JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1992, p. 359–361
0095-1137/92/020359-03$02.00/0

Vol. 30, No. 2

Cultivation of *Borrelia burgdorferi* from Erythema Migrans Lesions and Perilesional Skin

BERNARD W. BERGER,[1] RUSSELL C. JOHNSON,[2]* CARRIE KODNER,[2] AND LISA COLEMAN[2]

Department of Dermatology, State University of New York at Stony Brook, Stony Brook, New York 11794,[1] and Department of Microbiology, School of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

Received 17 September 1991/Accepted 12 November 1991

Skin biopsy specimens from the peripheral aspect of erythema migrans lesions (site 1) and from clinically normal perilesional areas (site 2) were compared as sources of *Borrelia burgdorferi*. This spirochete was isolated from the skin of 18 of 21 (86%) patients with untreated early Lyme disease at one or both biopsy sites. Site 1 specimens were superior to site 2 specimens for the isolation of *B. burgdorferi*. Site 1 specimens from 18 (86%) patients were culture positive, and site 2 specimens from 12 (57%) patients were culture positive. For patients whose site 2 specimens were culture positive, site 1 specimens were also found to be culture positive. *B. burgdorferi* was isolated from two patients with atypical lesions and from two patients with erythema migrans lesions that were less than 5 cm in diameter. This study demonstrates that the cultivation of *B. burgdorferi* from skin biopsy specimens from cutaneous lesions thought to be erythema migrans can be an efficacious procedure for confirming the diagnosis of Lyme disease and that the spirochete is present in clinically normal appearing perilesional skin.

The clinical diagnosis of Lyme disease can be established by correctly identifying its characteristic cutaneous marker, erythema migrans (EM) (1, 5, 8, 12, 18). This is particularly important because serologic tests are frequently not reactive during the first few weeks of the illness, a time when EM lesions are most likely to be present (14, 17).

There are times, however, when clinicians may encounter difficulty in recognizing EM lesions because they may not be familiar with the entity or may be presented with a small, early, or atypical lesion. Cultivation of the etiologic agent of Lyme disease, *Borrelia burgdorferi*, from the suspected EM lesions in these instances would provide a definitive laboratory diagnosis of the illness. Also, the isolation of *B. burgdorferi* from patients is of particular importance for confirming presumptive cases of Lyme disease in nonendemic geographic areas. This spirochete has been isolated and successfully subcultured from the blood (5, 13, 15), cerebrospinal fluid (CSF) (10, 15, 18), skin lesions (1, 9, 15), and a single synovial fluid specimen (16). However, this generally has been a low-yield procedure. *B. burgdorferi* may be present in the blood early in the infection, but the frequency of isolation has been low, ranging from 2 to 7% (13). Spirochetes were cultured from the CSF of 4 of 38 (11%) clinically selected patients with neuroborreliosis. All four patients had pleocytoses in their CSF and a history of neurological symptoms of only 4 to 10 days in duration (10). Skin biopsy specimens from the EM lesion appears to be the best source of *B. burgdorferi*, with the frequency of isolation ranging from 5% (18) to 43% (9).

The purpose of this study was threefold: (i) to compare the suitability of skin biopsy specimens from the peripheral aspect of the EM lesion and from the clinically normal perilesional area as a source of *B. burgdorferi*, (ii) to investigate the utility of culturing biopsy specimens from small and atypical cutaneous lesions for confirming the diagnosis of Lyme disease, and (iii) to evaluate the efficacy of culturing specimens transported from a distant site.

* Corresponding author.

