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Transformation of Cystic Forms of *Borrelia burgdorferi* to Normal, Mobile Spirochetes

Summary: The purpose of this study was to evaluate the behaviour of *Borrelia burgdorferi* under controlled conditions. The occurrence of cystic forms of *Borrelia burgdorferi in vitro* was noted, and these cysts were able to be transformed to normal, mobile spirochetes. *B. burgdorferi* was cultivated in a commercial culture medium without serum. The spirochetes multiplied only slowly in this medium, and transformation to encysted forms was observed after 1 week. When these cysts were transferred to the same culture medium with rabbit serum, the encysted forms developed into regular, mobile spirochetes after 6 weeks, and their regeneration time was normal. Examination of these cysts in the transmission electron microscope revealed transverse fission inside the cysts. It is probable that similar phenomena may occur *in vivo* under conditions unfavourable for spirochetes. These observations may help to explain why diagnosis and treatment of *B. burgdorferi* infections in humans can be difficult.

Introduction

Lyme borreliosis is a multisystem infection caused by three different genospecies of *Borrelia burgdorferi*: *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia sensu stricto* [1]. A conclusive diagnosis of Lyme borreliosis may be difficult to achieve for several reasons:

- Serological diagnosis is based mainly on tests depending on reference antigens from one strain of *B. burgdorferi*. Since the above-mentioned genospecies are divided into different subgroups, the sensitivity of the serological tests may show geographical variances [2].
- Antibodies are produced slowly [3, 4], particularly in an early stage of the disease. The antibodies may not be traceable in the serum because the few antibodies originally present are neutralised by the corresponding antigen when immune complexes are created [5].
- Antibodies may persist in the serum for a long time after having conquered the disease, and therefore one may observe positive serological results even if the patient is free from active Lyme borreliosis [6].
- The polymerase chain reaction (PCR) has shortcomings due to false-positive and false-negative reactions [7].
- Cultivation of this bacterium has shown variable sensitivity even when erythema migrans, an indisputable marker of infection, appears [8, 9]. Low sensitivity has also been observed in attempts to cultivate the bacteria from blood or cerebrospinal fluid [9].

Besides the points mentioned above, *B. burgdorferi* has been observed to appear as encysted forms in tissues [10, 11]. The phenomenon of encysted forms has occurred in *in vitro* experiments when these spirochetes were exposed to antibiotics [12, 13], changes in pH, depletion of metabolites or aging [14]. However, it is not known whether these encysted forms are low metabolic states of the spirochetes which can transform to normal, mobile bacteria, or if they are dead, degenerated bacteria [13]. As stated above, antibodies are produced slowly, and treatment with antibiotics may prevent the immune system from producing detectable antibodies [15]. Therefore the most conclusive way to diagnose Lyme borreliosis is to directly detect the infectious microorganism by cultivation, electron microscopy or PCR [4, 11, 15]. The culture medium has to be optimal and the cultivation time prolonged in order to maximize successful cultivation of *B. burgdorferi*. The aim of this study was to study the development of encysted forms and their ability to transform to normal spirochetes with appropriate manipulations of the culture medium.

Materials and Methods

The bacterial strain used in our experiments was *B. burgdorferi* ACA-1 (originally isolated in Sweden by *Eva Åsbrink*).
Two closely related culture media were used:
1) BSK-H medium (Sigma B3528) [16] adjoined with 6% rabbit serum (Sigma R 7136), which was tested to be free from *Borrelia* antibodies. We name this medium "BSK-H(a)."
2) The same BSK-H medium without rabbit serum, which we call "BSK-H(u)."
Both culture media had been stored for 6 months at –80°C before use. All culture media were sterile filtrated ensuring both sterility and absence of mammalian cells from serum. All cultures were cultivated at 33°C in closed 5 ml tubes (Nunc; Kamstrup, Roskilde, Denmark).
0.1 ml of borreliae suspension (10^6 per ml) were inoculated in 4.5 ml BSK-H(a) medium and cultured as described above. After 3 weeks this culture was examined by dark field microscopy, and

