

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 & Schrupf 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. BURGDORFERI*

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. BURGDORFERI* 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 μ l distilled water and resuspended in sample buffer to give a protein concentration of 30 μ g/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 μ g/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

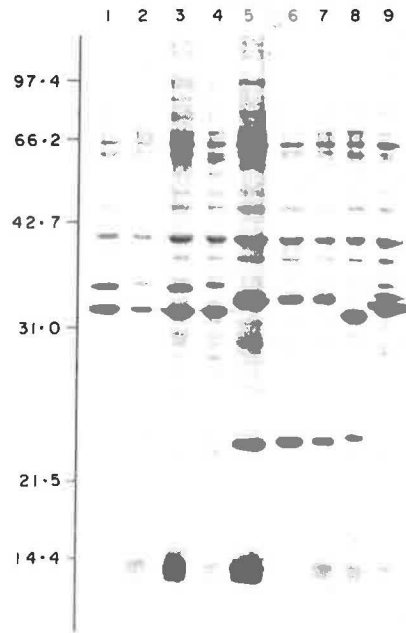


Figure 1. Eight Swiss strains of *B. burgdorferi* and North American isolate B31 were separated by SDS-PAGE and stained with Coomassie Blue R-250. Lanes 1 to 9: B31, NE56, B2, NE203, NE2, NE4, NE83, NE202, NE58. Molecular weight standards (MWS: kDa) are indicated on the left.

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).

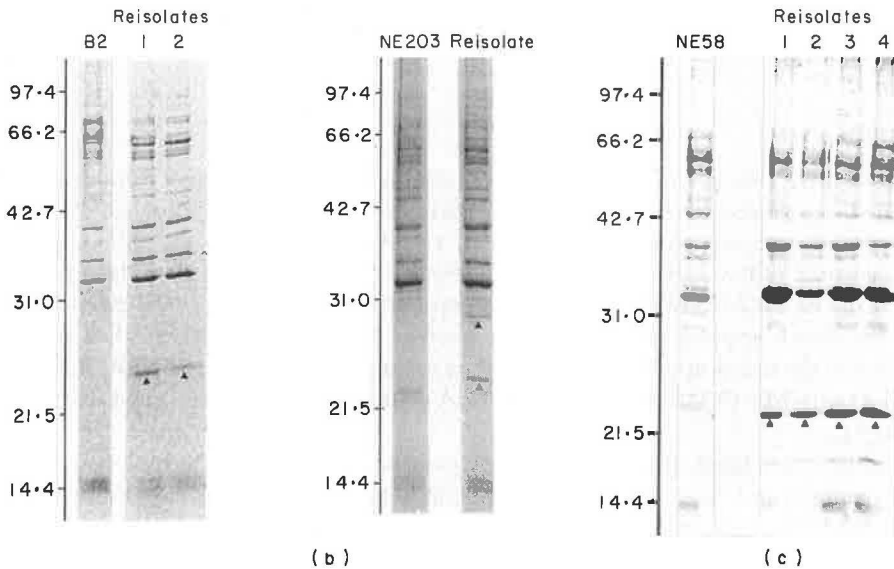
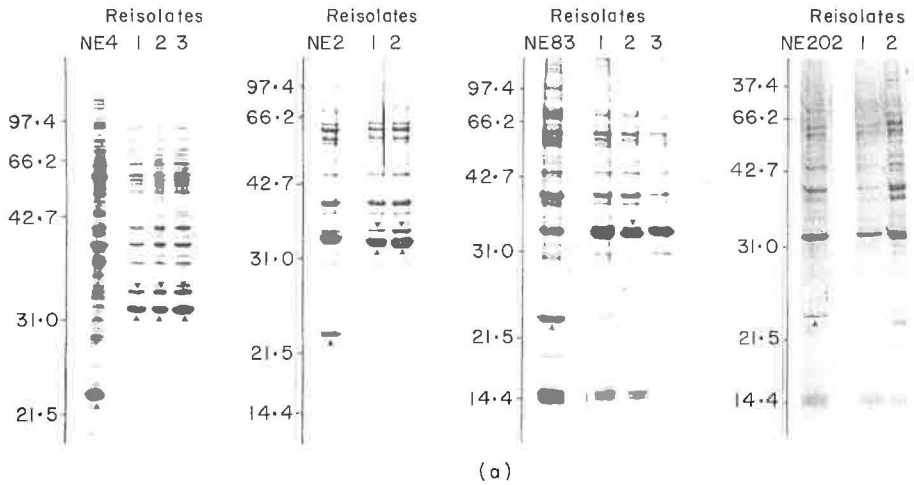
WESTERN BLOT

