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Expression des protéines de surface OspA et OspC chez *Borrelia burgdorferi* sensu lato pendant son cycle biologique

THESE

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Characterization of *Borrelia burgdorferi* isolated from different organs of *Ixodes ricinus* ticks collected in nature.

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Characterization of Borrelia burgdorferi Isolated from Different Organs of Ixodes ricinus Ticks Collected in Nature

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Abstract

Borrelia burgdorferi was isolated from 22 out of 133 adult *Ixodes ricinus* ticks collected from vegetation at two sites in Switzerland. From 17 ticks, spirochetes could be isolated from more than one organ. When the different isolates obtained from one tick were compared by SDS-PAGE analysis, differences in the protein profiles were observed in 8 cases. The isolates were further compared by immunological methods using mono- and polyclonal antibodies. Differences were observed in the proteins of 31–35 kDA and 18–25 kDa. Genetic divergence among isolates was evaluated by use of a *B. burgdorferi* specific gene probe for o*spA*. Correlation could be observed between immunological differences in OspA defined by monoclonal antibody LA31 and genetic variation of o*spA* as judged by restriction fragment length polymorphism (RFLP). Our findings indicate that systemic infection in unfed *I. ricinus* adults, as reflected by isolation of *B. burgdorferi* from multiple organs of one tick, is more frequent (8/22, 36%) than previously described (5%). Moreover, the presence of different *B. burgdorferi* phenotypes/genotypes in one tick is described for the first time. The findings may have bearings (i) on the time of tick attachment required for spirochete transmission since borreliae are already present in the salivary glands of systemically infected ticks at the beginning of the blood meal and (ii) perhaps also on the diversity of *B. burgdorferi* phenotypes inoculated by these ticks.

Introduction

Lyme borreliosis, a multisystemic disorder caused by *Borrelia (B.) burgdorferi,* is a tick-borne disease widely distributed over the northern hemisphere. In Europe, *B. burgdorferi* was isolated from its tick vector, *Ixodes (I.) ricinus* (2, 6). In Europe, human and tick isolates of *B. burgdorferi* present a variability with respect to their overall protein profiles and their outer surface proteins (Osp) A and B, whereas American isolates appear to be more homogeneous (3, 4, 13, 19, 20). In addition, some

European isolates present a major protein band of 20–23 kDa, designated pC or OspC (3, 15, 20, 21).

To date, most of the tick isolates were obtained from tick midgut. Because systemic infection with *B. burgdorferi* may be present in 5% of *I. ricinus* adults (6), we were interested in isolating spirochetes from different tick organs and in determining whether *B. burgdorferi* strains isolated from distinct organs may present distinctive protein and antigen profiles.

Material and Methods

Ticks and Borrelia isolation. I. ricinus (61 males and 72 females) were collected from the vegetation in two endemic areas in Switzerland (the Staatswald and Neuchâtel) between March and August 1991. The ticks were dissected in PBS (pH 7.4), the different organs were immediately inoculated individually into tubes containing 4 ml of BSK II medium (1,2) supplemented with antibiotics (Rimactan (Ciba-Geigy) 50 µg/ml and Phosphomycin (Boehringer) 50 µg/ml), and incubated at 34 °C.

SDS-PAGE analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (7). Briefly, whole cells were suspended in sample buffer (PBS containing 5 mM M_gCl_2) to yield a protein concentration of 30 µg/lane. The final pellet was resuspended (1 : 2) in sample buffer containing 250 µl Tris/HCl 0.5 M, 12.5% (v/v) glycerol, 4% (w/v) sodium dodecyl sulphate, 10% (v/v) mercaptoethanol and 0.1% (w/v) bromophenol blue.

The ratio of acrylamide to bis-acrylamide was 30 : 0.8. The pH of the separating gel buffer was 8.8 and the acrylamide concentration was 12.5%. A current of 20 mA was used to separate proteins for 30 min followed by 30 mA for 1.5 h. The gels were stained with Coomassie brilliant blue R-250. Molecular weight standards were run with each gel (low range Rad's SDS-PAGE standards).

Immunoblotting. Proteins separated by SDS-PAGE (15 µg of spirochetal proteins/lane) were transferred to a nitrocellulose paper by using an electrophoretic transfer unit (2117-250 Nova Blot Electrophoretic Transfer Kit, LKB AB Broma, Sweden), and Tris-glycine (pH 8.3) as transfer buffer, and using a current of 0.8 mA/cm^3 of gel for 1 h.

