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Bacteriophages and Ultrastructural Alterations of *Borrelia Burgdorferi* Induced by Ciprofloxacin

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In a former study, a lysogenic isolate of *Borrelia burgdorferi* harboring two different types of tailed A-1 and B-1 bacteriophages inducible by subinhibitory ciprofloxacin concentrations was described. In the present study, two further *Borrelia burgdorferi* isolates obtained by culture from a nymphal *Ixodes ricinus* tick and from human skin were exposed to increasing concentrations (0.125 to 8 μg/mL) of ciprofloxacin.
The *in vitro* minimal inhibitory concentration (MIC) was determined to be 1 μg/mL by a broth dilution method. In both isolates, belonging to the genospecies *Borrelia burgdorferi, sensu stricto* A-1 bacteriophages were discovered exclusively at subinhibitory concentrations of ciprofloxacin (0.125 to 0.5 μg/mL). After exposure of the isolates to ciprofloxacin concentrations coinciding with or exceeding the MIC, the following alterations of the borrelial ultrastructure became visible: (1) at a ciprofloxacin concentration of 1 μg/mL electron-lucent swollen areas within the protoplasmic cylinder complex of otherwise intact cells as well as very short borrelial cell fragments, (2) at a ciprofloxacin concentration of 2 μg/mL numerous small-membrane defects of the peptidoglycan layer, (3) at ciprofloxacin concentrations of 4 and 8 μg/mL disruption of the protoplasmic cylinder complex into many small particles. These ultrastructural alterations caused by high ciprofloxacin concentrations proved to be clearly different from the features of phage-induced cell lysis found at subinhibitory ciprofloxacin concentrations.

Key words: Borrelia burgdorferi, Ciprofloxacin, Ultrastructure, Bacteriophages, Electronmicroscopy

INTRODUCTION

Recently, we reported on the discovery of two different types of bacteriophages induced by subinhibitory concentrations of ciprofloxacin in a *Borrelia burgdorferi* skin isolate and described the typical phage-induced alterations of the borrelial morphology (1).

Ciprofloxacin is a fluorinated, piperazin-substituted quinolone related to nalidixic acid. By inhibiting the bacterial DNA-gyrase, this drug has a high *in vitro* activity against many gram-positive and gram-negative bacteria (2). In several reports, the ultrastructural alterations of ciprofloxacin treated gram-negative and gram-positive bacteria were described comprehensively (2–4). To our knowledge, there are only two studies dealing with the *in vitro* susceptibility of *Borrelia burgdorferi* to ciprofloxacin. Preac-Mursic et al. reported in 1987 that ciprofloxacin showed only low activity against *Borrelia burgdorferi* (5). Similar results were reported by Levin et al. (6) in 1993. So far, we are not aware of reports concerning ultrastructural alterations of spirochetes caused by ciprofloxacin.

In the present study, we determined the *in vitro* minimum inhibitory concentration (MIC) of ciprofloxacin for two *Borrelia burgdorferi* isolates and examined the morphological alterations of the borrelial cells after treatment with ciprofloxacin concentrations ranging from the MIC of 1 to 8 μg/mL.

Moreover, we examined borrelial cells exposed to subinhibitory concentrations of ciprofloxacin in order to look for the presence of further lysogenic isolates.

MATERIALS AND METHODS

Borrelia burgdorferi isolates

The *Borrelia burgdorferi* skin isolate was obtained by biopsy from an erythema migrans lesion located at the left mamma of a 63-year-old woman. The tick isolate was cultivated from a nymphal tick removed from a patient visiting our out-patient clinic.

Isolation and subcultivation of the borreliae were accomplished in BSK II-medium (7) modified by adding 0.15% agarose (Serva, Fine Biochemicals Inc., Paramus, New Jersey, No. 11397) (8). The two isolates were classified by nondenaturing polyacrylamide gel electrophoresis of RNA complementary to amplified *Borrelia burgdorferi*-specific gene segments (9, 10). Both isolates were found to belong to the genospecies *Borrelia burgdorferi* sensu stricto, according to the *Borrelia burgdorferi* subspecies classification delineated by Baranton et al. (11).

Evaluation of MICs of ciprofloxacin

In vitro susceptibility to ciprofloxacin (Bayer, Leverkusen, No. 521532) was determined via the broth dilution method (5). Here, 100 μL of an actively growing culture (log-phase) containing 10^7 cells/mL were added to tubes with 9.9 mL BSK II-medium, resulting in a final concentration of 10^5 cells/mL. Ciprofloxacin concentrations ranged from 0.125 to 8 μg/mL. Control tubes without antibiotics were inoculated with 100 μL of the log-phase culture. Each concentration was prepared in triplicate. Cultures were examined for the presence of spirochetes by dark-field microscopy after 5 days of incubation at 33°C. The MIC was defined as the lowest concentration of ciprofloxacin completely inhibiting growth, i.e., at which the spirochete count was 10^5 cells/mL or less.

The number of spirochetes was determined by using a Petroff Hauser counting chamber.

