Histological examination of *Borrelia burgdorferi* infections in unfed *Ixodes ricinus* nymphs

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ABSTRACT

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Borrelia burgdorferi, the agent of Lyme borreliosis, is vectored in Europe by *Ixodes ricinus*. In unfed ticks, the spirochaete resides primarily in the midgut, but a low percentage (5.5%; 4/73) of naturally infected ticks may present a systemic infection involving organs such as the salivary glands and central ganglion (Burgdorfer *et al.*, 1983).

In this study, we examined 79 unfed nymphs collected in two sites in Switzerland (Neuchâtel and Aarberg), and 35 unfed nymphs which were fed as uninfected larvae on 3 infected *Apodemus sylvaticus* mice. Dieterle silver staining was used to visualize the spirochaetes in the ticks.

Of the unfed field-collected nymphs, 21/79 (27%) were infected of which 2/21 (10%) had systemic infections. Taking account of the site of collection, we observed that 0/12 ticks from Neuchâtel were systemically infected whereas 2/9 (22%) from Aarberg had a disseminated infection. Out of the 35 unfed nymphs examined after an infectious blood meal on rodents, 14 (40%) were infected and 2 (14%) had a disseminated infection.

A total of 4/35 (11%) unfed infected nymphs presented a systemic infection which represents a higher percentage than previously described. The presence of spirochaetes in salivary glands of systemically infected ticks before the initiation of feeding may reduce the time delay generally recorded for the tick-borne transmission of *B. burgdorferi*.

INTRODUCTION

Since *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis, was first described in *Ixodes dammini* (renamed *I. scapularis* (Oliver *et al.*, 1993)) from Shelter Island, New York (USA) (Burgdorfer *et al.*, 1982) and *I. ricinus* from the Staatswald forest, near Ins (Canton Bern, Switzerland) (Burgdorfer *et al.*, 1983), the association between the spirochaete and its tick vectors has been extensively studied. In unfed ticks, the spirochaete is generally limited to the midgut tissues of most infected ticks (Benach *et al.*, 1987, Burgdorfer *et al.* 1988, Burgdorfer *et al.*, 1989, Zung *et al.*, 1989, Gern *et al.*, 1990). Nevertheless, spirochaetes may

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penetrate the gut wall to initiate and maintain a generalized infection since a small percentage of unfed *I. scapularis* adults (3.9%) (Burgdorfer *et al.*, 1988) and *I. ricinus* adults (5.5%) (Burgdorfer *et al.*, 1983) may harbor a systemic infection.

Histological studies of *B. burgdorferi* in immature tick stages are rare, despite the fact that nymphs are probably the most important transmitters of *B. burgdorferi* to humans. The presence of spirochaetes in salivary glands of ticks before the start of the blood feeding may have implications for transmission given that *B.burgdorferi* is transmitted via tick saliva (Ribeiro *et al.*, 1987, Zung *et al.*, 1989, Monin *et al.*, 1989, Gern *et al.*,1990). Zung *et al.* (1989) observed systemic infection of unfed *I. scapularis* nymphs but did not report the frequency.

The purpose of the present study was to investigate histologically the localization of spirochaetes in unfed field-collected and laboratory infected *I. ricinus* nymphs and to evaluate the frequency of systemic infections.

MATERIALS AND METHODS

Ticks

I. ricinus nymphs were collected by flagging vegetation in two sites on the Swiss Plateau: Bois de l'Hôpital forest close to Neuchâtel (Canton of Neuchâtel) (n=56) in May 1990, 1991 and 1992 and Karoline forest around Aarberg (Canton of Bern) (n=23), in May 1992.

Additionally, uninfected *I. ricinus* larvae from a laboratory colony bred at the Institut de Zoologie (Neuchâtel) according to the methods described by Graf (1978), were fed on 3 *B. burgdorferi* infected *Apodemus sylvaticus* (nos. G210, G149 and G185) captured in the Staatswald forest (Humair *et al.*, 1993). This tick colony has been shown to be free of spirochaete infection by successive controls using direct immunofluorescence test (Gern *et al.*, 1992). Engorged larvae were maintained at room temperature and saturated humidity following repletion. After molting to the nymphal stage, the ticks were prepared for histology 4 months after the infectious blood meal.

