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## Clinical implications of delayed growth of the Lyme borreliosis spirochete, *Borrelia burgdorferi*

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Lyme borreliosis, a spirochetal infection caused by *Borrelia burgdorferi*, may become clinically active after a period of latency in the host. Active cases of Lyme disease may show clinical relapse following antibiotic therapy. The latency and relapse phenomena suggest that the Lyme disease spirochete is capable of survival in the host for prolonged periods of time. We studied 63 patients with erythema migrans, the pathognomonic cutaneous lesion of Lyme borreliosis, and examined in vitro cultures of biopsies from the active edge of the erythematous patch. Sixteen biopsies yielded spirochetes after prolonged incubations of up to 10.5 months, suggesting that *Borrelia burgdorferi* may be very slow to divide in certain situations. Some patients with Lyme borreliosis may require more than the currently recommended two to three week course of antibiotic therapy to eradicate strains of the spirochete which grow slowly.

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Key words: *Borrelia burgdorferi*; Lyme borreliosis; Delayed growth

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### Introduction

*Borrelia burgdorferi*, a fastidious spirochetal pathogen, is the etiologic agent of Lyme borreliosis. Since Dr. Willy Burgdorfer's discovery of the spirochete in 1981 (Burgdorfer et al., 1982), relatively few cases of its isolation from human tissue or body fluid have been reported (Benach et al., 1983; Chengxu et al., 1988; Hoymark et al., 1986; Huppertz et al., 1986; Neubert et al., 1986; Pfister et al., 1984; Preac-Mursic et al., 1986; Preac-Mursic et al., 1984; Rawlings et al., 1987; Stanek et al., 1990; Steere et al., 1983; Stiernstedt et al., 1988). More than 13 000 cases of Lyme borreliosis from the United States have been recorded in the Centers for Disease Control Registry since 1982 (Centers for Disease Control, 1989). The infection is encountered by physicians in North America, Europe, the Soviet Union, China, and Australia. In certain humans, *B. burgdorferi* has caused disease after a period of clinical latency and this has prompted comparisons between this spirochete and *Treponema pallidum* (Pachner, 1988). A special example of prolonged survival of *B. burgdorferi* in humans is the condition acrodermatitis chronica atrophicans (ACA), from which skin

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lesions that have been present for 10 years have yielded *B. burgdorferi* in culture from biopsy material (Asbrink and Hovmark, 1985). Survival in the host tissue for a decade suggests that *B. burgdorferi* may, in certain circumstances, show very delayed growth in humans. We investigated the possibility that cultivation of biopsy specimens from erythema migrans for periods of up to one year might reveal strains of *B. burgdorferi* with very slow growth.

### Materials and Methods

From June 1986 to September 1987, biopsies of skin from the advancing edge of the centrifugally expanding erythema migrans lesions were obtained from patients in the Southampton area of Long Island, New York. Biopsies were transferred to sterile plastic vials containing 6.0 ml of modified Barbour-Stoenner-Kelly (BSK) medium, as previously described (Barbour, 1984; Berger et al., 1985) but lacking rabbit serum and gelatin. The biopsies obtained in 1986 were vortexed for one minute, left in the culture medium for 24 h, and then removed. Biopsies in 1987 were obtained using identical technique, but in contrast to the 1986 specimens, they were left in the culture medium for 12 months. Darkfield examination of each culture tube began 3 weeks after inoculation and was repeated at monthly intervals until spirochetes were detected or until 12 months had elapsed. All specimens were incubated at 35 °C for the first three weeks and were then maintained at 24 °C for the remainder of the study. Aliquots from positive cultures were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Barbour and Schrupf, 1986), and Western blot analysis using monoclonal antibody H5332 which is reactive with the outer surface protein A (OspA) unique to *B. burgdorferi* (Barbour et al., 1983).

### Results

Spirochetes were recovered after prolonged incubation of skin biopsies from 16 patients with erythema migrans lesions (Table 1). The time to detect motile spirochetes by darkfield examination averaged 181 days (6 months) with a range of 76–319 days (2.5–10.5 months). However, during 1987 when biopsied material was left in the culture medium, spirochetes were seen sooner than during 1986 with mean incubation times for detectable growth being 147 days and 208 days, respectively.

