ORIGINAL ARTICLE

Association of distinct species of *Borrelia burgdorferi* sensu lato with neuroborreliosis in Switzerland

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Objective: To evaluate by species-specific immunoblots the association of *Borrelia burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* with neuroborreliosis in Switzerland.

Methods: *Borrelia* strains isolated from the cerebrospinal fluid (CSF) of three children with neuroborreliosis were typed by phenotypic and genotypic analysis. The serologic reactions (IgG) of these three patients as well as those of 28 patients, including one of these three children, with confirmed neuroborreliosis were characterized and scored by immunoblots on the three individual *Borrelia* species antigens. Twenty patients with typical erythema migrans served as a control group.

Results: Phenotypic and genotypic analysis confirmed that all three CSF isolates were *B. garinii*. In the 28 patients with neuroborreliosis, the comparatively strongest reactions were as follows: 18 to *B. garinii*, three to *B. burgdorferi* sensu stricto and two to *B. afzelii*; five were inconclusive. In the control group (erythema migrans), the comparatively strongest reactions were as follows: six *B. garinii*, one to *B. burgdorferi* sensu stricto and five to *B. afzelii*; eight were indeterminate.

Conclusions: Typing of these three CSF isolates and characterization by immunoblots of the antibody reactions of patients with neuroborreliosis give additional evidence of the association of *B. garinii* and neuroborreliosis. Our serologic results suggest that *B. burgdorferi* sensu stricto and *B. afzelii* are also responsible for some neuroborreliosis cases in Switzerland. Our immunoblots and the scoring system proved particularly useful for the serologic typing of patients with late Lyme borreliosis.

Key words: Neuroborreliosis, Lyme borreliosis, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, immunoblots, isolation, typing, association

INTRODUCTION

Borrelia burgdorferi was discovered and recognized as the causative agent of Lyme borreliosis by Burgdorfer et al [1]. This spirochete is transmitted by ticks of the *Ixodes ricinus* complex. In Switzerland, up to 55% of *Ixodes ricinus* ticks are infected with *B. burgdorferi* sensu lato [2–4].

The first clinical manifestation of Lyme borreliosis is a distinctive skin lesion, erythema migrans (EM), which appears a few days to a few weeks after tick bite. This lesion may last from 3 days to several weeks and may be accompanied by fever, headache and malaise [5]. Patients seem to recover, but without antibiotic treatment some will suffer from complications such as neurologic disorders, cardiac manifestations, or articular migrating pain. In some patients, chronic diseases may develop which may affect the skin, such as acrodermatitis chronica atrophicans, a clinical manifestation primarily observed in Europe, or the joints with arthritis, which is more common in the USA [5]. Neurologic disease with chronic meningitis or severe degenerative cerebral lesions that may mimic multiple sclerosis may also develop. Neuroborrelioses are also more frequent in Europe, and differences in the clinical manifestations have been described compared to those

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in the USA [6]. Shortly after its discovery in ticks, the causative agent, later named *B. burgdorferi*, was isolated from the blood and, thereafter, from the skin, cerebrospinal fluid and synovial fluid of patients in many parts of the world [7–15]. More recent genetic and phenotypic analyses of *B. burgdorferi* isolates have revealed differences leading to the classification of *B. burgdorferi* sensu stricto, *B. garinii* [16] and *B. afzelii* [17]. Some studies described correlations between each of these species and specific clinical manifestations of the disease: *B. burgdorferi* sensu stricto with arthritic conditions, *B. garinii* with neurologic disorders, and *B. afzelii* with the skin disorder acrodermatitis chronica atrophicans [18–20]. However, controversial reports described a good match between the distribution of *Borrelia* in ticks and in patients of the same area, suggesting a lack of organotropism [21] or organotropism linked to strain-specific characteristics, not to genotypes [22].

These three species can be readily typed by Western blot according to immunoreactions with monoclonal antibodies H3TS, specific for *B. burgdorferi* sensu stricto, D6 for *B. garinii*, and I 17.3 and J 8.3 for *B. afzelii* [16,17,23] or by PCR restriction fragment length polymorphism analysis [24]. In this study, the relationship between neuroborreliosis and three species of *B. burgdorferi* sensu lato was investigated. The borreliae isolated from the cerebrospinal fluid (CSF) of three children with neurologic symptoms were characterized. The antibody responses of each patient to the three species of *B. burgdorferi* sensu lato and to their own isolate by immunoblot (Western blot) assays were compared. The particular antibody responses of these patients prompted us to look at a larger group of patients with confirmed neuroborreliosis. Sera from 28 patients, including one of these three children, with neuroborreliosis and intrathecal antibody synthesis were evaluated for reactions to *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* by immunoblot. A control group of 20 patients with EM was compared for reaction to the three species by the same method in order to check for the prevalence of the *Borrelia* species associated with the early stage of the disease. Patients from both groups came from the same geographic area, the western, or French-speaking, part of Switzerland.

