SEROLOGY

Advantage of recombinant borrelial proteins for serodiagnosis of neuroborreliosis

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Two enzyme immunoassay (EIA) systems were compared for their ability to detect *Borrelia burgdorferi sensu lato* specific IgG and IgM antibodies and to differentiate between symptomatic (83 patients with neuroborreliosis) and asymptomatic seropositive subjects (80 healthy controls). Antibody concentrations were determined by EIA; the antigens used were either a sonicate of *B. burgdorferi* or three recombinant borrelial proteins: the 14-kDa flagellin fragment, the outer surface protein C (22 kDa) and the high molecular mass protein p83 (83 kDa). In the sonicate, EIA, IgG or IgM antibodies to *B. burgdorferi*, or both, were detected in all patients with neuroborreliosis and in all controls. Pre-absorption of sera with *Treponema phagedenis* sonicate diminished the sensitivity of detection of borrelial specific IgG (IgG or IgM or both) antibodies in patients with neuroborreliosis from 80 to 57% (100 to 82%) and in the controls from 100 to 32% (100 to 37%). While being specific for *B. burgdorferi*, the recombinant EIAs proved to be significantly more sensitive than the sonicate EIA: IgG or IgM, or both antibodies against any of the recombinant antigens were detected in 92% of patients with neuroborreliosis and in 24% of controls. The increase in sensitivity in patients with neuroborreliosis was mostly due to the higher detection rate of IgM antibodies in the recombinant EIA (77% versus 48% in the sonicate EIA), while IgG antibodies were demonstrated with similar frequencies in both EIA systems (57% versus 60%). It was concluded that the recombinant EIAs are superior to the sonicate EIA with pre-absorption of cross-reactive antibodies in the confirmation of an acute borrelial infection and in the differentiation between symptomatic and asymptomatic infections.

Introduction

Lyme borreliosis is a multi-system disorder involving skin, joints, the heart and the central nervous system [1–4]. To differentiate diseases with similar clinical symptoms, highly sensitive and specific serological tests are needed. However, there are several basic problems associated with the evaluation of serological tests for borrelia infections. No individual test has been accepted as the reference method to demonstrate an infection with *Borrelia burgdorferi sensu lato* [3, 5–11]. A gold standard would be the isolation of *B. burgdorferi* from human body fluids or tissue, but this agent is difficult to culture from patients because of its fastidious nature and because the number of organisms in body fluids appears to be small. An approximate gold standard, which has yet to be defined, should

include not only typical clinical symptoms of Lyme borreliosis, but also additional laboratory findings such as evidence of inflammation in the cerebrospinal fluid in patients with neuroborreliosis.

At present, serological tests for Lyme disease usually include enzyme immunoassays (EIA) and indirect immunofluorescent assays (IFA). These tests employ whole cells or extracts of *B. burgdorferi* [3, 6, 11]. They have adequate sensitivity, but are lacking in specificity because of epitopes of *B. burgdorferi* that cross-react with other bacterial species [5, 12, 13]. The specificity of serological assays can be improved by adsorption of non-specific antibodies, but this procedure frequently reduces sensitivity [13, 14]. Alternatively, the diagnosis of Lyme borreliosis might be established by a two-test protocol: the first step would be a sensitive but not very specific serological test, such as a whole-cell extract EIA, and the second would be a confirmatory test such as immunoblotting (Western blot) [15]. Apart from immunoblotting with whole-cell extracts of *B. burgdorferi*, EIA with

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recombinant borrelial proteins represents a suitable method to confirm a borrelial infection [16–23].

The present study investigated the significance of three recombinantly expressed borrelial proteins: first, for the detection of *B. burgdorferi*-specific IgM and IgG antibodies; second, for the differentiation between symptomatic and asymptomatic infections; and third, as confirmatory antigens in the serodiagnosis of neuroborreliosis. The outer surface protein (Osp) C (22 kDa), the 14-kDa flagellin fragment and the high molecular mass protein p83 (synonyms: p93, p94, p100) were employed as antigens.

