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Research Article

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Simultaneous Expression of *Borrelia* OspA and OspC and IgM Response in Cerebrospinal Fluid in Early Neurologic Lyme Disease

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Abstract

Lyme disease is the major tick-borne disease, caused by *Borrelia burgdorferi* (Bb). Neurological involvement is common in all stages. In vivo expression of Bb antigens (Ags) and the immune response to them has not been well investigated in the cerebrospinal fluid (CSF). Upregulation of outer surface protein (Osp) C and concomitant downregulation of OspA before tick inoculation of the spirochete has been reported in skin and blood in animals. CSF OspA Ag in early disease suggests otherwise in CSF. Early Ag expression and IgM response in human CSF was investigated here. Paired CSF and serum was collected from 16 early, predominantly erythema migrans Lyme disease patients with neurologic problems, 13 late Lyme disease patients, and 19 other neurologic disease (OND) controls. Samples were examined for IgM reactivity to recombinant Bb-specific Osps using ELISA and immunoblot.

Of 12 early Lyme disease patients with neurologic involvement with both CSF and serum IgM against OspC, 7 (58%) had IgM to OspA ($n = 5$) or OspB ($n = 2$) that was restricted to the CSF, not serum. Overall, 12 of 16 (75%) of these early Lyme disease patients with neurologic involvement had CSF and serum IgM against OspC. Only 3 of 13 (23%) late Lyme disease patients and none of 19 OND controls had CSF IgM directed against OspC. In conclusion, in CSF, OspC and OspA can be coexpressed, and IgM response to them occurs in early Lyme disease patients with neurologic involvement. This biologic finding may also provide a discriminating marker for CNS infection in Lyme disease. (*J. Clin. Invest.* 1997. 100:763–767.) Key words: cerebrospinal fluid • IgM • OspC • *Borrelia burgdorferi* • neurologic Lyme disease

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Introduction

Lyme disease is a major infectious disorder, comprising 91% of vector-borne infections in the United States (1). This bacterial infection is due to a tick-borne spirochete, *Borrelia burgdorferi* (Bb)¹ (2). The nervous system is a very common target organ (3–9). The behavior of the invading Bb within the central nervous system (CNS) and the immune response in the cerebrospinal fluid (CSF) have not been described sufficiently. Although not studied in compartments, such as the CNS, several Bb genes have been reported to be induced selectively in vivo, including exported plasmid protein A (EppA), protein G (pG), outer surface protein (Osp) E and OspF homologues, and variable major protein-like sequence (VLS) (10–13). There are also reports of upregulation of OspC and concomitant downregulation of OspA before tick inoculation of the spirochete, and in skin and blood in animals (14–16). However, our finding of CSF OspA antigen (Ag) in neurologic Lyme disease (17) suggests that different regulatory factors may be operative in the CSF.

The lack of in-depth characterization of the host–spirochete interaction has made diagnosis of CNS involvement in Lyme disease difficult. This is particularly so when the hallmark rash, erythema migrans (EM), is absent, so that Lyme disease is not brought into the differential diagnosis. The spirochete is difficult to culture or stain from CSF (18), borrelial antigen and DNA assays are not widely available or standardized (17, 19, 20), and intrathecal borrelial antibody indices are most often negative in North American patients (21–23). In this study, CSF anti-Bb IgM was studied as an early biologic response to OspA, OspB, and OspC. This would enable detection of in vivo expression of these Ags, which appear to be unique to Bb. At the same time, it might lead to potential markers for early neurologic Lyme disease.

Methods

Subjects. We studied three patient groups. Early Lyme disease patients with EM and prominent neurologic features ($n = 14$) were defined as having a duration of illness of 3 mo or less. All met Centers for Disease Control and Prevention (CDC) criteria for Lyme disease (24); 11 were seropositive by ELISA for Bb exposure. Ages ranged

1. **Abbreviations used in this paper:** Ag, antigen; Bb, *Borrelia burgdorferi*; CSF, cerebrospinal fluid; EM, erythema migrans; OND, other neurological disease; Osp, outer surface protein; RT, room temperature.

from 10 to 44 yr, and illness ranged from 3 d to 12 wk. For this study, early Lyme disease patients with current or recent EM were also categorized based on time latency between onset of the skin lesion and lumbar puncture: 0–3 wk ($n = 7$), 3–6 wk ($n = 2$), and 6–12 wk ($n = 5$). Two other early patients (with facial nerve palsy and meningitis without prior history of EM) could not be categorized in this system and were considered separately as early disseminated Lyme disease. They also met CDC criteria. Combined, they accounted for 16 early Lyme disease patients.

