Pleomorphism and Viability of the Lyme Disease Pathogen *Borrelia burgdorferi* Exposed to Physiological Stress Conditions: A Correlative Cryo-Fluorescence and Cryo-Scanning Electron Microscopy Study

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To understand the response of the Lyme disease spirochete *Borrelia burgdorferi* exposed to stress conditions and assess the viability of this spirochete, we used a correlative cryo-fluorescence and cryo-scanning microscopy approach. This approach enables simple exposition of bacteria to various experimental conditions that can be stopped at certain time intervals by cryo-immobilization, examination of cell viability without necessity to maintain suitable culture conditions during viability assays, and visualization of structures in their native state at high magnification. We focused on rare and transient events e.g., the formation of round bodies and the presence of membranous blebs in spirochetes exposed to culture medium, host sera either without or with the bacteriolytic effect and water. We described all crucial steps of the workflow, particularly the influence of freeze-etching and accelerating voltage on the visualization of topography. With the help of newly designed cryo-transport device, we achieved greater reproducibility.

**Keywords:** cryo-fluorescence, cryo-scanning electron microscopy, *Borrelia burgdorferi*, Lyme disease, round body, pleomorphism, viability staining

**INTRODUCTION**

Certain spirochete genospecies belonging to the *Borrelia burgdorferi* sensu lato complex are causative agents of Lyme disease (LD) and are transmitted by hard ticks of the genus *Ixodes* (Barbour and Hayes, 1986). *Borrelia* consist of a protoplasmic cell cylinder surrounded by an outer membrane and a plasma membrane with a peptidoglycan layer (Barbour and Hayes, 1986). Both membranes enclose a periplasmic space in which the flagella are located (Goldstein et al., 1994). Flagella define the flat-wave morphology of spirochetes and are responsible for their motility. Motility is a crucial factor in *Borrelia* transmission and its efficient dissemination through host/vector tissues (Motaleb et al., 2000). Mutations in the major flagella protein B result in the
development of rod-shaped and non-motile spirochetes (Sultan et al., 2013). In addition to the flat-wave and rod-shaped morphological forms, the existence of non-motile atypical morphologies, such as looped/ring-shaped forms or spherical forms, has been previously described (Barbour and Hayes, 1986). Spherical cells are named in various ways, e.g., round bodies (RBs), spheroplast L-forms, cell wall-deficient, or cystic forms, and are described as large spherical structures with numerous flagella enclosed by an intact outer membrane (Hulínská et al., 1989, 1994; Mursic et al., 1996; Borson et al., 2001; Miklossy et al., 2008; for reviews see Stricker and Johnson, 2011; Berndtson, 2013; Lantos et al., 2014). The transformation of motile B. burgdorferi spirochetes into non-motile RBs has been demonstrated in vitro in response to a hostile environment, e.g., after incubation with water, serum starvation or antibiotic treatment (Borson and Borson, 1997, 1998a; Alban et al., 2000; Murgia and Cinco, 2004; Borson et al., 2009). Recently, Drecktrah et al. (2015) showed that during starvation, morphological conversion to RBs was dependent on the production of guanosine tetraphosphate and guanosine pentaphosphate. The transition of RBs back into the motile forms has been described after a short exposure of spirochetes to hypotonic conditions (Meriläinen et al., 2015). Next, spherical forms that were isolated from the spinal fluid converted back to the spiral form after cultivation in rich BSK-H medium (Borson and Borson, 1998b). Gruntar and colleagues showed the infectivity of B. garinii cystic forms prepared in dH2O that were intraperitoneally inoculated into mice (Gruntar et al., 2001). Cystic forms have been found in the brains of patients with neuropathologically confirmed Lyme neuroborreliosis (Miklossy et al., 2008), as well as in skin tissues (Aberer et al., 1996). Non-motile spirochetes have been visualized in vivo within the midgut of unfed Ixodes scapularis nymphs (Dunham-Ems et al., 2009).

