Diagnosis of Spirochetal Meningitis by Enzyme-Linked Immunosorbent Assay and Indirect Immunofluorescence Assay in Serum and Cerebrospinal Fluid

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The antibody response against a spirochetal strain isolated from Swedish Ixodes ricinus ticks was determined by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay of cerebrospinal fluid (CSF) and serum specimens from 45 patients with chronic meningitis. Samples of CSF, serum, or both from patients with various infections of the central nervous system, multiple sclerosis, syphilis, or infectious mononucleosis and from healthy individuals were used as control samples. Probable spirochetal etiology could be demonstrated for 41 of 45 (91%) patients with clinical symptoms of chronic meningitis. Approximately 25% of the patients had significantly elevated titers of antibody to the spirochete in CSF but not in serum. The highest diagnostic sensitivity, 91%, was demonstrated by measurement of CSF antibodies and calculation of a spirochetal CSF titer index, which is the ratio of (ELISA titer in CSF/ELISA titer in serum) to (albumin in CSF/albumin in serum) and which also considers the degree of blood-CSF barrier damage. The highest specificity, 98%, was obtained by calculation of a CSF titer index. Patients with short duration of disease were especially prone to be antibody negative in serum but positive in CSF. Significant rise in serum antibody titers was seldom demonstrated in patients treated with antibiotics. It is concluded that measurement of CSF antibodies, especially by ELISA, is a highly sensitive and specific method for the immunological diagnosis of spirochetal meningitis.

In Europe, neurological disease with radicular pain and chronic lymphocytic meningitis after tickbite was described in 1922 by Garin and Bujadoux (8) and in 1941 by Bannwarth (3, 4). In a few cases the neurological symptoms were preceded by erythema chronicum migrans, a skin lesion first described by the Swedish physician Alfred Afzelius in 1909 (2). In 1983, we reported persistent or progressive chronic meningitis (CM) in 21 patients without demonstrable infections or other disease. The patients had intrathecal synthesis of considerable quantities of immunoglobulin G, and they improved or recovered during treatment with high-dose intravenous penicillin (13). We discussed the possible connection with Lyme disease, first described in 1976 from the community of Lyme, Connecticut, by Steere et al. (16).

Lyme disease usually starts with erythema chronicum migrans and may then be followed by neurological and cardiac as well as arthritic complications (15). Recently, Burgdorfer and co-workers succeeded in isolating a spirochete from one vector of the disease, the tick, Ixodes dammini (6). This spirochete is now officially named Borrelia burgdorferi (9). The role of B. burgdorferi as the etiological agent of Lyme disease is supported by induction of a disease resembling erythema chronicum migrans in rabbits exposed to ticks, by high antibody titers to this spirochete in patients with Lyme disease (6), and by cultivation of spirochetes from blood, cerebrospinal fluid (CSF), or skin lesions from a few Lyme patients (5, 14). Steere in the United States (14) and Ackermann in the Federal Republic of Germany (1) have examined antibodies to B. burgdorferi by indirect immunofluorescence assay (IFA). Recently Russell (11) and Craft (7) published comparative studies between IFA and enzyme-linked immunosorbent assay (ELISA) performed on sera. To our knowledge, there have not been published any comparative studies between IFA and ELISA performed on both sera and CSF in parallel. We have recently presented preliminary data on serum antibodies against B. burgdorferi isolated from American I. dammini and against a spirochete isolated from Swedish Ixodes ricinus in sera from patients with CM measured by IFA (16a). The aim of the present investigation is to compare IFA and ELISA results with measurements of antibodies in serum and CSF for the laboratory diagnosis of I. ricinus-transmitted spirochetal infections of the central nervous system (CNS).

Materials and Methods

CSF and serum specimens. Table 1 shows the numbers and sources of specimens tested by IFA and ELISA.

