Failure of Newer β-Lactam Antibiotics for Murine Yersinia enterocolitica Infection

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Cefotaxime, imipenem, gentamicin, and doxycycline were active in vitro against the virulent serotype O8 Yersinia enterocolitica WA strain. Amoxycillin was inactive. The in vivo activity of these drugs was evaluated in a standardized and reproducible mouse model of systemic infection. Each single antibiotic was injected intravenously 30 h after intravenous inoculation of Y. enterocolitica WA. In vivo efficacy was measured by counting the viable bacteria recovered from the whole spleens of mice sacrificed at selected times. Doxycycline and gentamicin were active in stopping bacterial proliferation. Cefotaxime and imipenem, even at high doses (250 and 100 mg/kg of body weight, respectively), were totally ineffective, as was amoxycillin. Bacterial inocula (107), recovered from either the in vitro growth or the infected spleens, were plated on cefotaxime or imipenem concentration gradients in agar; no emergence of β-lactam-resistant organisms was detected. Based on these experiments it is not possible to explain, from any given property of the antibiotic, the bacteria, or the host, the discrepancy between the in vivo and in vitro activities of cefotaxime and imipenem. On the basis of these results, the use of newer β-lactam antibiotics should be delayed in the therapy of human Y. enterocolitica infections until further investigations are carried out.

The worldwide distribution of Yersinia enterocolitica and the life-threatening prognosis of septicemia due to this species in compromised hosts (24, 28, 32, 34) make investigations into the activity of new antibiotics mandatory. Y. enterocolitica appears to be susceptible to a wide range of antibiotics, especially aminoglycosides and tetracyclines, but the entire species exhibits some degree of resistance to ampicillin, cephalothin, and related β-lactam antibiotics because of the synthesis of β-lactamases (5, 6, 11, 13, 15, 16, 31, 40).

Marked in vitro activity against Y. enterocolitica of newer β-lactam antibiotics has been reported (15, 25, 33, 35, 39). A systematic trial of 126 clinical isolates belonging to various phenotypes of Y. enterocolitica clearly demonstrated the in vitro antibacterial efficacy of carbapenems and third-generation cephalosporins, not actually hydrolyzed by β-lactamases (13). These promising results might have represented a new and important therapy against severe Y. enterocolitica septicemia.

The present study was undertaken to compare the in vivo efficacy of the newer β-lactam antibiotics cefotaxime and imipenem against Y. enterocolitica with that of amoxycillin, gentamicin, and doxycycline in a reproducible mouse model mimicking natural systemic infection.

MATERIALS AND METHODS

Bacterial strain. Y. enterocolitica WA, biovar 1, serovar O8, phage type Xz, having a 42-megadalton virulence-associated plasmid, was originally received from P. B. Carter, The Trudeau Institute, Saranac Lake, N.Y. (4). It was regularly grown on tryptocasein-soy-agar (TCS; catalog no. 64557; Diagnostics Pasteur, Marnes-la-Coquette, France) overnight at 25°C from stock cultures frozen at −70°C. It was sensitive in vitro to all the antibiotics except the older β-lactams (ICS disk method). The presence of two types of β-lactamase was detected as initially reported (13). Inocula were checked to ensure that the plasmid-encoded properties were present, i.e., Ca2+ dependency (27) and autoagglutination (19), prior to injection of mice. Under these culture conditions the virulence of the strain was stable. It induced, after intravenous (i.v.) injection of standardized inocula into mice, a systemic infection of the reticuloendothelial system with reproducible growth kinetics.

Infection of mice. Swiss pathogen-free female mice, aged 5 weeks and weighing 22 ± 2 g at the beginning of each experiment, were purchased from the Ferme Expérimentale de Rennemoulon, Institut Pasteur, 78 Villepreux, France. Groups of five mice were injected i.v. with bacterial suspensions in sterile nonpyrogenic saline, adjusted to doses ranging from 1.3 × 108 to 2.4 × 108 CFU to ensure the induction of a reproducible systemic infection.

Antibiotics. The five antibiotics of known potency used in this study were kindly supplied by the manufacturers (or their subsidiary agents in France): amoxycillin (Beecham, Paris), gentamicin (Unilabo, Levallois-Perret), doxycycline (Pfizer, Paris), cefotaxime (Roussel, Romainville), and imipenem (Merck, Paris).

In vitro susceptibility tests. A strain of Escherichia coli (ATCC 25922) and two strains of Y. enterocolitica (no. 59, serovar O3, and no. 74, serovar O8 [13]) were checked as quality controls with each antibiotic in each experiment. The media used were Mueller-Hinton broth and agar (Diagnostics Pasteur).