Late Lyme disease patients ($n = 13$) had been diagnosed and had completed a course of antibiotic treatment for their infection a minimum of 6 mo before study. They had been treated originally for clinical syndromes of Lyme arthritis ($n = 6$), Lyme meningitis ($n = 3$), flu-like illness with documented seroconversion ($n = 3$), EM ($n = 2$), and Lyme-related facial nerve palsy ($n = 1$). All 13 were currently seropositive, and had persistent symptoms which prompted their reassessment. Ages ranged from 22 to 58 yr, and prior treatment ranged from 8 to 96 mo before testing. Other neurologic disease (OND) controls ($n = 19$) were patients who resided in our Lyme-endemic region and were seronegative for Bb exposure. Ages ranged from 20 to 70 yr. Their neurologic diagnoses were multiple sclerosis ($n = 8$), chronic fatigue ($n = 2$), Arnold-Chiari syndrome ($n = 1$), dementia syndrome ($n = 1$), dizziness ($n = 1$), headache ($n = 1$), lumbar radiculopathy ($n = 1$), peripheral neuropathy ($n = 1$), postinfectious encephalomyelitis ($n = 1$), recurrent meningitis ($n = 1$), and transverse myelitis ($n = 1$).

Samples. Paired CSF and serum were collected at the time of lumbar puncture. In all cases, lumbar puncture was carried out as part of the standard clinical evaluation of the patient.

Anti-Bb IgM. IgM was measured to recombinant Bb proteins, as well as to whole cell Bb proteins. For recombinant protein studies, the genes encoding OspA, OspB, and OspC for B31 strain Bb were cloned, sequenced, and expressed in *Escherichia coli* using previously reported techniques (25). For whole cell studies, B31 strain Bb sonicate Ags (Scripps Labs., San Diego, CA) were used.

Recombinant protein ELISA. IgM directed against recombinant OspA, OspB, or OspC fusion protein (Bb-specific Osp)s was examined by indirect ELISA. High-binding polystyrene microtiter plates (Costar Corp., Cambridge, MA) were coated with 1 $\mu\text{g}/\text{ml}$ of a single recombinant Osp fusion protein diluted in 0.1 M carbonate/bicarbonate coating buffer, pH 9.6. This concentration was determined to give optimal results in preliminary studies, as were the dilutions of assay samples and reagents. After overnight incubation at 4°C, plates were washed several times with PBS containing 0.05% Tween 20. Plates were blocked with 2% BSA in PBS-Tween for 1 h. Plates were then washed, and 50- μl samples were added to duplicate wells. CSF samples were added undiluted, while serum samples were diluted 1:25 in PBS-Tween. After overnight incubation at 4°C, plates were washed. Horseradish peroxidase conjugated to affinity-purified goat anti-human IgM, μ chain-specific (Tago, Inc., Burlingame, CA), was then added to each well (1:3,000 dilution in PBS-Tween) and incubated for 1 h. After washing, 3,3',5,5' tetramethylbenzidine peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. The reaction was stopped with 1 N H₂SO₄, and OD read at 450 nm in an automatic ELISA reader (Microplate Autoreader; Bio-Tek Instruments, Inc., Burlington, VT). Each plate included one or two positive controls and a panel of 10 negative control CSF samples. The negative control panel was used to generate the reactive OD cutoff (their mean OD reading +3 SD). Data were expressed as antibody indices (sample OD/cutoff OD), with an index of ≥ 1 considered positive.

Four CSF samples were used to determine the intra- and interassay variability of our optimized recombinant ELISA assays. For intraassay variation, the samples were each run three times in a single assay. The coefficients of variation for each sample were calculated from the SD of the antibody index divided by mean index, multiplied by 100. For interassay variations, the samples were run in five separate assays. Coefficients of variation were then calculated for each of the four samples. The intraassay coefficients of variation ranged from

6.5 to 8.7% for the anti-OspC IgM assay, 3.3 to 9.8% for the anti-OspA IgM assay, and 2.4 to 14.7% for the anti-OspB IgM assay. The interassay coefficients of variation ranged from 3.9 to 9.6% for the anti-OspC IgM assay, 3.8 to 10.5% for the anti-OspA IgM assay, and 5.0 to 11.2% for the anti-OspB IgM assay.

