

# Different B-cell populations are responsible for the peripheral and intrathecal antibody production in neuroborreliosis

András Lakos<sup>1</sup>, Emőke Ferenczi<sup>2</sup>, Sámuel Komoly<sup>3,4</sup> and Marta Granström<sup>5</sup>

<sup>1</sup>Center for Tick-borne Diseases, Visegrádi 14, H-1132 Budapest, Hungary

<sup>2</sup>Department of Virology, Johan Béla National Center for Epidemiology, Gyáli u 2-6, H-1096 Budapest, Hungary

<sup>3</sup>National Center for Neuroimmunology, Jahn Ferenc Teaching Hospital, Köves u. 2-4, H-1204 Budapest, Hungary

<sup>4</sup>Department of Neurology, University of Pécs, Rét u. 2, 7623 Pécs, Hungary

<sup>5</sup>Department of Clinical Microbiology, Microbiology and Tumorbiology Center (MTC), Karolinska Hospital, SE-171 76 Stockholm, Sweden

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## Abstract

**The diagnosis of neuroborreliosis (NB)—a serious complication of Lyme disease—relies on demonstration of intrathecal borrelia antibody production. We hypothesized that if a qualitative difference between the cerebrospinal fluid and the serum immunoblot-banding patterns was observed, then the borrelia antibodies found in the CSF could not be the result of leakage of serum antibodies to the CSF due to blood–brain barrier damage, but rather had to be produced intrathecally. CSF/serum pairs from 69 NB patients and from 85 control patients with other neurological disorders were investigated. All samples were tested blindly by immunoblot and a commercial capture ELISA kit for NB. The concordance between the two methods was 85.7%. When using the other method as reference, the accuracy of the two assays in the two patient materials was similar: 80% for sensitivity and 95% for specificity. Four types of comparative immunoblot-banding patterns that reflected intrathecal borrelia antibody synthesis were distinguished. The study showed that a simple comparison between the immunoblot pattern of serum and CSF samples allows for a reliable diagnosis of NB by demonstration of intrathecal antibody production. This is the first study to show that a qualitative difference of the antibody response between the immune response of serum and CSF is a rule. The findings also imply that partly different B-cell populations are responsible for the antibody production in the blood and in the central nervous system. In addition, our observation provides possible implications for other infectious diseases with CNS involvement.**

## Introduction

Lyme disease is the most common tick-borne disease in Europe and North America. Three decades after the description of the disease, the laboratory confirmation of the infection remains controversial (1). There is, however, a consensus regarding the demonstration of intrathecal antibody production as the currently most reliable diagnostic tool for Lyme disease with neurological involvement (2–7). A difficulty in the diagnosis of neuroborreliosis (NB) is to differentiate between intrathecal antibody production, diagnostic for the disease and a mere leakage of antibodies from the serum to the cerebrospinal fluid, due to damage to the blood–brain barrier.

Since the discovery of the Lyme spirochete *Borrelia burgdorferi* (Bb) *sensu lato* in 1982, several techniques and

calculations have been developed to demonstrate specific intrathecal antibody production. Many studies rely on quantitative methods for comparison of the relative amount of specific antibody in CSF and serum or in comparison to a reference Ig, e.g. tetanus antitoxin. These types of calculation usually necessitate four measurements, e.g. total IgG in serum and CSF and specific anti-borrelia IgG in serum and CSF, use of arbitrary formulas, equations and/or mathematical functions validated by empirical data (8–10).

Immunoblot has become a widely used diagnostic method for Lyme disease. A two-tier system, combining a quantitative assay, e.g. ELISA, followed by an immunoblot testing, is recommended in several countries as the optimal diagnostic

approach (11). Immunoblot has also been extensively used in research and several authors have noted that, in some cases of NB, the immunoblot showed different banding patterns in CSF and serum (10, 12–15). No systematic studies have, however been performed to evaluate the importance of the observed difference in banding pattern.