After transfer, the paper was cut into strips which were blocked with 5% fat-free milk during 2.5 h and washed with TBS (pH 7.5) (Tris-HCl 1M, NaCl 5M) at room temperature. The strips were incubated overnight at room temperature in 5% fat-free milk and TBS (1 : 4 – TBS-milk) with different monoclonal antibodies (moAbs) and polyclonal antibodies (poAbs). After three washings with 5% fat-free milk, the strips were incubated with peroxidase-labelled anti-mouse IgG antibodies or anti-rabbit IgG antibodies (1 : 1000, Nordic Immunological Laboratories, The Netherlands) for 2 h. Bound antibodies were visualized with 4 chloro-1-naphtol (Fluka). Finally, all strips were washed with distilled water.

Monoclonal and polyclonal antibodies. The monoclonal antibodies LA-31 (anti-OspA); LA-27, LA-25 (anti-OspB) and LA-7 (anti-20 kDa) (11) and the monoclonal antibodies H5332 specific for OspA and H9724 specific for the flagellin obtained from *Alan Barbour* (5) were used for immunoblotting.

The two polyclonal monospecific antibodies anti-22 kDa/NE4 and anti-B31 were produced by immunizing New Zealand rabbits, as described previously (10).

Southern blot hybridization. Total genomic DNA was extracted from *Borrelia* organisms as described previously (18). Approximately 5 µg of DNA was digested with 100 U of restriction nuclease (HindIII) according to the manufacturer's recommendations (Boehringer, Mannheim). Samples were subjected to electrophoresis using a 0.7% agatose gel. DNA fragments were transferred to Hybond^{TM}-N nylon membrane (Amersham) followed by UV-cross-linking and hybridization as described (18). Briefly, using ^{32}P-labelled *ospA* gene probe, hybridization was done overnight at 65 °C in 0.5 M $NaHPO_4$/7% $NaDodSO_4$,

pH 7.2. Afrer washing in 40 mM $NaHPO_4$/1% $NaDodSO_4$, pH 7.2 at room temperature for 30 min, the dry membrane was autoradiographed on Kodak XAR-5 film with intensifying screens at −80 °C for 1 to 12 h.

Results

One hundred thirty three *I. ricinus* adult ticks were collected by flagging the vegetation of two endemic areas in Switzerland (Staatswald n = 77 and Neuchâtel n = 56). Isolates were obtained from 22 out of 133 ticks (Table 1). In 17 out of 22 ticks, spirochetes were obtained from more than one organ. Since cross contamination between different organs during dissection could account for multiple isolations, we considered a tick as systemically infected when we obtained isolates from more than one organ and when these isolates presented different protein profiles. Among the isolates obtained from the 17 ticks, those from 9 ticks presented identical protein profiles and were thus not considered further. Isolates obtained from the remaining 8 ticks (3 from the Staatswald: NE6, NE9 and NE27 and 5 from Neuchâtel: NE8, NE20, NE21, NE24 and NE25) showed different protein profiles and were thus considered as having a systemic infection with phenotypically distinct strains of *B. burgdorferi* (Table 1).

Table 1. Systemic infection with *B. burgdorferi* of *I. ricinus* collected in nature

Sites	Number of collected ticks	Number of infected ticks (%)	Number of systemically infected ticks (%)*
Staatswald	77	13 (16.9)	3 (23.1)
Neuchâtel	56	9 (16.1)	5 (55.6)

* Ticks presenting isolates with different protein profiles.

These isolates were submitted to less than 5 *in vitro* subcultures with the exception of NE20M, NE21RA, NE21H, NE21U, NE24U, NE27M and NE27SG which were passaged 6 times. NE9H, NE20SG, NE20H, NE20S, NE20RA, NE25M, NE25T were submitted to 7 *in vitro* passages, and NE25H, to 10.

The isolates from these 8 ticks displayed differences in the protein profile in the 31–35 kDa and 18–25 kDa ranges. The differences were either qualitative (i.e. the proteins were either present or not) in one or more of the isolates from the same tick, or they were quantitative (i.e. the intensity of the protein band varied) (Fig. 1). No relation was observed between the organ of isolation and the protein or antigen patterns (Fig. 1).

Antigenic differences as revealed by reactivity with specific moAbs in immunoblotting were demonstrated in the spirochetes isolated of 5 out of 8 systemically infected ticks: 2/3 from the Staatswald (NE6 and NE9), and 3/5 from Neuchâtel (NE8, NE20 and NE24) (Fig. 1). The reactions of the various isolates with the poAbs (anti-22kDa/NE4 and anti-B31) showed also antigenic differences (data not shown). The number of reacted proteins varied between the different isolates of a tick. A six fold difference in