MATERIALS AND METHODS

Patients for *Borrelia burgdorferi* sensu lato isolation

Thirty-four CSF samples from children were cultivated for isolation of *B. burgdorferi* sensu lato. Complete case reports have already been published for the three children with isolates [11]. In short, the first patient (BP), a 4-year-old boy, was admitted to the hospital of Visp (Valais, Switzerland) in August 1990 for left facial paralysis. The second patient (BM), a 7-year-old boy, was hospitalized in summer 1991 at the same hospital. He had a diplopy, accompanied by loss of balance. Neither of these two patients had detectable antibody in their CSF. The third patient (DA), a 7-year-old boy, was admitted in September 1991 to the University Hospital of Lausanne (CHUV, Vaud, Switzerland) for epilepsy-like-crisis, and 4 weeks later for a papillary edema. High antibody titers to *B. burgdorferi* sensu lato were detected in serum as well as in CSF.

CSF was centrifuged at 10 000*g* for 10 min. The supernatant was collected for serologic testing. The pellet was resuspended in a few drops of the remaining supernatant and cultured in 5 mL of BSK-II medium (Barbour–Stoenner–Kelly medium) [25]. Dark field examination of the culture was performed twice, first after 10 days and then after 21 days. After 3 weeks, the culture was centrifuged at 10 000*g* for 10 min, the pellet was resuspended in fresh BSK-II medium, and the culture was kept for 3–4 additional weeks.

Preparation of antigens

The strains of *Borrelia* were: B31 reference strain isolated from *Ixodes scapularis* (*I. dammini*, Shelter Island, NY) (*B. burgdorferi* sensu stricto), VS 215 (*B. burgdorferi* sensu stricto from *I. ricinus*), VS 3, VS 102 (*B. garinii* from *I. ricinus*) and VS 461 (*B. afzelii* from *I. ricinus*) [23] and the three isolates from the patients [11]. The cultures were collected during their logarithmic growth phase, and washed twice in phosphate-buffered saline (PBS) (0.1 M, pH 7.45) with added $MgCl_2$ (5 mM). For the electrophoresis, the pellet was resuspended in distilled water. The protein concentration of the suspension was determined by the Biuret method, and adjusted to 1 mg/mL with distilled water; the suspension was then frozen until use. The isolate VS 3, used as antigen for the immunofluorescence assay, was washed first in PBS as above and then briefly in distilled water containing 0.05% sodium dodecylsulfate (SDS). The pellet was quickly resuspended in PBS containing 5% yolk sac.

Isolates used in this study were all low-passage strains (fewer than eight passages in BSK-II medium).

Genetic characterization of isolates

Isolates were identified to the species level by restriction enzyme analysis of *rrf*(5S)–*rrl*(23S) intergenic spacer amplicons as previously described [24]. Briefly, two oligonucleotides in the 3′ end of the first 5S rRNA (*rrf*) and the 5′ end of the second 23S rRNA (*rrl*) were used. The reaction occurred in a 50-μL reaction mixture containing 5 μL of bacterial thermolysate. Positive and negative controls were included in each reaction. The

PCR was carried out under the usual conditions, in an OmniGene thermocycler (Hybaid Limited, UK), for a total of 35 cycles (94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min). Amplified DNA was electrophoresed through 1% agarose gel and visualized by ethidium bromide staining.

PCR products were digested by the endonucleases *Mse*I (New England Biolabs, Beverly, MA, USA) and *Dra*I (Amersham International, Amersham, UK) according to the manufacturer's instructions. Electrophoresis of restricted DNA was carried out in a 16% acrylamide/0.8% bisacrylamide gel for 90 min at 130 V. Restricted DNA fragments from type strains were used as size controls.

Patients for serological study

Twenty-eight sera from symptomatic patients with neuroborreliosis proven by intrathecal antibody synthesis (index 2.2–144, cut-off value 2) were evaluated for immunologic (IgG) reaction to the three species of *B. burgdorferi* sensu lato. Sera from 20 patients with typical EM and a positive screening test ELFA (enzyme-linked fluorescent assay) (Vidas Lyme IgG and IgM bioMérieux, France) for *Borrelia* were randomly chosen as a control group.

