JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1988, p. 338–346
0095-1137/88/020338-09$02.00/0

Vol. 26, No. 2

Measurement of Antibodies to the *Borrelia burgdorferi* Flagellum Improves Serodiagnosis in Lyme Disease

KLAUS HANSEN,* PETER HINDERSSON, AND NILS STRANDBERG PEDERSEN

Borrelia Laboratory, Department of Treponematoses, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark

Received 8 September 1987/Accepted 3 November 1987

The isolation of *Borrelia burgdorferi* flagella and an enzyme-linked immunosorbent assay (ELISA) for detection of immunoglobulin G (IgG) and IgM to the *B. burgdorferi* flagellum are described. The diagnostic performance of the flagellum ELISA for serodiagnosis of Lyme disease was compared with the performance of a traditional whole cell *B. burgdorferi* sonic extract ELISA. We examined sera and cerebrospinal fluid (CSF) from 56 patients with lymphocytic meningoradiculitis (Bannwarth's syndrome), the most frequent secondary-stage manifestation of Lyme disease in Europe. Two hundred healthy individuals and patients with aseptic meningitis, encephalitis, Guillain-Barré syndrome, and syphilis served as controls. The flagellum ELISA was significantly more sensitive than the sonic extract ELISA. The diagnostic sensitivities were increased from 41.1 to 76.8% ($P < 0.01$) for IgG and from 35.7 to 67.9% ($P < 0.05$) for IgM detection in serum. The increase in sensitivity was most pronounced in patients with a short duration of disease (<20 days after onset). The diagnostic specificity increased for IgG detection but was almost unaltered for IgM. The flagellum ELISA did not improve the diagnostic sensitivity of measuring antibodies to borreliae in CSF, most likely owing to the low level of unspecific antibodies in CSF compared with serum. The cross-reactivity of sera and CSF from patients with syphilis decreased significantly. The flagellum antigen of *B. burgdorferi* shows no strain variation, is easy to purify in sufficient quantity, and is therefore a suitable reference antigen for routine serodiagnosis of Lyme disease.

Since the recent discovery of *Borrelia burgdorferi* as the causative agent of the tick-borne spirochetosis Lyme disease (11, 25), increasing numbers of human cases have been reported from almost every European country and the United States. Lyme disease includes a number of different clinical conditions. The primary manifestation is erythema chronicum migrans. If untreated, this condition may within weeks to months be followed by a secondary and tertiary stage, lymphocytic meningoradiculitis (LMR), also called Bannwarth's syndrome; arthritis; myocarditis; lymphadenosis benigna cutis; acrodermatitis chronica atrophicans (ACA); and chronic progressive encephalomyelitis (3, 4, 19, 20, 23, 25).

Although *B. burgdorferi* has been identified and cultured from almost all these lesions, the isolation of the spirochete from patients is difficult and not practicable for routine diagnosis. The clinical diagnosis has to be confirmed by measuring the antibody response to the spirochete. In the presently used serological tests, such as the immunofluorescense assay and the enzyme-linked immunosorbent assay (ELISA), whole cells or whole cell sonic extract is used as antigen (2, 5, 14, 24–26, 29). These tests are useful in later stages of the disease but yield unsatisfactory low diagnostic sensitivities (20 to 60%) in the first and early-secondary stages. A slow and late-appearing antibody response (13), but also inclusion of irrelevant cross-reacting antigens in the whole cell preparations, may be responsible for this.

The aim of this study was to develop a more sensitive serological assay using a single, *Borrelia*-specific, immunodominant antigen. Three observations made us believe that the *B. burgdorferi* flagellum might be a suitable candidate: (i) Western (immuno)-blotting (WB) studies with *B. burgdorferi* showed an early and strong immune response against the 41-kilodalton (kDa) band corresponding to the flagellum; (ii) a highly sensitive ELISA has been developed for diagnosis of syphilis which made use of the flagella of *Treponema phagedenis* as test antigen (22); and (iii) even high-titered sera from patients with Lyme disease reacted only occasionally and weakly in the *T. phagedenis* flagellum ELISA.

Thus, the *B. burgdorferi* flagellum was purified and used as the antigen in an ELISA. This assay was compared with a conventional ELISA that uses a whole spirochetal sonic extract as the antigen. Sera and cerebrospinal fluid (CSF) samples from 56 patients with LMR were used to evaluate the diagnostic performance of the tests.

MATERIALS AND METHODS

Patients. Paired serum and CSF samples were obtained from 56 consecutive patients with LMR hospitalized from 1984 to 1986 (34 males and 22 females aged 6 to 74 years; median age, 51 years). The LMR diagnosis was based on clinical evidence: the typical painful sensory radiculitis and lymphocytic pleocytosis in CSF. In many cases, the specificity of the clinical diagnosis was further strengthened by prior observation of a tick bite (15 patients), erythema chronicum migrans (35 patients), and the occurrence of the typical mononeuritis multiplex of Bannwarth's syndrome (37 patients) (1). Investigation of CSF revealed lymphocytic pleocytosis (16 to 702 cells per μl; median cell count, 200/μl) and an elevated protein concentration in most cases (0.16 to 6.4 g/liter, with a median elevation of 1.3 g/liter). All measurements were done on the first pretreatment sample taken 3 to 160 days (median, 27 days) after the onset of neurological symptoms.

Controls. Sera from 200 healthy controls were used for

* Corresponding author.

determination of the 95% specific cutoff level in both tests. Paired serum and CSF samples were obtained from 38 patients with abnormal CSF findings (aseptic menigitis [n = 11], encephalitis [n = 13], and Guillain-Barré syndrome [n = 14]). CSF samples without pathological changes were obtained from 54 patients undergoing myelography. Additionally, sera from patients with primary syphilis (n = 26), secondary syphilis (n = 29), and leptospirosis (n = 22) and CSF from 14 patients with neurosyphilis were investigated. All serum and CSF specimens were stored at −20°C.

***B. burgdorferi* test antigens.** The Swedish strain *B. burgdorferi* ACA-1 isolated from the skin of a patient with ACA by Eva Åsbrink (4) was used for all antigen preparations. Spirochetes were grown for 5 days in BSK medium (25) at 32°C to a cell density of 10^8/ml. Spirochetes used for the sonic extract ELISA were grown in 1 liter of medium, harvested by centrifugation (10,000 × g, 30 min), and washed three times in phosphate-buffered saline (pH 7.4; PBS) with 5 mM $MgCl_2$. The final pellet was suspended in 4 ml of PBS and sonicated on ice by seven 15-s blasts with an MSE 150 W ultrasonic disintegrator (Manor Royal, Crawley, England). The sonic extract was centrifuged (10,000 × g, 30 min), and the supernatant containing the soluble antigens was used for the ELISA (14).