MATERIALS AND METHODS

The culture medium used is that described by Kelly (11), fortified by Stoenner et al. (19), and further refined by Barbour (2). This medium is frequently referred to as the modified Kelly medium or the Barbour-Stoenner-Kelly (BSK) medium. Minor modifications have been made in the formulation of this medium; these modifications facilitate its preparation. Also, selection of the bovine serum albumin (BSA) component of the medium was based on pretesting results of several BSA lot samples. Medium prepared with a satisfactory lot of BSA should initiate the growth of a stock culture of *B. burgdorferi* from inocula of 1 to 10 cells and yield cell numbers that are easily visualized by microscopic examination within 2 to 3 weeks of incubation at 30°C. The broth medium is prepared by using glassware washed three times in distilled water. High-quality distilled water is used in the medium formulation. The following components are added sequentially to 900 ml of distilled water: 6 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, Mo.), 5 g of Neopeptone (Difco Laboratories, Detroit, Mich.), 0.7 g of sodium citrate, 5 g of glucose, 2.0 g of sodium bicarbonate, 2.5 g of TC Yeastolate (Difco), 0.8 g of sodium pyruvate, 0.4 g of *N*-acetylglucosamine (Sigma), and 50 g of pretested BSA, fraction V (Intergen Company, Purchase, N.Y.). After the solution is adjusted to pH 7.5 and sterilized by positive-pressure filtration, the following additions are made aseptically: 100 ml of sterile 10× CMRL 1066 medium without glutamine or sodium bicarbonate (GIBCO Laboratories, Grand Island, N.Y.), 60 ml of heat-inactivated (56°C, 30 min) sterile, trace hemolyzed rabbit serum (Pel-Freez Biologicals, Inc., Rogers, Ark.), and 200 ml of autoclaved (121°C, 20 min) 7% gelatin (Difco). The broth medium is aseptically dispensed into sterile containers to 75 to 90% of their capacity and tightly capped. The medium can be stored at 4°C for at least 2 months.

The isolation medium used in this study was prepared by the addition to the broth medium of ciprofloxacin and

rifampin to final concentrations of 0.4 and 40 μg/ml, respectively.

Patients and sites of skin biopsy specimens. Patients with early localized and early disseminated Lyme disease participated in the study. The presence of EM lesions alone or in combination with minor constitutional symptoms was considered to be early localized Lyme disease. If the EM lesion was accompanied by extracutaneous signs and symptoms of major intensity or if the EM lesions appeared in multiplicity, the patient was considered to have disseminated early Lyme disease (7). The most commonly experienced symptoms were fever, headache, fatigue, musculoskeletal discomfort, chills, and regional lymphadenopathy.

Cutaneous lesions thought to be EM were evaluated for the presence of *B. burgdorferi*. The peripheral border of the EM lesion was identified, and under sterile conditions, a 4-mm-diameter punch biopsy specimen was obtained from the peripheral aspect of the lesion (4 mm interior from the EM border) and from the clinically normal perilesional skin (4 mm beyond the EM border). The biopsy specimens obtained from the patient with multiple EM lesions (see Table 1) were from a secondary lesion because the primary lesion was fading and a distinct border could not be determined. The surgical wounds were closed with one suture.

These patients were treated with antibiotic regimens of either doxycycline (100 mg three times a day for 21 days) or amoxicillin (1,000 mg three times a day for 21 days) plus probenecid (500 mg three times a day for 21 days), which resulted in the resolution of the EM and any associated extracutaneous signs or symptoms of Lyme disease.

Processing of skin biopsy specimens. Each skin biopsy specimen was placed in a polystyrene tube (13 by 100 mm; Becton Dickinson Co., Lincoln Park, N.J.) containing 6 ml of isolation medium and was held at room temperature (21 to 23°C) for 1 to 11 days prior to overnight air express shipment to the University of Minnesota. Upon receipt of the skin biopsy specimen, it was transferred to polystyrene tubes containing 6 ml of broth medium without antibiotics. Both the tube of isolation medium from which the skin biopsy was removed and the tube with broth medium (antibiotic-free) containing the skin specimen were incubated at 30°C and examined for spirochetes by dark-field microscopy at 3-, 6-, and 12-week intervals.

Identification of spirochete isolates. The identities of the isolates in the skin biopsy specimens from 18 patients were determined by using the indirect fluorescent-antibody test. The test reagents were the murine monoclonal antibodies H5332 (4) and H3TS (3), which are specific for the 31-kDa outer surface protein of *B. burgdorferi*, and a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). *B. burgdorferi* ATCC 35210 and *Borrelia hermsii* ATCC 35209 served as the positive and negative controls, respectively. The monoclonal antibodies were provided by the Rocky Mountain Laboratories, Hamilton, Mont.

Preservation of spirochete isolates. All isolates in the skin biopsy specimens were preserved in liquid nitrogen by using 10% glycerol as the cryoprotective agent.

RESULTS

Between 29 May 1991 and 10 July 1991, the cutaneous lesions thought to be EM of 24 adults were biopsied. The patient population consisted of 15 females and 9 males ranging in age from 26 to 78 years (Table 1). All patients resided on the eastern end of Long Island, N.Y. The EM lesion was present from 2 to 14 days before biopsy of the skin. Twenty-three patients had solitary EM lesions ranging in size from 14 by 14 to 3 by 3 cm. One patient had multiple EM lesions (Table 1). Atypical solitary EM lesions were present on two patients. Sixteen patients were considered to have early localized Lyme disease (solitary EM lesions alone or with minor constitutional symptoms). Eight patients were characterized as having early disseminated Lyme disease (solitary or multiple EM lesions accompanied by marked constitutional complaints) (Table 1).

