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ORIGINAL INVESTIGATION

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Diversity of OspA and OspC among cerebrospinal fluid isolates of *Borrelia burgdorferi* sensu lato from patients with neuroborreliosis in Germany

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Abstract Neuroborreliosis is the most frequent manifestation of the second stage of Lyme borreliosis in Europe. However, only few isolates from the cerobrospinal fluid (CSF) have been characterized with controversial results. A large panel of 36 CSF isolates isolated over a 10-year period in Munich has now been analyzed for their OspA and OspC type, resulting in at least eight different types, respectively. Representatives of the different types cultivated from CSF in Munich have also been isolated from other geographical regions in Europe from CSF or ticks, suggesting a widespread distribution of pathogenic strains. A certain OspA type (type 4) was frequently observed in adults but rarely in children or ticks. Since OspA and OspC are the most promising candidates for a Borrelia vaccine, the considerable heterogeneity found among CSF isolates has important implications for development of a vaccine in Europe.

Key words Lyme borreliosis · Borrelia burgdorferi · Cerebrospinal fluid · OspA · OspC

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Introduction

Lyme borreliosis (LB) caused by the spirochete *Borrelia burgdorferi* sensu lato (s. l.) is the most frequent human tick-borne disease in the northern hemisphere [6]. Three different species, *B. burgdorferi* sensu stricto (s. s.), *B. garinii* and *B. afzelii*, are associated with LB in Europe [2]. LB is a multisystem disorder which develops in several stages [36]. However, erythema migrans, the early hallmark of the disease is missing in about 50% of the patients with neuroborreliosis the main manifestation of the second stage [28]. This raises the question of whether there are differences between strains causing erythema migrans and neuroborreliosis. It has been shown by analysis of large numbers of European isolates from the skin that OspA serotype 2 (or *B. afzelii*) is mainly associated with skin manifestations [9, 38, 41, 43]. Since it is difficult to cultivate *B. burgdorferi* s. l. from the cerebrospinal fluid (CSF), only a limited number of European CSF isolates have been analysed by molecular or immunological methods (1 Austrian, 3 French, 5 Scandinavian isolates, 3 isolates from the Netherlands and 11 from Germany [2, 23, 38, 43]). These analyses suggested that OspA serotypes associated with *B. garinii* prevail among European CSF isolates with the exception of France. In addition, our previous study from Germany [43] suggested differences between European isolates from CSF and from ticks with respect to the distribution of OspA types among *B. garinii* strains. However, in another study from Germany such differences could not be detected by *ospA*-specific polymerase chain reaction (PCR) in the CSF [15]. To investigate this issue further, we analyzed an enlarged panel of CSF isolates from Germany. Since OspA serotypes and *ospA* genotypes correspond completely [40, 43], the OspA-serotyping results of isolates from this study could be compared with the *ospA*-genotyping results obtained by PCR in the study from Eiffert et al. [15]. In addition to OspA serotyping, we analysed the isolates with respect to another major outer surface protein OspC using monoclonal antibodies. OspA and OspC are the main candidates for development of a *Bor-*

relia vaccine [16, 30, 34]. It is, therefore, necessary to analyze primarily human isolates to determine the heterogeneity of pathogenic strains.

We found that CSF isolates are considerably heterogeneous with respect to both OspA and OspC (eight OspA and OspC types, respectively). That the results of the previous German studies appear primarily contradictory might be due to differences in the age of the patients. Isolates from children are apparently similar in OspA type distribution to tick isolates. In contrast, it appears that in adult patients certain serotypes (OspA type 4) are more frequent, but others (OspA type 6) not as frequent as in ticks or children.

Material and methods

CSF isolates

Of the 36 German CSF isolates from patients with neuroborreliosis analyzed in this study (Table 1) 8 have been described [43] and are indicated by asterisk in Table 1. Since 1988 a total of 28 new isolates have been cultivated from CSF in our laboratoy using modified Kelly medium (MKP medium) [29].

Determination of the genospecies

Large restriction fragment patterns (LRFP) have been investigated by pulse-field gel electrophoresis (PFGE) to determine the genospecies of the CSF isolates. As described by Belfaiza et al. [4] PFGE of *Mlu*I-digested genomic DNA results in typical species-specific LRFP. The technique of Belfaiza et al. [4] was used with minor modifications [7].