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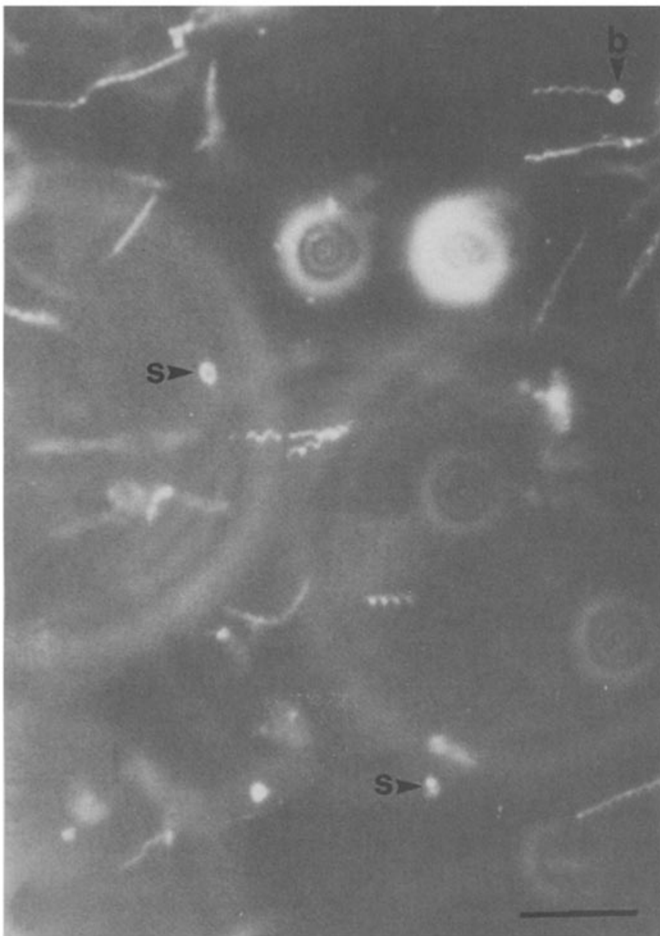

Figure 1: Dark field microscopy showing the BSK-H(u) culture after 1–2 weeks. Cystic structures were observed (s), and also blebs (b), which probably are beginning cysts. Mostly, normal, mobile spirochetes were present. Original magnification 400 x, bar = 5 μm.

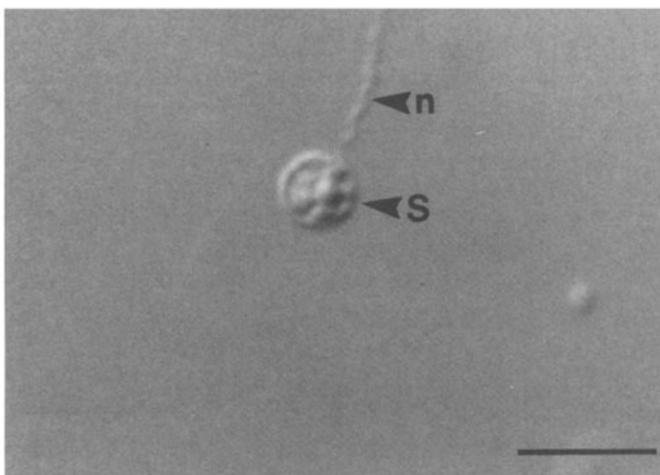

Figure 2: From BSK-H(u) culture after 3 weeks (interference contrast microscopy). The cyst (s) has irregular shape, perhaps due to an internal core (see also Figure 5) and internal spirochetal structures. A normal, mobile spirochete is also present (n). Original magnification 1,200 x, bar = 2 μm.

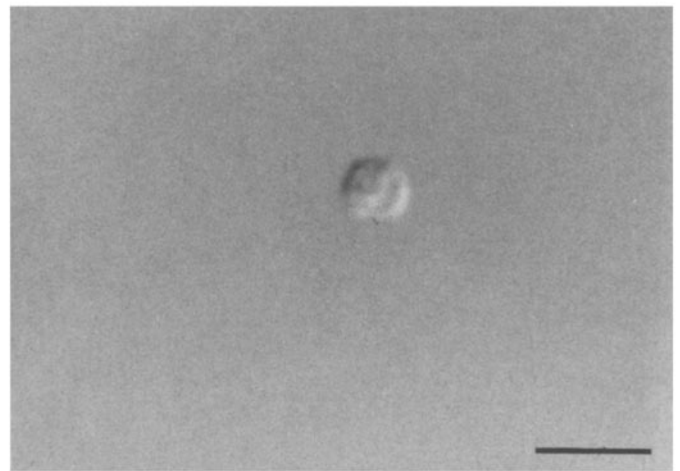

Figure 3: From a 6-week-old BSK-H(u)-culture where only cysts, and no mobile spirochetes were observed. The cyst present here seems to contain a spirochetal structure (interference contrast microscopy). Original magnification 2,000 x, bar = 1 μm.

