

FORMATION AND CULTIVATION OF BORRELIA BURGENDORFERI SPHEROPLAST-L-FORM VARIANTS

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SUMMARY: A clinical persistence of *Borrelia burgdorferi* in patients with active Lyme borreliosis occurs despite obviously adequate antibiotic therapy. In vitro investigations of morphological variants and atypical forms of *B. burgdorferi* were undertaken. In an attempt to learn more about the variation of *B. burgdorferi* and the role of atypical forms in Lyme borreliosis, borreliae isolated from antibiotically treated and untreated patients with the clinical diagnosis of definite and probable Lyme borreliosis and from patient specimens contaminated with bacteria were investigated. Furthermore, the degeneration of the isolates during exposure to penicillin G in vitro was analyzed. Morphological analysis by darkfield microscopy and scanning electron microscopy revealed diverse alterations. Persistors isolated from a great number of patients (60-80%) after treatment with antibiotics had an atypical form. The morphological alterations in culture with penicillin G developed gradually and increased with duration of incubation. Pleomorphism, the presence of elongated forms and spherical structures, the inability of cells to replicate, the long period of adaptation to growth in MKP-medium and the mycoplasma-like colonies after growth in solid medium (PMR agar) suggest that *B. burgdorferi* produce spheroplast-L-form variants. With regard to the polyphase course of Lyme borreliosis, these forms without cell walls can be a possible reason why *Borrelia* survive in the organism for a long time and the cell-wall-dependent antibody titers disappear and emerge after reversion.

INTRODUCTION: The etiologic agent of Lyme disease, *Borrelia burgdorferi* (1) has been isolated by culture from ticks of the *I. ricinus* complex, and from animals and humans. Immunological and molecular heterogeneity were demonstrated amongst isolates from the USA and Europe (2-5). On the basis of DNA homology, RNA-sequence analysis and r-RNA gene restriction profiles, the isolates were recently classified into three genospecies: *B. burgdorferi sensu strictu*, *B. garinii* and *B. afzelii* (6-8). Lyme disease, the most widespread disease transmitted by ticks, is characterized by various clinical stages, including dermatologic, neurologic, cardiac, and ocular manifestations.

Penicillin G, amoxicillin, doxycycline, the macrolides erythromycin, azithromycin, and the cephalosporins cefotaxime, ceftriaxone, ceftazidime and cefuroxime have been applied to prevent progressive clinical manifestation of Lyme borreliosis. The results of comparative in vitro and in vivo susceptibility studies of *B. burgdorferi* indicate efficacy of several antibiotics including doxycycline, amoxicillin, macrolides and cephalosporins (9-10). However, some patients develop late symptoms despite apparently adequate antibiotic treatment (11-15). The persistence of Bb even after therapy with antibiotics has been demonstrated in cerebrospinal fluid (CSF), in skin, iris, heart and joint biopsies (16-21).

The aim of the present study was to investigate cytomorphic variations of Bb isolates from patients with and without antibiotic treatment.

MATERIALS AND METHODS: The Bb strains used in this study were isolated from human CSF, skin biopsies and blood culture in MKP-medium after incubation at 33 degrees C for 2-15 weeks as previously described (22). The identification of isolates was performed by SDS-PAGE, immunofluorescence and Western blot with OspA-specific monoclonal antibodies (4). 1. The phenotypic and genotypic variations of Bb after serial in vitro passages in MKP-medium were investigated by darkfield microscopy, by SDS-PAGE and pulsed-field gel electrophoresis (PFGE), using strains PKo (5th and 303rd passage) and PBi (5th and 285th passage). The virulence and the loss of infectivity were investigated in gerbils as an animal model. The infectivity of these strains at low passage was demonstrated previously (23).

2. The effect of antibiotics on the morphological form of isolates was examined by darkfield and scanning electron microscopy during 6 days of cultivation of Bb strains (low passage) in MKP-medium with penicillin G (3 mg/l). The methods of cultivation and in vitro and in vivo susceptibility procedures were described elsewhere (10).

3. The degeneration of 18 Bb persistors, isolated from patients with definite and probable Lyme borreliosis after antibiotic therapy, was investigated by darkfield (18) and scanning electron microscopy (3) after growth in MKP-medium. We also investigated 20 isolates from untreated patients by darkfield microscopy (20) and by scanning electron microscopy (3). Case histories will be taken into consideration in a later publication.

4. The cytomorphic change from atypical nonmotile, "rigid" form to motile helical form of Bb was studied on specimens (2 CSF, 2 skin biopsies) from four patients primarily contaminated with bacteria. To eliminate bacterial contamination, cultures were filtered by 0.45 micrometer filter; the recoverable borreliae were subcultivated in MKP-medium at 33 degrees C and passaged repeatedly until motility. The origin of the "rigid" form was studied in vitro using an experimentally contaminated PKo strain. Cultures of this PKo strain (used as control strain) were contaminated with *Acinetobacter* sp. (1000/ml) and cultured in MKP-medium until the "rigid" form was observed. Cultures of "rigid" forms were manipulated as described above for patient strains. Darkfield microscopy and scanning electron microscopy were used to investigate the motility, morphology and structure of Bb cells.

