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Effects of Penicillin, Ceftriaxone, and Doxycycline on Morphology of *Borrelia burgdorferi*

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Antibiotic therapy with penicillin, doxycycline, and ceftriaxone has proven to be effective for the treatment of Lyme borreliosis. In some patients, however, it was noticed that borreliae can survive in the tissues in spite of seemingly adequate therapy. For a better understanding of this phenomenon, we investigated the different modes of degeneration of *Borrelia burgdorferi* suspensions during a 96-h exposure to various antibiotics. By dark-field microscopy and ultrastructural investigations, increasing blebbing and the gradual formation of granular and cystic structures could be followed during the exposure time. Although antibiotic concentrations at the MIC at which 90% of organisms are inhibited after 72 h were 80% or even greater, motile organisms were still present after incubation with penicillin and doxycycline but not after incubation with ceftriaxone. By transmission electron microscopy, intact spirochetal parts, mostly situated in cysts, were seen up to 96 h after exposure with all three antibiotics tested. According to experiences from studies with other spirochetes it is suggested that encysted borreliae, granules, and the remaining blebs might be responsible for the ongoing antigenic stimulus leading to complaints of chronic Lyme borreliosis.

Borrelia burgdorferi, the pathogenic agent of Lyme borreliosis, has been recognized as a bacterium that is susceptible to antibiotics. The long-term persistence of these bacteria in tissues, despite adequate treatment of infected patients, has been indicated to be responsible for late complications and a chronic course of disease (17, 26, 28). The withdrawal of borreliae, or parts of them, into privileged or secluded sites, where they are further inaccessible to antibiotics, raises the question of whether antibiotics themselves can be made responsible for transforming the organism into a persistent, viable, or nonviable but antigenically potent form.

Immobilization of bacteria has been seen as a result of incubation with antibiotics in vitro (21). By light microscopy, Preac-Mursic et al. (29) observed blebs, spherical structures, and granules in *B. burgdorferi* cultures during incubation with antibiotics. In ultrastrucutral studies the action of penicillin on cultures of *Borrelia hermsii* has been investigated (3).

Penicillin, doxycycline, and ceftriaxone are the most preferred antibiotics for treating Lyme borreliosis. Although certain differences in the actions of these antimicrobial agents have been evaluated, they seem to be equally effective (34).

Culture experiments were carried out to investigate the actions of these antibiotics on the motility, morphology, and survival of *B. burgdorferi* during exposure to antibiotic solutions. The course and mode of degeneration of these spirochetes were recorded on a videomicroscope and were photographed with an electron microscope.

MATERIALS AND METHODS

Culture experiments. Three strains of *B. burgdorferi* sensu lato, B31 (ATCC 35219) and two isolates from erythema migrans lesions on the skin of patients in Vienna, isolates high-passage H1 and low-passage H8, were used for the experiments. Isolate H1 was identified as *Borrelia afzelii*, and isolate H8 was identified as *Borrelia garinii* by Aurodye staining after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose by using 22 different OspC-specific monoclonal antibodies (20). The heterogeneity of OspC was further studied by analyzing the restriction fragment length polymorphisms among PCR-amplified OspC genes (20). The strains were cultured in BSK medium at 34°C over a period of 4 days (30).

Tubes of media containing the three drugs at 0.1-, 0.5-, 1.0-, and 2-fold the MICs at which 90% of strains are inhibited (MIC_{90}s) were used. Penicillin G (MIC_{90}, 4.0 μg/ml; Biochemie, Vienna, Austria), ceftriaxone (MIC_{90}, 0.06 μg/ml; Hoffmann-La Roche, Basel, Switzerland), and doxycycline (MIC_{90}, 2.0 μg/ml; Pfizer, New York, N.Y.) (27) were inoculated with 4-day-old cultures of *B. burgdorferi* to a final cell density of 10^6/ml, as determined in a Petroff-Hausser counting chamber, and were sealed with plastic caps. They were kept at 34°C for 96 h. Before examination they were gently mixed on a vortex mixer.

To avoid contamination during the observation period the tubes were opened only once for investigations and were then discarded.

For control purposes, *B. burgdorferi* cultures were also transferred to medium alone. *B. burgdorferi* cultures subjected to antibiotics and nonexposed cultures were transferred to BSK medium after 96 h for subculture.

Dark-field microscopy. Samples of *B. burgdorferi* B31, H1, and H8 in antibiotic solutions at each antibiotic concentration were transferred to glass slides. The movements and structures of the borreliae were examined by dark-field microscopy in a Leitz Diaplan microscope connected to a Philips monitor.