Here, we present results from the first in vitro study on the formation and viability of atypical morphologic forms of infectious spirochetes B. burgdorferi s.s. expressing green fluorescent protein (GFP) in response to water and after incubation with sera. We used either sera from impala (Aepyceros melampus) or African buffalo (Syncerus cifer), that were determined in our earlier work to exert different bacteriolytic activities against spirochetes (Tichá et al., 2016). Impala serum revealed the strongest borreliacidal effect against all tested spirochete species from the B. burgdorferi s.l. complex in the complement sensitivity test, whereas buffalo serum did not display any borreliacidal effect (Tichá et al., 2016).

Cryo-fluorescence is a powerful imaging technique used for the visualization of fluorophore-tagged molecules in the frozen-hydrated state that opens new possibilities for correlative light electron microscopy studies (CLEM) at cryogenic temperatures (Chang et al., 2014; Kaufmann et al., 2014a,b; Schorb and Briggs, 2014; Strnad et al., 2015). Main advantages are derived from the cryo-immobilization and visualization of vitrified specimens without the influence of any chemicals and presence of artifacts caused e.g., during dehydration and drying steps included in the conventional sample preparation (Vancová and Nebesárová, 2015). Next, the cryo-fluorescence can be (re-)evaluated after cryo-SEM observations if needed, even after freeze-etching and gold sputter coating (Strnad et al., 2015). Another benefit of the cryo-fluorescence described earlier is its substantial reduction of photo-bleaching at low temperatures (Schwartz et al., 2007; Li et al., 2015). However, the crucial factors are maintaining the temperature of samples below −140°C to avoid formation of crystalline ice and prevent contamination. Proper vitrification of bulk specimens is another limiting factor for observation in cryo-SEM. Biological cryo-specimens are highly sensitive to electron-beam radiation damage, therefore electron beam exposures must be minimized and observation at very low energy (e.g., 1 kV) enable collection only “topographic” surface information (Nebesárová et al., 2016).

**MATERIALS AND METHODS**

**Borrelia Strain and Culture Conditions**

*B. burgdorferi* Bb 914, a GFP-expressing virulent derivative of strain 297, was cultured in Barbour-Stoenner-Kelly medium (BSK-H, Sigma-Aldrich) containing 6% rabbit serum at 34°C until mid-log phase (~5 × 10^7 spirochetes/ml). The GFP-tagged strain *B. burgdorferi* was obtained from Dr. Caimano (Caimano et al., 2004).

**Serum**

Serum samples from male African buffalo (*Syncerus cifer*) and female impala (*Aepyceros melampus*) were obtained from the zoo in Hradec Kralove (Czech Republic; Tichá et al., 2016). Blood samples were taken during routine checks of the animals, following zoo ethics regulations.

**Cell Preparation and Viability Staining**

A summary of the experimental approach is provided in Figure 1. Pelleted cells (7.5 × 10^7) were washed in 0.1 M HEPES, centrifuged (820 × g, 10 min), resuspended in 100 µl of buffer, and immediately transferred onto freshly prepared 200-mesh index grids (e.g., Tedpella, Agar Scientific) coated with formvar-carbon film and glow discharged. After 1 min, either 3 µl of serum or culture media or dH2O was added. After incubation (1–5 min) at room temperature in a humidification chamber, 2 µl (1 µg/ml) of propidium iodide (PI, BioRad, λ excitation = 536 nm; λ emission = 620 nm) was added. After 30 s, excess solution was immediately removed with a piece of filter paper. Control cells were incubated in the presence of either water or BSK-H medium under the same conditions as described above.

