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Received 1 December 1995/Returned for modification 16 January 1996/Accepted 1 February 1996

RP 59500, a combination of the streptogramins quinupristin and dalfopristin, and sparfloxacin are new antibiotics with good in vitro activities against Enterococcus faecium, which is an increasingly important nosocomial pathogen with resistance to multiple antimicrobials. Since fluoroquinolones and related macrolides have displayed high intracellular concentrations inside host cells, we evaluated the intracellular activities of these agents inside neutrophils against three strains each of vancomycin-susceptible E. faecium (VSEF) and vancomycin-resistant Enterococcus faecium (VREF). At concentrations equal to four times the MIC, RP 59500 and sparfloxacin decreased the number of intracellular VSEF organisms, while both antibiotics were at best bacteriostatic against intracellular VREF strains. At concentrations equal to one-fourth of the MIC, both antibiotics were bacteriostatic against intracellular VSEF strains but were ineffective in inhibiting the growth of VREF strains. Despite their anticipated markedly higher intracellular human neutrophil (PMN) concentrations, RP 59500 and sparfloxacin activities in medium alone were equal to or greater than those inside PMNs against almost all strains. We conclude that the intracellular PMN concentrations of these antibiotics may not be directly related to their intracellular activities in our assay. The reason for the differences in their activities against VSEF versus VREF remains undefined.

The prevalence of enterococcal infections has increased dramatically (28). Enterococci are the second or third most common cause of nosocomial infections, accounting for 12% of the total (26), and a common cause of endocarditis (17). The development of vancomycin-resistant Enterococcus faecium (VREF) (2), which is often associated with resistance to all other currently available antibiotics, has produced infections for which there is no effective therapy. In addition, while Enterococcus faecalis was the predominant species found in clinical isolates in the past, the spread of vancomycin resistance has resulted in a dramatic population shift, such that E. faecium outnumbers E. faecalis in vancomycin-resistant isolates by 10:1 (6).

The lack of available antibiotics to treat infections due to VREF makes it even more important to develop new therapies. RP 59500, a new semisynthetic injectable streptogramin, is a combination of two compounds, quinupristin and dalfopristin (4), with excellent in vitro activities against many grammegative cocci, including E. faecium (5, 7, 12). Sparfloxacin, a new fluoroquinolone with two fluorinated substituents, has displayed very good in vitro activity against many gram-positive bacteria (21), especially in comparison with the limited activities of most other quinolones. RP 59500 (8) and sparfloxacin (11,34) are concentrated inside phagocytes, as are quinolones and most other quinolones. RP 59500 (8) and sparfloxacin (11, 34) displayed their activities to those of ampicillin and vancomycin.

We found that while RP 59500 and sparfloxacin both had modest intracellular killing activity against VSEF, they were at best bacteriostatic against VREF strains.

(Part of this work was presented at the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy [15].)

MATERIALS AND METHODS

Reagents. RP 59500 and sparfloxacin were obtained from Rhône-Poulenc Rorer (Witzy sur Seine, France, and Collegeville, Pa.). Other reagents, including ampicillin and vancomycin, were obtained from Sigma (St. Louis, Mo.). Stock antibiotic solutions were freshly prepared in sterile distilled water, except for sparfloxacin, which was prepared in 0.05 N NaOH, at 10 mg/ml.

Bacterial strains and susceptibility testing. MICs for a total of 24 strains from our culture collection were determined by microdilution as described by the National Committee for Clinical Laboratory Standards (20), except for the use of brain heart infusion (BHI) broth (Difco, Detroit, Mich.). The bacterial strains used to examine intracellular activities of antibiotics are listed in Table 1. All strains were susceptible to PMN-mediated phagocytosis after opsonization with normal human serum (1).

Isolation of PMN. PMNs were isolated from EDTA-anticoagulated blood of healthy volunteers by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of residual erythrocytes (25) and were resuspended in Hanks balanced salts solution (HBSS). Cells were ≥95% PMN by Diff-Quick (Baxter Scientific Products, Miami, Fla.) staining, and viability was ≥96% as determined by trypan blue exclusion.

Intracellular activity of antimicrobial agents. Bacteria were grown overnight at 37°C in BHI broth and then diluted 1:100 in fresh BHI broth and grown to logarithmic phase for 3 to 4 h at 37°C with tumbling. Bacteria were washed twice and resuspended in HBSS containing 1 mM CaCl2 and 1 mM MgCl2 in a final volume of 400 μl, and the mixture was tumbled for 30 min at 37°C to allow the bacteria to be phagocytosed. Extracellular bacteria were removed by washing twice, and PMNs containing intracellular bacteria were resuspended in 400 μl of Dulbecco’s modified Eagle’s medium containing 10% heat-treated (56°C for 30 min) fetal calf serum (pH 7.0). Antibiotics were added to a final concentration of one-fourth or four times the MIC, and initially and at 1, 2, and 18 h, samples were withdrawn and PMNs were lysed in distilled water for 10 min. Bacteria were serially diluted and viability was determined by the pour plate method with BHI agar. The viability of PMNs was...
92% after 18 h as determined by trypan blue exclusion. Samples without PMNs or without antibiotics were included, and all experiments were performed in duplicate.

