgence of symptoms months later. The lifetime prevalence of LD by history among children ages 9 through 17 in an endemic area may be at least 44.8/1000. In general, when LD is diagnosed early it responds well to treatment. Delayed diagnosis and treatment may lead to a chronic course.

INTRODUCTION

Lyme disease, a multisystem illness caused by the spirochete Borrelia (B.) burgdorferi, can cause neuropsychiatric problems (1). In adults with neurologic Lyme disease, common psychiatric problems include memory loss, word finding problems, depression, mood lability, and irritability. Paranoia, mania, schizophrenia-like states, and anxiety disorders may also occur (2).

Less is known about the neuropsychiatric profile of Lyme disease in children. Published case reports have associated Lyme disease with anorexia nervosa (3) and, in older teenagers, with obsessive compulsive disorder (2, 4), panic disorder (2), personality changes with aphasia and apraxia (5), and a catatonic-schizophrenic-like syndrome (6). In the latter case, B. burgdorferi was isolated from the cerebrospinal fluid (CSF) of a 19-year-old boy with no prior psychiatric history; the psychotic disorder resolved with antibiotic treatment.

Two studies have looked at children with late-stage Lyme disease. In one study of 46 children with Lyme arthritis, 10 children (22%) had had aseptic meningitis or facial palsy (7). In another study (8) of 96 seropositive children ages 3 through 19, who presented with new neurologic symptoms, the two most frequent features were headaches (71%) and behavioral or mood changes (38%). Listlessness, irritability, malaise, and decreased interest in play were common among preschoolers, while emotional lability was more common among school-age children.

Current studies are limited by sampling bias and the absence of standardized measures of psychiatric morbidity. In addition, because the published studies focused on late-stage Lyme disease, it is not known whether early Lyme disease is also associated with significant psychiatric problems.

In this study, two questions were addressed. First, by history, what is the lifetime prevalence of Lyme disease among children ages 9 through 17 in a Lyme endemic area? Second, what is the frequency of psychiatric disorders among children with carefully defined Lyme disease?

METHODS

In 1992, as part of a National Institute of Mental Health (NIHM) Collaborative study, probability samples of children ages 9 through 17 years and their parents (usually the mother) were interviewed at one of four sites across the United States. The purpose of the study (MECA Study) (Labey et al., unpublished manuscript) was to assess methods for use in psychiatric epidemiologic surveys of child and adolescent populations and the feasibility of carrying out such studies in the community for a national study. The data used in the present report were gathered from the New York State Psychiatric Institute/Columbia University Site (n = 357). Randomly selected children and their parents were drawn from households in 15 randomly selected communities in Westchester County, New York. The sample is representative of the population of Westchester County. The 2 hour interview consisted of a standardized psychiatric diagnostic assessment (the NIMH-Diagnostic Interview Schedule for Children (DISC), Version 2.3) (9) and a schedule that collected demographic data as well as data on medical and mental health service utilization and risk factors for childhood psychopathology. In general, the DISC asks about the behavior and mood of the child during the 6 month period prior to the interview. All interviews were conducted by trained lay interviewers.

As part of the health history, the following two questions

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were asked of the parent: "Has your child ever been diagnosed as having Lyme disease?" and "Would you be willing to be contacted by a researcher interested in interviewing children who have had Lyme disease and those who have not had Lyme disease?" Affirmative answers to both questions led to a telephone follow-up interview to explore further the diagnosis of Lyme disease.

RESULTS

In this study, 357 households participated. The racial composition of the children in this sample was mixed: 74.6% white, 17.5% black, and 7.9% other; 52% of the children were male, and 48% were female. These percentages proportionately reflect the racial and gender distribution of Westchester County.

Thirty-six of the 357 children's parents (10.1%) reported that the child had been diagnosed as having had Lyme disease. In the Northern Westchester communities, 27 of the 130 parents (20.8%) reported a past history of Lyme disease in the child. Of the 36 children in the total sample with a history of Lyme disease, the mean age was 13.2 (range 9 to 17). Half were female and half were male.

