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In Vitro Activity of Vancomycin against the Spirochete *Borrelia burgdorferi*

LISA L. DEVER,[1]† JAMES H. JORGENSEN,[2] AND ALAN G. BARBOUR[3,4]*

Division of Infectious Diseases, Department of Medicine,[1] Department of Pathology,[2] and Departments of Medicine[3] and Microbiology,[4] The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7881

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***Borrelia burgdorferi*, a spirochete and the causative agent of Lyme disease, has been reported to be susceptible to a variety of antimicrobial agents. In this investigation, the action of vancomycin, a glycopeptide antibiotic not previously known to have activity against spirochetes, against borrelias was examined. The in vitro activity of vancomycin against a variety of strains of *B. burgdorferi* and one strain of *Borrelia hermsii* was determined by use of a microdilution MIC method (L. L. Dever, J. H. Jorgensen, and A. G. Barbour, J. Clin. Microbiol. 30:2692–2697, 1992). MICs ranged from 0.5 to 2 μg/ml. MICs of the glycopeptides ristocetin and teicoplanin and the lipopeptide daptomycin against strain B31 of *B. burgdorferi* were all ≥8 μg/ml. Subsurface plating, time-kill studies, synergy studies, and electron microscopy were used to investigate further the activity of vancomycin against B31. The MBC of vancomycin was 2 μg/ml. Time-kill curves demonstrated ≥3-$\log_{10}$-unit (99.9%) killing of the final inoculum after 72 h by vancomycin concentrations twice the MIC. Synergy between vancomycin and penicillin was demonstrated at concentrations one-fourth the MIC of each drug. In electron microscopy, B31 cells exposed to vancomycin showed a disruption of cellular integrity and were indistinguishable from those exposed to penicillin. These studies demonstrate another class of microorganisms susceptible in vitro to vancomycin.**

Lyme disease is the most common arthropod-borne disease in the United States and Europe (36, 39). The disease, caused by the spirochete *Borrelia burgdorferi* and transmitted primarily by ticks of the genus *Ixodes*, may be acute and self-limited or may progress to a chronic state with skin, joint, cardiac, and neurological involvement. Although many antibiotics have been used successfully in the treatment of Lyme disease, the full spectrum of antibiotic susceptibility of *B. burgdorferi* has not been fully defined. *B. burgdorferi* has been reported to be susceptible in vitro to a variety of antibiotics, including penicillin, amoxicillin, ceftriaxone, cefotaxime, cefuroxime, doxycycline, tetracycline, erythromycin, clarithromycin, and azithromycin (2, 15, 18, 19, 31). Oral doxycycline, amoxicillin and, to a lesser extent, erythromycin have been the antibiotics recommended most often for the treatment of the early stages of Lyme disease (11, 27, 32, 36). Recent evidence suggests that oral cefuroxime and azithromycin are also effective in the early stages (23, 25). For central nervous system infection and for late complications of Lyme disease, intravenous penicillin G and intravenous ceftriaxone have been the antibiotic therapies most often used (13, 14, 36–38).

In the present study, we explored the action of vancomycin, a glycopeptide antibiotic, against borrelias. Although vancomycin is primarily thought of as an antibiotic for use against gram-positive bacteria, we pursued this investigational tack for the following reasons: (i) some gram-negative bacteria, such as certain strains of *Flavobacterium* species and *Neisseria gonorrhoeae*, have been shown to be inhibited in vitro by vancomycin (1, 24); (ii) spirochetes phylogenetically are no more closely related to gram-negative than to gram-positive bacteria (30); and (iii) there were no prior reports that vancomycin was in fact ineffective against spirochetes. To assess the activity of vancomycin against borrelias, we used a microdilution MIC method, a subsurface plating method for MBCs, time-kill studies, synergy studies, and electron microscopy.

