Clinical, diagnostic and immunological characteristics of patients with possible neuroborreliosis without intrathecal Ig-synthesis against Borrelia antigen in the cerebrospinal fluid

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Abstract

The diagnosis of neuroborreliosis is not always straightforward. Intrathecal immunoglobulin (Ig) synthesis against Borrelia antigen may not be detected, at least early in the disease course. Also other neurological and infectious diagnoses have to be considered. We have studied patients with clinical possible neuroborreliosis *without* intrathecal Ig synthesis against Borrelia antigen in the cerebrospinal fluid (CSF) (n=17). Diagnosis was based on typical clinical history and at least one of the following findings; mononuclear leucocytosis in the CSF (n=4); typical erythema migrans >5 cm in diameter in relation to debut of symptoms (n=8); prompt clinical response to antibiotic teratment (n=14). Also other possible diagnoses had to be excluded. Seventeen patients first investigated because of suspected neuroborreliosis but later confirmed with other diagnoses were used as controls. All patients had a lumbar puncture. Borrelia specific IFN-γ and IL-4 secretion was investigated in peripheral blood (PBL) and CSF with an ELISPOT assay. Polymerase chain reaction (PCR) was used to reveal any Borrelia antigen in the CSF. Six of 17 patients with possible neuroborreliosis showed high IFN-γ secretion in peripheral blood, otherwise we found no statistically significant differences between the groups. PCR did not reveal any Borrelia antigen in CSF. The diagnosis and treatment of possible but not confirmed neuroborreliosis is a clinical challenge. The clinical response to treatment may be the best option in these cases.

Introduction

Borrelia can affect many organ systems, i.e. skin, heart, joints and the nervous system.[1] Clinical symptoms are variable and depend on the subtype of Borrelia. Borrelia burgdorferi sensu latu typically cause arthritis. Borrelia garinii mainly causes neurological symptoms[2] whereas Borrelia afzelii give rise to the erythema chronicum migrans, common for all three subtypes.

The neurological symptoms are not specific for neuroborreliosis but may occur in many other neurological diseases.[3] This makes the clinical diagnosis difficult unless there is a preceding erythema migrans. Also laboratory parameters to diagnose neuroborrelia are uncertain because of a late and inconsistent occurrence of antibodies against Borrelia. Intrathecal synthesis of Borrelia antibodies is of diagnostic importance[4] and so is mononuclear leucocytosis in the cerebrospinal fluid (CSF) and signs of blood-brain barrier damage.[5] Polymerase chain reaction (PCR) has been utilised in several studies but is not routinely recommended due to the limited clinical sensitivity of this method.[6,7] When the diagnosis of neuroborreliosis is confirmed or highly likely treatment with antibiotics is initiated. Usually neurological symptoms are reduced and eventually disappear. Some patients, however, experience reappearing or persistent neurological symptoms some time after the treatment.[1] It is not clear if these late-symptoms are related to persistent symptoms of an Borrelia infection or if they are caused by other neuroimmunological reactions or both. Persistent symptoms may also be due to irreversible damage of the nervous system. The purpose of our study was to investigate patients with clinical possible neuroborreliosis but lacking intrathecal synthesis of antibodies against Borrelia antigen. In clinical practice there is a need for other methods to discriminate true neuroborreliosis patients from other with mimicking symptoms and laboratory parameters. We aimed at looking for levels of the type 1 cytokine IFN-γ and the type 2 cytokine IL-4 in CSF and blood.

Materials and Methods

Patients and controls

Clinical and laboratory supported definite neuroborreliosis[8] is characterized as; typical clinical neurological symptoms (meningitis, cranial neuritis, or radiculitis), CSF mononuclear leucocytosis and intrathecal synthesis of Borrelia specific antibodies (Daco kit for detection of anti-flagellin antibodies in serum and CSF was used).[9] We identified a subgroup of patients with typical neurological symptoms without intrathecal synthesis of Borrelia specific antibodies (n=17, 9 women and 8 men, mean age 50.5 years) but with intrathecal leucocytosis (n=4), erythema migrans in relation to symptoms (n=8) and/or response to antibiotic treatment (n=14), referred to as possible neuroborreliosis. Eleven of the 17 patients with possible neuroborreliosis, however, showed positive serology for IgG and/or IgM against Borrelia in serum. Seventeen patients first investigated because of suspected neuroborreliosis but later confirmed with other diagnoses were used as controls (8 women and 9 men, mean age 44.5 years). Characteristics of the patients are shown in Table 1.