The aim of the present study was to test the hypothesis that any difference between the immunoblot-banding pattern of CSF and serum in a pair would be diagnostic for intrathecal borrelia antibody synthesis, since leakage of antibodies to the CSF due to damage to the blood–brain barrier would result in a mirror of the serum-banding pattern. The hypothesis was tested in two separate sets of samples, with patient materials representing both NB cases and controls with other neurological disorders (ONDs) of importance for differential diagnosis.

## Methods

The study was approved by the Ethics Committee of the Hungarian Scientific Health Board.

### Subjects

We studied four groups of patients.

*NB patients selected on clinical symptoms and conventional immunoblot.* The patients, whose serum and CSF samples together with the detailed description of their illness had been sent to the Centre for Tick-borne Diseases, Budapest, were selected retrospectively. They all had had clinical symptoms strongly suggestive of NB and all fulfilled the case definition for NB (5). Their previous conventional immunoblot tests had been positive for Bb antibodies on serum and/or on CSF.

Twenty NB patients were included. The clinical diagnoses were: erythema migrans (EM) + lymphocytic meningitis (two patients), EM + lymphocytic meningoradiculitis + facial palsy (two patients), facial palsy + lymphocytic meningoradiculitis (nine patients) and chronic lymphocytic meningoradiculitis (>30 days; seven patients). For three patients, additional five consecutive serum/CSF pairs were available.

*NB patients identified by a commercial ELISA kit.* Forty-nine patients were retrospectively selected based on positive results in a commercial NB ELISA kit done at the Department of Clinical Microbiology, Karolinska Hospital, Stockholm, Sweden. Fifteen patients were lumbar punctured because of their headache and/or meningeal signs. Facial palsy was the main clinical symptom in six patients. Migrating radicular symptoms were revealed in six other patients. Two patients had also been diagnosed having NB 12 and 24 months previously to the present sampling, respectively. One patient had been treated for Lyme borreliosis 1 month prior to the current lumbar puncture, performed because of worsening symptoms. No clinical data were provided for 19 patients.

*Tick-borne encephalitis patients.* Samples from 48 serologically confirmed tick-borne encephalitis (TBE) cases, 24 patients collected in Hungary (at the Department of Virology, National Center for Epidemiology, Budapest) and 24 from

Sweden (sent for routine diagnostics to the Department of Clinical Microbiology, Karolinska Hospital) were retrospectively selected as controls. The patients were diagnosed by positive serum IgG and IgM in indirect fluorescent antibody assay, using Vero cells infected with TBE virus (16). Demonstration of IgM was performed in the IgM-rich fraction of serum, extracted by an ion-exchange chromatographic separation of the serum (17). FITC-conjugated polyclonal rabbit antibodies (DakoCytomation, Denmark) were used as second antibodies. The TBE patients were diagnosed in Sweden by positive IgM reaction in serum and/or CSF, using the commercial kits Immunozytm™ FSME IgM and IgG (Progen Biotechnik, Heidelberg, Germany).

*ONDs.* A second control group consisted of 37 patients. Five patients with neurosyphilis based on clinical symptoms and a positive serum antibody reaction in Serodia™-TP-PA, a commercial kit from Fugirebio (Breda, The Netherlands). Thirty-two patients (collected at the Jahn Ferenc Hospital) were selected at the Centre for Tick-borne Diseases based on clinical symptoms and conventional immunoblot borrelia serology—10 patients with headache, 8 patients with multiple sclerosis, 1 patient with neurosyphilis, four patients with polyneuropathy, two patients with fever of unknown origin and one case of each of the following: lymphocytic meningitis caused by enterovirus infection, paresis of nervi abducentis, myelitis, stroke, amyotrophic lateral sclerosis (ALS), migraine and acrodermatitis chronica atrophicans (ACA).