Of the 36 households in which the child had had the diagnosis of presumed Lyme disease, 29 (81%) of the parents allowed us to conduct a follow-up telephone interview. We used the following criteria for Lyme disease to confirm the diagnosis: (a) history of exposure to a Lyme endemic area; (b) physician-diagnosed Lyme disease; and either (c) a physician-diagnosed erythema migrans rash or (d) serologic evidence of Lyme disease with at least one sign/symptom of systemic illness. All of the children in our sample met the first of these criteria because they resided in Westchester County. Using the remaining criteria (b through d), 16 of the 29 follow-up interviews led to a confirmed diagnosis of Lyme disease. Of the 13 patients who were not included in our confirmed cases, seven had never had Lyme disease (e.g., treated for tick bite but never symptomatic) and five had had signs and symptoms suggestive of Lyme disease but they had not had an erythema migrans rash or positive serologic tests.

Of the 16 children with a history of confirmed Lyme disease, the median duration of illness was 24 days (range: 3 days to 5 years). Fifteen out of 16 youths had been treated within 1 month of symptom onset. Three received IV antibiotics. These three children missed between 2 and 6 weeks of school. Only one child was symptomatic at the time of the follow-up interview. This child began treatment 4 months after symptom onset. Although none of the other children had illness that extended beyond 4 months, this child's course was chronic. She experienced 5 years of intermittent arthritis with swollen knees, memory loss, severe fatigue, paresthesias, headaches, mood swings, depression, irritability, poor concentration, and poor school performance. She had received several courses of antibiotics over the 5 year period (a total of 11 months of oral and 1 month of IV), with an initial good response during each course followed months later by a resurgence of symptoms. On the DISC, she received a current diagnosis of major depression.

Of the 16 children who had had confirmed Lyme disease, five were diagnosed as having a current psychiatric disorder on the DISC. The diagnoses include oppositional defiant disorder (two children), agoraphobia (two children), social phobia (two children), attention deficit hyperactivity disorder (one child), Tic disorder (one child), and major depression (one child).

DISCUSSION

Several findings emerge from this study. First, Lyme disease is a frequently diagnosed condition among children in Westchester County. In this study, 1 in 10 of the parents from Westchester County reported that their child had been diagnosed with Lyme disease at some point. Second, in this study, all but one of the 16 confirmed Lyme cases had received antibiotic treatment within 1 month of symptom onset. Clearly, in this endemic area, parents seem alert to the symptoms of Lyme disease and rapidly seek treatment for their children. Third, this study suggests that in most cases, if treated early, Lyme disease in children is a benign illness with no long-term sequelae.

Using restrictive criteria for the diagnosis of Lyme disease to evaluate putative cases, this study suggests that the frequency of a history of Lyme disease among children ages 9 through 17 in Westchester County may be at least 44.8/1000. These criteria are restrictive in that confirmation of the diagnosis required either a physician-diagnosed erythema migrans rash or positive serologic tests and at least one systemic symptom. It is well known that the rash is only recalled in about two-thirds of the cases and that currently available serologic tests are not always reliable (10). For epidemiologic purposes, such restrictiveness is useful in order to ensure diagnostic conformity but limited in that the prevalence of Lyme disease will be underestimated.

The one persistently symptomatic child, diagnosed at age 12 with Lyme disease, had not been treated until 4 months after symptom onset. A chronic illness may have been prevented had she been treated earlier, as was the case with all of the other children in this study. Her fluctuating symptom profile was associated with severe pain, concentration problems, a deterioration in academic performance, irritability, and major depression. As reported among children with neurologic Lyme disease (8), behavioral and mood disturbances can be part of the Lyme disease profile. Psychiatric disturbances may be a secondary reaction to having a serious illness or a primary reaction induced directly or indirectly by the infection itself. Borrelia burgdorferi may initiate an immune reaction directed specifically against neural tissue (11) or it may trigger nonspecific inflammatory responses that cause neuropsychiatric symptoms (12). The immune response may remain active because B. burgdorferi antigens are still present or because an autoimmune process has been triggered against host tissue. Because of the combination of articular and psychiatric symptoms and the good response to antibiotics (although temporary), it is likely that this girl's symptoms were due to persistent infection.