The MICs were determined by the broth macrotitration system and an agar plate dilution method with a multipoint inoculator (Denley Instruments Ltd., Billingham, England) as described previously (20). The final antibiotic concentrations ranged from 0.016 to 512 μg/ml. Inocula of 1 × 108, 1 ×
10^6, 1 x 10^7, 1 x 10^8, 1 x 10^9, and 5 x 10^3 CFU/ml were prepared from overnight Mueller-Hinton broth cultures at 37°C. The final bacterial densities were determined by quantitative subcultures. Tubes and plates were incubated for 24 h at 37°C. The MIC was defined as the lowest concentration of antibiotic that inhibited development of visible growth on agar or in broth.

The MBCs were determined by the broth macrodilution method as described previously (20). It was used for all antibiotics and inocula, diluted 10-fold from 10^8 to 10^6 CFU/ml. Cultures were incubated for 18 h at 37°C. The MBC was defined as the lowest concentration of antibiotic producing at least a 99.9% reduction in the number of CFU.

In vivo antibiotic experiments. Antibiotic solutions were prepared by the manufacturers. Their in vitro activity was checked on the test strain grown on TCS. They were immediately injected into mice at 30 h after i.v. injection of the bacterial inoculum. The time of injection of the antibiotic was selected from the growth kinetics of strain WA in control mice; 30 h corresponded approximately to mid-exponential phase.

Antibiotics were injected i.v. at selected doses according to previously reported data: doxycycline (8), 125 mg/kg (the dose of 250 mg/kg was 100% lethal for mice in our experiment); gentamicin (26), 20 mg/kg; amoxycillin (3), 200 mg/kg; cefotaxime (18, 21), 25 and 250 mg/kg; and imipenem (18, 23, 4) and 400 mg/kg. All experiments were carried out at least twice. Additional experiments consisted of injecting cefotaxime (25 mg/kg) i.v. at 30, 48, and 54 h or at 30, 54, and 72 h postinfection.

Bacterial counts and expression of results. Viable bacteria, either in the inocula or recovered from spleen homogenates, were counted as CFU on TCS incubated for 18 h at 25°C as described previously (1,2). The times we selected for in vivo bacterial growth measurements were based on preliminary experiments that typically showed an early log phase of 6 h, followed by an exponential phase reaching a plateau at 54 h and leading to the death of the mice between 78 and 96 h. Data were obtained for the whole spleens without regard to the size increase of this target organ (1, 4). The geometric mean and the standard error of the mean were calculated from the data obtained for each group of five mice. In vivo growth curves expressed these results.

At the same time that the spleens were removed, blood samples were collected by retro-orbital puncture.

Detection of antibiotic-resistant bacteria. To check whether any resistant mutants might have been selected by antibiotics during the infection, we sampled the bacteria recovered from the spleens at 78 h postinfection. Spleen homogenates (0.1 ml in saline, 10^7 CFU) were plated on cefotaxime or imipenem concentration gradients (from 0 to 500 μg/ml) in agar (36) and on twofold dilutions ranging from 0.3 to 50 μg/ml in agar. Bacteria recovered from the in vitro growth were tested in the same manner. β-Lactamase production was tested as previously described (13).

RESULTS

In vitro susceptibility tests. MICs and MBCs were determined in both broth and agar; however, the MICs with inocula of 10^6 CFU/ml were determined only on agar. MICs in broth were equal to or only one dilution different from MICs in agar. We observed a slight inoculum effect on the MICs: the increase was no more than three dilution factors (log2) (Table 1). A significant inoculum effect on the MBCs appeared for 10^8 and 10^7 CFU/ml with all antibiotics (six or more log2 increase, except doxycycline, four log2).

Taking into account the MICs, all the drugs were active except amoxycillin. The MBC/MIC ratio (values expressed in log2) with 10^8 and 10^7 CFU/ml was equal to 1 or 2 for imipenem, 3 for gentamicin, 4 for cefotaxime and amoxycillin, and 5 or 7 for doxycycline.

In vivo antibiotic activities. Typical activities of the five antibiotics injected at the highest dose are illustrated in Fig. 1.

Y. enterocolitica WA was highly virulent for Swiss mice at the doses we selected for i.v. injection, as illustrated on the slope of the in vivo growth curves from 6 to 54 h. By 54 h, the number of CFU per entire spleen reached approximately 10^8. This value corresponded to the number of CFU recovered from the spleen of mice dying after 78 h (data not shown). Bacterial counts from blood samples (100 μl) showed low-level infection (less than 200 CFU/ml at 54 h). For this reason, the kinetics of bacterial growth and survival in the blood was judged unsuitable for demonstrating the effects of antibiotic therapy.