Four of the patients were selected because of high positive serum borrelia IgG antibody level in previous tests, but with typical signs of MS, ALS, migraine or ACA. Three of these latter four patients (with MS, ALS and migraine) were orienteers, regular tourists and country cottage owners, respectively, with numerous tick bites every year. The ACA patient was lumbar punctured because of polyneuropathy. The reasons for this patient to be included in this group, in spite of her peripheral neuritis possibly being caused by the chronic Lyme borreliosis, were that (i) ACA results in a very high serum IgG antibody level, but (ii) intrathecal antibody production has never been described in this condition. A syphilitic patient was selected because of moderate serum IgG reaction to Bb in previous ELISA and immunoblot tests. Paired serum and CSF samples were collected on the same day from all patients. CSF and serum samplings were done as routine diagnostic procedures in all cases. Samples were stored at –25°C until evaluation.

### Immunoblot

*Borrelia afzelii* (ACA1) were cultivated in Barbour-Stoenner-Kelly medium at 30 °C. The cultured cells were sonicated and solubilized and the proteins were separated by electrophoresis in 12% polyacrylamide minigels. The protein concentration in the gel was ~0.8 µg/cm<sup>2</sup>. Protein was transferred to polyvinylidene difluoride membrane (Immobilon P, pore size 0.45 µ—Millipore Corporation, Bedford, MA, USA) by semi-dry blotting. Instead of cutting the membrane into strips, a home-made frame was used for separating the samples. The purpose of the frame was to separate the test samples in such a way that each well could be filled with 1 ml liquid. Each

membrane yielded 26 tests. Pairs of samples, i.e. serum and the corresponding CSF drawn at the same visit, were filled into the neighboring wells. Each well contained 0.5 ml of diluted sample. Serum and CSF samples were diluted in 1:100 or 1:4, respectively, and incubated for 30 min at room temperature on a shaker. After washing, the membrane was incubated with alkaline phosphatase-conjugated anti-human IgG or IgM mAb (Sigma Chemical Company, St Louis, MO, USA) for 30 min at room temperature. The reaction was developed by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma Chemical Company).

Our immunoblot criteria, used to select the Hungarian NB patients, have previously been evaluated in 100 samples of each of the following three clinically defined groups: EM, lymphocytic meningoradiculitis and ACA for Lyme patients and three separate control groups; infants, healthy blood donors and patients with uveitis, caused by several types of autoimmune diseases (18). These bands investigated were antibodies against the 22 and 41 kD proteins in IgM, and the 22, 29, 35, 44, 47, 49 and 93 kD bands in IgG. The specificity of our test was 99% in infants, 97% in healthy blood donors and 92% in uveitis patients. The sensitivity of the test was 48% in EM, 75% in lymphocytic meningoradiculitis and 100% in the ACA group (18).

The sample pairs of the four groups of patients, i.e. two NB groups, TBE and OND, were randomly allocated to the blot membranes. The immunoblots were evaluated blindly by one of the authors (A.L.).

*Diagnostic criteria of the comparative immunoblot assay.* Intrathecal antibody production was considered to be positive if the immunoblot pattern observed clearly differed between the serum and the CSF sample in a pair. The difference could consist of bands present in CSF but not in serum or, conversely, bands that were present in serum were lacking in CSF, while at least one of the corresponding bands in the CSF was equally or more intense than in the serum. In addition to these differences, the intensity of the bands were also used as diagnostic criterion, if the difference was so disproportionate that a theoretical dilution or concentration of either the samples could not result in the same pattern as the other sample of the pair. In this test, all bands were evaluated for differences between the CSF and the serum-banding patterns, irrespective of any assumed specificity of the bands.

#### Capture ELISA by a commercial kit for NB

In the capture ELISA, the wells are coated with anti-human IgG or IgM, extracting the corresponding antibody class from the added test sample. In a next step, the enzyme-labeled antigen, in this case the flagellar antigen of *B. burgdorferi sensu lato*, binds to any specific antibody extracted from the test sample. Hence, the result will directly show the proportion of the specific and total IgG or IgM within a delimited proportion of the titration curve. A commercial kit, IDEIA™ Lyme Neuroborreliosis, (DakoCytomation Ltd, Ely, UK) was used for the capture ELISA at the Karolinska Hospital, Stockholm, following the instructions of the manufacturer (DakoCytomation Ltd). The assay is based on the demonstration of intrathecal antibody production by parallel testing of a serum and a CSF sample drawn on the same day. All samples were tested blindly.