TABLE 1. Patients from whom skin biopsy specimens were obtained and results of cultivation

Patient no.	Age (yr), sex[a]	EM lesion size (cm), pretreatment duration (days)	Culture disease category[b]	Cultivation from:	
				Inside EM	Outside EM
1	42, F	5 by 6, 5	L	−	−
2	49, F	4 by 5, 4	D	+	+
3	49, F	7 by 10, 5	L	+	−
4	69, M	3 by 3, 4	L	+	+
5	34, F	4 by 4, 5	L	−	−
6	39, F	5 by 15, 3	D	+	+
7	75, F	2 by 5, 3	L	+	−
8	47, F	8 by 10, 2	D	+	−
9	52, M	9 by 11, 14	L	+	+
10	69, F	14 by 14, 10	L	+	−
11[c]	47, M	10 by 15, 7	D	−	−
12	51, M	11 by 14, 7	L	+	+
13	70, F	11 by 18, 8	L	+	−
14[c]	54, F	3 by 11, 2	L	−	−
15	32, F	9 by 13, 5	D	+	+
16	78, M	8 by 13, 5	L	−	−
17	53, F	4 by 9, 2	L	+	+
18	26, M	6 by 8, 4	L	+	+
19[d]	60, F	Multiple, 4	D	+	−
20[c]	71, M	10 by 10, 8	L	−	−
21	73, M	2 by 4, 3	D	+	+
22	33, F	8 by 15, 7	D	+	+
23	38, M	5 by 5, 4	L	+	+
24	58, F	4 by 8, 2	L	+	+

[a] F, female; M, male.
[b] L, early localized disease; D, early disseminated disease.
[c] Patients received antibiotic treatment prior to skin biopsy.
[d] The biopsy specimen was obtained from a secondary lesion.

Three patients received antibiotic therapy prior to skin biopsy. Patient 11 received 100 mg of doxycycline twice a day for 2 days, patient 14 received 3,000 mg of amoxicillin and 1,500 mg of probenecid for 1 day, and patient 20 received 4,000 mg of amoxicillin and 2,000 mg of probenecid for 2 days. The skin biopsy specimens from these patients were culture negative for spirochetes (Table 1). Spirochetes were cultured from skin biopsy specimens of 18 of the 21 (86%) remaining patients. Specimens from the peripheral aspect of the EM lesion of 6 patients (33%) were culture positive. Specimens from both the peripheral aspect of the EM lesion and the clinically normal perilesional area of 12 patients (67%) were culture positive (Table 1). None of the skin biopsy specimen cultures were contaminated with other bacteria or fungi.

EM was confirmed by culture in two patients with atypical lesions. Patient 3 had a triangular erythematous, edematous plaque on the neck, and patient 10 had a violaceous patch on the thigh. Spirochetes were also isolated from EM lesions of patients 4 and 21; these lesions were less than 5 cm in

diameter, the minimal EM diameter accepted for surveillance purposes (20). The success of isolation could not be correlated with sex, age, size of EM lesion, or length of time the skin biopsy specimen was in the isolation medium before transfer to the antibiotic-free medium. Of the 30 positive cultures, spirochetes were detected in 25 (83%) of them at the 3-week observation time and in 5 (17%) of them at the 6-week observation time. Cultures that were negative for spirochetes at 6 weeks remained negative at 12 weeks. The spirochetes isolated from the 18 patients reacted with monoclonal antibodies H5332 and H3TS, which are specific for *B. burgdorferi* (3, 4).

DISCUSSION

EM is the most common and distinctive cutaneous manifestation of Lyme disease. It begins as a red macule which becomes papular and evolves into a centrifugally expanding red annular plaque (6). Recognition of an EM lesion can establish the diagnosis of Lyme disease, but considerable variation occurs in its configuration, size, color, central portion, and rate of expansion (7). The isolation of *B. burgdorferi* from the skin lesion provides a definitive diagnostic laboratory test for Lyme disease.