MATERIALS AND METHODS

Spirochetes. The *B. burgdorferi* strains studied were B31 (ATCC 35210), the type strain isolated from a pool of New York *Ixodes dammini* ticks; Ip90, an eastern Russian *Ixodes persulcatus* tick isolate; G1, a cerebrospinal fluid isolate from a patient with Lyme disease in Germany; HB19, a blood isolate from a patient with Lyme disease in Connecticut; and Sh.2, an *I. dammini* tick isolate from New York (15, 33). Cultures of strains B31 and HB19 had been passaged continuously from 1982 and 1983, respectively, and each had been cloned at least twice by limiting dilution (5, 9). These high-passage strains were designated B31-92 and HB19-92. The original strains B31 and HB19, which had not been passaged more than three times in vitro and were still infectious for rats, were designated B31-82 and HB19-83. Strains Ip90 and G1 had not been passaged more than 10 times in vitro. A single colony of Sh.2 was obtained directly from the plasma of infected mice. The first-passage clonal population was confirmed to be infectious for mice by peritoneal injection of 10^4 spirochetes. Strain HS1 of *Borrelia hermsii* (ATCC 35209) was also studied.

Antibiotics. The antibiotics tested were penicillin G, vancomycin, and daptomycin (Eli Lilly, Indianapolis, Ind.), ristocetin (Sigma, St. Louis, Mo.), and teicoplanin (Marion-Merrell Dow, Cincinnati, Ohio). Antibiotics were reconstituted in the diluents recommended by their manufacturers.

Broth microdilution susceptibility tests. MICs were determined in triplicate by a broth microdilution method that we

* Corresponding author. Electronic mail address: alan@taliesen.uthscsa.edu.

† Present address: The Rockefeller University, New York, NY 10021.

have described in detail elsewhere (15). In brief, antibiotics were diluted twofold in BSK II medium (5), and 100 μl of each concentration to be tested was dispensed into microtiter wells. The ranges of antibiotic concentrations tested were as follows, in micrograms per milliliter: penicillin, 0.015 to 8; vancomycin, 0.25 to 32; ristocetin, 0.25 to 32; teicoplanin, 0.25 to 32; and daptomycin, 0.25 to 32. All wells except negative control wells were inoculated with 10 μl of actively growing borrelias adjusted to yield a final inoculum of ca. 10^6 cells per ml, as determined by enumeration with a Petroff-Hausser chamber and phase-contrast microscopy. Microdilution trays were sealed with sterile adhesive plastic and incubated for 72 h at 34°C. The lowest concentration of antibiotic that resulted in an visual inhibition of growth and a lack of medium indicator color change from pink to yellow, compared with the growth control, was interpreted as the MIC. MBCs were determined for penicillin and vancomycin in duplicate by subsurface plating on BSK (see below) agar by a method that we have described elsewhere (15). The medium for subsurface plating consisted of 2× BSK II medium without gelatin, 3% bottom agarose, and 2% top agarose. At the time that microdilution trays were inoculated, the actual inoculum density for each MIC and MBC test was determined by subsurface plating. After 72 h of incubation and determination of MICs, 10 μl from each microdilution well determined to contain the MIC and above was mixed with 100 μl of 2× BSK II medium, and the mixture was subcultured by subsurface plating. After 10 to 12 days, plates were examined visually and colonies were counted. The lowest concentration of antibiotic resulting in ≥99.9% killing of the final inoculum was designated the MBC (28).

Broth macrodilution method. The MICs of vancomycin, ristocetin, teicoplanin, and daptomycin were also determined by a broth macrodilution method that we have described elsewhere (15). Tubes containing 5 ml of BSK II medium with antibiotics diluted to yield the same concentrations as for the microdilution method and control tubes without antibiotics were inoculated with B31 cells to yield a final inoculum of ca. 10^6 cells per ml. Tightly capped tubes were incubated at 34°C, and the contents were mixed by inversion at 24-h intervals. After 72 h of incubation, tubes were examined for inhibition of visual growth and medium indicator color change. The MIC was defined as the lowest concentration of antibiotic resulting in an inhibition of visual growth and a lack of color change of the medium indicator, compared with the control.

