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Laboratory Aspects of Lyme Borreliosis

ALAN G. BARBOUR

Departments of Medicine and Microbiology, The University of Texas Health Science Center, San Antonio, Texas 78284

INTRODUCTION

Lyme borreliosis (or Lyme disease) is a common arthropod-borne disease of humans in North America and Europe (147). More recently, it has been reported from the USSR (107) and Japan (100). There is also increasing evidence that Lyme borreliosis is a significant cause of morbidity among dogs and perhaps other domestic animals in some geographic areas (44, 45, 47, 104, 112, 118, 119, 120, 122).

This zoonotic spirochetal infection may be brief and inconsequential or chronic and severely disabling. Manifestations involve the skin, joints, nervous system, and heart. Like syphilis, Lyme borreliosis may mimic several other diseases, many of which are not infectious and, therefore, not ameliorated by antibiotics. A challenge for the clinician and diagnostic laboratory is identification of a case of chronic arthritis, meningoencephalitis, or persistent skin inflammation as Lyme borreliosis. If the clinical impression is confirmed by specific diagnostic procedures, appropriate antimicrobial therapy may gratifyingly reverse a long-standing pathologic process.

Many components of the Lyme complex of disorders have been recognized for several decades by European clinicians. The occurrence of these infections on the North American continent appears to be a more recent phenomenon. It is ironic, then, that the name we commonly use for this group of diseases comes from a town in New England. Steere and his colleagues investigated a cluster of arthritis cases in Lyme, Connecticut (166). Subsequent studies from Yale University defined the clinical parameters and epidemiology of "Lyme arthritis" (158, 164). The clinical similarity between the skin lesion that often preceded Lyme arthritis and erythema chronicum migrans (ECM), a disorder long-recognized in northern Europe, was established. However, not until a hitherto unknown spirochete was recovered from the presumptive Lyme disease vectors, *Ixodes dammini* and *I. ricinus* (15, 39), was it possible to prove that Lyme arthritis and ECM, as well as some other tick-associated syndromes, all had the same etiology (8, 9, 26, 40, 136, 161). The spirochete that causes Lyme disease was placed in the genus *Borrelia* on the basis of deoxyribonucleic acid (DNA) relatedness studies, morphology, and physiology (90, 92, 93, 148). In the six years following Burgdorfer's discovery of the etiologic agent, *Borrelia burgdorferi*, the literature on Lyme borreliosis and related disorders increased exponentially. A journal review of the literature up to 4 years ago could have comfortably encompassed all aspects of the subject. Now only a book would do justice to the body of studies that have since been carried out on Lyme borreliosis and its etiologic agent. Consequently, this review does not attempt comprehensiveness. Rather, it emphasizes those aspects of the clinical study of Lyme borreliosis that are most appropriately done in the laboratory.

First, though, background on clinical aspects of this disorder is provided. In the following section only representative literature citations are given. These references should allow access to the numerous other articles on the clinical manifestations of this infection. Those wishing further information on the biology of the etiologic agent could refer to reference 17 as a starting point.

CLINICAL ASPECTS

Transmission of *B. burgdorferi* from vertebrate to vertebrate depends on blood-feeding arthropods; infected vertebrate hosts are lightly spirochetemic for days to weeks (25, 38, 42, 95, 103, 154). During this time, the infection may spread to other organs. During the spirochetemic phase of illness, humans commonly have fever and constitutional symptoms. Some of these systemic effects may be the consequence of interleukin-1 production by leukocytes exposed to whole cells or released components of the borreliae (48, 75). Following spirochetemia, the organisms are to be found in various organs (61, 95). In many patients, the inflammation that follows may be due in part to the persistence of viable borreliae and in part to the host's immune response to the bacteria.

Lyme borreliosis is rarely fatal; thus, the knowledge of the pathology of human infection is not extensive and depends on the rare autopsy case, biopsies, and animal infections. The predominant finding in biopsy specimens is a lymphocytic and plasmocytic infiltrate, usually greatest in perivascular areas (31, 60, 61, 63, 98). Neither granulomas nor necrosis is found, but marked fibrin deposition and obliterative microvascular lesions have been noted.

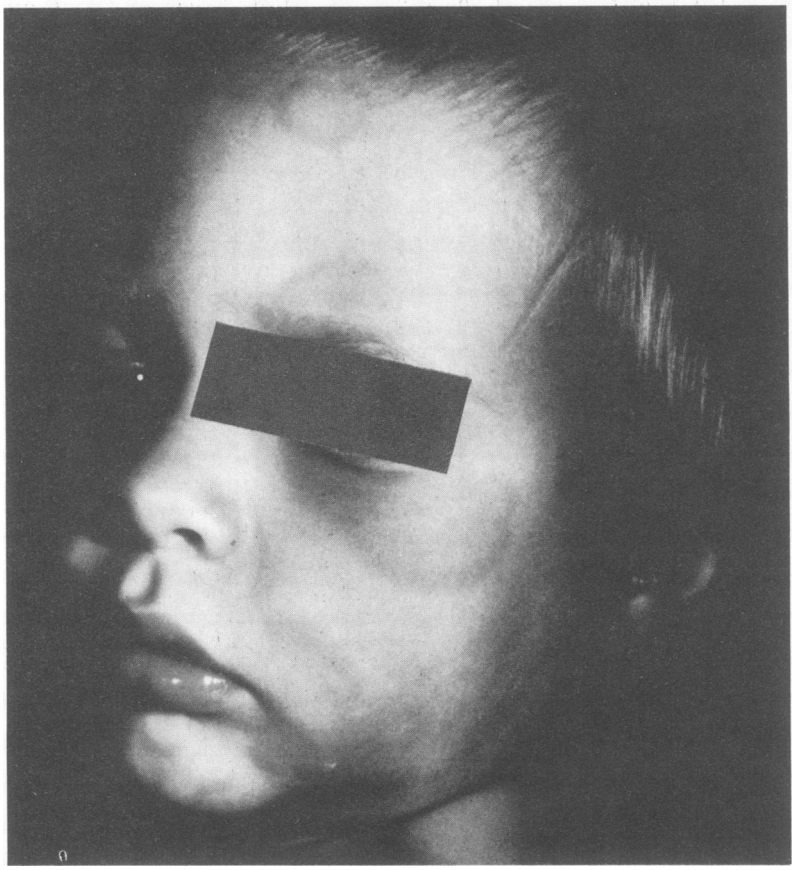

FIG. 1. A child with multiple ECM lesions on the face. Those above and below the left eye and on the forehead are most prominent. Photograph courtesy of Alan MacDonald, Southhampton Hospital, Southhampton, N.Y.

Early *B. burgdorferi* infection may be either asymptomatic or of such a nonspecific nature that it cannot be distinguished by respondents from an influenzalike illness. The disease can take many forms among those persons whose disorders are clearly attributable to *B. burgdorferi*. The manifestations can be roughly placed in one of three stages according to when they occur during the course of the infection. Some manifestations, such as acrodermatitis chronica atrophicans and lymphocytoma, are more common in Europe, and, conversely, Lyme arthritis appears to be more frequent in North America (10, 83, 158, 164).

ECM is the hallmark of the first stage and the best clinical and epidemiological marker of Lyme borreliosis (156, 164) (Fig. 1). It is analogous to the primary chancre of syphilis. Typically, this lesion appears at the site of a tick bite sustained 3 to 14 days previously. ECM is characterized by an advancing, slightly elevated, annular erythema which leaves a central clear area without scaling. The outer edge is usually more distinct than the inner edge of the ring. The primary skin lesions may not always take this classical form and may appear instead as an erythematous plaque which extends its margin. During early infection the patient may complain of low or moderate fever, headache, easy fatigueability, arthralgias, stiff neck, and myalgias. Approximately half of the patients with untreated ECM develop one or more metastatic annular lesions at sites distant from the original rash (Fig. 1); for some persons the second crop of skin lesions have been pruritic and urticarial (156). Examination of patients with early disease may reveal generalized or regional lymphadenopathy (156).

The leukocyte count and hepatic transaminases may be mildly elevated in the blood during acute disease (156, 164). When measured, low-to-moderate levels of circulating immune complexes have been found in ECM patients (77–79). Patients with elevated serum total immunoglobulin M (IgM) concentrations and cryoglobulins are more likely to have a complicated disease course (127, 162). Concentrations of total serum IgM correlate with the degree of disease activity (127).

