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ORIGINAL ARTICLE

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Intracellular persistence of *Borrelia burgdorferi* in human synovial cells

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Abstract To investigate if *Borrelia burgdorferi* can persist in resident joint cells, an infection model using cell cultures of human synovial cells was established and compared to the interaction of *Borrelia burgdorferi* and human macrophages. *Borrelia burgdorferi* were found attached to the cell surface or folded into the cell membrane of synovial cells analysed by transmission electron and confocal laser scanning microscopy. In contrast to macrophages, morphologically intact *Borrelia burgdorferi* were found in the cytosol of synovial cells without engulfment by cell membrane folds or phagosomes. *Borrelia burgdorferi* were isolated from parallel cultures. Treatment with ceftriaxone eradicated extracellular *Borrelia burgdorferi,* but spirochetes were reisolated after lysis of the synovial cells. *Borrelia burgdorferi* persisted inside synovial cells for at least 8 weeks. These data suggested that *Borrelia burgdorferi* might be able to persist within resident joint cells in vivo.

Key words *Borrelia burgdorferi* · Synovial cells · Intracellular persistence

Introduction

Lyme borreliosis transmitted by a tick bite is caused by the spirochete *Borrelia burgdorferi.* Clinical manifestations include erythema migrans, meningoradiculoneuritis and arthritis [1]. Lyme arthritis usually begins several months after the infection [2]. Lyme arthritis is treated by antibiotics, but approximately 25% of patients have ongoing arthritis after one or two antibiotic treatments [2, 3].

Although the aetiology of Lyme arthritis is known, the pathogenesis is far from clear. Several pathogenic aspects have been discussed including B- and T-cell responses against *Borrelia burgdorferi* [4, 5], autoimmune mechanisms [6] and phagocytosis of persisting spirochetes [2, 7]. Two patterns of phagocytosis have been described: (1) Conventional phagocytosis leading to the enclosure and degradation of *Borrelia burgdorferi* inside the phagosomes; (2) coiling phagocytosis by which spirochetes are enrolled by coiled pseudopods [8] and degraded inside the cytosol in the absence of phagosomes [9]. However, although their isolation and recultivation from joint fluid has rarely been successful [10], *Borrelia burgdorferi* can be detected in the joint by a variety of methods, even after antibiotic treatment [11–14]. Thus, chronic Lyme arthritis might be due to a persistent infection.

To investigate a possible long-term persistence of *Borrelia burgdorferi* in the joint, we established an in vitro model for human Lyme arthritis using non-phagocytic resident joint cells. A long-lasting cytosolic persistence of viable *Borrelia burgdorferi* in human synovial cells was demonstrated in vitro. This might be a key mechanism for *Borrelia burgdorferi* to escape from phagocytosis and antibiotic treatment.

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Material and methods

Cells

Human synovial cells (SC) were prepared from normal human joint tissue by tryptic digestion [15], and maintained in culture and passaged as described [16]. Cells were characterized by indirect immunoperoxidase staining of cytospin preparations using the following monoclonal mouse antibodies diluted in 0.5% bovine albumin (see also Table 1): anti-HLA-B, C antibody at 1 : 100 (Bethesda Research Laboratories, Neu-Isenburg, Germany); anti-HLA-DR antibody at 1 : 2000 (Becton-Dickinson, Heidelberg, Germany); KI-M 8 antibody

at 1:16000 (anti-CD68) [17]; anti-ICAM-1 antibody at 1:200 (Bender, Vienna, Austria). The cells were then incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark) diluted 1:50 in phoshate-buffered saline with 30% human AB serum for 30 min. Peroxidase-conjugated goat anti-rabbit IgG antibodies (1:50; Dako, Copenhagen, Denmark) were then applied for 30 min. Positive staining was identified by 0.01% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine (Serva, Taufkirchen, Germany). The cell preparations were counterstained with Mayer's haematoxylin (Sigma, Deisenhofen, Germany). In control samples the primary antibodies were replaced by buffer. For infection experiments, SC were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany; complete medium). Experiments were done between the sixth and the tenth passage of SC.

Human alveolar macrophages (AM) were isolated from lung tissue specimens of donors undergoing thoracic surgery for lung cancer who were free from rheumatic or infectious diseases [18]. Cells were cultured in complete medium and used for experiments 10 h after isolation [18]. Human peripheral blood macrophages (PBM) were isolated from heparinized blood of healthy adult volunteers by Ficoll-Hypaque gradient centrifugation (Nycomed, Oslo, Norway) followed by an overnight incubation in complete medium in plastic petri dishes (Nunc, Roskilde, Denmark). Non-adherent cells were washed off and the remaining adherent cells were used for experiments.