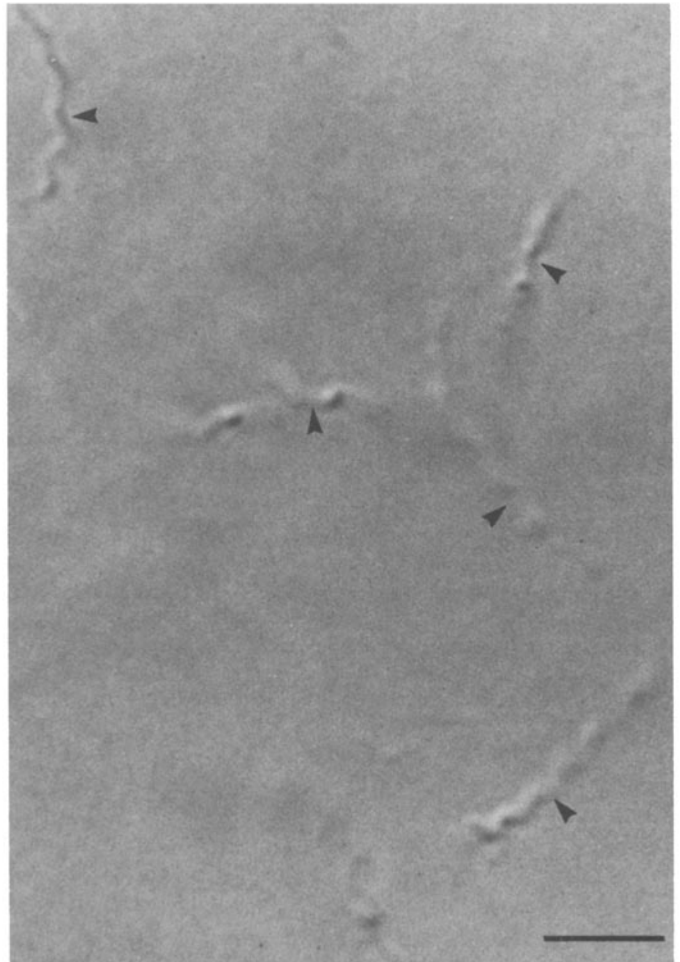

Figure 4: These normal, mobile spirochetes (arrows) have originated from cystic structures (as those in Figure 3) being transferred to BSK-H(a) medium. Original magnification 2,000 x, bar = 1 μm.