Materials and methods

Subjects

Serum samples were obtained from 83 patients with neuroborreliosis who had been admitted to the Department of Neurology of the University of Freiburg between 1990 and 1997. Sixty-seven patients had acute neuroborreliosis (duration of symptoms ≤ 6 months: lymphocytic meningoradiculitis, 56; encephalitis, 8; myelitis, 2; cerebral vasculitis, 1) and 16 had chronic neuroborreliosis (duration of symptoms > 6 months: encephalomyelitis, 14; polyneuritis in association with acrodermatitis chronica atrophicans, 2).

Except for two patients with peripheral polyneuritis, the diagnosis of neuroborreliosis was based on lymphocytic pleocytosis, and assessment of *B. burgdorferi*-specific IgM or IgG antibodies, or both, in serum by routine serological tests as follows: (1) a titre of > 320 in a passive haemagglutination assay with a mixture of antigens from *B. burgdorferi sensu lato* strain GeHo, *B. afzelii* (provided by K. Pelz, Institute of Medical Microbiology of the University of Freiburg, passage number > 25) and *B. garinii* (provided by B. Wilske, Max von Pettenkofer Institute, Munich); (2) a positive titre (IgM > 24 , IgG > 32) in an IFA. Specimens were tested at the Department of Immunology in the Institute of Medical Microbiology of the University of Freiburg. Intrathecal synthesis of IgG or IgM antibodies (or both) to *B. burgdorferi* in sonicate EIA was demonstrated in 71 patients (86%) and at follow-up examinations in 81 (98%) of 83 patients. The detailed CSF data are shown in Table 1.

All patients were treated with ceftriaxone 2 g daily for 2 weeks (acute) or 3 weeks (chronic).

Controls

In 1995, an epidemiological study was conducted to evaluate the frequency of *B. burgdorferi* seropositive subjects living in an area endemic for this infection [24]. Patients and their relatives who went to an ambulatory practice were questioned for symptoms compatible with Lyme borreliosis and asked to provide serum samples. Of all subjects who were seropositive (158/393; 40%), 80 persons did not recall any symptoms indicating a previous infection with *B. burgdorferi*. These individuals were either healthy or had diseases of other aetiology (diabetes mellitus, surgery).

Bacterial strains and antigens

B. burgdorferi sensu lato strain GeHo isolated from a patient with erythema migrans was used in this study [25]. Spirochaetes were grown in modified Kelly's medium according to standard techniques, centrifuged at 10 000 rpm for 30 min, and washed three times in phosphate-buffered saline (PBS). Spirochaetes were sonicated for 5 min and dissolved in 6 M urea to a final concentration of 2 mg/ml. DNA of strain GeHo was used for cloning of OspC, p83 and the 14-kDa flagellin fragment. The expression and purification of these proteins was performed according to standard methods as described previously [21, 26].

Enzyme immunoassay

Microtitration plates were coated with 100 μ l of *B. burgdorferi sensu stricto* strain GeHo sonicate (20 μ g/ml) in PBS or with individual preparations of recombinant proteins (14 kDa, 22 kDa, 83 kDa: 0.5 μ g/ml) in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.5) and incubated overnight at 4°C. The optimal antigen concentration for coating of EIA plates had been determined in preliminary experiments by checkerboard titration of two-fold dilutions of antigen and high-titre sera. Unspecified protein binding was blocked with 100 μ l of dilution buffer (BSA 1%, Tween-20 0.1% in PBS) for 1 h. Samples to be tested for IgG (IgM) antibodies were diluted 10 000-(2000-)

Table 1. Cerebrospinal fluid findings in patients with acute and chronic neuroborreliosis