In the second and third stages of Lyme borreliosis there may be skin, joint, nervous system, or cardiac involvement (10, 133, 142, 164). The second-stage manifestations usually start a few weeks to a few months after the initial ECM. Third-stage manifestations occur months or years after onset of infection.

The heart disorder in Lyme borreliosis is a diffuse myocarditis and is self-limited in almost all cases (123, 131). Nonetheless, Lyme carditis is the most potentially serious complication, for the usual presentation is one of heart block varying from first degree to complete. Cardiomegaly and heart failure are rare, but there may be evidence of mild ventricular dysfunction and electrocardiographic changes consistent with acute myopericarditis.

Second-stage arthritis initially is commonly migratory and polyarticular. Pains may also occur in the tendons, bursae, and muscles. A few weeks later, joint effusions with up to 100,000 cells per mm^3, mostly polymorphonuclear leukocytes, may be found (64). Immune complexes and cryoglobulins may be present in the synovial fluid when they are no longer detectable in the plasma (78). Antinuclear antibodies

have only rarely been found in adults (158, 164), but in some children with Lyme arthritis antinuclear antibodies that give a homogeneous staining pattern have been detected (64). By latex agglutination testing, rheumatoid factors are not usually found (78, 79). However, by a more sensitive enzyme-linked immunosorbent assay (ELISA) procedure, rheumatoid factors have been detected in patients with active Lyme arthritis, (108). Lyme arthritis patients have anti-Fab fragment antibodies in the IgG1 and IgG3 subclasses (J. S. Louie, J. Persselin, G.-Y. Shi, H. Jobe, M. Liebling, A. C. Steere, and R. Stevens, Abstr. Int. Conf. Lyme Dis., abstr. no. 15, 15 Sept. 1987). Rheumatoid factors have also been detected by ELISA in many patients with syphilis (49).

The frank arthritis typically involves a knee or other large joint (83, 91, 158, 164). Attacks of arthritis may last for weeks and recur several times. Some of these patients, if untreated, continue to have a chronic, destructive arthritis of one or more large joints; there may be erosion of the cartilage and bone and a proliferative synovium. The spine and small joints of the hands and feet usually are not affected. Chronic Lyme arthritis may last for years and is then considered part of the third stage of infection. The HLA haplotype, DR4, appears to be a risk factor for chronic Lyme arthritis (A. C. Steere, Ann. N.Y. Acad. Sci., in press).

Two skin disorders represent later stages of Lyme borreliosis (10, 86). Lymphocytoma is an erythematous swelling, typically of the ear lobe or around the nipple; it usually appears a few weeks after a tick bite. Biopsy reveals a heavy lymphocytic infiltrate and follicles. Acrodermatitis chronica atrophicans begins as a localized, acute inflammatory rash of the extremities or trunk and over the course of months and years progresses to fibrosis and atrophy of the affected region of the skin.

The second-stage neurologic disorders may appear suddenly a few weeks after appearance of ECM or advance insidiously over months (82, 84, 129, 142, 145, 168). Approximately 30 to 40% of patients with disease progressing beyond ECM have neurologic complaints. In the early stages there may be clinical and laboratory evidence of meningeal irritation; in endemic areas *B. burgdorferi* is a common etiology of "aseptic meningitis." Later a meningoradiculopathy with a lymphocytic pleocytosis and oligoclonal peaks in the cerebrospinal fluid (CSF) may come to the fore; this constellation has been termed lymphocytic meningoradiculitis or Bannwarth's syndrome (82, 84, 145). Patients typically complain of headache and sharp pains in the trunk or extremities; there may be sensory and motor deficits as well. Unilateral or bilateral Bell's palsy is often present, either by itself or associated with the radiculopathy. Some patients with chronic meningitis complain only of headache and extreme fatigue. During second-stage neurologic disease there may be evidence of encephalitis with altered mental status and diffuse slowing on the electroencephalogram. In the third stage of the disease, years after onset of infection, patients may present with such signs of diffuse or local cortical involvement as intellectual deterioration, hemiparesis, or unaccountable psychiatric abnormalities (3).

The organism, like other pathogenic spirochetes, is probably transmissible via the placenta to the fetus (17). *B. burgdorferi* infection of fetuses has been documented (114, 146).

Occasional patients have been coinfected with *Babesia microti* in eastern North America (28, 123) or with tick-borne viral encephalitis in central Europe (106). Neither humans nor dogs with *B. burgdorferi* infections have had elevated antibody titers to rickettsial antigens (175). However, some patients with ECM and neurologic disease in Europe had significant antibody titers to *Proteus* OX-2 antigens (174). In relapsing fever, some patients have shown reactions to the OX-K antigens (65).

Patients with first-stage disease are treated with oral antibiotics. For adults, phenoxymethyl penicillin or tetracycline has usually been recommended (163, 165), although there are reports of tetracycline failures (55, 64). Children receive phenoxymethyl penicillin or, in the case of penicillin allergy, erythromycin (163). Patients with neurologic disease or established arthritis of large joints usually benefit from intravenous penicillin G (159, 167). In cases of penicillin failure, chloramphenicol (59) or ceftriaxone (56) has been administered with apparent success. The longer a patient has had chronic arthritis, the less likely he or she is to have a favorable response to antibiotics (159).

Patients with early Lyme borreliosis may experience Jarisch-Herxheimer reactions shortly after the start of antibiotic therapy (163). This reaction can be experienced as increased warmth and irritation of the skin rash, lymphadenopathy, and an exacerbation or recurrence of fever. No instances of life-threatening, hypotensive episodes have been reported such as those that can follow the initial antibiotic doses in treatment of secondary syphilis or louse-borne relapsing fever.

Domestic animals in Lyme disease-endemic areas are also exposed to *B. burgdorferi*-bearing ticks. Some dogs appear to develop arthritis or renal disease during the course of *B. burgdorferi* infection (44, 104, 112, 118, 119). Other domestic animals that have had significant disease attributable to *B. burgdorferi* are horses (45, 120, 122) and cows (47). The extent of this health problem among domestic animals has not been sufficiently delineated.

DIFFERENTIAL DIAGNOSIS

A resident in an endemic area who first notices an enlarging ringlike rash in June and then polyarticular arthritis, Bell's palsy, and second-degree heart block in August could have little else but Lyme borreliosis. Diagnostic difficulties arise when the characteristic skin lesion, ECM, either never occurs or is overlooked by patient or physician. In this case, individual manifestations of late disease may suggest several other disorders, infectious and otherwise. Lacking characteristic skin lesions, acutely ill patients may be thought to have influenza, enteroviral aseptic meningitis, nonicteric hepatitis, or infectious mononucleosis (156). Moreover, if the early disease skin lesions are present but are not classical ECM, they can be considered to be an insect bite reaction, erythema nodosum, erysipelas, tularemic ulcer, erythema multiforme, or the urticarial rashes of serum sickness and hepatitis B (10, 156).

Lyme carditis has been diagnosed as viral myocarditis or atypical rheumatic fever. The initial joint involvement can mimic disseminated gonococcal infections or, in a more localized form, septic arthritis (91). Standard bacterial cultures of joint fluid would, in this case, be negative. Other arthritic presentations may be confused with Reiter's syndrome; the distribution of affected joints in the two disorders is similar. Some children in endemic areas have carried the diagnosis of pauciarticular juvenile rheumatoid arthritis before elevated titers to the Lyme spirochete were found. In endemic areas, *B. burgdorferi* may be the etiology of "seronegative rheumatoid arthritis."