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



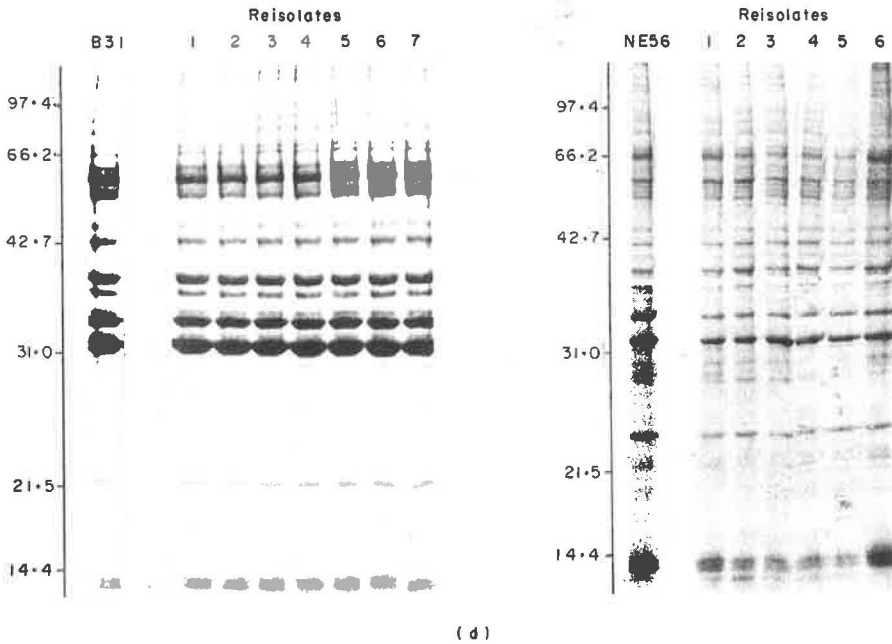


Figure 2. Coomassie Blue stained polyacrylamide gel of different *B. burgdorferi* strains and some reisolates. Figures 2a, b, c (opposite) and d concern respectively group I, II, III and IV of borrelia isolates. The initial isolate is on the first lane and the reisolates on the others. Molecular weights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