Preparation of mononuclear cells from blood and cerebrospinal fluid

Blood (heparinized) and CSF samples were obtained from patients with suspected NB. Peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation on Lymphoprep® (Medinor AB, Stockholm, Sweden) at 400 g for 30 minutes in room temperature, according to Bøyum.[10] Cells were counted under a phase-contrast microscopy using a Bürker chamber, and the lymphocyte concentration was adjusted to 1×10^6/mL. CSF-cells were counted using phase-contrast

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Table 1. Characterization of patients with possible neuroborreliosis without intrathecal Ig-synthesis against Borrelia antigen in the cerebrospinal fluid and controls with other diagnosesPat.

No.	Diagnosis	Sex	Age years	Tick bite	EM	Duration of symp-toms (days)	Borrelia serology[a] IgG	Borrelia serology[a] IgM	CSF MNC[b]	Sp/s alb ratio[c]	Intra-thecal total IgG-synthesis[d]	Symptoms	Antibiotic therapy[e]	Clinical response to antibiotic therapy
1	NB	F	66	-	-	43	+	-	+	N	+	Paresthesia, dysesthesia	Tetracycline	+
2	NB	F	51	+	+	17	+	+	-	N	-	Headache, numbness, arthralgia, fatigue	Tetracycline	+
3	NB	M	43	+	+	120	-	-	-	N	-	Fatigue, headache, paresthesia	Penicillin-V	+
4	NB	F	48	+	+	30	+	-	-	+	-	Headache, radiculitis, vertigo, double-vision	Ceftriaxone	+
5	NB	F	30	+	+	30	+	-	-	+	ND	Headache, fatigue	Tetracycline	-
6	NB	M	78	-	+	45	-	-	-	+	-	Numbness in the leg	tetracycline	+
7	NB	M	65	+	-	45	-	-	+	N	+	Fever, paresthesia, headache	Tetracycline	+
8	NB	M	34	+	+	60	-	+	-	+	-	Fatigue, vertigo, headache	Penicillin-V + tetracycline	+
9	NB	F	65	+	+	14	-	-	-	+	-	Facial paresis, neck pain	Teracycline	+
10	NB	M	58	-	-	270	+	-	-	N	-	Fatigue, vertigo, leg weakness, radiculitis	Tetracycline	+
11	NB	F	42	-	-	60	+	+	-	+	+	Facial paresis, numbness in half side of the face	Tetracycline	+
12	NB	F	55	-	-	30	+	-	-	N	-	Facial paresis, radiculitis	Tetracycline	+
13	NB	F	40	+	+	6	+	-	-	N	-	Myalgia, fatigue, headache	Penicillin-V	-
14	NB	M	68	+	-	14	-	-	+	N	-	Facial paresis, neck pain	Tetracycline	+
15	NB	M	38	+	-	30	+	-	-	+	-	Facial paresis, fatigue	Tetracycline	+
16	NB	F	47	+	-	360	-	-	-	N	-	Sensory disturbance in the foot	Ceftriaxone	+
17	NB	M	30	-	-	10 years	+	-	+	N	+	Paresthesia, numbness	-	
18	MS	M	40	-	-	4	-	-	-	+	+	Arm weakness	-	
19	MS	F	24	-	-	60	-	-	-	N	-	Facial paresis, numbness in the feet	-	
20	Facial palsy	M	52	+	-	6	-	-	-	N	-	Facial paresis, vertigo	-	
21	Facial palsy	F	57	-	-	7	-	-	-	N	-	Facial paresis	-	
22	Facial palsy	F	51	+	-	60	-	-	-	N	-	Facial paresis. fatigue	-	
23	Fatigue	M	37	+	-	150	-	+	-	+	-	Fatigue, concentration problem	-	
24	Fatigue	M	36	+	-	180	+	+	-	N	-	Fatigue, paresthesia	Tetracycline	-
25	Myalgia	F	48	+	-	70	-	+	-	N	-	Migrating muscle pain, low back pain	-	
26	Headache	F	56	-	-	240	-	+	-	N	-	Headache, vertigo, paresthesia, fatigue	-	
27	Vestibular neuritis	F	69	-	-	2.5 years	+	-	-	N	ND	vertigo	-	
28	Collagenosis	M	53	-	-	67	-	+	-	N	-	Fatigue, myalgia, arthralgia	Tetracycline	-
29	Meningitis	M	53	+	-	90	-	-	+	+	-	Facial paresis, vertigo, eye pain, nystagmus	-	
30	Paresthesia	F	26	-	-	60	-	+	-	N	-	Transient numbness in one arm	-	
31	Comotio medullae spinalis	M	29	-	-	60	-	-	-	+	-	Paresthesia in both hands	-	
32	Compression of thoracal vertebrae	M	35	+	-	120	+	-	-	N	-	Paresthesia and transient weakness of one leg	-	
33	Cerebral infarction	F	45	-	-	14	-	-	+	N	-	Headache, paresthesia, dysarthria	Ceftriaxone	-
34	Panic syndrome	M	46	-	-	20	-	-	-	N	-	Vertigo	-	