Isolation of *B. burgdorferi* flagellum. The *B. burgdorferi* flagellum was purified by modification of two previously described methods (8, 16).

Spirochetes from 1 liter of culture (approximately 10^{11} cells) were harvested and washed as described above. The cells were lysed by suspending the spirochetes in 20 ml of sarcosyl-TE (2% [wt/vol] *N*-lauroylsarcosine [sarcosyl; Sigma Chemical Co., St Louis, Mo.] in 10 mM Tris [pH 8] and 1 mM EDTA) and incubated at 37°C for 45 min. The lysate was centrifuged at 48,000 × g for 60 min at 25°C (fixed-angle rotor). The supernatant was discarded, and the pellet was suspended in 10 ml of sarcosyl-TE, incubated at 37°C for 10 to 15 min, and pelleted as described above. The pellet containing the detergent-insoluble material was dissolved in 10 ml of 0.15 M NaCl and sheared for 10 min on ice in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) at maximum speed. The sheared suspension was centrifuged at 220,000 × g at 15°C for 3 h (fixed-angle ultracentrifuge 60 Ti rotor; Beckman Instruments, Inc., Fullerton, Calif.). The pellet was suspended in 1 ml of sarcosyl-TE and for a final separation was subjected to CsCl density gradient centrifugation at 175,000 × g at 25°C for 15 h (vertical ultracentrifuge rotor TV 865; Sorvall). The CsCl gradient was adjusted to a density of 1.30 g/ml in the middle of the tube. Visible bands were collected separately, dialyzed against PBS, and examined by electron microscopy, WB, and crossed immunoelectrophoresis (CIE). Flagella-containing bands were pooled (final volume, approximately 1 ml) and stored at −20°C. The protein concentration was 7 mg/ml (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.).

ELISA procedure. The *B. burgdorferi* sonic extract ELISA and the flagellum ELISA were performed identically except for the antigen. Flat-bottom polystyrene microdilution plates (Immunoplates; code 2-69620; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of antigen diluted in PBS. The optimal coating concentration was defined as the antigen dilution resulting in the highest ratio of the optical densities (ODs) between a positive and negative control serum (P/N ratio). Unspecific protein binding was blocked with 1% (wt/vol) bovine serum albumin in PBS. The wells were washed, and 100 μl of serum diluted 1:200 or 100 μl of CSF diluted 1:25 in PBS with 0.5% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20 was added to the wells and incubated for 2 h at 20°C. After washing, 100 μl of peroxidase conjugate was added—either rabbit anti-human immunoglobulin G (IgG) or anti-human IgM (codes P-214 and P-215; Dakopats, Copenhagen, Denmark) diluted 1:10,000 and 1:1,000, respectively, in PBS with 0.05% (vol/vol) Tween 20. After incubation for 2 h at 20°C, the plates were washed and 200 μl of the substrate *o*-phenylenediamine (0.41 mg/ml; Sigma) in citrate buffer (pH 5) with 0.04% (vol/vol) H_2O_2 was added to each well. The enzymatic reaction was stopped by the addition of 50 μl of 3 N H_2SO_4. The OD at 490 nm was read by a colorimeter (Immuno Reader NJ 2000; Nippon InterMed, Tokyo, Japan). All washings were done three times with 0.56% (wt/vol) NaCl containing 0.05% (vol/vol) Tween 20. Positive and negative control sera were included on every plate. Samples were tested in duplicate, and the mean value was calculated. If the two values differed more than 10% from the mean, the sample was retested. To eliminate plate-to-plate and day-to-day variations, samples of three serum pools with low, medium, and high immunofluorescence assay antibody titers were included on every plate for construction of a standard curve. The OD value of every sample was adjusted to this standard curve.

The total assay precision of the *B. burgdorferi* flagellum ELISA was determined by testing negative and positive control sera in 20 independent assays. Examination of a positive control serum showed mean OD values of 0.456 (standard deviation [SD], 0.044) in the IgG assay and 0.302 (SD, 0.034) in the IgM assay. The negative control serum showed mean OD values of 0.066 (SD, 0.018) in the IgG assay and 0.075 (SD, 0.018) in the IgM assay.

Immunochemical techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and WB were performed essentially as previously described (17). The separating gel contained 12.5% acrylamide. Each lane was loaded with approximately 10^7 *B. burgdorferi* cells. A 20-μl sample of a 1:64 dilution of the flagellum preparation was applied to each lane of the gel.

Protein staining of the transferred antigens was done by a sensitive gold-staining technique (N. H. H. Heegård and O. J. Bjerrum, *in* O. J. Bjerrum and N. H. H. Heegård, ed., *Handbook of Immunoblotting of Proteins*, in press). The immunostaining of transferred antigens was performed with a high-titered serum from a patient with ACA and a negative control serum. Both sera were applied in a 1:200 dilution. The whole cell lysate and flagellum preparation were tested with *Borrelia*-specific monoclonal antibodies to the flagellum, H 9724 and H 604, diluted 1:10 (8, 9). The immunostaining technique was previously described in detail (17).

CIE was performed by standard methods (6). The antigen applied in each CIE was 10 μl of *B. burgdorferi* sonic extract (equivalent to 10^9 spirochetes) or 20 μl of the undiluted flagella-containing solution. The second-dimension gel contained 400 μl of polyspecific rabbit anti-*B. burgdorferi* immunoglobulin. Polyspecific rabbit antisera to *B. burgdorferi* was raised by subcutaneous inoculation of 5 · 10^9 washed and sonicated spirochetes in Freund incomplete adjuvant every third week. The most polyspecific sera were selected by CIE, and the immunoglobulin fraction was isolated by the method of Harboe and Ingild (15).