Spirochetes

Borrelia burgdorferi strains were cultured in BSK-H medium supplemented with 6% rabbit serum (RS; Sigma, Deisenhofen, Germany). Strain B 31 (*Borrelia burgdorferi,* sensu stricto) was obtained from ATCC (No. 35210); low passage strain LW 2 (*Borrelia burgdorferi,* sensu stricto) was kindly provided by Dr. G. Burmester, Berlin [13]. For infection, spirochetes were used at a logarithmic phase of growth and counted by phase contrast microscopy (Nikon, Düsseldorf, Germany). Viability was confirmed by corkscrew motility.

SC were grown for a few days in complete medium and then infected at a multiplicity of infection (MoI) of 1, 10 and 100 *Borrelia burgdorferi* per cell. Control cultures were processed in the same manner, but in the absence of *Borrelia burgdorferi.* The duration of the infection period was from 12 h up to 8 weeks.

Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM) SC were grown on circular glass slides (diameter of 8 mm) in 24-well polystyrene tissue culture dishes (Nunc, Roskilde, Denmark). After infection with *Borrelia burgdorferi* and the indicated interval of time, SC were fixed with methanol and stained with a polyclonal rabbit antibody against *Borrelia burgdorferi* strain B 31 as described [19]. Western blot analysis using supernatants of sonicated strain B 31 demonstrated specific binding of this antibody to bands at 93 kDa, 75 kDa, 41 kDa, 31 kDa and 21 kDa. For detection of bound antibodies, a FITC-conjugated goat anti-rabbit IgG antibody (Dianova, Hamburg, Germany) was used. Specimens were examined by a Leica TCS 4D confocal laser scanning microscope (Leica, Bensheim, Germany) using a FITC filter device. We analysed xy sections and xz sections of the cells by using a computerized image processing system. A rhodamine filter was used for visualization of the cell bodies by autofluorescence.

Transmission electron microscopy (TEM)

Cells were grown in complete medium and were fixed in 2.5% glutaraldehyde (Sigma, Deisenhofen, Germany) for 2 h at 4 °C. Subsequently, the monolayer was post-fixed by immersion in osmium tetroxide (Sigma, Deisenhofen, Germany). After dehydration in graded ethanol the monolayers were finally embedded in Epon 812 (Roth, Karlsruhe, Germany) via propylenoxide. Ultrathin sections were cut with an OMU 2 microtome (Reichert-Leica, Bensheim, Germany), contrasted with lead citrate, followed by uranyl acetate and examined with a Zeiss EM 10 transmission electron microscope (Zeiss, Oberkochen, Germany).

Antibiotic treatment of cocultures of *Borrelia burgdorferi* and SC

SC were cocultured with *Borrelia burgdorferi* for 72 h and then treated with fresh complete medium containing ceftriaxone at a concentration of either 1.0 or 4.0 µg/ml (Rocephin; Hoffmann-La Roche, Grenzach-Wylen, Germany). The minimum inhibitory concentration eradicating 90% of *Borrelia burgdorferi* in vitro (MIC_{90}) is 0.06 µg/ml. The maximum drug level present in cerebrospinal fluid 6 h after administration of a single i. v. dose of 50 mg/kg ceftriaxone is 4.7 µg/ml [20, 21]. The intra-articular drug level of ceftriaxone after an adult single i. v. dose is 98 µg/ml after 2 h, 53 µg/ml after 8 h and 24 µg/ml after 24 h in synovial fluid [22]. The concentration of either 1.0 or 4.0 µg/ml of ceftriaxone was chosen significantly higher than the MIC_{90} and according to the drug level in vivo. After 9 days parallel cell cultures were analysed by phase contrast microscopy, TEM and recultivation for the presence of *Borrelia burgdorferi* in BSK-H medium without antibiotics. Subsequently, media were changed routinely every 10 days and the supernatants were probed for recultivation of viable spirochetes. Ultrastructural analysis of SC by TEM was performed parallel to the recultivation experiments. After 9 weeks 50% of the SC were removed from the culture dish by rubber policeman, lysed with deionized water for 2 min and transferred to BSK-H medium for spirochete reisolation. The remaining half of the SC on the culture dish were analysed by TEM for the presence of *Borrelia burgdorferi.* SC without cocultivation of *Borrelia burgdorferi* cultured in complete medium with and without antibiotics served as negative controls. SC cocultured with *Borrelia burgdorferi* in complete medium without antibiotics served as positive controls.