Time-kill studies. Strain B31 of *B. burgdorferi* was used to determine generation times in BSK II medium and rates of killing by penicillin and vancomycin at concentrations one-eighth, one-fourth and twice the respective microdilution MICs. Penicillin and vancomycin were also tested in combinations at concentrations one-eighth and one-fourth the microdilution MIC of each agent. Polystyrene tubes containing 10 ml of BSK II medium with the antibiotics to be tested and one tube without antibiotics were inoculated with 100 μl of an actively growing culture adjusted to yield a final inoculum of ca. 10^6 cells per ml. Tubes were incubated at 34°C. At 0, 8, 24, 48, and 72 h, tubes were gently vortexed and spirochete numbers in each tube were estimated with a Petroff-Hausser counting chamber. The estimated numbers were used to determine the appropriate dilutions needed to provide plates with countable colonies following subsurface plating. For accomplishing this determination, 100-μl aliquots were removed from the tubes and serially diluted 10-fold in BSK II medium; 100-μl aliquots were also directly subcultured in duplicate by subsurface plating. Plates were examined, and colonies were counted after 10 to 12 days of incubation. A bactericidal effect was defined as ≥3-$\log_{10}$-unit (99.9%) killing of the final inoculum (28). A lack of antibiotic carryover was demonstrated for each antibiotic tested by subsurface plating of 100 μl of the highest concentration of antibiotic tested with a known inoculum of approximately 50 to 100 B31 cells.

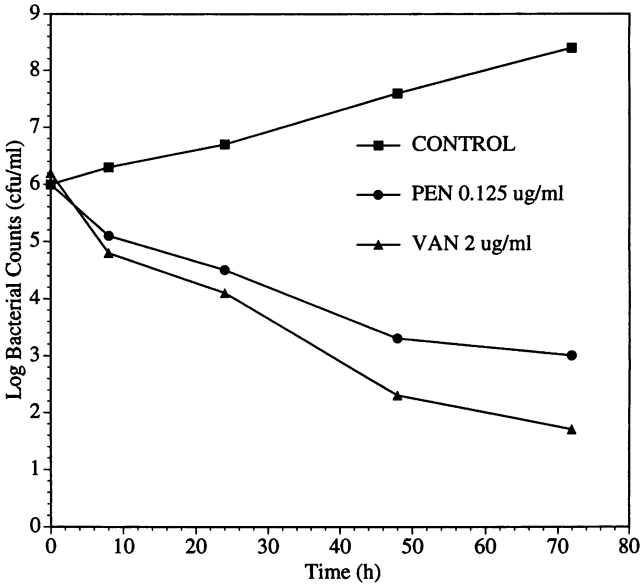

FIG. 1. Time-kill curves for *B. burgdorferi* B31 with concentrations of penicillin (PEN) (0.125 μg/ml) and vancomycin (VAN) (2 μg/ml) that were twice the respective MICs.

Electron microscopy. Cultures of B31 in log-phase growth and containing ca. 10^7 cells per ml were treated with 0.125 μg of penicillin and 2.0 μg of vancomycin per ml (concentrations representing twice the respective MICs). A growth control contained no antibiotics. After 24 h of incubation, 30 ml of each culture was centrifuged at 8,000 × *g* for 20 min at 24°C. The cell pellets were washed three times with 10 ml of phosphate-buffered saline (PBS) and resuspended in a final volume of 200 μl of PBS. Cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for a minimum of 1 h at room temperature. After this primary fixation, cells were washed three times with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The fixed samples were dehydrated through an ethanol series and embedded in Epon resin. Polymerization of the resin was accomplished at 60°C for 48 h. The specimens were then thin sectioned by use of a Sorvall MT-5000 ultramicrotome. Thin sections were stained with uranyl acetate and then lead citrate and examined in a JEOL 1200 EX transmission electron microscope.

RESULTS

In vitro antimicrobial susceptibility. We previously demonstrated the microdilution MIC method to be a reliable and reproducible method for antimicrobial susceptibility testing of borrelias (15). The microdilution MICs of penicillin and vancomycin for strain B31 of *B. burgdorferi* were 0.06 and 1 μg/ml, respectively. The microdilution modal MICs of van-