Electron microscopy. A microdrop of the undiluted flagella-containing solution was applied to a Formvar-coated carbon-reinforced copper grid (200 mesh; Veco, Eerbeek, The Netherlands). Samples were allowed to adsorb to the grids for 30 min at room temperature. Excess fluid was

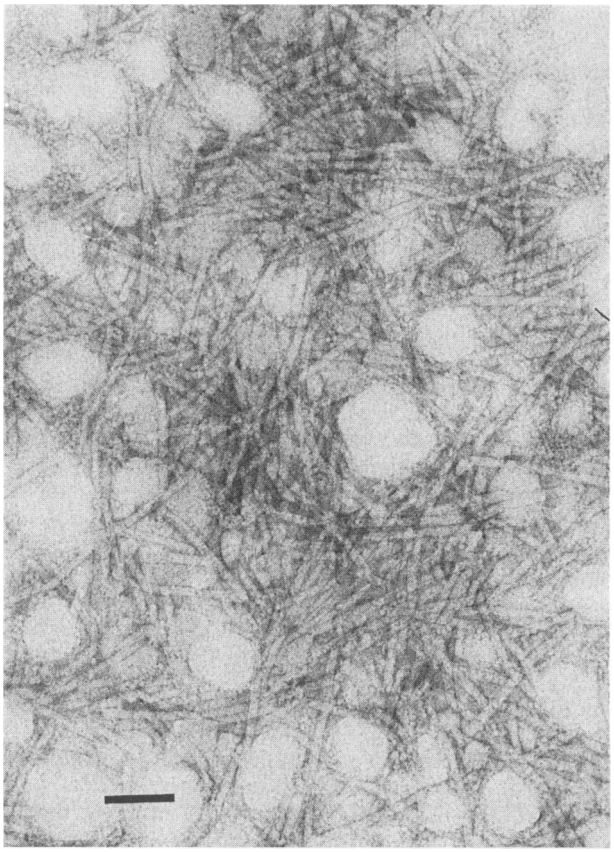

FIG. 1. Electron micrograph of purified *B. burgdorferi* flagella. Negative staining with 2% ammonium molybdate was used. The flagella are fragmented but morphologically preserved. No contaminants are seen. Bar, 0.1 μm.

removed, and the grids were washed with distilled water. Negative staining was done with 2% ammonium molybdate (pH 7). Electron microscopy was done on a Philips EM 201 C electron microscope.

Statistical analysis. The results of the sonic extract and flagellum ELISAs were compared by using exact confidence limits assuming a binomial distribution.

RESULTS

***B. burgdorferi* flagellum preparation.** The CsCl density gradient centrifugation yielded two bands: a broad, prominent band and, right underneath it, a narrow band. Both contained flagella of the same quality.

Electron microscopy (Fig. 1) of the preparation showed fragmented but otherwise morphologically preserved flagella. No contaminants, such as fragments of membranes, intact ribosomes, or other identifiable subcellular structures, were seen. Analysis by WB (Fig. 2) demonstrated a pure 41-kDa band analogous to the flagellin band in the whole cell lysate. Protein staining of the *B. burgdorferi* flagellum WB strip revealed one minor contaminant; traces of a 32-kDa band were seen. On the other hand, the immunostaining done with a high-titered polyspecific serum from a patient with ACA demonstrated a single 41-kDa band. Monoclonal antibodies H 9724 and H 604 reacted with the 41-kDa band in the whole cell lysate and flagellum preparation.

The purity of the flagellum preparation was further demonstrated by CIE (Fig. 3). The reference pattern of precipitates of whole cell sonic extract of *B. burgdorferi* allows the differentiation of about 20 precipitates. The flat precipitate designated Bb-fl in Fig. 3 is the *B. burgdorferi* flagellum. CIE of the purified flagella showed no contaminants.

Comparison of *B. burgdorferi* sonic extract and flagellum ELISAs. As shown in Fig. 4, the amount of antigen adsorbed to the microdilution plate increased with the concentration of sonic extract and flagellum antigens. An antigen dilution of 1:3,200 was chosen for both tests because this coating concentration gave an acceptably high P/N ratio. The OD values obtained by measuring IgG in the same high-titered ACA patient serum were almost identical. The essential difference between the two antigens was a significantly lower reactivity of the negative control serum when the flagellum was used. Thus, the P/N ratio of the flagellum ELISA showed a fourfold increase compared with that of the sonic extract ELISA, mainly owing to an increased specificity.

Measurement of IgG to the flagellum in sera of 200 healthy controls revealed a similar gain in specificity (Fig. 5). Using a 95% specific cutoff level in both tests, the diagnostic cutoff level could be lowered from 0.400 to 0.160 OD values by using flagellum as the ELISA antigen. Measuring IgG in serum samples from 56 patients with LMR, the flagellum

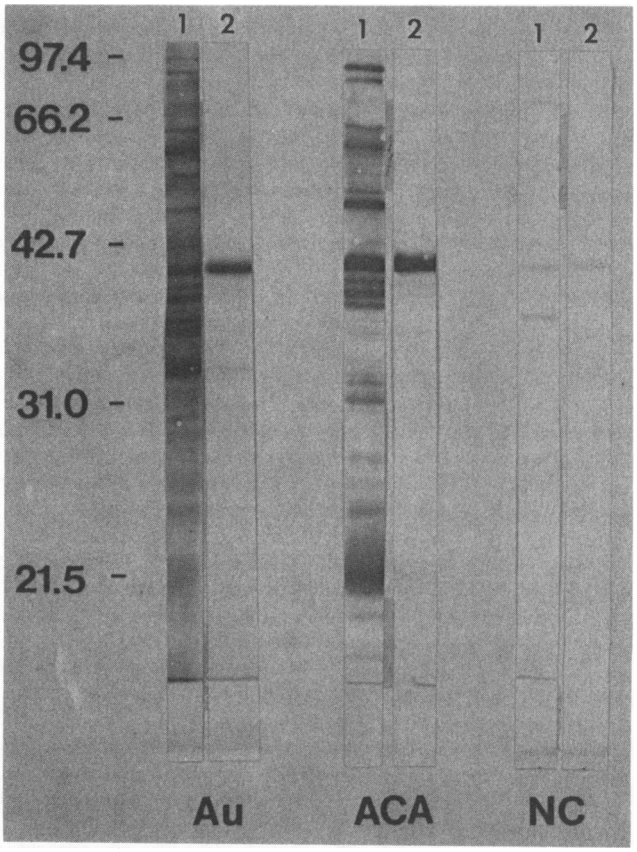

FIG. 2. WB of a *B. burgdorferi* whole cell lysate (lane 1) and the *B. burgdorferi* flagellum preparation (lane 2). Protein staining was done with gold (Au) and immunostaining was done with a high-titered ACA patient serum and a negative control (NC) serum. The single 41-kDa band in lane 2 shows the purity of the flagellum preparation. The negative control serum reacted very slightly with the 41-kDa band.

