

Lyme Borreliosis Spirochete and its Tick Vector

**Relationship of protein expression, antigenicity and
pathogenicity of *Borrelia burgdorferi* (Spirochaetales:
Spirochaetaceae) sensu lato and *Ixodes ricinus* ticks
(Acari: Ixodidae)**

THESE

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IMPRIMATUR POUR LA THÈSE

Relations entre les spirochètes de la borréliose de Lyme et
leur vecteur *Ixodes ricinus*: expression protéinique,
antigénique et pathogénicité
de *Borrelia burgdorferi sensu lato*.

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La Faculté des sciences de l'Université de
Neuchâtel sur le rapport des membres du jury,

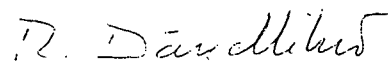
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Neuchâtel, le 30 janvier 1997

Le doyen:

R. Dändliker



献给我亲爱的父母
献给我亲爱的丈夫
献给我亲爱的儿女

To my parents,
To my husband,
To my children.

A mes parents,
A mon mari,
A mes enfans.

This thesis is based on the following papers:

(The complete thesis is deposited in the Central Library of Neuchâtel University)

- I. Comparison in the immunological properties of *Borrelia burgdorferi* isolates from *Ixodes ricinus* derived from three endemic areas in Switzerland.
Hu C. M., Leuba - Garcia S., Kramer M. D., Aeschlimann A. and Gern L.
Epidemiol. Infect. (1994) 112: 533 - 542
- II. Changes in the protein profile and antigenicity of different *Borrelia burgdorferi* strains after reintroduction to *Ixodes ricinus* ticks.
Hu C. M., Gern L. and Aeschlimann A.
Parasite Immunology (1992) 14: 415 - 427
- III. Antigenic variation in *Borrelia burgdorferi* after passage through *Ixodes ricinus* and *Ixodes hexagonus*.
Gern L., Hu C. M., Toutoungi L. A. and Kramer M. D.
In: Proc. of the 1st Intern. Conf. On Tick - Borne Pathogens at the Host - Vector Interface: An Agenda for Research. Munderloh U. G. and Kurtti T. J. (eds), Saint Paul, USA, 1992, pp 121 - 125
- IV. Proteinic, antigenic and pathogenic variations of a clonal *Borrelia burgdorferi* isolate from *Ixodes ricinus* hemolymph.
Hu C. M., Kramer M. D., Simon M. M. and Gern L.
In: Proc of VI International Conference on Lyme borreliosis. Cevenini R., Sambri V. and La Placa M. (eds), Bologna: Italy June 19 - 22, 1994, pp 19
- V. Tick factors and *in vitro* cultivation influence the protein profile, antigenicity and pathogenicity of a clonal *Borrelia garinii* isolate from *Ixodes ricinus* hemolymph.
Hu C. M., Simon M. M. Kramer M. D. and Gern L.
INFECTION (1996) 24 (3): 251/41-257/47
- VI. *Apodemus* sp rodents, reservoir hosts for *Borrelia afzelii* in an endemic area in Switzerland.
Hu C. M., Humair P. F., Wallich R. and Gern L.
Zbl. Bakt. Hyg. (1997) 285, 558-564

Comparison in the immunological properties of *Borrelia burgdorferi* isolates from *Ixodes ricinus* derived from three endemic areas in Switzerland

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SUMMARY

Borrelia burgdorferi isolates were obtained from *Ixodes ricinus* from three sites in Switzerland. They were examined by SDS-PAGE and immunoblotting. The phenotypes, in respect of three outer surface proteins (Osp), differed between the sites of collection. In site 1, most isolates had an OspA of 31 kDa and an OspB of 34 kDa; in site 2, isolates presenting an OspA of 33 kDa dominated and in site 3, the isolates with an OspA of 32 kDa and an OspB of 35 kDa were most frequent. This distribution differed significantly. About half of the isolates from sites 1 and 3 reacted with anti-OspA monoclonal antibody H5332 compared to 29% from site 2. Site 1 isolates reacted significantly more frequently (81%) with another anti-OspA monoclonal antibody LA-31 than isolates from site 3 ($P < 0.0001$). These findings have implications for the epidemiology of Lyme borreliosis, for the further development of serodiagnostic reagents and for the development of a vaccine.

INTRODUCTION

In humans, Lyme borreliosis is a disease caused by infection with *Borrelia burgdorferi* [1]. *B. burgdorferi* is transmitted by infected ticks belonging primarily to the *Ixodes ricinus* complex [2]. In Europe, *B. burgdorferi* can be isolated from infected ticks, animals and patients. The outer membrane of *B. burgdorferi* contains at least three outer surface proteins (Osp) A (31–33 kDa), B (34–36 kDa) and C (20–24 kDa) [3–6]. These lipoproteins are embedded in the fluid outer membrane of *B. burgdorferi* and are encoded by linear plasmids [6, 7]. Although their exact functions have not yet been defined, the outer surface proteins of *B. burgdorferi* are generally thought to have an important role in the host–parasite interactions during the course of infection. The European isolates are more heterogeneous with respect to their antigenic profiles than the American isolates [8–12].

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Table 1. *Characterization of B. burgdorferi isolates (n = 92)*

| Strains | Osp Mws (kDa) | | | MoAbs | | | | | | | | PoAb anti-22 kDa |
|---------|---------------|----|----|-------|------|------|-------|-------|-------|------|-----|------------------|
| | A | B | C | H5332 | LA-2 | LA-4 | LA-31 | LA-25 | LA-27 | LA-7 | p39 | |
| Site 1 | | | | | | | | | | | | |
| NE83 | 33 | a | 22 | - | - | - | + | - | - | - | + | + |
| NE84 | 33 | a | 22 | - | - | - | + | - | - | - | - | + |
| NE85 | 33 | a | 22 | - | - | - | + | - | - | - | - | + |
| NE190 | 31 | 34 | 23 | - | - | - | + | - | + | - | - | + |
| NE192 | 33 | a | 22 | - | - | - | - | - | - | - | - | - |
| NE193 | 31 | 34 | a | - | - | - | + | - | + | - | - | - |
| NE308 | 33 | 34 | a | - | - | - | + | - | - | - | - | + |
| NE317 | 32 | 34 | 22 | - | - | - | + | - | - | - | - | + |
| NE323 | 32 | 35 | a | - | - | - | - | - | - | - | - | + |
| NE15 | 31 | 34 | a | + | - | - | + | - | + | - | - | + |
| NE12 | 31 | 34 | 22 | + | + | + | + | + | + | + | + | - |
| NE14 | 31 | 34 | a | + | + | - | - | - | - | - | + | - |
| NE196 | 31 | 34 | a | + | + | - | + | - | + | - | + | - |
| NE303 | 32 | 35 | 23 | - | + | - | + | - | + | - | - | - |
| NE304 | 32 | a | 22 | - | - | - | - | - | - | - | - | - |
| NE305 | 31 | 34 | 22 | - | + | + | + | + | + | - | - | - |
| NE378 | 33 | a | 22 | - | - | - | + | - | + | - | - | - |
| NE20 | 32 | a | 23 | + | - | - | + | - | - | - | n | - |
| NE25 | 31 | 34 | 23 | + | + | - | + | + | + | + | n | + |
| NE26 | 33 | a | 22 | + | - | - | + | - | - | - | n | - |
| NE8 | 32 | a | 23 | + | - | - | + | - | - | - | n | - |
| NE19 | 32 | 35 | a | + | - | - | - | - | - | - | n | - |
| NE550 | 31 | 34 | a | + | + | - | + | + | + | + | n | - |
| NE21 | 32 | a | 22 | + | + | - | + | - | - | - | n | - |
| NE24 | 31 | 34 | 23 | + | + | + | + | + | + | + | n | + |
| NE23 | 32 | a | 22 | + | + | - | + | - | - | - | n | + |
| Site 2 | | | | | | | | | | | | |
| NE2 | 33 | a | 22 | - | - | - | + | - | - | - | - | - |
| NE4 | 33 | a | 22 | - | - | - | - | - | - | - | - | + |
| NE58 | 33 | a | a | - | + | - | + | - | - | - | + | - |
| NE60 | 33 | a | a | - | + | - | + | - | - | - | + | - |
| NE173 | 33 | a | 22 | - | - | - | - | + | - | - | - | + |
| NE352 | 32 | 34 | 23 | + | + | - | - | - | + | - | - | + |
| NE355 | 33 | a | 22 | - | - | - | + | - | - | - | - | + |
| NE363 | 32 | 35 | a | - | - | - | - | - | - | - | - | - |
| NE3 | 33 | a | a | - | - | - | n | n | n | - | n | n |
| NE9 | 32 | a | 22 | + | - | - | + | - | - | - | n | + |
| NE10 | 32 | a | 23 | + | - | - | + | - | - | - | n | + |
| NE22 | 33 | a | a | - | - | - | + | - | - | - | n | - |
| NE16 | 32 | a | 22 | + | - | - | - | - | - | - | n | - |
| NE1 | 32 | a | 22 | - | - | - | - | - | - | - | n | + |
| NE5 | 33 | a | 22 | - | - | - | - | - | - | - | n | + |
| NE6 | 33 | a | a | - | - | - | - | + | - | - | n | - |
| NE27 | 32 | 35 | 23 | + | - | - | - | - | - | - | n | - |
| Site 3 | | | | | | | | | | | | |
| NE413 | 33-5 | a | 22 | - | - | - | - | - | - | - | - | + |
| NE443 | 31 | a | 23 | + | - | + | - | - | - | - | - | + |
| NE450 | 32 | 35 | 23 | + | - | - | - | - | - | - | + | + |
| NE454 | 32 | 35 | 21 | - | - | - | - | - | - | - | - | + |
| NE456 | 31 | 34 | 22 | + | + | - | + | + | + | + | + | + |
| NE460 | 32 | 35 | 22 | + | + | - | - | - | - | - | - | - |

Table 1 (cont.)

| Strains | Osp Mws (kDa) | | | MoAbs | | | | | | | PoAb anti-22 kDa | | |
|---------|---------------|----|----|-------|------|------|-------|-------|-------|------|------------------|-----|---|
| | A | B | C | H5332 | LA-2 | LA-4 | LA-31 | LA-25 | LA-27 | LA-7 | | p39 | |
| NE461 | 32 | 35 | 22 | - | - | - | - | - | - | - | - | - | + |
| NE462 | 33 | a | 23 | + | - | + | - | - | - | - | - | - | + |
| NE472 | 32 | 35 | 23 | + | - | - | - | - | - | - | + | + | |
| NE474 | 31 | a | 23 | - | - | + | - | - | - | - | - | + | |
| NE477 | 32 | 35 | 22 | + | - | - | - | - | - | - | + | + | |
| NE478 | 32 | 35 | 22 | - | - | - | - | - | - | - | - | + | |
| NE485 | 32 | 35 | 22 | + | - | - | - | - | - | - | + | + | |
| NE490 | 32 | a | 22 | + | - | - | + | - | - | - | - | + | |
| NE496 | 32 | 35 | 23 | + | - | - | - | - | - | - | + | - | |
| NE506 | a | a | 22 | - | - | - | - | - | - | - | - | + | |
| NE507 | 32 | 35 | 22 | - | - | - | - | - | - | - | + | + | |
| NE517 | 32 | 35 | 22 | - | - | - | - | - | - | - | - | + | |
| NE519 | 32 | 35 | 22 | - | - | - | - | - | - | - | - | + | |
| NE558 | 32 | a | 22 | + | - | - | + | - | - | - | - | + | |
| NE601 | 32 | a | 21 | - | - | - | + | - | - | - | - | + | |
| NE603 | 32 | a | 22 | + | - | + | - | - | - | - | - | + | |
| NE606 | 32 | 35 | 22 | + | - | - | - | - | - | - | - | + | |
| NE607 | 32 | a | 22 | + | - | - | + | - | - | - | + | + | |
| NE608 | 32 | a | 22 | + | - | - | + | - | - | - | - | + | |
| NE623 | 32 | 35 | 22 | + | - | - | - | - | - | - | - | + | |
| NE624 | 32 | 35 | 22 | + | - | - | - | - | - | - | + | + | |
| NE629 | 32 | 35 | a | + | - | - | - | - | - | - | + | + | |
| NE630 | 32 | 35 | 22 | + | - | - | - | - | - | - | + | + | |
| NE632 | 32 | 35 | 23 | + | - | - | - | - | - | - | + | + | |
| NE418 | 32 | 35 | a | + | + | - | + | - | - | - | - | - | |
| NE426 | 33 | a | 22 | + | + | - | - | - | - | - | - | + | |
| NE429 | 32 | 35 | a | - | + | - | - | - | - | - | - | - | |
| NE435 | 32 | 35 | 22 | - | - | - | - | - | - | - | - | - | |
| NE438 | 33 | a | a | - | + | - | - | - | - | - | - | - | |
| NE463 | 31 | 34 | 21 | + | + | - | + | + | + | + | + | + | |
| NE467 | 32 | 35 | 21 | + | + | - | - | - | - | - | - | + | |
| NE471 | 32 | 35 | 22 | - | + | + | - | - | - | - | - | + | |
| NE470 | 32 | 35 | a | - | + | - | - | - | - | - | - | - | |
| NE493 | 31 | 34 | 21 | + | - | + | + | + | + | + | + | + | |
| NE508 | 32 | 35 | 22 | + | + | - | + | - | - | - | - | + | |
| NE537 | 32 | 35 | 22 | - | + | - | - | - | - | - | - | - | |
| NE605 | 32 | 35 | 22 | - | + | - | - | - | - | - | - | + | |
| NE200 | 32 | a | 22 | + | + | - | + | + | + | - | - | + | |
| NE207 | 32 | a | 23 | + | + | + | - | + | - | - | - | + | |
| NE201 | 32 | a | a | - | + | - | + | - | - | - | + | - | |
| NE202 | 31 | a | 22 | - | + | + | + | - | - | - | - | - | |
| NE203 | 32 | 35 | a | + | + | + | + | + | + | - | + | - | |
| NE204 | 32 | 36 | a | - | - | - | - | - | - | - | - | - | |

+, positive reaction; -, negative reaction; a, absent; n, not tested.

In this study, *B. burgdorferi* strains from *B. burgdorferi* ticks collected in three different endemic areas were screened for evidence of phenotypic differences using immunochemical methods.

MATERIAL AND METHODS

Collection of ticks and isolation of B. burgdorferi

I. ricinus ticks (adults and nymphs) were collected by flagging lower vegetation using a white cotton flannel flag (1 m²) which was dragged behind collectors in three endemic areas: site 1, Bois de l'Hôpital forest close to Neuchâtel; site 2, Staatswald forest and site 3, Karoline forest around Aarberg. Ticks were collected during April–June and August–November 1987 to 1992 in sites 1 and 2, and in 1989 and 1992 in site 3. For isolation of *B. burgdorferi*, the midgut of the tick was incubated for 10 days at 34 °C in individual culture tubes containing 4 ml BSK II medium [13] supplemented with rifampicin (50 µg/ml) and phosphomycin (50 µg/ml).

SDS-PAGE and immunoblot analysis

Each isolate was inoculated in 25 ml BSK II medium and after 10 days, the cultures were centrifuged and washed twice with PBS + 5 mM MgCl₂. Whole-cell lysates (equivalent to 10⁷ cells/lane) were separated by SDS-PAGE using a 12.5% polyacrylamide gel. The gels were stained with Coomassie brilliant blue R 250 [14].

The separated proteins were transferred onto nitrocellulose paper using a transit cell (2117-250 Nova Blot Electrophoretic Transfer Kit, LKB AM Bromma, Sweden) [14]. The monoclonal antibodies (MoAbs): H5332, LA-2, LA-4, LA-31 (anti-OspA) [8, 15], LA-25, LA-27 (anti-OspB) [15], LA-7 (anti-20 kDa protein) [15], and polyclonal antibodies (PoAbs): anti-22 kDa/NE4 [14] and anti-B31, produced by immunizing a New Zealand white rabbit with strain B31 [9], were used for immuno-blotting. Bound antibodies were visualized by using peroxidase labelled anti-rabbit IgG or anti-mouse IgG antibodies (1:1000, Nordic Immunological Laboratories, The Netherlands).

Statistical analyses

The Fischer's exact test was used to compare the distribution of the different characterized *B. burgdorferi* isolates derived from different areas. The difference was considered as significant if *P* value was < 0.017 [16].