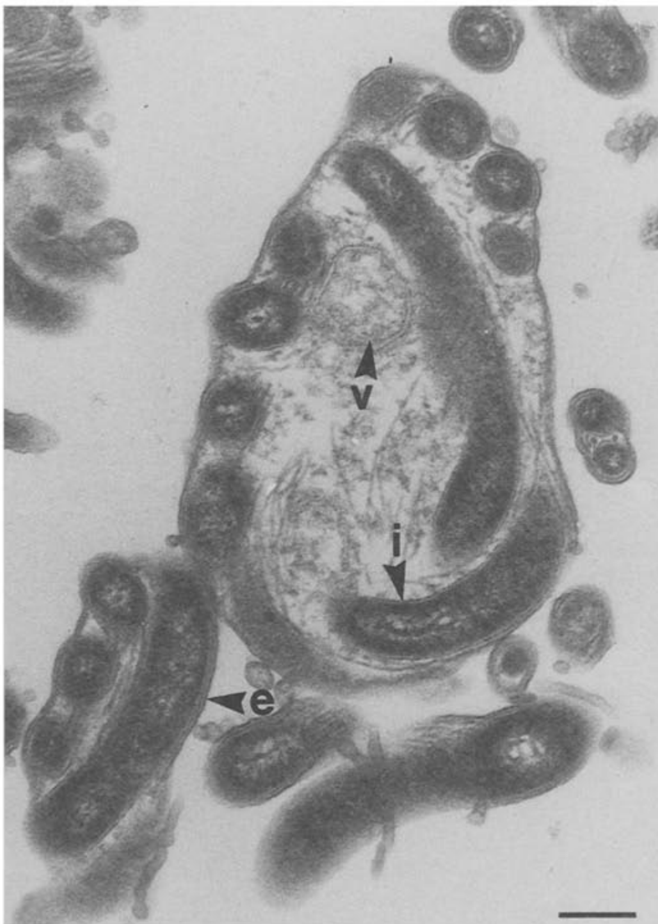

Figure 5: A cystic structure with spirochetes inside. Note the thin outer membrane of the bacteria inside the cyst (i) compared to the outer membrane of the extracystic *Borrelia* (e). Also note the membrane-enclosed structure inside the cyst which probably is a beginning core (v). Flagella and macromolecular aggregates are located inside the cyst. Original magnification 15,000 x, bar = 200 nm.

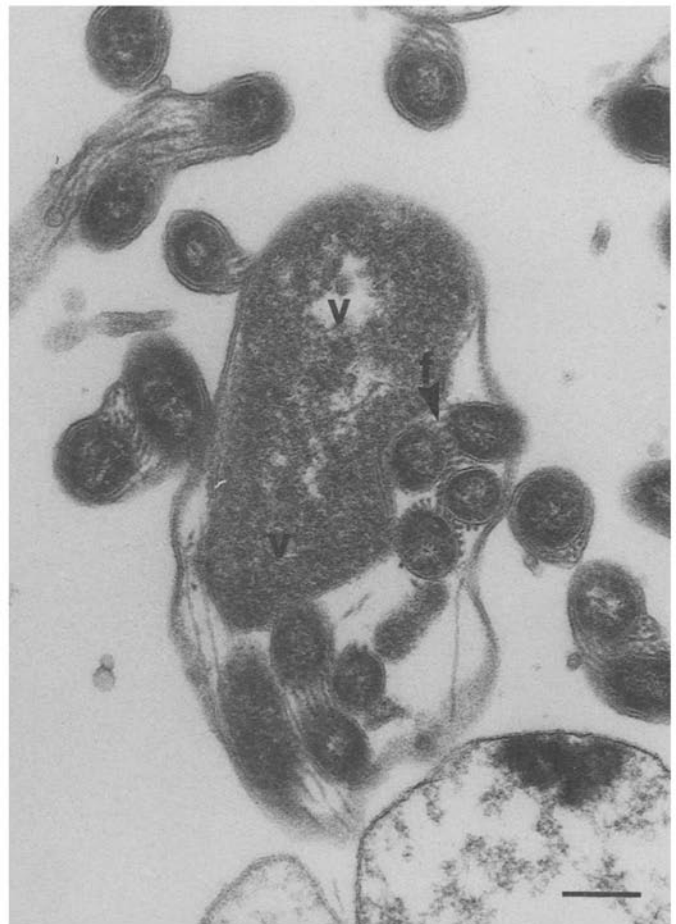

Figure 6: Inside the cyst there is a transverse fission (f). A membrane-encapsulated core (v) with electron dense material is also present inside this cyst. Original magnification 15,000 x, bar = 200 nm.

the vitality was confirmed by the presence of mobile spirochetes. 0.5 ml of this 3-week-old BSK-H(a) medium with spirochetes (10^7 borreliae per ml) was transferred to a tube with 4.0 ml BSK-H(u) medium to examine if normal spirochetes could be transformed to encysted forms in this scantier medium. The culture was routinely examined by dark field microscopy (Zeiss Axiophot; Carl Zeiss, Oberkochen, Germany) with magnification 400x once each week. If encysted forms were suspected, the culture was examined by interference contrast microscopy (2,000x). Contamination control was performed by dark field microscopy. This culture from BSK-H(u) medium was examined by transmission electron microscopy after 3 weeks of culturing (for details, see below).

After 2 months 0.5 ml of the BSK-H(u) medium was transferred to a tube with 4.0 ml BSK-H(a) medium to examine whether encysted forms were able to transform to normal spirochetes in this rich medium. We filtrated 0.5 ml of the BSK-H(u) medium by a 0.45 μm filter (Schleicher & Schuell FP 030/2; GmbH, Dassel, Germany). A volume of 0.5 ml of this filtrate was added to 4.0 ml BSK-H(a) medium and cultured for 3 months under the same conditions mentioned above in order to find out if there were any normal spirochetes present in the BSK-H(u) medium. The growth control was performed by dark field microscopy.