Neurologic involvement in its several forms resembles many other diseases, from the transitory to the inexorably

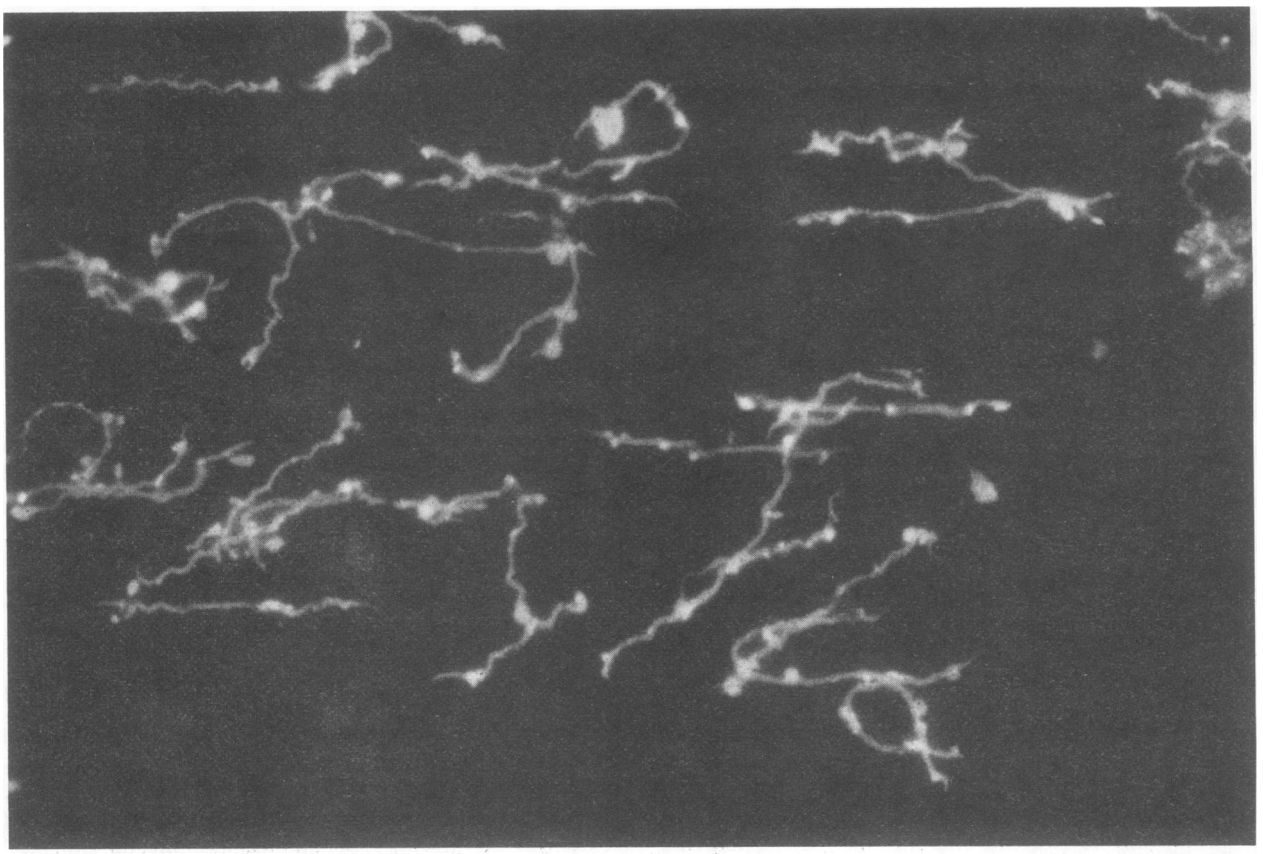

FIG. 2. Indirect immunofluorescence of *B. burgdorferi*. Strain B31 spirochetes were fixed in methanol on a slide as described in reference 21. Slides were incubated with a monoclonal antibody directed against the OspA protein (21). Bound antibody was detected with fluorescein-conjugated goat anti-mouse immunoglobulin. Numerous small and large blebs are associated with the spirochetes.

progressive. The salutary effect of antibiotics for Lyme borreliosis patients provides motivation for considering *B. burgdorferi* infection in cases suggestive of multiple sclerosis, Guillain-Barré syndrome, sarcoidosis, neurosyphilis, or tuberculous or fungal meningitis. In second-stage Lyme borreliosis of the nervous system, the CSF usually reveals lymphocytic pleocytosis with plasma cells, proteins elevated up to 100 mg/ml, and oligoclonal peaks on protein electrophoresis (84, 142). The presence of immunoblasts and plasma cells in CSF cytology studies can suggest lymphoma or the meningeal involvement in multiple myeloma (82, 141).

DIAGNOSIS

Direct Detection

The morphologic characteristics of *B. burgdorferi* are reviewed in reference 17. *B. burgdorferi* is about 200 mm wide and 10 to 30 μm long. There are 7 to 11 periplasmic flagella, the longitudinally transversing filaments that characterize spirochetes. Borreliae have both an inner (cytoplasmic) and an outer membrane. The outer membrane of *B. burgdorferi*, like those of other borrelial species, is easily disrupted (21, 52). The rigidity, which can be imparted by components such as the lipopolysaccharides of gram-negative bacteria, is not seen in the outer membrane of *B. burgdorferi* (21).

Stanek and colleagues were able to detect as few as 10^4 borreliae per ml of mouse blood by microscopic examination of a wet mount of blood (154). With the microhematocrit technique, in which the cellular elements at the interface between plasma and packed erythrocytes are examined, the limit of detection might be as low as 10^3 spirochetes (71). Lissman et al. used a variation of this microhematocrit technique to observe spirochetes in the blood of an infected dog (112). These investigators also detected spirochetes in the urine of about half of all field mice examined in an endemic area (35). The borrelia-like organisms could not be cultivated in the laboratory, however.

By either phase-contrast or dark-field microscopy of live organisms or standard light microscopy of stained, fixed organisms, *B. burgdorferi* can usually be distinguished from other borreliae by its looser and more irregular coiling. When immunologic probes, such as fluorescein-labeled antibodies, are used, large or small blebs may be associated with the cells (21) (Fig. 2). These blebs represent antibodies binding to outer membranes that have become disrupted during drying and fixation. In one report, a spirochete was seen by electron microscopy in the skin biopsy of a patient with ECM (173). The characteristic flagella (axial filaments) were not detectable in the published figure; therefore, the identity of the spiral structure remains in doubt.

Tinctorially, borreliae are gram negative. However, the Gram stain is not nearly as sensitive as Giemsa (39, 40) and silver (31, 58, 61) stains for demonstrating the organisms. Acridine orange was used to detect spirochetes in phagocytic cells (29) and in the CSF of a patient with Bannwarth's

syndrome (36). This dye was also used to stain what appeared to be spirochetes in the urine of field mice (35).

Warthin-Starry and modified Dieterle silver stains have been used to reveal the spirochetes in a variety of biopsy and autopsy materials that have been Formalin fixed and embedded in paraffin (31, 61, 62, 67, 99, 123, 130, 146). According to Duray and Johnson, a modified Dieterle stain is easier to perform than the Warthin-Starry stain (61). In various reports, silver stains have been used successfully to detect spirochetes in <1 to 100% of ECM lesion biopsies (31, 32, 63, 67, 130); for most investigators the success has been about 40 to 50%. The spirochetes are most easily found if the biopsy is taken from the advancing edge of the ECM lesion and the papillary dermis is examined. Usually fewer spirochetes are located in the center of the lesion and in the epidermis. A "positive control" slide prepared from *B. burgdorferi* cells suspended in an agar block should be included when biopsy material is examined by silver stains (50).

In exceptional cases, spirochetes have also been detected in synovial tissue biopsies with either the standard or modified Dieterle silver stain (63, 98). When seen, the numbers of spirochetes present were very low. The borreliae were seen within and close to small vessels displaying microangiopathic changes (98).

A modification of the Steiner silver stain is reported to further improve the sensitivity of histologic detection (58). In this method the tissues are treated with amylase after they have been fixed in Formalin and before the immersion in silver nitrate. When compared with the Warthin-Starry stain, this method has been reported to provide greater contrast between the spirochetes and the background tissues. Using this modified Steiner stain (Bosma-Steiner), de Koning et al. reported 100% sensitivity in demonstrating spirochetes in skin biopsies of ECM patients and in synovial biopsies of Lyme arthritis patients (58). The spirochetelike structures were not seen in synovial biopsies from patients with rheumatoid arthritis or bursitis or in skin biopsies of patients with granuloma annulare, eczema, or lymphoma.

Polyclonal antibodies have been used successfully in immunohistologic studies to demonstrate spirochetes in tissues (35, 102). However, with monoclonal antibodies not only are spirochete structures demonstrated, but also the particular type of spirochete can be determined (18, 20, 21). Using a monoclonal antibody to a borrelial flagellar antigen, Park et al. demonstrated spirochetes in a frozen section of skin biopsy from a patient with ECM (133). Monoclonal antibodies were also used by MacDonald and Miranda to reveal spirochetes in touch preparations of unfixed human brain tissues from an autopsy specimen (115), by Magnarelli et al. to identify *B. burgdorferi* in the kidney tissues of a dog with renal disease (118), and by Burgess et al. to show borreliae in organs of an infected cow (47).