RESULTS

Characterization of B. burgdorferi isolates by immunological methods

Twenty-six *B. burgdorferi* isolates were obtained from ticks of the Bois de l'Hôpital forest (site 1), 17 isolates from the Staatswald forest (site 2) and 49 isolates from the Karoline forest (site 3). OspA, OspB and OspC were expressed by 91 (99%), 50 (54%) and 70 (76%) of the 92 isolates (Table 1). Four different phenotypes could be distinguished on the basis of the expression pattern of the Osps, namely, A (11% of isolates), AB (16%), AC (41%) and ABC (32%).

Péter and colleagues [11] distinguished four typing groups of *B. burgdorferi* (I, II, III and IV) according to the molecular weight of OspA and OspB (Fig. 1*a*). Most of our isolates belong to groups I, II and III and only one, from site 3, is in group IV. Seven isolates did not fit into this classification and comprised three additional groups: V, VI and VII (Fig. 1*a*).

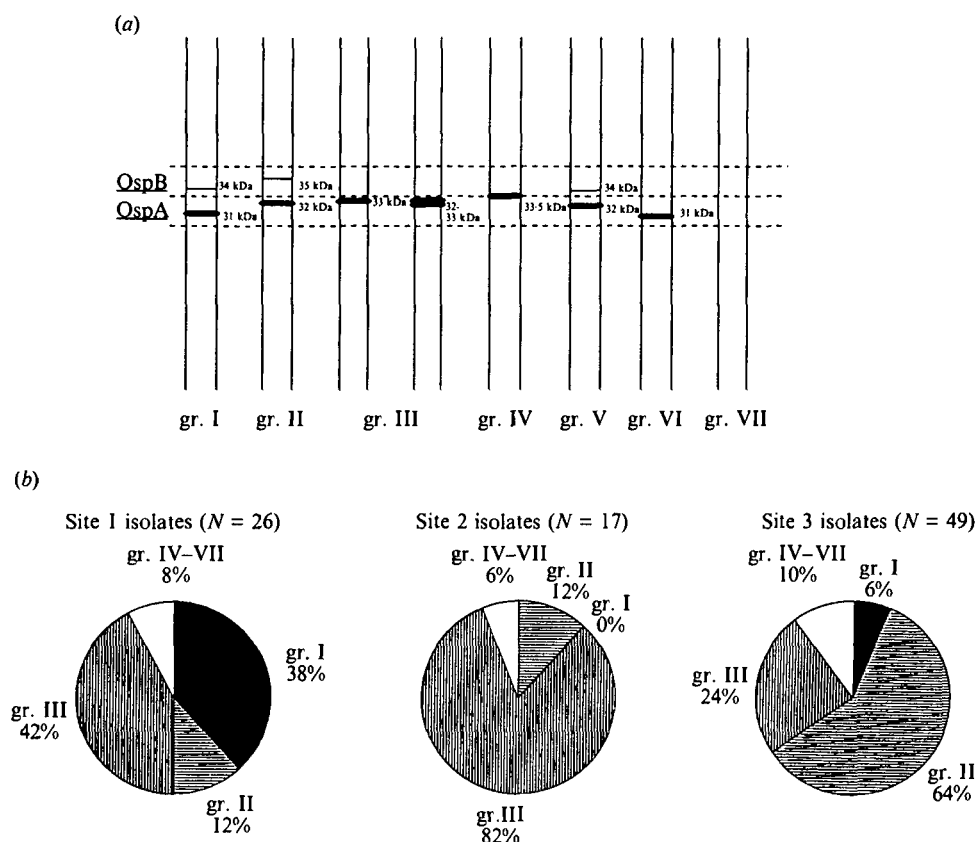


Fig. 1a and b. Typing groups of *B. burgdorferi* isolates and their geographical distribution. (a) Schematic description of the typing groups according to the molecular weights of OspA and OspB. The first four groups (gr. I-IV) were suggested by Péter and colleagues [11] and the groups V-VII were proposed by us. Group VII comprised one isolate (NE506) which did not express OspA and OspB. (b) Relative distribution of the different typing groups in the three sites of isolation: groups I and III prevailed in site 1, group III or II prevailed in site 2 and site 3, respectively.

Immunological characterization of these isolates revealed a heterogeneous reactivity with the different antibodies used (Table 1).

The different phenotypes obtained from April to November did not show any differences in their seasonal distribution (data not shown).

Distribution of the characterized isolates in the three studied sites

The isolates from the three different geographic sites of isolation were compared for: (1) the apparent molecular weights (M_w) of OspA, OspB and OspC; (2) their classification into the respective typing groups; (3) differences in the Osp phenotypes; (4) their reactivity with mono- and polyclonal antibodies specific for *B. burgdorferi* antigens.

The apparent molecular weights of OspA, OspB and OspC

The results are summarized in Table 2. OspA was present in nearly all isolates;

Table 2. *The presence of the major proteins in isolates derived from three different sites*

| | OspA | | | OspB | | | OspC | | |
|----------------------------|--------------------|--------------------|----------------|--------------------|--------------------|-----------|------------|-------------|------------|
| | 31 kDa | 32 kDa | 33 kDa | 34 kDa | 35 kDa | 36 kDa | 21 kDa | 22 kDa | 23 kDa |
| Site 1 (<i>N</i> = 26) | 10 (38%) | 9 (35%) | 7 (27%) | 12 (46%) | 3 (12%) | 0 (0%) | 0 (0%) | 12 (46%) | 6 (23%) |
| Site 2 (<i>N</i> = 17) | 0 (0%) | 7 (41%) | 10 (59%) | 1 (6%) | 2 (12%) | 0 (0%) | 0 (0%) | 8 (47%) | 3 (18%) |
| Site 3 (<i>N</i> = 49) | 6 (12%) | 38 (78%) | 3 (6%) | 3 (6%) | 29 (59%) | 1 (2%) | 5 (10%) | 28 (57%) | 8 (16%) |
| Statistical results | | | | | | | | | |
| <i>P</i> 1* | 0.0072 | 0.7521 | 0.0565 | 0.0062 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.7223 |
| <i>P</i> 2* | 0.0158 | 0.0004 | 0.0170 | 0.00008 | 0.00007 | 1.0000 | 0.1567 | 0.4669 | 0.5398 |
| <i>P</i> 3* | 0.1889 | 0.0081 | 0.00002 | 1.0000 | 0.0014 | 1.0000 | 0.3165 | 0.5753 | 1.0000 |

* *P*1: was as compared between site 1 isolates to site 2 isolates.

*P*2: was between the site 1 isolates to the site 3 isolates.

*P*3: was between the site 2 isolates to site 3 isolates.

Bold type: significant differences between both.

however, they varied in molecular weight with the 31, 32 and 33 kDa protein predominating in isolates from sites 1, 3 and 2 respectively (Table 2). OspB was more prevalent among isolates from sites 1 and 3 than site 2; the 34 kDa protein predominated in site 1 and the 35 kDa protein in site 3. OspC was detected in 69, 65 and 83% of isolates from sites 1, 2 and 3 respectively with the 22 kDa protein being the most prevalent in all three sites.

The distribution of typing groups in different areas

Between the sites of isolation, the distribution of the main typing groups differed significantly (Fig. 1*b*). In site 1, group I and III were most frequent (38 and 42%) and group I was more prevalent than in site 2 (0%, $P = 0.0072$) and in site 3 (3%, $P = 0.0008$). Group III was the main group in site 2 (87%). Its presence differed significantly from site 1 (42%, $P = 0.0058$) and site 3 (24%, $P = 0.00004$). Group II was more frequent in site 3 (60%) than in site 1 (12%, $P = 0.00007$) and site 2 (12%, $P = 0.0014$). About 8% of isolates from each site belonged to groups IV, V, VI or VII.

The distribution of the different Osp phenotypes

Differences in the distribution of the Osp phenotypes according to the expression pattern of the different Osps were observed between the three areas studied (Fig. 2). Phenotype AC (OspA + OspC) was the most frequent in sites 1 and 2, whereas phenotype ABC (OspA + OspB + OspC) predominated in site 3 (54%) and was less frequent in site 2 (12%) than in site 3 ($P = 0.0038$). Phenotype A (OspA only) was more prevalent in site 2 (29%) than in the other sites.

The immuno-reactivity with different MoAbs and PoAbs

About half of isolates from sites 1 and 3 reacted with MoAb H5332. The isolates

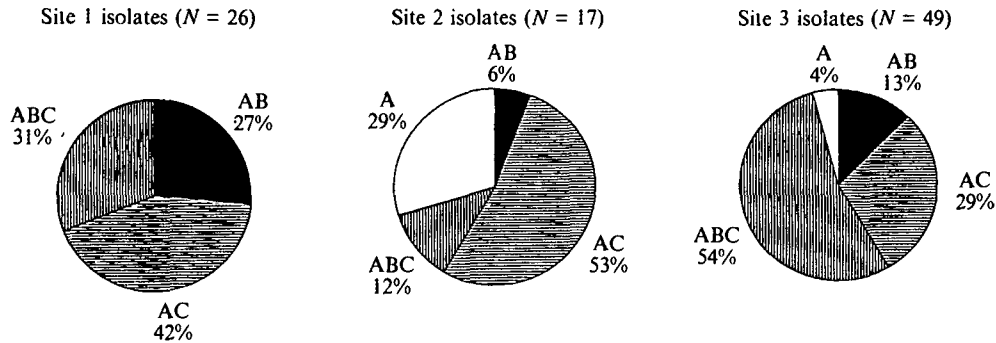


Fig. 2. Relative distribution of the Osp phenotypes (in percentage) in *B. burgdorferi* isolates derived from the three sites of isolation. A, expression of OspA only; AB, expression of OspA and OspB; AC, expression of OspA and OspC; ABC, expression of OspA, OspB and OspC.

Table 3. The reaction of *B. burgdorferi* isolates derived from three different sites with specific mono- and polyclonal antibodies

| | MoAbs | | | | | | | PoAb |
|------------------------|-------------|-------------|------------|--------------------|------------|--------------------|------------|--------------------|
| | H5332 | LA-2 | LA-4 | LA-31 | LA-25 | LA-27 | LA-7 | anti-22 kDa |
| Site 1 (N = 26) | 13 (50%) | 10 (30%) | 3 (12%) | 21 (81%) | 5 (19%) | 11 (42%) | 4 (15%) | 11 (42%) |
| Site 2 (N = 17) | 5 (29%) | 3 (18%) | 0 (0%) | 7 (41%) | 2 (13%) | 1 (6%) | 0 (0%) | 8 (50%) |
| Site 3 (N = 49) | 29 (59%) | 18 (37%) | 9 (18%) | 14 (29%) | 6 (12%) | 5 (10%) | 3 (6%) | 37 (76%) |
| Statistical results | | | | | | | | |
| P1* | 0.2194 | 0.1874 | 0.2658 | 0.0200 | 0.6897 | 0.0149 | 0.1498 | 1.0000 |
| P2* | 0.4733 | 1.0000 | 0.5256 | 0.00002 | 0.4984 | 0.0023 | 0.2270 | 0.0059 |
| P3* | 0.0490 | 0.2273 | 0.0977 | 0.3566 | 1.0000 | 1.0000 | 0.5692 | 0.0386 |

* P1: was as compared between the site 1 isolates to site 2 isolates.

P2: was between the site 1 isolates to site 3 isolates.

P3: was between the site 2 isolates to site 3 isolates.

Bold type: significant differences between both.

MoAbs: monoclonal antibodies.

PoAbs: polyclonal antibodies.

from site 1 reacted more frequently (81%) with MoAb LA-31 (anti-OspA of B31) than isolates from site 3 (29%; $P = 0.00002$) (Table 3). Reactions of the isolates with MoAb LA-27 (anti-OspB) were more frequent in site 1 (42%) as compared to site 2 (6%) and site 3 (10%); the differences were statistically significant ($P = 0.0149$ and $P = 0.0023$). In contrast, 76% of site 3 isolates reacted with anti-22 kDa/NE4 (PoAb) and this was significantly different from isolates from site 1 (42%, $P = 0.0059$).

To reveal the number B31-like strains in each area, we also compared our isolates with the *B. burgdorferi* strain B31, a prototype strain isolated from *I. dammini*. Five of the site 1 isolates had the same Osp phenotype as B31. Five additional site 1 isolates and three site 3 isolates, all of which expressed the OspC

protein, were similar to the B31 strain in the protein Mw of their OspA (31 kDa) and OspB (34 kDa). However, only two of the site 1 isolates displayed the same reactivity as B31 with the MoAbs described by Barbour and colleagues [8], Kramer and colleagues [15] and Wallich and colleagues [12].

DISCUSSION

Our study confirms the antigenic heterogeneity of the European *B. burgdorferi* isolates [8, 9, 11, 12]. In this study we compared isolates from ticks from three endemic areas and showed significant differences in the distribution of the different phenotypes among the isolates of these sites.

According to the molecular weight of OspA and OspB (Fig. 2), each endemic area presented a main typing group which differed significantly from the two other sites: group I prevailed in site 1, group III in site 2 and group II in site 3. A recent study demonstrated that the molecular weights of OspA and OspB and the representative phenotypes of borrelia isolates from patients with disseminated Lyme borreliosis were different from those obtained from patients with the cutaneous form of Lyme borreliosis [20]. It was reported that most skin isolates presented proteins of 32 kDa (OspA) and 35 kDa (OspB) (group II in our study), whereas the isolates from the disseminated Lyme borreliosis patients expressed an OspA of 32.5 kDa and an OspB of 33–34 kDa (groups III and V in our study). In view of our results, it may be suggested that the clinical manifestations of Lyme borreliosis may differ in different geographical areas. This hypothesis remains to be confirmed.

Immunoreactivity of the isolates varied between the different sites of isolation. Site 1 isolates reacted most with MoAbs LA-31 and LA-27. Site 3 isolates reacted most frequently with the MoAb H5332 and PoAb anti-22 kDa/NE4. In addition, the frequency of isolates reacting with MoAb LA-2, a MoAb which recognizes a protective epitope against *B. burgdorferi* infection [21], was different in each site. On the other hand, the comparison of our isolates with the strain B31 showed that only two isolates presented exactly the same reactivity with the MoAbs described by Barbour and colleagues [8], Kramer and colleagues [15] and Wallich and colleagues [12]. In view of this, it is suggested that European isolates could elicit specific antibody responses during infection, which differ from one site to another site and which is different from that induced by the strain B31. Therefore, a correct selection of the antigen or antigens seems to be necessary for the serodiagnosis of Lyme disease.

The different protein profile and immunoreactivity of the isolates from different geographical locations may account for seroconversion in people who do not develop Lyme borreliosis, as described in Aarberg (site 3, in this study) where 26% of an asymptomatic population was seropositive by ELISA and Western blotting [22, 23]. Among isolates from the Aarberg area (site 3), 83% expressed the 21–23 kDa proteins and 76% reacted with PoAb anti-22 kDa/NE4 and this was significantly different from the isolates from site 1. Several studies showed that these proteins are important immunogens which elicit the antibody response early after the tick bite [5, 17–19]. It remains to be elucidated whether these proteins could be responsible for the presence of asymptomatic seropositive people in

Aarberg by eliciting a protective antibody response. Another explanation could be that some of the strains present in this area were less pathogenic or non-pathogenic and that could depend on their antigenic profiles.

Heterogeneity observed among *B. burgdorferi* isolates from different sites which are fairly close to each other may have implications for human health if some strains are capable of producing early or late manifestations of Lyme borreliosis, on serodiagnosis and also on the production of protective vaccine since OspA and a 22 kDa protein are actually vaccine candidates. In fact, several studies showed that these antigens elicit protective antibody responses in animal models [21, 24–26].

The reasons leading to such a geographic diversity remain unknown but could be due to differences in the reservoir hosts in these three areas. However, our results suggest that studies on the local distribution of *B. burgdorferi* strains in each endemic area should represent an important step toward understanding the epidemiology of Lyme borreliosis, toward improving serological testing and toward developing an efficient vaccine for populations exposed to bites by infected ticks.

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Changes in the protein profile and antigenicity of different *Borrelia burgdorferi* strains after reintroduction to *Ixodes ricinus* ticks

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Summary Eight Swiss strains of *Borrelia burgdorferi*, with various protein profiles and the North-American strain B31 were artificially introduced into *Ixodes ricinus* ticks and reisolated 10 days later. All isolates were subsequently examined by SDS-PAGE analysis. Comparing initial isolates with the reisolates, we observed that 7 out of 9 strains changed their protein pattern with respect to the major proteins OspA, OspB and the 22 kDa protein after passage in the tick. The strains NE2, NE4 and NE83 with the initial phenotype of OspA and 22 kDa proteins changed to the phenotype of OspA and OspB, the strains B2 and NE202 with the initial phenotype of OspA acquired an additional protein of 22 kDa and the strain NE58 with the initial phenotype of OspA also acquired a protein of 22 kDa. Examination of these isolates by Western blot analysis demonstrated that the reaction with the monoclonal antibody H5332 and a monospecific polyclonal antibody PoAb/anti-22 kDa differed between the initial isolates and the reisolates.