Screening tests

The indirect microimmunofluorescence assay (IgG and IgM) was performed as described previously [26] and adapted for *B. burgdorferi* sensu lato (isolate VS 3, *B. garinii*). Sera were diluted starting at 32 (reciprocal titer) and CSF at 2. In the sera, IgG antibody titers of 256 or more were considered as positive, as were antibody titers of 8 or more in the CSF. Immunoglobulins M and A at a titer of 32 were defined as positive in the absence of rheumatoid factor. The index of intrathecal antibody synthesis was calculated as follows: IgG-specific CSF/serum divided by albumin CSF/serum (negative below 2.0).

Sera were screened for antibody to *B. burgdorferi* sensu lato by ELFA VIDAS Lyme IgG and IgM (BioMérieux, France). This commercial test used a *B. burgdorferi* sensu stricto strain (B31). The tests were performed and interpreted according to the manufacturer's instructions. Relative fluorescence values (RFV) <0.75 were negative, 0.75–1.00 equivocal and >1.00 positive.

Electrophoresis and immunoblot

The SDS-PAGE and immunoblot assay were performed as previously described [23]. In short, the suspension of washed borrelia (protein concentration of 1 mg/mL) was electrophoresed (constant voltage 170 V) on a 12.5% polyacrylamide gel. The standard molecular weights (MW) of BioRad (low-range protein molecular weight standards) were used as references for the calculation of relative molecular weights. The gels were either stained with Coomassie blue or were transferred by Western blot to polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore, Kloten, Switzerland).

Immunoreactions were performed with human serum diluted 1:200 or with monoclonal antibodies diluted 1:500 for H3TS (Symbicon, Sweden), 1:500 000 for I 17.3 and J 8.3 (kindly provided by G. Baranton, Institut Pasteur, Paris), and 1:100 for D6. Fixed antibodies were revealed by a secondary antibody (anti-human or antimouse IgG γ chain specific or IgM μ chain specific) conjugated to alkaline phosphatase, followed by the addition of substrate BCIP/NBT (5-bromo-4-chloro-3-indoyl-*p*-toluidine phosphate/*p*-nitroblue tetrazolium chloride) (Kirkegaard and Perry Lab., Gaithersburg MD, USA).

Criteria for a positive IgG immunoblot with human serum were a minimum of five bands, including flagellin and two of the following specific bands: OspC, OspA, OspB, p39, 93 kDa (p100). Our criteria were almost identical to those of Engström et al [27].

Immunoblots for IgG to local isolates belonging to the three species were performed with three strips, one for each species (VS 215, *B. burgdorferi* sensu stricto; VS 102, *B. garinii* and VS 461, *B. afzelii*). They were prepared and incubated with the sera of individual patients and were processed at all steps in the same tray strip.

Immunologic reactivity scoring

For comparison between the three immunoblots, scores were allocated (0–3 points) depending on the presence and intensity of the reaction to seven proteins of *Borrelia* as shown in Figure 1: 93 kDa (p100), flagellin, p39, OspA, OspD, OspC and 18 kDa. All but 18 kDa proteins were first identified in the three *Borrelia* species by monoclonal antibodies: 181.1 (p93), H9724 (flagellin), p39, H5332 (OspA), H1C8 (OspD), LA22 2B8 (kindly provided by A. G. Barbour, University of California, Irvine CA; W. T. Golde, CDC, Fort Collins CO; T. Schwan, Rocky Mountain Lab., Hamilton MT, USA; B. Wilske Max von Pettenkofer Institute, München, Germany). Readings were performed by two independent persons. A total score superior by two points for one individual species compared with the other species was considered as a specific reaction (for example, B 13, A 12, G 16 was typed as *B. garinii* (Figure 1)). This criterion was based on the significant differences observed in the statistical analysis of the mean scores. Since only minor divergences were observed for the typing of the