Patients with CM. Forty-five patients were hospitalized from 1975 to 1983 with CM. CM was defined as a disease which failed to improve or worsen clinically in its CSF abnormalities during at least a 2-week course (13, 16a). Infectious CNS diseases caused by Toxoplasma gondii, Cryptococcus neoformans, Mycobacterium tuberculosis, Listeria monocytogenes, and Treponema pallidum were excluded in all 45 patients. Forty-three of the 45 patients had been treated with high-dose intravenous antibiotics.

Controls. As meningitis controls, CSF and serum samples from patients with different infectious diseases of the CNS were studied. The infections included tick-borne encephalitis (TBE), varicella-zoster virus, herpes simplex virus, cytomegalovirus, Epstein-Barr virus, measles or echovirus, Mycoplasma pneumoniae, and acute bacterial and tuberculous or cryptococcal infections. We also examined CSF and...
serum samples from some patients without demonstrable CNS disease.

In other control groups, we analyzed samples of CSF or serum or both from healthy individuals aged 0 to 90 years or from patients with multiple sclerosis, infectious mononucleosis, syphilis, or leptospirosis. All controls (except patients with syphilis) having positive titers to *I. ricinus* spirochete in CSF or serum or both were negative by syphilis serological tests (Wasserman, Venereal Disease Research Laboratory, fluorescent treponemal antibody-absorption test).

**Serological methods.** (i) IFA. The antigen used was a preparation of spirochetes from Swedish *I. ricinus* (strain G 152) and was grown in modified Kelly medium (14), centrifuged at 10,000 × g for 30 min, washed three times in phosphate-buffered saline (PBS), and fixed to glass slides by methanol fixation. Twofold dilutions of serum or CSF were made from initial dilutions of 1:20 for serum and 1:5 for CSF. The slides were covered by these dilutions for 30 min, washed, and then stained with fluoresceinated goat anti-human immunoglobulin (polyvalent conjugate; National Bacteriological Laboratory, Stockholm, Sweden) for 30 min. After repeated washing, the slides were read in a fluorescence microscope. The endpoint was defined as the highest titer at which all organisms still showed a bright fluorescence. The same high-titer and low-titer control serum samples were included in each test.

(ii) ELISA. The antigen preparation was performed essentially by the method of Craft et al. (7). Spirochetes isolated from Swedish *I. ricinus* (strain G 152) were grown in modified Kelly medium for 4 to 7 days. The spirochetes were then pelleted by centrifugation at 10,000 × g for 30 min and washed four times in PBS with 5 mM MgCl₂. The final pellet was suspended in PBS without MgCl₂ and then sonicated on ice four times for 30 s each. After a final centrifugation at 10,000 × g for 30 min, the supernatant was used as antigen. The protein content was determined by the method of Lowry (10). The coating concentration was determined by testing a serum with a high concentration of spirochetal antibodies against serial dilutions of antigen. The optimal coating concentration was found to be 5 μg of protein per ml for immunoglobulin G (IgG) and 10 μg/ml for IgM. Antigens were diluted in PBS (pH 7.2).

Microplates (129 B; Dynatech Laboratories, Plochingen, West Germany) were coated overnight at room temperature. After repeated washings, 100 μl of serum diluted 1:1,000 or 100 μl of CSF diluted 1:500 in PBS with 0.05% Tween 20 was added, and the plates were incubated for 1 h at room temperature for IgG and 2 h at 37°C for IgM. Alkaline phosphatase-conjugated swine anti-human IgG (100 μl; Orion Diagnostica, Helsinki), diluted 1:250, or IgM, diluted 1:200 in PBS with 0.05% Tween 20, was added to each well, and the plates were incubated overnight at room temperature. *p*-Nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml in 1 M diethanolamine buffer (pH 9.8) containing 5 mM MgCl₂, was added to each well. Absorbance was determined after 25 to 65 min in an ELISA reader (Flow Laboratories, Irvine, Scotland). All washings were done at least three times with 0.9% NaCl containing 0.05% Tween 20. Positive and negative controls were included in each test, and the time for substrate incubation was adjusted to these controls to eliminate day-to-day variations.