Keywords: *Ixodes ricinus*, *Borrelia burgdorferi*, antigen, electrophoresis

Introduction

Borrelia burgdorferi, the causative agent of Lyme Borreliosis, is directly inoculated into the vertebrate by a tickbite. This means that the spirochaetes spend part of their life cycle within the arthropod, an environment strikingly different from that found within the vertebrate hosts. The necessity for micro-organisms to adapt to different environmental conditions has often been underestimated in laboratory research.

In Europe, as well as in the USA, many *B. burgdorferi* isolates have been established from ticks and vertebrates. The two outer surface proteins OspA (31 kDa) and OspB (34 kDa), of the North American isolates are more homogeneous than the European ones which has been demonstrated with various monoclonal antibodies (MoAbs) specific to these proteins by Western blot analysis (Barbour & Schrumphf 1986, Barbour, Heiland & Tessin 1985, Barbour, Tessier & Hayes 1984, Kramer *et al.* 1990, Wilske *et al.* 1986). In some of the European isolates, OspA and OspB could not be detected. Moreover, a

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number of them showed another major protein band of approximate molecular weight of 20–23 kDa, designated 'pC' (Barbour *et al.* 1985, Wilske *et al.* 1986, 1988).

Most of the studies on *B. burgdorferi* have been done after serial *in vitro* passages of the spirochaetes. Under these conditions, changes in protein profile and antigenicity as well as the loss of infectivity to animals have been reported in a tick isolate (Schwan and Burgdorfer 1987, Schwan, Burgdorfer & Garon 1988).

To determine if the passage into *in vivo* models of *in vitro* cultivated strains, influences (a) the protein profile and antigenicity of the strains, and (b) the pathogenicity and infectivity of the strains, we reintroduced various isolates of *B. burgdorferi* to *Ixodes ricinus*, the most important vector in Europe (Barbour *et al.* 1983c). If such modifications occurred within ticks, then this passage into the arthropod, which represents a prerequisite condition for the transmission to human and animal host, may be of great importance in the epidemiology of Lyme borreliosis.

We present data which suggest changes in the protein profiles and in the antigenicity of the strains during tick passages. The influence of the tick on the pathogenicity of *B. burgdorferi* will be the subject of another paper.

Materials and methods

STRAINS

B. burgdorferi strains NE2, NE4, NE56, NE83, NE202, NE203 were isolated from *I. ricinus* tick midguts incubated individually in BSK II medium (Barbour *et al.* 1985). Ticks were collected from the Neuchâtel area of Switzerland. The isolate B2, derived from synovial fluid of a Lyme disease patient (kindly provided by Dr J. Schmidli, Bern Hospital, Switzerland) (Schmidli *et al.* 1988) and the North American strain B31 were examined in parallel. These strains were maintained in our laboratory in BSK II culture medium as described by Barbour *et al.* (1985). Strains B31 and NE4 are high-passage strains (more than 100 *in vitro* passages) and the other strains were low-passaged (less than 6 passages). None of these strains developed changes after serial *in vitro* passages.

CLONING OF *B. BURGDOFFERI*

To isolate clonal populations of *B. burgdorferi*, the two strains NE2 and NE83 (10^7 cells/ml; Helber count cell chamber) were serially diluted 10-fold from 10^{-3} to 10^{-8} in BSK II medium (Bundoc & Barbour 1989). Three tubes containing 5 ml BSK II were used for each dilution. All dilutions were examined after 1, 2 and 4 weeks of incubation using dark field microscopy. The spirochaetes were tested by SDS-PAGE as described later.

REINTRODUCTION OF *B. BURGDOFFERI* TO *I. RICINUS* AND REISOLATION

The borrelia isolates (10^8 cells/ml) were reintroduced to uninfected *I. ricinus* females bred in our laboratory (Graf 1978), by the capillary method (Gern, Zhu & Aeschlimann 1990, Monin, Gern & Aeschlimann 1989). One week after infection, *B. burgdorferi* was reisolated from the tick midgut and maintained in BSK II medium at 34 °C for 7 to 10 days. Each reisolate was named by adding the letter R to the parental name, e.g., R2NE58 designates the reisolate number 2 from strain NE58.

SDS-PAGE

All isolates and their reisolates were incubated in 25 ml BSK II medium. Ten days later, the borrelia cultures were centrifuged and washed twice with PBS + 5mM MgCl₂ (10 000 g., for 20 min at 20°C). For polyacrylamide gel electrophoresis (SDS-PAGE), whole cells were suspended in 15 µl distilled water and resuspended in sample buffer to give a protein concentration of 30 µg/lane (Wilske *et al.* 1988) or 10⁷ cells/lane. The pH of the separating gel buffer was 8.8 and the acrylamide concentration was 12.5%. The gels were stained with Coomassie brilliant blue R-250. Molecular weight standards were low range specification of Bio-Rad's SDS-PAGE standards.

ANTIBODIES

The monoclonal antibodies H9724 specific for the flagellin and H5332 specific for the OspA of *B. burgdorferi* strain B31 were obtained from Alan Barbour (Barbour, Tessier & Todd 1983a). The polyclonal monospecific antibody (PoAb) anti-22kDa/NE4 was produced by immunizing a New Zealand white rabbit with the 22 kDa protein of strain NE4 as described by Wilske *et al.* (1988).

WESTERN BLOT ANALYSIS

Proteins of whole cell lysates, separated by SDS-PAGE (15 µg/lane) (Wilske *et al.* 1988) were transferred to nitrocellulose in a transit cell (2117-250 Nova Blot Electrophoretic Transfer Kit, LKB AB Bromma, Sweden). Blots were incubated with rabbit specific antibodies (1:200), monoclonal antibodies (MoAbs) H5332 (1:10) (Barbour *et al.* 1983a), H9724 (1:200) or polyclonal monospecific antibodies (PoAbs) anti-22 kDa/NE4 (1:200). The immunocomplexes were detected using peroxidase labelled anti-rabbit IgG antibodies or anti-mouse IgG antibodies (1:1000; Nordic Immunological Laboratories, The Netherlands).

Results

SDS-PAGE

Eight *B. burgdorferi* strains from Switzerland and the North American isolate B31 were subjected to SDS-PAGE (Figure 1). The protein patterns of these isolates were heterogeneous. The strain NE58 presented only one major molecular mass of 33 kDa and the strain NE202 contained a 31 kDa protein and in addition one with a protein mass of 22 kDa. Some of the isolates showed similar major surface proteins, for example, the three strains NE56, B2 and NE203 expressed the protein pattern as the strains B31, and the strains NE2, NE4 and the NE83 expressed the 33 kDa and the 22 kDa proteins.

These isolates were introduced to *I. ricinus* ticks and reisolated (Table 1). The reisolates were again examined by SDS-PAGE. Some showed different protein profiles after passage in *I. ricinus*. Therefore, we grouped the isolates according to their major surface proteins before and after passage into ticks (Table 2).

Striking modifications were observed in the molecular weight ranges of the outer surface proteins A or B. The initial isolates included in group I presented two major

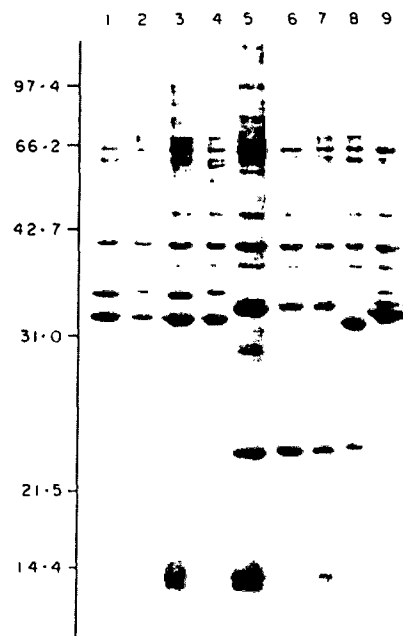


Figure 1. Eight Swiss strains of *B. burgdorferi* and North American isolate B31 were separated by SDS-PAGE and stained with Coomassie Blue R-250. Lanes 1 to 9: B31, NE56, B2, NE203, NE2, NE4, NE83, NE202, NE58. Molecular weight standards (MWS: kDa) are indicated on the left.

proteins with molecular masses of 31 kDa and 22 kDa, or 33 kDa and 22 kDa. All reisolates from the tick midgut were found to have lost the 22 kDa. Some of them have shifted the 33 kDa band. In three reisolates a 34 kDa band and in two reisolates a 31 kDa band appeared. Thus, through the passage in tick, the strains NE4 and NE2 changed into another phenotype with respect to OspA and OspB (B31-like). The protein patterns of the two strains NE202 and NE83 remained unchanged as far as the proteins of 31 and 33 kDa were concerned (Figure 2).

The initial isolates of group II presented proteins with molecular masses of 31 and 34 kDa. Reisolates of these strains showed an additional protein band of 22 kDa. The major surface proteins OspA and OspB remained unchanged (Figure 2).

Only one strain (NE58) was included in group III. The main protein of the initial isolate revealed by SDS-PAGE is a 33 kDa protein, perhaps OspA, which remained unchanged after tick passage. In contrast, a new protein band with a molecular mass of 22 kDa appeared in the reisolates (Figure 2).

Table 1. Borrelia isolates and their reisolates

| Strains | NE2 | NE4 | NE56 | NE58 | NE83 | NE202 | NE203 | B2 | B31 |
|-----------------------------|-----|-----|------|------|------|-------|-------|-----|-----|
| No. of ticks infected | 10 | 11 | 10 | 11 | 10 | 8 | 7 | 7 | 19 |
| No. of reisolates | 5 | 5 | 6 | 9 | 7 | 4 | 4 | 3 | 7 |
| Ratio of changed reisolates | 2/5 | 3/5 | 0 | 4/9 | 3/7 | 2/4 | 1/4 | 2/3 | 0 |

Table 2. Groups of *Borrelia* isolates and their major surface proteins

| Groups | Strains | Initial MSP* | Reisolate's MSP | Rate |
|-----------|---------|-------------------------|-------------------------------|------|
| Group I | NE2 | 33 kDa, 22 kDa | 31 kDa, 34 kDa | 2/5 |
| | NE4 | 33 kDa, 22 kDa | 31 kDa, 34 kDa | 3/5 |
| | NE83 | 33 kDa, 22 kDa | 33 kDa, 34 kDa | 3/7 |
| | NE202 | 31 kDa, 22 kDa | 31 kDa | 2/4 |
| Group II | B2 | 31 kDa, 34 kDa | 31 kDa, 34 kDa, 22 kDa | 2/3 |
| | NE203 | 31 kDa, 34 kDa | 31 kDa, 34 kDa, 22 kDa | 1/4 |
| Group III | NE58 | 33 kDa | 33 kDa, 22 kDa | 4/9 |
| Group IV | B31 | 31 kDa, 34 kDa, 22 kDa† | 31 kDa, 34 kDa, 22 kDa† | 0 |
| | NE56 | 31 kDa, 34 kDa, 23 kDa† | 31 kDa, 34 kDa, 23 kDa† | 0 |

* MSP: Major surface protein.

† Trace on the SDS-PAGE.

Bold type: lost or acquired proteins in reisolates.

All reisolates of the strains NE56 and B31 gathered in group IV did not show any changes with respect to the proteins with molecular masses of 31, 34 and 22 kDa to 23 kDa (Figure 2).

In addition, we repeated these experiments using two reisolates of NE58, i.e., R2NE58 (Figure 3a), a modified reisolate (appearance of a new 22 kDa protein) and R5NE58 (Figure 3b) an unmodified reisolate comparable to NE58. The second passage into *I. ricinus* did not influence R2NE58: the resulting reisolates presented the same protein pattern as R2NE58. In contrast, the passage of R5NE58, ended up with 2 out of 7 reisolates showing modifications (appearance of a new 22 kDa protein) comparable to the modifications presented by R2NE58 after the first passage into ticks. (Figures 3a and 3b).

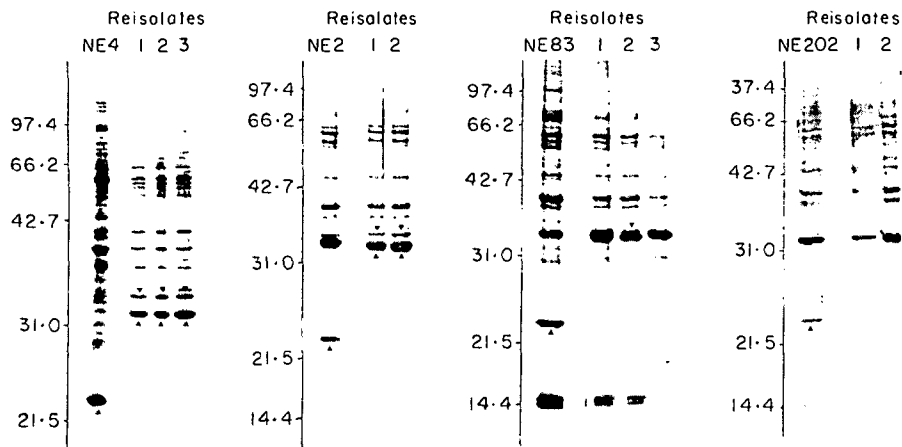
WESTERN BLOT

To investigate changes of the antigenic determinants, *B. burgdorferi* isolates and their modified reisolates were examined by Western blot. Western Blot analyses using monoclonal and polyclonal antibodies to OspA, flagellin and the 22 kDa protein demonstrated differences of the *B. burgdorferi* isolates before and after their passage in ticks. Loss of the 22 kDa protein of the group I reisolates resulted in the lack of staining with the PoAb specific for the 22 kDa protein (PoAb/anti 22 kDa/NE4) (Table 3). Moreover, the reaction of the three modified reisolates from NE4 (R5NE4; R7NE4 and R9NE4) with MoAb H5332 specific for OspA changed from negative to positive staining (Table 3).

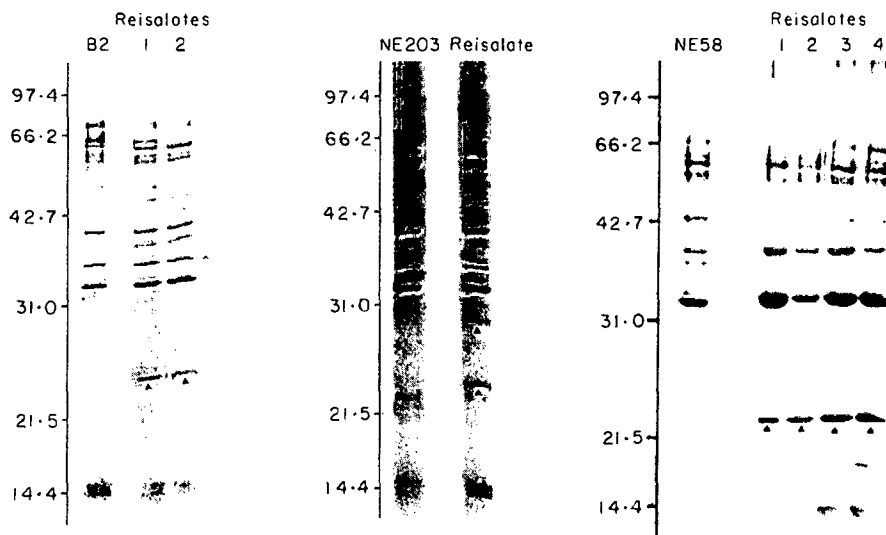
On the other hand, the additional new protein of 22 kDa of the modified reisolates from groups II and III reacted with the PoAb 22 kDa/NE4 (Table 3). In addition, OspA of strain NE58 did not react any more with MoAb H5332 after reisolation from the tick midgut (Table 3).

All strains examined in this study did not change their reactivity with MoAb H9724 specific for the flagellum associated protein of *B. burgdorferi*, after tick passage (Table 3).