The culture from BSK-H(u) medium was examined by transmission electron microscopy after 3 weeks of culturing according to the following procedure: The culture was centrifuged by 6,000 x g for 30 min; too fast a centrifugation may destroy the S-layer [11]. The medium was removed and replaced with 2% glutaraldehyde in 0.2 M cacodylate buffer (pH = 7.3), and the bacteria were fixed in this fixative for 2 h. The bacteria were postfixed in 1% osmium tetroxide in 0.2 M cacodylate buffer for 2 h. The pellet was dehydrated, infiltrated and embedded in epoxy resin (LX-112; Ladd Inc., Burlington, VT, USA) by a method described earlier [17]. Ultrathin sections were cut with a diamond knife (Jumdi; Juniper ultra Micro, Stockholm, Sweden) on an ultramicrotome (LKB 2088 Ultrotom V), and mounted on 200 mesh copper grids. The sections were stained with 5% uranyl acetate in 30% ethanol for 20 min and Reynolds lead citrate for 5 min. The sections were examined in the electron microscope (Jeol 1200 EX) to identify bacterial structures.

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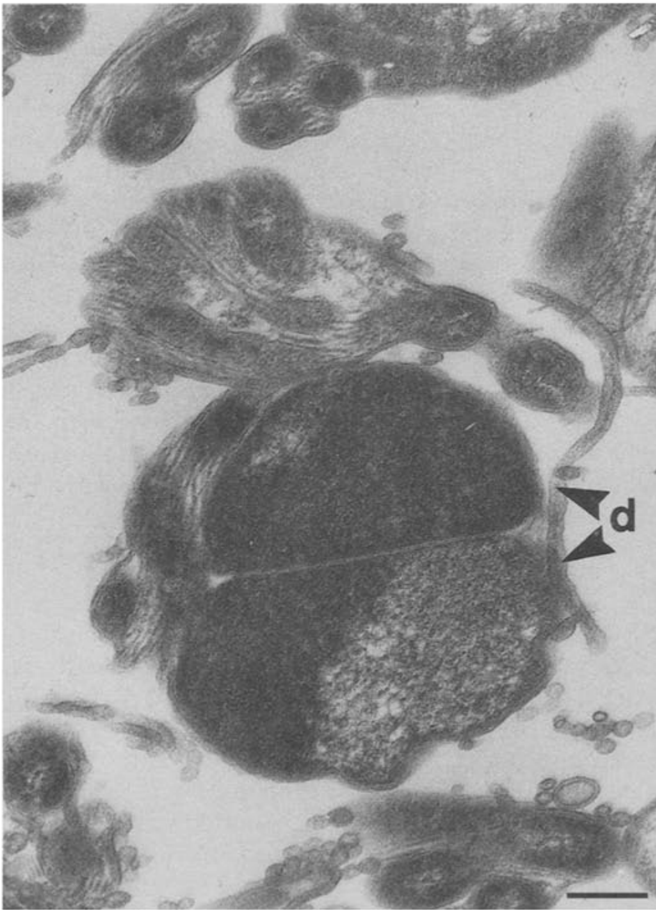


Figure 7: The cyst (d) on this micrograph is in the process of dividing. There is no bacteria visible inside in this plane of sectioning; only the core seems to be present. Original magnification 12,000 x, bar = 200 nm