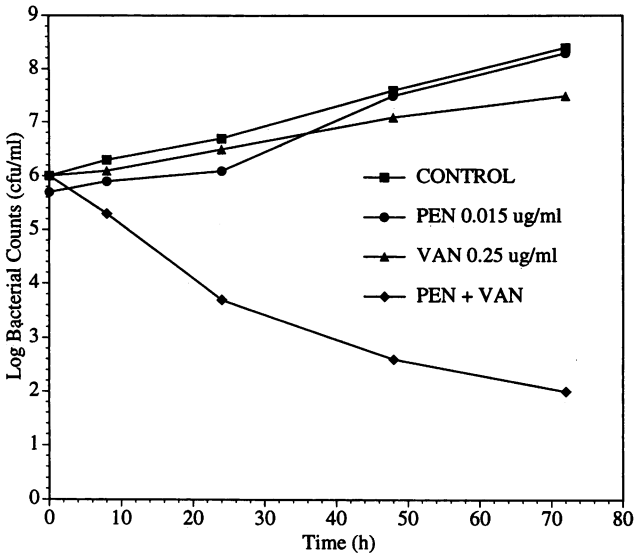

FIG. 2. Time-kill curves for *B. burgdorferi* B31 with concentrations of penicillin (PEN) (0.015 μg/ml) and vancomycin (VAN) (0.25 μg/ml) that were one-fourth the respective MICs.

comycin for the other strains of *B. burgdorferi* (B31-82, B31-92, HB19-83, HB19-92, G1, Ip90, and Sh.2) and *B. hermsii* (HS1) tested were 1, 1, 1, 1, 1, 2, 1, and 0.5 μg/ml, respectively (determined in triplicate). The MIC of vancomycin for B31, as determined by the broth macrodilution method, was 1 μg/ml. After determining that vancomycin had good in vitro activity against B31, we tested the peptide antibiotics ristocetin, teicoplanin, and daptomycin and found that both the macrodilution and the microdilution MICs were 16, 8, and 16 μg/ml, respectively. The MBCs of penicillin and vancomycin for strain B31 of *B. burgdorferi*, as determined by subsurface plating, were 0.125 and 2 μg/ml, respectively.

Time-kill studies of B31 with penicillin and vancomycin at concentrations twice the respective MICs demonstrated ≥3-$\log_{10}$-unit (99.9%) killing of the final inoculum after 72 h (Fig. 1). Under test conditions, the generation time of B31 was approximately 9 h. Penicillin and vancomycin did not exhibit bactericidal activity when tested at concentrations one-fourth (Fig. 2) or one-eighth the respective MICs (data not shown). In combination, penicillin and vancomycin at concentrations one-fourth the respective MICs demonstrated synergy, with ≥3-$\log_{10}$-unit killing of the final inoculum (Fig. 2). In contrast, at a concentration one-eighth the MIC of each antibiotic in combination, there was a 1.8-$\log_{10}$-unit increase in the number of CFU (data not shown).