Immunological analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and determination of OspA serotype by immunoblot using a panel of eight monoclonal antibodies (mAb; Table 2) were performed as described previously [43]. Instead of the mAb LA 26 that was used previously, the isolates were investigated with mAb L32 14G7 (Fig. 1) which was obtained from BALB/c mice immunized with purified recombinantly expressed OspA derived from *B. afzelii* strain PKo. For immunological characterization of OspC the borreliae were analyzed with an assortment of OspC-specific mAb [44]. An mAb (L22 A16) that has not been described previously was included in the study. This mAb was produced by immunization of BALB/c mice with washed whole cells of *B. garinii* strain PBi according to previous protocols [43]. The same fusion resulted in another mAb (L18 A14) that recognizing an undefined 18-kDa protein of *B. garinii.*

Table 1 Immunological and molecular analysis of cerebrospinal fluid (CSF) isolates from Germany (*mAb* monoclonal antibody, *B. burgdorferi*, s. s. *Borrelia burgdorferi* senso stricto)

Patient	Strain	Age of patient	OspA serotype	Species	Immunoblot with mAb	
					L32 14G7	L18 A14
1	PKa2[a]	63	1	*B. burgdorferi* s.s.	–	–
2	PFra	62	1	*B. burgdorferi* s.s.	–	–
3	PHas	40	1	*B. burgdorferi* s.s.	–	–
4	PStm	Unknown	1	*B. burgdorferi* s.s.	–	–
5	PKle[a]	72	2	Not done	+	–
6	PSpe[a]	78	2	*B. afzelii*	+	–
7	PAlt	61	2	*B. afzelii*	+	–
8	PHa	59	2	*B. afzelii*	+	–
9	PKr	48	2	*B. afzelii*	+	–
10	PBr[a]	34	3	*B. garinii*	–	+
11	PFe	23	3	*B. garinii*	–	+
12	PMek	18	3	*B. garinii*	–	+
13	PBi[a]	14	4	*B. garinii*	–	+
14	PFei[a]	50	4	*B. garinii*	–	+
15	PHoe[a]	49	4	Not done	–	+
16	PFin	64	4	*B. garinii*	–	+
17	PBaEII	52	4	*B. garinii*	–	+
18	PWa	76	4	*B. garinii*	–	+
19	PMue	26	4	*B. garinii*	–	+
20	PSh	64	4	*B. garinii*	–	+
21	PFlk	65	4	*B. garinii*	–	+
22	PScf	60	4	*B. garinii*	–	+
23	PHei[a]	8	5	*B. garinii*	–	+
24	PLi	2	5	*B. garinii*	–	+
25	PHe	2	6	*B. garinii*	–	+
26	PSoR	35	6	*B. garinii*	–	+
27	POhm	4	6	*B. garinii*	–	+
28	PSeS	Unknown	6	*B. garinii*	–	+
29	PBeS	6	7	Not done	–	+
30	PRef	44	7	*B. garinii*	–	+
31	PBo[a]	16	0	*B. afzelii*	–	+
32	PLap	62	0	*B. afzelii*	+	–
33	PStb	58	0	*B. afzelii*	(+)	–
34	PKi	44	X	*B. garinii*	–	+
35	PLa	3	X	*B. garinii*	–	(+)
36	PFCa	26	2 and 4	*B. afzelii* and *B. garinii*	+	+

[a] Old study [43]

Table 2 Immunoreactivity of *B. burgdorferi* senso lato with OspA-specific monoclonal antibodies

OspA serotype	L32 1F11	L32 1C8	H5332	H3TS	L32 14G7	L32 1F7	L32 1G3	L32 1D11
1	+	+	+	+	–	–	–	–
2	+	–	+	–	+	–	–	–
3	+	+	+	–	–	+	–	–
4	+	+	–	–	–	–	+	–
5	+	+	–	–	–	–	–	–
6	+	+	+	–	–	–	–	–
7	+	+	–	–	–	+	–	+
8	+	–	+	–	–	–	–	–

