

Interactions between *Borrelia burgdorferi* and Mouse Fibroblasts

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Abstract

Borrelia burgdorferi spirochetes are an infectious agent of Lyme borreliosis. The aim of our studies was to investigate the fate of engulfed *B. burgdorferi* cells in L-929 mouse fibroblasts and to observe development of intracellular infection *in vitro* after 2 and 48 h. Electron microscopic studies reveal consecutive stages of *B. burgdorferi* spirochetes penetration to mouse fibroblasts *in vitro*. It has been observed, as a first step attachment and engulfment of spirochetes followed by formation of vacuoles. After 48 hours of infection, vacuoles of fibroblastic cells have been seen full of *B. burgdorferi* bacteria and latter they have been released from infected cells to extracellular space. It can be the evidence that *B. burgdorferi* multiply intracellularly.

Key words: *Borrelia burgdorferi*, fibroblasts, interaction

Introduction

Borrelia burgdorferi spirochetes are an infectious agent of Lyme disease, also known as borreliosis, which is the most common tick-borne disease in the northern hemisphere. Early manifestations of infection include fever, headache, fatigue, and a characteristic skin rash called erythema migrans. Untreated, it can cause late symptoms involving tissue of the joints, heart, and nervous system (Stanek *et al.*, 1996).

Microscopic studies indicate that the bacteria can bind to the cell surface and enter the cytoplasm directly after inducing local engulfment and fragmentation of the plasma membrane. Several reports have described interactions between *B. burgdorferi* bacteria and different host cells. It has been shown that the spirochetes can enter mammalian immune cells and other cells as well as tick tissue. This probably allows the pathogen to survive in host tissues, to infect them and to escape the host defense (Hu *et al.*, Linder *et al.*, 2001, Sigal, 1997, Szczepanski *et al.*, 1990, Thomas *et al.*, 1989). However, there is no information on the fate of *B. burgdorferi* spirochetes inside eukaryotic cells and the way they leave the host cells.

Various modes of bacterial entry into the host cell have been described as an essential pathogenic factors.

Legionella pneumophila uptake is completed by a process termed “bacteriophage”, followed by engulfment of the organisms with microvilli in association with intracellular cytoplasmic filaments (Oldham *et al.*, 1985). Another pathogen, *Rickettsia prowazekii*, can enter endothelial cells *via* induced phagocytosis and it is then released into the cell cytoplasm by disruption of the phagosomal membrane (Walker, 1984). Transmission electron microscopy has shown that viable or killed *Candida* organisms were attached to endothelial cells, then enveloped by cell membrane and incorporated into the endothelial cells within phagosomes (Rotrosen *et al.*, 1985).

The ability of *Borrelia burgdorferi* to attach to and invade human fibroblasts was investigated by confocal and scanning electron microscopy. Scanning electron microscopy has revealed that *B. burgdorferi* are tightly attached to fibroblast monolayers after 24–48 h. Spirochetes were observed in the perinuclear region within human fibroblasts by laser scanning confocal microscopy (Klempner *et al.*, 1993).

The aim of our studies was to investigate the fate of engulfed *B. burgdorferi* cells in mouse fibroblasts and to observe the development of intracellular infection *in vitro* in electron and fluorescence microscopy.

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Experimental

Material and Methods

Borrelia afzelii VS461 strain (ATCC 51567) was grown in BSK-H medium Complete (Sigma Aldrich, St. Louis, USA) supplemented with 6% of rabbit serum for 7 days at 35°C in 5% CO₂ atmosphere. The number of bacteria per 1 ml was counted in a Thoma counting chamber.

Line L-929 (ATCC CCL-1, USA) were propagated in Eagle's minimum essential medium (MEM) with L-glutamine and NaHCO₃ (Biomed, Lublin, Poland), supplemented with 4% of fetal calf serum (ATCC, USA) at 37°C in 5% CO₂ for 2 days. Cells were grown in shell-vials on glass coverslips inside tubes with screw caps (Sterilin, United Kingdom) until a confluent monolayer was obtained.

Bottles containing cell line monolayer were inoculated with spirochetes culture containing 10⁸ bacteria per 1 ml. L929 cells infected with *B. burgdorferi* (initial density 10⁶ organisms/ml medium) were incubated for 2 and 48 hours at 35°C in 5% CO₂ atmosphere.

Infected mouse fibroblasts were fixed with acetone, washed three times with PBS and incubated 30 minutes with anti-*B. burgdorferi* human immune serum at 37°C. Next they were washed three times with PBS followed by incubation with rabbit anti-human immunoglobulins conjugated with FITC (DAKO, Denmark). Immunofluorescence was observed in the fluorescence microscope Eclipse E 400 (NIKON, Japan) at 500X magnification.