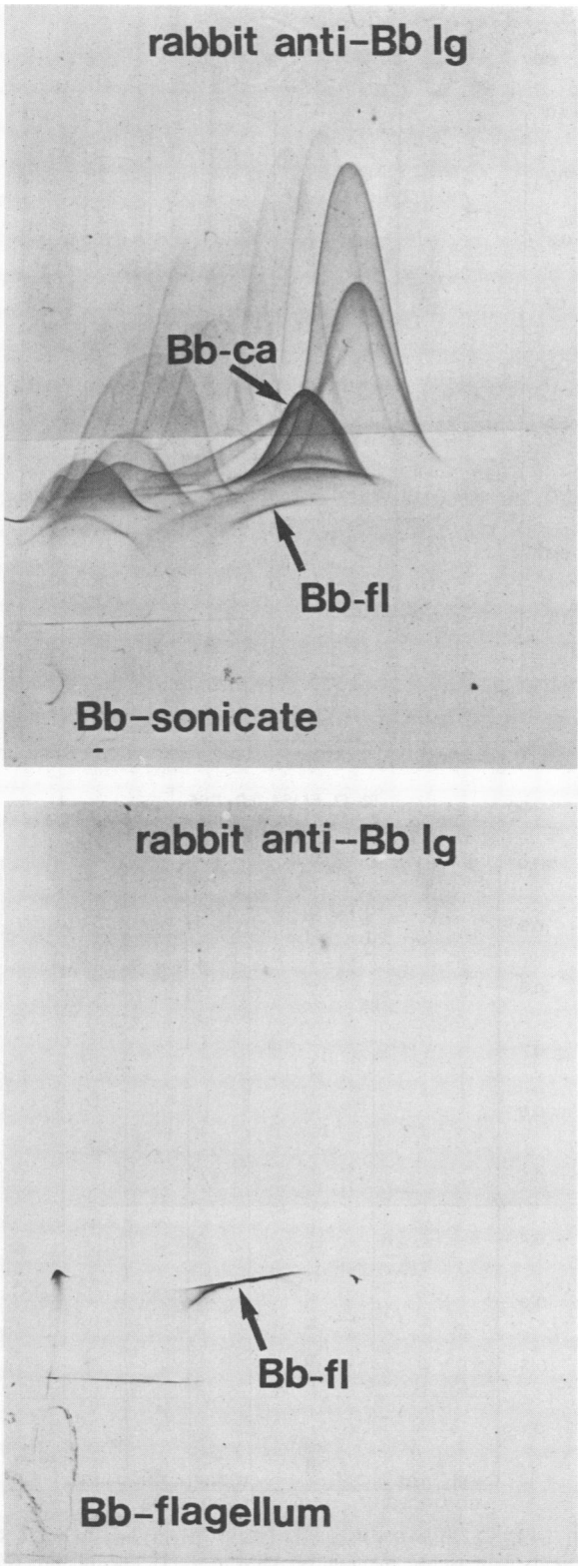

FIG. 3. CIE of a *B. burgdorferi* whole cell sonic extract (top) and purified *B. burgdorferi* flagella (bottom). The second-dimension gel contained polyspecific rabbit anti-*B. burgdorferi* immunoglobulin. The intermediate gel was blank. Precipitates marked Bb-fl correspond to the flagellum, and those marked Bb-ca correspond to the common antigen of *B. burgdorferi*.

ELISA showed an overall increase in diagnostic sensitivity from 41.1 to 76.8% ($P < 0.01$) (Table 1) compared with the sonic extract ELISA. The OD values of the LMR patient serum samples were either unchanged or even higher (Fig. 5). When the 56 patients with LMR were divided into three groups according to the duration of the disease, the increased sensitivity of the flagellum ELISA was most pronounced in the early phase. In 19 patients tested within 20 days after onset, it increased from 10.5 to 57.9% ($P < 0.05$) (Table 1). All sera reactive in the sonic extract ELISA were also reactive in the flagellum ELISA.

The flagellum ELISA did not increase the diagnostic specificity of serum IgM measurement. The 95% specific cutoff based on the same 200 healthy controls could be lowered from an OD value of 0.260 to 0.230 only, when the sonic extract and flagellum ELISAs were compared (Fig. 6).

Measuring IgM to the flagella in sera of the 56 patients with LMR showed an overall increase in diagnostic sensitivity from 35.7 to 67.9% ($P < 0.05$; Table 1). The increase was most prominent in early disease (26.3 to 63.2%; Table 1). The highest frequency of IgM-positive sera occurred in both tests from 21 to 40 days after onset (Table 1).

To obtain an acceptable specificity of the CSF serology, a high (>100 percentile) cutoff is necessary to prevent false-positive results caused by the leakage of serum antibodies through a disturbed blood-CSF barrier. Therefore, when antibodies in CSF were measured, the same arbitrary diag-

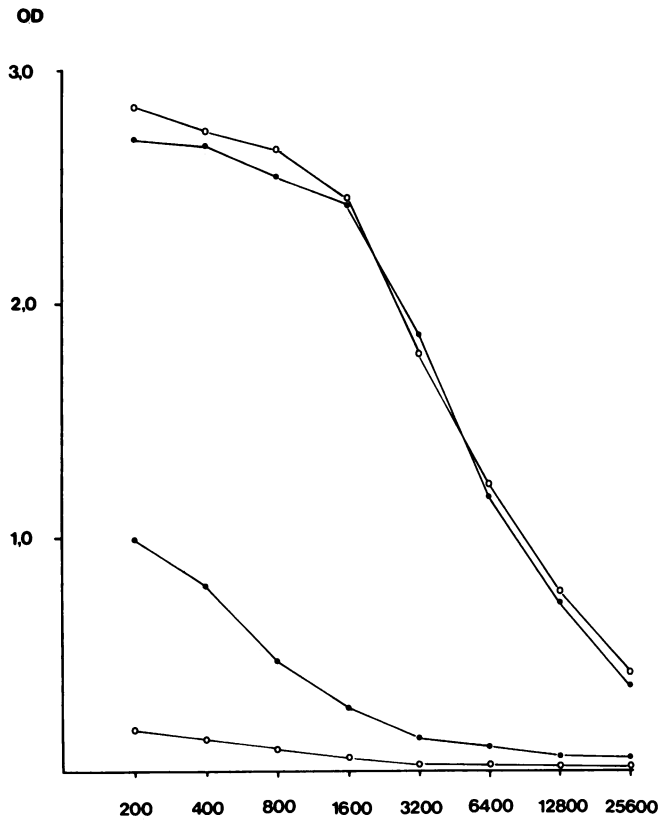

FIG. 4. ELISA antigen dilution curve of *B. burgdorferi* sonic extract (●) and purified *B. burgdorferi* flagella (○). IgG antibodies were measured in a high-titered serum from a patient with ACA and a negative control serum. Each point is the mean OD value of two determinations.