Preparation for electron microscopy. Electron microscopy was performed with *B. burgdorferi* B31 cultures exposed to the antibiotics at the MIC_{90}s for 24 and 96 h. Samples were centrifuged at 8,000 × *g* for 20 min at 4°C. Negative-contrast staining was done by placing 300 copper mesh grids (Balzers Union, Balzers, Fürstentum Liechtenstein) on the suspensions for 5 min (23). Fixation was performed with 2.5% glutaraldehyde for 15 min; this was followed by washing steps with distilled water and counterstaining with 1% aqueous uranyl acetate dihydrate for 30 s. For transmission electron microscopy (TEM), the pellets were fixed with 2% paraformaldehyde–2% glutaraldehyde dissolved in 0.1 M cacodylate buffer–0.1 mM $CaCl_2$ (pH 7.4) for 1 h at 4°C, rinsed, and incubated with 1% OsO_4 in 0.1 M buffer at room temperature for 1 h. After the pellets had again been rinsed in buffer and distilled water, they were incubated with dimethoxypropan (Merck, Darmstadt, Germany) and one droplet of concentrated sodium chloride per 10 ml twice for 10 min each time. These pellets were embedded in Spurr resin (Serva, Heidelberg, Germany) for 1 h at room temperature and were allowed to polymerize for 12 h at 70°C. The preparations were cut into ultrathin sections (Ultracut; Reichert), applied on 200 copper mesh grids, counterstained with 1% uranyl acetate dihydrate in methanol, and examined with an electron microscope (Jeol EM 100 S).

SDS-PAGE analysis. The proteins of *B. burgdorferi* B31 cultures were characterized by SDS-PAGE and were compared with borrelia cultures after 24 and 96 h of exposure to penicillin G, ceftriaxone, and doxycycline at the MIC_{90}s. After being washed twice in phosphate-buffered saline solution with 5 mM $MgCl_2$, spirochetes were boiled for 5 min and were tested on a vertical gel (Miniprotean II; Bio-Rad). Gels were stained with Coomassie brillant blue R 350, and the major proteins were compared with molecular weight standards (Bethesda Re-

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search Laboratories, Inc., Gaithersburg, Md.). Quantification of the proteins was achieved by computerized analysis.

Determination of the stabilities of the antibiotics. To investigate the stabilities of the antibiotics under culture conditions, samples were prepared and incubated in the same way as those for morphological investigations. Time points for sampling were at 0, 12, 24, 48, and 72 h. The samples were taken in duplicate for penicillin G, doxycycline, and ceftriaxone. The samples were stored at −20°C until processing. Quantitative analysis was performed by high-pressure liquid chromatography (HPLC) method. Aliquots of 0.3 ml of *B. burgdorferi* B31 suspensions in BSK II medium were taken after vortexing. Protein precipitation was performed by adding 0.3 ml of acetonitrile and subsequent vortexing and centrifugation.

For determination of the penicillin G concentration in *B. burgdorferi* B31 suspensions, 10 μl of the clear supernatant was loaded onto the HPLC column (an HP 1090 M chromatograph [Hewlett-Packard] with a variable UV detector 3100[LDC]; wavelength, 210 nm; column, nucleosil 120 3 C_{18} [80 by 4 mm, inner diameter]; mobile phase, 25% acetonitrile with 75% [vol/vol] 0.02 M phosphoric acid; retention time, approximately 3.1 min). Validation of the method described above gave linearity in the range of 0 to 0.6 μg/ml of suspension. The recovery rate was 91 to 100%, with a coefficient of variation of 0.8 to 4.8%; accuracy was between −2.2 and +2.9% for the range tested.

For doxycycline concentration determinations, 200 μl of a 1:3 dilution with water of the clear supernatant described above was loaded onto the column (nucleosil 100 5CN [125 by 4 mm, inner diameter]; wavelength, 350 nm; mobile phase, 10% acetonitrile and 10% tetrahydrofuran in 80% [vol/vol] phosphate-citrate buffer; retention time, approximately 2.1 min). The linearity of the method was in the range of 0 to 4.1 μg/ml of suspension, with a recording rate of between 79 and 89% (coefficient of variation, 1.3 to 3.9%), the accuracy for the range tested was 0.8 to 14.5%.

Because of the very low MIC_{90}s of ceftriaxone and interference with the culture medium, no reliable analysis method could be developed for this antibiotic.

RESULTS

Dark-field microscopy. There were no obvious morphological differences between the three strains analyzed, strains B31, H1, and H8. The degree of alteration was strongly dose and time dependent. After exposure to penicillin a few individual motile *B. burgdorferi* organisms could be detected at any time of the 4-day observation period. The morphological alterations developed gradually; initially, after 17 h of incubation, granules of up to 0.8 μm adhering to the end and/or middle regions of the spirochetes developed in cultures incubated with concentrations at the MIC_{90} or greater. Their numbers increased with the time of incubation, and they formed paired as well as multiple granules after 24 h of incubation. In incubations with concentrations less than the MIC_{90}, these granules arose about 12 h later and at a lower percentage. After 48 h of incubation with 1.0 or 2.0 times the MIC_{90}, these granules were transformed into up to 1.8-μm vesicle-like structures. During the development of granules, the motility of the spirochetes appeared to be accelerated, reminiscent of "cramping" or "tetania," resulting in the aggregation of single or multiple organisms to amorphous clusters with residual slow motility. Formation of small colonies undergoing degeneration was observed after 48 to 72 h of incubation. The motilities of altered organisms then consecutively decreased. Singule borrelia or immotile borrelia associated with 0.8-μm spherical bodies or 2- to 3-μm short rods, both with prominent self propelling, appeared in the medium after 72 h. After 4 days of incubation with antibiotics at concentrations greater than the MIC_{90}s, immotile aggregates or immotile granula- or vesicle-bearing organisms were predominantly present. At less than the MIC_{90}s higher numbers of motile and morphologically intact spirochetes were present.