**Cryo-Fluorescence and Cryo-Scanning Electron Microscopy**

Grids were immediately frozen by plunging into liquid ethane cooled by liquid nitrogen using a homemade plunger. The excess of liquid ethane was blotted with filter paper and transferred to a Leica EM Cryo CLEM (Leica Microsystems, Vienna, Austria) system, comprising a shuttle device, a fluorescent light microscope (Leica DM6000 FS), a cryo CLEM objective (Leica HCX PL APO 50x/0.9), and a camera (Leica DFC310FX). Viability was determined by counting the cells using cryo-fluorescence. For topography, grids were transferred to a FESEM.
The cartridge with the mounted grid (Figure 2A, arrow) was transferred to a pre-cooled stainless steel cup placed in a polystyrene box (Figure 2B, inset). The block was transferred from the Leica cryo-transfer shuttle into the pre-cooled homemade stainless steel cup placed in a polystyrene box (Figure 2C). The interior of the cup comprised areas separated by metal walls (Figure 2C, black arrow) that were filled with liquid nitrogen and a small central cup (Figure 2C, white arrow) for the transport of the metal block in nitrogen gas. The stainless cup with the metal block was transported to a CryoALTO slushing chamber (Figure 2D), where the cap was removed, and the chamber was evaporated. Finally, the holder was transported under vacuum (Figure 2E) to the preparation chamber using a transfer rod. Here, ice contaminants caused by the condensation of water vapors (Figures 3A,B) were removed by a 10-min sublimation at a range from −98°C to −95°C under high vacuum (Figures 3C,D). However, the surface topography of frozen spirochetes embedded into the thin layer of either serum or water was revealed after an additional 10-min sublimation under the same conditions (Figures 3E–H). Despite the extensive sublimation of the ice, neither specimen damage nor the presence of holes in the structure was observed. Next, the specimen surface was sputter-coated for 10–20 s with gold (cold sputter coater is a part of CryoALTO 2500), and images were recorded using the conventional Everhart-Thornley (ET) detector of secondary electrons at 1–3 kV at −140°C (Figures 3F–H). A graded increase in magnification enabled navigation to the area of interest. Fluorescence and SEM images were aligned using Adobe Photoshop.

RESULTS

Morphological Transformation and Viability of Spirochetes after Incubation with Sera

After a 2-min incubation with impala serum, the majority of spirochetes were viable (81%, 237 cells) and had a flat-wave appearance (73%, 212 cells, Figure 4A). Other viable morphological forms, irregularly shaped spirochetes (5%, 16 cells) and viable RBs (3%, 9 cells) were observed rarely. Non-viable cells (19%, 54 cells, Figure 4A) had either a regular flat-wave shape (or were stretched, 11%, 31 cells), or an irregular cell morphology, e.g., coiled parts (5%, 14 cells), and 3% represented red-stained RBs (9 cells; Figure 5). RB structures were formed by either three irregular or three regular folds of one bacterium (Figure 5, white arrows). A representative image shows a PI-stained spirochete with a flat-wave morphology (Figures 5D, yellow arrow) with membranous blebs or leaking cell content (Figure 5E, yellow arrow). An overload of several areas with a high amount of Borrelia prevented the recognition of individual cells (Figure 5A, asterisks). After the incubation time was extended to 5 min, all spirochetes were positively stained with PI (Figure 4B) and were spiral-shaped (72%, 238 cells), although straight spirochetes and bacteria with coiled ends (27%, 89 cells) and, rarely, RBs (1%, 4 cells) were observed (Figures 5G–K).

In contrast to the impala serum, the majority of spirochetes incubated with African buffalo serum for 5 min were viable and had a flat-wave shape (73%, 169 cells, Figure 4C). Rarely, irregularly shaped forms (4%, 9 cells) or RB forms (3%, 8 cells) were found. PI-stained spirochetes (Figure 4C) exhibited either spiral (11%, 25 cells), irregular (6%, 13 cells) or RB forms (3%, 7 cells). Spirochetes with coiled ends were defined among irregularly shaped/PI-positive cells (Figures 5G–H). Similarly, the majority of spirochetes incubated in the presence of culture media (supplemented with 6% rabbit serum) for 5 min were viable (97%, 95 cells) and had a flat-wave morphology (87%). Individual cells were also irregular (7%) and in the form of RBs (3%). We rarely found spirochetes with atypical coiled ends that were counterstained with PI (Figures 6A–C).
Morphological Transformation and Viability of Spirochetes after Short Exposure to Osmotic Stress

Spirochetes exposed to short hypotonic stress for 1–3 min showed significant pleomorphism (Figures 4E, 6D, A–G). Only 24% (45 cells) of all viable cells (68%, total 127 cells) had a spiral morphology (Figure 4E), whereas 28% of spirochetes were irregularly shaped (53 cells), and 15% were spherical (29 cells). Cell viability was affected in 32% of cells (61 cells) that were either in the spiral (5.8%), irregular (13.8%), or RB forms (12.7%). These numbers also included spirochetes that were partially PI-positive, e.g., PI-stained spiral-shaped parts of the protoplasmic cylinders with green centers of coiled parts, as shown in Figure 6G (details shown in the insets).