The ELISA titer was defined as the titer of the serum or CSF that gave a two-fold increase in optical density over that of the negative control. Each sample was tested in duplicate, and the mean value was calculated. The difference in two values differed more than 10% from the mean, the sample was reretested. If the difference was more than 1.5 or less than 0.1, the sample was retested in a higher or lower dilution. Each sample was tested in two uncoated wells (background), and if the value in these uncoated wells was higher than approximately 25% of the value for coated wells, the sample was reretested. All specimens from each patient were tested simultaneously. All serum specimens having antibody titers above the 95 percentile for healthy individuals were considered significantly elevated, i.e., positive. An increase in titer of fourfold or greater in IFA or twofold or greater in ELISA was considered significant. A CSF/serum titer ratio was expressed as the ratio of the ELISA titer in CSF to the ELISA titer in serum. Albumin and IgG levels in CSF and serum samples were measured by a nephelometric method, and a spirochetal CSF titer index was calculated as the ratio of (ELISA titer in CSF/ELISA titer in serum) to (albumin in CSF/albumin in serum) (17).

**RESULTS**

IFA. Spirochetal antibody levels measured by IFA in serum and CSF samples are shown in Fig. 1. The 95 percentile serum level in healthy individuals was 320. With this limit, 23 of 45 (51%) CM patients were seropositive when the sample with the highest titer was considered.

CSF samples with detectable antibodies, i.e., a titer of ≥1:5, were considered positive. Positive antibody titers in CSF were found in 38 of 45 (84%) CM patients and in none of 32 meningitis control patients.

**ELISA.** (i) Serum antibodies. Spirochetal IgG and IgM antibody titers in serum measured by ELISA are shown in:

<table>
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<tr>
<th>Subject, disease</th>
<th>IFA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients</td>
<td>No. of CSF samples tested</td>
</tr>
<tr>
<td>Patient (CM)</td>
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<td>90</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBE</td>
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<td>3</td>
</tr>
<tr>
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<td>21</td>
</tr>
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<td>8</td>
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<tr>
<td>Multiple sclerosis</td>
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<td>0</td>
</tr>
<tr>
<td>Syphilis</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Leptospirosis</td>
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<td>0</td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
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<td>0</td>
</tr>
<tr>
<td>None (healthy individuals)</td>
<td>63</td>
<td>0</td>
</tr>
</tbody>
</table>

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The 95 percentile of titer level for healthy individuals was 450 for IgG and 580 for IgM. With these limits, 11 of 120 (9%) healthy controls were seropositive for IgG or IgM with one patient positive for both IgG and IgM. No significant differences were found in different age groups.

The 95 percentile of titer levels in a healthy population was set as the upper limit for normal values. Of 45 CM patients, 28 (62%) were IgG positive, 10 (22%) were IgM positive, and 8 (18%) were both IgG and IgM positive. Thus, 30 of 45 patients (67%) were positive for IgG or IgM or both (Fig. 2; see Fig. 4). The median titer levels were not significantly different between healthy individuals and patients with TBE or meningitis of various etiology. None of 16 patients with multiple sclerosis was seropositive, whereas 10 of 20 patients with mononucleosis were IgM seropositive and 2 of 20 were both IgG and IgM positive. Among patients with other spirochetal diseases, 19 of 25 (76%) patients with syphilis and 1 of 4 patients with leptospirosis had significantly elevated antibody titers against I. ricinu pirochete.

(ii) CSF antibodies. The spirochetal IgG and IgM antibody levels in CSF measured by ELISA are shown in Fig. 3. A titer level of 10 was set as the upper limit for normal values for both IgG and IgM. With these limits, elevation of IgG antibody titers in CSF was found in 39 of 45 CM patients (87%), elevation of IgM antibody titers was found in 29 of 45 (64%), and 41 of 45 patients (91%) showed elevation of either IgG or IgM antibodies or both (Fig. 3 and 4). Significantly elevated antibody titers in CSF were found in 29 of 30 seropositive CM patients (data not shown in figures). Among the meningitis controls, 3 of 53 (6%) were positive for IgG or IgM or both. All three patients were seropositive and showed high CSF/serum albumin ratios, indicating a high degree of damage to the blood-brain barrier. Two of these three patients suffered from tuberculoid meningitis, and one patient suffered from varicella-zoster meningoencephalitis. None of 16 patients with multiple sclerosis had elevated antibody titers in CSF, whereas 3 of 12 patients with syphilis were IgG positive.