The alterations in the *B. burgdorferi* organisms incubated with ceftriaxone were identical to those in organisms incubated with penicillin. However, the onset of the alterations was al-

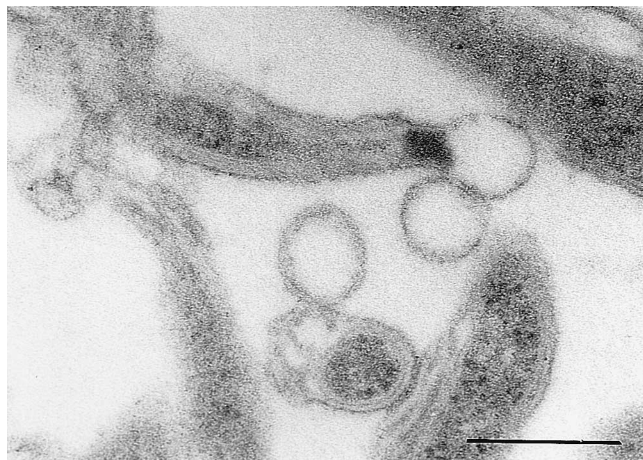

FIG. 1. Two membrane blebs arise at the end of a borrelia organism induced by penicillin after 24 h of incubation, as shown by TEM. Bar, 0.2 μm.

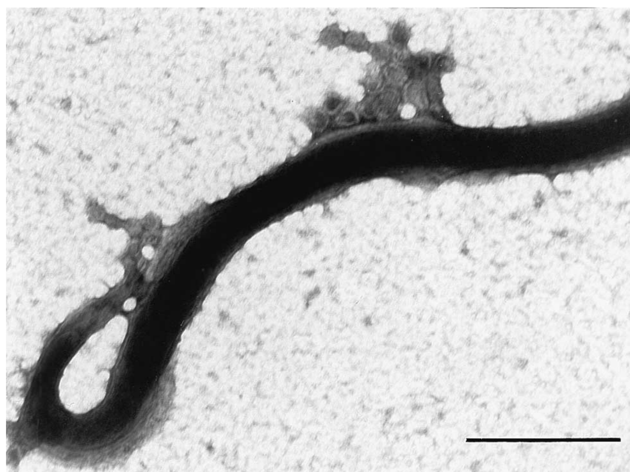

FIG. 2. Shedding of membrane blebs is the initial morphological characteristic of membrane alteration. Blebs tend to aggregate to pearls or tubules, as shown by TEM after negative-contrast staining. Bar, 0.1 μm.

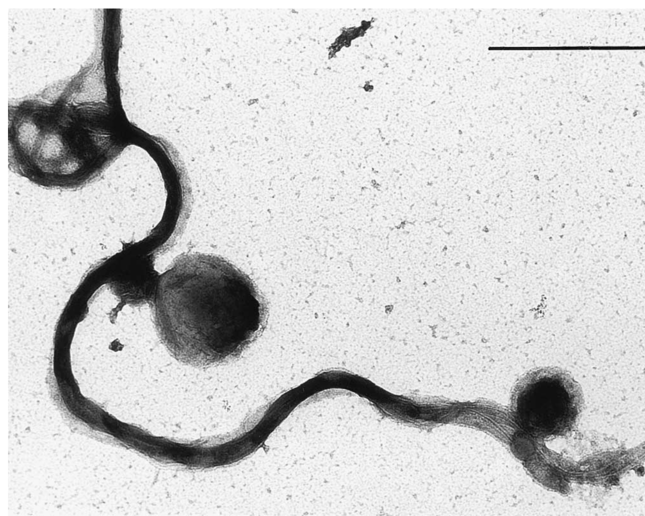

FIG. 3. Several granules along borreliae after 24 h of incubation with ceftriaxone at the middle and end regions, as shown by TEM following negative-contrast staining. Bar, 1.4 μm.

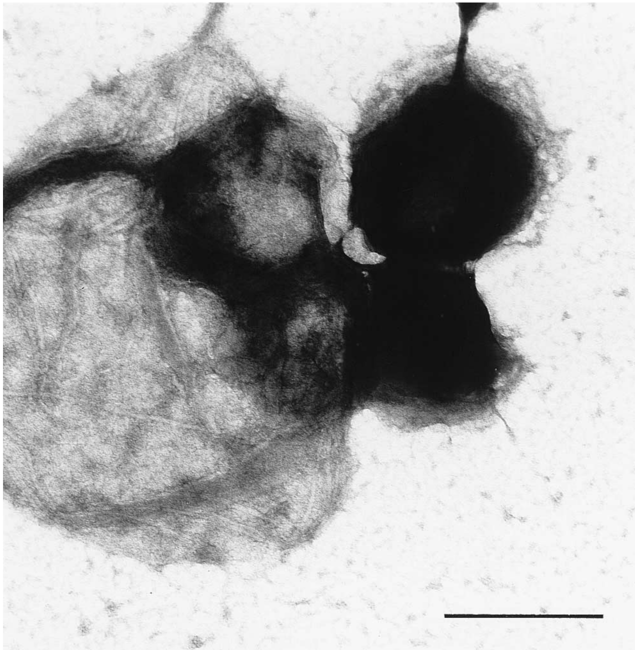

FIG. 4. Formation of aggregated granules and vesicles of different sizes (0.4 to 1.4 μm) on borreliae after exposure to penicillin for 96 h is seen. The large vesicle contains loose loops of flagellae and a convoluted spirochete, as shown by TEM following negative-contrast staining. Bar, 0.6 μm.