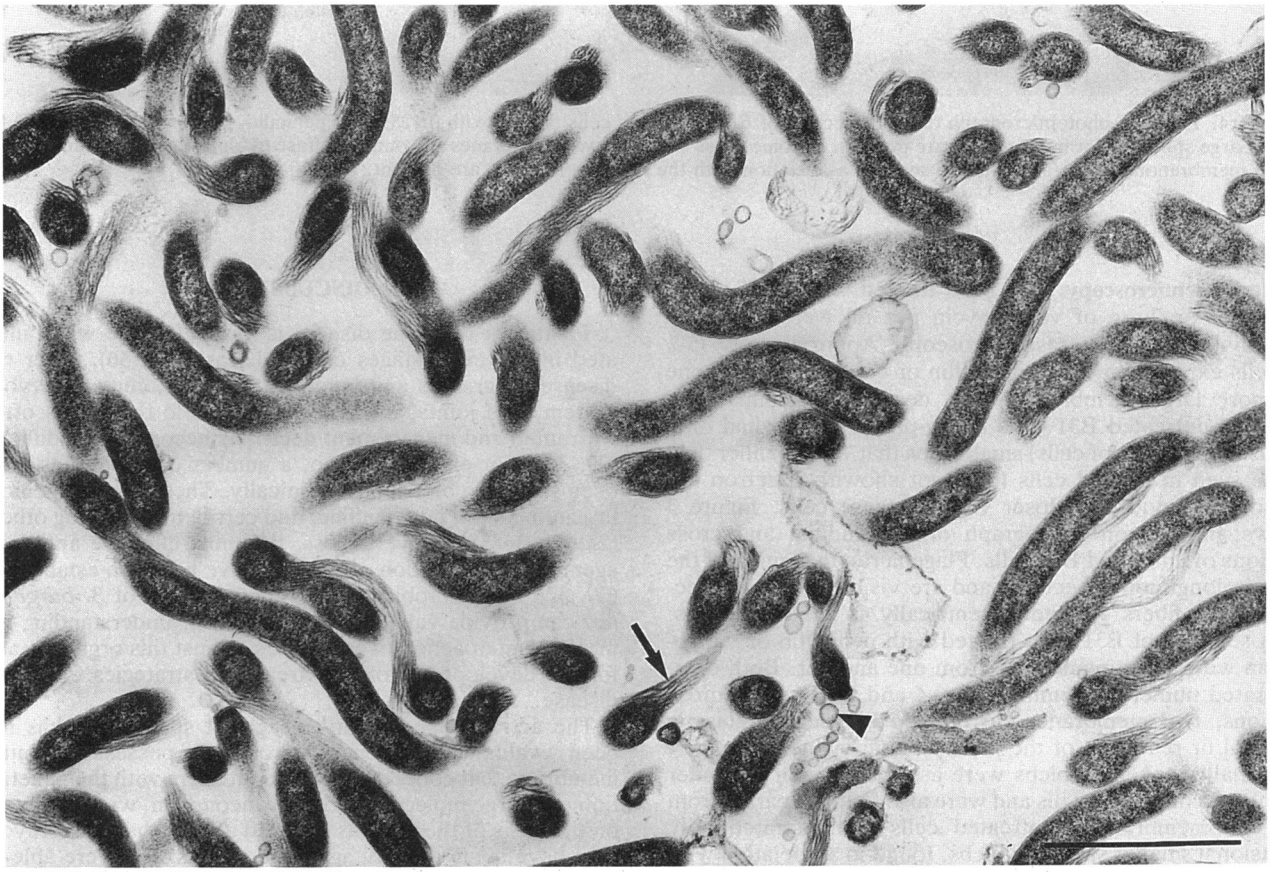

FIG. 3. Electron photomicrograph of a thin section of untreated *B. burgdorferi* cells. Longitudinal and cross sections are shown. Flagella run the length of the cells in longitudinal sections and are visible as small, electron-dense fibers located eccentrically in cross sections (arrow). Small spherical membranous blebs (arrowhead) are present in association with and separate from outer membranes. Bar, 10 μm.