Direct and indirect immunofluorescence assays with antiborrelial antibodies have been used to determine the prevalence of infected ticks in different geographic areas (7, 43). Although this approach has proved useful in field studies, laboratory experiments with ticks have shown that some borreliae in the ticks may either not react at all with certain monoclonal antibodies or react more weakly than they usually do with polyclonal antisera (43, 110). This phenomenon suggests that antigenic variation occurs.

Another strategy for direct detection of organisms is with DNA probes, using cloned *B. burgdorferi* genes (75a, 87, 88). Schwan and colleagues used a probe for an outer membrane protein gene to detect varying numbers of borrelial cells attached to a membrane in a variation of the dot blot (T. G. Schwan and A. G. Barbour, Ann. N.Y. Acad. Sci., in press). Whether in situ hybridization can be used to identify organisms in tissues remains to be determined.

The presence of *B. burgdorferi* may also be suggested by the detection of borrelial antigens in body fluids. Using an immunoassay, Benach et al. found evidence of an outer membrane protein in the urine of infected hamsters (27). Endotoxin is an indicator of gram-negative bacterial infection, but endotoxin-like activities have not been found in the blood of patients with Lyme borreliosis (149). Beck et al. isolated a lipopolysaccharide fraction with endotoxin activity from *B. burgdorferi* (24), and Fumarola et al. found that endotoxinlike activity was associated with killed *B. burgdorferi* cells (69). However, in biochemical analysis of *B. burgdorferi*, Takayama et al. failed to confirm the presence of an endotoxin similar to the lipid A-containing lipopolysaccharides of gram-negative bacteria (171). A previous study had failed to show endotoxin in the closely related relapsing fever borreliae (80).

In Vitro Cultivation

Koch's postulates are partially fulfilled by isolating the offending organism from the affected patient; this has been done in several cases of Lyme borreliosis as described below. The culture medium is complex and expensive and has a short shelf life. Only a minority of cultures from definite cases of Lyme borreliosis yield spirochetes. Under these circumstances, *B. burgdorferi* cultivation can hardly be considered the diagnostic method of choice, but this approach remains the only way to confirm a diagnosis. Recovery of *B. burgdorferi* from a patient indicates an active or latent disease state and not simply an inconsequential colonization.

Although several microbiologists apparently have been successful in cultivating relapsing fever borreliae in the laboratory (see reference 17), modern formulas date from Kelly's report of the successful cultivation of the North American tick-borne relapsing fever spirochete *B. hermsii* (101). Kelly added the amino sugar, *N*-acetylglucosamine, to previous concoctions, which specified serum, glucose, and an albumin source.

Stoenner and co-workers subsequently "fortified" Kelly's medium by adding a yeast extract and a concentrated tissue culture mixture (CMRL 1066; GIBCO Diagnostics, Madison, Wis.) containing amino acids, vitamins, nucleotides, and other growth factors (169). These supplements permitted growth of cultures inoculated with a single *B. hermsii* organism. The critical growth-enhancing ingredients of CMRL 1066 supplement have not been determined as yet.

We first recovered a spirochete from *I. dammini* ticks by using Stoenner's version of Kelly's medium (39). This, or a closely related formulation, was then used to recover identical spirochetes from the blood, skin, and CSF of patients with Lyme disease (26, 161). By additional modifications of Stoenner-Kelly medium to improve the buffering capacity and make preparation easier (BSK medium), we were able to isolate a borrelia from *I. ricinus* ticks of Europe and to grow *B. burgdorferi* from a single organism (15). This culture capability allowed us to clone the newly isolated spirochetes by limiting dilution. For details of the current medium formulation used in our laboratory, BSK II, see reference 11.

Kanamycin and 5-fluorouracil have been added to BSK medium for the selective isolation of the spirochetes from

ticks (96). Others have used neomycin, gentamicin, rifampin, or kanamycin alone to reduce contamination (9, 42, 43, 130, 161). We currently use rifampin (50 μg/ml) and phosphomycin (100 μg/ml) to prevent the growth of other bacteria (A. Barbour and A. MacDonald, unpublished results). Successful antimicrobial inhibition and killing studies have been carried out in modifications of Kelly's medium (32, 94, 97, 138). In most studies, the initial borrelial inoculum has been 10^5 cells per ml. The minimum inhibitory concentration has been considered to be that concentration of antimicrobial agent that prevents growth after 48 h or more of incubation. The minimum bactericidal concentration has been the antimicrobial concentration that prevents growth of spirochetes in subculture. Studies with *B. hermsii* have shown that it is possible to determine the minimum bactericidal concentration by injecting cultures exposed to different levels of antibiotic into mice and observing for spirochetemia a few days later (22). The in vitro susceptibility studies have shown that *B. burgdorferi* is susceptible to penicillin, ampicillin, erythromycin, tetracycline, doxycycline, minocycline, chloramphenicol, mezlocillin, cefotaxime, and ceftriaxone (32, 94, 97, 138). The level of susceptibility between strains may vary (32), but individual isolates that show significant in vitro resistance to any one of these agents have not as yet been encountered. *B. burgdorferi* is resistant in vitro to the aminoglycosides, rifampin, metronidazole, and trimethoprim-sulfamethoxazole (92, 97, 138).

B. burgdorferi is grown at temperatures between 30 and 37°C in the laboratory. At temperatures above 38°C, borrelial growth slows substantially (11). Most investigators use temperatures of 32 to 34°C. The cap or lid of the culture vessel is usually tight or sealed to prevent loss of carbon dioxide from the medium. The generation time is 8 to 24 h, and culture-adapted strains achieve cell densities of about 10^8 spirochetes per ml (11). The microaerophilic character of the borrelia is indicated by its preference for the bottom portion of the culture medium during initial growth (11, 92). Addition of low concentrations (0.1 to 0.2%) of agarose to further thicken the medium improves the recovery of *B. burgdorferi* from animal fluids and tissues (4, 6, 94, 95). In some cases reducing agents, such as L-cysteine, dithiothreitol, and superoxide dismutase, have been added as supplements. However, in one report there was no difference in spirochete isolation rates between sets of culture media with or without the reducing agents (4).

A more substantial change has been the deletion of serum from BSK medium (32). When serum is left out, the peptone source must be heated first and a less purified source of bovine albumin must be used (32). Apparently, the heating step and the cruder albumin preparation provide growth factors usually present in serum. These steps would save some money in medium production expenses. Nevertheless, it has not been shown that serumless BSK medium provides an advantage over or is equivalent to serum-containing formulations.

Our early studies showed that *B. burgdorferi* would grow as a lawn on BSK medium containing 0.8% agarose (11). Kurtti and colleagues subsequently grew these organisms as isolated colonies by increasing the agarose concentration to 1.3% and doubling the amount of gelatin (109). The plates were incubated for 2 to 3 weeks in a candle jar. Using this solid medium, these investigators demonstrated growth of at least two different colony types of *B. burgdorferi*.

As stated above, only a minority of specimens from Lyme disease patients have yielded *B. burgdorferi* isolates. Steere and colleagues isolated *B. burgdorferi* from only 2 of 65 patients who had large volumes of blood cultured (160). Benach et al. reported two successful blood sample isolations from blood cultures of 36 patients (26). The chances of recovery are increased if the patient has early or first-stage disease; most successful blood isolations have been from patients with ECM and some evidence of systemic illness (26, 160, 161). *B. burgdorferi* can survive in citrated blood stored at 4°C for 25 days (G. Baronton and I. Saint-Girons, Abstr. Int. Conf. Lyme Dis., abstr. no. 55, 16 Sept. 1987), but most cultures have been inoculated soon after blood collection. Citrated or heparinized blood is lightly centrifuged to separate the plasma from the cellular blood elements. The plasma is then centrifuged at a higher force, and the plasma pellet is suspended in growth medium. The plasma pellet should contain platelets as borrelia are usually found in the platelet-rich fraction of blood. Cells in the CSF have also been concentrated by centrifugation to improve the odds of recovery (136, 138, 139). *B. burgdorferi* has been successfully recovered from the blood of animals (4, 6, 25, 42) and from two human cases (26) without such enrichment. All human lymph node aspirate cultures have been negative to date (161).