Whole cell ELISA. IgM directed against whole cell Bb proteins was examined by both indirect and antibody capture ELISA. The indirect ELISA was performed as above, except that 1 $\mu\text{g}/\text{ml}$ whole cell B31 Bb sonicate Ag (instead of recombinant Osp fusion protein) was used to coat microtiter plates. For antibody capture ELISA, plates were coated overnight at 4°C with 100 μl affinity-purified goat anti-human IgM at a concentration of 2 $\mu\text{g}/\text{ml}$ (Tago, Inc.) in carbonate/bicarbonate buffer, pH 9.6. Plates were then washed, and the unbound sites were blocked for 1 h with 3% BSA-PBS-Tween. Plates were then incubated sequentially with 50 μl of the following samples diluted in PBS-Tween: patient serum (diluted 1:100) or CSF (undiluted), added to duplicate wells for 2 h at room temperature (RT); sonicated Bb Ags (10 $\mu\text{g}/\text{ml}$), added for 1 h at RT; the F(Ab')₂ fragment of rabbit polyclonal IgG antibodies to B31 strain Bb (diluted 1:2,000), added for 1 h at RT; and a 1:2,000 dilution of horseradish peroxidase-conjugated goat antibody to rabbit IgG (Sigma Chemical Co., St. Louis, MO). After washing, 100 μl of 3,3',5,5' tetramethylbenzidine peroxidase was added, followed by 1 N H₂SO₄ to stop the reaction. Each assay included one or two human positive samples, and a panel of 10 negative controls to generate the assay reactive cutoff.

Immunoblot. IgM reactivity to OspC in early Lyme disease serum samples was also examined by immunoblot. 10 μg recombinant OspC was added to Laemmli gel sample buffer consisting of 3% SDS, 10% 60 mM Tris-HCl (pH 6.8), 0.005% bromophenol blue, and 1 mM PMSF. The protein was boiled for 5 minutes, then loaded onto a preparative 12% SDS-polyacrylamide gel. This was a 7 \times 8 cm minigel, 0.75 mm thick, with a stack. The gel was run using a Bio-Rad apparatus (Bio-Rad Laboratories, Hercules, CA), then transferred to an 0.2- μm nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) at 30 constant V at 4°C overnight. The membrane was blocked with 2% BSA/1% normal goat serum in TBS/0.05% Tween (50 mM Tris-HCl, 0.2 mM NaCl, 3 mM KCl, pH 7.5) for 1 h at RT. A single strip was cut from the membrane to use as a positive control, and the rest of the membrane was placed in a 28-well miniblitter apparatus (Immunetics, Cambridge, MA). After serum samples were diluted 1:50 with TBS-Tween, 60 μl was pipetted into each well. The miniblitter was then slowly rocked for 2 h at RT.

After washing with TBS-Tween, alkaline phosphatase-conjugated goat anti-human IgM, μ chain-specific and affinity-purified (Jackson ImmunoResearch Labs., Inc., West Grove, PA), was diluted 1:4,000 in TBS-Tween and added to the membrane for 1 h. The positive control strip was incubated for 1 h at RT with a 1:100 dilution of mouse mAb to OspC, purified from the B31 strain of Bb (antibody 75.27, IgG2a isotype). The strip was washed and then incubated for 1 h with affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG, diluted 1:10,000 in TBS-Tween. After a final washing, the membrane and strip were developed using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate system (Kirkegaard & Perry Laboratories).

Other CSF tests. Additional CSF tests included standard cell count and protein determination, and intrathecal borrelial antibody production, as performed by the clinical laboratory (26).

Results and Discussion

The majority (12/16, 75%) of early Lyme disease patients had detectable IgM to OspC in both CSF and serum (Table I). A striking finding in 5 of these 12 (42%) was IgM OspA reactivity restricted to the CSF and not the serum (Table II). Also, 2 of these 12 had IgM against OspB limited to the CSF compartment. 7 of these 12 (59%) early infection patients also showed CSF IgM reactivity to both OspA and OspB in addi-

Table I. CSF IgM Reactivity to Bb by ELISA to Recombinant OspA

Reactivity	Early LD* patients (n = 16)			Disseminated (n = 2)	Late LD patients (n = 13)	OND patients (n = 19)
	EM 0–3 wk (n = 7)	EM 3–6 wk (n = 2)	EM 6–12 wk (n = 5)		Number of patients P value [‡] of early LD compared to this group	Number of patients P value [‡] of early LD compared to this group
Recombinant OspA	3(43%)	1(50%)	1(20%)	2(100%)	3(23%) P = NS	0 P = 2 × 10 ⁻³
Recombinant OspB	3(43%)	1(50%)	1(20%)	2(100%)	0 P = 7 × 10 ⁻³	0 P = 2 × 10 ⁻³
Recombinant OspC	4(57%)	1(50%)	5(100%)	2(100%)	3(23%) P = 7 × 10 ⁻³	0 P = 2 × 10 ⁻⁶

*Early patients were divided based on time interval between EM skin lesion and lumbar puncture; the two patients without prior EM were considered to be early disseminated. LD, Lyme disease. [‡]P values by Fisher's Exact test.

tion to OspC. IgM reactivity against recombinant OspC was detected in some patients with negative indirect or antibody capture ELISA assays measuring IgM against whole cell Bb proteins.