To exclude the possibility that a simple cloning effect was responsible for the reisolation of modified spirochaetes, we cloned the two strains NE2 and NE83 (10^7 cells/ml (both strains had lost the 22 kDa protein after passage into ticks) by limiting dilution from 10^{-3} to 10^{-8} . After cultivation for 4 weeks, NE2 spirochaetes were found in all tubes at the 10^{-6} dilution, in 2/3 tubes at the 10^{-7} dilution and in 1/3 tubes at the 10^{-8} dilution; NE83 spirochaetes were found in 2/3 tubes at the 10^{-6} dilution, in 2/3 tubes at the 10^{-7} dilution and in 1/3 tubes at the 10^{-8} dilution. All the clones obtained were examined by SDS-PAGE analysis (data not shown). Compared to the uncloned strains NE2 and

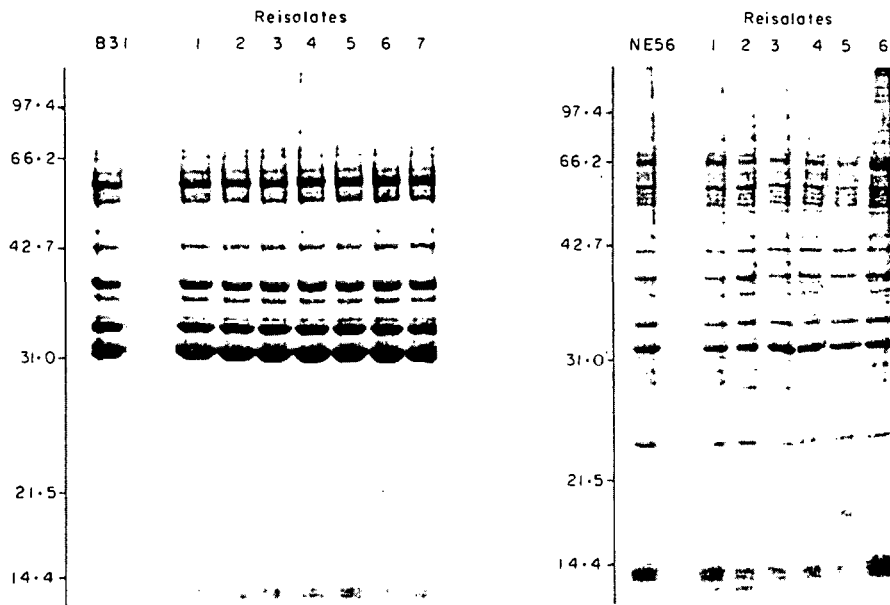


(a)



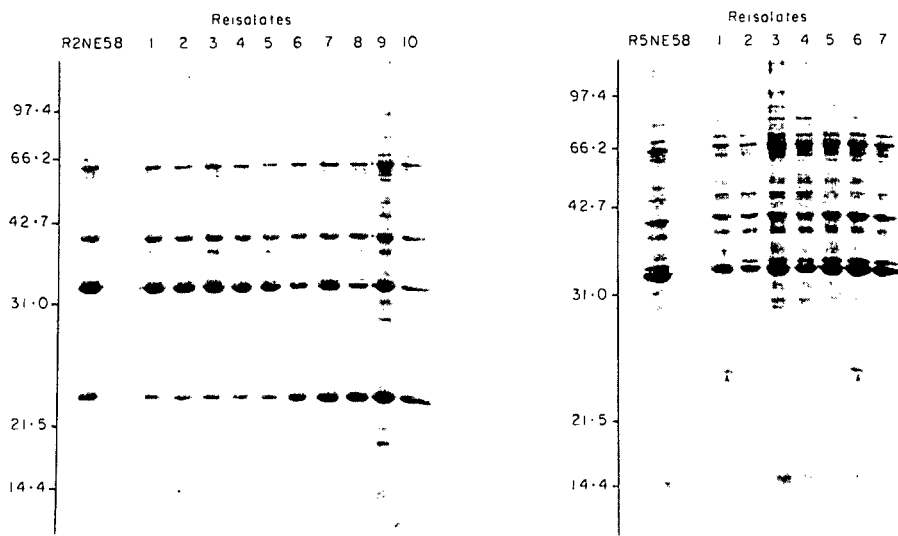
(b)

(c)



(d)

Figure 2. Coomassie Blue stained polyacrylamide gel of different *B. burgdorferi* strains and some reisolates. Figures 2a, b, c (opposite) and d concern respectively group I, II, III and IV of borrelia isolates. The initial isolate is on the first lane and the reisolates on the others. Molecular weights are indicated on the left. The arrows indicate the modified proteins after passage into *I. ricinus*.



(a)

(b)

Figure 3. Coomassie Blue stained polyacrylamide gel of two reisolates R2NE58, R5NE58 and their reisolates from tick midgut. (a) R2NE58 and its 10 reisolates (lanes 1-10). (b) R5NE58 and its 7 reisolates (lanes 1-7). Two reisolates of R5NE58 modified their protein profiles after the second passage into *I. ricinus* as indicated by the arrows.

Table 3. Reaction of borrelia isolates with different antibodies

| Strains | PoAb/anti- 22 kDa/NE4 | MoAb/anti- OspA/H5332 | MoAb/anti- flagellin/H9724 | Group |
|---------|--------------------------|--------------------------|-------------------------------|-------|
| NE2 | + | - | + | I |
| R3NE2 | - | - | + | |
| R4NE2 | - | - | + | |
| NE4 | + | - | + | I |
| R5NE4 | - | + | + | |
| R7NE4 | - | + | + | |
| R9NE4 | - | + | + | |
| NE83 | + | - | + | I |
| R3NE83 | - | - | + | |
| R4NE83 | - | - | + | |
| R5NE83 | - | - | + | |
| NE202 | + | - | + | I |
| R1NE202 | - | - | + | |
| N5NE202 | - | - | + | |
| B2 | - | - | + | II |
| R1B2 | + | - | + | |
| R3B2 | + | - | + | |
| NE203 | - | - | + | II |
| R3NE203 | + | - | + | |
| NE58 | - | + | + | III |
| R2NE58 | + | - | + | |
| R3NE58 | + | - | + | |
| R4NE58 | + | - | + | |

* Results obtained by B.Wilske.

NE83, no differences were observed after cloning. The 22 kDa protein did not disappear suggesting that what happened in tick midguts is not a phenomenon of cloning.

Then, we repeated our experiment and infected 20 ticks with the cloned strain NE83 (at dilution 10^{-8}), named cNE83. Eleven ticks were dissected 1 week after infection to reisolate the spirochaetes from different organs: midgut, salivary glands and synganglion. Twenty one reisolates were obtained and were examined by SDS-PAGE. Results showed that 2 midgut and 3 salivary glands reisolates (from 5 different ticks) present now only a weak band of 33 kDa protein suggesting that some modification may also occur in reisolates from cloned strain after passage into ticks (Figure 4). No change was observed with respect to the 22 kDa protein.

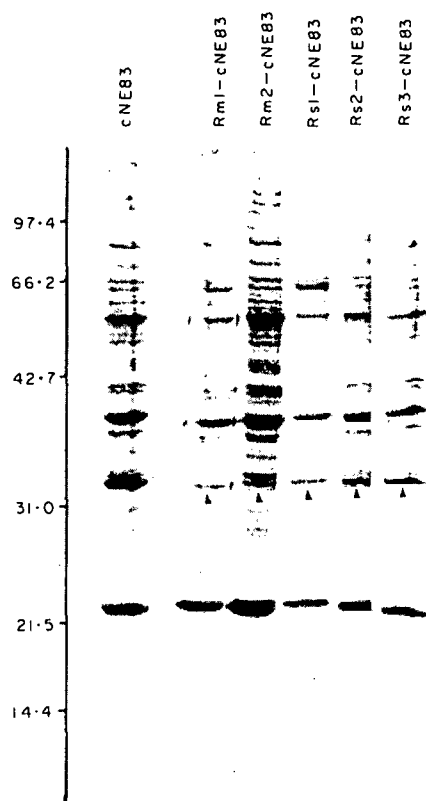


Figure 4. The cloned strain cNE83 (lane 1) and its reisolates from 5 different ticks were examined by SDS-PAGE. Two reisolates from tick midguts (Rm1-cNE83, Rm2-cNE83; lanes 2 and 3) and three reisolates from tick salivary glands (Rs1-cNE83, Rs2-cNE83 and Rs3-cNE83; lanes 4, 5 and 6) were modified after passage into ticks. The arrows indicate the modified proteins and the molecular weights (MW: kDa) are indicated on the left.

Discussion

The heterogeneous protein patterns observed in the Swiss isolates correspond to those described for other European borrelia isolates (Barbour *et al.* 1985, Wilske *et al.* 1986, 1988). Their phenotypes differed in the amount and the molecular weight of their OspA (31 kDa) and OspB (34 kDa) proteins. In addition a protein of 22 kDa was expressed in 5/8 isolates.

In this study, 8 Swiss strains and the North American strain B31 were artificially introduced to *I. ricinus*. One week later, they were reisolated from the tick midgut, examined by SDS-PAGE and Western blot and compared to the initial strains. In most of the cases, modifications of the protein profiles were observed. The major surface proteins OspA (31–32 kDa) and OspB (34–35 kDa), and a protein with a molecular mass of 22 kDa, were mainly involved in these changes.

The protein band of 22 kDa was either 'lost' or 'acquired' in the reisolates. These modifications were accompanied by shifts of the molecular masses of the 33 kDa protein

and in some reisolates a 31 kDa and a 34 kDa protein were present. Thus reisolated spirochaetal populations contained new phenotypes expressing different surface proteins through the influence of residence in the tick midgut. In fact, the changes of OspA and the 22 kDa protein found by SDS-PAGE were supported by Western blot analysis which revealed loss or appearance of epitopes detected by specific monoclonal and polyclonal antibodies.

Previous reports suggested that clonal polymorphisms of the major outer surface antigen B (OspB) of *B. burgdorferi* occurred (Bundoc & Barbour 1989). The modifications obtained in the tick reisolates could be explained by such a clonal polymorphism but not by a cloning effect. This is suggested by the fact that the strains NE2 and NE83 lost their 22 kDa protein after tick passage, a phenomenon which was not observed after *in vitro* cloning.

Reisolates obtained from an uncloned (NE83) and a cloned (cNE83) strain presented different protein patterns after tick passage: RNE83 lost the 22 kDa protein and presented with 33 kDa protein whereas the contrary was observed with cRNE83. This could be explained by a selection, through cloning, of a variant which after passage into ticks is more susceptible to express the 22 kDa protein than the 33 kDa protein. A comparable phenomenon was described by Bundoc & Barbour (1989): an increase in expression of the 21 or 18.5 protein was associated with a decrease or halted production of OspB in different variants. Additional experiments using cloned strains are in process.

The number of *in vitro* passages of the strains did not influence the occurrence of a change. This occurrence seems to depend on the strain (e.g. B31 and NE56 did not change) and on the ticks (all the reisolates from the same starting population did not always indicate change in the isolate). Changes are repeatable and constant for the same starting population as it was shown: reisolates of NE58 were either modified (appearance of a 22 kDa protein) or unmodified; if such an unmodified re isolate is introduced again to ticks, this induces a modification comparable to that presented by the reisolates which were modified after the first passage. Why changes occur in some ticks and not in all, could be due to different physiological conditions or to other unknown factors present in ticks.

Previous studies showed that *B. burgdorferi* spirochaetes multiply in the midgut of *I. ricinus* females. Only in some infected ticks, a small number of spirochaetes were found to escape from the midgut and to penetrate the midgut wall leading to systemic infections (Gern *et al.* 1990, Monin *et al.* 1989). This phenomenon could be somehow linked to the antigenic changes observed in tick reisolates which also took place only in some ticks. A selection process may occur in the midgut. The modifications observed in the antigenicity of OspA and OspB in our reisolates may play a role in this selection: only the 'adapted' spirochaetes could be able to adhere to midgut epithelial cells and traverse the midgut wall. The adherence of *B. burgdorferi* to the tick midgut cell surfaces has already been described (Benach *et al.* 1987). The OspA and OspB have been suggested to play a role in the adherence of spirochaetes to endothelial cells (Benach *et al.* 1988, Comstock & Thomas 1989, Thomas & Comstock 1989) and adherence to cells is considered an important step for bacterial invasion and in pathogenesis (Finlay & Falkow 1989). Therefore, the antigenic shift of OspA and OspB and may be the 22 kDa protein, revealed after passage into tick midgut, might play an important role for the pathogenicity and infectivity of *B. burgdorferi* in the vertebrate host.

In experimentally infected mice, the first antibodies to be detected are those directed against OspA and OspB (Benach *et al.* 1988, Schwan *et al.* 1989). Antibodies specific for

the outer surface protein A (OspA) can protect immunodeficient *Scid* mice (Schaible *et al.* 1990, Simon *et al.* 1991) as well as immunocompetent *C3H* mice (Fikrig *et al.* 1990) against clinical manifestations. However, in patients, antibodies against OspA and OspB are not expressed until the late stages of the disease (Barbour *et al.* 1983b, Craft *et al.* 1986, Dattwyler *et al.* 1988). The differences observed in the immune response in mice and patients could be related to the mode of inoculation of the spirochaete as it has already been suggested in a previous study in dogs (Greene *et al.* 1988). The authors found that experimentally infected dogs expressed antibodies directed to OspA, whereas naturally infected dogs did not. Our data could support the suggestion that the different immune responses in experimentally and naturally infected hosts like humans are influenced by the way of inoculation.

Our observations might explain the protein patterns of European isolates showing such a heterogeneity with respect to their major surface proteins A and B (Figure 5). The fact that the phenotype of a *B. burgdorferi* population changed during residence in the tick suggests that ticks not only transmit the micro-organism but provide also the environment which leads to the variation of one or the other epitope of *B. burgdorferi* antigens. The mechanisms responsible for such changes in the arthropod environment remain unknown. Experiments concerning pathogenicity and the molecular genetics of these isolates and their modified reisolates are in process.

In vertebrate host, antigenic variation is also suggested but not established at this point. Additional investigations of its occurrence in animals and human infections, as well as in ticks, should be done.

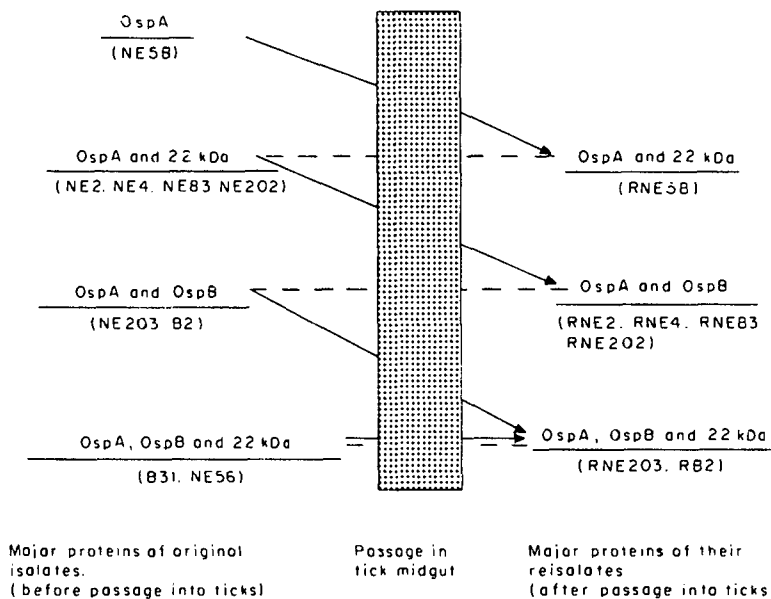


Figure 5. Schema of the modifications of the major proteins of *B. burgdorferi* after passage into *I. ricinus*. It is shown that changes from a phenotype (major proteins) to another one occur in the tick midgut (indicated by arrows) and the reisolated phenotypes (left) are similar to other original phenotypes of *B. burgdorferi* strains (right).

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Antigenic variation in *Borrelia burgdorferi* after passage through *Ixodes ricinus* and *Ixodes hexagonus* ticks

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Introduction

Borrelia burgdorferi, the causative agent of Lyme borreliosis is transmitted to humans and animals by infected ticks. In Europe, *Ixodes ricinus* is the main vector of this spirochete (Burgdorfer *et al.*, 1983), and recently the hedgehog tick, *I. hexagonus* has proved to transmit *B. burgdorferi* (Gern *et al.*, 1991).

The spirochetes spend part of their life cycle within the arthropod. In laboratory research, this aspect has often been underestimated. Most of the studies on *B. burgdorferi* have been performed with bacteria passaged *in vitro*. Under these conditions, changes in the antigen profile as well as loss of infectivity for animals have been reported (Schwan *et al.*, 1987, 1988).

In this study, various *in vitro* grown isolates of *B. burgdorferi* were reintroduced into *I. ricinus* and *I. hexagonus* and afterwards recultivated to determine whether the passage through ticks influences the protein and antigen profile of the spirochetes.

Materials and Methods

Strains

B. burgdorferi strains NE2, NE4, NE56, NE58, NE83, NE202, NE203, NE196 were isolated from *I. ricinus* midguts in BSKII medium (Barbour, 1984). Ticks were collected from the Neuchâtel area of Switzerland. The isolate B2 derived from synovial fluid of a patient (Schmidli *et al.*, 1988) and the North American strain B31 were also examined. To isolate clonal populations of *B. burgdorferi*, strains NE2 and NE83 were cloned by limiting dilution (Bundoc and Barbour, 1989).

