Assessment of a Fluoroquinolone, Three β-Lactams, Two Aminoglycosides, and a Cycline in Treatment of Murine Yersinia pestis Infection

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Amoxicillin, cefotaxime, cefeiximine, gentamicin, doxycycline, and ofloxacin were active in vitro, like the reference drug streptomycin, against the virulent strain Yersinia pestis 6/69M. The comparative efficacies of these drugs in vivo were evaluated in a standardized and reproducible mouse model of systemic infection. Each antibiotic was injected intravenously once, at 24 h postinfection, and then repeatedly during 48 h. In vivo results were measured by counting the viable bacteria recovered from the whole spleens of mice sacrificed at selected times. All the drugs were manifestly successful; ceftriaxone, ofloxacin, and the reference drug were the most effective. Therefore, gentamicin and doxycycline could be used, depending on the clinical forms of the Y. pestis infection. Further investigations on β-lactams, especially those used in the present study, could be carried out to confirm or not confirm their activities against Y. pestis. Ofloxacin appeared to be as active and to perform as rapidly as streptomycin in the treatment of murine Y. pestis infection, which is in agreement with the previous successes obtained with the use of fluoroquinolones in the treatment of murine infections caused by other pathogenic yersiniae.

Three pathogenic species are included in the genus Yersinia. Y. enterocolitica and Y. pseudotuberculosis essentially cause gastroenteritis and occasionally septicemia. Y. pestis, which was discovered in 1894 by Alexandre Yersin, is the agent of plague. Despite the efforts of public health workers to control plague, this infection still has a widespread distribution in the world (7, 20, 22, 25, 35). During the last decade (1980 to 1989), 21 countries notified the World Health Organization of almost 10,000 cases of plague, and the global fatality rate was 11.5%, with peaks in some countries exceeding 35% (35). If, at the moment, the worldwide incidence seems to be stable, a few countries regularly report an increasing number of cases. In the four past decades (the 1950s, 1960s, 1970s, and 1980s), the United States has recorded 10, 28, 105, and 179 cases of plague, respectively (6, 35). Septicemic plague, which is difficult to recognize, may represent 20 to 25% of the cases of plague in the United States (17, 34). Secondary localizations, for example, pulmonary or meningeal, may complicate 6% of cases of bubonic or septicemic plague (3, 5) and up to now have been characterized by a high death rate, in some reports rising to 33% (2, 17).

Forty years ago, Meyer (23) considered streptomycin, chloramphenicol, and tetracyclines to be reference drugs for the treatment of plague, and they are still recommended for that use today (3). This limited antibiotic choice can be a serious handicap because of (i) localization of the disease (e.g., meningitis), (ii) underlying disease, or (iii) antibiotic side effects.

In the face of this dilemma, it seemed to us that it would be useful to evaluate the in vivo comparative efficacies of newer antibiotics like fluoroquinolones and extended-spectrum cephaplosporins against Y. pestis, as we did previously against the two other pathogenic yersiniae (19, 26, 28); we also tested another aminoglycoside (gentamicin), an aminopenicillin (amoxicillin), and another cycline (doxycycline). In the study described here, we used a reproducible systemic Y. pestis infection in mice caused by a virulent isolate from a human.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strain used to infect mice was Y. pestis 6/69M, which was isolated in Madagascar from a patient suffering from bubonic plague. This strain belongs to the subgroup Y. pestis var. orientalis (gycerol negative, nitrous acid positive) (1) and harbors the three usual plasmids (21) and the associated virulence ipr2 gene (8). Its 50% lethal dose by intravenous (i.v.) injection in mice is less than 10 CFU.

A collection of 18 Y. pestis strains was selected at the French National Yersinia Center, Institut Pasteur, Paris, France. Thirteen strains belonging to the subgroup Y. pestis var. orientalis were isolated in India, Java, Madagascar, Senegal, Turkey, the United States, and Vietnam. Two strains isolated in Iran and three strains isolated in Kenya belonged to the subgroups Y. pestis var. mediaeivalis and Y. pestis var. antiqua, respectively. These isolates were stored in semisolid conservation agar at room temperature in the dark.