The frequency of recovery of borrelia from skin biopsies of ECM lesions has ranged from about 6 to 45% (8, 32, 139, 140, 160). Most biopsies yielding positive cultures have been taken from the expanding edge of the ECM lesion where histologic stains have shown that the spirochetes are in highest numbers. *B. burgdorferi* has also been isolated from skin biopsies of patients with acrodermatitis chronica atrophicans of several years duration (9, 130) and patients with lymphocytoma (86). A skin biopsy was stored frozen at −80°C for 2 years before successful recovery of borreliae in culture medium (130). The etiologic agent has been isolated only infrequently from affected joints (140, 153); synovial fluid cultures of humans and dogs with Lyme arthritis have usually been negative (83, 104, 160). Nevertheless, the ameliorating effect of antibiotics on Lyme arthritis strongly suggests that viable organisms are required for disease progression (64, 83, 159).

Lyme disease spirochetes have been recovered from several types of feral and domestic animals. These include field mice (*Peromyscus leucopus*), raccoons, voles (*Microtus pennsylvanicus*), dogs, horses, cows, and a bird (4, 5, 7, 34, 47, 70, 113). Most isolates, especially from small animals, have been from blood, kidneys, or spleen (4, 6, 113). In addition, *B. burgdorferi* has been isolated from the brain of a horse with encephalitis (45) and the lungs and liver of a cow with arthritis (47). Although *B. burgdorferi* can be cultured from the kidneys of infected animals, to date no successful serially passaged cultures have been obtained from the urine of animals (30, 95).

B. burgdorferi has been isolated from *Ixodes* spp. ticks (2, 7, 15, 39, 43, 96, 161). The culture success rate is close to prevalence of infection in the tick population as established by immunofluorescent detection. Although organisms have been isolated from whole ticks ground up and inoculated into culture medium, the midgut is the site most likely to contain cultivable spirochetes (39, 40, 43). Dissection of the midgut out of the tick reduces the chance of contamination of the cultures (15, 39). Preparing serial dilutions of the inoculated medium can also reduce the chance of contamination; enough spirochetes are usually present in the tick to permit up to 1,000-fold dilutions of the original inoculum (15). In some studies, antibiotics have been added to the medium to prevent overgrowth of bacteria colonizing the exterior and interior of the tick (43, 96). Isolation of spirochetes from

other tissues of ticks that have generalized borrelial infections has, for unknown reasons, been much more difficult to achieve (43, 110).

Once in culture the borreliae may undergo change in one or more traits. Alteration in both the size of outer membrane proteins and the reactivity of these proteins with monoclonal antibodies has been seen after an isolate has been passaged as few as 10 to 20 times (70 to 140 generations) in the laboratory (20, 151, 176). With continuous in vitro passage, *B. burgdorferi* lose the ability to infect laboratory animals (95, 151a) and have shown loss of plasmids from the cells (13, 90, 151a). Whether this is purely coincidental or indicative of plasmid-conferred virulence factors is as yet unknown.

In Vivo Cultivation

The ability of *B. burgdorferi* to infect many different types of mammals, and even birds, indicated that infections of laboratory animals could be established. This has been the case for there is evidence of infections of laboratory mice (*Mus musculus*) (154), Syrian hamsters (95), cotton rats (*Sigmodon hispidus*) (42), rabbits (25, 38, 39, 103), field mice (*P. leucopus* and *P. maniculatus*) (46), dogs (44), rats (23), splenectomized gerbils (154), and chickens (5). The animals have been infected by direct intradermal, intravenous, or intraperitoneal inoculations (42, 95, 103) or by placing spirochete-carrying ticks on the animals to feed (25, 38, 103, 154). When direct injection has been used, the inocula have usually been at least 10^3 borreliae (94). Neubert et al. implanted skin biopsies from patients with ECM in nude mice and observed spirochetes in the blood of these animals a few days later (130). Infected dogs became detectably spirochetemic only after administration of corticosteroids (44).

Some experimental animals, such as the hamster, cotton rat, and mouse, do not suffer apparent illness, even though the infection is shown to be systemic. The rabbit develops skin lesions that are similar in gross appearance and histology to ECM (25, 38, 39, 103), but neurologic or arthritic disorders have not been noted. Dogs living in endemic areas have arthritis attributable to *B. burgdorferi* (104, 112, 119, 120), but as yet there are no reports of arthritis produced in experimentally infected dogs (44). Infant rats inoculated by the intraperitoneal route with *B. burgdorferi* have developed the greatest range of clinical signs (23). Of particular importance is the finding that infected infant rats have an arthritis that resembles in its timing and characteristics the secondary stage polyarthritis of human infections.

In most animals, spirochetemia lasting from 1 to 4 weeks has been shown by direct examination of the blood (103, 154), cultures in BSK medium (25, 42, 95, 154), or xenodiagnosis (38, 41, 42). A peak spirochetemia of about 10^5 borreliae per ml in experimental animals infected with *B. burgdorferi* (154) is 10- to 100-fold lower than that noted in some animals infected with tick-borne relapsing fever borreliae (17, 171).

In the xenodiagnosis approach, spirochete-free ixodid ticks feed on infected animals (38, 41, 42). The ticks are later examined by dark-field or immunofluorescence microscopy for the presence of spirochetes. Whether some attractant or adherent material is associated with the feeding mouth parts of the tick, leading to enrichment of spirochetes in the blood meal taken from the host, is not known. Conceivably, xenodiagnosis could also work in humans and would be a fairly simple, if unorthodox, way to determine if there is spirochetemia.

Studies of infected rabbits, cotton rats, and mice have shown a biphasic pattern to the spirochetemia (38, 42, 154). That is, after an initial peak in the numbers of borreliae in the blood, the spirochete concentrations decrease, only to rise again a few days later. The borreliae then gradually disappear from the blood completely. One explanation of this pattern is that some form of antigenic variation occurs as in relapsing fever (17).

After the spirochetemia has passed, *B. burgdorferi* can still be recovered from the host spleen and kidneys. In cotton rats (42), but apparently not hamsters (95), the borreliae commonly persist in the brain. The eye has been involved in some animals as well as in humans (157).

Borreliae may be present in the kidneys and urine of animals but usually not in humans. Little overt renal disease has ever been documented in humans with Lyme borreliosis, and the human urine specimens that have been cultured have been negative (160). Nevertheless, in one case spirochete-like structures were demonstrated in the urine of a man with suspected Lyme borreliosis (1).

The possibility of viable spirochetes in the urine of infected animals has significance for care of experimentally infected animals. Burgess et al. suggested that contact transmission of *B. burgdorferi* from one animal to another occurs, possibly via the urine (46).

In one published study, hamsters were used to assess the efficacy of different antimicrobial agents on *B. burgdorferi* (94). The inhibitory and bactericidal concentrations were established by culturing the spleen and kidneys of the animals several days after a course of parenteral antibiotics. A potentially important consideration for choice of animals for in vivo antibiotic studies is whether *B. burgdorferi* commonly persists in the brain of the animal. An antibiotic that successfully eliminates the spirochete from the blood, kidneys, and spleen may not reach bactericidal concentrations in the CSF.

Cell-Mediated Immunity

Several studies have been carried out on the role of cell-mediated immunity in Lyme disease and, specifically, the interaction between *B. burgdorferi* cells and the cellular elements of the immune system. Although much of this work does not have immediate relevance for clinical microbiology, a brief consideration of pertinent immunologic findings might provide useful background for understanding the disease and future developments.

The *B. burgdorferi* cells associate with macrophages and polymorphonuclear leukocytes even in the absence of immune serum (29, 135). Phagocytosis has been demonstrated under these conditions, but it is still unclear whether the association between the borreliae and the macrophages in this situation is entirely due to uptake of the spirochetes into the phagocyte. The spirochetes that are taken up may be those already damaged during the centrifugation and washing steps of cell preparation. Moreover, the finding that killed *B. burgdorferi* cells adhere to T- and B-cell lymphocytes suggests that the spirochete has binding sites on its surface for eucaryotic cell ligands (68). The addition of specific antiserum significantly increases the association of the spirochetes with macrophages and other phagocytic cells; this appears to be due to phagocytosis of the borreliae (29, 135). The borreliae appear to be killed once inside a phagocytic cell (29). Uptake appears not to be mediated by heat-labile plasma components such as complement (29, 135).