The limitations of this study need to be recognized. First, the diagnosis of Lyme disease was made based on information supplied by the parent. Biased or incorrect recall about symptoms or serologic tests may have influenced the results of this study. However, it should be noted that information about the one persistently symptomatic child was confirmed by discussion with the child's treating physician. Second, because our research found that in the majority of cases Lyme disease was recognized and treated promptly, our study conclusions must be limited to children with early Lyme disease; i.e., the favorable results of early administered treatment in our study should not be generalized to children with later-stage illness. The troubling course of the one persistently symptomatic child suggests that more research needs to be conducted on the diagnosis, treatment, and pathophysiology of late-stage Lyme disease. Third, because only one of the 16 confirmed cases displayed phys-
ical symptoms of Lyme disease during the 6 month period covered by the DISC interview, no conclusions can be drawn about the frequency of psychiatric disorders among children with currently active Lyme disease.

In conclusion, childhood Lyme disease when treated early appears to be associated with good outcome. When treatment is delayed, a chronic, relapsing illness may emerge associated with disabling physical, cognitive, and emotional sequelae.

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Letters to the Editor

Persistence of *Borrelia burgdorferi* Despite Antibiotic Treatment

*To the Editor:* It has been suggested that Lyme disease may trigger fibromyalgia and that antibiotic therapy beyond 30 days is almost always unnecessary (1). Recently, two cases demonstrating persistence of *Borrelia burgdorferi* despite lengthy antibiotic treatment were noted.

Case Number 1: In October 1991, a 35-year-old Caucasian female, registered nurse, was referred for evaluation. She had reported a lesion compatible with erythema chronicum migrans about one year earlier. After a short course of oral antibiotics, she noted fatigue, myalgia, and arthralgias and was given 2 weeks of intravenous ceftriaxone 1 g daily with resolution of her symptoms. Over the next several months, however, her symptoms gradually returned. An ELISA titer was elevated, and she was started on ceftriaxone 2 g intravenously daily. After 10 days, the patient developed a vigorous Jarisch-Herxheimer reaction and was referred to the author. The patient was switched to cefotaxime 3 g intravenously every 12 hours with improvement in symptoms. After 6 weeks, the intravenous cefotaxime was changed to oral clarithromycin 500 mg daily for 6 more weeks with complete resolution of all signs and symptoms. One week later, the patient discovered that she was 1 month pregnant and, after a normal gestation, delivered a healthy male infant. The placenta was examined at Brigham and Women’s Hospital in Boston, Massachusetts, where several spirochetes were noted in perivascular and intervillous spaces on modified Dieterle silver stain.

Case Number 2: A 47-year-old Caucasian female was well until an untreated tick bite in 1985. She subsequently developed a progressive arthritis diagnosed as rheumatoid. After failing treatment with nonsteroidal anti-inflammatory and remittive agents, the author saw the patient for the first time in 1990. Aspiration of fluid from the right knee was positive by specific antibody ratio for Lyme disease at the University of Medicine and Dentistry of New Jersey—Robert Wood Johnson University Hospital Lyme Disease Research Center. The patient was started on ceftriaxone 2 g intravenously daily for 4 weeks. She had a significant objective response to treatment but quickly relapsed after it was discontinued. A second 4-week course of ceftriaxone was given with only moderate improvement. The patient then sought treatment at several university centers, where she received experimental treatment for rheumatoid arthritis including monoclonal antibody therapy. There was no improvement in her condition. By July 1992, the patient developed bilateral aseptic necrosis of the hips. A right total hip replacement was performed and histopathologic examination revealed several spirochetes on modified Dieterle silver stain of synovial tissue performed at the Brigham and Women’s Hospital. The patient was then started on continuous oral antibiotic treatment with azithromycin 250 mg daily. Approximately 6 months later, the patient underwent left total knee replacement and once again spirochete-like structures were observed in synovial tissue on modified Dieterle silver stain.

These two cases suggest that despite lengthy courses of both intravenous and oral antibiotics, *Borrelia burgdorferi* may persist. The presumption that residual symptoms are due to fibromyalgia may not always be true and is not assured simply because a patient has received 30 days of treatment. Careful histopathologic examination by modified Dieterle silver stain may suggest otherwise.

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