All specimens were prepared according to standard technics (Glauert, 1975). The cultures were washed twice with PBS, fixed overnight with 2.5% glutaraldehyde. On the next day the cells were centrifuged at 750 g at 4°C and the pellet was washed with 2 ml 0.2 M sodium cacodylate buffer (pH 7.4) and re-centrifuged as above. After three washes, the cells were

fixed for 2 hours in 1% OsO₄ in 0.1 M sodium cacodylate buffer. The cells were then washed three times in 0.1 M sodium cacodylate buffer and stained for 30 minutes by being resuspending in 1% aqueous uranyl acetate (Roth, Karlsruhe, Germany) solution. For embedding, fibroblasts were centrifuged and then dehydrated through a wash series in methyl alcohol solutions (from 25% to 100%) and embedded in epoxy resin and incubated overnight at 65°C. Transverse thin sections were cut and transferred to copper mesh 300 grids (Polysciences, St. Goar, Germany), stained with lead citrate and uranyl acetate and dried. The cells were observed in a JOEL 100C electron microscope (Japan) at magnifications from ×6000 to ×35000.

Results

Various stages of *B. burgdorferi* spirochetes infection in L-929 fibroblasts were observed. After two hours of incubation spirochetes were seen outside of the host cells. Their position suggested that most of them were motile. The first observed interaction step was attachment of the bacteria to the surface of the fibroblasts. In fluorescence microscopy it has been seen as adhesion to the cell surface. After two hours of incubation the spirochetes were bound and entered the mammalian cells. Most of them were bound apically. This contact triggered the engulfment of the bacteria in the cytoplasmic host membrane (Fig. 1, Fig. 2). This process initiated the cellular uptake of single bacteria into phagocytic-like vacuoles. Within two hours of incubation such bacterial cell, surrounded by host cell membrane, were seen inside the fibroblast (Fig. 3). Some bacteria entered fibroblast cells in a different way. Fibroblast pseudopods bent around single spirochetes in the intercellular spaces. The pseudopods had a characteristic hook-like form, turned back to the fibroblasts membrane and slid along the



Fig. 1. Attachment of spirochetes to the cytoplasmic membrane of L-929 cell (X 30 000).



Fig. 2. Cell membrane penetration (X 30 000).

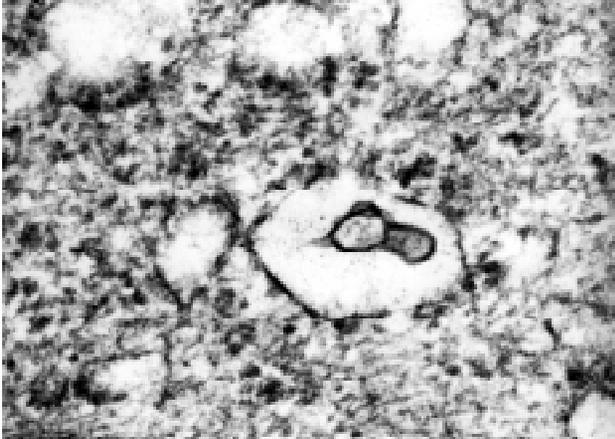


Fig. 3. Single *B. burgdorferi* bacteria in vacuole in L-929 cytoplasm (X 33 000).

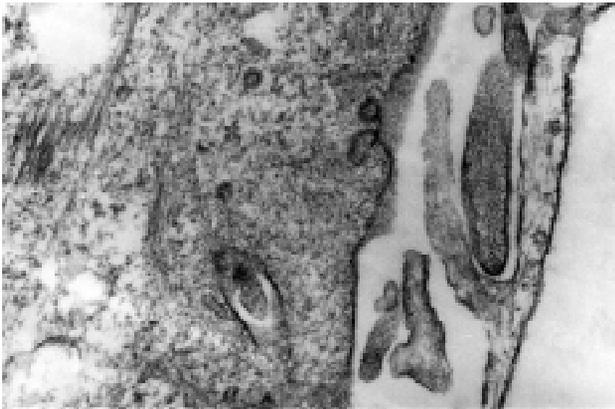


Fig. 4. Endocytosis of *B. burgdorferi* spirochetes (X 27 000).

cell membrane. The observed manner of penetration resembled endocytosis however characteristic for coiling phagocytosis long appendages wrapping the spirochetes were also observed (Fig. 4).

On the second day of infection the fibroblasts were seen to be vacuolized. Many vacuoles with bacteria

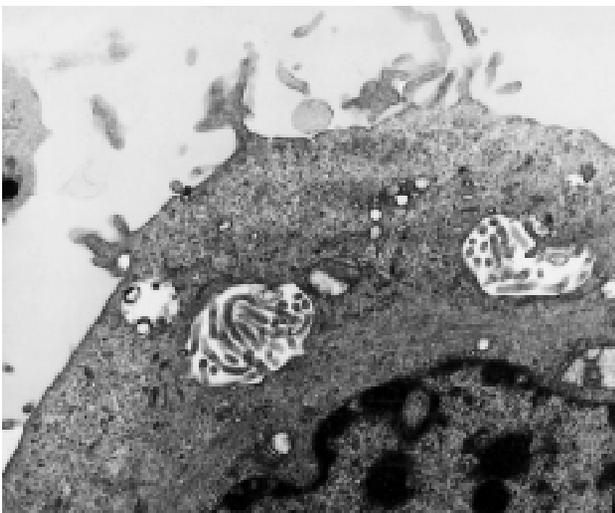


Fig. 5. Multiplication of spirochetes within vacuoles (X4 000).