MS, multiple sclerosis; EM, erythema migrans in relation to symptoms; N, normal; ND, not done; F, female, M, male. Duration of symptoms in days if not stated otherwise. [a]+= patients positive in the Borrelia ELISA for IgG or IgM in blood. [b]Mononuclear leucocytosis in the cerebrospinal fluid ≥5×10⁶/L. [c]Spinal/serum albumin ratio (Sp/s alb ratio) (+=elevated). [d]+=IgG index ≥0.7. [e]Tetracycline was given as an oral dose of 200 mg daily for 14 days; penicillin-V 2 gr orally daily for 10 days; ceftriaxone 2 gr intravenously daily for 14 days.

microscopy, by use of a Jessen chamber, before undergoing 10 minutes of centrifugation at 200 g at 4ºC, followed by gentle resuspension in cell culture medium (TCM).

Preparation of outer surface protein fraction *Borrelia* antigen

An outer surface protein (osp) enriched fraction (OF), mainly containing OspA and OspB, was prepared from of *Borrelia garinii* strain Ip90, as previously described.[11-13] OF stimulation of PBMC and CSF-MNC was shown in adults to discriminate between patients with neuroborreliosis and patients with other neurological diseases as well as healthy controls in the IFN γ and IL 4-ELISPOT-assay.[14,15]

ELISPOT for analysis of IL-4 and IFN-γ producing cells

The ELISPOT assay, which was originally described by Czerkinsky *et al.*,[16] was used for analysis of Borrelia (OF)-stimulated and unstimulated cytokine secreting cells. The method was slightly modified and optimized for IL-4 and IFN-γ spots as described previously.[11-13] The spots were counted under a dissection microscope. One spot resembles one cytokine secreting cell.

DNA extraction and polymerase chain reaction amplification

A QIAamp tissue kit (Qiagen) was used for DNA extraction according to the protocol of the manufacturer. Samples were eluted with 50 μL of AE buffer and stored in – 20°C. For detection of Borrelia antigen the 16S rRNA sequence was amplified by a nested PCR. Primers16S-F and 16S-R and LD1 and LD2 were used in the amplification. The products were visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. A negative control and a positive control were included in all PCR runs. The method is previously described by us.[17]

Data handling and statistics

The mean of triplicates or duplicates was used in the analysis of cytokine results. To determine *Borrelia specific secretion*, the number of spots in the unstimulated wells was subtracted from the number in the OF-stimulated wells. For CSF-cells, data were recalculated to the number of spots/100 000 lymphocytes. For comparison of cytokine secreting cell counts between multiple groups or intervals, Kruskal-Wallis was used as a pre-test, and Mann-Whitney U test as *post-hoc*. P<0.05 were considered significant.