Statistical analysis. Statistical analysis was performed by Student’s two-tailed t test.

RESULTS

MICs. The MICs of RP 59500 for 14 VSEF and 10 VREF strains ranged from 0.25 to 4.0 μg/ml (MIC at which 90% of the strains are inhibited [MIC\textsubscript{90} = 2.0 μg/ml]). The MICs of sparfloxacin ranged from 0.125 to 32 μg/ml (MIC\textsubscript{90} = 8 μg/ml). Three strains each of VSEF and VREF, which were clonally distinct by pulsed-field gel electrophoresis (18), were used to examine the intracellular activities of antimicrobial agents (Table 1).

Intracellular activity against VSEF strains. VSEF-strains (UWHC 2145, GE-1, and 758) were phagocytosed by PMNs and exposed to antibiotics at one-fourth or four times the MIC. As seen in Fig. 1A, B, and C, RP 59500 at one-fourth of the MIC was typically bacteriostatic (defined here as <0.5 log change in viability) for intracellular organisms, while exposure to four times the MIC decreased bacterial viability, especially over the first 2 h, with a reduction in log (CFU per milliliter) after 18 h of incubation varying from 0.32 log units for E. faecium GE-1 to 1.60 log units for UWHC 2145. The activity of sparfloxacin at one-fourth of the MIC was similar to that at four times the MIC; the loss of viability after 18 h ranged from 0.43 to 1.41 log units for both levels of antibiotic. PMNs alone, in the absence of antibiotics, were ineffective in killing E. faecium in this model system (Table 2).

To compare the intracellular activities of these two antibiotics with those of antibiotics that do not reach similar intracellular levels or that are thought to have poor intracellular activities, we also exposed these three strains of E. faecium to one-fourth or four times the MIC of ampicillin or vancomycin. The activities of these two antibiotics were not appreciably different at one-fourth or four times the MIC (data not shown). As seen in Table 2, the loss of viability after 18 h due to ampicillin ranged from 0.20 to 0.81 log units, while that due to vancomycin ranged from 0.06 to 0.55 log units.

Intracellular activity against VREF strains. In contrast to our results with VSEF strains, neither RP 59500 nor sparfloxacin at one-fourth the MIC could inhibit the growth of intracellular VREF strains (SH-4, VREH-1, and E-1), with an increase in viability after 18 h ranging from 0.8 to 1.45 log units with RP 59500 and from 0.64 to 1.71 log units with sparfloxacin (Fig. 1D, E, and F). Even at levels equal to four times the MIC, both antibiotics were at best bacteriostatic against intracellular organisms; the increase in viability after 18 h ranged from 0.11 to 0.61 log units for RP 59500 and from −0.12 to 0.43 log units for sparfloxacin. Ampicillin and vancomycin were not tested with VREF strains because the concentration of antibiotic equal to four times the MIC for these resistant strains would be too high for clinical relevance.

Antimicrobial activity in the absence of PMNs. To compare the activities of these antimicrobial agents intracellularly with those seen in the absence of PMNs, bacteria were also resuspended in Dulbecco’s modified Eagle’s medium–10% fetal calf serum alone without PMNs and were exposed to four times the MIC of antibiotics. For VSEF strains, four times the MIC of RP 59500 in medium alone was equally active as or more active than it was intracellularly inside PMNs for all three strains (Table 2), while sparfloxacin was more active in medium alone for two of the three strains. Against VREF strains, both RP 59500 and sparfloxacin were about equally effective in medium alone versus intracellularly, with at best bacteriostatic activity.

TABLE 1. Bacterial strains used to examine intracellular activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UWHC 2145</td>
<td>2.0</td>
<td>18</td>
</tr>
<tr>
<td>GE-1</td>
<td>0.25</td>
<td>10</td>
</tr>
<tr>
<td>SH-4</td>
<td>1.0</td>
<td>14</td>
</tr>
<tr>
<td>E-1</td>
<td>0.5</td>
<td>16</td>
</tr>
</tbody>
</table>

DISCUSSION

Both RP 59500 (5, 7, 12) and sparfloxacin (21) have been reported to have excellent in vitro activities against gram-positive organisms with relatively low MICs, which was confirmed in the present study for VSEF and VREF. However, RP 59500 and sparfloxacin had only modest intracellular activities inside PMNs against VSEF and even less intracellular activities against VREF. This occurred despite our expectation that intracellular levels for these two antibiotics should far exceed the MICs for the organisms tested. The reason for this discrepancy is not known, but there are a few potential explanations.