ready observed after 8 h of incubation. The number of viable bacteria could not be evaluated in penicillin- and ceftriaxone-containing media because of the aggregation of motile organisms and the formation of microcolonies. After 48 h no motile borreliae were present even in the presence of concentrations as low as 1/10 the MIC_{90}, but self-propelled rods or granules were evident, and these were sometimes associated with dead borrelia.

In contrast, doxycycline-treated cultures revealed single organisms with gradually decreasing motilities after 18 h of incubation at concentrations greater than MIC_{90}; after 24 h there was a loss of motility without marked morphological alterations. After 4 days of incubation 90% of the bacteria were immotile. In cultures grown in the presence of concentrations

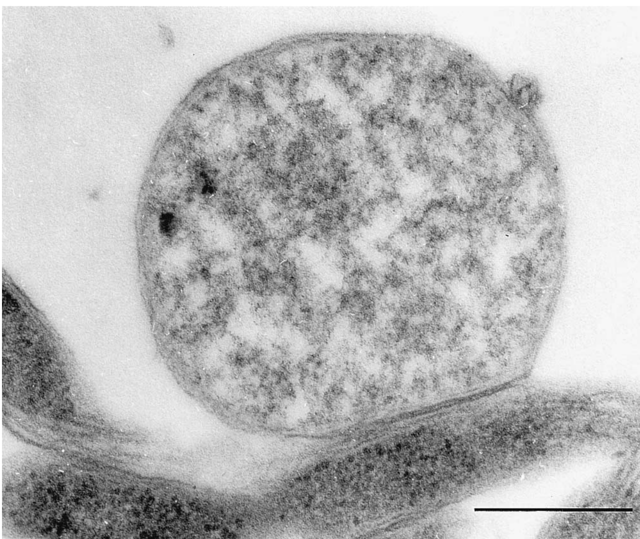

FIG. 5. *B. burgdorferi* exposed to peniciilin for 24 h. A vesicle is shown by TEM to adhere to the outer surface of a spirochete. The outer membrane encloses amorphous material. Bar, 0.4 μm.

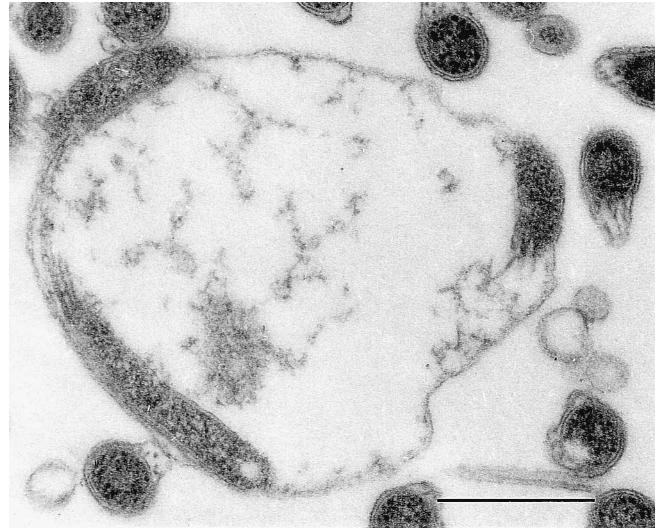

FIG. 6. Formation of vesicles by ceftriaxone after 24 h. The vesicle contains disarranged flagellae and residual protoplasmic cylinder, as shown by TEM. Bar, 0.6 μm.