Reintroduction of B. burgdorferi into I. ricinus and I. hexagonus and reisolation

The isolates NE2, NE4, NE56, NE58, NE83, NE202, NE203, B2, B31 and the cloned strain NE83 (cNE83) (10^8 cells / ml) were reintroduced into uninfected *I. ricinus* females, by the capillary method (Gern *et al.*, 1990). One week thereafter, spirochetes were reisolated from the tick midgut. All isolates and reisolates were submitted to SDS-PAGE and Immuno Blot as described elsewhere (Hu *et al.*, 1992). The monoclonal antibodies (MoAb) H9724 and H5332 specific for the flagellin and OspA, respectively, of *B. burgdorferi* strain B31 were obtained from Alan Barbour (Barbour *et al.*, 1983). Other monoclonal antibodies (LA-31, LA-4, LA-28; LA-25, LA-32, LA-27; LA-7) recognizing epitopes of OspA, OspB and 20 kDa antigens (Kramer *et al.*, 1990) were used for Immuno Blot as well as polyclonal monospecific antibodies against a 22 kDa protein of strain NE4 (PoAb) (anti-22 kDa/NE4; Hu *et al.*, 1992).

The isolate NE196 (B31 type; 10^7 cells / ml) was introduced into 10 flat *I. hexagonus* females using the capillary method and was reisolated one week later. The cloned strain cNE83 (10^7 cells / ml) was used to infect 30 engorged *I. hexagonus* nymphs by the same method and was reisolated after tick moulting.

Results

I. ricinus, I. hexagonus and uncloned strains

Some reisolates showed altered protein profiles after passage in *I. ricinus*. Striking modifications were observed in the molecular weight ranges of OspA and OspB. We grouped the isolates according to their major surface proteins before and after passage into ticks. In group I, four isolates (NE2, NE4, NE83 and NE202) presented two major proteins with molecular masses of 33 kDa and 22 kDa, or 31 kDa and 22 kDa. All modified reisolates from the tick midgut were found to have lost the 22 kDa band. Some of them have shifted

and altered the 33 kDa band: In three reisolates a 34 kDa band and in two a 31 kDa band appeared. The strains NE4 and NE2 changed into a B31-like phenotype with respect to OspA and OspB. Loss of the 22 kDa protein of the group I reisolates resulted in lack of staining with PoAb specific for the 22 kDa protein of NE4. Moreover, the reaction of the modified reisolates from NE4 with moAb H5332 changed from negative to positive. The initial isolates of group II (B2 and NE203) presented proteins with molecular masses of 31 and 34 kDa and reisolates showed an additional protein band of 22 kDa. Only one strain, NE58, was included in group III. The main protein of this isolate is a 33 kDa protein, perhaps OspA, which remained unchanged after tick passage. In contrast, a new protein band of 22 kDa appeared in the reisolates. The additional new protein of 22 kDa of the modified reisolates from groups II and III reacted with PoAb 22 kDa/NE4. In addition, OspA of strain NE58 did not react any more with moAb H5332 after reisolation from the tick. All reisolates of group IV, NE56 and B31, did not show any changes.

After passage through *I. hexagonus*, reisolates from NE196 (type B31) did not show any changes.

I. ricinus, *I. hexagonus* and the cloned strain cNE83

To exclude the possibility that a simple cloning effect was responsible for the reisolation of modified spirochetes, we cloned the strains NE83 and NE2 (both strains had lost the 22 kDa protein after passage through *I. ricinus*). When compared to the uncloned strains, no differences were observed after cloning: the 22 kDa did not disappear suggesting that loss of the 22 kDa protein (as observed in tick midguts) is merely not a phenomenon of cloning.

The cloned strain cNE83 was reintroduced into *I. ricinus* and *I. hexagonus*. Five reisolates from *I. ricinus* presented only a weak band of OspA with 33 kDa and the 22 kDa protein was unchanged (Fig.1). The 33 kDa was not detectable in 3/6 reisolates from *I. hexagonus* (Fig.1). Immuno Blot results of cNE83 and its reisolates from *I. ricinus* and *I. hexagonus* are summarized in Table 1.

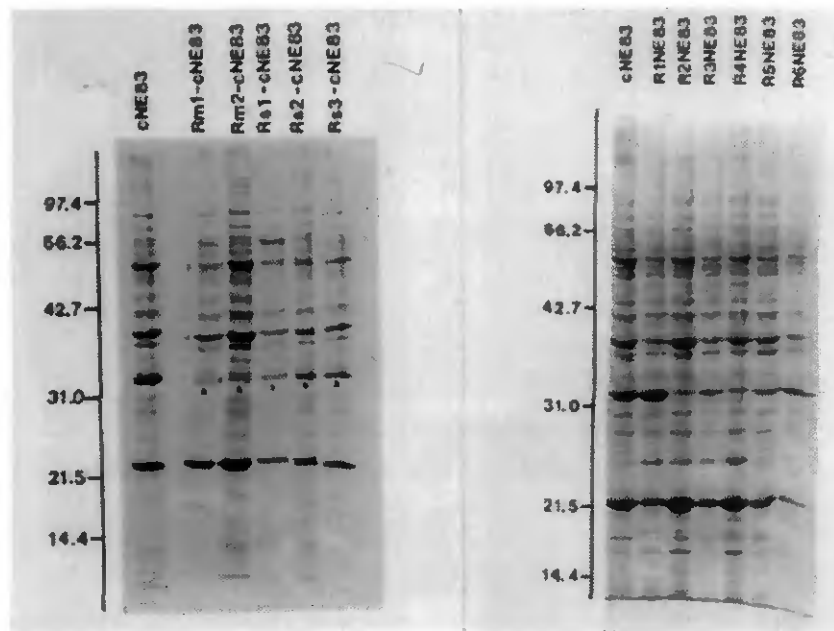


Fig.1. Protein profiles of the *B. burgdorferi* cloned strain cNE83 before passage through ticks and its reisolates from *I. ricinus* (left) and *I. hexagonus* (right). R: reisolates.

Table 1 Immuno-reactions of cNE83 before and after passage through *I. ricinus* and *I. hexagonus*

| Specific antibodies | cNE83 | cNE83 (Ir) 5 reisolates | cNE83 (Ih) 6 reisolates |
|---------------------|-------|----------------------------|----------------------------|
| anti-OspA | | | |
| H5332 | - | ND | -/-/-/-/- |
| LA-2 | - | ND | -/-/-/-/- |
| LA-4 | + | ND | -/-/-/-/- |
| LA-31 | + | -/+/-/+/- | +/-/-/+/>+ |
| anti-OspB | | | |
| LA-25 | + | -/-/-/-/- | -/-/-/-/- |
| LA-27 | - | ND | -/-/-/-/- |
| LA-32 | - | ND | -/-/-/-/- |
| anti-OspC | | | |
| 22kDa/NE4 | + | +/+/+/+/+ | +/+/+/+/+/+ |
| anti-20kDa | | | |
| LA-7 | - | ND | -/-/-/-/- |
| anti-flagellin | | | |
| H9724 | + | +/+/+/+/+ | +/+/+/+/+/+ |

ND: not done

Ir: *Ixodes ricinus* Ih: *Ixodes hexagonus*

Discussion

In this study, 9 Swiss strains and the North American strain B31 were artificially introduced into *I. ricinus* or *I. hexagonus* and reisolated from their midguts. In most cases, modifications of the protein and antigen profiles were observed. OspA, OspB and a protein with a molecular mass of 22 kDa were mainly changed. The 22 kDa band was either "lost" or "acquired" in the reisolates and these modifications were accompanied by shifts of the 33 kDa protein: i.e. in some reisolates a 31 and a 34 kDa protein was present. Changes were also found by Immuno Blot analysis which revealed loss or appearance of specific epitopes. Reisolates obtained from an uncloned (NE83) and cloned strain (cNE83) presented different pattern after tick passage: reisolates from NE83 lost the 22 kDa protein and presented the 33 kDa whereas the contrary was observed with the reisolates of cNE83. Selection of a *B. burgdorferi* variant that is more prone to express the 22 kDa protein than the 33 kDa protein, possibly induced by the cloning process, could account for this phenomenon. A comparable observation was described by Bundoc and Barbour (1989): an increase in expression of the 21 or 18.5 protein was associated with a partial or complete loss of OspB in different variants. Moreover Immuno Blot analysis of the reisolates of cNE83 from both tick species revealed a loss of epitopes detected by OspA- and OspB-specific antibodies (Table 1).

Previous studies showed that *B. burgdorferi* multiplies in the midgut of *I. ricinus* females. Only in some infected ticks, few spirochetes escape from the midgut and induce a systemic infection (Gern *et al.*, 1990). It is tempting to speculate that this is somehow linked to the antigenic changes observed in tick reisolates which also appeared only in some ticks.

Moreover, recent studies revealed that spirochetemia in mice infected via tick bites is higher than in experimentally infected animals suggesting that the passage of the spirochetes in the tick environment enhances the infectivity of *B. burgdorferi* (Gern *et al.* submitted). In vertebrate hosts, antigenic variation has also been suggested (Schwan *et al.* 1991). Additional investigations in animals, ticks and humans should be addressed to this matter.

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**PROTEINIC, ANTIGENIC AND PATHOGENIC VARIATIONS OF
A CLONAL *BORRELIA BURGDORFERI* ISOLATE FROM
IXODES RICINUS HEMOLYMPH**

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In this study, we obtained a *B. burgdorferi* isolate from tick hemolymph which was highly pathogenic for SCID mice and expressed two main proteins in the molecular weight range ~ 20 kDa, i.e. 22 kDa and 23 kDa, detected by a specific immune serum. Continuous cultivation *in vitro* resulted in a successive loss of the 22 kDa and 23 kDa molecules, loss of reactivity with the immune serum and a loss of pathogenicity in the SCID mice. After cloning, this non pathogenic strain was reintroduced into ticks by capillary feeding. Most of the reisolates from various tick organs again reacted with the specific immune serum. Two out of four isolates which re-expressed the 22/23 kDa proteins induced clinical arthritis in SCID mice. These results suggest that tick factors influence the protein profiles, antigenicity and pathogenicity of *B. burgdorferi* spirochetes.

In Europe, *Borrelia burgdorferi* is transmitted by *Ixodes ricinus* ticks. In most host-seeking ticks, spirochetes are limited to

the midgut. During the blood meal, they penetrate the midgut wall and induce a systemic infection via hemolymph (Gern et al., 1990).

Immunochemical analysis of the European isolates derived either from tick midgut or from different animal hosts showed that their outer surface proteins (OspA, OspB and OspC) are variable in their molecular weights and reactivity with different antibodies (Kramer et al., 1992). The great majority of tick isolates investigated so far were obtained from either tick midgut or tick homogenates. Previous studies have shown that the reintroduction of *B. burgdorferi* into ticks resulted in changes of protein profile and antigenicity (Hu et al., 1992). In an attempt to further study the influence of the tick's physiology on the phenotypic variability of *B. burgdorferi*, spirochetes were isolated from *I. ricinus* hemolymph. Here we describe one isolate, and its clonal derivative and report on its variations in protein profile, antigenicity and pathogenicity.

Ixodes ricinus adults were collected by flagging vegetation in a forest near Neuchâtel (Bois de l'Hôpital, Switzerland). The tick hemolymph was collected using capillaries after cutting the coxa I from the first leg and was inoculated in a tube containing 4 ml of BSK II medium. The culture tubes were examined after 10 days by dark-field microscopy.

One isolate was obtained and was subcultured in BSK II medium for 15 passages. In addition, the isolate was cloned at passage 3 and reintroduced into uninfected *I. ricinus* adults using the capillary feeding technique. One week later, the spirochetes were reisolated from various tick organs including midgut, salivary glands and genital tissues. The protein profiles and antigenicity of the isolate, its clonal derivative as well as the reisolates were examined by SDS-PAGE and by immunoblotting using

a panel of monoclonal antibodies and an immune serum (IS) specific for 22/23 kDa proteins. In addition, the pathogenicity of all spirochetal preparations were examined in adult SCID mice by monitoring for clinical signs of arthritis in the tibiotarsal joints for 70 days post inoculation.

Results showed that the original isolate expressed four major protein bands corresponding to 33 kDa, 32 kDa and two proteins in the 20 kDa range, 22/23 kDa. Such a protein pattern has never been observed before in our laboratory among ~ 200 tick isolates from the midgut which is the primary organ site for spirochetes in unfed ticks. This suggests that the particular phenotype of the present isolate is related to the particular hemolymph environment.

During serial subcultivation *in vitro*, the original isolate lost the 22 kDa protein after 5th passage and the 23 kDa protein after 15th passage as well as its reactivity with the IS. After cloning of the original isolate at passage 3, the cloned strain lacked the 22 kDa protein and presented a phenotype similar to that of the 5th *in vitro* passage of the original isolate.

When the cloned *B. burgdorferi* strain was reintroduced into ticks and reisolates from various tick organs were analysed, variations in the protein profile were observed. Two of the reisolates reexpressed the 22 kDa protein. These results indicate that the expression of the 22/23 kDa proteins is influenced by tick factors. Moreover, we demonstrate that proteins and epitopes of *B. burgdorferi* which are lost during *in vitro* cultivation might be re-acquired after re-exposure to its former environment.

The original isolate was highly pathogenic for SCID mice (Schaible *et al.*, 1993) but lost this activity after continuous *in vitro* passages. Moreover, the clonal strain was also unable to induce clinical arthritis in SCID mice. Whereas, after passage through ticks, two out of four

reisolates again induced disease in SCID mice. Together the results suggest that tick factors may influence both the phenotype and the pathogenicity of *B. burgdorferi*.

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Tick Factors and *In Vitro* Cultivation Influence the Protein Profile, Antigenicity and Pathogenicity of a Cloned *Borrelia garinii* Isolate from *Ixodes ricinus* Hemolymph

Summary: A *Borrelia garinii* isolate (NE11H) was obtained from the hemolymph of infed *Ixodes ricinus*. NE11H expressed four major proteins of 33 kDa, 32 kDa, 23 kDa and 22 kDa. During *in vitro* culture, NE11H successively lost the expression of the 22 kDa and 23 kDa proteins and the NE11H variant (NE11Hp15) was not recognized by an immune serum specific for the OspC protein (anti-OspC IS). However, when reintroduced into tick midguts, NE11Hp15 spirochetes present in the midgut again reacted with anti-OspC IS. A clone derived from the wild type line, cNE11H, lacked the 22 kDa but not the 23 kDa protein. The 23 kDa protein of cNE11H was recognized by anti-OspC IS. In addition, the two descendant lines (NE11Hp15 and cNE11H) lost their capacity to induce clinical arthritis in SCID mice. When cNE11H was reintroduced into ticks and reisolated from various tick organs, most reisolates presented the same reaction with anti-OspC IS as cNE11H. Interestingly, two reisolates obtained from the tick midgut reexpressed large amounts of the 22 kDa protein which was recognized by anti-OspC IS and these two reisolates induced clinical arthritis in SCID mice. The results confirm that proteins of 22/23 kDa are differentially expressed during *in vitro* subcultures and in ticks, and show that proteins which are not detectable after *in vitro* culture may be reexpressed after reexposure of *B. burgdorferi* to its former environment in the tick. The data suggest that the pathogenicity of *B. burgdorferi* for mice might be influenced by environmental factors via differential expression of 22/23 kDa proteins.

Introduction

The Lyme disease spirochete, *Borrelia burgdorferi* [1] is transmitted in Europe by *Ixodes ricinus* [2]. In most host-seeking infected ticks, spirochetes are localized in the midgut. The salivary route of *B. burgdorferi* transmission is now generally accepted for *I. ricinus* [3] and for the American tick vector *I. scapularis* [4, 5]. This implicates that spirochetes penetrate the midgut wall and via hemolymph infect other tick organs during the blood meal. After molting, some ticks may have a systemic infection [6, 7]. To our knowledge, only a few isolates of *B. burgdorferi* have been obtained from tissues other than tick midgut and most tick isolates previously described have been derived from tick midgut or tick homogenates [6, 8, 9].