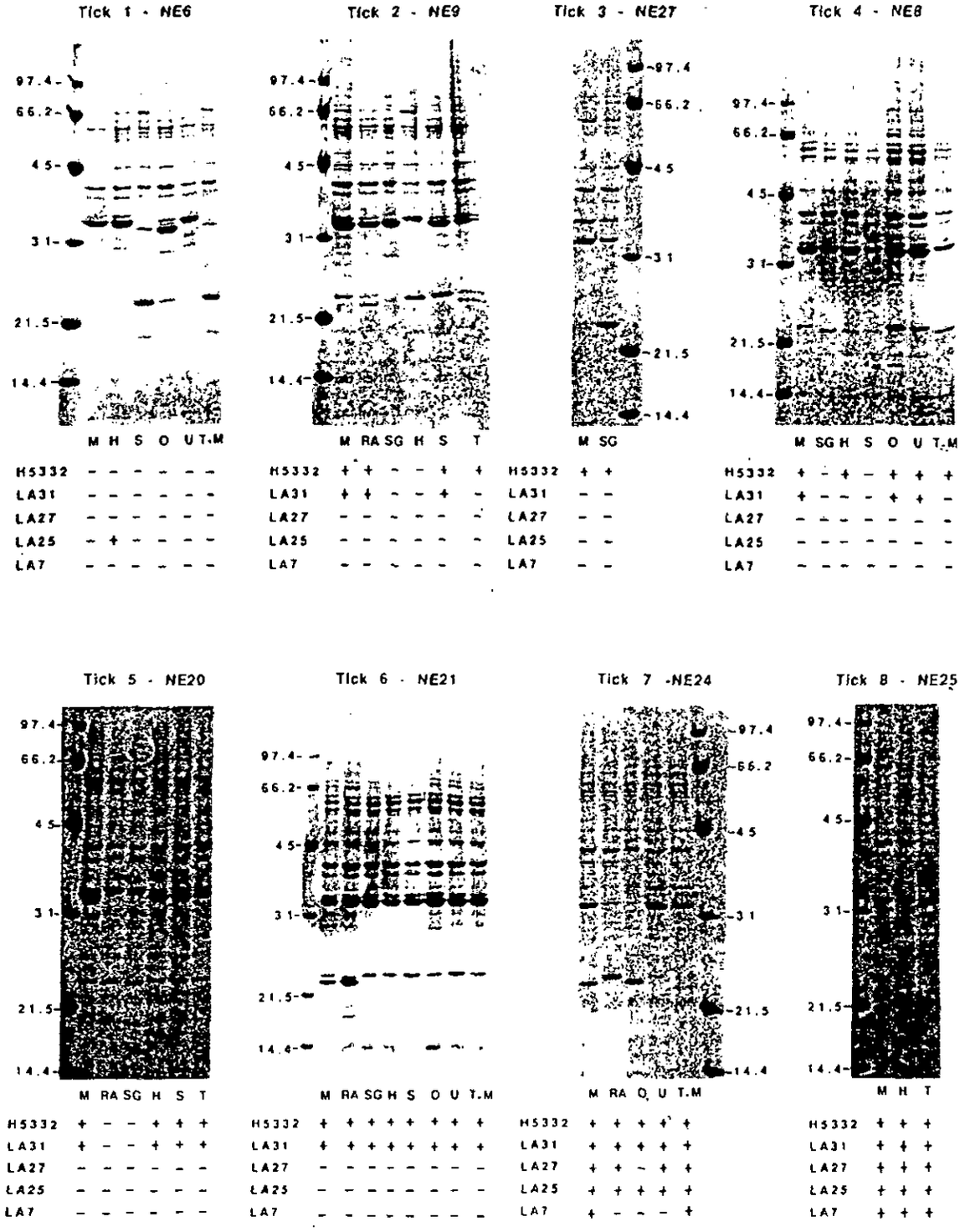

Figure 1. SDS-PAGE analysis of *Borrelia burgdorferi* isolates derived from ticks collected in two areas (the Staatswald: NE6, NE9, NE27 and Neuchâtel NE8, NE20, NE21, NE24, NE25) and their reactions with monoclonal antibodies.
Organs of isolation: (*) M = midgut, RA = rectal ampoula, SG = salivary glands, H = hemolymph, S = synganglion, O = ovary, U = uterus, T = testis, T+M = trachea and Malpighian tubules.
The different monoclonal antibodies used in this analysis were H5332, LA-31 (anti-31 kDa), LA-25, LA-27 (anti-34 kDa), and LA-7 (anti-20 kDa).
The molecular weight standards are marked on the left and on the right of the SDS gel.
+/–: positive or negative reactions with the respective monoclonal antibody

the number of reacting proteins could be observed in the 31–35 kDa and 20–24 kDa regions.

Previous studies have shown the existence of distinct OspA genomic groups (18, 19). Anti-OspA moAb LA31 reacted exclusively with OspA types I and II (18). As shown in Table 2, those isolates that stained positive with mAb La31 expressed OspA genotype II (HindIII fragments of 0.9 and 0.4 kb). These data proved a strict correlation between phenotypic and genotypic analysis.