The efficiency of each antibiotic was quantified from comparative bacterial counts in the spleens of treated mice and nontreated controls. A single i.v. injection of a moderate dose of gentamicin (20 mg/kg) or a high dose of doxycycline (125 mg/kg) was effective in stopping bacterial proliferation. In contrast, amoxycillin (200 mg/kg), cefotaxime (25 or 250 mg/kg), and imipenem (4 or 100 mg/kg) had no influence on the in vivo growth of Y. enterocolitica WA. To confirm these results, we did two experiments with cefotaxime (25 mg/kg) repeatedly injected at 30, 48, and 54 h or 30, 54, and 72 h postinfection; they also failed to demonstrate any antibacterial effect (data not shown).

Detection of antibiotic-resistant mutants. No bacteria resistant to cefotaxime or imipenem were detected among suspensions of 10^7 CFU recovered from the spleen homogenates at 78 h on concentration gradients or agar dilutions of these antibiotics.

No change in β-lactamase production was detected.

### Table 1. Effect of inoculum size on in vitro activity of five antibiotics against Y. enterocolitica WA

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MBC/MIC (μg/ml) with inoculum (CFU/ml) of:</th>
<th>5 x 10^3</th>
<th>10^6</th>
<th>10^9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxycillin</td>
<td>NT/0.125</td>
<td>NT</td>
<td>0.50</td>
<td>1/0.125</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>NT/0.125</td>
<td>NT</td>
<td>0.50</td>
<td>1/0.25</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NT/1</td>
<td>32/2</td>
<td>1/0.25</td>
<td>1/0.25</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>NT/0.06</td>
<td>1/0.125</td>
<td>1/0.125</td>
<td>16/0.25</td>
</tr>
<tr>
<td>Imipenem</td>
<td>NT/0.25</td>
<td>0.5/0.25</td>
<td>0.5/0.25</td>
<td>2/0.5</td>
</tr>
</tbody>
</table>

* NT, Not tested.
DISCUSSION

In the present study, we selected a reproducible mouse model of systemic infection with a highly virulent strain (4) representative of the majority of the Y. enterocolitica strains isolated in human pathology in the United States (17). We observed that cefotaxime or imipenem, injected i.v. during the phase of irreversible establishment of the septicemic process, even at high doses of 250 and 100 mg/kg of body weight, respectively, were totally ineffective. In contrast, a single injection of gentamicin or doxycycline expressed the activities revealed in vitro in the infected mice effectively. The inefficacy of cefotaxime was confirmed in two experiments in which infected mice received three repeated i.v. injections of this antibiotic at 25 mg/kg, a dose highly effective on other enterobacteria (18).

Several possibilities may be considered to explain the discrepancies between the antibacterial activities of cefotaxime and imipenem in vitro and in the animal model. The in vitro susceptibility of Y. enterocolitica WA to cefotaxime or imipenem appeared to be at least as great as to the classically active gentamicin and doxycycline. Although the MBC/MIC ratio of cefotaxime was quite high, the MBC/MIC ratio of imipenem was quite compatible with a bactericidal mechanism. Moreover, no β-lactam-resistant mutants were selected by antibiotics during the murine infection even if bacteriological mechanisms could be implied, such as induction of β-lactamases or changes in outer membrane permeability or in affinity with penicillin-binding proteins (7, 14, 29, 30, 37, 38).

In experiments on murine systemic infections due to different enterobacteria (18, 21, 23), the 50% effective doses were obtained with doses of cefotaxime or imipenem lower than those we used. Imipenem, which is hydrolyzed by renal enzyme, is active in systemic infections (18, 23). However, host factors that may influence antibiotic activity in the spleen, such as tissue enzyme or pH, were not measured in our experiments and should be planned for future ones.

One could argue that β-lactam antibiotics are poorly endocytosed and thus are mainly effective against extracellular bacterial cells (10) and that pathogenic yersiniae for the most part replicate intracellularly (1, 2, 4). However, gentamicin, whose action is mainly extracellular (10), administered at a moderate dose of 20 mg/kg of body weight, was effective in stopping bacterial proliferation in the host. A high dose of doxycycline (125 mg/kg), a drug which has intracellular activity, had the same result.

Pathogenic yersiniae are known to undergo phenotypic variations, depending on the expression of plasmid genes coding for structural changes in the outer membrane proteins (9, 12, 27). Most of these phenotypic changes are expressed in vivo but not on standard culture media (22). They are implied in the resistance to host defenses, but further studies would be needed to clarify their possible role in the susceptibility of virulent yersiniae to antimicrobial agents.

We studied only a single isolate of Y. enterocolitica. Even though the WA strain has a phenotype representative of prominent human isolates in the United States (17), we cannot determine that all cases of human septicemia due to Y. enterocolitica are resistant to chemotherapy with cefotaxime or imipenem.

However, on the basis of the conclusions we came to here, it would be wise to delay the use of these newer β-lactam antibiotics in the therapy of human Y. enterocolitica infections, even if they appear highly active on cultures, until further investigations into their failure in the infected host are carried out.

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