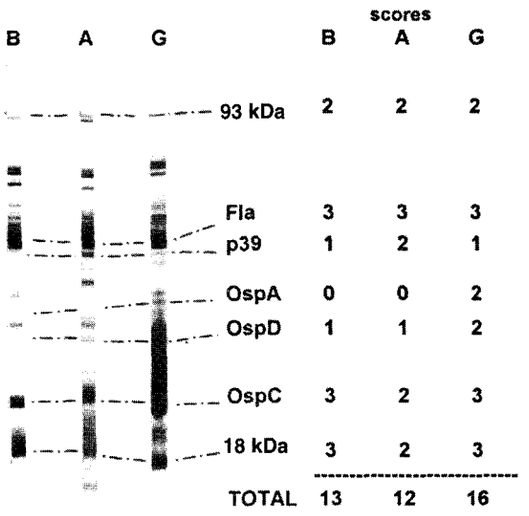

Figure 1 Immunoblots (IgG) with the serum of a patient with neuroborreliosis and reaction specific to *B. garinii*. Strip B: VS 215—*B. burgdorferi* sensu stricto. Strip A: VS 461—*B. afzelii*. Strip G: VS 102—*B. garinii*. Score points are allocated to proteins: 93 kDa, flagellin, p39, OspA, OspD, OspC and 18 kDa.

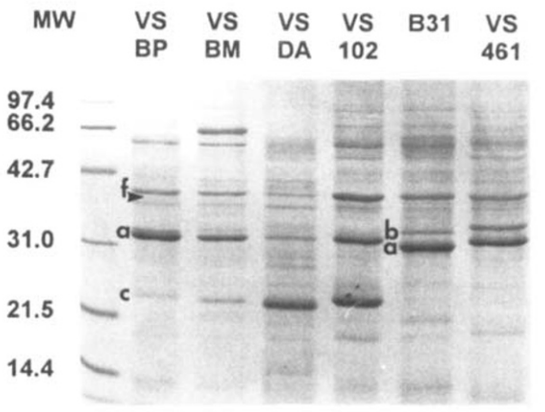

Figure 2 Coomassie blue-stained SDS-PAGE of whole cell lysates of *B. burgdorferi* isolates. VS BP, VS BM, VS DA: CSF isolates. VS 102, *B. garinii* tick isolate; B31, *B. burgdorferi* sensu stricto reference strain; VS 461, *B. afzelii* reference strain, tick isolates. MW: Molecular mass in kilodaltons. f=flagellin; arrow=p39; a=OspA; b=OspB; c=OspC.

serologic reactions between the two readers, we used the classification of one reader only.

Statistical analysis

A statistics software package (Statsoft, Phoenix, AZ, USA) was used to analyze differences between the EM and neuroborreliosis groups (chi-squared test) and differences between individual paired reactivity scores for *Borrelia* species (Wilcoxon matched paired test).

RESULTS

Isolation and characterization of *Borrelia burgdorferi* sensu lato

Thirty-four CSF samples from children were cultivated and three of them provided isolates. The culture characteristics of the three isolates varied. The first isolate (VS BP) was obtained after 6 weeks of culture, whereas the isolate VS BM grew within 10 days, and the culture was highly positive after 7 days with the isolate VS DA.

The protein patterns of all the isolates, as detected by Coomassie blue staining (Figure 2), revealed many identical bands, mainly above the flagellin (41 kDa). The electrophoretic mobility of the OspA (32.5 kDa) of the three human isolates was comparable to that of the tick isolate VS 102. Distinct protein patterns were observed with the three isolates. The VS DA isolate demonstrated a strong expression of OspC (23 kDa) and a low amount of OspA. The VS BM isolate showed a strong band around 66 kDa. The VS BP isolate expressed OspA strongly in comparison to other CSF isolates. The immunoreactivity of the isolates with the monoclonal antibody D6 (no reactions were detected with monoclonal antibodies H3TS and I 17.3) confirmed that these three isolates belonged to the species *B. garinii*.

PCR amplification of the spacer region between the two *rrf* and *rrl* genes generated a fragment of about 250 bp. The patterns produced by these isolates after cleavage by *Mse*I and *Dra*I were compared to those of the type strains of each of the species *B. garinii*, *B. burgdorferi* sensu stricto and *B. afzelii* (Figure 3). The three strains VS BP, VS BM and VS 102 exhibited the same pattern as the type strain 20047 of *B. garinii*, with three fragments of 108, 95 and 50 bp (Figure 3A). The *Mse*I pattern generated by the strain VS DA is slightly different, in that the fragment of 95 bp is about 5 bp longer. However, the *Dra*I pattern exhibited by this strain is indistinguishable from patterns of other *B. garinii* strains (Figure 3B) with these running conditions. Based on the analysis of restriction fragment length polymorphism of the amplified product of the *rrf–rrl* spacer, the four strains were assigned to the species *B. garinii*.