Assay	Normal	Acute neuroborreliosis (n = 67)		Chronic neuroborreliosis (n = 16)	
		Number of patients with abnormal values	Median (range)	Number of patients with abnormal values	Median (range)
Leucocytes/ μ l	< 5	67	241 (7–600)	14	60 (10–135)
Q-albumin ($\times 10^{-3}$)	< 7.5	47	20 (4–58)	14	35 (10–70)
Oligoclonal IgG bands	None	54		14	
IgG synthesis (%)	0	42	20 (10–50)	14	40 (5–75)
IgM synthesis (%)	0	50	50 (10–95)	7	15 (10–80)
IgA synthesis (%)	0	19	10 (5–50)	8	30 (15–80)

and 1000-(200-)fold. For pre-absorption of cross-reactive IgG antibodies, 100 μ l of 100-fold pre-diluted sera were incubated with 100 μ l of *Treponema phagedenis* 1.0 mg/ml. After incubation at 37°C for 1 h, samples were pelleted by centrifugation at 13 000 rpm for 20 min. The supernate was used for further dilution steps. Control samples were treated in the same way. Antibodies to *B. burgdorferi* were tested on the same plate with and without pre-absorption with *T. phagedenis*. Before performing the IgM antibody assay, total IgG was immunoprecipitated by the method of Müller *et al.* [27]. Control samples were treated in the same way. Specimens were incubated for 2 h. After washing, 100 μ l of peroxidase-conjugated goat anti-human IgG (1 in 10 000, Dianova, Hamburg, Germany) or anti-human IgM antibodies (1 in 4000, Dianova) were added. After 1.5 h at room temperature in a dark chamber, the plates were washed and 100 μ l of *o*-phenylenediamine in citrate buffer (pH 5) containing H₂O₂ 0.04% were added to each well. The enzymic reaction was stopped after 20 min by the addition of 30 μ l of 2.5 N H₂SO₄. The optical densities at 492 nm (OD₄₉₂) were measured with a Titertek Multiscan reader. The cut-off value was defined as the mean OD₄₉₂ of 80 negative controls (healthy students with no history of Lyme borreliosis and negative findings in Western blotting) plus two SD. Samples were tested in duplicate and the mean value was calculated. Negative controls were included on every plate. The cut-off values are shown in Table 2.

Statistical analysis

The frequency of positive results in both groups was examined by the χ^2 test, with SPSS/PC⁺ software. A *p* value < 0.05 was considered significant.

Results

Sonicate EIA and absorption of cross-reactive IgG antibodies

The sonicate EIA showed that 67 (80%) of the 83 patients with neuroborreliosis had IgG antibodies and 40 (48%) had IgM antibodies to *B. burgdorferi* in serum; all patients displayed either IgG or IgM antibodies, or both. After pre-absorption with *T. phagedenis*, 47 (70%) of 67 samples were still positive for IgG-antibodies and 68 (82%) of 83 samples were still positive for IgG or IgM antibodies, or both. Of 20 patients who became negative after absorption of cross-reactive antibodies, recent infection with *B. burgdorferi* was demonstrated by intrathecal synthesis of borrelial-specific IgG or IgM antibodies (or both) in 15, and by increasing titres of specific IgM antibodies in five.

IgM antibodies to *B. burgdorferi* were detected more frequently in patients with acute neuroborreliosis (38/67; 57%) than in those with chronic neuroborreliosis (2/16; 12%). In contrast, IgG antibodies were demonstrated in all patients with chronic neuroborreliosis, but in only 31 (46%) of 67 patients with acute neuroborreliosis.

In seropositive healthy subjects, IgG antibodies were present in all and IgM antibodies in 8 (10%) of 80 samples. After pre-absorption with *T. phagedenis*, only 26 (32%) of 80 samples were still positive. The detailed data are shown in Table 3.

Recombinant proteins

The frequency of IgG and IgM antibodies to individual recombinant borrelial antigens is shown in Tables 4–6.

Table 2. Cut-off values in EIA

Immunoglobulin	OD ₄₉₂ cut-off value			
	<i>B. burgdorferi</i> sonicate	14 kDa	22 kDa	83 kDa
IgM	0.4	0.15	0.17	0.10
IgG	0.3	0.31	0.34	0.26
IgG (after pre-absorption)	0.1	...	...	...