Table 2. Characteristic genotypic features of *B. burgdorferi* strains

Strains	Origin(s)	Source(s)	Southern blot results[a] OspA		LA31
			Type	Fragment size(s)	
ZS7	Germany	Ixodes tick	I	1.2, 0.3	+
19857	United States	Rabbit	III	4.0, 0.5	−
ACA-1	Sweden	Human	IV	1.7	−
20047	France	Ixodes tick	V	3.0	−
S90	Germany	Ixodes tick	VI	1.1	−
ZQ1	Germany	Ixodes tick	II	0.9, 0.4	+
NE4	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE58	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE11H	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE6M	Switzerland	Ixodes tick	VII	0.45	−
NE6H	Switzerland	Ixodes tick	VII	0.45	−
NE6S	Switzerland	Ixodes tick	n.t.	n.t.	−
NE6O	Switzerland	Ixodes tick	VII	0.45	−
NE6U	Switzerland	Ixodes tick	VII	0.45	−
NE6TM	Switzerland	Ixodes tick	VII	0.45	−
NE8S	Switzerland	Ixodes tick	VIII	1.8, 0.5	−
NE8O	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE8U	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE8H	Switzerland	Ixodes tick	VIII	1.8, 0.5	−
NE8TM	Switzerland	Ixodes tick	VIII	1.8, 0.5	−
NE8SG	Switzerland	Ixodes tick	VIII	1.8, 0.5	−
NE9H	Switzerland	Ixodes tick	VII	0.45	−
NE9T	Switzerland	Ixodes tick	n.t.	n.t.	+
NE9S	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE9M	Switzerland	Ixodes tick	II	0.9, 0.4	+
NE9SG	Switzerland	Ixodes tick	VIII	1.8, 0.5	−
NE9RA	Switzerland	Ixodes tick	II	0.9, 0.4	+

[a] HindIII fragment sizes are given in kilobases.
n.t.: not tested

Discussion

To our knowledge, it is the first time that *B. burgdorferi* spirochete of distinct phenotypes/genotypes are isolated from different organs of systemically infected ticks. Our results also indicate that the rate of systemic infection in *I. ricinus* adult ticks may be higher (8/22, 36%) than 5% as previously described by *Burgdorfer* et al. (6). In view of these findings, it can be anticipated that the time of attachment that is required for effective transmission of spirochetes may be shorter than previously expected. In that respect, *Ribeiro* et al. (14) demonstrated spirochetes in experimentally obtained saliva not before 3 days after attachment of *I. dammini* (at present *I. scapularis* (12)) ticks. Our own histological studies showed that during feeding of *I. ricinus*, a period of 3 days is required for the spirochetes to emigrate from the midgut and to invade the salivary glands (9). In systemically infected unfed ticks, the transmission may be quicker since spirochetes are already present in the salivary glands at the beginning of the blood meal.

The results suggest that *B. burgdorferi* spirochetes of more than one phenotype/genotype may be harboured by different organs of one tick. A previous study showed that antigenic variation in cloned and uncloned *B. burgdorferi* strains occurred after the spirochetes had been reintroduced into tick midguts and were then reisolated (8, 10). Whether or not phenotypic shifting of the spirochetes in ticks relates somehow to their localization in the different tick tissues, cannot be decided on the basis of our data.

Our study indicates that there is a substantial potential for OspA antigenic variation. The possibility that recombination between *ospA* and *ospB* genes could have generated such phenotypic diversity is supported by results obtained by Southern blot analysis (19). Whether chimeric genes or additional molecular mechanisms resulted in the generation of antigenic OspA variants has to be elucidated by sequence analysis of the *ospA/B* gene locus.

Quantitative alterations between OspA, OspB and OspC, or loss of OspA and OspB have been described for subcultures of European isolates. Wilske *et al.* (20, 21), Schwan and Burgdorfer (16) and Schwan *et al.* (17) have described loss or antigenic alterations of OspB during subculture of *B. burgdorferi*. In our study, differences in the protein pattern of the various isolates from one tick did not correlate with a difference in the number of subcultures.

Further studies are needed to clarify the mechanisms that account for the presence of different spirochetal phenotypes in the organs of systemically infected ticks. Since it remains unclear whether regurgitation of midgut content may occur as a mode of transmission in addition to the salivarial route, the question whether systemically infected ticks can inoculate different spirochete phenotypes during their blood meal still awaits an answer.

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Expression of outer surface proteins A and C of *Borrelia afzelii* in *Ixodes ricinus* ticks and in the skin of mice.