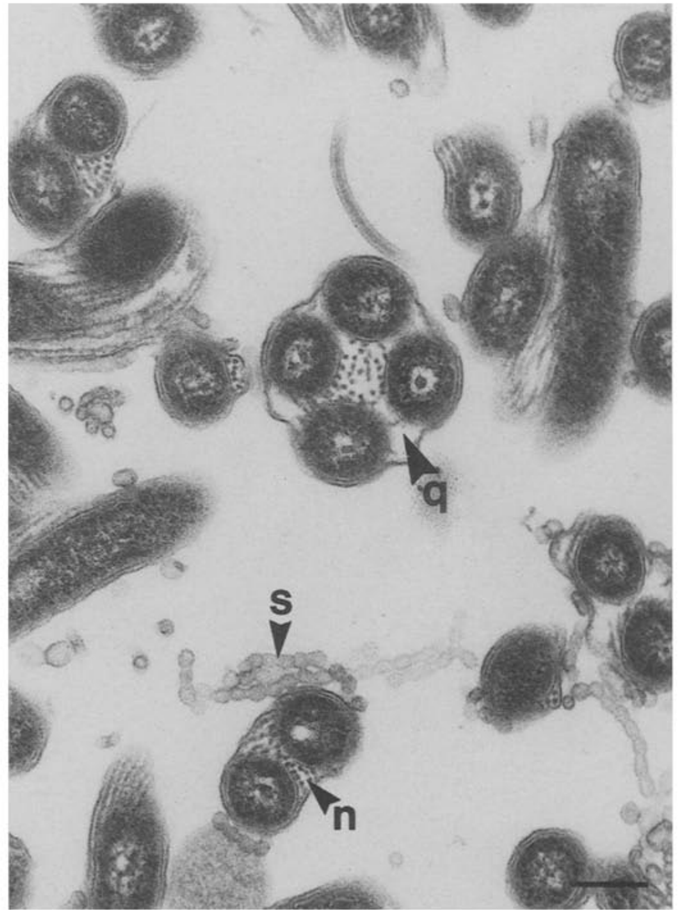


Figure 8: An extracystic (normal) transverse fission (n). A beginning cyst containing four spirochetes (q) is also present, probably as a result of repeated bacterial fissions where the two outer layers of the membrane were not pinched off. "String of pearls" (s) is also present. Original magnification 20,000 x, bar = 200 nm.

Results

In the original culture with BSK-H(a) medium, some unusual cystic structures were observed after 1 month of culture. These unusual structures had a diameter of about 0.5–2μm. The cystic forms were observed by dark field microscopy (400x) as round-shaped structures, and some of them had irregular movements as if something were moving inside the cysts. Interference microscopy (2,000x) showed barely coiled bacteria and granula inside a cystic structure. When the culture was younger than 1 month, only normal, mobile spirochetes were observed.

The same cystic structures occurred after only 1 week's incubation in BSK-H(u) medium (Figure 1). The encysted forms often had irregular shapes, probably due to an internal core and internal spirochetal structures (Figure 2). All normal, mobile bacteria disappeared after 6 weeks, and only cystic structures were left (Figure 3). In BSK-H(u) medium with only cystic forms we did not observe any change of colour as a result of decreased pH. We verified the absence of normal *Borrelia* bacteria in this BSK-H(u) medium by filtrating the medium with a 0.45 μm filter. Spirochetes will pass through the filter [18], and if any normal borreliae were present, they would multiply when being passed to BSK-H(a) medium (This filtration technique does not damage the bacteria, and is very sensitive, since one borrelia per ml will pass through the filter [18]). We did not observe any spirochetes after cultivating the filtrate for 3 months in BSK-H(a) medium [7–9, 15], even if we added fresh BSK-H(a) medium each month (The regeneration time of borreliae was found normal in 2-month-old BSK-H(a) medium by 30°C. This confirms that monthly addition of fresh medium is sufficient.). When the cystic forms (which were confirmed to be free from normal spirochetes) were passed to BSK-H(a) medium, normal, mobile spirochetes were produced after about 6 weeks (Figure 4).

The BSK-H(u) medium (where cystic structures were observed by interference microscopy) was examined by transmission electron microscopy (TEM). The TEM-examination was performed on the culture at the age of 3