Homologous and heterologous antibody reactions

The first patient, BP, showed a very weak antibody response by immunoblot. A reciprocal titer of 64

Table 1 Serologic results of the serum and CSF from the three patients by enzyme-linked fluorescence assay (ELFA), indirect immunofluorescence assay (IFA) and immunoblot (IB)

Patients	BP	BM	DA
Serum			
ELFA (IgG+IgM)	0.80	1.07	5.72
IFA IgG	64	256	512
IFA IgM	<32	<32	<32
IB IgG	(+)	(+)	+++
IB IgM	–	–	–
CSF			
IFA IgG	<2	<2	256
IFA IgA	<2	<2	<2
IB IgG	–	–	+++
IB IgA	–	–	–

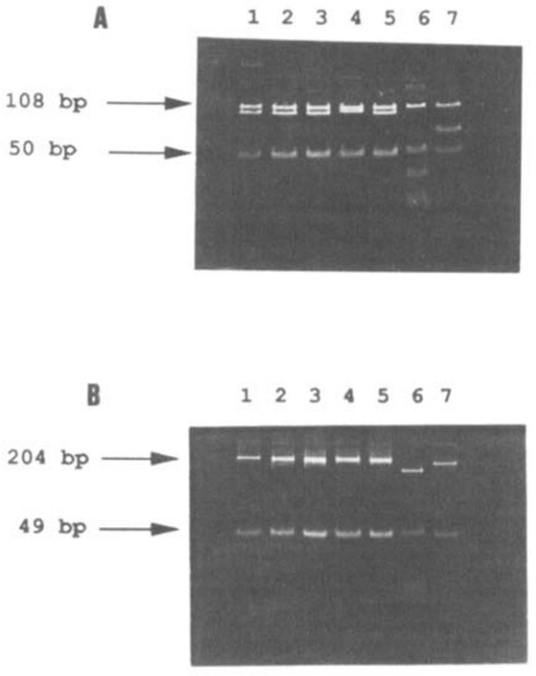

Figure 3 Restriction patterns of *B. burgdorferi* sensu lato strains, after digestion of PCR products by *Mse*I (A) and *Dra*I (B). DNA was electrophoresed on a 16% acrylamide gel, stained with ethidium bromide and UV illuminated. Lane 1: *B. garinii* strain 20047 (reference strain). Lanes 2–5: VS BP, VS BM, VS DA (CSF isolates) and VS 102 (tick isolate). Lane 6: *B. burgdorferi* sensu stricto strain B31. Lane 7: *B. afzelii* strain VS 461.

(insignificant) was defined by the indirect immunofluorescence assay (IFA) test and ELFA results were equivocal (Table 1). Patient BM developed specific antibodies detectable by IFA and ELFA, and the immunoblot was considered weakly positive. The serologic response of patient DA was clearly positive by IFA, ELFA and immunoblot. Specific IgG antibodies were also detected in the CSF by immunoblot and by IFA at a titer of 256 without IgA. The CSF samples of