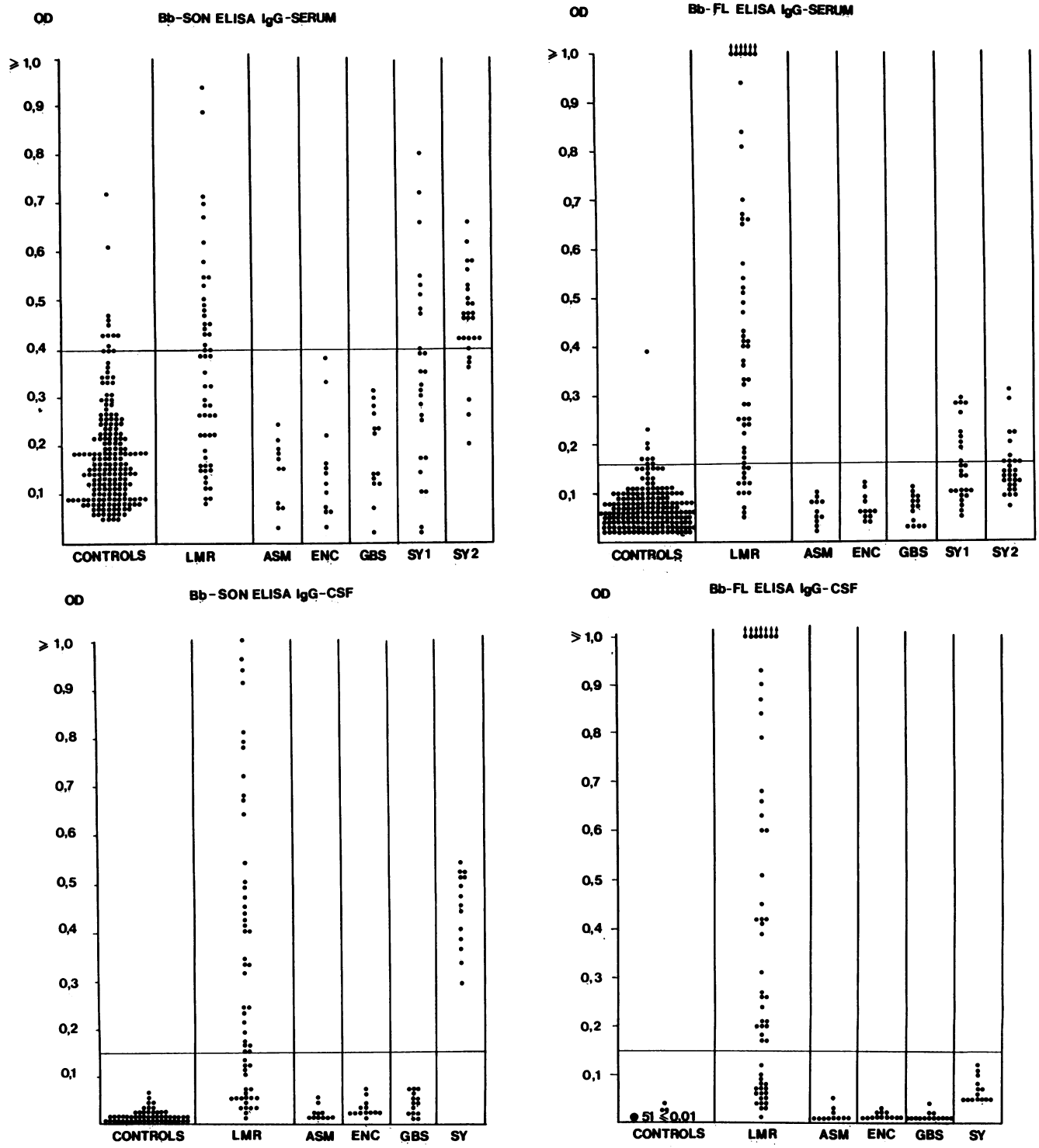

FIG. 5. IgG levels measured by *B. burgdorferi* sonic extract ELISA (left) and *B. burgdorferi* flagellum ELISA (right) in sera and CSF of 56 patients with LMR. Serving as controls were 200 healthy individuals; patients with aseptic meningitis (ASM; $n = 11$), encephalitis (ENC; $n = 12$), Guillain-Barré syndrome (GBS; $n = 14$), primary syphilis (SY1; $n = 26$), and secondary syphilis (SY2; $n = 29$); CSF from patients undergoing myelography ($n = 54$); and patients with neurosyphilis (SY; $n = 14$). The horizontal lines mark the diagnostic cutoff levels.