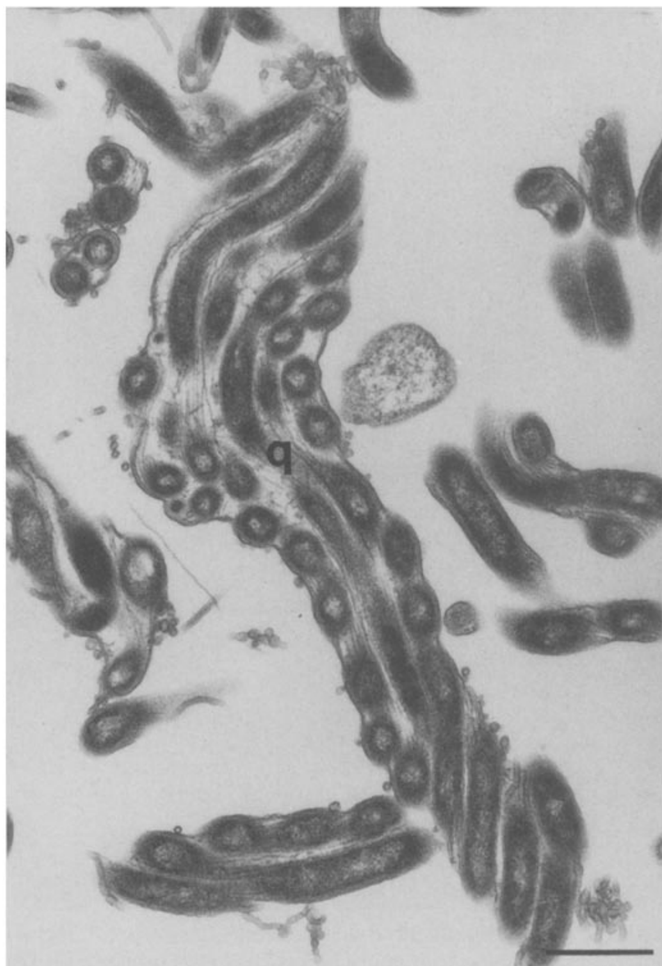


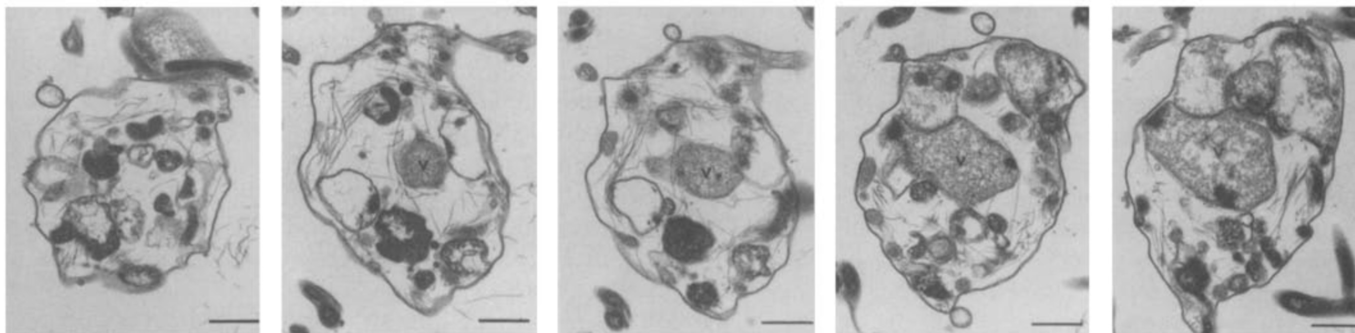
Figure 9: A beginning cyst containing four spirochetes (q) is present, as explained in Figure 8, but these borreliae are sectioned parallel to their longitudinal axis. Original magnification 10,000 x, bar = 500 nm

weeks when both normal spirochetes and cystic structures were observed by the usual microscopical techniques. Ultrastructurally we observed cystic structures with coiled spirochetes inside, and the cysts were limited by a double bilayer membrane (Figure 5). In addition to the spirochetes, the cysts contained flagella and macromolecular substances. The spirochetes inside the cysts were not surrounded by a trilaminar membrane as they are when not inside a cyst; they seemed to have lost one membrane layer. Transverse fissions of bacteria were detected inside some cysts (Figure 6), and several cysts seemed to contain more than one spirochete. Some cysts contained a membrane-encapsulated core in proximity with more or less well-preserved spirochetal structures (Figures 5, 6). We also observed fission of the cyst itself (Figure 7). Beside the encysted forms we observed normal spirochetes, many of them in the fission process (Figure 8). Some mini-cysts contained four spirochetes where these seemed to be in the process of fission (Figures 8, 9). Serial sections showed that cysts without a core in one plane could have a core in neighbouring planes, which illustrates that cores are more frequent than what is observed by examining one section (Figures 10–14).

Discussion

Our *in vitro* experiments with *B. burgdorferi* demonstrated the transformation of normal, mobile spirochetes to encysted forms. These cystic forms (Figures 3, 5, 7) seem to be an alternate morphologic state to which *B. burgdorferi* resorts when the environment becomes too unfavourable. In our study the unfavourable conditions were created by a culture medium (BSK-H), which was too scanty because of the absence of rabbit serum. From the electron microscopical observations it appears that the encysted forms of *B. burgdorferi* arise as a result of bacterial fission where the two outer layers of the membrane are not pinched off. We thereby obtain primarily a cyst containing two spirochetes with thinner outer membranes. When these two bacteria divide, the cyst will have four spirochetes inside (Figures 8, 9), and so on. Further, it seems that several spirochetes in the same cyst may assemble their contents into a core, which may grow to fill up the whole cyst (Figures 2, 5, 6, 7, 11–14). Serial sections show that most cysts have a core (Figures 10–14). It also seems that filled-up cysts may divide (Figure 7).