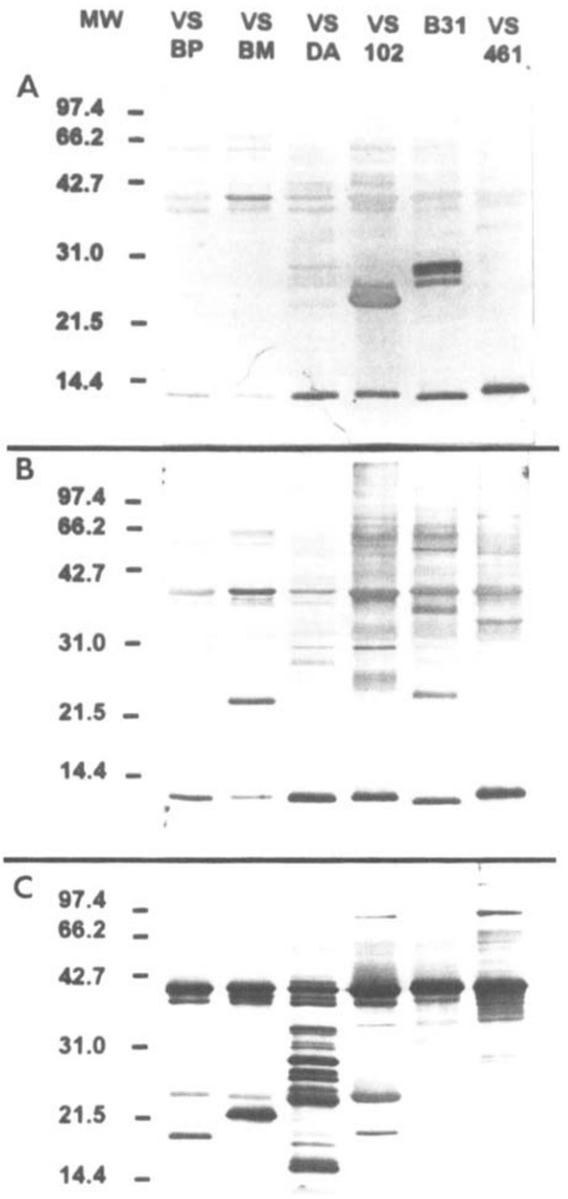

Figure 4 (A) Immunoblot IgG of serum from patient BP using the isolates in the same order as in Figure 2. (B) Immunoblot IgG of serum from patient BM using the same isolates. (C) Immunoblot IgG of serum from patient DA using the same isolates.

the other two patients were negative by both tests. No IgM was detected by immunoblot in the sera of the three patients.

We characterized the patients' antibody responses by immunoblot to their own isolates and to representative isolates of each of the species *B. burgdorferi* sensu stricto (B 31), *B. garinii* (VS 102) and *B. afzelii* (VS 461). Patient BP (Figure 4A) demonstrated a weak positive antibody response to VS 102 and to B 31, with detectable reaction to OspC and to a 12-kDa antigen. The reaction to flagellin was weak with all the isolates, except VS BM. The serologic reaction of patient BP to his own isolate would be considered as negative. Patient BM (Figure 4B) developed a stronger antibody response, mostly to isolates VS 102, B 31, VS DA and his own isolate VS BM. Antibodies to OspC, OspA, p39, flagellin and the 12-kDa antigen were detectable. Weaker reactions were observed to VS 461 and VS BP. The serum of patient DA showed a strikingly high reactivity to isolate VS DA (Figure 4C). This patient also developed a stronger serologic reaction to *B. garinii* isolates than to *B. burgdorferi* sensu stricto (B 31) and to *B. afzelii* (VS 461).

Serologic reactivity of patients with neuroborreliosis and with EM

Sera from patients with neuroborreliosis or EM were evaluated for IgG reactivity against local tick isolates belonging to the three species of *B. burgdorferi* sensu lato.

Table 2 Attribution of serologic reaction to one particular species using the criteria of a total score superior by two points compared with the other two *Borrelia* species

	No. of patients	G	B	A	Inc.
Neuroborreliosis	28	18 (64%)[a]	3 (11%)	2 (7%)[b]	5 (18%)
EM	20	6 (30%)[a]	1 (5%)	5 (25%)[b]	8 (40%)

[a]Chi-square $p<0.02$.
[b]Chi-square $p<0.01$.
G=*Borrelia garinii*; B=*Borrelia burgdorferi* sensu stricto; A=*Borrelia afzelii*; Inc.=inconclusive.

The serologic responses of the 28 patients with confirmed neuroborreliosis gave the following total scores (mean of the two readers): *B. garinii*, 353; *B. burgdorferi* sensu stricto, 283; and *B. afzelii*, 291. Scores for patient DA included in this group were: *B. garinii*, 11; *B. burgdorferi* sensu stricto, 8; and *B. afzelii*, 7. Scores for the control group (20 patients with EM) were: *B. garinii*, 155; *B. burgdorferi* sensu stricto, 143; and *B. afzelii*, 162. Median scores are shown for comparison in Figure 5. In the group of patients with neuroborreliosis, the differences between the scores of *B. garinii* and *B. burgdorferi* sensu stricto, as well as *B. garinii* and *B. afzelii*, are statistically significant