The cell concentration of Bb was determined by total counts and by viable counts using counting chamber and darkfield microscopy (22). cultures were routinely monitored for bacterial contamination. Specimens were prepared from 2-, 4- and 6-day cultures. Drops of culture medium were either placed on slides for darkfield microscopy or prepared for scanning electron microscopy. For colony morphology analysis, cultures on PMR-agar were done on the basis of information presented in a previous study (24). Subcultures from agar to agar were made by loop inoculation and by the "push block" technique used for mycoplasma. Dienes staining technique was used for staining agar colonies (25).

----- NOTE: SEE ORIGINAL FOR REST OF M & M, PHOTOS AND RESULTS -----

DISCUSSION: The role of different atypical bacterial forms, spheroplasts and L-forms in infectious disease is considered an important problem in microbiology. spheroplasts and growth into L-forms occur in many bacterial species in liquid and on various solid media. These forms are products of partial or complete removal of the cell wall by enzymatic digestion (27) and partial or incomplete inhibition of cell wall synthesis (28). The morphology and reproductive processes of bacteria with defective cell walls was studied extensively by numerous investigators (29, 30, 25).

Many factors have been shown to induce the formation of these fragile bacterial forms in vitro: inadequate culture media, alteration of pH, enzymatic activity (lysozymes), antibodies and complement as well as antibiotics. The morphology of bacteria, the mode of multiplication and the structure of colonies may be altered.

An important discovery was that L-forms can be induced in vivo by treatment with penicillins and that microbial variants may persist in vivo and may thereby contribute to the establishment of subclinical or chronic infection. The recovery of spheroplasts, L-forms and other bacterial variants was reported from blood, body fluid and tissues of humans and animals. Dolman et al. (31) described the isolation of the L-form of *Streptobacillus moniliformis* from the blood of one patient with rat bite fever.

The recurrence of endocarditis and the persistence of L-forms of *Corynebacterium* sp. was reported by Wittler et al. (29): the persistent and antibiotic resistant L-form was associated with the latent stage of infection. Furthermore, the chronicity of *Brucella* infection may be due to *Brucella* L-forms (32). Demonstration of atypical forms of *Haemophilus influenzae*,

Listeria sp., Streptococcus sp., Staphylococcus sp., E. coli, Klebsiella sp. in human disease was made by Charache (33).

About atypical forms of Bb and their role in Lyme borreliosis infection there is scarcely any information. As persistence of Bb and recurrence of the disease occur despite adequate antibiotic therapy, in vitro investigations of Bb morphological variants and atypical forms were undertaken by the present authors.

We found Bb forms in four biological variations in cultures:

- 1) cell forms tending to regain formal organism status in first/second subculture and growing in MKP-medium as well as on PMR-agar;
- 2) cell forms reverting to normal helical form of the organism after subculturing in MKP + AB (10 p.c albumin bovine) and growing on PMR-agar;
- 3) cell forms of most organisms reverting to normal (viable) after numerous subcultures in MKP + AB; the colonies may grow to resemble mycoplasma;
- 4) cell forms with no reversion to normal helical cells, no replication, and no growth on PMR-agar.

In older cultures we found mostly type 1 and 2 cells, in patients treated with antibiotics type 2 and 3, and after growth of cells in the presence of penicillin G in vitro we found type 1, 2 and 3. In contaminated patient specimens and in the experimentally contaminated strain PKo incubated with Acinetobacter sp. we found type 3 and 4. Furthermore, the colony morphology on PMR-agar varied. We obtained smooth, rough, rough-diffused and spherical-granular forms.

Very interesting are the membrane blebs which have been observed and described earlier by Barbour and Hayes (34) and Preac Mursic et al. (22). Many years ago, Swain (35) demonstrated the encysted form of pathogenic spirochetes by electron microscopy. Transmission electron microscopic (TEM) studies showed the presence of large bubbles (1.0-1.6 micrometers) and encysted forms of leptospirae and borreliae. Encysted forms were occasionally adherent to the end of the middle of the organism or were separated (Leptospira). A number of investigators later began extensive studies on the ultrastructure of spirochaetes obtained from artificial culture media and directly from pathological lesions (36,37).

In recent studies performed by transmission and scanning electron microscopy, the membrane structure and the presence of extracellular vesicles of Bb were discussed by several investigators (38-40).