Investigations of cell-mediated immunity showed a specific response by T cells of Lyme borreliosis patients to *B. burgdorferi* antigens (57, 124, 132, 152). This response has been demonstrated for T cells obtained from the peripheral blood, the diseased synovium, and the CSF. The specific proliferative response of T cells from either CSF or synovial fluid is greater than that of peripheral blood T cells obtained at the same time from a Lyme disease patient (132, 152). The greatest reactivity is at the site of the localized infection, be it the nervous system or a joint. Antigen-specific T-cell responses have also been demonstrated in the synovial fluids of patients with postinfectious Reiter's syndrome (66). T-cell clones that respond specifically to *B. burgdorferi* antigens have been recovered from the CSF of a patient with Lyme borreliosis (124).

Patient's T cells were significantly stimulated by whole cells as well as soluble components of the spirochete (57, 132, 152). T-cell blastogenesis was more vigorous in the presence of whole borreliae than when a sonicated supernatant was used (R. J. Dattwyler, D. J. Volkman, J. Thomas, P. A. Falldorf, and M. G. Golightly, Ann. N.Y. Acad. Sci., in press). The active response to whole cells of *B. burgdorferi* indicates that patients are responding to cell surface components.

The stimulation index of T-cell responsiveness to whole borreliae has been used at one institution to confirm the diagnosis of Lyme borreliosis (57). Some patients demonstrate a significant cell-mediated immune response to the borreliae when they have only borderline or slightly elevated antibody titers to the organisms (57). Family members of Lyme disease patients have higher stimulation indices than unrelated controls, indicating either a hereditary predisposition or shared exposure to the infectious agent (152). The T-cell responses of patients show less apparent cross-reactivities than the corresponding antibody responses to relapsing fever borreliae antigens (152).

In affected tissues and organs, there is usually a prominent lymphocytic infiltration. Among T cells, there are more helper/inducer cells than cytotoxic/suppressor cells (57, 127, 152); this is also the case in joint fluids in rheumatoid arthritis patients (72). In the skin, large numbers of dendritic Langerhans cell have also been seen (37). In biopsies of ECM lesions, HLA-DR markers have been found on keratinocytes; patients acrodermatitis chronica atrophicans have both HLA-DR and HLA-DQ markers displayed on keratinocytes in their chronic skin lesions (172).

Antibody Response

Currently, the usual method for establishing a diagnosis of Lyme borreliosis is serologic testing. The most commonly used assays for human and animal serologic studies are indirect immunofluorescence assays (IFA) and ELISA (2, 14, 26, 28, 39, 54, 121, 126, 128, 144, 161, 168, 178). The assays utilize either whole *B. burgdorferi* cells or crude fractions of sonicated organisms. Several public and private laboratories offer one or the other of the tests. However, neither the antigens nor the methods have been standardized between laboratories. A survey of four different laboratories offering the *B. burgdorferi* IFA revealed an unacceptable number of discrepancies between test results on identical samples (81). Some investigators have found the ELISA to be more sensitive and specific than the IFA in detecting early disease (54; L. E. Mertz, G. H. Wobig, J. Duffy, and J. A. Katzmann, Abstr. Int. Conf. Lyme Dis., abstr. no. 39, 16 Sept. 1987). However, in laboratories that have experience with the assay and specify a conservative cutoff titer, the IFA is at least as predictive as the ELISA in first- as well as second- and third-stage disease (2, 121, 144).

Infected humans produce IgM, IgG (14, 54, 82, 116, 161, 168, 178), and IgE antibodies (30) that recognize *B. burgdorferi* antigens. There does not seem to be a significant specific IgA response (82). The bulk of the IgG-reactive antibodies are of the IgG1 and IgG3 subclasses (B. Vandvik, Abstr. Int. Conf. Lyme Dis., abstr. no. 43, 16 Sept. 1987; K. E. Hechemy, H. L. Harris, and M. J. Duerr, Abstr. Int. Conf. Lyme Dis., abstr. no. 18, 14 Sept. 1987).

In the IFA, whole borrelial cells are invariably used. The cells are dried on the slide with or without yolk sac material. Our laboratory uses washed sheep erythrocytes mixed with the borreliae to evenly distribute the spirochetes on the smear and to provide a convenient reference point for microscope focusing (14, 21). Once dried on the slide, the spirochetes are fixed with methanol or acetone; some investigators freeze the slides without the organic solvent fixation step. Diagnostic cutoff IgG titers range between 64 and 256 in different laboratories, depending in part on the accepted criterion for intensity of fluorescence. Whereas one laboratory may consider spirochetes emitting fluorescence at an intensity equal to the background as a "nonreactive" result, another laboratory may call such detectably fluorescing borreliae still reactive. Consequently, the latter laboratory would, likely, have a higher cutoff point than the former one. Another factor that lowers the titer and is considered diagnostic is whether a "sorbent" material is used. In most reports the adsorbing material was derived from *Treponema phagedenis* biotype Reiter cells (2, 54, 89, 126, 128, 130, 177, 178). While the adsorption step reduces antibody titers to both *B. burgdorferi* and *T. phagedenis* in Lyme disease patients, the magnitude of the drop is greater against *T. phagedenis*. Adsorption with the relapsing fever species *B. hermsii* also reduces the antibody titer to other spirochetes (54, 117). However, because these closely related spirochetes share several antigens (14, 18, 117, 130, 176), more may be lost in sensitivity than gained in specificity (54, 117). At some institutions, human serum samples are also treated with anti-human IgG antibodies before testing for *B. burgdorferi*-binding IgM antibodies (83, 168). Adaptions of the IFA are the FIAX test (International Diagnostic Technology, Inc., Santa Clara, Calif.) (134), which measures the fluorescent emission from antibody-bound antigens on nitrocellulose membranes, and the slide immunoperoxidase assay (150). The advantage of the latter assay is the use of a simple light microscope instead of a fluorescence microscope. The results of the slide immunoperoxidase assay appear to correlate closely with the results of the IFA. The FIAX test requires no microscope at all, but the published study reports lack of specificity for the assay as it is now formulated (134).

The ELISA tests reported on specify use of either whole cells (117, 121) or the supernatant of sonicated cells (54, 144). The diagnostic cutoff points are ELISA absorbances that are 2.5 to 3 standard deviations above the mean for a group of healthy control patients.

In an early serologic study of Lyme disease patients, we used a solid-phase radioimmunoassay incorporating a detergent-soluble fraction of *B. burgdorferi* as the antigen and radioiodinated protein A as the second ligand (14). For the sample of patient sera evaluated, the radioimmunoassay was more sensitive and specific than the IFA.

While many laboratories use the original Shelter Island, N.Y., isolate of *B. burgdorferi*, strain B31 (ATCC 35210),

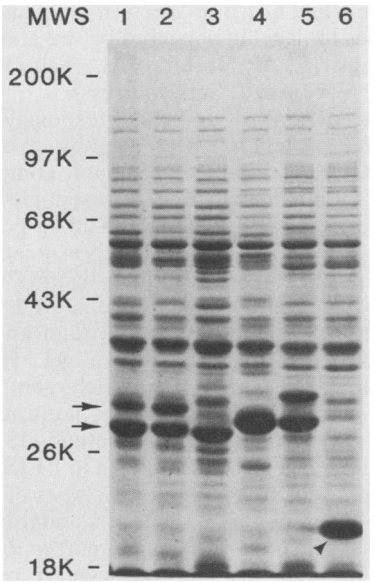

FIG. 3. Coomassie blue-stained proteins in whole-cell lysates of six isolates of *B. burgdorferi* from North America and Europe. The components were separated by polyacrylamide gel electrophoresis (acrylamide concentration, 10%). The electrophoretic migrations of molecular weight standards (MWS) are shown on the left. The two arrows point to the OspA (31K) and OspB (34K) proteins of strains 1 and 2. The arrowhead indicates the pC protein of strain 6. The flagellin protein is the major band with a relative migration of 41 kDa.

several other isolates are also used. This practice of using different strains may have little consequence for testing within North America, where strains are very similar to one another in their antigenic makeup (12, 20, 43). On the other hand, European strains are more heterogeneous in the types of major outer membrane proteins they possess (Fig. 3) (19, 155, 176; B. Wilske, V. Preac-Mursic, G. Schierz, R. Kuhbeck, A. G. Barbour, and M. Kramer, Ann. N.Y. Acad. Sci., in press). The differences between strains are not great enough to completely invalidate use of one strain, even a North American one, like B31, in all geographic areas. This strain has been used successfully in Europe for serologic testing (2). Nevertheless, a one- or two-tube difference between the reactivity of a particular immune serum against one strain versus another could, in some instances, mean that a sample could be called falsely negative. There is also the question whether continuous passage of the test strain could result in loss of certain critical antigens for IFA and ELISA. Changes in the major outer membrane proteins OspA and OspB (Fig. 3) have been noted during serial in vitro cultivation (20, 33, 151, 151a, 176; Wilske et al., in press).