Results

Characterization of SC and macrophages (Table 1)

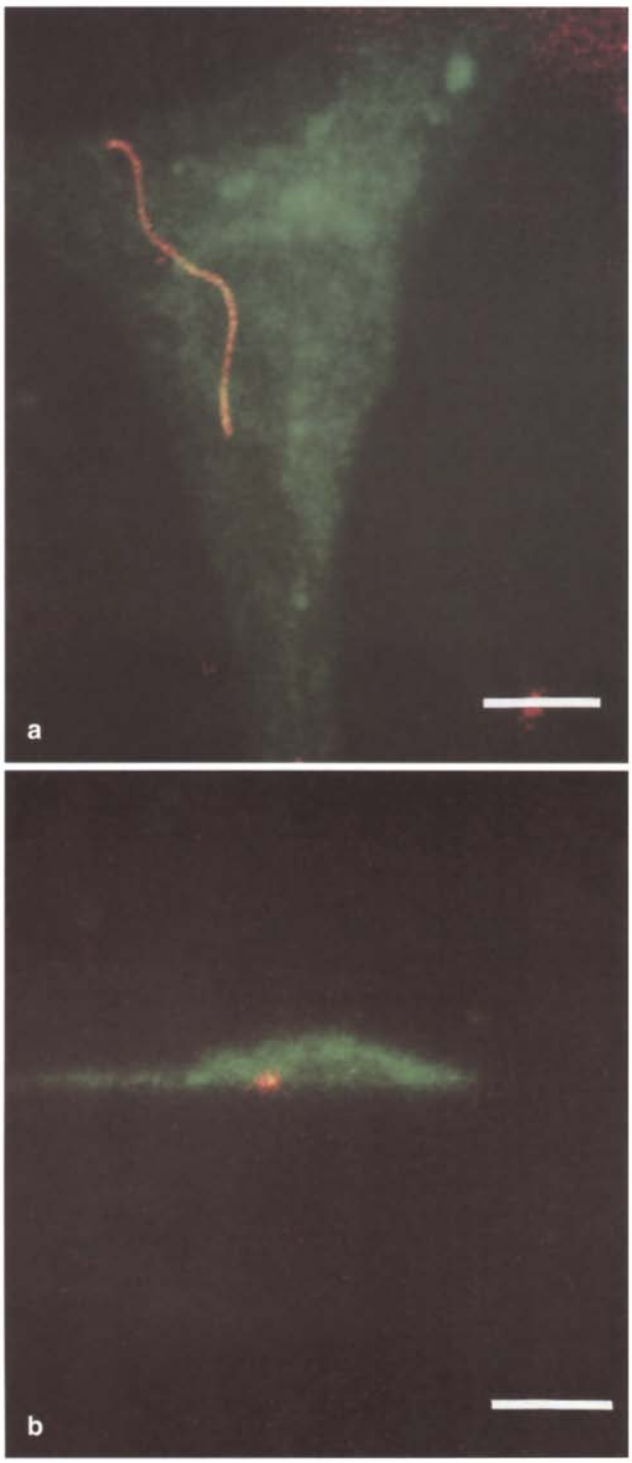
In primary cell cultures of SC, about 20–30% of the cells were macrophage-like cells showing phagocytosis vacuoles by TEM and expressing HLA-DR and CD 68. The rest of the cells were fibroblast-like cells. After two passages the macrophage fraction of the SC were no longer detected. AM isolated from human lung tested positive for CD 68 and non-specific esterase, characterizing these cells as macrophages. PBM were positive for HLA-DR and CD 68.

Table 1 Immunocytochemical characterization of human synovial cells (SC), peripheral blood macrophages (PBM) and alveolar macrophages (AM)

Cellular antigen	Antibody	SC	PBM	AM
HLA class I	a-HLA-B,C	+	+	n.d.
HLA class II	a-HLA-DR	–	+	n.d.
CD 68, 110 kDa (macrophages)	Ki-M 8	–	+	+
ICAM-1, CD 54, (intercellular adhesion molecule)	a-ICAM-1	–	–	n.d.