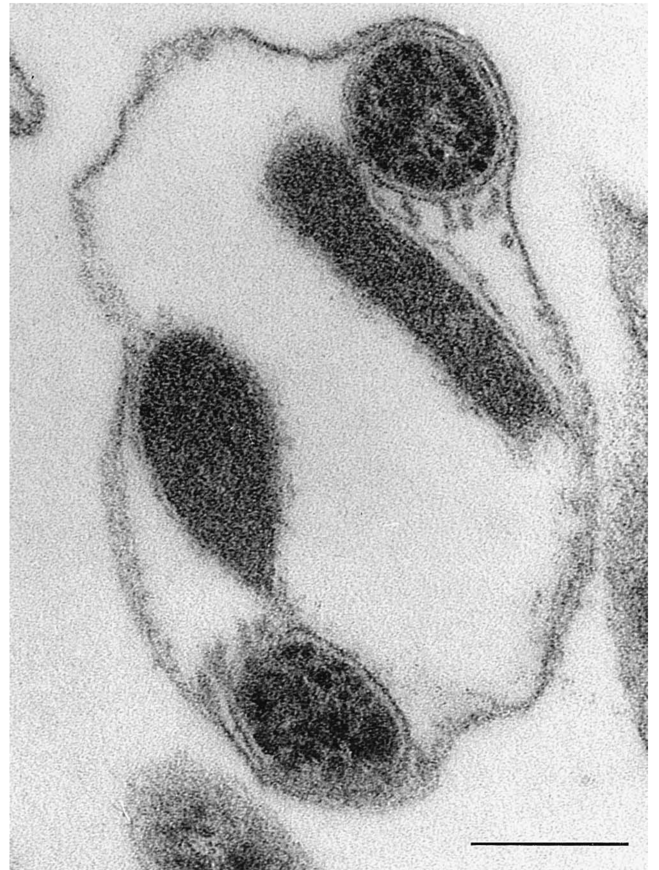

FIG. 7. Cross-sectioned spherical body revealing intracellular localized, coiled spirochetal parts. Typical unfolding of the two central cross-sectioned cytoplasmic cylinders is shown by TEM. Bar, 0.2 μm.

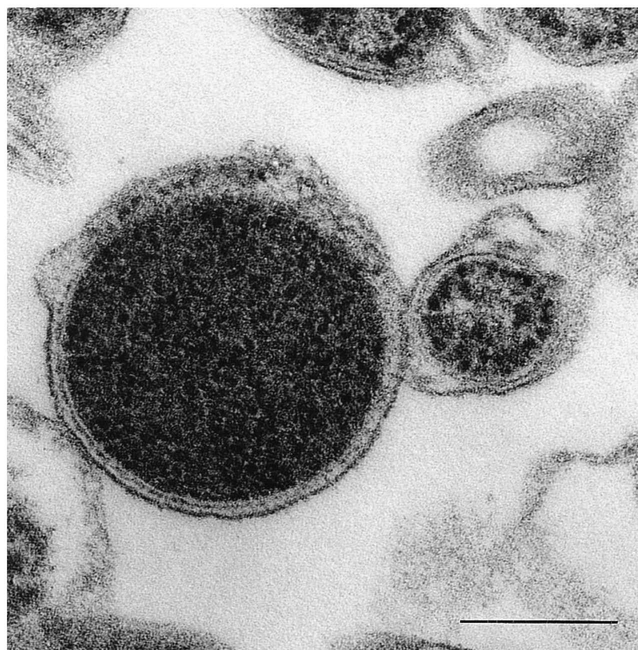

FIG. 8. Spherical body developing after 96 h of incubation in cultures exposed to doxycycline, as shown by TEM. Electron-dense material is enclosed by an outer membrane identical to the spirochetal membrane. Bar, 0.2 μm.

less than the MIC_{90}, the proportion of motile spirochetes was 25%. Morphological alterations similar to those induced by penicillin or ceftriaxone developed only occasionally after 4 days of incubation. Spherical bodies of 0.8 to 1.4 μm were found free in the culture after 18 h of incubation in all culture experiments.

Subcultures from all investigated samples containing antibiotics carried out on day 4 and examined by dark-field microscopy once weekly for up to 2 weeks only revealed small granules and self-moving rods but no borrelia organisms with regular morphologies.

Controls. During the period of incubation, the organisms in untreated cultures remained morphologically unaltered and showed regular motility. In subcultures intact borreliae were seen.

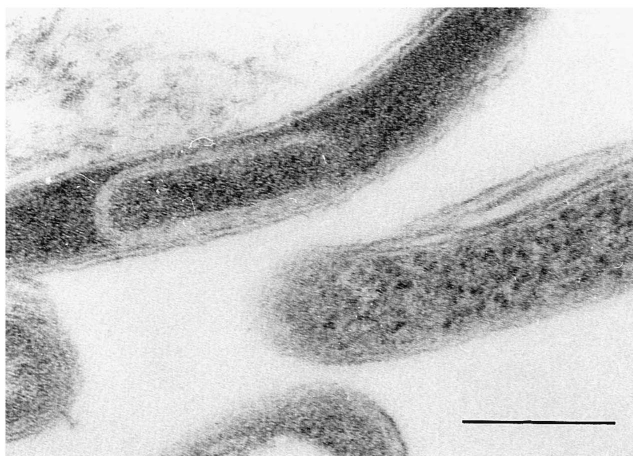

FIG. 10. Bazilla-like mesosome shown by TEM in doxycycline-incubated borrelia culture suspensions after 4 days. Bar, 0.25 μm.