CSF/serum ELISA titer ratio and titer index for CSF. An IgG titer in CSF/IgG titer in serum ratio of 0.04 and an IgM titer in CSF/IgM titer in serum ratio of 0.02 were chosen as the upper limits for normal values. The corresponding values for titer indexes for CSF were 2.0 for IgG and 1.0 for IgM. With these limits, CSF/serum titer ratios were positive for 36 of 45 (80%) CM patients for IgG, 32 of 45 (71%) for IgM, and 39 of 45 (87%) for either IgG or IgM or both. The corresponding results for the spirochetal titer index for CSF were as follows: of 45 CM patients, 39 (87%) were positive for IgG antibodies, 32 (71%) were positive for IgM, and 41 (91%) were positive for IgG or IgM or both (Fig. 4).

Sensitivity and specificity of the different assays. The results of the different antibody assays and index calculations in CM patients (sensitivity) and meningitis control patients (specificity) are shown in Fig. 4. Measurement of CSF antibodies was more sensitive and specific than measurement of serum antibodies in both IFA and ELISA. The highest diagnostic sensitivity with 41 of 45 positive CM patients was achieved by measuring IgG and IgM antibodies in CSF by ELISA or by calculating the IgG and IgM spirochetal ELISA titer index for CSF. Three CM patients were negative in all antibody assays and index calculations. One further CM patient was seropositive by ELISA for both IgG and IgM but negative in all other assays and index calculations. Spirochetal etiology of disease could thus be found in 41 of 45 (91%) CM patients. The corresponding specificity found for measurement of CSF antibodies by ELISA was 94%, and the specificity found for the titer index for CSF was 98%. Only one meningitis control patient was positive by the titer index calculation for CSF. This patient suffered from tuberculoid meningitis and had a slightly elevated IgM index of 1.03.

Influence of duration of disease and antibiotic treatment on titer levels. Levels of ELISA titer to spirochete in relation to duration of disease and antibiotic treatment are shown in Fig. 5. Of 39 pretreatment samples, 21 (54%) were seropositive for IgG, 8 (21%) were positive for IgM, and 23 (59%)

![Graph showing antibody levels measured by IFA in patients with CM and control patients. Each dot represents the highest titer noted in one individual. Horizontal bars mark the median titer in each group. The 95 percentile of titers in serum for healthy individuals used as the upper limit of normal values was 320. In CSF, titers of ≥5 were considered positive.](http://jcm.asm.org/)

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**Fig. 2.** The 95 percentile titer level for healthy individuals was 450 for IgG and 580 for IgM. With these limits, 11 of 120 (9%) healthy controls were seropositive for IgG or IgM with one patient positive for both IgG and IgM. No significant differences were found in different age groups.

**Fig. 3.** CSF antibody titers in CM patients and meningitis control patients. The median titer levels were not significantly different between healthy individuals and patients with TBE or meningitis of various etiologies. None of 16 patients with multiple sclerosis was seropositive, whereas 10 of 20 patients with mononucleosis were IgM seropositive and 2 of 20 were both IgG and IgM positive. Among patients with other spirochetal diseases, 19 of 25 (76%) patients with syphilis and 1 of 4 patients with leptospirosis had significantly elevated antibody titers against I. ricinu pirochete.