Phenotypic and genotypic analysis of European *B. burgdorferi* isolates from *I. ricinus* ticks showed that there are at least three species: *B. burgdorferi* sensu stricto (ss), *B. garinii* and *B. afzelii* [8, 10, 11]. *B. burgdorferi* ss expresses outer surface protein (Osp) A of 31 kDa and OspB of 34 kDa, *B. garinii* has an OspA of 32–33 kDa and *B. afzelii* expresses an OspA of 32 kDa and an OspB of 35 kDa. In addition to OspA and OspB, many European *B. burgdorferi* sensu lato (sl) isolates express a major protein with a molecular weight of about 22 kDa described as OspC [8, 12]. Most American isolates belong to *B. burgdorferi* ss and lack the expression of OspC protein [12, 13].

Previous studies have shown that cultivation of tick-derived *B. burgdorferi* sl isolates may result in differential

expression of cell surface proteins and/or antigen variation and that the altered phenotype is associated with a loss of virulence in animal hosts [14, 15]. In addition, it was shown that both expression of OspA, OspB and/or OspC as well as immunogenicity of *B. burgdorferi* sl isolates were influenced by passage through ticks [16, 17]. These results indicate an environmental influence on the phenotype and/or infection potential of *B. burgdorferi*.

In the present study, we employed a *B. garinii* isolate (NE11H) obtained from the hemolymph of a systematically infected *I. ricinus* to examine the protein profile, antigenicity and pathogenicity of its uncloned and cloned descendants after *in vitro* passages and reintroduction into ticks. The data demonstrate that spirochetes express proteins differently under *in vivo* from *in vitro* conditions and suggests that environmental factors are critical in the control of their pathogenic potential.

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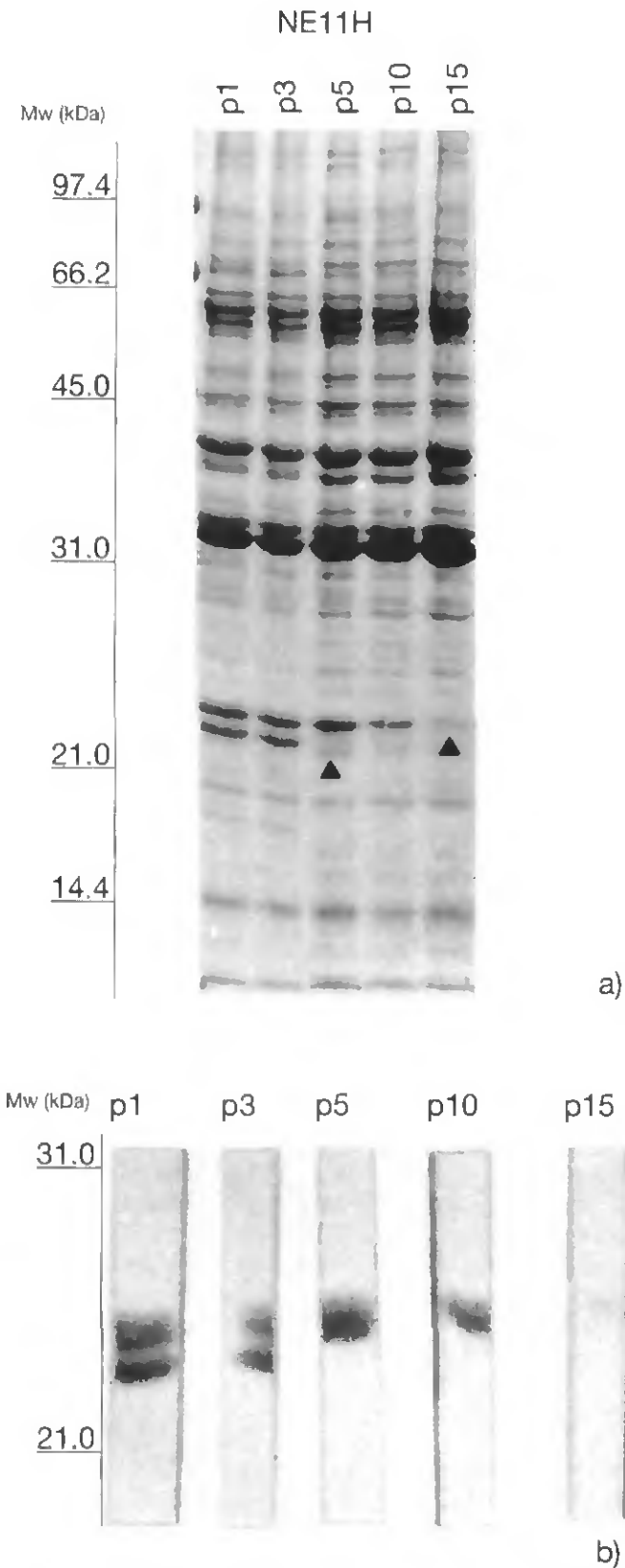


Figure 1. a) SDS-PAGE stained with Coomassie blue of NE11H and its *in vitro* passages (p1, p3, p5, p10 and p15); b) reaction of NE11H *in vitro* passages with IS anti-OspC. The protein molecular weight standards are shown on the left.

Materials and Methods

Ticks and *B. burgdorferi* isolates: Twenty *I. ricinus* adults were collected by flagging vegetation in a forest near Neuchâtel (Bois de l'Hôpital, Switzerland) in June 1990. Ticks were washed in 70% ethanol and rinsed in sterilized PBS pH 7.4. Tick hemolymph was collected using capillaries after cutting the first leg and was immediately inoculated in tubes containing 4 ml of BSK II medium [18] and maintained at 34°C. After 10 days of incubation the culture tubes were examined by dark-field microscopy over a period of 6 weeks. NE11H was cultivated in BSK II until the fifteenth serial *in vitro* passage (p) (NE11Hp1–NE11Hp15). The third passage (p3) containing 10⁷ cells/ml (Helber counting cell chamber) was cloned by limiting dilution [16, 19]. Serial ten-fold dilutions from 10⁻³ to 10⁻⁹ were carried out in BSK II medium and three tubes containing 5 ml BSK II were used for each dilution. After 3 weeks of cultivation, none of the tubes at the 10⁻⁹ dilution contained spirochetes and only one of three tubes was positive at the 10⁻⁸ dilution for *B. burgdorferi*.

This cloned strain, termed cNE11H, was reintroduced into 20 uninfected *I. ricinus* adults from our laboratory colony, using the capillary feeding technique as described previously [16, 20]. During capillary feeding all ticks were maintained at 34°C for 2 hours. Infected ticks were maintained at room temperature and dissected 10 days later. The midgut (m), salivary glands (s), genital tissues (g) as well as hemolymph (h), removed as described above, were incubated individually in tubes containing 4 ml of BSK II medium for regrowth of spirochetes. Each reisolate was designated with a R (reisolate) followed by the tick number and the organ of origin (m, s, g or h).

In addition, the fifteenth *in vitro* passage (p) of NE11H (NE11Hp15) was reintroduced into five uninfected *I. ricinus* adults. Ten days later, these ticks were dissected and each tick midgut was examined for the presence of spirochetes by direct immunofluorescence analysis (DIFA). Moreover, the expression of OspC on spirochetes in the tick midgut was tested by indirect immunofluorescence analysis (IDIFA).

Immunofluorescence analysis: The midguts of ticks infected with NE11Hp15 were smeared on two glass microscope slides, dried and fixed with acetone for 10 min. One slide was used to detect the presence of *B. burgdorferi* spirochetes by direct immunofluorescence using a polyclonal antibody serum against *B. burgdorferi* si conjugated with fluorescein isothiocyanate [21]. The second slide was used to detect the expression of OspC using a rabbit anti-OspC antiserum [22] kindly obtained from T. Schwan (Rocky Mountain Laboratories, Montana, USA) and visualized by a goat anti-rabbit fluorescein isothiocyanate conjugate. Each incubation lasted 1 h at 34°C. The slides were then examined at x 400 magnification by fluorescence microscopy.

SDS-PAGE and Western blotting analysis: Serial *in vitro* subcultures of NE11H (p1–p15), cNE11H and its reisolates from different tick organs were examined by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Whole cells underwent electrophoresis on a 12.5% acrylamide gel with 10⁷ cells/lane and stained with Coomassie brilliant blue R-250 [16]. Western blotting was carried out as described previously [16] using the following monoclonal antibodies (MoAbs): H5332, LA-31, LA-4, LA-28 reactive with OspA [23, 24]; LA-25, LA-27, LA-32 reactive with OspB [24] and LA-7 reactive with 20 kDa protein [24]. Moreover, one rabbit polyclonal immune serum (IS): anti-OspC obtained from T. Schwan [22] was used.

Pathogenicity: Adult SCID mice were bred under pathogen-free conditions at the Max-Planck Institut für Immunbiologie in Frei-

burg, Germany. Mice were inoculated subcutaneously into the base of the tail (3 mice *per* isolate) with 1×10^8 viable spirochetes of NE11Hp3, NE11Hp15, its clonal derivative (cNE11H) and of the reisolates of cNE11H from tick organs: R5g, R8m, R10m, R11m. Mice were inspected under blinded conditions every 3 days over 70 days after inoculation for clinical signs of arthritis in the tibiotarsal joints as described in detail [25].

Results

SDS-PAGE and Immunoblot analysis of in Vitro Passage of NE11H and its Pathogenicity

In the molecular weight range below 35 kDa, NE11Hp1 (first *in vitro* passage of NE11H) expressed four major protein bands corresponding to 33 kDa, 32 kDa, 23 kDa and 22 kDa (Figure 1). After the fifth *in vitro* passage (p5), the 22 kDa protein was no more detectable, followed by the subsequent decline of expression of the 23 kDa band during the further 10 passages (shown after p15, Figure 1a).

All isolates from the *in vitro* passages of NE11H reacted with mAbs H5332 and LA-31 directed against OspA. None of them reacted with mAbs LA-4, LA-28 (specific for OspA), LA-25, LA-27, LA-32 (specific for OspB) and LA-7 (specific for 20 kDa protein) generated against *B.*

Table 1: Pathogenicity of NE11Hp3 and its variants in SCID mice.

| Isolates | Arthritis in mice* | | | | | | | | | | |
|------------|--------------------|----|----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 8 | 12 | 15 | 19 | 21 | 26 | 29 | 33 | 36 | 50 | 69 |
| | days** | | | | | | | | | | |
| NE11Hp3*** | - | - | - | - | - | - | - | - | - | - | - |
| NE11Hp15 | - | - | - | - | - | - | - | - | - | - | - |
| cNE11H | - | - | - | - | - | - | - | - | - | - | - |
| R5g | - | - | - | - | - | - | - | - | - | - | - |
| R8m | - | - | - | - | - | - | - | - | - | - | - |
| R10m | - | - | - | - | - | - | - | - | - | - | 1/3 |
| R11m | - | - | - | 1/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |

*Each isolate was inoculated into three mice; **Mice were inspected about every 3 days after inoculation; ***Shown previously (Schuible et al. 1993); -: No arthritis.

burgdorferi ss [15]. During serial *in vitro* subcultivations, spirochetes of strain NE11H lost the ability to bind antibodies from anti-OspC (Figure 1b): the 22 kDa and 23 kDa proteins of NE11H (p1) and p3 spirochetes reacted with anti-OspC IS whereas only the 23 kDa protein of p5 and p10 reacted. Spirochetes of NE11H (p15) did not react at all with anti-OspC IS.

In contrast to the potential of the low passage strain NE11Hp3 to induce arthritis in SCID mice [26], NE11Hp15 (after 12 additional passages) lost this ability (Table 1).

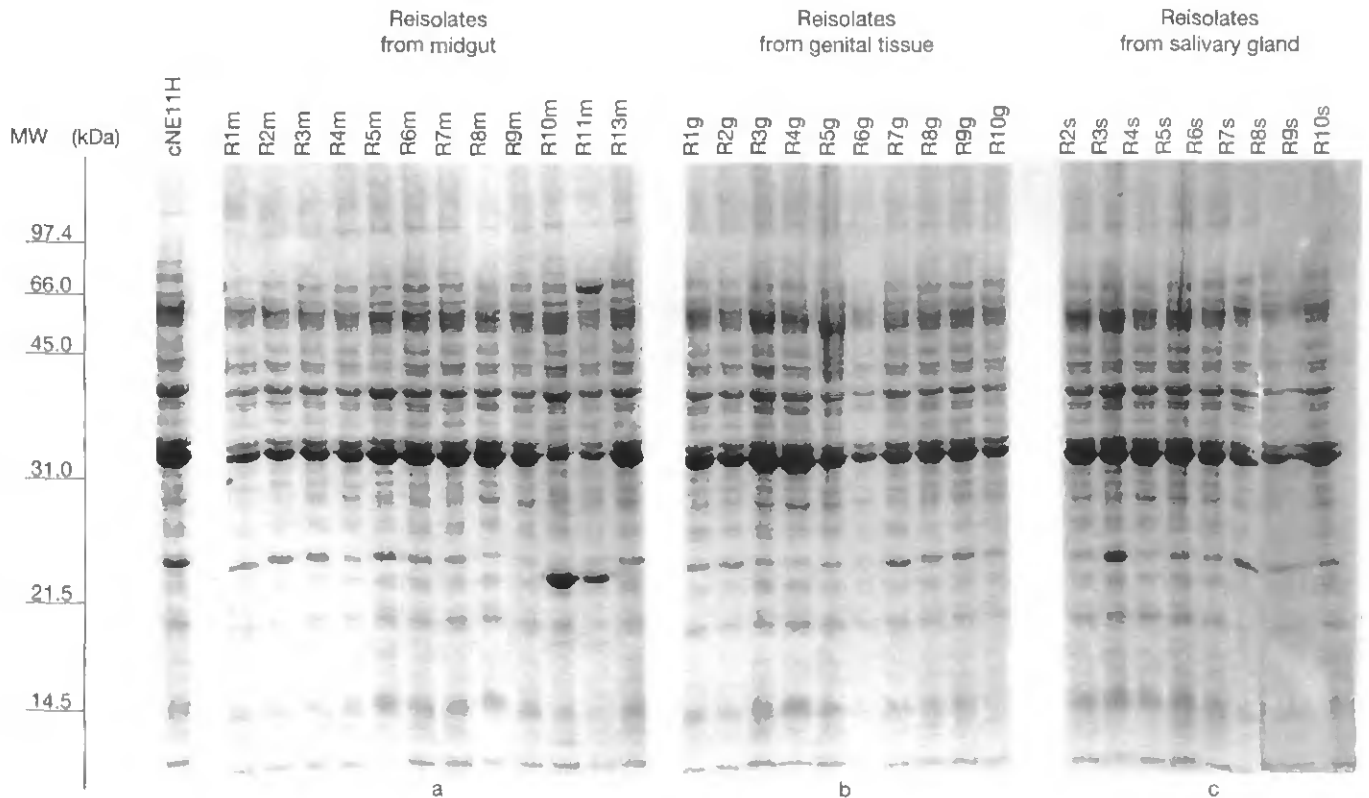


Figure 2. SDS-PAGE (Coomassie stain) of cNE11H reisolates from different organs of *I. ricinus*. a) reisolates from tick midgut (Rm); b) raiisolates from genital tissue (Rg); c) reisolates from salivary glands (Rs). The protein molecular weight standards are shown on the left.

Table 2: Immuno-reactions of cNE11H and its reisolates from ticks.

| | cNE11H | Reisolates from ticks | | | | | | | | | | | | | | |
|-------------|--------|-----------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|
| | | R4m | R4g | R4s | R5m | R5g | R5s | R6m | R6g | R6s | R8m | R8g | R8s | R10m | R10g | R11m |
| Antibodies* | | | | | | | | | | | | | | | | |
| H5332 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| LA-31 | + | + | + | + | - | - | + | + | + | + | - | + | + | + | + | + |
| anti-OspC | 23 | 23 | - | 23 | 23 | 23 | 23 | 23 | 23 | 23 | - | 23 | 23 | 22 | 23 | 22 |

*+: Positive reaction; -: Negative reaction; 22: Reaction with the 22 kDa protein; 23: Reaction with the 23 kDa protein; m: midgut, g: genital tissues, s: salivary glands.

Analysis of the 22–23 kDa Protein Expression in the Tick Midgut

As shown above, *in vitro*-cultured NE11Hp15 spirochetes lack 22 kDa and 23 kDa proteins as revealed by SDS-PAGE (Figure 1a) and they do not react with anti-OspC IS (Figure 1b). To see whether cultured NE11Hp15 spirochetes reexpress the 22 kDa protein when present in the tick midgut the *in vitro* variant NE11Hp15 was reintroduced into ticks by capillary feeding. Analysis by DIFA revealed that 4/5 tick midguts were infected with spirochetes. The spirochetes in 3/4 tick midguts also reacted with the anti-OspC IS by IDIFA. However, the number of OspC-expressing spirochetes in tick midgut was less (one to two per spot of 4 mm diameter) than the number of spirochetes detected by antiserum against *B. burgdorferi* sl (> 10 per spot).