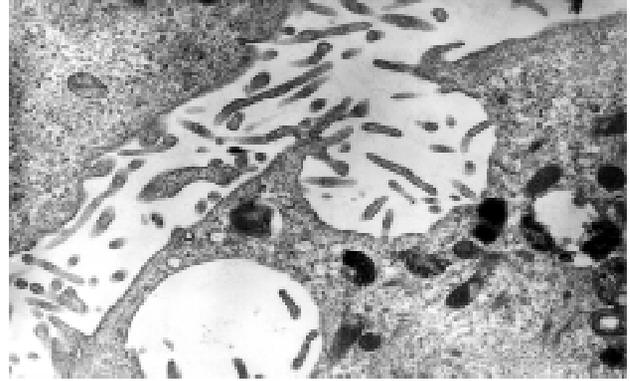


Fig. 6. Translocation of spirochetes to the intracellular space (X 6 700).

were located in the peripheral part of the host cell adjacent to the cell membrane. Strong immunofluorescence of vacuoles and bacteria was observed in fluorescence microscopy. The vacuoles contained 15 to 20 bacterial cells as it has been seen on a cross sections. The number of spirochetes in the host vacuoles observed after two days of infection indicated that *B. burgdorferi* multiplied inside the fibroblasts (Fig. 5). Some of the vacuoles were disrupted and *B. burgdorferi* spirochetes were released to the extracellular space (Fig. 6).

Discussion

Electron microscopic studies revealed the consecutive stages of *B. burgdorferi* spirochetes penetration into mouse fibroblasts *in vitro*. The first observed step was the attachment and engulfment of the spirochetes, followed by the formation of vacuoles and multiplication of bacteria inside vacuoles followed by release from the infected cells to the extracellular space. After 48 hours of infection, vacuoles of fibroblastic cells containing dozens of *B. burgdorferi* bacteria were seen. This can be taken as evidence that *B. burgdorferi* multiply intracellularly similarly to *Legionella pneumophila*, *Coxiella burnetii* and other obligatory intracellular parasites (Baca *et al.*, 1983, Oldcham *et al.*, 1985, Walker *et al.*, 1984, Rotrosen *et al.*, 1985).

Studies by Comstock *et al.*, (1989) with electron microscopy revealed that borreliae entered the endothelial cells and suggested that the organisms penetrated the host monolayers primarily by passing through them. Attachment of spirochetes is time and temperature dependent and pretreatment with heat, immune human serum or monoclonal antibodies to OspB reduce the binding to the endothelial cells (Thomas *et al.*, 1989). Examination of spirochete-endothelial interactions demonstrated the presence of spirochetes in the intercellular junctions between endothelial cells as well as beneath the monolayers. Scanning electron microscopy identified a mechanism of transendothelial

migration whereby spirochetes pass between cells into the amniotic membrane at areas where subendothelium is exposed (Szczepanski *et al.*, 1990).

Other study with Vero cells revealed that essential for the attachment process is metabolic activities of the spirochaete, not viability (ability to grow) (Hechemy *et al.*, 1992). After entry of untreated *B. burgdorferi*, most of the spirochaetes were either free in the cytoplasm or tightly bound to the host membrane. In contrast, heat treated spirochaetes remained bound to host membrane in large phagosome-like vesicles (Comstock *et al.*, 1989).

It seems that several eukaryotic cells provide *B. burgdorferi* spirochetes with a protective environment contributing to their long-term survival (Peters *et al.*, 1997, Rittig *et al.*, 1998, Rittig *et al.*, 1992). *B. burgdorferi* have been protected in fibroblasts for at least 14 days of exposure to ceftriaxone. In the absence of fibroblasts, organisms did not survive. They were not protected from ceftriaxone by glutaraldehyde-fixed fibroblasts or fibroblast lysate, suggesting that a living cell was required. The ability of the organism to survive in the presence of fibroblasts was not related to its infectivity (Georgilis *et al.*, 1992). Mouse keratinocytes, HEP-2 cells, and Vero cells showed a similar protective effect. Doxycycline or erythromycin were more effective in killing *B. burgdorferi* when they were grown in the presence of eukaryotic cells (Brouqui *et al.*, 1996). Our findings show that in fibroblasts could occur process of spirochetes multiplication.

Difficulties with the isolation of *B. burgdorferi* from clinical material when cultured on artificial media also indicate that spirochetes are very fastidious bacteria, which require the presence of certain substances in the host cells for their growth. These observations allowed us to isolate several strains by inoculating cerebrospinal fluids, synovial fluids and blood of Lyme borreliosis patients into cell line culture (Chmielewski *et al.*, 2003, Tylewska-Wierzbowska *et al.*, 1997).

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