False-positive IFA and ELISA reactions can occur in patients with syphilis or relapsing fever, two other spirochetal diseases (89, 116, 117). Lyme borreliosis patients occasionally have reactive fluorescent treponemal antibody-absorption and treponemal agglutination tests for syphilis (89, 117, 128); in these as well as treponemal antibody-negative cases, reagin antibody-assays, such as the Venereal Disease Research Laboratory and rapid plasma reagin tests, are negative. As demonstrated by IFA, syphilis, yaws, and pinta patients have had high titers of antibodies that react with *B. burgdorferi* (14, 117, 126). The immunofluorescence reactions of sera from syphilis patients are qualitatively different from those seen with sera from Lyme disease patients. The cross-reactive antibodies of syphilis patients do not bind to the outer membrane blebs of the spirochete, and, consequently, the stained spirochetes appear thinner and less irregular than fixed organisms bound by antibodies recognizing outer membrane antigens (14). In serologic tests, some Lyme disease patients have equivalent titers to *B. burgdorferi* and *B. hermsii*, a relapsing fever agent in North America (117). Patients with tick-borne and louse-borne relapsing fever have cross-reactive antibodies to *B. burgdorferi* (117). Although this is a potential problem, the clinical presentation and the epidemiologic features of the case would usually allow discrimination between Lyme borreliosis and relapsing fever. There is much less cross-reactivity between *B. burgdorferi* and the leptospires (14, 117). Only a few serovars of *Leptospira interrogans* seem to be weakly cross-reactive with *B. burgdorferi* (118).

Some patients with rheumatic diseases, such as rheumatoid arthritis and systemic lupus erythematosus, which the physician may be trying to distinguish from Lyme borreliosis, have false-positive reactions in *B. burgdorferi* serologic tests (14, 53, 116, 144). These reactions appear due to the nonspecific sticking of rheumatoid factor aggregates or immune complexes to the borrelial antigens. One indicator of a false-positive IFA reaction among rheumatic disease patients is the beaded appearance of the spirochetes (128). This type of staining reaction is also seen in false-positive fluorescent treponemal antibody-absorption test results of patients with systemic lupus erythematosus and other autoimmune diseases (125).

IgM antibody determinations tend to be less specific than those for IgG antibodies, but may be useful in early disease or when reactivation or reinfection is suspected (54, 126). Patients with infectious mononucleosis often have false-positive IgM tests (126, 161, 168). Magnarelli and Johnson found that 5 of 16 patients with Rocky Mountain spotted fever and three of 7 patients with rheumatoid arthritis had a positive IgM-specific ELISA for *B. burgdorferi* (116).

While patients with second- or third-stage Lyme borreliosis almost always have elevated IgG titers, those with early disease often have serum antibody titers below the diagnostic threshold for 6 weeks or more after onset (2, 54). Only about 50 to 60% of patients with early disease, i.e., ECM, have diagnostic titers as measured by either IFA or ELISA (2, 14, 54, 126). Antibiotic therapy of first-stage disease may blunt the immunoglobulin response to the point that diagnostic thresholds are never reached (161). In cases of reinfection, the antibody titers to *B. burgdorferi* may show a fourfold rise from the previous convalescent value (137).

In patients with a neurologic disorder attributable to Lyme borreliosis, the antiborrelia antibody concentrations in the CSF are usually higher than could be accounted for by leakage of circulating antibodies into the CSF. Any CSF titer above 5 is probably significant (168). However, antibodies may be present in the CSF as a consequence of disturbance of the blood-brain barrier. One indication of nervous system involvement is the presence of oligoclonal immunoglobulin peaks in the CSF but not in the serum (82, 84, 129, 143). To further establish that the antiborrelia antibodies were produced intrathecally, a comparison of serum antibodies and CSF antibodies can be carried out. CSF/serum-specific antibody ratios can be adjusted by using factors that take into account the total IgG, IgM, or albumin concentrations in the CSF and serum. The resultant indices serve to identify those patients with antibody produced locally in the central nervous system (82, 84, 85, 105, 168, 170, 178). Calculations

of such an index may be needed, for example, to accurately diagnose the disease in a patient who has a neurologic disorder resembling multiple sclerosis and an elevated titer to *B. burgdorferi* in the serum.

Western blot (immunoblot) assays have been performed on a research basis to determine to which protein antigens patients are responding with antibody (12, 14, 51, 53, 74, 176, 178; Wilske et al., in press). These studies have confirmed the finding of IFA and ELISA studies that there is a delay in production of detectable amounts of antibody to the borreliae. Once antibody production begins, it is usually in the form of IgM antibody to flagellin (18), a 41,000-dalton (41-kilodalton [kDa]) protein that is the predominant component of the flagella (53, 74). With time, both IgM and IgG antibodies to a variety of other antigens appear; these include proteins with apparent molecular weights of 15,000, 27,000, 55,000, 60,000, 66,000, and 83,000 (12, 14, 53, 177, 178). The 66-kDa protein appears to be another protein associated with the outer membrane (20, 51). The more chronic and complicated the disease, the greater the number of antigens to which the patients respond. Almost all patients with Lyme disease of more than a few weeks duration have IgG antibody to the 41-kDa flagellin protein (14, 51, 53, 74). Some patients have IgE antibodies to the 41-kDa protein (30).

Other abundant proteins of the *B. burgdorferi* cell are the major surface-exposed proteins OspA and OspB (87, 88). In most North American strains, the apparent molecular weights of these proteins are 31,000 and 34,000, respectively (Fig. 3) (20, 21). These proteins appear to be highly immunogenic in experimental animals that have been injected with whole organisms (20, 21; unpublished observations). Paradoxically, humans with Lyme borreliosis develop antibody against OspA and OspB, if they develop them at all, only late in the course of the disease (14, 51, 53).

Sera from patients with other spirochetal disease have shown cross-reactions in Western blots to the 41- and 60-kDa proteins of *B. burgdorferi* (14, 74, 176). Considering the known antigenic relatedness between the flagella of the different *Borrelia* spp. (18), one might expect some degree of cross-reactivity to the flagellin protein. Epitopes of the 60-kDa protein antigen appear to be conserved among various spirochetes (75a, 176).

Qualitative as well as quantitative differences may be seen in Western blots and other immunoblotlike assays that use serum and CSF obtained from the same patient with neurologic involvement (129, 178). To date, these differences have not been noted when paired serum and synovial fluid specimens from patients with arthritis have been examined (53; unpublished observations). The B-cell response to *B. burgdorferi* has been demonstrated by studies of immunoglobulin synthesis and specificity on an individual B-cell level (111).

The antibody responses of dogs to protein antigens of *B. burgdorferi* have also been examined by Western blot. Greene et al. found that the patterns of reactivities to the various components of the spirochete differed between experimentally and naturally exposed dogs (73). While both groups of dogs had IgG antibodies specific for the 41-kDa flagellar protein, the naturally exposed dogs recognized a much wider variety of antigens than did dogs infected through intravenous inoculations of borreliae. Another difference between the two groups of dogs was the finding that experimentally infected dogs demonstrated antibodies to the OspA and OspB proteins, but dogs naturally exposed through tick bites in the community did not. Bosler et al. have also noted that experimentally infected dogs had antibodies to OspA and OspB in Western blots and that detectable antibodies to flagellin appeared before antibodies to the surface proteins (E. M. Bosler, T. L. Schulze, and D. P. Cohen, Ann. N.Y. Acad. Sci., in press).