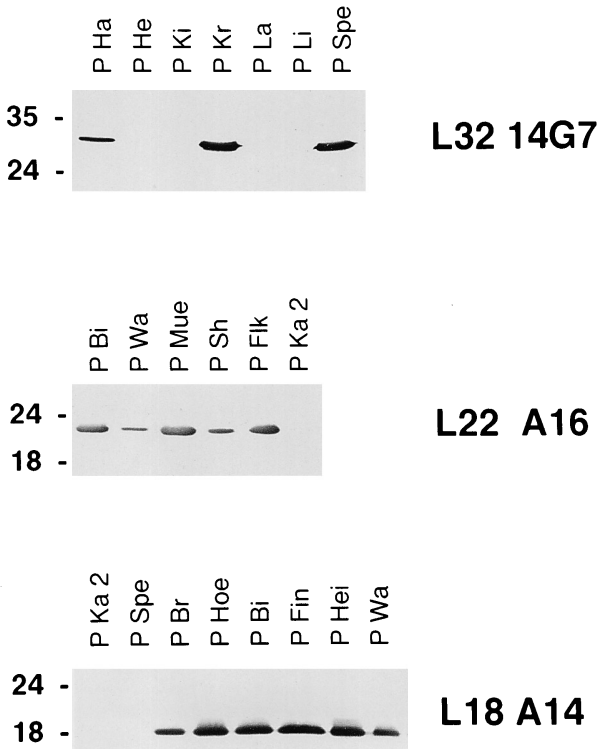

Fig. 1 Immunological characterization (Western blot) of *Borrelia burgdorferi* sensu lato strains with monoclonal antibodies L32 14G7, L22 A16 and L18 A14. Size standards were pepsin (35 kDa), trypsinogen (24 kDa) and β-lactoglobulin (18 kDa) (Sigma, St. Louis, Mo.)

Partial sequencing of *ospA* genes

ospA genes were amplified by PCR from genomic DNA using previously described primers [15]. PCR fragments were sequenced, using the same primers, by the dideoxynucleotide chain-termination method on an ABI 373 DNA sequencer (cycle sequencing reactions) following the manufacturer's instructions.

Sequence analysis and accession numbers

For comparison of sequences the GCG programm package version 7.1 [13] and Clustal V (German Cancer Center, Heidelberg, Germany) were used. The sequences were assigned the following EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the following accession numbers: strains PSpe (×95353), PKi (×95354), PLa (×95355), POhm (×95356), PHe (×95357), PLi (×95358), PFe (×95358), PFra (×95360), PBes (×95361), PRef (×95362).

Results

Determination of the genospecies

As shown in Table 1 we found a significant prevalence of *B. garinii* (64.9%). The remaining strains were classified as *B. burgdorferi* s. s. (10.8%) or *B. afzelii* (24.3%). One isolate (PFCa) showed *B. afzelii*- as well as *B. garinii*-specific bands, suggesting that the isolate contains a mixture of both species. Details of this study will be reported elsewhere.

Molecular analysis of OspA

According to SDS-PAGE analysis 33 of the 36 isolates expressed OspA and could be reliably analyzed for OspA serotype (Table 1). They were considerably heterogeneous: 31 strains belonged to serotypes 1–7. Two *B. garinii* strains could not be assigned to one of the previously described OspA serotypes. They were not reactive with mAb L32 1C8, which is usually reactive with *B. garinii*, thus representing a new type. Since they also differed significantly in the OspA sequence from types 1–7 (see below) they were designated OspA serotype 8. All OspA-expressing *B. afzelii* strains (or OspA serotype 2) were specifically recognized by mAb L32 14G7. This antibody was not reactive with the *B. garinii* and the *B. burgdorferi* s. s. strains, but it was reactive with 40 OspA serotype 2 strains selected from a previous study [43] (data not shown). Isolate PFCa appears to be a mixture of OspA-serotype 2 and 4 since it was reactive with the type-specific L32 14G7 and L32 1G3 mAbs.

Cluster analysis of partial OspA sequences confirmed the strong association between OspA serotype and OspA sequence (Fig. 2). Besides partial OspA sequences from strains analyzed in this study, those from CSF isolates PBr, PBi and PHei, the skin isolate PKo and the tick isolates P31, TN and T25 [43] were also included and compared to sequences obtained by PCR from CSF (A, B, C, D, H) and ticks (A–G) previously published by Eiffert et al. [15]. Cluster analysis of the partial OspA sequences shows that OspA serotypes 1–6, and 8 correspond to PCR types E, B, D, C, G, A, and H, respectively. Type 7-*ospA*, however, was not detected by Eiffert and colleagues in Göttingen using PCR, and strains corresponding to type F have not so