#### Statistical analysis

Fisher's exact test was used for statistical evaluation of different banding patterns.

#### Results

There was an 85.7% overall agreement between the results of the two assays for intrathecal antibody production, comparative immunoblot assay and the commercial NB kit (Table 1). When using the other method as reference, the sensitivity of the two assays in the two patient groups (Hungarian and Swedish) was similar, i.e. ~80%. The specificity in the TBE controls was 94% for the immunoblot and 100% for the NB kit. In the group of patients with ONDs, the specificity of the immunoblot was 97% and that of the NB kit was 89%.

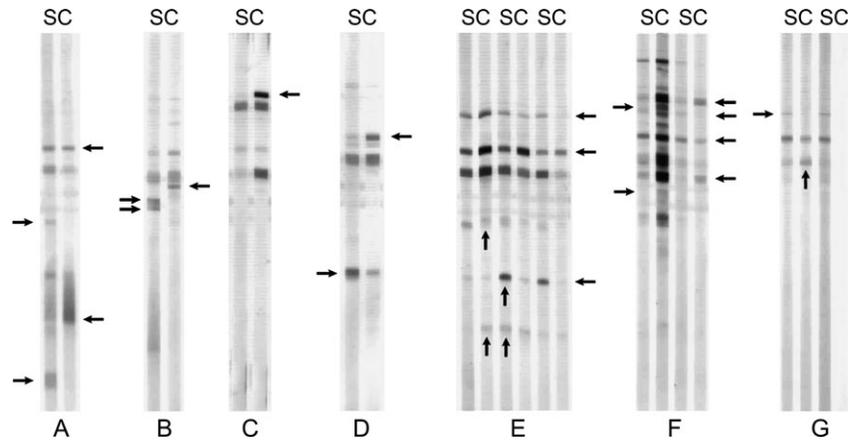
We distinguished four types of banding patterns indicative for intrathecal antibody production in the immunoblot:

- (i) Serum immunoblot showed one or more bands that were missing from the CSF, while some of the bands in the CSF were similar or even more intense than the corresponding bands in the serum sample. This type of banding pattern was seen in 21 tests, 8 in IgM and 13 in IgG (Fig. 1A).
- (ii) Serum immunoblot revealed bands that were missing from the CSF, while the CSF immunoblot showed bands that were missing from the serum. This type of banding pattern was seen in 23 tests, 8 in IgM and 15 in IgG (Fig. 1B).

**Table 1.** Concordance of the comparative immunoblot assay and the NB kit

	Hungarian NB patients ( <i>n</i> = 20)	Swedish NB patients ( <i>n</i> = 49)	TBE patients ( <i>n</i> = 48)	OND patients ( <i>n</i> = 37)
COMPASS IgM	10	34	0	0
COMPASS IgG	20	29	3	1
COMPASS all	20	39	3	1
NB kit <sup>a</sup> IgM	12	38	0	1
NB kit IgG	13	42	0	3
NB kit all	16	49	0	4
Concordance	16/20 (80%)	39/49 (79.6%)	45/48 (93.7%)	32/37 (85.1%)

Number of intrathecal borrelia antibody (IgM and IgG) producing patients tested by the comparative immunoblot assay (COMPASS) and the NB kit in the groups of patients. <sup>a</sup>IDEIA™ Lyme Neuroborreliosis kit (Dako).