The present study was approved by the ethics committee of the Faculty of Health Sciences at Linköping University (Linköping, Sweden). Informed consent was obtained from each of the patients included in the study.

Results

Six of 17 patients with possible neuroborreliosis had the highest IFN-γ secretion in peripheral blood upon Borrelia antigen stimulation. The median values between the groups were not statistically significant, however. We found no differences in IL-4 or IFN-γ secretion in the CSF (Table 2 and Figure 1). PCR did not reveal any Borrelia antigen in CSF.

Borrelia serology in blood seems unspecific and could mirror an earlier infection or in case of IgM antibodies be an unspecific reaction (10 had IgG and 3 IgM of the neuroborreliosis patients and 3 had IgG and 6 IgM of the controls). Also mononuclear leucocytosis in the CSF, blood-brain barrier damage, and intrathecal immunoglobulin synthesis were seen in both possible neuroborreliosis patients and controls with other diagnoses. In the control group mononuclear leucocytosis in the CSF was seen in one patient with viral meningitis and one with cerebral infarction; blood-brain barrier damage in one patient with multiple sclerosis (MS), fatigue, viral meningitis and comotio medullae spinalis, respectively; intrathecal immunoglobulin synthesis in one MS patient.

Symptoms are often non-specific such myalgia, paresthesia, fatigue and headache and were reported equally in the possible neuroborreliosis patients and controls with other diagnoses. In the control group 3 patients with facial palsy (Bells palsy) and 3 with unspecific fatigue or myalgia and 1 with transient paresthesia and 1 with tension headache without preceding erythema migrans or mononuclear leucocytosis in CSF were included. Otherwise the diagnoses in the control group were diverse but specific (Table 1).

The most reliable clinical feature of a possible neuroborreliosis was a preceding erythema migrans in 8 out of 17 patients in contrast to none of the 17 controls. Tick bite did not discriminate between the groups.

Clinical response to antibiotic treatment was seen in 14 out of 16 treated patients with possible neuroborreliosis (12 were treated with tetracycline 200 mg orally for 14 days; 2 with ceftriaxone 2gr iv for 14 days; 3 with penicillin-V 2 gr orally for 10 days, one of which also treated with tetracycline) and could be taken as an indication of at least an infectious etiology. Patient no 5 and 13 in table 1 did not respond to treatment. The first patient had persistent headache and fatigue after tetracycline treatment and the second patient was treated early after debut of symptoms (6 days) and received only penicillin-V orally. In the control group 3 patients were treated on the early suspicion of neuroborreliosis (2 with tetracycline and one with ceftriaxone) but later confirmed with other diagnoses.

Patient no 10, 12, 15, and 16 were judged as possible neuroborreliosis (Table 1) because of typical neurological symptoms (2 radiculitis, 1 facial palsy and one with a radicular distribution of sensory disturbance in the foot) and prompt response to antibiotic treatment. These 4 patients, however, did not report any erythema migrans or had any CSF findings (except one with a non-specific elevated spinal/serum albumin-ratio).

One patient (no 17) judged as possible neuroborreliosis was not treated because of long-lasting symptoms (10 years). These patients are referred to as post-Lyme disease syndrome (PLDS) and therapy has no clear impact of the symptoms. Three of our patients with possible neuroborreliosis had their symptoms for more than 6 months, often classified as late neuroborreliosis,[18] but the majority of our patients had early possible neuroborreliosis.

Discussion

The most common diagnostic tool to the diagnosis of neuroborreliosis is ELISA analysis for antibodies against the Borrelia antigen. We

Table 2. Net secretion of IFN-γ and IL-4 spots in patients with possible neuroborreliosis and patients with other diagnoses.