Table 3. Prevalence of IgG and IgM antibodies to *B. burgdorferi* in sonicate EIA

Immunoglobulin	Number (%) of positive results		
	Neuroborreliosis: (n = 83)	Controls: (n = 80)	<i>p</i> value
IgG	67 (81)	80 (100)	
IgG and absorption	47 (57)	26 (32)	0.002
IgM	40 (48)	8 (10)	0.0001
IgM or IgG or both	83 (100)	80 (100)	NS
IgM or IgG or both and absorption	68 (82)	30 (37)	0.0001

In patients with neuroborreliosis, employment of the three recombinant borrelial proteins increased the sensitivity of detection of specific IgM antibodies from 48% in sonicate EIA to 77% in recombinant EIA, while in seropositive controls the frequency of IgM antibodies fell from 10% to 7.5% (Table 4).

In patients with neuroborreliosis, the sensitivity of detection of specific IgG antibodies was similar in the recombinant (60%) and in the sonicate (57%) EIA method, if the latter was performed after pre-absorption of cross-reactive antibodies. Of 40 patients with neuroborreliosis that were IgM positive in the sonicate EIA, 31 (77%) were also positive in the recombinant EIA. On the other hand, a further 33 patients with neuroborreliosis were positive in the recombinant EIA, but negative in the sonicate EIA. The frequency of IgM antibody reactions in these 33 patients did not differ significantly between the various proteins (14 kDa, $n = 18$; 22 kDa, $n = 15$; 83 kDa, $n = 19$).

In seropositive controls without clinical symptoms, the sensitivity of detection of IgG antibodies fell from 32% in the sonicate EIA (after pre-absorption of cross-reactive antibodies) to 18% in the recombinant EIA ($p < 0.05$) (Table 5).

Evaluation of the prevalence of IgM or IgG antibodies, or both, to any of the three recombinant borrelial proteins revealed a sensitivity of 92% in patients with neuroborreliosis, but only 24% in asymptomatic controls ($p < 0.0001$) (Table 6).

Differences between patients with acute and chronic neuroborreliosis concerning the frequency of antibody reactions to any recombinant protein were detected for only two combinations: IgG antibodies to the 83-kDa protein were detected more frequently in patients with chronic neuroborreliosis (56%) than in those with acute neuroborreliosis (30%; $p = 0.046$), and IgM antibodies to the 14-kDa protein were more frequent in patients

Table 4. Prevalence of IgM antibodies to individual recombinant borrelial proteins

Proteins	Number (%) of positive results		p value
	Neuroborreliosis: (n = 83)	Controls: (n = 80)	
14 kDa	41 (49)	4 (5)	< 0.0001
22 kDa	39 (47)	0	< 0.0001
83 kDa	32 (39)	4 (5)	< 0.0001
14/22 kDa	58 (70)	4 (5)	< 0.0001
14/83 kDa	55 (66)	6 (7)	< 0.0001
22/83 kDa	50 (60)	4 (5)	< 0.0001
14/22/83 kDa	64 (77)	6 (7)	< 0.0001

Table 5. Prevalence of IgG antibodies to individual recombinant borrelial proteins

Proteins	Number (%) of positive results		p value
	Neuroborreliosis: (n = 83)	Controls: (n = 80)	
14 kDa	28 (34)	6 (7)	< 0.0001
22 kDa	9 (11)	1 (1)	0.008
83 kDa	29 (35)	12 (15)	0.003
14/22 kDa	33 (40)	6 (7)	< 0.0001
14/83 kDa	45 (54)	14 (18)	< 0.0001
22/83 kDa	36 (43)	12 (15)	< 0.0001
14/22/83 kDa	50 (60)	14 (18)	< 0.0001

Table 6. Prevalence of IgM/G antibodies to individual recombinant borrelial proteins

Proteins	Number (%) of positive results		p value
	Neuroborreliosis: (n = 83)	Controls: (n = 80)	
14 kDa	52 (63)	10 (12)	< 0.0001
22 kDa	40 (48)	1 (1)	< 0.0001
83 kDa	49 (59)	16 (20)	< 0.0001
14/22 kDa	64 (77)	10 (12)	< 0.0001
14/83 kDa	70 (84)	19 (24)	< 0.0001
22/83 kDa	64 (77)	16 (20)	< 0.0001
14/22/83 kDa	76 (92)	19 (24)	< 0.0001

with acute neuroborreliosis, (53%) than in chronic neuroborreliosis (31%; $p = 0.1$, not significant).