Short-term interaction of *Borrelia burgdorferi* with SC

Borrelia burgdorferi and SC were cocultured at a MoI (bacteria/cell) of 1, 10 or 100 using *Borrelia burgdorferi* strains LW 2 and B 31. SC were analysed after 90 min, 12 h, and 1 and 5 days by CLSM and TEM. Attachment of *Borrelia burgdorferi* to SC and partial or complete internalization of the spirochetes into SC were observed by CLSM after 90 minutes (Fig. 1 a, b). Intracellular spirochetes seemed to be morphologically intact. However, an enclosure of *Borrelia burgdorferi* into superficial or deep membrane folds could not be excluded by this method. Therefore, parallel cultures were analysed by TEM. *Borrelia burgdorferi* were found attached to SC and engulfed in cell membrane folds at the cell surface after 90 minutes (Fig. 2 a). Moreover, *Borrelia burgdorferi* were found to penetrate the cell membrane (Fig. 2 b). Finally, morphologically intact spirochetes were found within the cytosol in the absence of phagosomes, lysosomes or engulfing membrane structures (Fig. 2 c). Intracellular bacteria showed the characteristic ultrastructure of *Borrelia burgdorferi* (Fig. 2 c). *Borrelia burgdorferi* were often found in proximity to cytoplasmic filaments, confirming the cytosolic localization. Of 200 SC, 10 contained intracellular *Borrelia burgdorferi* at a MoI of 100 after 5 days of infection. With a lower MoI detection of intracellular spirochetes by TEM was possible, even at a MoI of 1, but limited to only single cells. Neither conventional or coiling phagocytosis nor degradation of *Borrelia burgdorferi* was observed in SC. The number of *Borrelia burgdorferi* taken up per cell did not differ between the high passage strain B 31 and the low passage strain LW 2.



Phagocytosis of *Borrelia burgdorferi* by human PBM and AM

To characterize the interaction of SC and *Borrelia burgdorferi* more closely, we compared it to the interaction of *Borrelia burgdorferi* with macrophages from peripheral blood and tissue. PBM and AM were cocultured with *Borrelia burgdorferi* strains LW 2 and B 31 at a MoI of 1, 10 or 100. By CLSM we found *Borrelia burgdorferi* inside the macrophages, and in contrast to SC, already degraded after 90 minutes (Fig. 3 a). The ultrastructure of PBM and AM showed the phagocytic morphology with an abundance of membrane-bound cytoplasmic inclusions and "empty" vesicles, none of which were found in SC (Fig. 3 b). In contrast to SC, *Borrelia burgdorferi* were found enclosed in phagosomes and phagolysosomes of PBM and AM (Fig. 3 b). Coiling phagocytosis of *Borrelia burgdorferi* was mainly present in PBM (Fig. 3 d). Of 200 PBM, 50 showed coiling phagocytosis after 1 day. In

Fig. 1 **a** Confocal laser scanning microscopy of synovial cells after 24 h of coculture with *Borrelia burgdorferi*. Compound computerized image of a series of xy sections through a synovial cell (green autofluorescence of the cell body) bearing an intracellular spirochete detected by indirect immunofluorescence using a polyclonal rabbit anti-*Borrelia burgdorferi* antibody. Colours were subject to the computerized image processing system and are not linked to the chosen fluorescence filter device. Original magnification ×1000; bar = 7 µm, multiplicity of infection (MoI) = 100. **b** xz section through the same cell: the cross-sectioned *Borrelia burgdorferi* is lying in the cytoplasm of the synovial cell close to the nucleus. The synovial cell is residing on a glass plate. Original magnification ×1000; bar = 7 µm. Although the combination of Fig. 1 a and b suggest that the spirochete is located within the cytoplasm, definite proof of intracellular location is by ultrastructural analysis (Fig. 2 and 4)