Preparation for electron microscopy

Each tube was centrifuged at 4000 × *g* for 20 minutes at 33°C. The resulting pellets were suspended in SMC [0.03% sucrose in redestilled water with 0.01 M $CaCl_2$ and 0.01 M $MgCl_2$ added (12)]. Two drops of each suspension were placed on grids for electron microscopy. In some experiments, the samples were negatively stained with 2% phosphotungstic acid for 30 seconds. In other experiments, the samples were first fixed with 1.5% glutaraldehyde (pH 7.2, in 0.1 M PO_4-buffer) and then negatively stained with 1% phosphotungstic acid for 30 seconds.

We decided to examine fixed and unfixed borrelial cells of each ciprofloxacin concentration, as the specific ciprofloxacin-induced cell alterations were better visible in the fixed samples; the bacteriophages, however, were better visible in the unfixed samples.

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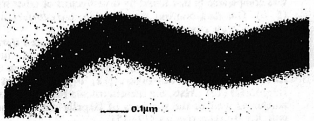

FIG. 1. Untreated Borrelia burgdorferi *with a (a) smooth-structured outer cell membrane without blebs and (b) unchanged peptidoglycan layer. Phosphotungstate stain,* × *60,000, fixed. Bar = 0.1 µm.*

FIG. 2. (a) Unassembled tails and (b) heads of A-1 bacteriophages inside of a Borrelia burgdorferi *cell. Phosphotungstate stain,* × *330,000, unfixed. Bar = 30 nm.*

RESULTS

Ciprofloxacin susceptibility

The mean MIC of both isolates was 1 μmL.

Ultrastructure of untreated Borrelia burgdorferi cells

The untreated spirochete in Fig. 1 confirms the often-described structural characteristics of borrelial cells (12–15).

No phages were observed in borreliae grown in the untreated control cultures.

A-1 bacteriophages induced by subinhibitory ciprofloxacin concentrations

While the majority of the cells presented a regular shape, approximately 20% of the borrelial cells of both isolates showed severe abnormalities of ultrastructure when exposed to subinhibitory ciprofloxacin concentrations, ranging from 0.125 to 0.5 μg/mL. In the phage-carrying and morphological-altered borreliae, the outer envelope appeared to be undamaged, while the protoplasmic cylinder showed at least three different stages of destruction (1):

(1) numerous irregular constrictions of the normally smooth-structured peptidoglycan layer,
(2) disruption of the protoplasmic cylinder into several segments within a largely intact outer envelope, and
(3) small plasmolyzed protoplasma cylinder debris particles within an enlarged and irregularly shaped outer envelope.

In both isolates, plasmolyzed cells were filled with clusters of numerous unassembled heads and tails of bacteriophages (Fig. 2) showing an A-1 morphology (1, 16–18). According to the classification of Ackermann (17), this type consists of an isometric head (30 nm), a thin collar, and a long contractile tail (length 50 to 64 nm, width 13 to 19 nm) with a baseplate. In contrast to our former study (1), only unassembled heads and tails of A-1 bacteriophages could be observed within the borrelial cells. We detected no phages in borrelial cells exposed to ciprofloxacin concentrations equal to or higher than the MIC or in the untreated controls.

ULTRASTRUCTURE OF BORRELIA BURGDORFERI EXPOSED TO CIPROFLOXACIN CONCENTRATIONS ≥ 1 μg/mL

As previously described, nearly 20% of the cells showed severe *phage-induced* morphological alterations at subinhibitory ciprofloxacin concentrations. In the remaining borreliae, which presumably were not infected by temperate phages, no ultrastructural changes were seen when exposed to ciprofloxacin concentrations from 0.125 to 0.5 μg/mL.

The majority of borrelial cells exposed to a ciprofloxacin concentration of 1 μg/mL showed irregular constrictions of the peptidoglycan layer, which were located near the end of the cell (Fig. 3a). Obviously, as a result of these irregularly located constrictions, abnormally short distinct fragments of borrelial cells (Fig. 3b) became visible. The size of these fragments ranged from 0.6 to 0.8 μm. Moreover, the protoplasmic cylinder complex of *Borrelia burgdorferi* cells exposed to 1.0 μg/mL of ciprofloxacin showed electron-lucent swellings (Fig. 4a).

At a ciprofloxacin concentration of 2 μg/mL, approximately 75% of the treated cells revealed numerous defects of the peptidoglycan layer (Fig. 5a). The diameters of the protoplasmic cylinder complex varied from 0.09 to 0.20 μm (Fig. 5 arrows). Finally, at 4 and 8 μg/mL (Fig. 6a), in almost all cells the protoplasmic cylinder complex and the outer envelope were disrupted into many small plasmolyzed particles.

At ciprofloxacin concentrations from 0.125 to 2 μg/mL, spherical structures were seen (Fig. 7). Coiled up spirochetes were lying within these spheres.