Detection of spirochaetes

For histology, ticks were fixed in a 10% formaldehyde solution (extra pure) in phosphate buffered saline pH 7.4. To facilitate rapid penetration of the fixative, tick legs were removed and a cut was made on the side of the tick body. Dissections were carried out in cold phosphate buffered 3.5% formaldehyde, pH 7.4, at 4°C. In addition, a prolonged time (overnight) for fixation and infiltration at 4°C in fresh fixative was necessary.

Ticks were dehydrated in alcohol, cleaned with chloroform, incubated in paraffin and finally embedded in fresh paraffin at 58–60°C.

The embedded ticks were sectioned at a thickness of 8 μm using a microtome (E. Leitz, Wetzlar). Paraffin sections were then deparaffinized with xylol and alcohol, and stained using modified Dieterle silver stain according to the methods

described by Van Orden and Greer (1977) and Gern *et al.* (1990). All serial tick sections were examined; spirochaetes stain dark brown and the background stains yellow to light tan.

RESULTS

Of the 56 *I. ricinus* nymphs collected in Neuchâtel, 12 (22%) were infected by spirochaetes, which were limited to the midgut (Table 1).

In Aarberg, 23 ticks were investigated and 9 (39%) harboured spirochaetes (Table 1); two of them (22%) were systemically infected. In one nymph, spirochaetes were found in high numbers in the midgut, the central and peripheral regions of the synganglion (Figure 1), the hypodermis, the salivary glands (agranular (type I) acini and glandular salivary acini); they were present in lower numbers in muscles. The synganglion was highly infected with up to 50 spirochaetes per section (Figure 1). In the second tick, borreliae were observed in the midgut, the synganglion, the agranular and glandular salivary acini and in the muscles (Figure 2), but the spirochaete density was lower than in the previous tick in organs other than midgut.

The infection status of the ticks which fed as larvae on infected field captured *Apodemus* mice is presented on table 1. A total of 35 ticks were examined and 14 (40%) were infected, two of which (14%) presented systemic infections. In the systemically infected tick fed on animal number G210, high numbers of spirochaetes were found in the midgut, and lower numbers in the hypodermis and salivary glands, whereas in the systemically infected nymph fed on animal number G185, the infection was only present in the midgut (high density of spirochaetes) and in the salivary glands, where the infection level was low.

Three out of four (75%) *I. ricinus* nymphs which fed as larvae on the rodent

TABLE 1

Frequency of *B. burgdorferi* infection in unfed *I. ricinus* nymphs (n=114).

Tick origin	Infected ticks/ collected ticks	Systemic infection/ infected ticks	Infection degree in the midgut		
			+	++	+++
Neuchâtel	12/56 (22%)	0/12	6	4	2
Aarberg	9/23 (39%)	2/9 (22%)	4	2	1 + 2*
G210	8/26 (31%)	1/8 (12%)	6	1	1*
G149	3/4	0/3	1	0	2
G185	3/5	1/3	1	1	1*

* Ticks presenting a systemic infection.

+: Moderate degree of infection: ~1–20 spirochaetes/tick.

++: Mild degree of infection: ~21–100 spirochaetes/tick.

+++: High degree of infection: > 101 spirochaetes/tick.