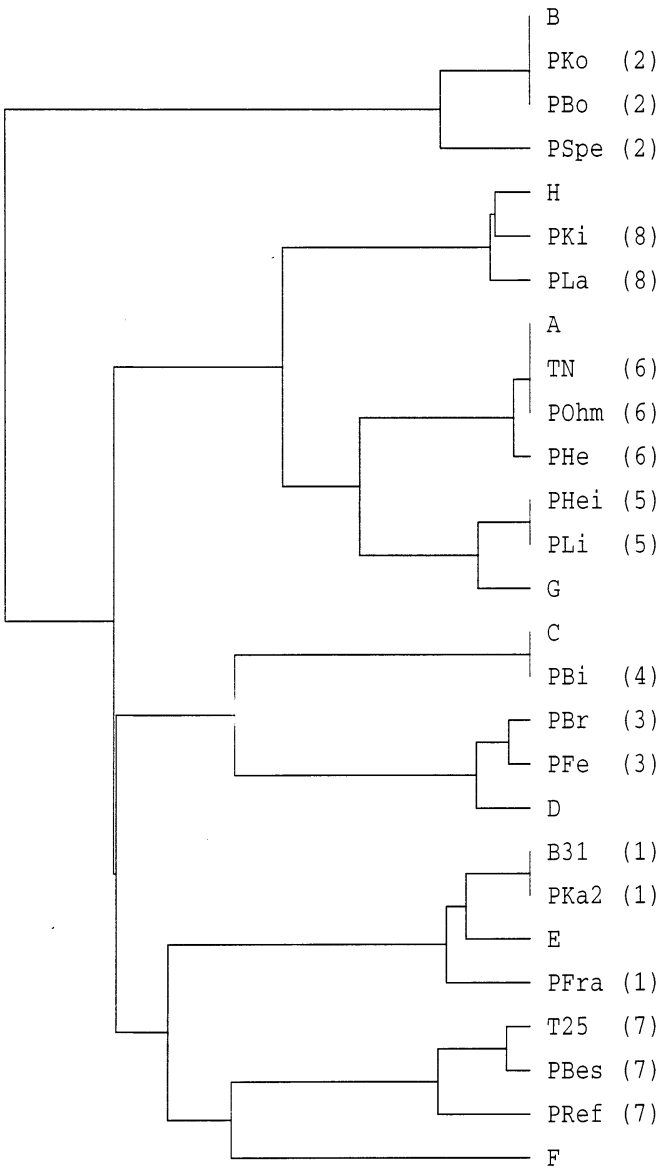

Fig. 2 Cluster analysis of partially deduced OspA sequences [amino acids (aa) 27–156, B31 numbering]. Sequences were derived from *Borrelia* isolates analyzed in this study (PSpe, PKi, PLa, POhm, PHe, PLi, PFe, PFra, PBes, and PRef) and in previous studies (PKo, PBo, TN, PHei, PBi, PBr, P31, PKa2, and T25). They are compared with aa sequences (A–H) deduced from PCR amplificates published by Eiffert et al. [15]

far been isolated in Munich. Taken together, OspA types 1–8 were found in ticks as well as in CSF from patients. OspA types 1–3 and 5–7 could be cultured from ticks but OspA type 4 could only be detected by PCR in the tick [15] but not by culture (Wilske et al., unpublished results from typing >90 European isolates from ticks).

Age and OspA serotype

Of the CSF isolates 6 have been cultivated from children, 28 from adults. For the remaining 2 isolates, the age of the patients is unknown. These findings and the results from the study in the CSF in children found by Eiffert are summarized in Table 3. OspA type 4 was present in 11 of 29 (37.9%) adults and only in 1 of 18 children (5.6%), OspA type 6, in contrast, was found in the CSF from 7 of 18 (38.9%) children but only in the CSF from 1 adult.

Table 3 Distribution of OspA-types among CSF isolates from children and adults

OspA type	Children		Adults	
	No.	%	No.	%
1 (E)[a]	0	0.0	3	10.3
2 (B)	4	22.2	9	31.0
3 (D)	1	5.6	3	10.3
4 (C)	1	5.6	11	37.9
5 (G)	2	11.1	0	0.0
6 (A)	7	38.9	1	3.4
7	1	5.6	1	3.4
8 (H)	2	11.1	1	3.4
	18	100.0	29	100.0

[a] OspA type determined by PCR [10] is given in parentheses

Immunological analysis of OspC

According to SDS-PAGE analysis OspC was expressed by 25 of the 36 isolates. Immunoblot analysis with a panel of 11 mAb revealed 8 different OspC antibody patterns corresponding to 6 different OspA types (OspA serotype 3 and 5 did not express OspC). With the exception of the OspA serotype 1 and 6 strains, OspCs were conserved among strains of a given OspA serotype. Notably, this is true for all 11 OspA type 4 strains as well as the 5 OspC-expressing *B. afzelii* strains. Isolate PFCa had two OspC bands reactive with mAb L22 1F8; one band reacted with mAb L22 1F10, the other with L22 A16, another indication that this isolate is a mixture of two strains (see also Table 4).