No bacteriophages became visible in borrelial cells treated

FIG. 3. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 1 µg/mL. (a) Irregular constriction of the peptidoglycan layer at the cell periphery. (b) Abnormal short fragments of borrelial cells showing a length of 0.8 µm. Phosphotungstate stain,* × *97,000, fixed. Bar = 0.1 µm.*

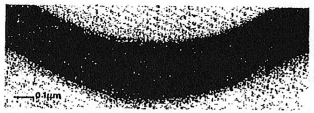

FIG. 4. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 1 μg/mL. (a) Electronlucent swelling of the protoplasmic cylinder complex. Phosphotungstate stain, × 97,000, fixed. Bar = 0.1 μm.*

FIG. 5. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 2 μg/mL. (a) Numerous defects of the peptidoglycan layer. Note the different diameters of the protoplasmic cylinder complex (arrows). Phosphotungstate stain, × 97,000, fixed. Bar = 0.1 μm.*

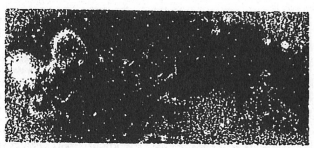

FIG. 6. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 8 μg/mL. (a) Debris of the disrupted protoplasmic cylinder complex partially enclosed by fragments of the outer envelope. Phosphotungstate stain, ×90,000, unfixed. Bar = 0.2 μm.*

FIG. 7. Ultrastructural alterations in Borrelia burgdorferi *after being exposed to a ciprofloxacin concentration of 0.125 μg/mL. Coiled-up spirochete forming a spherical structure (spheroplast). Phosphotungstate stain, × 37,000, unfixed. Bar = 1 μm.*

with ciprofloxacin concentrations of 1 μg/mL (MIC) and more.

DISCUSSION

The MIC of 1 μg/mL for our *Borrelia burgdorferi* strains was comparable to that found by investigators of other studies (5, 6). Our data confirm that *Borrelia burgdorferi* shows only moderate susceptibility to ciprofloxacin.

The ultrastructural morphology of our untreated *Borrelia burgdorferi* isolates (Fig. 1) corresponded with former morphological descriptions by Barbour and Hayes (13), Hovind-Hougen and coworkers (12, 14), and Hayes and Burgdorfer (15). Also, the measurements for length and diameter as well as the numbers of flagella were characteristic for Borrelia species (12–15).

Both *Borrelia burgdorferi* isolates examined in this study contained temperate bacteriophages showing an A-1 morphology that were inducible exclusively by subinhibitory ciprofloxacin concentrations (Fig. 2). These phage-carrying *Borrelia burgdorferi* cells showed severe ultrastructural alterations of their morphology (1), which completely differed from the ciprofloxacin effects on borreliae observed at concentrations of 1 to 8 μg/mL. Induction of prophages occurred only at subinhibitory ciprofloxacin concentrations, presumably as production and release of bacteriophages depend on an undisturbed metabolism of the host organism. Including our former study (1), we examined two erythema migrans isolates and one tick isolate for the presence of bacteriophages. All lysogenic borreliae contained A-1 bacteriophages, the first skin isolate in addition a B-1 bacteriophage (1).

Besides these phage-induced morphological alterations of borrelial cells, other severe ciprofloxacin-induced ultrastructural changes could be observed at concentrations of 1 μg/mL (MIC) and more. The normal cell division was considerably disturbed at a ciprofloxacin concentration of 1 μg/mL (MIC). Multiplication of *Borrelia burgdorferi* occurs by binary transverse fission (13). Usually, cell division is started by constriction of the peptidoglycan layer in the middle of a long cell (13). Obviously, as a result of the irregular constriction of the peptidoglycan layer in the periphery of abnormal elongated borrelial cells, very short cell fragments became visible (Fig. 3). The damaging effect of ciprofloxacin first led to swellings (Fig. 4a), after that to membrane defects (Fig. 5a), and finally to the disruption of the protoplasmic cylinder complex (Fig. 6).

At ciprofloxacin concentrations ranging from 0.125 to 2 μg/mL, large spherical forms filled with remnants of the protoplasmic cylinder complex, as described before (Fig. 7), were observed (13, 15), but the significance and function of such structures are still unknown. In comparison with the results of Voigt and Zeiler (2), Elliott et al. (3), and Rodgers et al. (4), who demonstrated that ciprofloxacin primarily affected areas located in the cell wall of gram-negative and gram-positive bacteria, we found severe morphological alterations concerning mainly the protoplasmic cylinder complex of *Borrelia burgdorferi*.

In contrast to penicillin-treated borreliae, which showed morphological alterations even at subinhibitory concentrations (Schaller M, Neubert U. Morphology of *Borrelia burgdorferi* exposed to benzylpenicillin. Infection, in press), in nonlysogenic borreliae, no ciprofloxacin-induced changes were visible at concentrations below the MIC.

This may be a further explanation why ciprofloxacin does not show the same *in vivo* efficacy (Meisel C. personal

communication) in comparison to the β-lactam antibiotics preferentially used in treatment of early Lyme-borreliosis (5, 6).

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