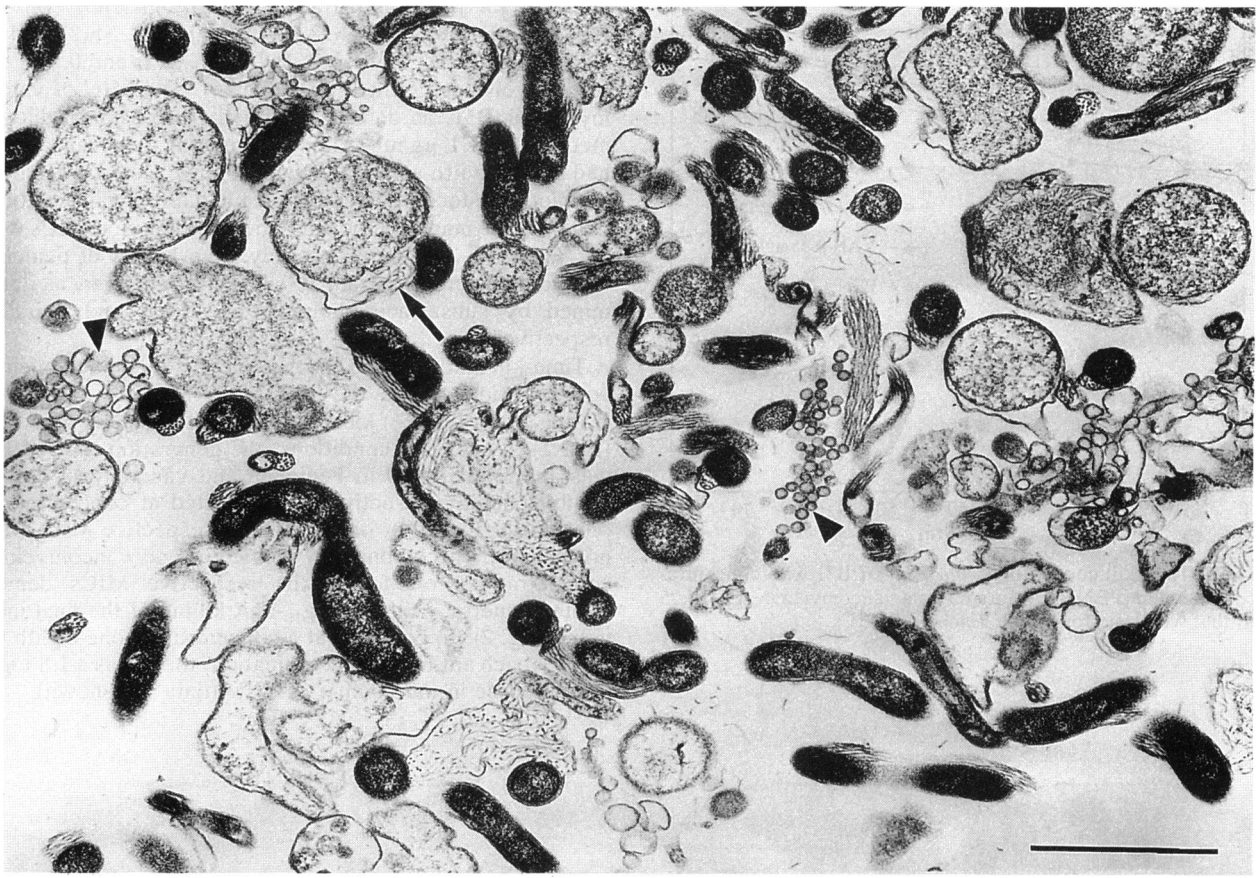

FIG. 4. Electron photomicrograph of a thin section of *B. burgdorferi* cells treated with 0.125 μg of penicillin per ml (twice the MIC) for 24 h. Large spherical forms (gemmas) are present. In some cross sections, two membranes surround a diffuse cytoplasmic reticulum (arrow). Small membranous blebs (arrowhead), some in association with the outer membrane, are present. Bar, 10 μm.

Electron microscopy. B31 cells treated with 0.125 μg of penicillin or 2 μg of vancomycin per ml were examined initially by phase-contrast microscopy. Approximately 75% of cells exposed to either penicillin or vancomycin had one or more large membrane blebs, designated gemmas (6), whereas untreated B31 cells in log-phase growth had only occasional (<20% of cells) small blebs that were smaller than those seen in treated cells (data not shown). Electron microscopy provided a closer view of these cells. Figure 3 shows an electron micrograph of longitudinal and cross sections of untreated B31 cells. Flagella run the length of the cells in longitudinal sections and are visible as small, electron-dense fibers located eccentrically in cross sections. Thin sections of B31 cells treated with penicillin or vancomycin were indistinguishable from one another. Both demonstrated numerous gemmas (Fig. 4 and 5). In some cross sections, two membranes surrounded diffuse cytoplasmic material or remnants of the protoplasmic cylinder. Numerous smaller spherical blebs were associated with the outer membrane of treated cells and were also found separate from the cell membranes. Untreated cells demonstrated only occasional smaller spherical blebs, found in association with and separate from the outer membrane. Rod-shaped forms or extremely long spirochetes were not observed in treated cultures.

DISCUSSION

Treatment of Lyme disease is most effective when initiated in the early stages of the disease (32, 36). After the disease progresses and involves the heart, central nervous system, and joints, intravenous antibiotic therapy is often warranted and management decisions become more difficult (29, 36). For either situation, a number of antibiotics have been shown to be effective clinically. These include penicillin, amoxicillin, doxycycline, and ceftriaxone, among others (23, 25, 36–38). Nevertheless, the most effective antibiotic agent and the duration of therapy have not been established (29, 32). Antimicrobial susceptibility studies of *B. burgdorferi* can provide valuable information for understanding the mechanism of action of antibiotics against this organism and for the development of new treatment strategies for Lyme disease.