**Fig. 4.** CSF/serum ELISA titer ratio and titer index for CSF. An IgG titer in CSF/IgG titer in serum ratio of 0.04 and an IgM titer in CSF/IgM titer in serum ratio of 0.02 were chosen as the upper limits for normal values. The corresponding values for titer indexes for CSF were 2.0 for IgG and 1.0 for IgM. With these limits, CSF/serum titer ratios were positive for 36 of 45 (80%) CM patients for IgG, 32 of 45 (71%) for IgM, and 39 of 45 (87%) for either IgG or IgM or both. The corresponding results for the spirochetal titer index for CSF were as follows: of 45 CM patients, 39 (87%) were positive for IgG antibodies, 32 (71%) were positive for IgM, and 41 (91%) were positive for IgG or IgM or both.

**Fig. 5.** Influence of duration of disease and antibiotic treatment on titer levels. Levels of ELISA titer to spirochete in relation to duration of disease and antibiotic treatment are shown in Fig. 5. Of 39 pretreatment samples, 21 (54%) were seropositive for IgG, 8 (21%) were positive for IgM, and 23 (59%)
were positive for IgG or IgM or both. With increasing duration of disease before the start of treatment with antibiotics, the percentage of positive CM patients and their IgG titers increased and their IgM titers decreased. After treatment, the titers in CSF and serum usually decreased, with the exception of the IgG titers in serum. These titers showed a slight increasing trend during the first 5 weeks posttreatment, especially in patients with a short duration of disease pretreatment. Among CM patients with paired serum samples, a significant rise of ELISA titers was found in only 5 of 35 (14%). In all five cases, the rise was in the IgG antibody class. Both of the patients not treated with antibiotics showed a significant rise in titers. All 32 CM patients with both pre- and posttreatment CSF samples showed higher

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**FIG. 2.** Antibody levels of IgG and IgM in serum measured by ELISA in patients with CM and in control patients. Each dot represents the highest titer noted in one individual. Horizontal bars mark the median titer in each group. The 95th percentile of titers in serum for healthy individuals used as the upper limit of normal values was 450 for IgG and 580 for IgM.

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**FIG. 3.** CSF antibody levels of IgG and IgM measured by ELISA in patients with CM and in control patients. Each dot represents the highest titer noted in one individual. Horizontal bars mark the median titer in each group. A titer level of 10 was used as the upper limit of normal values for both IgG and IgM.
antibody titers in their pretreatment samples than in their posttreatment samples.

By IFA (data not shown), 21 of 39 (54%) pretreatment samples from CM patients were seropositive. With increasing duration of disease before the start of antibiotic treatment, the percentages of CSF- and serum-positive CM patients increased. The kinetics of the titer decrease during the posttreatment period followed essentially the pattern described for ELISA IgG antibodies. Only 1 of 35 CM patients with paired serum samples showed a significant increase in serum titer. None of 32 CM patients with both pre- and posttreatment CSF samples had higher titers in their posttreatment samples compared with their pretreatment samples.

**DISCUSSION**

A probable spirochetal etiology could be demonstrated by ELISA in almost all patients with CM examined. The
sensitivity of the different assays might however be underestimated, since sensitivity had to be evaluated against a clinical diagnosis of the disease. The possibility of nonspirochetal etiology in some patients with CM can therefore not be completely excluded. With the upper limit of normal values established in healthy controls, the assays also showed a high diagnostic specificity in patients with meningitis of nonspirochetal etiology. However, measurement of CSF antibodies was necessary for diagnosis in approximately 25% of the CM patients. This approach was especially important in patients with short duration of disease, since these patients often were seronegative. Generally, measurement of CSF antibodies was a more sensitive method than measurement of serum antibodies by either IFA or ELISA. In addition, measurement of CSF antibodies was a more specific method than measurement of serum antibodies.

The spirochetal antibody response seemed to be rather slow, and high antibody levels in serum were usually reached between 5 and 10 weeks after the onset of symptoms. Treatment with high-dose intravenous antibiotics seemed to influence the antibody response in both serum and CSF. This might be due to an interruption of the antigenic stimulation of the immune system resulting in stationary or decreasing titers. In patients receiving antibiotic treatment, a significant rise of serum antibody titers was seldom found. A significant rise of titers was found in 2 of 3 untreated patients compared with 3 of 33 patients receiving antibiotics.