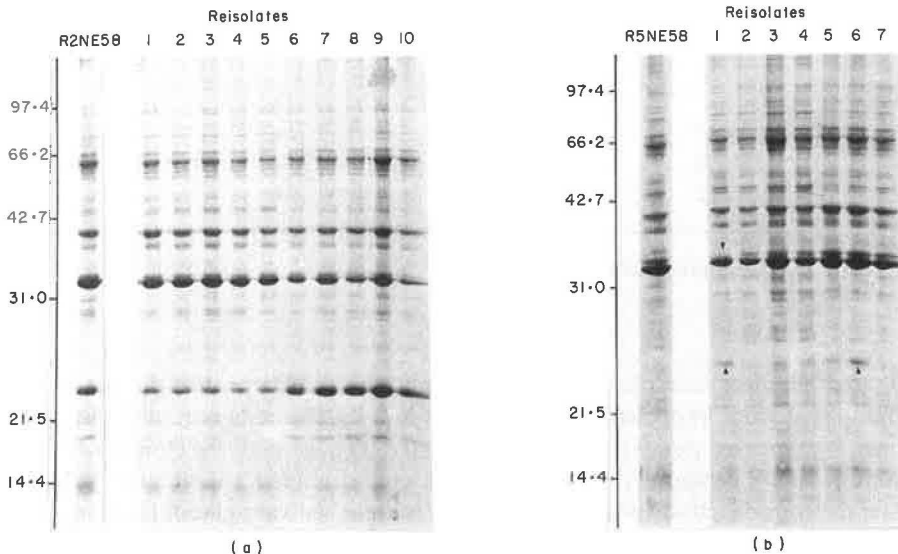


Figure 3. Coomassie Blue stained polyacrylamide gel of two reisolates R2NE58, R5NE58 and their reisolates from tick midgut. (a) R2NE58 and its 10 reisolates (lanes 1–10). (b) R5NE58 and its 7 reisolates (lanes 1–7). Two reisolates of R5NE58 modified their protein profiles after the second passage into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

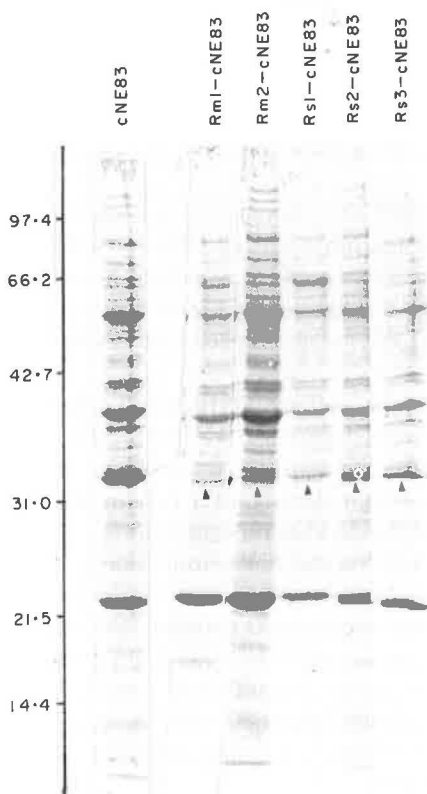


Figure 4. The cloned strain cNE83 (lane 1) and its reisolates from 5 different ticks were examined by SDS-PAGE. Two reisolates from tick midguts (Rm1-cNE83, Rm2-cNE83; lanes 2 and 3) and three reisolates from tick salivary glands (Rs1-cNE83, Rs2-cNE83 and Rs3-cNE83; lanes 4, 5 and 6) were modified after 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 an 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.

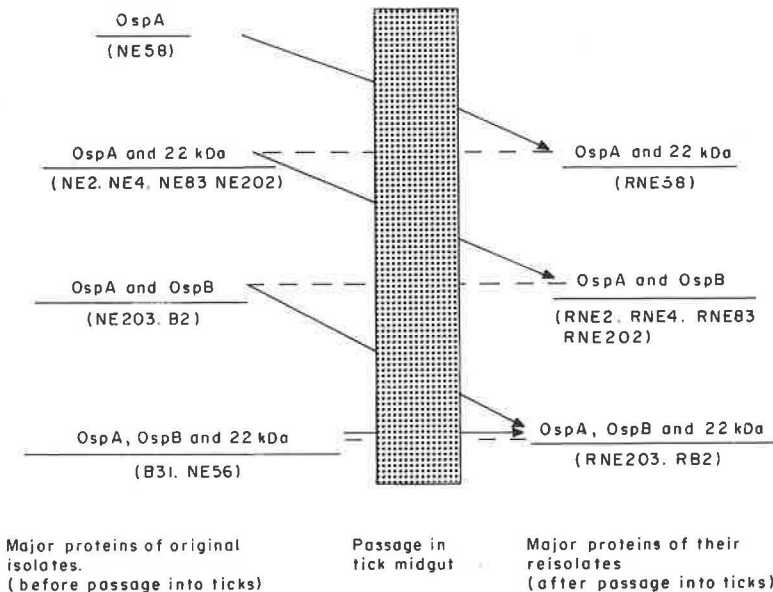


Figure 5. Schema of the modifications of the major proteins of *B. burgdorferi* after passage into *I. ricinus*. It is shown that changes from a phenotype (major proteins) to another one occur in the tick midgut (indicated by arrows) and the reisolated phenotypes (left) are similar to other original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

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