Electron microscopy. In organisms in all culture preparations exposed to penicillin and ceftriaxone, increased blebbing was obvious after 24 h of incubation (Fig. 1). Membrane blebs of 0.05 to 0.2 μm produced by the outer envelope appeared to be either adherent to the outer surface of the spirochete or shed off into the culture medium. Negative staining illustrated their tendency for self-aggregation, forming pearls or even tubules (Fig. 2). The underlying spirochetal surface was not altered. Negative staining revealed granules and larger, vesicle-like structures (gemmae) ranging in size from 0.5 to 1.8 μm (Fig. 3 and 4). As shown by TEM, gemmae were surrounded by an outer membrane enclosing either amorphous material (Fig. 5) or residual, coiled, intact, or unfolded protoplasmic cylinders and disarranged flagellae (Fig. 4, 6, and 7). In addition to the structures described above, 0.8-μm solitary spherical bodies composed of an outer membrane and electron-dense internal material could be shown after 24 to 96 h. By TEM, a ribosomal pattern of the cytoplasmic cylinder suggested vitality (Fig. 8). The majority of spherical bodies, however, showed lysis after 96 h; the whole intracellular contents were released

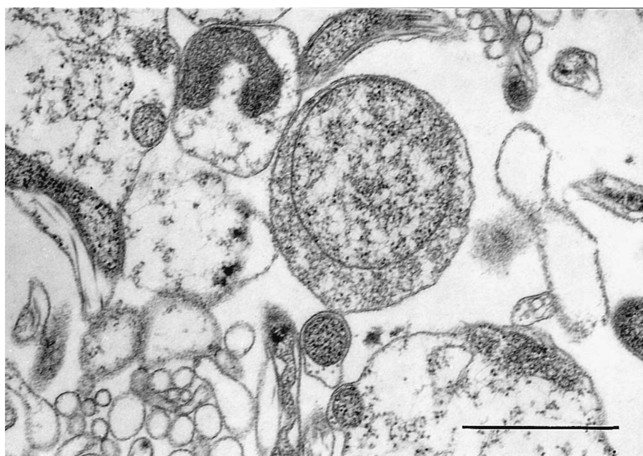

FIG. 9. Ultrathin section of *B. burgdorferi* incubated with penicillin for 4 days, as shown by TEM. Lysis of cells with residual membranes and empty protoplasmic cylinders and numerous membrane blebs are shown. Bar, 0.8 μm.

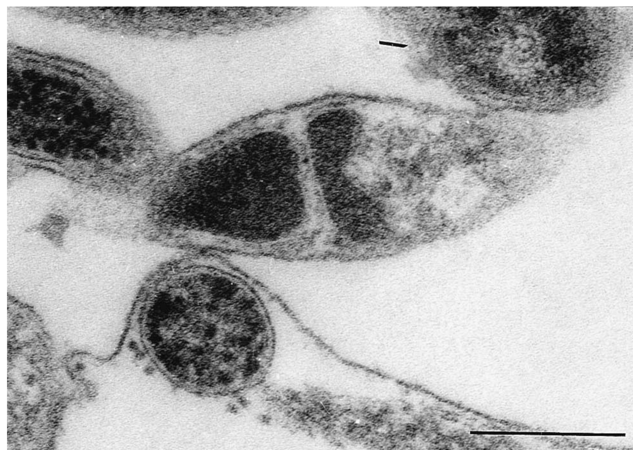

FIG. 11. Two cytoplasmic cylinders surrounded by a single outer membrane arising after 96 h of incubation with doxycycline, as shown by TEM. Bar, 0.3 μm.

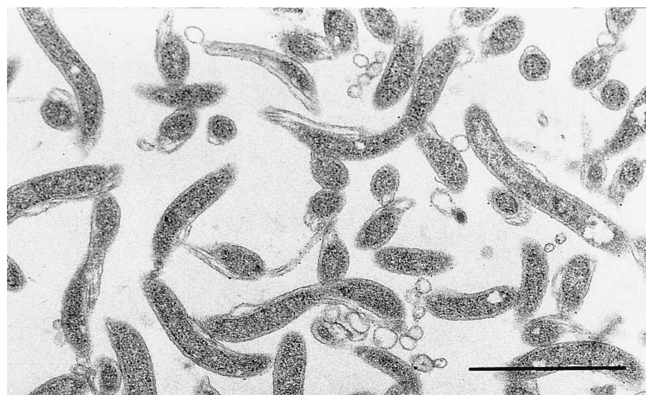

FIG. 12. Ultrathin section of *B. burgdorferi* in control experiments after 96 h examined by TEM. Intact borreliae with regularly arranged flagellae, axial filaments, and membranes are shown. Bar, 1.4 μm.

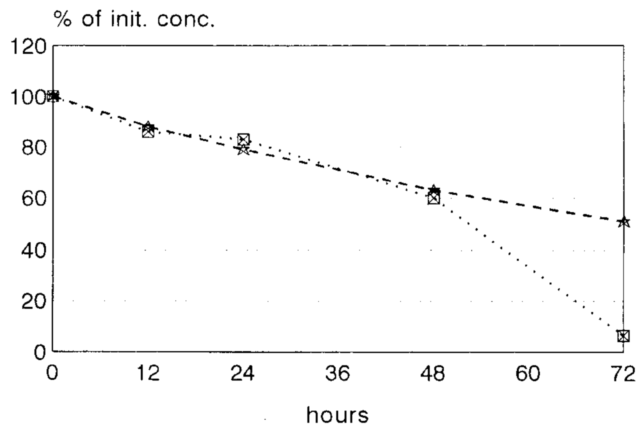

FIG. 14. Concentrations of penicillin G during incubation in BSKII medium with a *B. burgdorferi* B31 suspension for 72 h. ⊠, MIC_{90}; ☆, two times the MIC_{90}.

into the medium, leaving empty protoplasmic cylinders or cell membranes (Fig. 9).