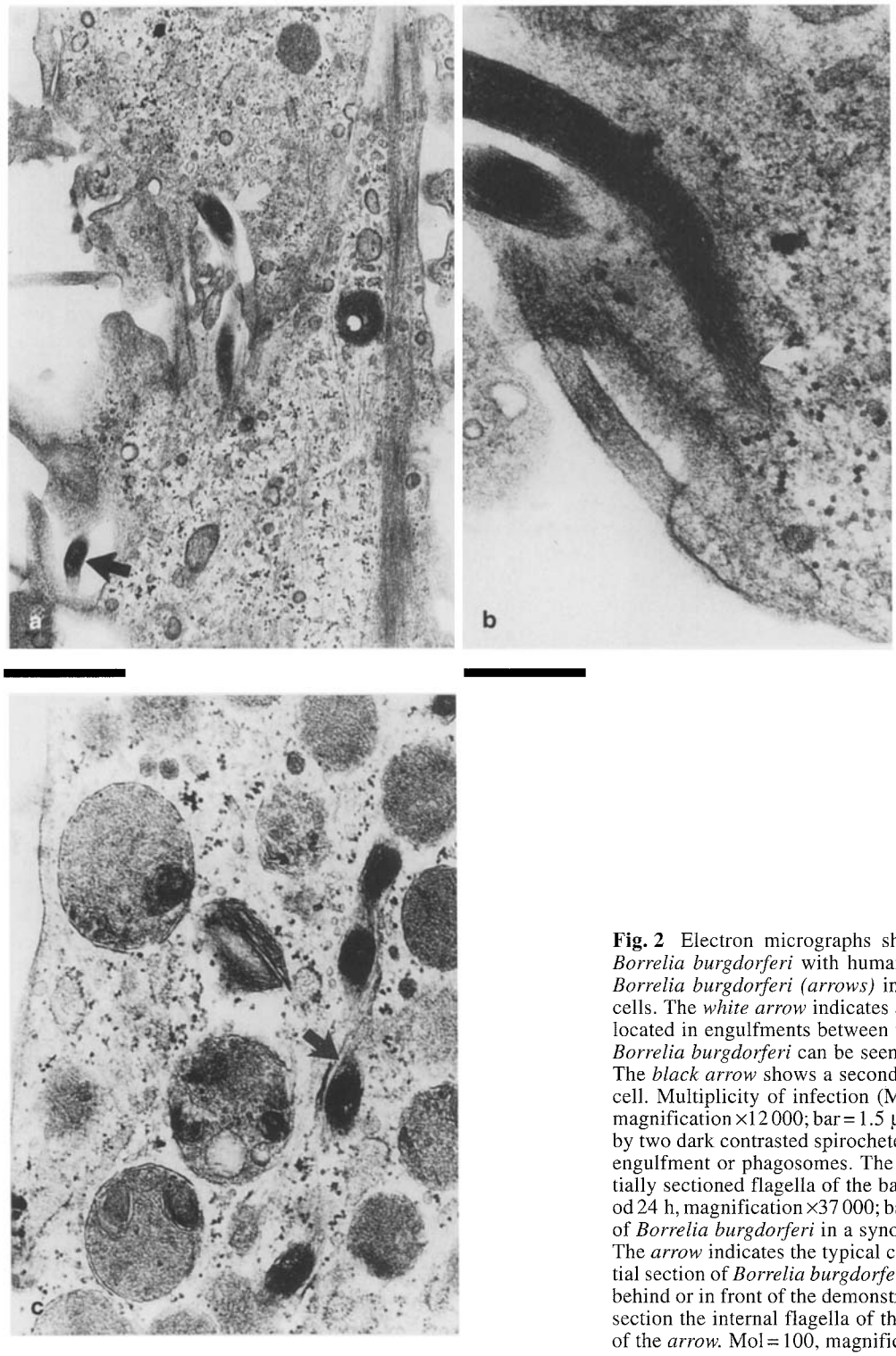


Fig. 2 Electron micrographs showing short-term interactions of *Borrelia burgdorferi* with human synovial cells. **a** Engulfment of *Borrelia burgdorferi (arrows)* into the cell membrane of synovial cells. The *white arrow* indicates a tangentially sectioned spirochete located in engulfments between two synovial cells. The flagella of *Borrelia burgdorferi* can be seen to the bottom of the *white arrow.* The *black arrow* shows a second spirochete engulfed in a synovial cell. Multiplicity of infection (Mol) = 100, incubation period 24 h, magnification ×12 000; bar = 1.5 μm. **b** Penetration of a synovial cell by two dark contrasted spirochetes in the absence of cell membrane engulfment or phagosomes. The *white arrow* indicates the tangentially sectioned flagella of the bacteria. Mol = 100, incubation period 24 h, magnification ×37 000; bar = 0.5 μm. **c** Cytosolic persistence of *Borrelia burgdorferi* in a synovial cell after 5 days of coculture. The *arrow* indicates the typical corkscrew morphology of a tangential section of *Borrelia burgdorferi.* Other parts of the spirochete are behind or in front of the demonstrated section. Due to the tangential section the internal flagella of the spirochete can be seen at the tip of the *arrow.* Mol = 100, magnification ×24 000; bar = 0.8 μm

a parallel experiment, AM (number of cell analysed by TEM = 200) showed only occasional coiling phagocytosis, whereas conventional phagocytosis into phagosomes and membrane-enclosed particles followed by degradation of *Borrelia burgdorferi* was abundant (Fig. 3 b, c). During the next 5 days secondary phagolysosomes were formed and the characteristic spirochete morphology was destroyed.

Long-term interaction of *Borrelia burgdorferi* with SC

Due to the longer life span of SC compared to phagocytes, the cocultures of SC and *Borrelia burgdorferi* were analysed for long-term intracellular persistence of *Borrelia burgdorferi* by CLSM and TEM. CLSM after 18 and 35 days of infection demonstrated intact intracellular spiro-