**Fig. 1.** Different patterns of intrathecal antibody production by comparative immunoblot in patients with NB (S = serum, C = CSF). Each letter (A–G) below the lanes represents samples from one patient. The 'D' pair of lane presents IgM and the others IgG reactions. (A) The left arrows show bands in the serum sample not present in the CSF, while the right arrows point to bands equally or more intense in the CSF than the serum. This banding pattern was the most common, listed as category (i) in the Results. (B) The left arrows show the bands in the serum sample not present in the CSF sample, right arrow points to a band in the CSF missing from serum. This banding pattern corresponds to category (ii) in the Results. (C) The arrow shows a band in the CSF that is missing from the serum. This banding pattern corresponds to category (iii) in the Results. (D) Almost the same banding patterns are seen in the serum and the CSF, but there is one band that is more intense in the serum than in the CSF (arrows). This banding pattern was the least common, listed category (iv) in the Results. (E) Three pairs from the same patient drawn at different time points. The first pair of samples presents the banding pattern pre-treatment. All but one (upper vertical arrow) bands are more intense in the CSF, and there is a band in the CSF (lower vertical arrow) that is missing from the serum ('C'-type). The second pair was drawn 4 months after treatment. The vertical arrows point to the bands representing the serological progression ('D'-type). The third pair was drawn 8 months after treatment. Intrathecal antibody production is still visible. The right lower arrow shows one band that is more intense in the serum sample, while there is a band in the CSF that is at least equally strong as the corresponding band in the serum (right middle arrow). There is a band in the serum missing from the CSF (right upper arrow, 'A'-type). (F) The left arrows show the bands that are missing from the serum but present in the CSF (C-type). The right arrows show the difference between the band pattern of the CSF and serum drawn 1 year after treatment (A-type). (G) The arrow on the left shows a serum band missing from the CSF, while the vertical arrow shows the band that is stronger in the CSF than the corresponding band in the serum (A-type). The convalescent CSF sample shows no bands, indicating an absence of intrathecal antibody production.

- (iii) CSF immunoblot showed bands that were missing from the serum (Fig. 1C). This was the most frequent banding pattern, seen in 36 tests, 19 in IgM and 17 in IgG.
- (iv) Serum and CSF immunoblot showed the same pattern but the intensity of the corresponding bands was disproportionate (Fig. 1D). This was the least frequent observation, noted in 17 tests, nine in IgM and eight in IgG.

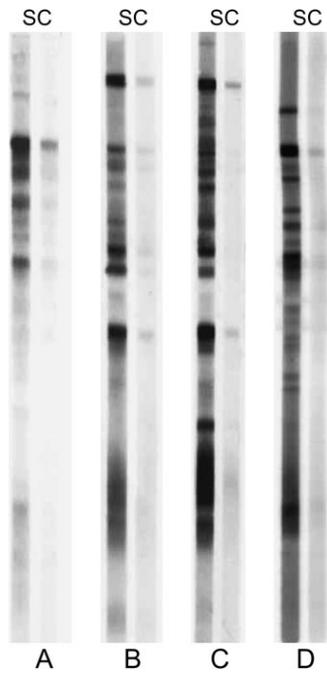
No statistically significant differences between the four types were found by antibody class, clinical symptoms or duration of the symptoms before sampling.

Among the three patients with follow-up samples, one patient suffered from a chronic encephalomyelitis. This patient's CSF showed one band more in comparison to the corresponding serum in the first pair. In the first follow-up sample, 4 months after the ceftriaxone treatment, this band also appeared in the serum. While the serum showed serological progression, there was a minimal improvement in CSF. Eight months after treatment, there were still clear signs of intrathecal borrelia IgG (Fig. 1E) and IgM antibody production by immunoblot. Three years after the first symptoms, the patient still has ataxia and low motorneuron symptoms. In the case of the second patient with available follow-up samples, the ceftriaxone treatment was initiated 53 days after debut of bilateral facial palsy accompanied by lymphocytic meningitis. The first pair showed some IgM and IgG bands in CSF missing from the serum. Most of the CSF and serum bands disappeared during the follow-up but intrathecal antibody synthesis was

still evident 1 year after treatment (Fig. 1F). The patient became symptom-free only 5 months after completing the antibiotic treatment. The third patient was treated on the third day after onset of facial palsy accompanied by lymphocytic meningitis. By the next sampling 1 month later, the bands had disappeared from the CSF and the bands in the serum had weakened (Fig. 1G). Clinical symptoms resolved in a week.