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Expression of Outer Surface Proteins A and C of *Borrelia afzelii* in *Ixodes ricinus* Ticks and in the Skin of Mice

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Summary

Several studies have described changes in the expression of proteins, especially of OspA and OspC, of *B. burgdorferi* sensu stricto during tick feeding. In this study, the expression of OspA and OspC of *B. afzelii* in unfed and feeding *I. ricinus* nymphs and in the subsequent adults was followed by means of the immunofluorescence test. Spirochaetes expressing OspA and OspC were observed in 70% and 80%, respectively of the unfed nymphs. In feeding and in fully engorged ticks, spirochaetes expressed OspC, while OspA disappeared 24 hours after the beginning of the blood meal. Spirochaetes expressing OspC in salivary glands were observed in one engorged tick. After molting, in unfed adults spirochaetes again expressed OspA and OspC but did so less frequently (6% and 13%, respectively). The mouse strain (AKR/N or BALB/C) on which ticks had their infectious blood meal influenced OspC expression in the following tick stage. In the skin of AKR/N mice, at the tick feeding site, *B. afzelii* expressed OspC only, as was shown by immunostaining.

Introduction

In Europe, the causative agent of Lyme borreliosis, *Borrelia burgdorferi* sensu lato (sl), is associated with the vector, *Ixodes ricinus* (3). In its European distribution, *B. burgdorferi* sl has been classified into five genotypes: *B. burgdorferi* sensu stricto (ss), *B. garinii, B. afzelii,* spirochaetes of group VS116 and of group Poti-B2 (25).

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In unfed infected *I. ricinus* ticks, spirochaetes are always found in the midgut (10, 14, 19, 20). During feeding, spirochaetes penetrate through the midgut epithelium and colonize different organs and the salivary glands, from which they are transmitted via saliva to vertebrate hosts (10, 14). This implies that spirochaetes are confronted with different physiological environments during their life cycle. Previous studies demonstrated that the expression of various outer surface proteins (Osp's), mainly OspA and OspC, can vary in the tick (16, 17) and in the host (5, 30, 33). Besides *Schwan* et al. (31) described that in unfed *I. scapularis,* OspA was expressed by *B. burgdorferi* sensu stricto (ss) spirochaetes, but not OspC, whereas spirochaetes in the midgut of feeding and fully engorged ticks expressed OspC but little or no OspA. Changes in OspA and OspC expression of *B. burgdorferi* ss seemed to be regulated by temperature and other undetermined factors related to the absorption of blood meals (31).

In order to determine whether the same phenomenon occurs with *B. afzelii,* OspA and OspC expression of *B. afzelii* was followed in *I. ricinus* nymphs before, during and after the blood meal, and also in unfed adults derived from the former. We also studied OspA and OspC expression of *B. afzelii* at the tick attachment site in the skin of mice.

Materials and Methods

Spirochaetal strain and tick infection

A *B. afzelii* isolate (NE496) obtained from the midgut of a free-living *I. ricinus* adult tick collected in an endemic area in Switzerland (Karoline forest, Aarberg) was used in this study (16). Infected *I. ricinus* nymphs were obtained by allowing uninfected larvae from a spirochaete-free tick colony to feed on BALB/C mice (H-2^d haplotype, Igh allotype: a) (29) infected by *B. afzelii.* Ticks were held at room temperature and saturated humidity and allowed to molt to the next stage.

Ticks were tested by immunofluorescence for the presence of spirochaetes and the detection of OspA and OspC expression as unfed nymphs and at different times (12 h, 24 h, 55 h and 72 h after tick attachment) during blood feeding on AKR/N mice (H-2^k haplotype, Igh allotype: d) (29), at drop-off and after the molt.

Collection of tick saliva and faeces

Salivation was induced by application for one minute of 1 µl of 5% (w/v) pilocarpine hydrochloride (Sigma-Aldrich, Deisenhofen, Germany) solution in methanol on the dorsal part of each nymphal tick fed for 72 h (28). Finally, ticks were rinsed in distilled water, dried and a clean glass slide was applied directly on the hypostome to collect saliva.

Faeces were collected from nymphs when attached to the mice, 12 h after the beginning of feeding and rehydrated in 50 µl phosphate-buffered saline (PBS) 0.15 M pH 7.35. Ten µl of this mixture were smeared on a glass slide and examined using direct immunofluorescence (DFA) and another 10 µl were tested by the polymerase chain reaction (PCR) (see below).