Three-dimensional reconstruction of Borrelia cells by serial sectioning and transmission electron microscopy is very difficult, if not impossible; because a high portion of the sections shows tangentially sectioned details (small cell diameter, helical winding); severely limiting reconstruction. Scanning electron microscopic investigations by taking stereo pairs clearly reveal that the cell profiles are still cylindrical. Flattening by squashing of normally growing cells is not observed indicating that freezing of the specimen is a rather gentle and structurally preservative method. Three-dimensional imaging shows that the cells are spindle shaped with maximum diameter of 300 nm. The helical winding may change within one cell from clockwise to counter-clockwise. After destruction of the outer bacterial membrane, bundles of flagellae become clearly visible. The real rate of destruction of Borrelia cells as well as the form and details of blebs can be interpreted only by scanning electron microscopy.

The blebs (cyst) forms induced with penicillin G during growth of Bb in MKP-medium are similar to encysted Spirochaeta duttoni demonstrated by Swain (35). In our "encysted" form we have not seen evidence of borrelia fragments, but we found "whole" or "intact" and "empty" forms as described by Garon et al. (38). We could not directly compare our findings of atypical forms in Bb strains with results of other investigators because different conditions

and organisms were tested. Nevertheless, we speculate that these atypical *Borrelia* forms are spheroplast-L-phase forms as can be found in other bacterial species. It is currently held that conversion to L-form as well as formation of spheroplasts may be a universal property of bacteria (41, 42). Pleomorphism, the presence of elongated forms, the inability of cells to replicate, the induction of exposing Bb to penicillin G, the long period of adaptation to growth in MKP-medium and the mycoplasma-like colonies after growth on PMR-agar suggest that Bb produce spheroplast-L-phase forms (SL-forms).

Penicillin G was the most effective inducer of SL-forms. The reversion of this form to the helical parental form was mostly achieved by cultivation of isolated SL-colonies in penicillin G-free medium. The atypical forms isolated from patients treated with antibiotics show similar features. The same effect is probably obtained with all other beta-lactam antibiotics. Furthermore, atypical forms were also induced in patient specimens contaminated with gram-positive as well as gram-negative bacteria and by simultaneous growth of Bb and bacteria in MKP-medium. Here the nonmotile "rigid" form was more stable, the reversion to the helical parental form was often impossible; the organisms were unable to replicate. A long period of growth adaptation in modified Kelly medium was necessary to obtain an adequate concentration of viable borreliae. This may be the reason for negative culture results in contaminated patient specimens with small numbers of borreliae. Our data suggest that bacterial toxins may also play a role in the overgrowth of Bb in contaminated cultures. The release of toxins and enzymes during growth or after cell lysis of fast growing gram-positive and gram-negative bacteria can induce SL-forms in the slow growing Bb culture. Obviously, the most important question is what the actual role of these SL-forms may be. Some recent studies with bacterial spheroplast-L-forms in vivo suggest a role in pathogenicity and host-parasite interaction.

Very interesting are the studies by Hoyer and King who demonstrated the loss of a portion of the chromosomal DNA in an L-form of *Enterococcus* (43).

The pathogenicity of bacterial SL-forms and their sensitivity to antimicrobial agents have been controversially discussed. However, findings suggest that SL-forms may play a role in the microbial persistence of various chronic infections (31-33).

The role of spheroplast-L-forms in LB has not yet been established because the in vitro and in vivo studies are scarce. Preliminary data about morphological changes of cells in vitro, and isolation of persisters with atypical form from patient specimens after treatment with antibiotics (18, 20, 34) suggest that SL-forms may be involved in LB disease.

The efficacy of induction of SL-forms in our in vitro study varied greatly among Bb strains. In some cultures only one colony of 20 was shown to produce SL-transformation. This transformation probably reflects phenotypic and genetic differences among different isolates.

The Bb persisters isolated from patient specimens and two induced SL-forms tested showed no differences in the antibiotic sensitivity pattern. The biological functions of the membrane vesicles remain unclear. The demonstration of DNA in blebs by Garon et al. (38, 41) has led to the hypothesis that these structures may play a role in the protection and transfer of genetic markers. Shoberg et al. (40) proposed that Bb vesicles may provide an important tool for elucidation of borreliae adhesion antigens or structures. Radolf et al. (39) support the hypothesis of Garon and co-workers. Very interesting are the results concerning spheroplasts demonstrated by Bruck and co-workers and Kersten et al. (44, 45). We support their hypothesis that with the outer membrane damage and flagellar release, spheroplasts may provide a model that mimics cells under attack by the host's immune system during infection. In conclusion, the findings about atypical forms allow us to speculate about the formation of spheroplast-L-forms in Bb.

Further in vitro and in vivo studies with respect to Bb protoplasts, spheroplast-L-forms, biochemical and genetic composition, and the role in LB disease are necessary. Of particular importance are their persistence and significance for immune response and treatment of the disease. It would be important to find different antibiotic groups or combinations in respect to spheroplast-L-form production.

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