The four patients in the group of ONDs who were selected because of the high serum antibodies for borrelia were unequivocally negative for intrathecal antibody production with both tests, in spite of some faint bands also seen in the CSF. These bands in the CSF mirrored exactly the bands in the serum, indicating that their appearance in the CSF was due to leakage through the blood–brain barrier. (Fig. 2)

Discrepant results between the comparative immunoblot and the NB kit were seen in a total of 22 patients. Ten NB patients selected by the NB kit were negative by the comparative immunoblot. For five of them no clinical data were available. Two other patients had had NB 1 and 2 years, respectively, before the current sampling. Three patients had meningitis with an onset 2–11 days prior to sampling. All four patients who were positive by comparative immunoblot but negative by the NB kit had lymphocytic meningoradiculitis. One of them also had EM, while another also had a facial palsy. Among these four patients, symptoms started 7, 39, 40 and 44 days prior to sampling, respectively. In the control group with ONDs, the NB kit identified four positives that were negative by the comparative immunoblot. CSF showed no



**Fig. 2.** Immunoblots of serum/CSF pairs from patients with ONDs, selected because of high serum borrelia antibody levels. Faint bands are seen in all CSF blots but these bands mirror the banding pattern of the serum. According to the evaluation criteria of the comparative immunoblot, none of these four patients had intrathecal borrelia antibody production and the antibodies in the CSF thus originated from the serum by leakage through the blood–brain barrier. All four sample pairs were also negative by the capture ELISA NB kit. (A) ALS, (B) migraine, (C) multiple sclerosis and (D) ACA. All four patients had chronic borrelia infection without central nervous involvement.

elevation of protein and cell number in any of these four patients. Three of them revealed IgG reaction by the NB kit and had been lumbar punctured because of headache. One of these three had had EM 2 years before sampling. The fourth patient only had a positive reaction in IgM by the NB kit, but the onset of her polyneuropathy occurred 3 years prior to sampling. The patient in the OND group who was positive in the comparative immunoblot had neurosyphilis.

## Discussion

The aim of the present study was to investigate the observed qualitative difference in banding pattern when testing in parallel serum and CSF pairs from patients with a clinical and/or laboratory diagnosis of NB. The comparative immunoblot and a commercial NB kit gave concordant results in 85.7% of cases. The specificity of both tests was 95%. The relatively low sensitivity, i.e. 80% for both tests, could be due to the difficulties in case ascertainment.

A weakness in any study of diagnostic methods in Lyme borreliosis is the lack of a 'gold standard' for case ascertainment. In most other bacterial infections, culture of the pathogen is the gold standard but culture of the spirochete in Lyme disease is difficult and rare, especially from the CSF. Early studies of diagnostic methods relied on clinical diagnosis

as reference method (10, 19, 20) while later studies usually compare the performance of a new method, e.g. capture ELISA, immunoblot using monoclonal antigens or DotBlot, with that of diagnostic methods in current use (21–23). In the present study, we used both approaches for patient selection and tested both patient materials by both methods.

In some previous studies, where immunoblot pictures were published, intrathecal antibody synthesis was clearly visible, but the authors did not seem to have recognized the diagnostic implications of the observation (13, 24). Others have discovered the qualitative difference between CSF and serum-banding patterns in NB and other neuroinfections, but this finding was thought to be a rare event (12, 25). In previous studies, the criterion for intrathecal borrelia antibody production was that the amount (and/or the number of the bands) of specific borrelia antibodies in the CSF must exceed that in the serum when the total IgG is equalized by appropriate dilutions (10, 12, 14, 26, 27). This method—especially when the sample dilution is high—may however result in undiagnosed cases (28).

The comparative immunoblot is not influenced by the total amount of Ig's and needs therefore no adjustment of the sample dilution to the total IgG as in some previous immunoblot studies. Also, only two tests are needed, one for serum and one for CSF. Immunoblot is also simpler than isoelectric focusing and other methods that have previously been used to demonstrate intrathecal antibody production. The introduction of capture ELISA made the analysis easier as the specific and the total antibody proportions may directly be measured, but it also needs arbitrary mathematical formulas for determination of the cut-off level (2, 4). Moreover the capture ELISA is less reliable in higher or lower antibody ranges. In comparison to the capture ELISA, a drawback of the immunoblot is the subjective reading. Due to the comparative nature of the evaluation, the problem may however be less pronounced in this case.