Conventional CSF abnormalities pointed infrequently to CNS invasion by Bb: CSF culture for Bb (n = 1/16, 6%), intrathecal borrelial antibody production (n = 4/16, 25%) (Table III). IgM directed against Bb was frequent in both the serum and CSF of early Lyme disease patients, in contrast to late Lyme disease or OND subjects (Table I). IgM reactivity to OspC gave a much higher reading in CSF than in serum (Fig. 1). Antibody reactivity ranged as high as 30 times the reactive cutoff in CSF, compared to only 4 times the reactive cutoff in serum. These results suggest intrathecal production of specific OspC antibody. Immunoblot of serum confirmed IgM reactive against OspC in 11 early Lyme disease patients (Fig. 2).

We found that the majority of early Lyme disease patients had IgM to OspC in both CSF and serum. Early infection patients (7/12) also had CSF IgM to both OspA and OspB, which suggests coexpression in CSF of three major Bb Ags. Suggestive of different (CSF vs. peripheral) regulatory mechanisms governing either the expression of these Ags or at least the early IgM response to them are the data showing restriction to compartments. This occurred in five patients in whom IgM reactivity to OspA was limited to CSF, and in two patients in whom IgM reactivity to OspB was limited to CSF. An alternative possibility, for which we have no evidence here, is that some of these cases could have been caused by a neurotropic-restricted strain; however, the serum OspC reactivity would

suggest otherwise. IgM reactivity against recombinant OspC was detected in some patients with negative ELISA assays measuring IgM against whole cell Bb proteins. Collectively, these findings suggest that expression and regulatory factors for the spirochete may differ in the CSF compartment compared to peripheral tissue and blood. They also demonstrate that OspA and OspB may be expressed preferentially in the CSF compared to the peripheral system.

Although not as secluded as the CNS, the joints represent another compartment of the body where Bb may exhibit differential tissue expression of genes. One study found serum IgG to OspA and OspB associated with persistent Lyme arthritis in some humans (27); however, the immune response in the synovial fluid was not reported. Further investigation of gene expression and the immune response in the synovial compartment may provide insights into important pathogenic in vivo selective expression of Ags.

Detection of organism-specific IgM in CSF is useful for indicating CNS infection (28). IgM is a pentamer with a molecular weight of ~ 800,000. Very little of this large molecule normally leaks into CSF from serum, so CSF IgM levels are generally < 0.01% of serum IgM levels (29, 30). Typically, IgM is the earliest host antibody response to an invading organism, but does not persist once IgG is formed. The utility of documenting an early CSF IgM response to in vivo expressed Bb Ags as a marker of CNS infection has not been investigated previously in North American Lyme disease patients.

In our study, to assure specificity, we used Bb organism-specific recombinant proteins. These abundant Osp lipopro-

Table II. Compartmental Distribution of IgM Reactivity Among 12 Early Lyme Disease Patients with OspC Reactivity

Reactivity to	CSF alone	Serum alone	Both CSF and serum
Recombinant OspA	5	0	7
Recombinant OspB	2	0	7
Recombinant OspC	0	0	12

Table III. CSF Antibody Abnormalities

CSF study	Early LD patients (n = 16)	Late LD patients (n = 13)	OND patients (n = 19)
Intrathecal borrelial antibodies	4/14(29%)	3(23%)	0

LD, Lyme disease.