In vivo Passage of cNE11H and its Pathogenicity

cNE11H, a clone derived from NE11Hp3, was shown to still express, besides the 33 kDa and 32 kDa proteins, the 23 kDa protein but not the 22 kDa protein (Figure 2). As expected, only one band of 23 kDa was detectable in the Western blot with anti-OspC IS (Table 2).

cNE11H was reintroduced into 20 ticks and spirochetes were reisolated from various organs 10 days later. Most ticks (10/12; tick number 1–10) had a systemic infection with spirochetes isolated from midgut and one or two additional organs (Figure 2). No reisolates were obtained from the hemolymph whereas 11 reisolates (R) were derived from the midgut (m), 10 from the genital tissues (g) and 8 from the salivary glands (s) of a total of 12 infected ticks (tick number 1–11 and 13) (Figure 2). The protein profiles of reisolates were not significantly different from that of cNE11H with respect to the 23 kDa protein (Figure 2). However, Rm10 and Rm11, two midgut reisolates, re-expressed a protein of 22 kDa (Figure 2a).

Western blot analysis of the tick reisolates also showed a different reaction pattern particularly with the anti-OspC IS and MoAbs LA-31 (anti-OspA). All reisolates showed the same reactions of the 23 kDa protein with anti-OspC IS as cNE11H except reisolates R4g and R8m which did not react at all and R10m and R11m whose 22 kDa protein reacted with anti-OspC IS (Table 2). On the other hand, R5m, R8m and R5g lost their ability to react with MoAbs LA-31.

Experimental infection of SCID mice with cNE11H showed that cNE11H was unable to induce disease in these mice (Table 1). Four reisolates R5g, R8m, R10m and R11m, which were phenotypically different from cNE11H either by expression of a very low level of 23 kDa protein and by a lack of reaction with mAb LA-31 directed against OspA (R5g and R8m) (Table 2 and Figure 2) or by expression of a very high amount of the 22 kDa protein (R10m and R11m) (Figure 2), were tested for their ability to induce arthritis in SCID mice. Whereas R5g and R8m were unable to induce clinical symptoms in SCID mice, R10m and R11m were shown to induce either moderate (R10m, 1/3 SCID mice) or severe (R11m, 3/3 SCID mice) clinical arthritis (Table 1).

Discussion

The main finding of this study is that a clonal isolate of *B. garinii* derived from tick hemolymph which had lost its pathogenic potential during *in vitro* culture regained this capacity upon reintroduction into ticks. The alteration of pathogenic potential is associated with a differential expression of proteins in the range of 22/23 kDa.

The *B. burgdorferi* isolate NE11H was obtained from the hemolymph of a systematically infected unfed *I. ricinus* female and belongs to OspA genotype II (*B. garinii*) based on Southern blot analysis [6]. This isolate expresses two protein bands in the molecular weight range of 22 kDa and 23 kDa which reacted with an immune serum directed against OspC (anti-OspC) [22]. This phenotype has been observed only twice so far among about 200 tick isolates obtained in our laboratory [6, 8, 16]. These three isolates, which express two anti-OspC reactive proteins, were obtained from unfed ticks which were systematically infected by *B. garinii* [6]. The relation between expression of the 22 kDa and 23 kDa proteins of *B. garinii* and the presence of a systemic infection in ticks remain to be studied.

During serial *in vitro* subcultivation, NE11H successively lost the expression of the 22 kDa and 23 kDa proteins after the fifth and fifteenth passages respectively, together with the respective epitopes recognized by a monospecific IS directed against OspC [22]. Phenotypic changes in spirochetal populations during their *in vitro* propagation have been described before by others [13, 27] and are most probably induced by culture conditions. It is therefore

possible that the *in vitro* conditions lead to the generation of genotypic/phenotypic variants and that this process results in differential expression of 22 kDa and 23 kDa molecules [12, 19, 28]. In fact, this could be proven for the 22 kDa molecule of NE11H by both *in vitro* passage and cloning procedures: p5 and cNE11H did not express the 22 kDa protein.

The finding that cNE11H, after re-introduction into ticks, could be reisolated at a very high rate from organs of infected ticks other than midgut demonstrates the capacity of this variant to induce systemic infections in this vector. However, the possibility that this was due to the high concentration of spirochetes introduced into the midgut by capillary or to a contamination between organs during tick dissection cannot be excluded.

The re-expression of the 22 kDa protein in two reisolates of cNE11H from ticks and of the epitope (22 kDa protein) recognized by anti-OspC IS indicates an influence of the tick environment on the cell surface phenotype of spirochetes. Indeed, when NE11Hp15 was reintroduced into ticks, the 22 and 23 kDa proteins, which were not expressed in cultured NE11Hp15 were expressed in the tick midgut as detected by IDIFA using anti-OspC IS [22]. Variation of OspC expression was described to be associated with the number of *in vitro* passages [29], with passage into ticks [16] as well as with temperature variations [30]. Schwan et al. [30] observed that OspC was produced by *B. burgdorferi* ss spirochetes when grown *in vitro* at 32°C–37°C or in engorged tick midguts. When *B. burgdorferi* ss spirochetes were grown at 24°C very little OspC was expressed, as well as when spirochetes were present in unfed ticks. In fact, the contrary was observed in our study. When spirochetes were cultivated at 34°C they lost the expression of OspC, whereas they expressed OspC while present in the tick midgut. An explanation might be found in the *Borrelia* species: OspC of *B. burgdorferi* ss is not expressed in tick midgut [30] whereas OspC of *B. garinii* spirochetes is. In order to see whether OspC is expressed in unfed *I. ricinus* ticks, nymphal and adult ticks were collected from vegetation in the Neuchâtel area and examined by IDIFA using anti-OspC IS. When examined for the presence of spirochetes, only spirochetes found in adult ticks reacted with anti-OspC IS (unpublished data). Data from our laboratory show that *B. garinii* has been more frequently isolated from adult ticks (30/51) than from nymphs (5/21) ($p=0.009$). The significance of this relationship between expression of OspC and the presence of *B. garinii* in unfed field collected adult *I. ricinus* remains to be explored.

The present study is in line with our previous results, which demonstrated that the variations of OspC of *B. garinii* were related to tick passage [16, 17], and suggests that expression of the 22/23 kDa proteins and/or immunological determinants of *B. garinii* might be influenced by environmental conditions. In the tick, the 22 kDa protein of *B. burgdorferi* may play a role in penetrating different tick tissues and furthermore, in the transmission of spirochetes to the animal hosts. In fact, antibodies directed against the

22/23 kDa protein are detected early after infection in patients [31, 32] and in animals infected via tick bites [33].

The mechanism responsible for changes in the protein expression of *B. burgdorferi* sl after passage into ticks is not known. There are at least two hypotheses to explain this phenomenon: variants may reflect the presence of genetically independent clones as shown for the *Osp* locus [19, 28], on the other hand, they may be generated by regulatory processes at the genetic level. A recent study [34] identified plasmid sequences homologous to the genes for two purine biosynthesis enzymes (*guaA* and *guaB*). These genes are adjacent to the outer surface protein OspC (22 kDa) gene of *B. burgdorferi*. Margolis et al. [34] suggest that this would allow the spirochetes to readily adapt to the different purine levels in vertebrates (low level) and ticks (high level). A more detailed genetic analysis of cNE11H and its reisolates is required to elucidate the mechanisms responsible for our observation.

The changes observed in the expression of proteins and their antigenic determinants of NE11H and cNE11H after serial *in vitro* subcultures and re-introduction in ticks seem to influence their pathogenicity in SCID mice. Similar to the strain B31 [14], the low passage strain NE11H was highly pathogenic for SCID mice but lost its pathogenicity after prolonged *in vitro* subcultivation. Most notably, after re-exposure of cNE11H to tick environment two reisolates from their midgut (R10m and R11m) acquired pathogenicity in SCID mice, though to various degrees. Both reisolates expressed the 22 kDa protein after passage through ticks whereas the two nonpathogenic re-isolates only expressed a very low level of this protein. These results indicate that factors of the tick which allow the expression of the 22 kDa protein also might influence the pathogenicity of *B. burgdorferi*. A relationship between infectivity and OspC expression was described previously [29]. Here we show that the 22 kDa protein correlates with the pathogenicity of *B. garinii* and that the expression of this protein is influenced by tick factors. This association is in line with finding that the infectivity of spirochetes for hamsters is maintained for a longer period upon their co-cultivation with tick cells as compared to BSK II medium alone [35]. These findings warrant further studies on the association between the 22 kDa protein and the pathogenicity of *B. burgdorferi*.

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Zusammenfassung: Faktoren der Zecke und *in vitro*-Kultivierung beeinflussen das Proteinnmuster, die Antigenität und die Pathogenität eines aus Hämolymphe gewonnenen klonierten *Borrelia garinii* Isolats. Ein *Borrelia garinii* Isolat (NE11H) wurde aus der Hämolymphe nicht gefütterter *Ixodes ricinus* Zecken gewonnen. NE11H exprimiert vier Hauptproteine mit einem Molekulargewicht von jeweils 33, 32, 23 und 22 kDa. Im Laufe der *in vitro* Kultur entstand eine Variante (NE11Hp15), die durch den Verlust der 22 und 23 kDa Proteine gekennzeichnet war. NE11Hp15 zeigte keine Reaktion mit einem Immunsereum gegen OspC. Wurde NE11Hp15 in Zecken passagiert, fand sich wieder eine Reaktion mit dem anti-OspC-Immunsereum. Ein Klon der Ausgangslinie NE11H (cNE11H) war durch das Fehlen des 22 kDa, nicht jedoch des 23 kDa Proteins, gekennzeichnet. Das 23 kDa Protein von cNE11H wurde durch anti-OspC-Immunsereum erkannt. Sowohl NE11Hp15 als auch cNE11H waren nicht in der Lage, eine klinisch nachweisbare Arthritis in SCID-Mäuse zu induzie-

ren. Nach Reinokulation von cNE11H in Zecken und anschließender Reisolierung aus verschiedenen Zeckenorganen, zeigten die meisten Reisolare eine Reaktion mit anti-OspC-Immunsereum wie der Ausgangsklon cNE11H. Interessanterweise fand sich bei zwei Reisolaten aus dem Zeckenmitteldarm eine starke Expression des 22 kDa Proteins, welches durch das anti-OspC-Immunsereum erkannt wurde. Darüberhinaus induzierten diese beiden Reisolare eine klinisch nachweisbare Arthritis in SCID-Mäusen. Zusammengefaßt zeigen unsere Resultate, daß die spirochätalen 22/23 kDa Proteine während der Zeckenpassage differentiell exprimiert werden. Darüberhinaus zeigen sie auch, daß spirochätale Proteine, die nach Kultivierung *in vitro* nicht mehr nachweisbar sind, dann reexprimiert werden können, wenn *B. burgdorferi* der Mikroumgebung des Zeckenorganismus ausgesetzt wird. Wir postulieren, daß die Pathogenität von *B. burgdorferi* in Mäusen durch umgebungsbedingte differentielle Expression der 22 und 23 kDa Proteine beeinflußt werden könnte.

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Book Review

A. Bauernfeind, M. I. Marks, B. Strandvik (eds.)
Cystic Fibrosis Pulmonary Infections: Lessons from Around the World
 352 pages
 Birkhäuser, Basel 1995
 Price: DM 268.

This book is very interesting and unique since it gathers for the first time clinically very important information about the prevalence of the different microbial pulmonary infections in cystic fibrosis patients, treatment regimes, survival curves, lung-function studies etc. from all over the world. There is no other source of this wealth of information currently available, and it is therefore highly recommended to clinicians and scientists interested in cystic fibrosis. The book was initiated after the International Congress of Chemotherapy in Berlin 1991 by the three editors from Germany, USA and Sweden. They have recruited over 90 authorities from all over the world to contribute, including authors from 21 different countries: Canada, USA, Mexico, Argentina, Venezuela, Uruguay, Australia, Japan, Russia, Israel, Turkey, Greece, Italy, The Czech Republic, Spain, Hungary, Germany, United Kingdom, The Netherlands, Sweden and Denmark. All authors work in well-known centers, and therefore all clinical aspects of diagnosis and treatment of CF pulmonary infection are covered.

The first section contains six chapters on viral, fungal, and atypical bacterial infection in cystic fibrosis, pharmacokinetics of antibacterials in cystic fibrosis, antimicrobial pharmacotoxicity, microbial resistance, microbial virulence and pathogenesis in cystic fibrosis, and lung transplantation.

The second section contains 22 chapters describing the general approach to cystic fibrosis and pulmonary infection in 22 different countries (West and East Germany are represented by two different chapters). These chapters are highly interesting and instructive. They show the results of many years of intensive efforts in CF centers in Europe and North America, where the median survival of patients is now over 30 years, in contrast to the problems which still exist in other countries, for instance in South America where intensive effort in CF centers only started some 10 years ago, and where the survival is much shorter. It is therefore possible, for the first time in one publication, to compare the present situation in countries where centers have just started with the results in countries where centers were established over 30 years ago, and realize how much the care of CF patients can be improved by intensive team-work over many years. The book is filled with useful clinical details with regard to regimes, surveillance etc. and it can be recommended as a standard book for any CF center in the world.

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Bioprosthetic Valve Endocarditis Caused by *Neisseria elongata* Subspecies *nitroreducens*

Summary: A new case of *Neisseria elongata* ssp. *nitroreducens* bacteremia and endocarditis in a 74-year-old woman who had undergone aortic valve replacement in 1992 is reported in detail. *N. elongata* ssp. *nitroreducens* differs from the other subspecies of *N. elongata* in the additional reduction of nitrate without gas formation. Like most *Neisseria* spp. except *Neisseria meningitidis* and *Neisseria gonorrhoeae*, this *N. elongata* ssp. *nitroreducens* is usually classified in the group of "non-pathogenic" *Neisseria* spp. This case report indicates that the presence of subspecies of this group is significant when isolated from normally sterile sites and can cause severe disease in susceptible individuals.

Introduction

Neisseria elongata subspecies *nitroreducens* is a non-motile, short, gram-negative coccobacillus. It is considered to be part of the normal human oral flora. Despite the fact that only *Neisseria gonorrhoeae* and *Neisseria meningitidis* are universally accepted as pathogens in the genus *Neisseria*, some of the so-called "non-pathogenic" *Neisseria* species, in particular *N. elongata* ssp. *nitroreducens*, can cause serious infections. The most common illnesses noted with this subspecies were endocarditis, bacteremia and osteomyelitis [1].

We report in detail on the isolation of a *N. elongata* ssp. *nitroreducens* from blood cultures of a patient with a previous history of valve damage and replacement, hospitalized with the diagnosis of possible infective endocarditis.

Case Report

A 74-year-old woman was admitted to hospital because of repeated episodes of fever. In 1988 a pacemaker was implanted because of a high-degree atrioventricular heart blockage, and in 1992 she underwent a biological prosthetic aortic valve implantation because of severe aortic stenosis. The medication for ventricular arrhythmia consisted of flecainide. The patient had been well until 1 month before admission, when she presented with fever and vague discomfort. There was no anamnestic or clinical clue to point to the cause of the fever, so the general practitioner started a treatment course of amoxicillin (500 mg t.i.d. for 4 days). Initially the fever disappeared, but 4 days after withdrawal of the antibiotic, temperature rose again and a quinolone (ofloxacin 200 mg b.i.d. for 10 days) was administered. Despite an initial benefit, the fever soon returned after withdrawal of ofloxacin. The patient was then admitted to the hospital.

On physical examination she appeared well. Her temperature was 36.4°C. There were no signs of heart failure and the skin was normal (no signs of vasculitis, no splinter hemorrhages). Laboratory data included a leucocyte count of 11,900/mm³ (with 86.3% neutrophils), a platelet count of 159,000/mm³, a CRP of 15.5 g/dl and an ESR of 54 mm/h and no microscopic hematuria. Prothrombin time, immune complexes and complement were normal.

An electrocardiogram presented normal pacemaker activity on demand. A radiograph of the chest showed cardiac enlargement,

a slight left pleural effusion and signs of chronic bronchitis. Fundoscopy showed neither Roth spots nor a clue for the cause of fever. Transthoracic cardiac ultrasound examination did not reveal any vegetation. The prosthetic biological aortic valve appeared normal. The left and right ventricular functions were normal. The atria were slightly dilated. There was a peak instantaneous pressure gradient over the aortic valve of 55 mmHg and a slight central aortic regurgitation, a moderate mitral regurgitation and a tricuspid regurgitation without pulmonary hypertension. Transoesophageal cardiac ultrasound examination revealed degenerative changes of the mitral valve, a slight thickening of the aortic wall at the prosthetic valve implantation, but no signs of abscess formation and no vegetation.