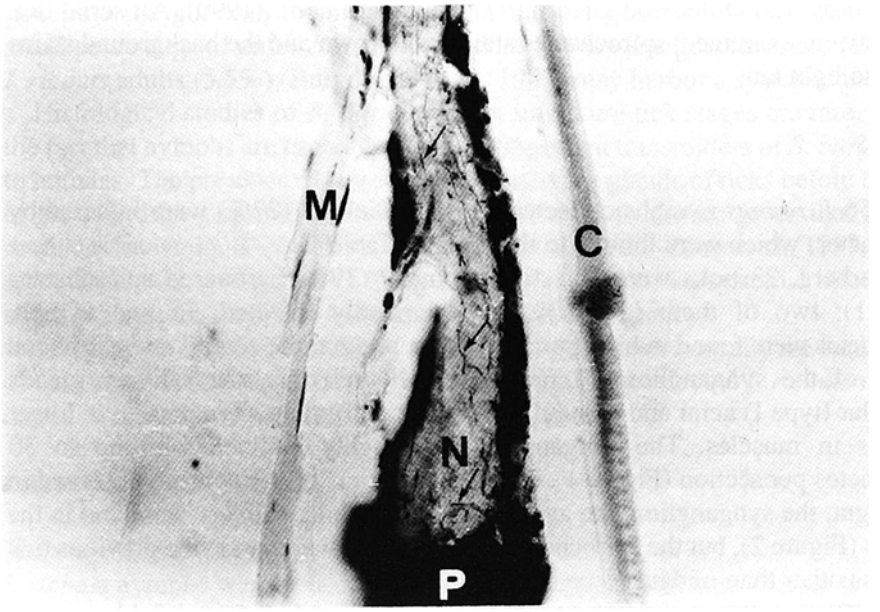

Fig. 1. *B. burgdorferi* (arrows) diffusely distributed in the synganglion of an unfed *I. ricinus* nymph from Aarberg. N: neuropile region; P: perikaryo-region; C: cuticle; M: muscle. Dieterle silver stain. ×600.

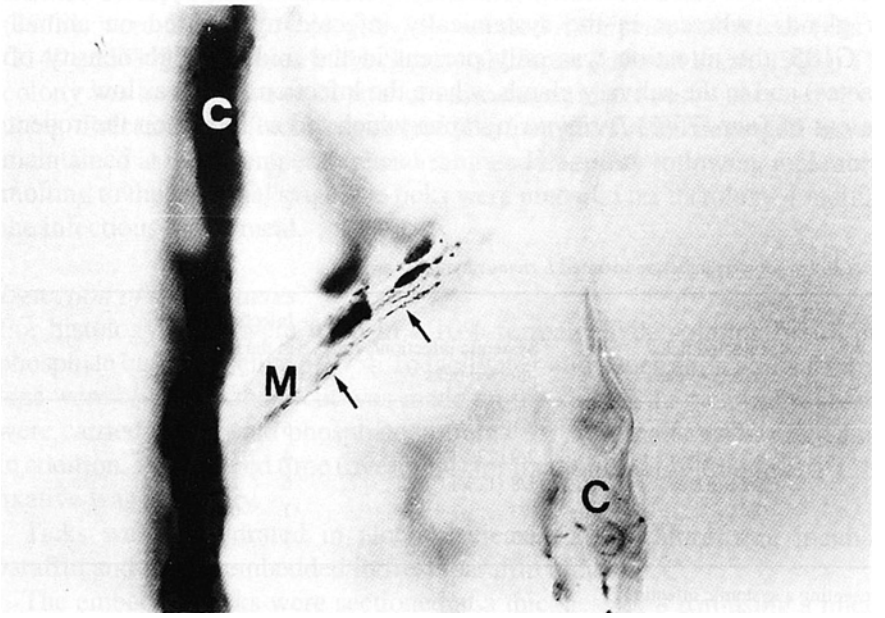

Figure 2: *B. burgdorferi* (arrows) in the muscle (M) of an unfed *I. ricinus* nymph from Aarberg. C: cuticle. Dieterle silver stain. ×600.

G149 were infected, all of which presented an infection limited to the midgut; the infection was at high levels in two of them.

In many infected ticks (18/35; 51%), the number of borreliae in the midgut was low (table 1). In contrast, all ticks having disseminated infections, had high numbers of *B. burgdorferi* in the midgut, and the salivary glands always harboured spirochaetes.