Similar to penicillin and ceftriaxone, membrane blebbing was observed after exposure to doxycycline, but granules or gemmae developed only occasionally after 3 to 4 days of incubation. Multiple mesosome-like structures were apparent after 24 h of incubation. These ovoid bodies, 0.07 to 0.35 μm in size, to bazilla-like bodies up to 0.4 μm in length, were located in the center of the protoplasmic cylinder, being partially surrounded by a double-layered cytoplasmic membrane (Fig. 10). After 96 h of incubation transverse fission of the cytoplasmic cylinder was also seen, but these parts were partly undergoing degeneration (Fig. 11).

Controls. Besides membrane blebs that were frequently seen at any site of the organism in control cultures, no ultrastructural alterations were observed after 96 h (Fig. 12).

SDS-electrophoresis. Exposure of *B. burgdorferi* B31 to antibiotics for 24 or 96 h revealed polypeptide patterns identical to those of organisms in control cultures (Fig. 13).

Analysis of antibiotic concentrations. Penicillin concentrations (two times the MIC_{90} and the MIC_{90}) decreased by about 20% during the first 24 h (Fig. 14). At 48 h more than 60% of the initial drug levels were detectable at all concentrations tested. The remaining penicillin concentrations after 72 h were 50% of the initial concentration at two times the MIC_{90} and 20% of the initial concentration at the MIC_{90}.

The investigations with concentrations less than the MIC_{90} presented high variabilities; interference with the culture medium may have been the cause (data not shown). Doxycycline was rather stable under the culture conditions described here. After 48 h of incubation drug concentrations were more than 80% of the baseline levels. At 72 h drug concentrations of between 64 and 76% of the initial values could be detected (Fig. 15).

The biochemical analyses of ceftriaxone could not be evaluated because their low concentrations ranged between 0.006 and 0.12 μg/ml.

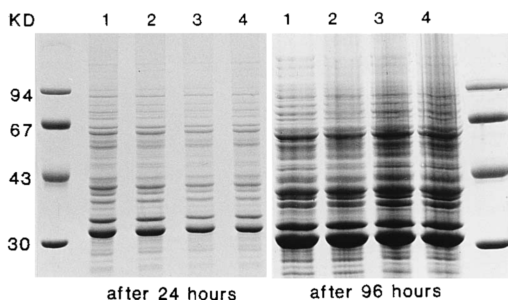

FIG. 13. SDS-PAGE showed identical polypeptide distributions after 24 or 96 h of exposure of *B. burgdorferi* B31 solutions with antibiotics. Lanes: 1, penicillin; 2, ceftriaxone; 3, doxycyline; 4, *B. burgdorferi* suspension without antibiotics.

DISCUSSION

Although the spectrum of antibiotics capable of eradicating *B. burgdorferi* in vitro has been enlarged, treatment failures continue to be a problem for clinicians in the management of patients with chronic Lyme borreliosis. Beta-lactam antibiotics such as penicillin and ceftriaxone are recommended for therapy and act on the cell wall by inhibiting the assembly of insoluble peptidoglycans, leading to bacteriostasis (22). Studies in group A streptococci have shown that penicillin also induces a specific loss of total cellular RNA in the absence of hydrolysis of the cell wall (22). Besides the loss of integrity of the outer cytoplasmic cell membrane, this latter action of antibiotics can also be suspected in *B. burgdorferi* since several penicillin-binding proteins have been identified (33) in association with

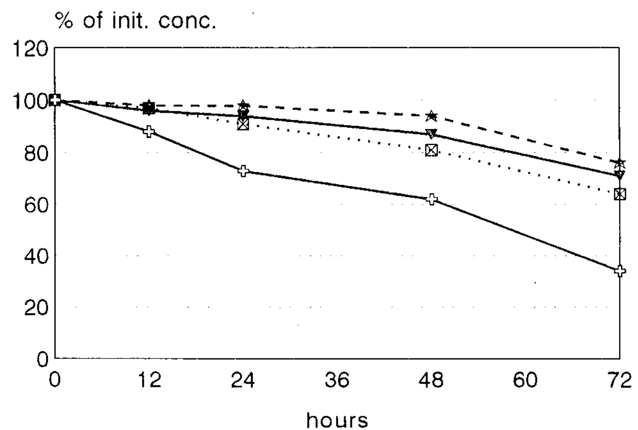

FIG. 15. Concentrations of doxycyline during incubation in BSKII medium with a *B. burgdorferi* B31 suspension for 72 h. ♧, 0.1 time the MIC_{90}; ⊠, 0.5 time the MIC_{90}; ☆, MIC_{90}; ▼, two times the MIC_{90}.

the insoluble cytoplasmic cylinder, as shown after Triton X-114 pretreatment (5).