TABLE 1

Number of isolations of *Borrelia burgdorferi* from human skin biopsies and incubation periods for cultures to become positive

Year	Numbers of cultures		Total	Incubation (days)	
	Positive	Contaminated		Mean	Range
1986	9	2	23	208	155–319
1987	7	7	40	147	76–235
Total	16	9	63	181	76–319

Specimens processed in 1986 yielded 9 primary isolates (39%) while in 1987 only 7 isolates were recovered (17%). Spirochetes were not found in any of the cultures that became contaminated. None of the isolates in subculture grew as rapidly as the prototype strain of *B. burgdorferi*, B31, (American Type Culture Collection #35210). SDS-PAGE of whole cell lysates for 8 of the 16 isolates showed protein profiles typical of *B. burgdorferi* (Fig. 1). Although these eight isolates showed very similar protein profiles, some differences were evident. For example, isolate number 5 had a dominant protein with an apparent molecular mass of 24 kDa which was not as pronounced in any of the other isolates examined. These eight isolates all showed the dominant outer surface protein OspA, which has an apparent molecular mass of approximately 31 kDa. By Western blot analysis, monoclonal antibody H5332 reacted with OspA of the eight isolates (Fig. 2), which along with the SDS-PAGE profiles, identify these isolates as *B. burgdorferi*. We did not examine the other eight isolates by SDS-PAGE or reactivities with monoclonal antibodies. However, the fact that these other isolates were also cultured in BSK media from active erythema migrans lesions from patients living in an endemic Lyme borreliosis area, makes it unlikely that these other spirochetes were something other than *B. burgdorferi*.

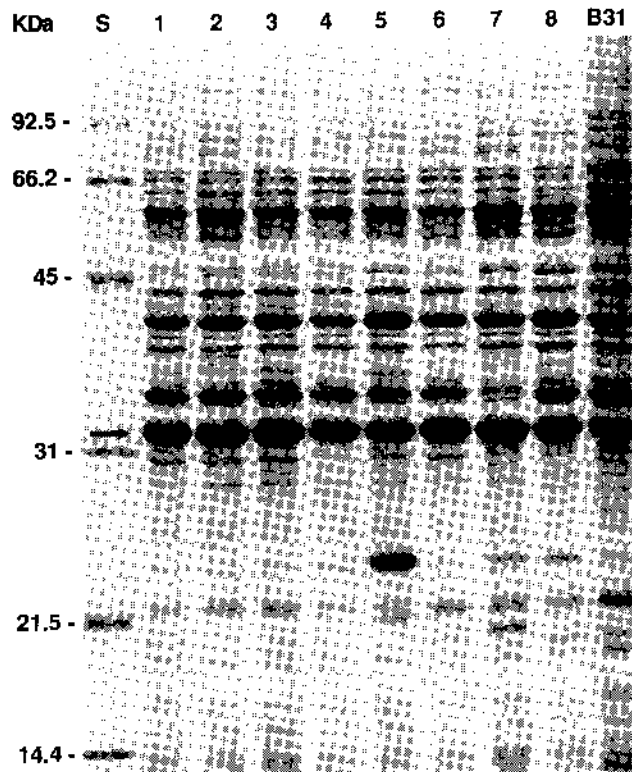


Fig. 1. SDS-PAGE profiles of whole-cell lysates of *Borrelia burgdorferi* after prolonged incubations from skin biopsies taken from erythema migrans lesions of eight human patients with Lyme borreliosis (lanes 1-8) and the prototype strain B31. Arrow indicates position of outer surface protein A. Molecular mass standards (S) are from Bio-Rad Laboratories, Richmond, Calif. Gel stained with Coomassie Brilliant Blue.

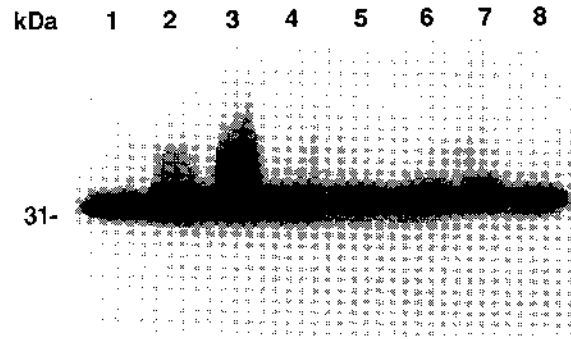


Fig. 2. The Western blot reactivity of isolates 1-8 with monoclonal antibody H5332, specific for outer surface protein A of *B. burgdorferi*.