Discussion

In this study we analyzed a large panel of *B. burgdorferi* s. l. CSF isolates from Germany. We found that representatives of the three species, *B. burgdorferi* s. s., *B. afzelii* and *B. garinii*, present in isolates obtained before 1988 [43] were also isolated in the following years.

A new and most important finding is the considerable heterogeneity of the major outer surface protein OspA among the *B. garinii* isolates. We identified eight different OspA types (six among *B. garinii*), including two not previously found among CSF isolates. OspA serotype 7 has been only isolated from *Ixodes ricinus* [43] and the OspA serotype 8 strains represent a new type which had previously only been detected in *I. ricinus* by *ospA* PCR [15].

Table 4 Immunoreactivity of OspC with monoclonal antibodies

Isolate	Species	OspA serotype	OspC-specific monoclonal antibodies										
			L22 2B8	L22 6C4	L22 22C11	L22 1F8	L22 7G5	L22 10C5	L22 2E3	L22 12E5	L22 1F1	L22 6C8	L22 A16
PKa2	*B. burgdorferi* s. s.	1	+	+	–	+	+	+	+	+	–	–	–
PStm	*B. burgdorferi* s. s.	1	+	+	–	+	+	–	+	+	+	–	–
PAlt	*B. afzelii*	2	+	+	+	+	+	–	–	–	+	–	–
PHa	*B. afzelii*	2	+	+	+	+	+	–	–	–	+	–	–
PBi	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PFei	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PHoe	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PFin	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PBaE II	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PWa	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PMue	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PSh	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PFlk	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PScf	*B. garinii*	4	+	+	+	+	+	–	–	–	–	–	+
PHe	*B. garinii*	6	+	+	+	–	+	–	–	–	–	–	+
PSor	*B. garinii*	6	+	+	+	–	+	–	–	–	–	–	+
POhm	*B. garinii*	6	+	+	+	–	+	–	–	–	–	–	–
PBeS	*B. garinii*	7	+	–	+	–	–	–	–	–	–	–	–
PRef	*B. garinii*	7	+	–	+	–	–	(+)	–	–	–	–	–
PBo	*B. afzelii*	OspA negative	+	+	+	+	+	–	–	–	+	–	–
PLap	*B. afzelii*	OspA negative	+	+	+	+	+	–	–	–	+	–	–
PStb	*B. afzelii*	OspA negative	+	+	+	+	+	–	–	–	+	–	–
PKi	*B. garinii*	8	+	+	+	–	+	–	–	–	–	–	–
PLa	*B. garinii*	8	+	+	+	–	+	–	–	–	–	–	–
PFCa	*B. garinii*	2 and 4	+	+	+	+	+	–	–	–	+	–	+

Another notable new finding is the high frequency of OspA type 4 among isolates from adult patients, whereas this type has been rarely observed among isolates from children or ticks. In contrast, OspA type 6 was detected most frequently in the CSF from children and in ticks. With some exceptions [1, 38], *B. garinii* strains have been primarily isolated from *I. ricinus* from different regions in Europe [2, 5, 27, 43]. The fact that we could not identify OspA type 4 among >90 tick isolates from various regions in Europe (unpublished results) suggests that culture isolation from the tick may be difficult. It is possible that OspA type 4 is present in mixed infections in the tick and is overgrown in culture by other types, which may explain why this type has only been isolated by PCR [15]. Another possibility is that OspA type 4 is present in certain organs of the tick outside the midgut, the organ that is normaly examined in tick surveys. Phenotypical differences have been described for isolates from different organs of *I. ricinus* [24]. The geographical distribution of OspA type 4 in ticks is also not known and deserves further examination, preferentially by PCR. However, OspA type 4 strains have been also isolated from the CSF of patients from the Netherlands [43] and Denmark [23], indicating that this type is widespread.