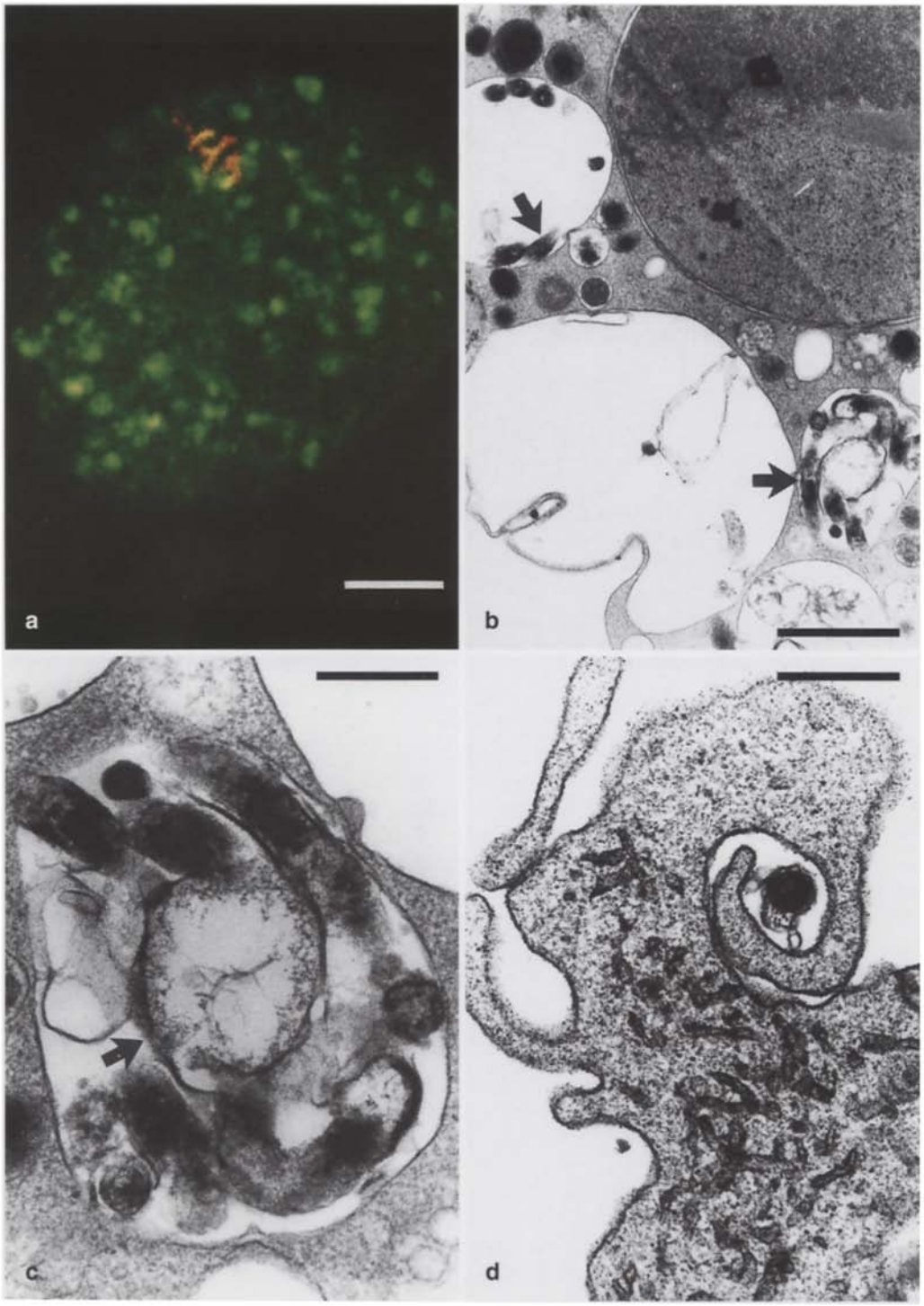


Fig. 3 Confocal laser scanning microscopy and electron micrographs showing interactions of *Borrelia burgdorferi* with human macrophages. **a** Confocal laser scanning microscopy of alveolar macrophages after 24 h of coculture with *Borrelia burgdorferi*. Compound computerized image of a series of xy sections through an alveolar macrophage (green autofluorescence of the cell body) bearing an intracellular spirochete. The spirochete is coiled up inside the cytosol. Methods were according to those in Fig. 1 a. Original magnification ×1000; bar = 5.5 μm, multiplicity of infection (Mol) = 100. **b** Electron micrograph of an alveolar macrophage bearing several spirochetes (arrows) inside phagosomes. The picture shows the typical macrophage morphology with an abundance of phagosomes and lysosomes. Mol = 100, incubation period 24 h, magnification ×9250; bar = 2.1 μm. **c** Electron micrograph of the same alveolar macrophage showing a phagolysosome containing a coiled spirochete at a higher magnification. The spirochete shows signs of degradation including a hydropic protoplasma body (arrow) and a disruption of the typical double membrane structure. Mol = 100, incubation period 24 h, magnification ×37 000; bar = 0.5 μm. **d** Electron micrograph showing coiling phagocytosis of spirochetes by macrophages. A peripheral blood macrophage enrolls a cross-sectioned spirochete with a cytoplasmatic protrusion. The cross-sectioned flagella can be recognized at the bottom of the protoplasma body of the spirochete. Mol = 100, incubation period 5 days, magnification ×33 500; bar = 0.6 μm