Direct and indirect immunofluorescence tests

Unfed nymphs and partially engorged nymphs (12 h and 24 h after tick attachment) were squashed and spread *in toto* on glass slides. Nymphs fed for 55 h and 72 h, fully engorged nymphs and unfed adults were dissected. Salivary glands and midgut were removed. Each organ was distributed and smeared on three different glass slides and tested with three different antibodies as described below. Smears were dried at 34–37 °C for one night and fixed for 10 min in acetone. The direct fluorescent antibody test (DFA) was used in order to detect borreliae in the tick smears. Tick smears were treated with fluorescein isothiocyanate-labelled conjugate prepared from high-titre sera of Lyme borreliosis patients (12, 23). Expression of OspA and OspC was detected by using the indirect immunofluorescent antibody test (IFA). Tick smears were incubated with anti-OspA monoclonal antibody H5332 (2) followed by goat anti-mouse fluorescein isothiocyanate conjugate (Sigma, Biosciences, St. Louis, USA) or with a rabbit polyclonal anti-OspC antiserum (kindly provided by Tom Schwan) (32) followed by goat anti-rabbit fluorescein isothiocyanate conjugate (Sigma). All smears were examined by epifluorescence microscopy at 400 × magnification.

Tissue preparation and immunohistochemical labelling

Skin biopsies were taken from four AKR/N mice at the attachment site of nymphs a few hours after drop-off and were embedded in Optimal Cutting Temperature compound (OCT, Bayer-Pharma, Zürich, Switzerland). Biopsies were frozen in isopentane chilled in liquid nitrogen and stored at −80 °C. Cryostat sections (6–8 μm) were placed on poly-L-lysine-coated slides (18) and allowed to dry. Sections were fixed in acetone for 10 min and tested by DFA (with an anti-*B. burgdorferi* sl conjugate) and by IFA as described above.

PCR and RFLP

Tick faeces were examined for *Borrelia* using the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) according to the method described by *Postic* et al. (25). All samples were boiled at 100 °C for 10 min. PCR was performed in 50 μl of a solution containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3, 5 pmol primer 1 (5′-CTGCGAGTTCGCGGGAGA-3′) (MWG Biotech, Ebersberg, Germany), 5 pmol primer 2 (5′-TCCTAGGCATTCACCATA-3′) (MWG Biotech, Ebersberg), 200 μM dNTP's mix (Sigma) and 1.5 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany). Forty μl of this PCR solution were mixed with 10 μl of the tick faeces sample or 10 μl ultrafiltered water for negative control or 1 ng DNA of *B. burgdorferi* ss (strain B31), *B. garinii* (strain NE11H) (17) and *B. afzelii* (strain NE632) (16) for each positive control. The amplification reaction was carried out twice for 35 cycles under the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The DNA amplification was controlled by agarose gel electrophoresis.

To cleave PCR (25 μl) products, endonuclease *MseI* (New England Biolabs, USA) was used as recommended by the manufacturer. Electrophoresis was carried out in 16% acrylamide/bisacrylamide gel, and DNA fragments were separated at 120 V for 1.5–2 h. Marker V (Boehringer, Mannheim) was used as molecular weight marker. DNA fragments were visualized under UV with ethidium bromide.

Statistical analysis

Fisher's exact test (2-tailed) was used to compare the number of infected ticks using DFA and cultivation. *P* values of less than 0.05 were regarded as significant.

Results

In unfed *I. ricinus* nymphs, spirochaetes were detected in 9/10 ticks by DFA (Table 1). During feeding, a significant decrease in the number of infected ticks from 9/10 (90%) in unfed nymphal ticks to 5/16 (31%) in nymphs fed for 55 h was observed (Fisher test, p = 0.005). When the infection rate of unfed nymphs (9/10) was compared with the infection rate of engorged nymphs (drop-off) (9/17, 53%), there was no significant difference (Fisher test, p = 0.091). In ticks fed for 72 h and in engorged ticks atypical, comma-shaped spirochaetes as described by *Aberer* & *Duray* (1) and *Gern* et al. (10) were observed by DFA.

Spirochaetes were detected in the salivary glands of 1/5 (20%) infected ticks fed for 55 h, in 2/10 (20%) fed for 72 h and in 3/9 (33%) infected ticks observed at drop-off. After the molt, in unfed adults, spirochaetes were limited to the midgut. Examination of saliva collected from 20 nymphal ticks fed for 72 hours using DFA showed no spirochaetes. Faeces were collected from 11 nymphs still attached to the mice, 12 h after placement on the host, rehydrated in PBS, and examined by DFA and PCR. No spirochaetes were observed in the rehydrated faeces examined by DFA. However, using PCR, spirochaetal DNA was detected in the rehydrated faeces of 9/11 (82%) ticks. Digestion of the amplified DNA by *Msel* showed the typical fragment length pattern of *B. afzelii*.