The activity of vancomycin against spirochetes has not been evaluated. Although spirochetes possess an outer membrane that conceivably could interfere with the penetration of a large molecule such as vancomycin, we postulated, on the basis of the reasons outlined above, that vancomycin may have some activity against borrelias. We were able to demonstrate that vancomycin had excellent in vitro inhibitory activity against multiple isolates of *B. burgdorferi*, including low-passage strains, and against *B. hermsii*. The

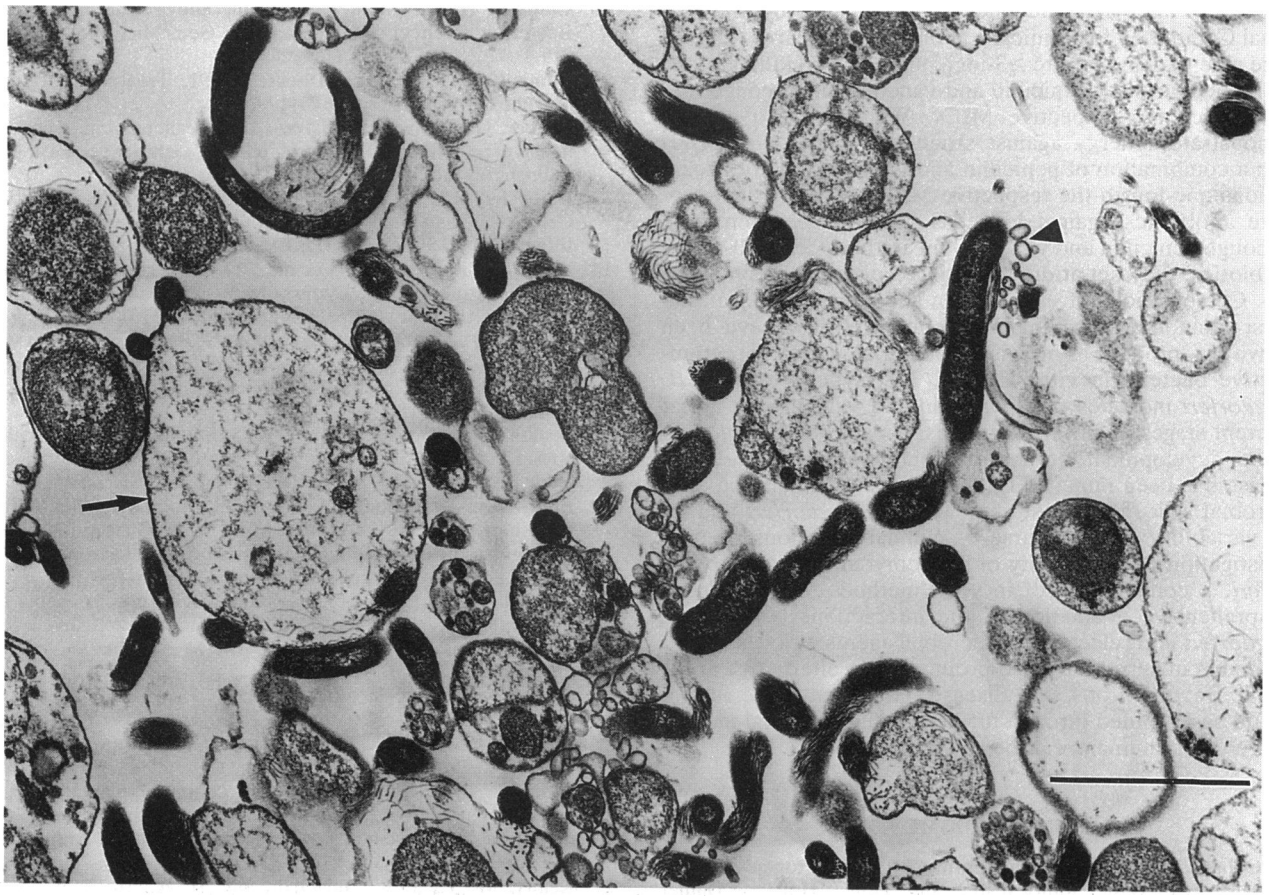

FIG. 5. Electron photomicrograph of a thin section of *B. burgdorferi* treated with 2 μg of vancomycin per ml (twice the MIC) for 24 h. Large spherical forms (gemmas) in cross sections (arrow) and membranous blebs (arrowhead) are indistinguishable from those seen in Fig. 4 (penicillin-treated cells). Bar, 10 μm.

microdilution MICs ranged from 0.5 to 2 μg/ml. Using a subsurface plating technique for MBC determinations, we were also able to demonstrate good in vitro bactericidal activity of vancomycin against strain B31 of *B. burgdorferi*: the MBC was 2.0 μg/ml, one dilution higher than the MIC.