DISCUSSION

We used a cryo-fluorescence and cryo-SEM approach for the rapid and close-to-native assessment of both the viability and morphological changes of GFP-expressing spirochetes exposed to physiological stress. The SEM approach allows the reliable differentiation of pleomorphic forms of *Borrelia* from other artifacts (e.g., background signals and unbound PI dye) and the visualization of the spatial organization of pleomorphic variants. Viable cells were distinguished from dead cells based on cell membrane integrity and PI permeability. This combination of green fluorescence emitted by cellular GFP and red fluorescence emitted by PI has been used previously to measure bacterial viability and has several advantages: the short amount of time required to perform the experiment, highly reliable results and no competition for binding sites in contrast to SYTO9/PI staining (Lehtinen et al., 2004). We found PI-positive cells that were either simultaneously GFP-positive (spirochetes incubated in the presence of impala serum for 5 min, Figures 5G–K) or only partially positive (control cells, spirochetes incubated in the presence of serum from African buffalo or water for 5 min, Figures 5L–O, 6D–G). A similar observation, in which a proportion of the dead GFP-tagged strain of *Pseudomonas* retained some GFP at a lower concentration, has been previously described (Lowder et al., 2000). We believe that this observation could be caused by a combination of different factors: (a) the extent of damage to the cell membrane, (b) the time of cell death/damage and/or (c) the difference in the molecule sizes of both GFP (27 kDa, the shape of a cylinder with a length of 4.2 nm and a diameter of 2.4 nm) and PI (0.668 kDa). The mechanism of cell death induced by hypo-osmotic shock probably involves cell burst after the extensive absorption of water and later leakage of GFP proteins. In contrast, during complement-mediated killing, terminal complement components form pores in the membranes, leading to damage, and death of the pathogen (Kurtenbach et al., 1998). The membrane-attack complex is ~10 nm in diameter (Janeway et al., 2001), which may explain the lower release of GFP from dying spirochetes after incubation with impala serum.

The influence of serum on the morphology of LD spirochetes has been previously described by de Taeye and Meriläinen...
FIGURE 3 | Influence of ice sublimation time and accelerating voltage on the visualization of surface topography of spirochetes. The same areas were imaged without sublimation (A,B) or after 10 min (C,D) and 20 min (E–H) of sublimation. Sputter-coated specimen was imaged with secondary electrons at 1 kV (E,F), 2 kV (G) and 4 kV (H). Images (A–E), 1 kV. Bars 10 µm.

FIGURE 4 | GFP/PI viability assay performed by counting bacteria using Leica Cryo CLEM fluorescence microscopy. Borrelia were incubated under different conditions: serum isolated from impala for 2 min (A) or 5 min (B), or from African buffalo for 5 min (C). BSK-H supplemented with 6% rabbit serum for 5 min (D) or with H₂O for 2 min (E). Three morphological variants were distinguished: spiral forms (dark green/red), irregularly shaped spirochetes (medium light green/red) and round body forms (light green/red). Viable bacteria (green), PI-stained bacteria (red).