Although skin biopsy specimens of the EM lesion are a better source of *B. burgdorferi* than the blood (5, 13, 15), CSF (10, 15, 18), and synovial fluid (16) are, a frequency of isolation of 5 to 43% (9, 18) from EM lesions is relatively low. We attempted to improve the efficiency of isolation from the skin by culturing skin biopsy specimens from clinically normal skin peripheral to the margin of the EM. Selection of the perilesional site was based on the possibilities that the organisms' lateral migration through the skin preceded the centrifugally expanding EM and that they could be more readily isolated before the inflammatory response was manifested. Selection of the peripheral aspect of the EM was based on our past experience with biopsy specimens obtained for histopathologic examination using Warthin-Starry staining, which demonstrated the presence of spirochetes more commonly in biopsy specimens obtained from the peripheral aspect of the lesion than in the central portion, and previous isolation of *B. burgdorferi* from this site (6, 9). The frequency of isolation from the perilesional site in the 21 patients was less (57%) than that achieved from the margin of the EM (86%). However, this study established that *B. burgdorferi* is present in the clinically normal appearing perilesional skin and that isolation of the spirochete from the marginal area of the EM can be a high-yield diagnostic procedure. In addition, the isolation procedure can be used to confirm the fact that small and atypical lesions are caused by *B. burgdorferi*. This study also demonstrated that it is feasible to successfully culture specimens transported from distant sites.

ACKNOWLEDGMENT

This research was supported in part by Public Health Service grant AR41522 from the National Institutes of Health (to R.C.J.).

REFERENCES

1. **Asbrink, E., and A. Hovmark.** 1988. Early and late cutaneous manifestations in Ixodes-borne borreliosis. Ann. N.Y. Acad. Sci. **539**:4–16.
2. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:521–525.
3. **Barbour, A. G., R. A. Heiland, and T. R. Howe.** 1985. Heterogeneity of major proteins in Lyme disease borreliae: a molecular analysis of North American and European isolates. J. Infect. Dis. **152**:478–484.
4. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41**:795–804.
5. **Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow.** 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. **308**:740–742.
6. **Berger, B. W.** 1984. Erythema chronicum migrans of Lyme disease. Arch. Dermatol. **120**:1017–1021.
7. **Berger, B. W.** 1989. Lyme disease. Rheum. Dis. Clin. N. Am. **15**:627–633.
8. **Berger, B. W., O. J. Clemmensen, and A. B. Ackerman.** 1983. Lyme disease is a spirochetosis. Am. J. Dermatopathol. **5**:111–124.
9. **Berger, B. W., M. H. Kaplan, I. R. Rothenberg, and A. G. Barbour.** 1985. Isolation and characterization of the Lyme disease spirochete from the skin of patients with erythema chronicum migrans. J. Am. Acad. Dermatol. **13**:444–449.
10. **Karlsson, M., K. Hovind-Hougen, B. Svenungsson, and G. Stiernstedt.** 1990. Cultivation and characterization of spirochetes from cerebrospinal fluid of patients with Lyme borreliosis. J. Clin. Microbiol. **28**:473–479.
11. **Kelly, R.** 1971. Cultivation of *Borrelia hermsii*. Science **173**:443–444.
12. **MacDonald, A. B., B. W. Berger, and T. G. Schwan.** 1991. Clinical implications of delayed growth of the Lyme borreliosis spirochete, *Borrelia burgdorferi*. Acta Trop. **48**:89–94.
13. **Nadelman, R. B., C. S. Pavia, L. A. Magnarelli, and G. P. Wormser.** 1990. Isolation of *Borrelia burgdorferi* from the blood of seven patients with Lyme disease. Am. J. Med. **88**:21–26.
14. **Rahn, D. W., and S. E. Malawista.** 1991. Lyme disease: recommendations for the diagnosis and treatment. Ann. Intern. Med. **114**:472–481.
15. **Rawlings, J. A., P. V. Fournier, and G. J. Teltow.** 1982. Isolation of *Borrelia* spirochetes from patients in Texas. J. Clin. Microbiol. **25**:1148–1150.
16. **Schmidli, J., T. Hunziker, P. Moesli, and U. B. Schaad.** 1988. Cultivation of *Borrelia burgdorferi* from joint fluid three months after treatment of facial palsy due to Lyme borreliosis. J. Infect. Dis. **158**:905–906. (Letter.)
17. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321**:586–596.
18. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308**:733–740.
19. **Stoenner, H. G.** 1974. Biology of *Borrelia hermsii* in Kelly medium. Appl. Microbiol. **28**:540–543.
20. **Wharton, M., T. L. Chorba, R. L. Vogt, D. L. Morse, and J. W. Buehler.** 1990. Case definitions for public health surveillance. Morbid. Mortal. Weekly Rep. **39**:19–21.