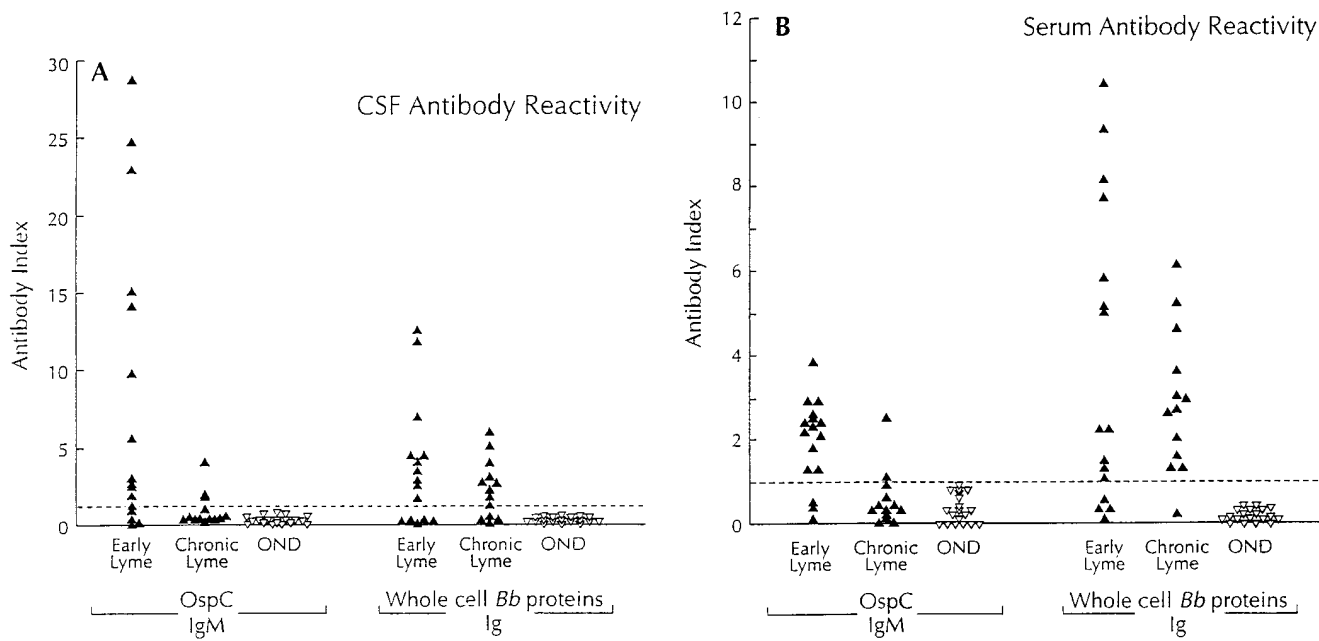


Figure 1. IgM reactivity to OspC, a Bb-specific protein, and the total Ig reactivity to whole cell Bb proteins are shown for paired CSF (A) and serum (B) from early Lyme disease ($n = 16$), late Lyme disease ($n = 13$), and OND patients ($n = 19$). Reactivity is expressed as the antibody index (sample OD/reactive cutoff), where an index of ≥ 1 is considered positive.

teins can be very immunogenic and have been implicated in immunoprotection in Lyme disease. OspA, a 31-kD protein, and OspB, a 34-kD protein, are cotranscribed and carried on a 49-kb linear plasmid of Bb (31). They share 53% sequence homology (32). OspC, a 23-kD protein, is transcribed from a 27-kb circular plasmid (33). OspC has provoked a very early and strong IgM response in serum (34–36).

Schwan et al. have provided evidence that in the feeding tick, OspA may be downregulated and OspC upregulated before spirochetal inoculation into the host (14). There is even data that expression of OspC by the organism is upregulated by the infected host (37). Our CSF data show that upregula-

tion of OspC does not necessarily occur in the face of downregulation of OspA within the CNS. This is supported to a certain degree by a study of Fikrig and colleagues which showed that such a relationship was not an all-or-nothing phenomenon, because one-third of the spirochetes inoculated in mice still expressed OspA in the peripheral system (15).

This study suggests that certain Bb Osp proteins can be simultaneously expressed and provoke a CSF IgM response. This may provide a new inroad for CSF diagnosis of early neurologic Lyme disease.

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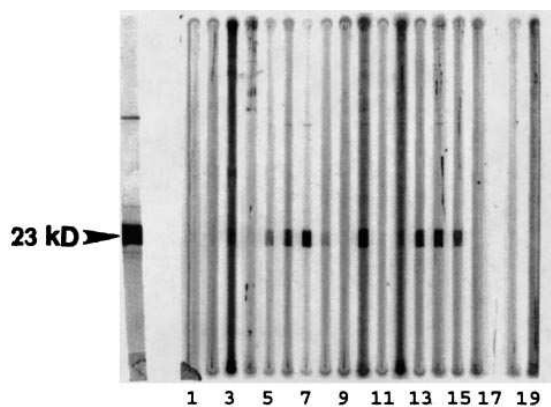


Figure 2. Recombinant OspC (10 μ g) was electrophoresed, transferred to nitrocellulose, and then probed with sera from the 16 early Lyme disease patients (lanes 1–16) and 2 negative control (lanes 18 and 19). A positive control lane (left) was probed with mAb to OspC. Staining of a 23-kD band was noted in 11 of 16 early Lyme disease patients (lanes 4 and 8 had faint but visible bands).

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