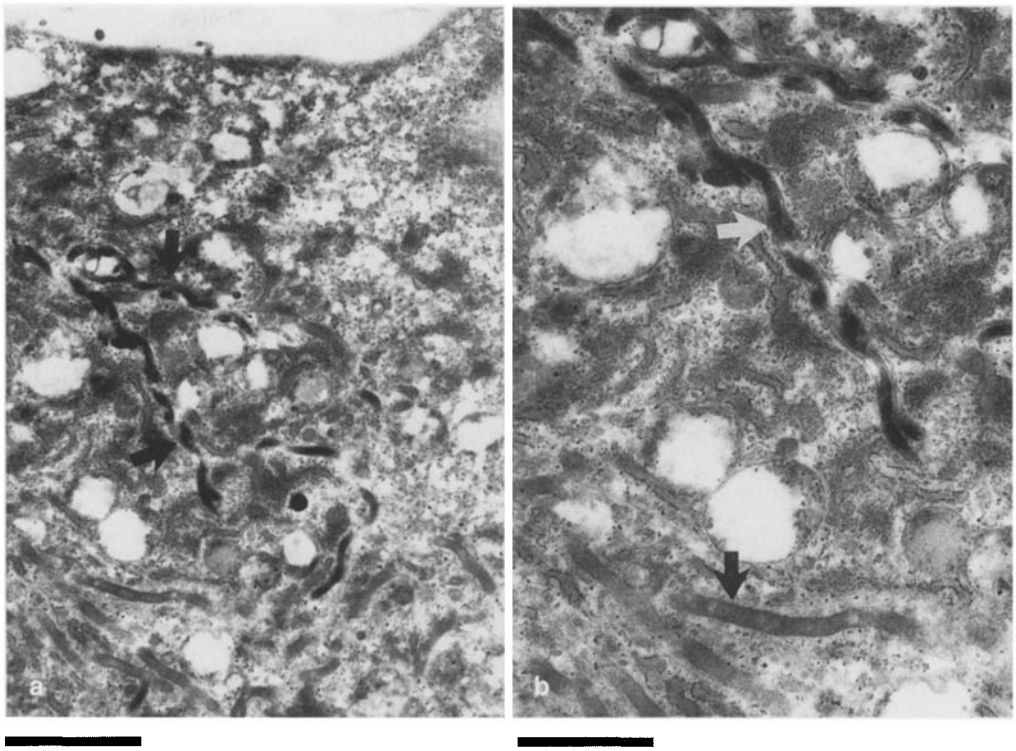


Fig. 4 Electron micrographs showing long-term interactions of *Borrelia burgdorferi* with human synovial cells. **a** Cytosolic persistence of several spirochetes in a synovial cell after 8 weeks of coculture. The two *arrows* indicate the cytosolic location of the dark-stained *Borrelia burgdorferi* without signs of degradation. Mol = 100, magnification ×4800; bar = 4.4 μm. **b** Higher magnification of the synovial cell shown in Fig. 4 a. Two dark-stained bacteria are located in the cytosol without signs of degradation (*white arrow*). Mitochondria are less contrasted than the spirochetes and can easily be distinguished by their typical ultrastructure from the bacteria, which show mitochondrial cristae (*black arrow*). Magnification ×10 250; bar = 1.9 μm

chetes of normal length (18 – 20 μm) or occasionally coiled up at one end of the bacteria. In contrast to the short-term interaction, SC bearing more than one spirochete were visualized by TEM after 56 days of infection (Fig. 4 a). There were no signs of degradation of the spirochetes (Fig. 4 b). The cocultures were split with trypsin for 7 weeks at weekly intervals and viable *Borrelia burgdorferi* were reisolated after each passage.

Antibiotic treatment of SC infected with *Borrelia burgdorferi*

To assess the viability of cytosolic spirochetes, we cocultured SC with *Borrelia burgdorferi* (Mol = 100) for 3 days followed by antibiotic treatment with ceftriaxone at a concentration of either 1.0 or 4.0 μg/ml for 9 days. Cultures were analysed by phase contrast microscopy (PCM) for the presence of *Borrelia burgdorferi* in the supernatant. After 6 days no extracellular *Borrelia burgdorferi* were detected by PCM. After cessation of antibiotic treatment (day 10) no extracellular *Borrelia burgdorferi* were found by TEM or PCM, in contrast to cultures not treated with antibiotics. However, *Borrelia burgdorferi* were localized within the cytosol of SC in about 10 of 200 cells at that time by TEM analysis. There was no difference between the two concentrations of ceftriaxone. SC were kept in culture up to 63 days without cell passages. Cell media supernatants were probed for viable spirochetes once a week by PCM and recultivation experiments up to 63 days, but recultivation of *Borrelia burgdorferi* failed. No extracellular spirochetes were detectable by PCM. However, cytosolic *Borrelia burgdorferi* were still detectable by TEM. After lysis of the SC at day 63, viable *Borrelia burgdorferi* were identified 7 days later by PCM and recultivation from the cell lysates; this did not occur in the uninfected controls.