	Diagnosis	N	Mean rank	P[a]
Net IFN-γ in PBL Spots/100 000 cells	Neuroborreliosis	15	16.7	0.12
	Other diagnosis	13	11.9	
	Total	28		
Net IL-4 in PBL Spots/100 000 cells	Neuroborreliosis	15	15.9	0.33
	Other diagnosis	13	12.9	
	Total	28		
Net IFN-γ in CSF Spots/100 000 cells	Neuroborreliosis	12	12.10	0.72
	Other diagnosis	10	11.0	
	Total	22		
Net IL-4 in CSF Spots/100 000 cells	Neuroborreliosis	8	6.4	0.54
	Other diagnosis	3	5.0	
	Total	11		

[a]Mann-Whitney

used the Daco kit for detection of anti-flagellin antibodies in serum and CSF, previously shown to be a sensitive and specific test.[19] None of the 17 patients with possible neuroborreliosis had antibody titers against the flagellin antigen in CSF, but 11 had elevated IgG or IgM titers in serum. PCR did not reveal any Borrelia antigen in CSF and the method is often regarded as of low sensitivity, especially in blood and in late neuroborreliosis cases.[18]

The earliest indication of Borrelia infection is usually erythema migrans.[20] Eight patients (47%) with possible neuroborreliosis had noticed an erythema migrans in relation to symptoms in the present study. Only 15 (14%) of 106 patients with definite neuroborreliosis had noted an erythema migrans in a previous study.[21] A typical erythema migrans with a diameter of >5 cm in conjunction with symptoms is usually regarded as more or less proof of a Borrelia infection.[22]

Leucocytosis in the CSF is a common finding in neuroborreliosis but may be a finding in many other infectious or inflammatory diseases. All patients were lumbar punctured and 4 of 17 showed leucocytosis in the present study. Also one patient with cerebral infarction and one with viral meningitis showed leucocytosis. We have previously shown that 85 of 106 patients with ELISA positive neuroborreliosis had leucocytosis (5-2330×10^6/L) in the CSF. It is possible that the lower number of patients with leucocytosis in the CSF in the present study reflects a weak immune response against the Borrelia antigen. Another possibility is that the lumbar puncture was taken too early in the disease process. Only 6 were taken one month or earlier after the debut of symptoms, however. A diagnosis and treatment of neuroborreliosis is not always straightforward and proposed guidelines for the management of this has recently been published.[18,23]

We previously showed a compartmentalized interferon-gamma (IFN-γ) response in the central nervous system (CNS) in patients with neuroborreliosis[15] and that an IFN-γ predominated response was associated with a good prognosis in asymptomatic sero-positive individuals.[24] In the present study we did not find any significant difference in IFN- γ or IL-4 production in the CSF, however. Recently elevated levels of the B-lymphocyte attracting chemokine (CXCL13) in the CSF in early neuroborreliosis patients have been found.[25] It has also been seen in the CSF of multiple sclerosis patients[26] making the specificity of the test uncertain. Most of our patients with possible neuroborreliosis were treatment with doxycycline reported to have a downregulatory effect on proinflammatory cytokines.[27] If this has any significant relevance is uncertain and furthermore little is known about the effects on the IFN- γ or IL-4 responses.

The diagnosis and treatment of possible but not confirmed neuroborreliosis is a clinical challenge. Even though we found the highest IFN-γ levels in 6 out of 17 patients with possible neuroborreliosis in blood, the test is unspecific and may be elevated also in other inflammatory conditions. Further improvements in diagnostic tests are warranted to establish early diagnosis and to avoid late complications. The clinical picture may raise the suspicion of neuroborreliosis, which can be further strengthened by a preceding erythema migrans and/or elevated mononuclear cell count in the cerebrospinal fluid. A pragmatic approach has been put forward in patients with