Low biological activity was demonstrated by the absence of change in pH in the culture medium, suggesting a tor-



Figures 10–14: Five photographs showing serial sections of an encysted form of *Borrelia burgdorferi*. Figure 10 does not show a membrane encapsulated structure (core) inside the cyst, but Figures 11–14 show a growing core (v). Original magnification 10,000 x, bar = 500 nm.

por state. When BSK-H medium with serum was added to cystic forms only (as shown in Figure 3), they seemed to wake from this torpor state, and once again became metabolically active (Figure 4). Cultivation of cystic forms of *B. burgdorferi* has been attempted in previous studies, but unsuccessfully [11]. Some studies [15, 19, 20] indicate that unfavourable conditions represented by the presence of antibiotics stimulate creation of cystic forms. We assume that this low-activity state of the bacteria is important for their survival in a negative environment. The effectiveness of antibiotics requires active metabolism by the bacteria, and therefore it is likely that cystic forms of *B. burgdorferi* may be resistant to antibiotic treatment. This may explain why Lyme borreliosis can be difficult to treat in some patients [15, 19]. It is also possible that the membrane surrounding the encysted forms will protect the bacteria against external stress. DNA has been demonstrated in blebs [21], and it is therefore possible that these structures may participate in the protection and transfer of genetic markers. The observation of transverse fission of spirochetes inside the cysts indicates a more complex regeneration of *B. burgdorferi* than assumed earlier, and may give the bacteria quantitative advantages when they finally escape from the encysted forms.

Our observations of encysted forms of *B. burgdorferi* emphasize the importance of using the correct culture medium for this spirochete. The existence of encysted forms in infected patients may explain why we often have to cultivate *B. burgdorferi* for up to 3 months in order to observe growth. Even if a conclusive infection is present, there are few indisputable positive results after a 3-month culture. The occurrence of these cysts may clarify why attempts to cultivate *B. burgdorferi* may be unsuccessful despite the presence of infection [11] (The cystic forms will be especially difficult to discover when cultivated from blood, since thrombocytes have a similar size and shape.). If so, an improvement of the culture media, prolonged cultivation time, and microscopic examination including TEM is necessary.

In vivo these encysted forms may explain why *Borrelia* infection can be temporarily dormant, why a reactivation of the disease may occur when the conditions suit *B. burgdorferi*, and why the infection may relapse after treatment with antibiotics. Cystic forms have been observed earlier both intra- and extracellularly from biopsies with Lyme borreliosis [10, 11]. It has been hypothesized that encysted structures may provide a model that mimics cells under attack by the host's immune system during infection [22]. The transformation from the cysts to normal, mobile spirochetes, as far as we know, has not been previously reported for *B. burgdorferi*. For the above reasons, a role for these forms in the life cycle of these pathogens in human disease should be sought. However, encysted forms of *Borrelia recurrentis* may arise at a low temperature, and these encysted forms may be cultivated to normal, mobile spirochetes by increasing the temperature [14]. Other researchers have demonstrated that the cell wall enclosing the encysted *B. burgdorferi* does not react with antibodies directed against normal surface antigens of this bacterium [11]. Therefore, to detect if cystic forms are present in infected patients, electron microscopy [11, 23] or immunocytochemistry with cyst-specific monoclonal antibodies or lectins [11] (either directly from biological material or after cultivation and centrifugation) may be required.

For the above reasons it will be of interest to undertake further studies to examine the antigenic properties of the cysts in order to develop improved serological tests, as well as to find methods of treatment that may destroy the cysts in an effective manner.

Acknowledgement

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Information

Stipendium der Walter-Marget-Vereinigung zur Förderung der Infektiologie e.V. gemeinsam mit dem SmithKline Beecham-Stipendium-1997 „Infektiologie“

Ausschreibung eines Stipendiums

Die Walter-Marget-Vereinigung zur Förderung der Infektiologie e.V. vergibt zusammen mit SmithKline Beecham Pharma zur Förderung der Ausbildung junger Ärztinnen/Ärzte ein von zahlreichen Förderern finanziertes Stipendium „Infektiologie“.

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