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ADVANCES IN PLEOMORPHIC FORMS OF BORRELIA

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Lyme Disease is the most frequently reported tick-borne infection in North America and Europe and is endemic in many other areas in the world. Various in vitro studies confirm that B. burgdorferi sensu lato is pleomorphic in morphology. The bacteria can be seen in the parent spirochete form, cell wall deficient forms such as the cyst or L forms, round bodies, and spheroplasts, or blebs, and loops as well as biofilm colonies. Clinically relevant in vivo pleomorphic forms of Borrelia such as cysts have been documented in astrocytic cultures, or granular and swollen forms identified in skin biopsies containing infections of Borrelia in lichen schlerosus, cysts in erythema chronicum migrans, and atypical forms of Borrelia in fresh fetal brain autopsy. Although immense in vitro and few in vivo data suggest the existence of the pleomorphic forms, there is a long-standing debate regarding if these forms in situ exists and if these forms are actually pathogenic in nature. A recent limited systematic review of B. burgdorferi on the morphological variants even suggested that these forms do no support a role in chronic Lyme disease when analyzing 6 studies. However, antibiotic resistance in long-term antibiotic treatment in Rhesus monkeys has been reported. Generally speaking, in order for the bacteria to survive from interference of the cell wall or RNA synthesis during antibiotic treatment, it has to be protect itself. Pleomorphic forms could be one explanation why B. burgdorferi can survive during long antibiotic treatments. Actions to conduct a larger cohort in situ studies in human specimens regarding pleomorphic forms are definitely needed and requested.

In our study we investigated the induction of Borrelia pleomorphic forms in adverse environmental conditions, characterize thoroughly their structural components, and assayed the bioactivity of these forms. Initially, pleomorphic forms were screened in normal physiologically relevant culture conditions. In addition, B. burgorferi was exposed to additional environmental pressures such as H20, rabbit serum-free BSK-II media, mammalian culture media RPMI-1640, doxycycline, and human sera, in order to study the morphological changes in these conditions. Pleomorphic forms were characterized using LIVE differential interference contrast microscopy and the reversions back to motile vegetative spirochetes were evaluated. Our results indicated that pleomorphic forms such as spirochetes, few round bodies, blebs as well as biofilms are seen in normal culture conditions. Growth curve analysis also indicated that the biofilm formation is reached already at early stages of growth and is not caused only because of

the overgrowth of the bacteria. In addition, Borrelia treated with doxycycline or cultured in RPMI media display immense outer envelope damage compared to normal cultures. When grown in the presence of human sera or H20, the blebs and round bodies increased to significant levels. These results indicated that when the Borrelia is exposed to unfavourable conditions such as human sera, the amount of blebs is increased that lead to more round body formation.

Reversion and metabolism studies followed to investigate if the induced round bodies were destroyed or were bioactive. Induced round bodies were isolated and reintroduced into normal Borrelia culture media. At 8 days, reversion is seen that indicated that the bacteria were able to exponentially replicate. As a control, bystander spirochetes from induced cultures were filtered first and then reintroduced into normal Borrelia culture media. As a result, the possible bystander spirochetes did not grow suggesting that the induced round body culture was homogenous. Similarly when the pleomorphic forms were introduced to normal culture media for 1 hr or for 1 day, the ATP synthesis analysis indicated that the round bodies in this form are not metabolically active, but when reintroduced to normal culture media for several days, they become biologically active again. This data supports that notion that round bodies are sleath in nature, however, they can regain their bioactivity and revert back to their normal parent form.

To further characterize these pleomorphic forms, various labeling protocols were conducted. Initially, biochemical stains such as propidium iodine (PI), wheat germ agglutinin (WGA-555), Nile red, and Acid Fuchsin to indicate DNA, polysaccharides, lipids and collagen, respectively, were used to label the bacteria. LIVE cell confocal microscopy was employed to evaluate the staining. The parent form of spirochetes displayed similar compositions of DNA and lipids as for the round bodies, blebs, and biofilms. Here we appreciate the unique nature of the cell envelope of the Borrelia in that exclusion stains such as PI were able to enter living cells. PI is commonly used to label dead bacteria, but we demonstrated that it can label living Borrelia. The round bodies was seen to have very specific binding with WGA indicating that there are differences in the polysaccharides of this form to spirochetes and biofilms. Additionally, biofilms had prominent staining of collagen as typical for such structures. The specificity of the biochemical stains was supported with observed increased staining in methanol fixed bacteria. Immuno-labelling for p41 protein of the flagella also indicated that flexibility of the outer membrane in the round bodies as the flagella was also present in these forms. Similarly transmission electron microscopy micrographs of these pleomorphic forms indicated that uniqueness of the cell envelope of the Borrelia. The typical double membrane of the outer and inner envelope is apparent in both spirochetes and round body forms indicating that the outer envelope is not as rigid in round bodies as once proposed. Instead, it is elastic in nature allowing the protoplasmic cylinder to fold within its circumference. In addition we found that round bodies have the flagella inside indicating

that round bodies are able to maintain structures related to motility during round body formation.

To examine further the bioactivity of round body forms ATP production of 10 min and 2 h round bodies were measured. The reversion of round bodies back to spirochetes was observed and the production of ATP of reversion cultures in early and mid log-phases were assayed. ATP determination experiments indicated that round body forms do not have ATP production while parent spirochetes in mid or late log-phase of growth had. During the reversion back to spirochete form, no ATP activity was detected after 1 h or 24 h of culturing in normal medium. However, round bodies were able to revert and become active spirochetes at day 5. In addition, ATP experiments provided more evidence that there are no viable bystander spirochetes present in the round body cultures, since no ATP production was detected.

In addition, the morphology and the cell wall characteristics of different B. burgdorferi forms, spirochetes, round bodies and blebs were analyzed using transmission electron microcropy (TEM). Interestingly, TEM images clearly illustrate that round bodies have an intact double outer membrane and inner membrane around the protoplastic cylinder, like in the parental spirochete form. Blebs have a partly expanded outer envelope usually on one end the lateral part of the spirochete, but these forms still have a partly spirochaetal morphology. This data also indicated that the round bodies are not cyts nor are they cell wall deficient. These pleomporphic forms have an intact cell membrane similar to the parent spriochaetal form. The TEM micrographs also indicated a model for round body formation in that B. burgdorferi has an elastic outer membrane that expands and allows folding of the protoplastic cylinder inside the outer membrane during round body formation.

Taken together, the results indicated that B. burgdorferi can change morphology very quickly, adapt and survive in adverse environments, and have pleomorphic forms consisting of DNA as well as antigenic relevant proteins that are freely recognized by the immune system. In addition, B. burgdorferi and its pleomorphic forms have atypical cell wall characteristics and that these sleath round bodies have the ability to become viable spirochetes.