The primary alterations of borrelia cultures exposed to penicillin and ceftriaxone started with the overproduction of the surface membrane. Although membrane blebbing has been interpreted as an initial characteristic of degenerating bacteria, as tested in *Escherichia coli*, the release of altered membrane segments as a protective mechanism has not been excluded (11). The proliferation of the outer surface membrane also resulted in pronounced stretching and budding of the outer envelope, giving rise to vesicles and spherical gemma bodies that formed in association with the borreliae or, finally, that were shed off into the culture medium (4, 10). In some spherical bodies or gemmae, convoluted cytoplasmic cylinders with a loss of typical spirochetal coiling were evident. Spirochetes in these were partly unfolded and lacked the typical ribosomal pattern of the normal protoplasmic cylinder. Spirochetes which had lost their ribosomes tended to degenerate. Unfolding is due to a defect of the peptidoglycan layer induced by antibiotics. This results in the loss of one layer of the cytoplasmic membrane (14, 16). From our studies it could not be determined whether the cystic structures seen after 96 h of incubation with antibiotics were able to survive. Gemmae of *Treponema pallidum* can, for example, be seen intracellularly in the macrophages of rabbits with experimental syphilis half a year after penicillin therapy as well as in human chancre tissue (19, 25). Spirochetal gemmae are known to occur in tick midguts, arising on solid culture medium (4, 18), and also develop under stress conditions like penicillin treatment (2, 3, 6, 7, 25). Also under experimental conditions, such as by the action of emzymes like lysozyme, the formation of spirochetal gemmae has been observed in vitro (12).

Only a 40% reduction in the concentration of penicillin used at the MIC_{90} was seen in our experiments after 48 h of incubation, and after 72 h the concentration in the borrelia suspension with penicillin at two times the MIC_{90} was reduced by only 50%. Whereas most gemmae structures showed degeneration after 96 h, motile borreliae, self-moving rods, and granules with intact cytoplasms were still present. Subculture of these suspensions, however, was not successful. It was interesting that in *B. burgdorferi* cultures containing penicillin or doxycycline at 0.1 time the MIC_{90} high numbers of intact borreliae were present. This situation must be considered when low doses of antibiotics are prescribed or when an irregular antibiotic treatment is performed by the patient.

Tetracyclines act on the bacterial ribosome by inhibiting bacterial protein synthesis (31). The changes in the borreliae after incubation with doxycycline were completely different. Primary immobilization was seen, along with the development of multiple ovoid structures (mesosomes) whose function has not yet been clarified. They have been described as ovoid condensations of the protoplasmic cylinder and were shown by Ovcinnikov and Deletorskij (24), Holt (12), and Hovind-Hougen (13) to differ in their structures and functions, but they could not yet be classified further. They were seen to communicate with the exterior and were interconnected by long channels. In our investigations they were either ovoid or bazillary. They were separated from the surrounding protoplasmic cylinder by a double-layered membrane and represented an internal part of the cytoplasmic cylinder separated by a small cleft. This gave the impression that a new borrelia developed and separated in the protoplasmic cylinder after 1 day. After 4 days two protoplasmic cylinders were occasionally seen surrounded by a single outer membrane. Mesosomes or spores have been observed in *T. pallidum* and were shown to be associated with those parts of the spirochete undergoing transverse fission (12, 13). On the other hand, coccoid, vibrio-like, or even bazillary bodies have been identified in the blood of patients with recurrent fever at the "crisis" and in tick eggs after infection with *Borrelia duttoni* (8). These bodies can cause spirochetemia and death after inoculation into laboratory mice (8).

In the present study it could not be evaluated whether the immotile *B. burgdorferi* organisms are only paralyzed after exposure to doxycyline, similar to *T. pallidum* in immobilization tests (15), or whether they are killed. Culture constituents other than BSK medium, tissue elements, or cells might be responsible for keeping borrelia parts viable and capable of division. Because motility was demonstrated to be one important factor for the invasion of tissue, such as the endothelium (32), doxycycline possibly prevents the intracellular invasion of spirochetes or active dissemination.

It has been indicated that persistent symptoms of Lyme borreliosis are due to residual spirochetal structures like vesicles or membrane blebs (9). In previous studies identical granules and vesicles corresponding to gemmae were detected in skin biopsy specimens of erythema migrans and acrodermatitis chronica atrophicans lesions by immunohistochemical methods with a flagellar antibody (1). The effect of extensive blebbing on the human organism is still unknown. In studies by Garon and colleagues (9, 35), membrane blebs have not only been seen to contain *B. burgdorferi* plasmids but could also be made responsible for an intense B-cell mitogenesis when they were incubated with peripheral blood mononuclear cells.

The exposure of borrelia cultures to antibiotics in vitro for 96 h cannot be compared with the actions of antibiotics given for 2 or more weeks in vivo. Morphologically intact borrelia parts seen after 4 days of incubation with antibiotics, however, may also persist in humans during antibiotic treatment.

Because the pathogenesis of *B. burgdorferi* infections is not completely elucidated, degenerative products, granules, and encysted *B. burgdorferi* should be investigated further in view of their long-term persistence in infected tissues and their contribution to the pathogenesis of Lyme borreliosis.

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