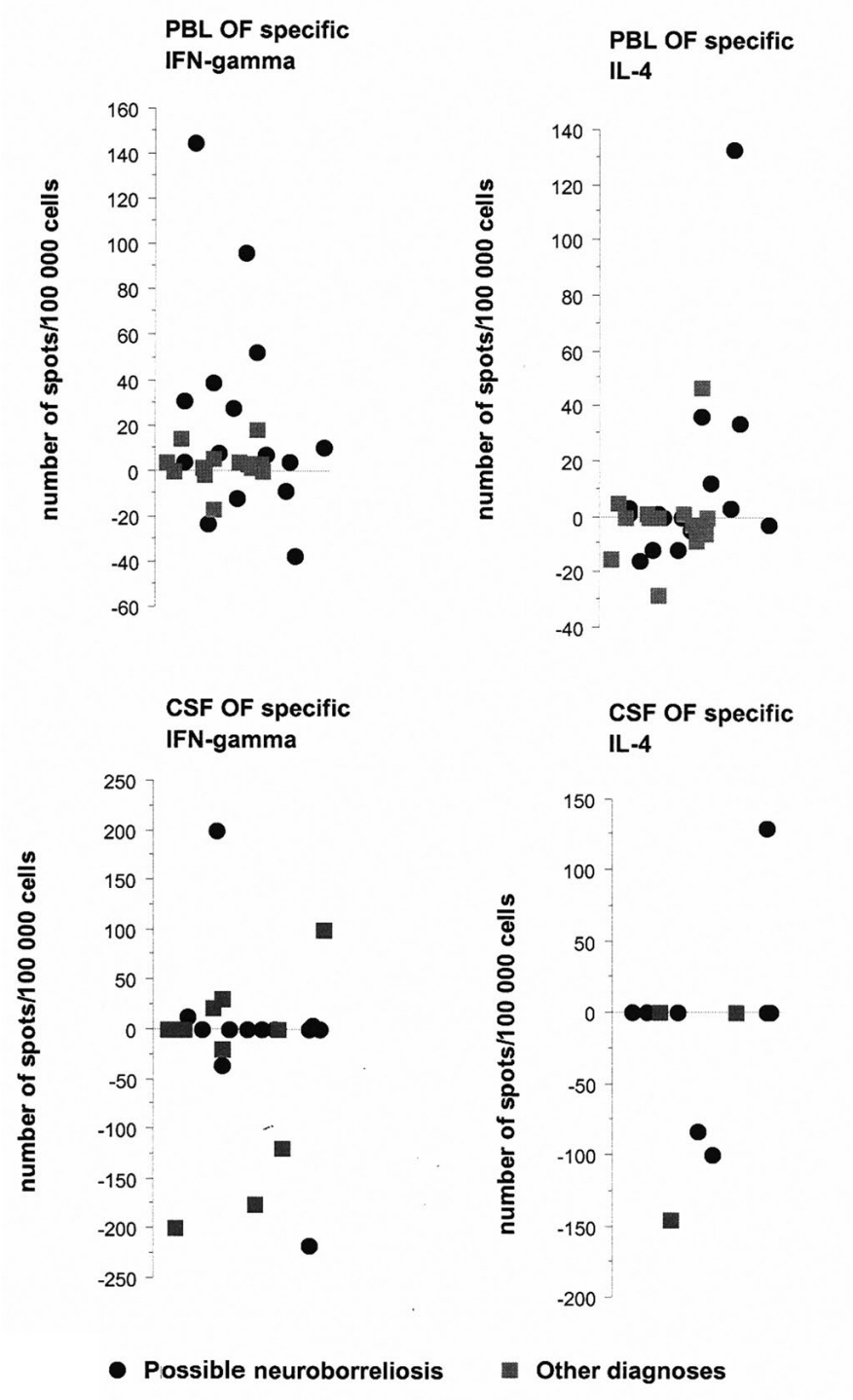

Figure 1. Number of Borrelia specific IFN-γ and IL-4 secreting cells per 100.000 lymphocytes in cerebrospinal fluid and peripheral blood in patients with probable neuroborreliosis and patients with other diagnoses as measured by ELISPOT. The Borrelia specific secretion was obtained by subtracting the number of spots in unstimulated wells from the number in Borrelia OF-stimulated wells.

possible neuroborreliosis, namely a favorable response to antibiotic treatment.[28] Our study also indicates that the clinical response to treatment may be the best option in patients with possible neuroborreliosis.

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