The results in this study also indicate that the performance of IgM serology adds little to the total diagnostic sensitivity of the ELISA test. However, it is possible that with a tendency towards earlier admission and treatment of the patients, the IgM serology could become more important.

Serum antibodies to *B. burgdorferi* in Lyme disease and related disorders have been analyzed by Burgdorfer (6) and subsequently by Steere (14) in the United States and by Ackermann in the Federal Republic of Germany (1), using IFA. They all found about 90% sensitivity and almost 100% specificity in the neurological stage of the disease. Recently Russell et al. (11) and Craft et al. (7) have compared the IFA with ELISA. In both studies, ELISA was considered somewhat more sensitive and specific than IFA. The sensitivity of ELISA in serum was >90% in the neurological stage, which is higher than the sensitivity found in this study, possibly reflecting differences in the selection of patients and controls and also possibly reflecting differences in the nature of the diseases seen in the United States and in Europe.

The high antibody levels in CSF indicated intrathecal production of spirochetal antibodies in the CNS. This observation agrees well with our previous finding of elevated synthesis of IgG in the CNS of our patients with CM (13). Also, by immunofluorescence of electrofocused and sequentially sampled CSF and serum pairs from one patient with CM, intrathecal production of oligoclonal specific IgG antibodies to *B. burgdorferi* has been demonstrated (16a).

The demonstration of intrathecal synthesis of specific antibodies has been utilized notably in chronic neurologic disorders, especially multiple sclerosis, but also in acute CNS infections, especially herpes simplex encephalitis (12, 18, 19). The importance of considering the degree of blood-CSF barrier leakage, measured by reference antibodies or albumin or both, has been pointed out (13).

Against this background we found it relevant to focus interest on a spirochete-CSF titer index. Such an index has some advantages. A serum antibody titer to spirochetes per se does not need to be related to the etiology of the actual disease. A significantly high spirochete-CSF titer index, however, supports the spirochetal etiology of the CNS disease. By calculation of a specific CSF titer index, the degree of blood-CSF barrier damage may be considered.

An elevated spirochete-CSF titer index was found in a total of 91% of 45 CM patients and also in 1 of 4 patients with tuberculous meningitis. Two of the four patients with tuberculous meningitis also had positive titers to our spirochete strain in their serum samples. This finding might indicate some degree of antigenic cross-reactivity between this spirochete strain and *Mycobacterium* sp.

A cross-reactivity between sera from patients with syphilis and patients with CM was found in this study. This finding is in agreement with other studies showing cross-reactivity between sera from patients with Lyme disease, syphilis, and relapsing fever (7, 11, 14). This cross-reactivity is easily managed in routine diagnostic serological work by testing all specimens positive by specific spirochetal serology by routine syphilis serology as well. Although patients with spirochetal disease were negative by syphilis serology tests, weakly positive fluorescent treponemal antibody-absorption test results may be observed in patients with Lyme disease and related disorders.

In Europe, diagnostic separation of spirochetal meningitis from viral TBE is of special interest because of the common vector. Furthermore, it may be difficult to separate the two diseases on clinical grounds. In this study there was no cross-reactivity between antibodies to our spirochetal strain and to TBE viral antigen. We did not find any definite evidence of dual infection, but unfortunately serological specimens were not available from the patients with TBE who also had high spirochetal titers in serum.

In conclusion, the present study shows that the serological diagnosis of spirochetal or Lyme meningitis becomes more sensitive and specific if CSF serology is performed, especially in patients with a short duration of disease. The most specific method is to calculate a spirochetal titer index for CSF that takes the degree of blood-CSF barrier damage into consideration. Antibiotic treatment seems to interrupt the increase of spirochetal antibody levels and in most cases eliminates the possibility of serological diagnosis by determination of significant titer increase.

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LITERATURE CITED