Vancomycin prevents the polymerization of phosphodisaccharide-pentapeptide-lipid complexes during the second stage of cell wall synthesis (12, 26). It binds tightly to the terminal D-alanyl-D-alanine residues at the free carboxyl ends of the pentapeptide and stearically interferes with the elongation of the peptidoglycan backbone (12). In electron microscopy, B31 cells treated with vancomycin were indistinguishable from those exposed to penicillin. In a previous study of *B. hermsii*, Barbour and colleagues showed that gemma formation in spirochetes treated with cell wall-active antibiotics is associated with the inhibition of penicillin-binding proteins (7). These findings provide evidence that vancomycin acts at the level of cell wall synthesis in borrelias.

Because vancomycin had significant in vitro activity against borrelias, we also determined the MICs of ristocetin and teicoplanin (glycopeptides) and daptomycin (lipopeptide) against strain B31 of *B. burgdorferi* and found that these drugs were less active than vancomycin, with MICs ranging from 8 to 16 μg/ml. Teicoplanin and ristocetin have also been shown to inhibit the polymerization of peptidoglycan by binding to terminal D-alanyl-D-alanine residues (10, 35). Structural and kinetic analyses of the binding of vancomycin, ristocetin, and teicoplanin to cell wall peptides suggest that there are differences in binding affinities and forces for these three glycopeptides that could explain differences in their activity against bacteria (4, 40) and might explain differences in their activity against spirochetes as well. Differences in the susceptibility of gram-positive organisms to teicoplanin and vancomycin have been reported (21).

Although it is a peptide antibiotic, daptomycin is a calcium-requiring cyclic lipopeptide with a mechanism of action against susceptible bacteria different from that of glycopeptide antibiotics. The disruption of membrane energization appears to be responsible for the antimicrobial effects of daptomycin (3). Inadequate calcium concentrations in susceptibility test medium have been shown to reduce the potency of daptomycin and raise MICs (20). The latter case was not a factor in our studies, as the concentration of calcium in BSK II medium exceeds that recommended by the National Committee for Clinical Laboratory Standards (27).

Time-kill curve techniques show the best correlation with cure in animal models (8, 16, 28). In addition, this methodology is useful for determining synergy for combinations of antibiotics (28). Luft and coworkers previously demonstrated that 0.1 μg of penicillin per ml killed 99% of *B. burgdorferi* cells in a culture after 72 h (22). However, these investigators used motility as the measure of spirochete

viability. Following guidelines recommended by the National Committee for Clinical Laboratory Standards (28), we were able to demonstrate ≥3-$\log_{10}$-unit (99.9%) killing of the final inoculum with penicillin and vancomycin at concentrations twice the respective MICs. We were also able to demonstrate synergy against strain B31 of *B. burgdorferi* with a combination of penicillin and vancomycin at concentrations one-fourth the respective MICs. Synergy of two or more antibiotics against borrelias has not been reported. Although penicillin and vancomycin are both cell wall-active antibiotics, they act at different stages of cell wall synthesis (12). Combinations of vancomycin and several other antibiotics, including penicillin and cephalosporins, have been shown to produce synergistic bacterial killing of gram-positive bacteria in vitro (12, 17, 34). Synergy against *B. burgdorferi* indicates that penicillin and vancomycin act at different stages of cell wall synthesis in spirochetes as well.

The development of antimicrobial therapies for Lyme disease has been impeded by difficulties in performing antimicrobial susceptibility studies with *B. burgdorferi* and by the variability of the course of clinical infections. In this investigation of the activity of vancomycin against *B. burgdorferi*, a combination of in vitro methods that provide a comprehensive evaluation of the interactions between *B. burgdorferi* and selected antimicrobial agents was described. Vancomycin appears to hold promise as an alternative agent for the treatment of Lyme disease. However, these results should be extended through further testing in animal models before use in humans can be recommended.

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