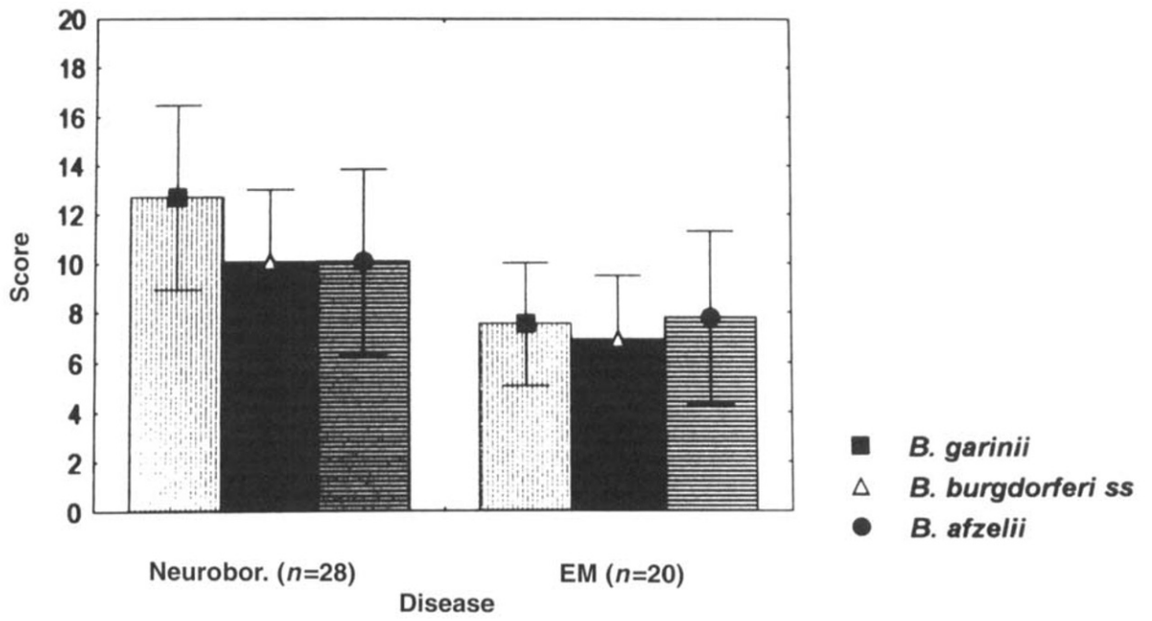

Figure 5 Mean scores for each *Borrelia* species in the group of patients with neuroborreliosis (Neurobor) and with erythema migrans (EM). Differences between the scores of *B. garinii* and *B. burgdorferi* sensu stricto ($p<0.001$) and *B. afzelii* ($p<0.005$) are significant for the neuroborreliosis group and are not significant for the control group (EM).

($p<0.001$ and $p<0.005$, respectively), whereas they are not significant in the control group. The criterion of total score equal or greater than 2 for one particular species allowed us to type the serologic reactions of patients with neuroborreliosis as follows: 18 *B. garinii*; three *B. burgdorferi* sensu stricto; two *B. afzelii*; and five inconclusive. In the control group, six reactions were typed as *B. garinii*, one as *B. burgdorferi* sensu stricto, and five as *B. afzelii* and eight were inconclusive (Table 2). Minor discrepancies were observed between the two readers, particularly with sera having a borderline score. For example, in the neuroborreliosis group, one inconclusive was grouped as *B. garinii* by the other reader, and one *B. garinii* plus one *B. burgdorferi* sensu stricto were found inconclusive by the other reader. The mean scores were also very close for the two readers; in the neuroborreliosis group, mean scores were *B. garinii* 12.7 and 12.5, *B. burgdorferi* sensu stricto 10.0 and 10.2, and *B. afzelii* 10.3 and 10.5, respectively. The comparison of relative incidences of *Borrelia* species showed a significant difference in favor of *B. garinii* in the neuroborreliosis group ($p<0.02$). On the contrary, there was a significant difference in favor of *B. afzelii* in the group EM ($p<0.01$), allowing us to observe the relative incidence of infecting *Borrelia* species in the early stage of the disease in our region.

DISCUSSION

Borrelia bacteria were cultured from three of 34 CSF samples from children in whom Lyme borreliosis was suspected. The three isolates were typed as *B. garinii* by phenotypic and genotypic analysis. Serum from one of these three patients, with more prolonged neurologic disease and intrathecal antibody synthesis, clearly reacted more strongly towards *B. garinii* (score 11 points) than towards *B. burgdorferi* sensu stricto (8 points) or *B. afzelii* (7 points) in immunoblot assays. Additionally, 17 sera out of 27 other sera from patients with neuroborreliosis confirmed by intrathecal antibody synthesis demonstrated a predominant reaction to *B. garinii*.

The association between *B. garinii* and neuroborreliosis in Europe has been described in few reports and always with a small number of isolates [10,19,20]. In 1990, Karlson et al [10] suggested that isolates with a particular OspA profile were responsible for neuroborreliosis in Europe. Although this is the only typing information available for the four Swedish isolates discussed, their protein profiles indicate that they are *B. garinii* isolates. Other reports of genotypic and phenotypic analysis have not clearly shown the *B. garinii*–neuroborreliosis association, as they included only a selection of CSF isolates [28–30]. However, they added important data showing that all three *Borrelia* species may be isolated from the CSF. Busch et al [9] confirmed on a large panel of 36 CSF isolates that all three species were present, but with a clear predominance of *B. garinii* (58%).