Three days after admission the patient developed several episodes of high fever (39.3°C). During each episode, blood cultures were taken and in 19 vials of 24 blood culture sets *N. elongata* ssp. *nitroreducens* was found. Stomatological examination revealed poor dental hygiene, and on the orthopantomogram a small radiolucent spot could be detected at the apex of tooth 36.

Following Durack's new criteria for diagnosis of infective endocarditis [2], since one major and two minor criteria were met, the diagnosis of possible infective endocarditis could be made.

The patient received gentamicin (160 mg – 240 mg p.d.) for 14 days and ampicillin (6 x 2 g p.d.) for 6 weeks. The fever disappeared completely within 12 h. The inflammatory parameters normalised progressively. The pleural effusion also cleared after 1 week. A weekly control of the transthoracic cardiac ultrasound did not reveal any change. Meanwhile, tooth 36 was extracted.

Microbiological Examination

For 7 successive days we received a total of 24 blood culture sets from this patient at our laboratory. They were examined daily with the Bactec System (Bactec 730; high volume resins, Bactec plus 26 (aerobic) and 27 (anaerobic), Becton-Dickinson). Out of

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Apodemus sp. Rodents, Reservoir Hosts for *Borrelia afzelii* in an Endemic Area in Switzerland

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Summary

Borrelia burgdorferi is maintained in nature in transmission cycles alternatively involving ticks and reservoir hosts. Small rodents like *Apodemus* mice and *Clethrionomys* voles are the primary reservoir of Lyme disease in Europe. In this study, we analyzed by SDS-PAGE and Western blot 20 borrelial isolates from xenodiagnostic ticks fed on four *Apodemus* sp. mice captured in the Staatswald forest (Switzerland). All isolates but one showed a homogeneous protein pattern expressing an outer surface protein, (Osp) A of 32 kDa and an OspB of 35 kDa and reacted with monoclonal antibody (mAb) I 17.3 specific for *B. afzelii*. One isolate expressed an OspA of 32.5 kDa and an OspB of 35 kDa and did not react with species-specific mAbs I 17.3, D6 and H3TS, but was shown to belong to *B. afzelii* by Southern blot analysis. The possibility exists that non-cultivable borreliae are present in xenodiagnostic ticks. However, our results clearly show that *Apodemus* sp. are reservoir hosts for *B. afzelii*, since this genospecies is transmitted from *Apodemus* sp. to feeding larval ticks.

Introduction

The etiologic agent of Lyme disease, *Borrelia* (*B.*) *burgdorferi*, is maintained in enzootic cycles involving wild vertebrate hosts and different species of ticks belonging primarily to the *Ixodes* (*I.*) *ricinus* complex (12). Ticks acquire the infection during a blood meal on infected animals, maintain transstadially spirochetes, and transmit these bacteria to other animals during the next feeding (3). In Europe, *Apodemus* mice have been shown to be important reservoirs for *B. burgdorferi* sl being infective for *I. ricinus* (5, 10, 14).

Various studies have shown the phenotypic and genotypic heterogeneity of *B. burgdorferi* isolates with respect of their abundant outer surface proteins (Ops) A, B and C (19, 20, 21). Now, three genospecies are known to cause Lyme borreliosis in Europe: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* (2, 4). *B. burgdorferi* ss expresses

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an outer surface protein (Osp) A of 31 kDa and an OspB of 34 kDa, *B. garinii* has an OspA of 32–33 kDa and *B. afzelii* expresses an OspA of 32 kDa and an OspB of 35 kDa (2, 4). The expression of OspC with an approximate molecular weight of 22 kDa is more variable than that of the OspA and OspB among *B. burgdorferi* sl strains (21).

The transmission cycle of the three genospecies in nature needs to be clarified. Recently, Humair et al. (11) demonstrated that ears of wild rodents were exclusively infected by *B. afzelii*. A preferential association seems to exist between rodents and *B. afzelii* and rodents may be important reservoir hosts for this *Borrelia* species in nature. In order to evaluate the ability of *Apodemus* sp. to transmit Lyme borreliosis genospecies to feeding ticks, we used tick xenodiagnosis on naturally infected *Apodemus* rodents and analyzed isolates from xenodiagnostic ticks.

Materials and Methods

Mice and ticks

Four naturally infected rodents, three *Apodemus* (*A.*) *sylvaticus* (G149, G185 and G210) and one *A. flavicollis* (G220) captured in the Staatswald forest (Berne, Switzerland) (10), were used as sources of infection for *I. ricinus* larvae. In a previous study (5), we demonstrated that G149 transmitted *B. burgdorferi* sl. to 62.8% of *I. ricinus* larvae, G210 to 31.3% and G220 to 81.4%.

I. ricinus larvae derived from our laboratory colony (7), being free of borrelial infection, were used for xenodiagnosis. Approximately 50 *I. ricinus* larvae were placed on the head of each *Apodemus* rodent. Engorged larvae were maintained in glass vials at room temperature and at 100% relative humidity until moulting had become completed.

B. burgdorferi isolations

Unfed nymphs resulting from the engorged larvae were used for isolation of *B. burgdorferi* sl.: 10 nymphs from G149, 14 nymphs from G185, 12 nymphs from G210 and 12 nymphs from G220. Nymphal ticks were washed in 70% ethanol for 1 min and rinsed in sterile PBS pH 7.4. Each nymph was cut into two pieces using sterilized scissors and placed into tubes containing 4 ml BSK II medium (1) supplemented with phosphomycin (50 µg/ml, Boehringer, Mannheim, Germany) and rifampin (50 µl/ml, Ciba-Geigy, Basel, Switzerland) (8). Culture tubes were incubated at 34 °C and examined by dark-field microscopy every 5–7 days for two months. All positive cultures (1 ml) were inoculated into 25 ml BSK II medium for further growth and then prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

SDS-PAGE and immunoblot analysis

Isolates were subcultured 2–3 times before analysis by SDS-PAGE and Western blot. SDS-PAGE and immunoblot analysis were carried out according to the methods described previously (8). All isolates were centrifuged at 10 000 g for 20 min and washed twice with PBS supplemented with MgCl₂ (5 mM). Whole-cell lysates of each isolate were electrophorized on a polyacrylamide gel at 12.5% for the separating gel and 6% for the stacking gel with a borrelial concentration of 10⁷ cells/lane (Helber cell counting chamber). The gels were stained with Coomassie brilliant blue R 250.

The proteins from whole cell lysates separated by SDS-PAGE were transferred to nitrocellulose paper using a transit cell (2117-250 Nova Blot Electrophoretic Transfer Kit. LKB AB Bromma, Sweden). The following monoclonal antibodies (mAbs) were used to identify the three genospecies as previously described (11): H3TS against OspA protein of *B. burgdorferi* ss (2); I 17.3 against OspB of *B. afzelii* (4) and D6 against 12 kDa protein of *B. ga-*

rinii (2). An immune serum (IS anti-22kDa/NE4) was used to examine the expression and the antigenicity of the 22 kDa protein (8). IS anti-22 kDa/NE4 was produced by immunizing a New Zealand white rabbit with the 22 kDa protein of NE4 strain (8) identified as OspC by *Wilske* (personal communication). Immunocomplexes were detected using peroxidase-labelled anti-mouse IgG antibody (for H3TS and I 17.3), IgM antibody (for D6) and anti-rabbit IgG antibody (for IS anti-22KDa/NE4) diluted 1:1000 (Nordic Immunological Laboratories, Netherlands).

Southern blot hybridization

Total genomic DNA was extracted from NE388, an isolate which did not react with H3TS, I 17.3 and D6, and digested with 100 units of restriction endonuclease *Hind* III. Southern blot was performed as described previously (19).

Statistical test

Fischer's exact test was used to compare the tick infection rates determined by immunofluorescence of ticks fed on G149, G210 and G220 (5) with the tick infection rate determined by *Borrelia* isolation. The difference was considered as significant when $p < 0.05$.

Results

A total of 20 isolates was obtained from 48 nymphs fed as larvae on four infected *Apodemus* rodents. Three isolates were obtained from the cultivation of 10 nymphs (30%) fed on G149, 5 from 14 nymphs (35%) fed on G185, 3 from 12 nymphs (25%) fed on G210 and 9 from 12 (75%) nymphs fed on G220 (Fig. 1). In order to elucidate whether non-cultivable *Borrelia* could be present in the xenodiagnostic ticks, we compared the success of borrelial isolation obtained in this study with the infection rate of xenodiagnostic ticks fed on G149 (155/247, 62.8%), G210 (88/281, 31.3%) and G220 (219/269, 81.4%) described previously (5) and determined by immunofluorescence. No significant differences were observed for G210 and G220 ($p = 0.759$ and $p = 0.704$, respectively) and a weakly significant difference was observed for G149 ($p = 0.048$). The infection rate of ticks fed on G185 is unknown.

All tick isolates but one (NE388) show similar protein profiles expressing an OspA of 32 kDa and an OspB of 35 kDa. Western blot analysis showed that all isolates except NE388 reacted only with mAb I 17.3 that identifies *B. afzelii* (Fig. 1). Isolate NE388 which had an OspA of 32.5 kDa and an OspB of 35 kDa (Fig. 1) did not react with any of the 3 mAbs but was identified as *B. afzelii* by Southern blotting (Fig. 2).

The expression of the 22 kDa (OspC) protein was variable among the 20 tick isolates and it presented heterogeneous reactions with Is anti-22 kDa/NE4 (Fig. 1).

Discussion

Lyme borreliosis spirochetes isolated from ticks and humans show a great phenotypic and genotypic heterogeneity (2, 19, 20). At present, eight *Borrelia* genotypes have been described (16). In Europe, at least three *B. burgdorferi* genospecies can coexist in the tick population and circulate between tick vectors and reservoir hosts (9). At present, the transmission of these *Borrelia* genospecies from different reservoir hosts to feeding ticks is still unclear.

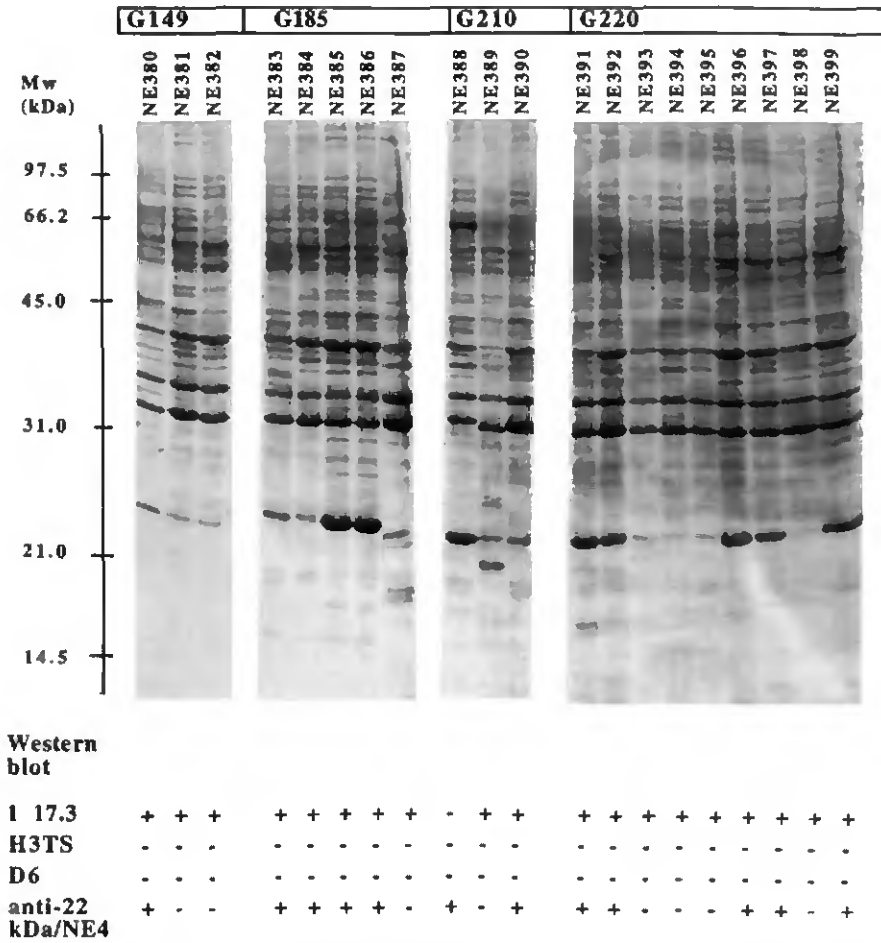


Fig. 1. Protein patterns of *B. burgdorferi* isolates from ticks fed on the four infected *Apodemus* rodents, G149, G185, G210 and G220. The gels were stained by Coomassie brilliant blue R 250 and the protein molecular weight standards are shown on the left.

In Europe, *Apodemus* mice and *Clethrionomys* voles are important reservoirs for *B. burgdorferi* s.l, transmitting the infection to a great number of larvae (5, 10, 14). These rodents are infested with *I. ricinus* larvae and nymphs harbouring one or more *Borrelia* genospecies (11, 15, 17). Recent studies have shown that different *B. burgdorferi* genospecies can infect the internal organs of small mammals (6, 13). In contrast, Humair et al. (11), described only *B. afzelii* to be prominent in the ears of rodents captured in two endemic areas and these authors suggested that rodents could be reservoirs for *B. afzelii*.

In the present study, we showed that all isolates obtained from xenodiagnostic *I. ricinus* ticks, fed on naturally infected rodents, were identified as *B. afzelii*. The possibil-

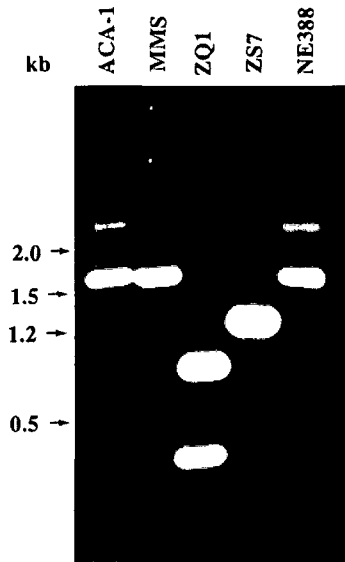


Fig. 2. Southern blot hybridization of NE388 and other *B. burgdorferi* species. The sources of DNA are indicated above the lanes. ACA-1 and MMS represent *B. afzelii*, ZQ1 represents *B. garinii* and ZS7 represents *B. burgdorferi* sensu stricto. NE388 showed a single *Hind* III DNA fragment of 1.7 kb for the *ospA* gene as *B. afzelii* strain ACA-1.

ity exists that borreliae non-cultivable are present in xenodiagnostic ticks. However, the success of isolation was not significantly different from the infection rates of ticks fed on G210 and G220 and determined by immunofluorescence, and only weakly significantly different for ticks fed on G149.

All rodents were captured in an area where isolates from unfed field-collected ticks belonged to *B. burgdorferi* ss, *B. garinii*, *B. afzelii* and group VS116 (9, Humair, personal communication). This assumes that various *Borrelia* species circulate between reservoirs and ticks in this area. Since the main role of a reservoir host is to transmit infection to feeding ticks, we have shown in the present study that rodents are reservoirs for *B. afzelii*. Rodents are important hosts for *I. ricinus* larvae (10) which implicates that *B. afzelii* might mainly be found in unfed nymphs. In fact, studies have demonstrated that most of *B. burgdorferi* isolates from field-collected nymphs belonged to *B. afzelii* (11, Humair, personal communication). This seems to support our viewpoint.

Protein and antigenic variations among the *B. afzelii* isolates were observed: an isolate (NE388) presented an OspA of 32.5 kDa and did not react with mAb I 17.3 and moreover, a great heterogeneity in the expression of OspC was present among isolates from *I. ricinus* ticks fed on the same rodent. In contrast, *B. afzelii* isolates obtained from *Apodemus* and *Clethrionomys* ear biopsies by Humair et al. (11) were much more homogeneous. Successive isolations from the same host did not show variation in the expression of Osps. The reason of the difference observed in Osp expression between rodents isolates and rodent-feeding tick isolates remains unknown but could be related to the tick environment as suggested in previous studies (8). These results also show that variation in the Osps expression exists during the natural transmission cycle.

In conclusion, our study shows that small rodents are reservoirs of *B. afzelii* since they transmit this genospecies to feeding ticks. The exact reason for such an association remains unknown. *B. afzelii* is observed in human skin lesions (4, 18) and in rodent skin biopsies (11) and since ticks attach to animal skin, *I. ricinus* larvae infesting rodents pick up *B. afzelii*.

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