It is also notable that (with the exception of type 7) each OspA type (1 – 6, and 8) has not only been isolated in Munich from the CSF of patients but also from at least one other geographical region [France (type 1), Austria (type 5), the Netherlands (type 4), Denmark (type 4), Sweden (type 6), Göttingen in Germany (types 2 – 6, and 8)] [2, 23, 43]. This is an important finding since it suggests that pathogenic strains are not only heterogeneous but each type also widespread. With the exception of type 4 we also found the whole panel of OspA types in isolates from ticks collected in at least two different regions in Europe (Wilske et al., unpublished results).

We found that the OspC-expressing OspA types (1, 2, 4, 6, and 7) also differed in their OspC phenotype, whereas the two OspA-type 8 strains revealed the same OspC-mAb reactivity as one out of the three OspA type 6 strains. Surprisingly, OspC heterogeneity was not as pronounced as could be expected from a previous study including isolates from ticks and human skin [44]. In particular it is notable that the *B. afzelii* strains were conserved in their OspC phenotype, which is in contrast to previous findings with skin isolates. This possibly indicates that a special type of *B. afzelii* is associated with neuroborreliosis. In addition, all OspA type 4 strains had identical OspC phenotypes, which corresponds to nearly identical *ospC* sequences of the three OspA serotype 4 strains analyzed so far [19, 37]. The OspA serotype 4 strains may represent a recently emerged variant of *B. garinii*, which may have developed a mechanism leading to a better chance of survival in hosts immune to other more frequently occuring types. Such an escape mechanism could also explain the increasing prevalance of OspA type 4 with the age of the patients and the decreasing frequency of OspA type 6, the most frequent tick isolate. Subtyping of the OspA serotype 4 strains (for example by sequence analysis of OspAs, OspCs, and p83/100 homologues – proteins which were highly con-

served among the few hitherto analyzed OspA type 4 strains [14, 19, 32, 37]) should be performed on all OspA type 4 isolates. Interestingly, the p83/100 homologues (which have been shown to be conserved among the OspA type 4 strains so far characterized) are rich in Arg-Gly-Asp (RGD) peptides [32], which are ligands for integrins (adhesion molecules) that have been shown to be receptor molecules on platelets for *B. burgdorferi* [10]. Thus, differences in the structure of p83/100 homologues may account also for differences in adhesion.

The considerable heterogeneity of OspA amongst the pathogenic *B. garinii* strains may indicate that immunoselection of OspA variants is more effective than for the other two species which are apparently highly conserved in their OspA-proteins [9, 12, 14, 39, 40, 43]. One might speculate that OspA is better expressed in the host by strains that disseminate, since it has been shown that OspA binds plasminogen on the surface of the *Borrelia,* thus leading to enhanced dissemination of the spirochetes in the host [11, 18, 22].

Differences in the expression of Osp proteins may account for differences in the organotropism of strains. Such differences in expression of variable major proteins has been associated with different clinical manifestations in the *B. turicatae* scid mouse model, in which one type caused neuroborreliosis and the other arthritis [8]. Since differences in the expression of OspA and OspC may be strain-specific and not simply associated with the type of species (for example such differences in Osp expression have been also observed between two different *B. afzelii* strains [21]), differences in Osp expression may also explain the fact that not only *B. garinii* causes neuroborreliosis.

OspA and OspC are the major candidates for a *Borrelia* vaccine [16, 30, 31, 34]. It has been shown by Johnson et al. [20] that whole cell vaccines derived from different strains are not necessarily cross-protective. Various authors demonstrated lack of cross-protection for OspA vaccines derived from different strains, some demonstrated that this was associated with differences in the OspA sequence [17, 25, 26, 35]. In addition, it has been shown that a neutralizing OspA epitope is recognized by a type-specific mAb [34, 42]. However, the latter topic has not been investigated intensively. Our defined panel of type-specific and cross-reactive mAb against OspA and OspC will enable a systematic analysis of specific and conserved neutralizing epitopes. Also of interest is whether epitopes recognized by the *B. garinii*-specific mAb L18 A14 are neutralizing, this question is important in the light of the considerable heterogeneity of OspA in *B. garinii* and the recent findings that antibodies to low molecular weight proteins are also borreliacidal [3, 33].

We have characterized a broad panel of strains of human origin that display a considerable heterogeneity of OspA and OspC. Before a European *Borrelia* Osp vaccine for use in humans can be establihed, it is necessary to show that the vaccine protects animals against the various Osp types from the broad panel of human isolates.

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