The finding of almost universal responsiveness to the 41-kDa flagellar protein has been used by investigators as a point of departure for studies of subunit components of *B. burgdorferi*. Coleman and Benach used purified flagellin protein eluted from sodium dodecyl sulfate-polyacrylamide electrophoresis gels (51); the protein presumably was denatured during purification. These investigators found that an ELISA based on a cruder but undenatured "flagellin-enriched" fraction was more sensitive than an ELISA that used purified flagellin eluted from a gel. Hansen et al. isolated whole flagella through mild detergent disruption of the cells and subsequent density gradient ultracentrifugation (76); flagella remain intact by this method (18). This group used the isolated flagella as the antigen in ELISA testing and found improved sensitivity in serologic tests of patients with early disease when compared with a standard ELISA (76). The heightened sensitivity was due in part to lowering of the cutoff point between positive and negative reactions. The assay of Hansen and co-workers appeared to provide greater discrimination between patients with Lyme borreliosis and those either without disease or with nonspirochetal disorders.

The outer membrane OspA and OspB proteins are other isolated components of the borrelial cell that have been examined by Coleman and Benach for use in immunoassays (51). This study, which used eluted proteins in an ELISA, confirmed the Western blot analyses that showed antibodies against these antigens appearing later in the course of the disease. Although these outer membrane proteins may not be useful for immunodiagnosis of early disease, they could have a role as components of a very specific assay in secondary or tertiary disease in humans. A patient with Lyme arthritis had antibodies that bound to recombinant OspA and OspB proteins (88), and, thus, it is likely that patients are responding to the proteins themselves and not carbohydrate or glycolipid moieties that might be associated with them.

The Western blot analysis has been proposed as a practical clinical laboratory test for Lyme borreliosis (74). The advantage to this procedure is that the response to individual components can be examined. Grodzicki and Steere found the Western blot to be the most sensitive test in early Lyme borreliosis (74). Kirsch et al. used the Western blot to diagnose Lyme disease in a patient with a fatal illness (102).

Almost all immunodiagnostic assays reported on have used culture-grown borreliae that were washed and centrifuged at least twice before use in the assay. Whether or not loosely associated spirochetal antigens, such as a slime layer, could be dislodged from the cell surface during antigen preparation is not known. Neubert and colleagues used borreliae obtained directly from the blood of an infected mouse for their IFA; however, the *Borrelia* species used in the test was not *B. burgdorferi* (130). Another area that has been little investigated is whether there are important nonproteinaceous antigens of *B. burgdorferi*.

TYPING STRAINS

As the number of isolates of *B. burgdorferi* from different human and animal sources and from different parts of the world increases, greater attention is being paid to strain distinctions. Several options are available, including poly-

acrylamide gel electrophoresis profiles of cellular proteins, reactivities of monoclonal antibodies, and plasmid analysis.

The initial isolates of *B. burgdorferi* from the United States were almost identical in their polyacrylamide gel electrophoresis profiles (12, 20, 21, 43). They all had major proteins of 31 (OpsA) and 41 (flagellin) kDa. A large majority had an abundant 34-kDa surface protein, OspB, but some isolates either lacked this protein or had an OspB with a slightly different electrophoretic migration (12, 20). As more isolates from Europe were examined, differences in the OspA and OspB proteins were noted (19, 155, 176; Wilske et al., in press). The OspA-like proteins varied from approximately 30 to 33 kDa in apparent size. OspB-like proteins also varied; some European strains had no major protein that could be considered the equivalent of OspB. Some strains, especially those from regions of Germany, Austria, and Scandinavia, lacked even an OspA-like protein. Instead, they had a major protein of about 22 kDa. This protein has been designated "pC" by Wilske et al. until its surface localization can be confirmed (176; in press). A single United States strain with a major surface protein of about the same size as pC has been isolated from a tick in California (33).

When antisera prepared against whole cells of different strains have been compared by IFA, too few differences in the reactivities of the various isolates have been seen to justify a serologic typing scheme based on use of antisera against whole cells (7). Polyclonal antibodies to isolated cell components, such as OspA and pC protein, offer better discrimination between strains (Wilske et al., in press), as do monoclonal antibodies. The monoclonal antibodies are directed against single epitopes in one protein, usually either OspA- or OspB-like proteins (20, 21; Wilske et al., in press). Using criteria of polyacrylamide gel electrophoresis profiles, polyclonal antisera reactivities, and monoclonal antibody binding, Wilske et al. (in press) identified seven distinct types of *B. burgdorferi* among a panel of European strains.

Another way to characterize *B. burgdorferi* isolates is to analyze their plasmid content; both circular and linear plasmids have been identified (13, 16, 90, 151a). A relatively simple extraction procedure can be used to enrich for plasmids in the DNA preparation (13, 16). The plasmid species are then separated on low-percent agarose gels. Analyses have shown considerable heterogeneity in plasmid profiles among strains, even those from North America (13). Plasmids either undergo rearrangement or are lost from the cell during serial in vitro cultivation (13, 90, 151a).

DNA hybridization of whole chromosomal DNA has shown that *B. burgdorferi* is a distinct species in the genus *Borrelia* (90, 92, 93, 148) and that strains within the species differ in the amount of DNA relatedness. These differences may not be great enough, however, to use genomic DNA hybridization as a routine typing procedure for *B. burgdorferi*. Its most appropriate use is still as a tool for determining whether an unknown arthropod-associated spirochete is a member of the genus *Borrelia* and to what species it is most closely related. Use of DNA probes for specific genes, such as the *ospA* gene, may offer more advantages for distinguishing between strains within the species (19).

LABORATORY SAFETY

B. burgdorferi is a blood-borne pathogen of humans and domestic animals that can cause significant and prolonged disease. It may be confused with a variety of other chronic, noninfectious disorders. *B. burgdorferi*, like the relapsing fever borreliae, has been considered a biocontainment level 2 organism, and it is appropriate to continue to treat it as such. Although there have been no documented examples of laboratory-acquired Lyme borreliosis in humans, there clearly has not been enough experience with the organism to be complacent about its risk to laboratory workers. The most likely routes of infection would be through a break in the skin, the conjunctiva, and the oral mucosa; experience with the closely related relapsing fever borreliae indicates that infection can occur through these routes (17). Infected blood and cultures pose the greatest potential risk, but animal and laboratory workers may also be infected through contact with urine of infected animals (35, 46) and through handling live ticks. There is probably little chance of infection through aerosolization or contact with spirochetes that have dried on animate or inanimate materials.

CONCLUDING REMARKS

The clinician and clinical microbiologist attempt to confirm a diagnosis of Lyme borreliosis based on clinical findings and epidemiologic circumstances. Several approaches to the laboratory diagnosis of *B. burgdorferi* infection have been discussed in this review. *B. burgdorferi* can be detected directly in tissue or body fluid, isolated in culture medium, or identified in experimental animals inoculated with patient specimens. Alternatively, the etiologic agent can be used as the basis of a serologic test. At present, only the last option, that is, diagnosis based on antibody titers to the spirochete, is practical in most situations. An exception is ECM during first-stage Lyme borreliosis. In this case, a skin biopsy subjected to silver stain and microscopic examination has a reasonable likelihood of revealing spirochetes. For other clinical manifestations, such as meningoradiculitis or arthritis, direct detection of the spirochetes in tissues has proven to be more difficult. We await the development of methods of even greater sensitivity to rapidly detect the presence of *B. burgdorferi* in CSF and the joint.

For the great majority of cases confirmed as Lyme borreliosis through laboratory examination, the successful assay was either an IFA or ELISA test. Both types of immunoassay incorporate either whole cells or a crude fraction of the spirochetes as the test antigen. Although these assays for antibody to *B. burgdorferi* have proven of great value in the diagnosis of Lyme borreliosis in its various forms, the specificity of currently available assays is not high. Consequently, the present-day serologic assays should only be used to confirm a strong clinical impression of Lyme borreliosis. If the serum is obtained too early in the course of infection or if a patient with first-stage disease has been treated with appropriate antibiotics, the serologic test result may be falsely negative. Laboratory assays that provide greater specificity to reduce the risk of false positivity and greater sensitivity to improve the laboratory diagnosis of early or treated Lyme borreliosis are needed.

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