Expression of OspA and OspC in I. ricinus

In unfed *I. ricinus* nymphs *B. afzelii* spirochaetes positive for OspA were observed in 7/10 ticks when tested with the monoclonal antibody (H5332) and spirochaetes positive for OspC were present in 8/10 unfed nymphs when tested with the polyclonal antibody (anti-OspC) (Table 1).

A significant decrease in OspA (p = 0.029) and OspC (p = 0.001) expression was seen between unfed nymphs and nymphs fed for 12 h. In fact, in nymphs fed for 12 h, spirochaetes positive for OspA but not for OspC were observed in 2/11 (18%) of infected ticks and spirochaetes positive for OspC but not for OspA were present in 1/11 (9%) of infected nymphs (Table 1). Twenty-four hours after the beginning of blood feeding, OspC expressing spirochaetes were detected in 1 tick, and OspA and OspC expressing spirochaetes were detected in 2 additional ticks. Spirochaetes positive for OspA were no more detected in ticks attached to the host for more than 24 h, whereas OspC-expressing spirochaetes were detected in the midgut of 2/5 (40%) infected ticks fed for 55 h and of 4/10 (40%) infected ticks fed for 72 h. At drop-off, spirochaetes expressing OspC were detected in the midgut of 3/9 (33%) of infected ticks. Atypical spirochaetes always expressed OspC.

Table 1. Detection of *B. afzelii* in *I. ricinus* nymphs during tick feeding and after molting, and OspA and OspC expression by *B. afzelii*

	DFA	IFA	
Feeding status	Infection rate (%)	Number of nymphs with spirochaetes expressing OspA (%)	Number of nymphs with spirochaetes expressing OspC (%)
Unfed nymphs	9/10 (90)	7/10 (70)	8/10 (80)
Nymphs fed for 12 h	11/13 (85)	2/11 (18)	1/11 (9)
Nymphs fed for 24 h	8/15 (53)	2/8 (25)*	3/8 (38)*
Nymphs fed for 55 h	5/16 (31)	0/5 (0)	2/5 (40)
Nymphs fed for 72 h	10/21 (48)	0/10 (0)	4/10 (40)
Nymphs at drop-off	9/17 (53)	0/9 (0)	3/9 (33)
Unfed adults	16/29 (55)	1/16 (6)	2/16 (13)

* In two ticks, spirochaetes expressing OspA and OspC were detected.
DFA: direct immunofluorescence test.
IFA: indirect immunofluorescence test.

After the molt, in unfed adults, spirochaetes less frequently expressed OspC (2/16, 13%) ($p = 0.001$) and OspA (1/16, 6%) ($p = 0.001$), than in unfed nymphs (8/10 and 7/10, respectively). One explanation for the low ability to detect OspA and OspC in unfed adults was that unfed adults had a previous blood meal on AKR/N mice in contrast to unfed nymphs which had had a previous blood meal on BALB/C mice. To examine the possibility that mice strains might influence OspA and OspC expression in unfed ticks, additional uninfected larvae were fed on infected AKR/N mice and examined as unfed nymphs. This demonstrated that *B. afzelii* expressed OspC significantly less frequently ($p = 0.023$) (OspA expression was not changed) in the midgut of unfed ticks when the previous blood meal had been taken on infected AKR/N mice (2/10 expressed OspC and 7/10 expressed OspA) than on infected BALB/C mice (8/10 expressed OspC and 7/10 expressed OspA).

Expression of OspA and OspC at the tick attachment site of the skin

Using DFA, spirochaetes were detected in sections of skin biopsies taken at the tick feeding site from 4 AKR/N mice (Table 2). Spirochaetes were observed mainly in the dermis and sometimes between dermis and hypodermis. The number of spirochaetes in the sections was always very low ($n \leq 5$). Analysis by IFA showed that spirochaetes present in the skin of AKR/N mice expressed OspC but not OspA.

Table 2. Detection of *B. afzelii* in skin biopsy sections and determination of the OspA and OspC expression of *B. afzelii* in mice (AKR/N)

Mice	No. of biopsies	No. of infected/examined skin sections reacting with		
		anti-B.b.	anti-OspC	anti-OspA
1	1	0/91	n.d.	0/111
	2	0/71	n.d.	0/115
	3	1/50	0/194	n.d.
	4	0/136	n.d.	0/71
2	1	0/114	n.d.	0/47
	2	0/70	n.d.	0/136
	3	0/72	n.d.	n.d.
	4	1/77	0/135	0/57
3	1	0/100	n.d.	0/157
	2	1/25	1/115	0/98
	3	0/50	n.d.	0/21
4	1	1/72	1/129	0/52
	2	0/27	n.d.	0/28
	3	0/73	n.d.	0/49
Total	14	4/1028	2/573	0/942

n.d. = no done.