First, we did not actually measure intracellular levels of antibiotics in this study, and the intracellular levels in this model system may have been less than those predicted from previous reports. The two components of RP 59500, quinupristin and dalfopristin, were concentrated 50-fold and 34-fold, respectively, inside a murine macrophage-like cell line (8), but differences between murine and human cells and also between the uptake of antibiotics by macrophages and PMNs (29) may provide one reason for a potentially lower intracellular concentration of RP 59500 in the human PMNs used in the present study. On the other hand, intracellular levels of sparfloxacin have been measured in human PMNs, with an intracellular-to-extracellular level ratio of about 8:1 reported (11). Other factors present in the incubation mixture also may have played a role in intracellular antibiotic levels. Uptake of quinolones and related macrolides may be dependent on pH values (11, 23) and extracellular calcium concentrations (19), and neither of these factors may have been optimal for maximal antibiotic uptake in this study.

Second, even though antibiotics may be concentrated inside phagocytes, their intracellular location may not necessarily be the same as the intracellular location of phagocytosed bacteria (29, 31). For example, macrolides are typically concentrated in lysosomes by trapping of organic bases (31) and thus may be located in close proximity to ingested bacteria, while quinolones are typically found in the cytoplasm. However, preexisting infection of murine macrophage-like cells with bacteria apparently alters the subcellular distribution of sparfloxacin, with more directed to the phagolysosomes (27). Third, the intracellular environment around the ingested bacteria may not be conducive to killing by the antibiotic. For example, the low phagolysosomal pH may inhibit the activities of related macrolides (23). In addition, the presence of serum has been reported to reduce the activity of RP 59500 (10). While multiple factors related to the model system used in this study could explain the relatively modest intracellular activity seen, we have used this model system previously to demonstrate good intracellular activity of a related macrolide, azithromycin, against intracellular enteric pathogens (22), and others have demonstrated intracellular killing of Staphylococcus aureus by sparfloxacin inside human PMNs (11), although the latter...
study was able to show killing only with extracellular concentrations of sparflaxacin that were greater than or equal to four times the MIC. The differences between the present results and those described previously may be dependent on multiple factors, including the type of phagocytic cell used, the species of bacteria examined, and the particular antibiotic tested. Our results in the present study suggest once again that the intracellular concentration of an antibiotic may not be directly related to its intracellular activity (13, 32).

Overall, we found that the intracellular activities of RP 59500 and sparflaxacin against VREF were different from those against VSEF. The reason for this difference is unclear, but it was also evident in the activities of these antibiotics in the absence of PMNs. While the antimicrobial agents could have lost activity over the 18-h incubation period, this would still not explain the relative differences due to vancomycin resistance, and the MICs of both antibiotics for the strains of VREF used were generally similar to those for the strains of VSEF. Since vancomycin-resistant enterococci contain altered peptidoglycan (3), it is possible that changes in the bacterial cell wall associated with vancomycin resistance affect the activities of other antibiotics both intracellularly and in an extracellular environment. Alternatively, since the VREF and VSEF strains used here were unrelated, a factor other than resistance to

FIG. 1. Intracellular activities of RP 59500 and sparflaxacin. Bacteria inside PMNs were exposed to antibiotics, and at the indicated times viability was determined. Data are the means of two experiments performed in duplicate. □, one-fourth the MIC of RP 59500; ■, four times the MIC of RP 59500; ○, one-fourth the MIC of sparflaxacin; ●, four times the MIC of sparflaxacin.

Figure 1.
vancomycin could have been responsible for their differing susceptibilities to intracellular killing. Of note, the VREF and VSEF strains also differed in their susceptibilities to killing by PMNs alone, and this could have contributed to the differences seen in intracellular antibiotic activities.

Against the majority of the strains studied, especially VSEF, both RP 59500 and sparfloxacin were more active in medium alone than intracellularly. Since PMNs themselves were ineffective in killing organisms in this model system, this suggests that intracellular E. faecium actually may have been protected against killing by antibiotics, despite the predicted higher intracellular concentrations. Whether intracellular E. faecium have a better rate of survival against antibiotic exposure in vivo is unknown.

Even with only modest intracellular activities, both RP 59500 and sparfloxacin may be effective in treating patients infected with VREF. Peak levels in serum have been about 5 μg/ml for RP 59500 (following intravenous infusion of 7 mg/kg of body weight) (9) and 1.4 μg/ml for sparfloxacin (following oral administration of 400 mg) (24), which are equal to or greater than the typical MICs for E. faecium, and ongoing clinical trials should indicate whether these agents are clinically useful for infections due to these organisms. In patients, a combination of the human host defense system and antimicrobial agents may be successful in overcoming E. faecium infections when neither one alone is effective. Clearly, efforts to develop alternative treatment strategies for infections due to multiply resistant VREF are important.

ACKNOWLEDGMENTS

This work was supported by a grant from Rhône-Poulenc Rorer, I.H.-I. was supported by a grant from the University Complutense of Madrid.

REFERENCES