By serologic analysis, others confirmed that *B. garinii* was more frequently associated with neuroborreliosis in Europe than any other *Borrelia* species. Assous et al [18] were the first to propose this association on the basis of Western blot. They observed a percentage of 46.6% (7/15) of patients with neuroborreliosis who had a stronger reaction to *B. garinii*. This moderate percentage may reflect a difference between patients with early and late neuroborreliosis. Indeed, we observed in our three patients that only the one with prolonged neurological disease and intrathecal antibody synthesis developed a specific antibody response to *B. garinii*. This assumption motivated our selection of patients with neuroborreliosis and intrathecal antibody synthesis. In another study, Anthonissen et al [31] used a very efficient but time-consuming method of Western blot of each *Borrelia* species, testing for residual reactivity after absorption of sera with antigens of representative genospecies. Their 18 Belgian patients with neuroborreliosis showed preferential reactivity to *B. garinii*.

The prevalence of *Borrelia* species in one particular geographic area may represent a selection bias for their relative recognition in association with a particular symptom. We have chosen to compare the serologic status of patients with early infection (EM) with that of a group of patients from the same area with late neurologic symptoms.

The scores applied in the present study were simple to achieve and showed a good reproducibility between the two readers. They allowed us to determine on a semi-quantitative basis the differential reactivities to *Borrelia* species in most patients. We selected a number of proteins for the scoring method, on the basis of high immunogenicity, such as flagellin or OspC, and five other high-, medium- and low-molecular-weight proteins. Some of these proteins appeared to better differentiate the specific serologic reaction, in particular the 18-kDa, OspC, p39 and 93-kDa (p100) proteins in our group of patients with neuroborreliosis. The comparison of relative incidences of infections probably due to one particular *Borrelia* species showed a significant difference, with the group of patients with neuroborreliosis favoring *B. garinii* ($p<0.02$) and the control group favoring *B. afzelii* ($p<0.01$). Total scores and differences in the control group were less important, since the serologic reaction of patients with EM was restricted to fewer proteins, particularly flagellin, OspC and 18 kDa. In fact, the scores as presented seem more useful for quantifying the

serologic reaction of patients with late Lyme borreliosis, since the number of reactive proteins is larger and allows a better total score differentiation. We have found a similar, and even better, association of *B. afzelii* and acrodermatitis chronica atrophicans. Out of 30 patients' sera, 28 (93%) were more reactive to *B. afzelii* using the same typing method (unpublished). Furthermore, in a group of patients with arthritis, about 70% showed a stronger reactivity to *B. burgdorferi* sensu stricto. This study is in progress and includes the fourth *Borrelia* species (group VS 116) present in the area. So far, serology has been an essential tool for the diagnosis of Lyme borreliosis, mainly in the late stages, but its usefulness is less in endemic areas because of high seroprevalence in the population. When conclusive data are available on the organotropism of the *Borrelia* species, typing of antibody reactions to the three *Borrelia* species by immunoblot or by other serologic tests will be of great interest and will provide further information for the definite diagnosis of late Lyme borreliosis.

In the literature there is more and more evidence that clinical manifestations such as arthritis, neurologic symptoms and acrodermatitis chronica atrophicans are increasingly associated with *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* respectively [18–20,28,29,31]. These differences of association between *Borrelia* species and clinical manifestations may reflect a particular tropism of each *Borrelia* species for a tissue or organ. The mechanism of such tropism remains to be investigated. In addition, a fourth genospecies of *Borrelia*, called group VS 116 [23,24] occurs in ticks of the area under study as well as in Europe [32]. Up to now, strains belonging to this group have never been isolated from humans. However, these strains may also be more difficult to grow from patient material. With regard to the possible role of this genomic group in Lyme borreliosis, we are investigating this issue, using the same immunoblots, including this fourth species, with sera from patients having defined manifestations of Lyme borreliosis.

Culture is not recommended for routine diagnosis of Lyme borreliosis [33] but the isolation of *Borrelia* species from CSF may be very useful for the diagnosis of neuroborreliosis, since a weak serologic response is sometimes obtained from patients with early neurologic symptoms. In this regard, PCR may become an essential tool for the early diagnosis and typing of the causative agent in the future [32,34].

In conclusion, typing of these three CSF isolates and characterization by immunoblot of the antibody reactions of patients provide additional evidence of the association between *B. garinii* and neuroborreliosis. Our serologic results suggest that *B. burgdorferi* sensu stricto and *B. afzelii* are also responsible for some neuroborreliosis cases in Switzerland.

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