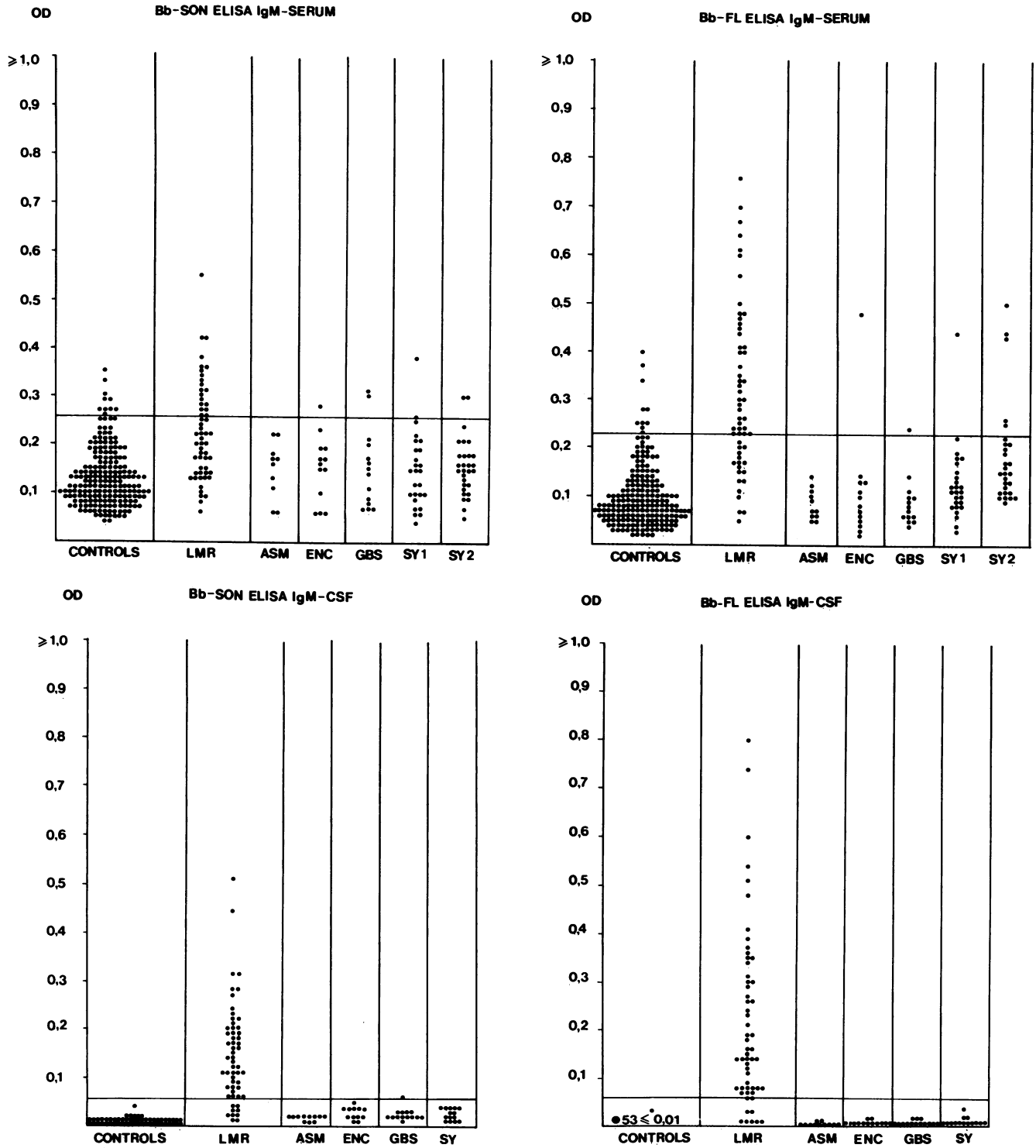

FIG. 6. IgM levels measured by *B. burgdorferi* sonic extract ELISA (left) and *B. burgdorferi* flagellum ELISA (right) in sera and CSF of 56 patients with LMR and the same controls as in Fig. 5. The horizontal lines mark the cutoff levels. See the legend to Fig. 5 for definitions.

TABLE 1. Diagnostic sensitivity of *B. burgdorferi* sonic extract ELISA and flagellum ELISA in serum and CSF

Days after onset of neurological symptoms (no. of patients)	Sensitivity (%) of indicated test with:							
	Serum				CSF			
	Sonic extract ELISA		Flagellum ELISA		Sonic extract ELISA		Flagellum ELISA	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
<20 (19)	10.5	26.3	57.9	63.2	21.1	63.2	26.3	78.9
21–40 (24)	41.7	50.0	79.2	83.3	75.0	95.8	83.3	95.8
41–160 (13)	84.6	23.1	100	46.2	100	92.3	92.3	84.6
Total (56)	41.1	35.7	76.8	67.9	62.5	83.9	66.1	87.5

nostic cutoff level at OD values of 0.150 for IgG and 0.060 for IgM was chosen in the sonic extract and flagellum ELISAs. A lower cutoff for CSF IgM detection is justified because the extent of IgM leakage from blood to CSF is less than for IgG.

The results of antibody measurement in CSF by the sonic extract and flagellum ELISAs are shown in Fig. 5 and 6 and Table 1. The overall diagnostic sensitivities were almost identical for the two assays: 62.5 and 66.1% for IgG detection and 83.9 and 87.5% for IgM detection. Combining the results of the IgG and IgM measurements in CSF yielded overall diagnostic sensitivities of 89.0 and 94.6% with the sonic extract and flagellum ELISAs, respectively. In the sonic extract ELISA, 15 patients (27%) had diagnostic antibody levels in CSF and not in serum, whereas this was seen in only 2 patients tested with the flagellum ELISA. In only one case was a CSF sample that was positive in the sonic extract ELISA not positive in the flagellum ELISA.

As in serum, the OD values of CSF samples from the different control groups were generally lower in the flagellum ELISA, indicating an increased specificity.

Only serum and CSF samples from patients with syphilis showed considerable cross-reactivity with *B. burgdorferi* (Fig. 5 and 6). The cross-reacting antibodies belonged mainly to the IgG class. The flagellum ELISA lowered the OD values significantly, leading to reductions of seropositive samples from 75.9 to 34.5% ($P < 0.05$) in secondary syphilis and from 100 to 0% ($P < 0.01$) in CSF from patients with neurosyphilis. The number of false-positive sera from patients with primary syphilis was unaltered, although they showed a marked decline in OD values.

When 22 high-titered sera from patients with acute leptospirosis were tested, four and two patients were IgG seropositive and six and three patients were IgM seropositive in the sonic extract and flagellum ELISAs, respectively (results not shown).

DISCUSSION

This study shows that an ELISA using the *B. burgdorferi* flagellum as test antigen significantly improves serodiagnosis of Lyme disease.

Recently, it was reported that the application of purified *B. burgdorferi* flagellin (the reduced protein subunit of the flagellum [8]) in an ELISA did not increase diagnostic performance (12). The most likely explanation for the failure was the use of sodium dodecyl sulfate detergent lysis and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis for isolation of the antigen. Sodium dodecyl sulfate probably denatures important flagellum epitopes and furthermore interferes with the binding of the antigen to microdilution plates.

In our study, the purification of *B. burgdorferi* flagella was achieved by using the milder detergent sarcosyl for lysis of the cells, followed by shearing of the detergent-insoluble material and final banding on a CsCl density gradient. Electron microscopy of our preparation showed that sarcosyl did not disintegrate the flagella, which may be of great importance in preserving the diagnostically essential epitopes.