### Discussion

Previous investigators may have underestimated the amount of time that is required for certain wild strains of *B. burgdorferi* to divide *in vivo* or *in vitro*. In the laboratory, strain B31, adapted to *in vitro* cultivation, divides rapidly (every 6 to 12 h) (Barbour and Hayes, 1986), and has been viewed as a reasonable model for the study of the antimicrobial chemotherapy of Lyme borreliosis and the humoral immune response of various hosts to infection by *B. burgdorferi*. *In vitro* cultivation of other strains, however, has demonstrated the loss of spirochetal proteins and possible virulence factors, rendering them incapable of producing infection in experimentally inoculated animals (Schwan and Burgdorfer, 1987; Schwan et al., 1988).

Species of *Borrelia* other than *B. burgdorferi* may survive for remarkably long periods of time in various hosts. Felsenfeld (1971) reviewed reports by several investigators showing that *Borrelia* species may survive in *Ornithodoros* ticks in the laboratory for 2 to 12 months. Survival of *Borrelia* species in the brains of guinea pigs for 1 to 3 years without detectable disease in these hosts was exploited by investigators earlier in this century (Felsenfeld, 1971) to preserve strains of *Borrelia* prior to the discovery by Kelly (1971) of an artificial medium for *in vitro* cultivation.

A minimal essential medium for *in vitro* cultivation of *B. burgdorferi* has not been defined. The original formula of Kelly (1971) has been modified several times and each modification has been used successfully to cultivate *B. burgdorferi* from human tissue or body fluids. Irrespective of the BSK modification, however, wild strains of *B. burgdorferi* are difficult to recover and for this reason attempts to culture the organism cannot be expected to be a practical diagnostic tool for the practicing physician. BSK medium without rabbit serum has been used (Berger et al., 1983; Berger et al., 1985; Asbrink et al., 1984) to isolate *B. burgdorferi* from skin lesions. Johnson et al. (1984) added antibiotics to suppress the growth of nonspirochetal microbes, and Russell C. Johnson (unpublished) has added reducing substances to enhance the medium. Bovine serum albumin (Fraction V) from commercial sources should be tested each time a new shipment is received because some preparations may be unsuitable for *in vitro* cultivation of *B. burgdorferi* and could influence the time for a culture to show growth. We chose not to add antibiotics, rabbit serum or a viscosity

enhancer such as gelatin or agarose, none of which is required for the in vitro growth of *B. burgdorferi* (Barbour and Hayes, 1986). The incubation temperature of 35°C for the first three weeks was identical to the temperature used by most other workers presented in Table 1. However, we realize that the media and cooler temperatures used after 3 weeks in our study may have had some effect on retarding the growth of the freshly isolated spirochetes. Yet strain B31 grew well under these conditions. Also, media in which skin biopsies were left produced more rapidly growing spirochetal cultures compared to media in which biopsies remained for only the first 24 h. Therefore, it is unlikely that skin biopsies retarded the growth of *B. burgdorferi* but may have actually enhanced the media. Our results also demonstrate that holding cultures for isolation attempts for longer than 3 weeks may be warranted, although we certainly are not suggesting that such prolonged incubation periods of up to 1 year have any practical role in the diagnosing of active *B. burgdorferi* infection.

Aggressive antibiotic therapy of Lyme borreliosis may fail to eradicate the clinical symptoms of the infection (Dattwyler et al., 1988). Some cases that appear to be treatment failure may actually be due to reinfection. However, at least two well documented cases of Lyme borreliosis have been described which demonstrate that the infection may relapse in spite of parenteral antibiotic therapy administered over a time course which would be expected to kill all spirochetes with a cell division cycle of between 6 to 12 h (Diringer et al., 1987; Case report in New Eng. J. Med., 1988). Antibiotics are only able to kill actively dividing *Borrelia*. If a borrelial cell does not divide at least once during the period of antibiotic therapy, it may persist in the host and produce relapse or recrudescence of disease. Therefore, some cases of Lyme borreliosis may require prolonged periods of antibiotic therapy to influence those cell lines of *B. burgdorferi* with a very slow cycle of cell division.

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