DISCUSSION

In unfed nymphal and adult *I. scapularis* and *I. ricinus* adults, spirochaetes are generally confined to the midgut (Burgdorfer *et al.*, 1983, 1988, Benach *et al.*, 1987, Zung *et al.*, 1989, Gern *et al.*, 1990). In this study, we have shown that unfed field-collected nymphal *I. ricinus* may present generalized infections involving various tick organs such as the midgut, salivary glands, synganglion, muscles and hypodermis. Four out of 35 (11%) infected nymphs presented such infections, which represents a much higher percentage than the 5.5% (4/73) previously described for *I. ricinus* adult ticks (Burgdorfer *et al.*, 1983). In the systemically infected nymphs examined in this study, spirochaetes were always observed in the salivary glands although their number varied.

The salivary route of *B. burgdorferi* transmission is now generally accepted for *I. scapularis* and *I. ricinus*. Spirochaetes were observed in salivary glands two or three days after tick attachment in *I. scapularis* and *I. ricinus* sections (Zung *et al.*, 1989, Monin *et al.*, 1987, Gern *et al.*, 1990), and in *I. scapularis* saliva (Ribeiro *et al.*, 1987). Prompt removal of ticks reduces the risk of transmission of *B. burgdorferi* by *I. scapularis* (Piesman *et al.*, 1987; 1991; Piesman 1993; Shih and Spielman, 1993). Implied delay in transmission appears to result from the time it takes for the spirochaetes to disseminate from the midgut, through hemolymph to the salivary glands. The presence of borreliae in salivary glands of systemically infected ticks before the commencement of feeding may greatly reduce this delay.

We observed that the number of *B. burgdorferi* in *I. ricinus* nymphs was relatively low, 18/35 (51%) infected ticks harbouring less than 20 spirochaetes/tick. Similar results were obtained for nymphal and adult *I. ricinus* collected in southern Moravia (Hubalek *et al.*, 1991), the United Kingdom (Livesley *et al.*, in press), and in *I. ricinus* nymphs which were infected as larvae on *Apodemus* mice (Gern *et al.*, in press). In a recent study, Piesman (1993) suggested that the spirochaetal burden in unfed ticks may be too low to infect the hosts and that ~2–3 days are necessary for the spirochaetes to reach a sufficient number to infect the host. Interestingly in this study all systemically infected ticks presented very high levels of infection in their midgut. Benach *et al.* (1987) and Gern *et al.* (1990) reported that in systemically infected *I. scapularis* and *I. ricinus* adults, *B. burgdorferi*, although abundant in the midgut, usually established a mild infection in other tissues. Here, we found a great number of spirochaetes in synganglion and salivary glands of the two systemically infected nymphs from Aarberg. The

spirochaetal burden of nymphs fed on rodents G210 and G149 was notably different: most of the G210-derived nymphs presented a low degree of infection whereas the G149-derived nymphs contained a great number of spirochaetes. The strains infecting these small mammals (Gern *et al.*, in press) and the various strains infecting ticks in Aarberg and Neuchâtel (Hu *et al.*, in press) may partly explain why some ticks have higher levels of infection than others. Other unknown reasons related to individual rodents may also play a role.

Zung *et al.* (1989) assumed that systemic infection in unfed *I. scapularis* nymphs was related to the age of the ticks or to an inherited infection due to transovarially transmitted spirochaetes. Our results suggest that systemic infection is not related to age since all the ticks fed on rodents had an infectious blood meal 4 months prior examination. Moreover, our study supports the fact that disseminated infections in unfed nymphs are not dependent on a previous infection resulting from transovarially transmitted spirochaetes in the unfed larvae since larvae which were fed on infected rodents were from our breeding colony which is free of spirochaetes.

The proportion of systemically infected ticks and their degree of infection differ in each group of ticks, and the geographic origin as well as the strains of *B. burgdorferi* seem to be important factors influencing the presence of spirochaetes in more than one organ, although the number of systemically infected ticks examined in this study was not very high.

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