Discussion

The present study investigated the significance of recombinantly expressed borrelial proteins for serodiagnosis of neuroborreliosis. As no individual test has been accepted as the reference method for demonstration of an infection with *B. burgdorferi*, the most critical aspect in such investigations is designation of a gold standard [3, 5–11]. The 'approximate gold standard' in the present study was a group of patients who presented with typical clinical symptoms of neuroborreliosis, inflammatory findings in the cerebrospinal fluid and an intrathecal synthesis of borrelia-specific antibodies.

The main finding of this study was an improved sensitivity of the recombinant EIAs over the sonicate EIA to detect borrelia-specific IgM antibodies in patients with neuroborreliosis. This finding might be explained by higher concentrations of IgM-relevant epitopes in the wells of the recombinant EIAs when compared with the variety of different antigens in a whole-cell sonicate EIA.

The frequent finding of IgM antibodies to the OspC and the 14-kDa flagellin fragment in this study is in line with the findings reported by Wilske *et al.* who estimated sensitivities of 43% for IgM antibodies against OspC in a recombinant immunoblot and of 46% for IgM antibodies to the 14-kDa flagellin fragment [28]. Gerber *et al.* found sensitivities of 48% for a recombinant OspC-IgM EIA and of only 28% for a whole-cell-IgM EIA in 82 patients with erythema migrans [17]. The high incidence of IgM antibodies to the 83-kDa antigen has not been described previously in neuroborreliosis. Wilske *et al.* reported sensitivities of a recombinant immunoblot for detection of IgM antibodies to the p100 antigen (the p83 homologue in their study) in neuroborreliosis to be 7% and 12% [23]. A similar result was reported by Rauer *et al.*, with 9% sensitivity achieved by a recombinant p83-IgM EIA in neuroborreliosis [22]. The present study confirmed the positive results of the p83-IgM EIA by recombinant and native immunoblot (data not shown). Differences in case definition and the time of collection of sera with respect to clinical course might be the most appropriate reasons for the discrepancies revealed. As shown in a previous study, the choice of species (e.g., *B. burgdorferi sensu stricto* versus *B. afzelii*) probably does not influence the sensitivity of the assay [22]. Both strains show a high degree of sequence homology (89%) of the antigens [29, 30].

Borrelia-specific IgG antibodies may persist for many years in the sera of patients previously infected with

B. burgdorferi, while IgM antibodies appear mostly during early infection and thereafter decline gradually. In patients with atypical clinical symptoms, it is difficult to differentiate between an 'anamnesic IgG antibody titre' and a relevant titre related to active disease. In this situation, increased sensitivity of the recombinant EIA in the detection of IgM antibodies might be of advantage in differentiating between active and recent disease.

Another finding in the present study was the advantage of the recombinant EIAs over the sonicate EIA in detecting *B. burgdorferi*-specific antibodies. In sonicate EIA, IgG antibodies specific for this agent can be demonstrated only after absorption of samples with Reiter spirochaetes. However, this procedure is time-consuming and expensive. Antibody testing by recombinant EIAs requires no pre-absorption of cross-reactive antibodies and in this study was even a little more sensitive than testing by sonicate EIA [18, 22, 31].

In many cases, screening for antibodies to *B. burgdorferi* is requested for patients with ambiguous symptoms. If both the screening and confirmatory assays are positive, the clinician has to consider a borrelial infection as a possible cause of the patient's complaints. However, if the confirmatory assay is negative, other reasons for the symptoms are more likely. The higher sensitivity of the recombinant EIAs for the detection of borrelial antibodies in patients with neuroborreliosis and the lower detection rate of this assay for borrelial antibodies in healthy subjects, support its use in patients with uncertain clinical symptoms because of its increased predictive value.

In conclusion, the overall sensitivity of the three recombinant antigens for detection of IgM antibodies was significantly better when compared with the sonicate EIA. Sensitivity of the recombinant EIAs decreases if any of the three antigens is excluded. High specificity of the recombinant antigens has been demonstrated in earlier studies [18, 22, 32, 33]. Consequently, when neuroborreliosis is suspected, the use of the recombinant EIAs will be helpful in confirming the suspected diagnosis.

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