A major issue in the diagnosis of NB is to distinguish between the leakage of borrelia-specific antibodies from the serum to the CSF and genuine intrathecal antibody synthesis. In particular, the severe encephalitis caused by the TBE virus, transmitted by the same vector as Lyme disease, may cause leakage of antibodies from the serum to CSF. Since Lyme disease is some 100 times more common than TBE (29), and affects people with the same outdoors type of activities, it is not unlikely that TBE patients have suffered a previous borrelia infection with remaining, high serum antibody concentrations. In this control group, the immunoblot identified three patients as having NB but who were negative by the NB kit. Although these cases are likely to represent false positives in the immunoblot, double infections cannot be entirely ruled out without follow-up samples. In the control group with ONDs, the NB kit identified four positives that were negative by the comparative immunoblot.

Due to the inherent difficulty caused by the lack of a gold standard reference method in NB and/or a study design with long-term follow-up samples, it remains however difficult to conclude whether one of the assays lacked sensitivity or the other specificity. In the three patients with follow-up samples, the comparative immunoblot indicated a rapid decrease in intrathecal antibody production. A significant decline in the NB

kit was noted in only one out of the three patients. This drawback of the capture ELISA used in the NB kit was already noted in the original description of the method. Hansen *et al.* stated that since intrathecal antibody synthesis persists for a lengthy period of time, measurement of Bb-specific intrathecal antibody synthesis is not suitable as a follow-up procedure (20). In their study, the specific IgG index decreased to the normal level during the 3–10 months follow-up in only one out of 10 patients. Wilske *et al.* found that CSF and serum antibody levels declined at apparently the same rate, and therefore the ratio of specific anti-borrelia to total IgG remained unchanged after treatment (10).

The slow decrease in the specific IgG index of the NB kit may also in part explain an apparent lack of sensitivity of the comparative immunoblot. Three of the Swedish NB patients, positive in the kit but negative by the immunoblot, were diagnosed based on the NB kit as having an NB. It was only when going back to the referral sheets that the information that the patients had suffered a previous NB, 1 month, 1 year and 2 years, respectively, prior to the current sampling was noted, thus indicating a likely false-positive diagnosis for a current disease in at least some of these cases. Although the number of patients investigated was limited, the comparative immunoblot seems to be promising for follow-up of treatment in NB patients.

The observations in the present study seem to support the conclusions of Kaiser and Lucking (14) on the nature of antibody response in the CSF as compared with serum. They stated that 'The autochthonous antibody response in the CSF might be directed against other antigens of the infectious agent than the serum response. In this case the western blot would detect an autochthonous immune reaction, even when the relative amounts of specific IgG antibodies in the CSF and serum are very similar and calculation methods fail to indicate specific intrathecal synthesis'. Our study is the first to show that a qualitative difference of the antibody response between the immune response of serum and CSF is a rule. If this phenomenon has general validity then a comparative immunoblot could be of value in other infection with neurological involvement where an immunoblot is available.

In conclusion, the study has provided interesting insights into the immune response in the CSF. The findings imply that non-identical B-cell populations are responsible for the antibody production in the blood and the central nervous system. In addition, the observation could be translated into a sensitive and specific tool for the diagnosis of NB, and with possible implications for other infectious diseases with CNS involvement.

## Abbreviations

ACA	acrodermatitis chronica atrophicans
ALS	amyotrophic lateral sclerosis
Bb	<i>Borrelia burgdorferi</i>
CNS	central nervous system
CSF	cerebrospinal fluid
EM	erythema migrans
MS	multiple sclerosis
NB	neuroborreliosis
OND	other neurological disorder
TBE	tick-borne encephalitis

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