(de Taeye et al., 2013; Meriläinen et al., 2015). RBs and blebs (large membrane bulges) were induced in vitro after incubation with culture media supplemented with human serum (de Taeye et al., 2013; Meriläinen et al., 2015). However, blebs (defined as small outer membrane vesicles) are released from microbial cell surfaces as a general response to stress (e.g., after antibiotic
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FIGURE 5 | GFP-expressing B. burgdorferi spirochetes incubated for 2 min (A–F) or 5 min (G–O) in the presence of serum showing the strong (A–K) or weak (L–O) bacteriolysis capacity. Animal sera were isolated either from Aepyceros melampus (A–K) or Syncerus cifer (L–O). After incubation, spirochetes were stained with propidium iodide (PI, red). (A–F) Cryo-fluorescence and cryo–SEM images merged with viability staining results and with the structural information of spirochetes at a different magnification. Areas overloaded by a high number of bacteria (A, asterisk), PI-stained damaged/dead bacteria in the form of the round body (B, D) and with a spiral-shaped morphology (D, E). Regular (F, arrows) or irregular (C, inset-arrows) coils of the round body forms. Leakage of cellular content from the red-stained spirochete (E, yellow arrow). G–K: Flat-wave-shaped spirochetes with a frequent presence of blebs (G–I, arrows) that represent coiled ends/irregular parts of spirochetes as observed at high magnification (J, K). (L–O) Representative image at low (L, M) and high (N, O) magnification. Irregularly shaped PI-positive parts of cells (O, arrows) shown on a merged secondary electron image and a fluorescence image.
treatment, prolonged cultivation, addition of specific antibodies, and complement to culture) and are referred to as an initial sign of membrane alteration (Barbour and Hayes, 1986; de Taeye et al., 2013). We demonstrated here that in the presence of host sera (with and without a bacteriolytic effect), *B. burgdorferi* spirochetes do not change their typical flat-wave shape, even though RBs and spirochetes with bulges (which were often identified at high magnification as damaged coiling parts of the protoplasmic cylinder) were observed (Figure 5). We repeatedly observed low amount of viable and non-viable RBs forms (3%) in the control cultures and similarly after short exposure to host sera. In contrast, various viable pleomorphic forms of LD spirochetes developed after short exposure to osmotic shock; 68% of cells were still viable after 2 min; we observed 15% of viable and 13% of non-viable RBs (Figure 4). These results are consistent with the previous studies of Meriläinen and colleagues (Meriläinen et al., 2015), who showed that 85% of RBs were formed after a 10-min incubation with H$_2$O and that spirochetes later reverted to their parent vegetative form after incubation in culture media.

These round morphological variants likely represent stressed viable bacteria that can revert, in some circumstances, back to the motile spiral form. The presence of atypical morphological forms of LD spirochetes *in vivo* and their direct association with chronic persistent infection in human or animal models has been reported in a small number of studies (Hulinská et al., 1989, 1994; Brorson et al., 2001; Miklossy et al., 2008). However, the significance of RBs in LD pathogenesis remains unclear (Lantos et al., 2014). The formation of different spirochetal forms can hypothetically explain the persistence of spirochete infection or the presence of unusual symptoms lasting for several months despite antibiotic treatment (Kersten et al., 1995; Golovchenko et al., 2016; Rudenko et al., 2016; Sapi et al., 2016). Recently, different bactericidal drugs targeting RBs and biofilm-like forms were tested *in vitro*, however, further *in vivo* studies are needed to evaluate their significance for treatment of “chronic” LD (Feng et al., 2016a,b,c). Similarly, findings on the viability of various morphological forms (e.g., RBs, blebs) of spirochetes can provide valuable insight into their role in the LD. For that purpose, we used (for the first time) a combination of cryo-fluorescence and cryo-scanning electron microscopy. In contrast to standard viability studies at ambient (or culture) temperature, the cryogenic conditions allowed us to freeze all dynamic events in the certain period, to stop the mobility of...
spirochetes, and to observe their morphology without influence of chemicals or unfavorable conditions that could be potential sources of artifacts. Cryo-fluorescence microscope Leica EM cryo CLEM, equipped with a HCX PL APO 50x objective with NA 0.9, is now commercially available and, in combination with cryo-EM, is becoming a powerful tool for imaging biological specimens.

**AUTHOR CONTRIBUTIONS**

MV carried out the experiments; JV optimized the cryo-transfer and carried out SEM observations; MG and LT prepared the sera; MS contributed to protocol optimization, RR cultivated the GFP-MV carried out the experiments; JV optimized the cryo-transfer specimens. of dissemination of *Borrelia burgdorferi* within ticks. J. Clin. Invest. 119, 3652–3665. doi: 10.1172/JCI39401

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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