Our flagellum preparation was essentially pure when analyzed by electron microscopy, WB, and CIE. The only contaminant was a minor trace of a 32-kDa band probably corresponding to the major surface protein described by Barbour et al. (7). It was visible only in the protein stain of the flagellum preparation, and it is not expected to have any impact on the quality of the flagellum antigen for serological use, because the 32-kDa protein is specific for *B. burgdorferi* and elicits only a slow and weak immune response (7, 13, 27). In accordance with this, the immunostaining with a high-titered polyspecific ACA patient serum did not stain the 34-kDa band.

The present data showed that the main advantage of the IgG flagellum ELISA was increased diagnostic specificity. After adjusting the diagnostic cutoff level to 95%, this could be converted into a significant increase in diagnostic sensitivity. In the IgM flagellum ELISA, the specificity was almost unaltered, whereas the diagnostic sensitivity increased significantly.

The improved diagnostic performance of the flagellum ELISA is most likely due to the early and strong antibody response to this antigen (13) and to the loss of irrelevant cross-reacting antigens that are included in the whole cell preparations. *B. burgdorferi* shares important antigens not only with spirochetes but also with many other bacteria. One such example is the so-called common antigen, a protein that has been identified in more than 50 different bacteria (18). We have identified the common antigen of *B. burgdorferi* by CIE (Fig. 3) and WB as a 60-kDa band (K. Hansen, N. S. Pedersen, and P. Hindersson, submitted for publication). It explains the frequent finding of unspecific reactions to the 60-kDa band of *B. burgdorferi* in control sera (7, 27). Serum antibodies to such cross-reacting antigens due to common bacterial infections are expected to belong primarily to the IgG class. This may explain why the diagnostic specificity of the flagellum ELISA increased primarily in the IgG assay, corresponding to the findings of Wilske et al., who found unspecific-IgG but never unspecific-IgM reactivity to a 60-kDa band (27).

The use of a single purified antigen, such as the *B. burgdorferi* flagellum, as the test antigen eliminates the detection of such unspecific antibodies. This explanation is further supported by the observation that the flagellum ELISA did not improve the measurement of antibodies in

the CSF as much as in serum. Compared with serum immunoglobulins, the CSF immunoglobulins in LMR consist mainly of specific intrathecally produced antibodies (28), because normal CSF contains very low levels of immunoglobulin and, thus, low levels of unspecific antibodies.

In a whole cell sonic extract ELISA, 27% of the patients with LMR had significant antibody levels in the CSF only (all but one patient was diagnosed within 6 weeks after onset). This observation is in accordance with a previous study with a sonic extract ELISA (26). The greater sensitivity of serum antibody detection by the flagellum ELISA reduced this discrepancy, since now only two patients were CSF positive while being negative in serum. In any event, a spinal tap is still recommended because the finding of CSF pleocytosis and a high CSF antibody titer provides significant proof for the clinical LMR diagnosis. In contrast to a positive CSF antibody titer, an elevated serum titer may be a coincidental finding, since high serum titers can persist for a long time, even after asymptomatic infections.

The early and strong immune response to the spirochetal flagellum shows that the search for a valuable test antigen should not be limited to surface proteins. In the case of *B. burgdorferi* infections, the human antibody response to the flagellum is much stronger than it is to the 31- to 34-kDa major surface proteins (7, 13, 27).

WB studies have shown that the 41-kDa flagellin band is not completely specific. Sera from healthy controls may react slightly with the *B. burgdorferi* flagellum, as seen in Fig. 2, 5, and 6. The specificities of these cross-reacting antibodies are unknown. Exposure to oral spirochetes could be an explanation. Flagella from different *Borrelia* species are antigenically related (8). The flagellum ELISA is therefore not expected to improve serological discrimination between patients with relapsing fever and patients with Lyme disease. However, this is of minor practical importance because relapsing fever does not occur in Europe and the northeastern United States, where Lyme disease is most prevalent. The main limitation of the diagnostic specificity of the 41-kDa band is the reactivity with sera from patients with syphilis. Despite this observation, we found that the flagellum ELISA significantly diminished the cross-reactivity of serum and CSF specimens from syphilis patients. This is most likely a consequence of the elimination of other cross-reacting spirochetal antigens. The exposure of different epitopes of the morphologically intact flagella and the flagellum protein flagellin may explain the stronger reactivity of syphilis sera with the 41-kDa band in WB compared with the flagellum ELISA. In any event, the cross-reactivity between *Treponema pallidum* and *B. burgdorferi* does not constitute a major problem because patients with syphilis and Lyme disease are easily differentiated clinically and serologically by the nontreponemal syphilis serological tests (24). The limited number of sera from patients with leptospirosis investigated does not permit any conclusions as to whether the flagellum ELISA is more specific than the sonic extract ELISA in terms of serological discrimination between Lyme disease and leptospirosis.

Preabsorption of sera with fluorescent treponemal antibody-ABS sorbent (21), *T. phagedenis* (29), or *Borrelia hermsii* (14) has been used to increase the specificities of conventional assays. Although unspecific reactions can be avoided, preabsorption always leads to a certain loss of specific antibody activity and is furthermore difficult to standardize. The present approach, using a single antigen test, is therefore preferable. The antigenic profile of *B. burgdorferi* shows strain variation (9) and may change during subcultivation. Furthermore, some *Borrelia* antigens possibly undergo genetically determined antigen variation (10). Considering these observations, the advantage of the flagellum is that this antigen is phenotypically stable and without strain variation.

We therefore believe that the *B. burgdorferi* flagellum may become a suitable and needed reference antigen, making the serological results from different laboratories comparable. The cost benefit of the flagellum ELISA is acceptable, since a preparation from 1 liter of culture (approximately 10^{11} spirochetes) yielded flagellum antigen for about 300 96-well microdilution plates.

In conclusion, this study showed that the *B. burgdorferi* flagellum ELISA is superior to presently used serological tests and improves IgG and IgM serodiagnosis significantly, especially in early cases of Lyme disease. The improved diagnostic performance is most likely due to the removal of widely cross-reacting antigens which are present in whole cell antigen preparations. Pure flagella, retaining the epitopes important for serodiagnosis, can be isolated in sufficient quantity without difficulty.

ACKNOWLEDGMENTS

Klaus Hansen was supported by a grant from the University of Copenhagen and the Thorvald Madsen foundation.