Discussion

Several previous studies have described changes in the expression of proteins, mainly of OspA and OspC, by *B. burgdorferi* ss during tick feeding. *Schwan* et al. (31) and *de Silva* et al. (6, 7) observed that *B. burgdorferi* ss in unfed *I. scapularis* nymphs expressed OspA but not OspC. Loss of OspA expression of *B. burgdorferi* ss occurred between 0 h and 24 h after *I. scapularis* attachment to the host (6) whereas spirochaetes synthetizing OspC were observed in the tick during the blood meal and in engorged ticks (7, 31).

In the present study, a similar evolution in OspA and OspC expression of *B. afzelii* in *I. ricinus* nymphs was detected but the mice strain on which ticks had their previous blood meal influenced OspC expression in unfed ticks. In fact, spirochaetes present in unfed nymphs which fed as larvae on infected BALB/C mice more frequently expressed OspC (80%) than did spirochaetes in unfed nymphs fed on infected AKR/N mice (20%) ($p = 0.023$). Expression of OspC in unfed *I. ricinus* was also observed by *Hu* et al. (16, 17) when cul-

tivated spirochaetes had been reintroduced into the tick midgut and by *Fingerle* et al. (9) who observed OspC expressing spirochaetes in unfed *I. ricinus*. Factors influencing OspC expression in unfed ticks remain unknown but the immune system specific to each strain of mice may be one of these factors (29).

In feeding ticks, expression of OspA and OspC significantly decreased during the first 12 hours. OspA of *B. afzelii* was no more expressed in ticks fed for more than 24 h, as described for *B. burgdorferi* ss by *Burkot* et al. (4), *Schwan* et al. (31) and *de Silva* et al. (6), whereas spirochaetes expressing OspC were detected during the entire observation period.

The absence of spirochaetes in saliva did not allow us to obtain information on the outer surface protein expression of *B. afzelii* when spirochaetes were transmitted to the host. However, spirochaetes transmitted via tick bites expressed OspC, but not OspA, at the tick attachment site in the AKR/N mouse skin as shown by in-situ immunostaining. Previous indirect evidence showed that, in animals infected via tick bites and at the early stage of the disease in humans, the immune response was principally directed against OspC, but not against OspA (5, 8, 13, 15, 22). The OspC expression by spirochaetes in the mice skin, as shown in the present study, suggests that OspC may play a role in the infectivity and the pathogenicity of *B. burgdorferi* sl in the vertebrate host as already suggested by *Masuzawa* et al. (21) and *Hu* et al. (17). It remains unclear whether spirochaetes which express OspC in ticks more than 24 h after tick attachment represent spirochaetes which produce OspC instead of OspA in the tick midgut during feeding or whether they represent spirochaetes which have been transmitted to the host, have expressed OspC in the host skin, as shown here, and have been picked up by feeding ticks during the last days spent on the host.

In unfed *I. ricinus* nymphs and in nymphs fed for 12 h, a high *B. afzelii* infection rate was observed (90% and 85%, respectively). During feeding, a significant decrease in the infection rate followed by an increase in the number of infected ticks at the end of the attachment to the host was observed. Similar dynamics of the tick infection rate during feeding have been previously described using various methods (4, 10, 24, 27). *Gern* et al. (10) suggested that the decrease in tick infection rate was due to loss of infection in some ticks by excretion of *Borrelia* in the faeces, and that the increase in the infection rate was due to ingurgitation of spirochaetes localized at the site of inoculation at the end of feeding during the rapid engorgement phase (11, 26, 34). In the present study, we were not able to observe spirochaetes in tick faeces using DFA, surely because faeces had been excreted many hours before they have been collected and examined. However, *B. afzelii* infection was detected by PCR/RFLP in faeces collected from 9/11 (82%) nymphs.

In nymphs fed for 72 h, spirochaetes were observed in salivary glands (2/10, 20%) but never in saliva collected from 20 ticks. Explanations for our inability to observe spirochaetes in saliva might be that spirochaetes are not very abundant in salivary glands of *I. ricinus* (10, 14, 19 and in the present study, data not shown) and might not be continuously transmitted via saliva.

In summary, expression of both OspA and OspC of *B. afzelii* was observed in unfed *I. ricinus* followed by a variation in the expression of OspA and OspC during *I. ricinus* feeding. However, expression of OspC in *B. afzelii* in unfed ticks depended on the mice strains (AKR/N vs BALB/C) on which ticks had their previous infectious blood meal. At the tick attachment site, i. e. in the skin of AKR/N mice, *B. afzelii* expressed only OspC as shown by *in situ* immunostaining. A variation in the infection rate of ticks during feeding with a strong decrease in the number of infected ticks followed by an increase in the number of infected ticks at the end of feeding was observed.

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