Discussion

This study shows that *Borrelia burgdorferi* can invade and persist in the cytosol of resident joint cells in vitro. Several authors have investigated the possibility of an intracellular persistence of *Borrelia burgdorferi* using in vitro models. Internalization of *Borrelia burgdorferi* into a trypsin resistant compartment of human umbilical vein endothelial cells (HUVEC) has been reported [23]. Immune pre- and post-fixation staining techniques suggest the intracellular location of spirochetes. However, the authors could not exclude a persistence of spirochetes in deep membrane folds [23]. In vitro data suggest that *Borrelia burgdorferi* do not persist in endothelial cells, but can penetrate them [24]. Little is known about virulence factors that mediate the entry of *Borrelia burgdorferi* into nonphagocytic cells. Non-motile mutants that do not express the internal flagella are unable to penetrate HUVEC monolayers [25]. Our data suggested that resident tissue cells including SC may be a target for the intracellular persistence of *Borrelia burgdorferi* after the bacteria have passed through the endothelial barrier.

It has been reported that human foreskin fibroblasts mediate protection of spirochetes from antibiotic treatment with ceftriaxone [26]. After initial antibiotic treatment,

Borrelia burgdorferi can be recultivated after cell lysis from cocultures with human fibroblasts, suggesting an intracellular persistence of viable *Borrelia burgdorferi* for at least 7 days [27]. These authors could not exclude the possibility that spirochetes might be engulfed deeply within the cell membrane where the spirochetes might not be accessible to antibiotic drugs. In our in vitro model, engulfment in cell membrane folds, as opposed to intracellular persistence of *Borrelia burgdorferi,* was ruled out by ultrastructural analysis. In contrast to phagocytes, SC did not phagocytose or degrade *Borrelia burgdorferi.* We were able to demonstrate long-term cytosolic persistence of *Borrelia burgdorferi* in SC for up to 8 weeks in vitro. Comparing the survival of *Borrelia burgdorferi* in a fibroblast coculture [27] with our in vitro model, cytosolic spirochetes were also sheltered from antibiotic treatment in SC. The distribution of ceftriaxone in the extracellular space might explain why ceftriaxone did not reach these intracellular bacteria in vitro. In contrast, clinical experience shows that 75% of patients with Lyme arthritis benefit from antibiotic therapy [3]. However, 25% of patients still have ongoing arthritis even after one or two courses of antibiotic treatment [3]. Whether or not intracellular spirochetes in SC play a role in the pathogenesis of Lyme arthritis in these patients needs further investigation.

There are reports on the persistence of *Borrelia burgdorferi* in the joint in vivo [10, 12]. Isolation of viable *Borrelia burgdorferi* from synovial fluid has been reported twice, but subcultivation and subsequent analysis have failed [14, 28]. However, borrelial DNA has been detected in synovial fluid by polymerase chain reaction (PCR) [29, 30]. Moreover, detection of *Borrelia burgdorferi* DNA can be improved by probing synovial tissue rather than synovial fluid [31]. In vivo, the number of infected resident joint cells would certainly be small and, in fact, a small number of *Borrelia burgdorferi* is sufficient to maintain inflammation in Lyme arthritis [10]. Interestingly, the long-term interaction of *Borrelia burgdorferi* with SC in our model showed that SC bearing more than one spirochete were present. Whether or not this was due to an intracellular proliferation of bacteria needs further investigation. In our model a subsequent infection of SC by several individual spirochetes could be possible. Cytosolic spirochetes might release spirochetal products and maintain the inflammatory response. In some patients chronic stimulation by intracellular *Borrelia burgdorferi* might even cause an autoimmune response. This scenario might lead to the clinical manifestation of recurrent or chronic Lyme arthritis [6].

Our in vitro data demonstrated long-lasting cytosolic persistence of viable *Borrelia burgdorferi* within resident joint cells. This might be an important clue to the pathogenesis of chronic recurrent Lyme arthritis. Further studies are required to determine the exact role of the persistence of viable *Borrelia burgdorferi* inside resident joint cells.

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