Hanna Hansen, Dorthe Søeborg Petersen, Anna Hammer, and Helle Fjordvang are thanked for perfect technical assistance. We thank Eva Åsbrink, Department of Dermatology, Södersjukhuset, Stockholm, Sweden, for supplying *B. burgdorferi* ACA-1; Allan G. Barbour, Department of Microbiology, University of Texas, San Antonio, for monoclonal antibodies H 9724 and H 604; and Aksel Birch-Andersen, Department of Biophysics, Statens Seruminstitut, Copenhagen, Denmark, for performing the electron microscopy.

LITERATURE CITED

1. **Ackermann, R., P. Hörstrup, and R. Schmid.** 1984. Tick borne meningopolyneuritis (Garin-Bujadoux, Bannwarth). Yale J. Biol. Med. **57**:485–490.
2. **Ackermann, R., J. Kabatzki, H. P. Boisten, A. C. Steere, R. L. Grodzicki, S. Hartung, and U. Runne.** 1984. Spirochaeten aetiologie der Erythema chronicum migrans Krankheit. Dtsch. Med. Wochenschr. **109**:92–97.
3. **Åsbrink, E., B. Hederstedt, and A. Hovmark.** 1984. The spirochetal etiology of erythema chronicum migrans Afzelius. Acta Dermato. Venereol. **64**:291–295.
4. **Åsbrink, E., A. Hovmark, and B. Hederstedt.** 1984. The spirochetal etiology of acrodermatitis chronica atrophicans Herxheimer. Acta Dermato. Venereol. **64**:506–512.
5. **Åsbrink, E., A. Hovmark, and B. Hederstedt.** 1985. Serologic studies of erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans with indirect immunofluorescence and enzyme-linked immunosorbent assays. Acta Dermato. Venereol. **65**:509–514.
6. **Axelsen, N. H.** 1983. Intermediate gel immunoelectrophoresis. Scand. J. Immunol. **17**(Suppl. 10):141–149.
7. **Barbour, A. G., W. Burgdorfer, E. Grunwaldt, and A. C. Steere.** 1983. Antibodies of patients with Lyme disease to components of the Ixodes dammini spirochete. J. Clin. Invest. **72**:504–515.
8. **Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier.** 1986. A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. **52**:549–554.
9. **Barbour, A. G., R. A. Heiland, and T. R. Howe.** 1985. Heterogenity of major proteins in Lyme disease borrelia: a molecular analysis of North American and European isolates. J. Infect. Dis. **152**:478–484.
10. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect.

Immun. **45**:94–100.
11. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216**:1317–1319.
12. **Coleman, J. L., and J. L. Benach.** 1987. Isolation of antigenic components from Lyme disease spirochete: their role in early diagnosis. J. Infect. Dis. **155**:756–765.
13. **Craft, J. E., D. K. Fisher, G. T. Shimanto, and A. C. Steere.** 1986. Antigens of Borrelia burgdorferi recognized during Lyme disease. J. Clin. Invest. **78**:934–939.
14. **Craft, J. E., R. L. Grodzicki, and A. C. Steere.** 1984. Antibody response in Lyme disease: evaluation of tests. J. Infect. Dis. **149**:789–795.
15. **Harboe, N., and A. Ingild.** 1983. Immunisation, isolation of immunoglobulins and antibody titer determination. Scand. J. Immunol. **17**(Suppl. 10):345–351.
16. **Hardy, P. H., Jr., W. R. Fredericks, and E. E. Nell.** 1975. Isolation and antigenic characteristics of axial filaments from the Reiter treponeme. Infect. Immun. **11**:380–386.
17. **Hindersson, P., J. Knudsen, and N. H. Axelsen.** 1987. Cloning and expression of Treponema pallidum common antigen (Tp4) in Escherichia coli K12. J. Gen. Microbiol. **133**:587–596.
18. **Hindersson, P., C. S. Petersen, N. S. Pedersen, and N. H. Axelsen.** 1984. Immunological cross-reactions between antigen Tp-4 of Treponema pallidum and an antigen common to a wide range of bacteria. Acta Pathol. Microbiol. Scand. Sect. B **92**: 183–188.
19. **Hovmark, A., E. Åsbrink, and I. Olsson.** 1986. Spirochetal etiology of lymphadenosis benigna cutis solitaria. Acta Dermato. Venereol. **66**:479–484.
20. **Kohler, J., J. Kasper, U. Kern, U. Thoden, and B. Rehse-Küpper.** 1986. Borrelia encephalomyelitis. Lancet **ii**:35.
21. **Muhlemann, M. F., D. J. M. Wright, and C. Black.** 1982. Serology of Lyme disease. Lancet **i**:553–554.
22. **Pedersen, N. S., C. S. Petersen, M. Vejtorp, and N. H. Axelsen.** 1982. Serodiagnosis of syphilis by an enzyme-linked immunosorbent assay for IgG antibodies against the Reiter treponeme flagellum. Scand. J. Immunol. **15**:341–348.
23. **Pfister, H. W., K. Einhäupl, V. Preac-Mursic, B. Wilske, and G. Schierz.** 1984. The spirochetal etiology of lymphocytic meningoradiculitis of Bannwarth (Bannwarth's syndrome). J. Neurol. **231**:141–144.
24. **Russell, H., J. S. Sampson, G. P. Schmid, H. W. Wilkinson, and B. Plikaytis.** 1984. Enzyme-linked immunosorbent assay and indirect immunofluorescence assay for Lyme disease. J. Infect. Dis. **149**:465–470.
25. **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.
26. **Stiernstedt, G. T., M. Granström, B. Hederstedt, and B. Sköldenberg.** 1985. Diagnosis of spirochetal meningitis by enzyme-linked immunosorbent assay and indirect immunofluorescence assay in serum and cerebrospinal fluid. J. Clin. Microbiol. **21**: 819–825.
27. **Wilske, B., V. Preac-Mursic, G. Schierz, and K. V. Busch.** 1986. Immunochemical and immunological analysis of European Borrelia burgdorferi strains. Zentralbl. Bakteriol. Hyg. A **263**: 92–102.
28. **Wilske, B., G. Schierz, V. Preac-Mursic, K. von Busch, R. Kühbeck, H.-W. Pfister, and K. Einhäupl.** 1986. Intrathecal production of specific antibodies against Borrelia burgdorferi in patients with lymphocytic meningoradiculitis (Bannwarth's syndrome). J. Infect. Dis. **153**:304–314.
29. **Wilske, B., G. Schierz, V. Preac-Mursic, K. Weber, H. W. Pfister, and V. K. Einhäupl.** 1984. Serological diagnosis of erythema chronicum migrans disease and related disorders. Infection **12**:331–337.