Y. pestis easily grew on tryptocasein soy (TS) agar at 28°C. However, 0.025% hemin in 0.01 M NaOH (TSH) was added to the medium to obtain optimal growth.

The following strains were used to carry out the bioassays: Staphylococcus aureus ATCC 25923 for doxycycline and amoxicillin, Bacillus subtilis ATCC 6633 for streptomycin, and Escherichia coli ATCC 25922 for cefotaxime, ceftriaxone, gentamicin, and ofloxacin.

Mice. Pathogen-free female OF1 mice (age, 6 weeks; weight, 25 ± 2 g at the time of the experiment) were used throughout

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the study; they were obtained from Ifa Credo, Lyon, France. They were allowed free access to food and water. Five days of acclimatization was systematically observed between their arrival and the beginning of experimentation.

**Antibiotics.** The following seven antibiotics, of known potencies, used in the study were kindly supplied by the manufacturers (or their subsidiary agents in France): amoxicillin (Beecham, Paris, France), cefotaxime (Roussel, Romainville, France), ceftriaxone (Roche, Neuilly-sur-Seine, France), streptomycin and ofloxacin (Diamant, Paris, France), gentamicin (Unicet, Levallois-Perret, France), and doxycycline (Pfizer, Paris, France). All the antibiotics were dissolved in sterile physiological saline (except for amoxicillin usable per os, which was dissolved in phosphate buffer [pH 7]) and were administered in a volume of 0.2 ml.

**In vitro susceptibility tests.** The MICs of the seven antibiotics were determined for the 19 strains of *Y. pestis* by the dilution method in Mueller-Hinton (MH) agar with a multipoint inoculator (Denley Instruments Ltd., Billingham, England) as described previously (16, 27). The final antibiotic concentrations ranged from 0.016 to 16 mg/liter. The standard inoculum was approximately 10^4 CFU per spot; it was obtained from a 48-h TS agar culture at 28°C and was diluted in MH broth. The MIC was defined as the lowest concentration of drug that inhibited the development of visible growth after 48 h at 28°C.

The MICs of the seven antibiotics were determined for *Y. pestis* 6/69M by the macrodilution method in MH broth as described previously (16). The initial inoculum, which was prepared as described above, was approximately 10^6 CFU. The MIC was defined as the lowest concentration of drug that permitted at least a 99.9% (10-fold) reduction in the number of CFU. The cultures were enumerated after 6 and 24 h at 28°C.

*E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as quality controls with each antibiotic in each experiment.

**Pharmacokinetic assays in infected mice.** The doses of antibiotics administered to the mice were those used in previously reported experimental infections in mice (4, 10, 12, 13, 29, 32). They were selected to achieve about the same peak levels (or, more exactly, early concentrations) in the sera of mice as are achieved in the sera of humans (see Table 2). The individual antibiotics were administered to the mice at 24 h postinfection. Groups of 12 mice each were used for each antibiotic. At selected time points (at least three each) after antibiotic administration, three mice were anesthetized by exposure to chloroform, and blood was obtained from the inferior vena cava. The samples were centrifuged at 10,000 rpm for 5 min (Sorvall RC5C, rotor SS34), and then the serum specimens were removed and frozen at −20°C for 5 days at the most. The serum specimens were assayed by the microbiological technique (diffusion in agar) (9). Antibiotic concentrations were derived from a standard curve obtained from standard solutions diluted in pooled mouse serum specimens. The early concentration of each drug was determined, and the serum elimination half-life was estimated by the expression log_{10} 2/β, where β is the slope of the serum elimination regression curve (log_{10} serum concentration versus time).

**Establishment of infection.** The mice were infected i.v. with 400 ± 200 CFU of *Y. pestis* (>20 times the 50% lethal dose) in a volume of 0.5 ml. The inoculum was prepared in sterile physiological saline; first, a solution was obtained from a 48-h TS agar culture at 28°C and was adjusted to an optical density at 600 nm equal to 0.8 (corresponding to 8 × 10^8 CFU/ml) with a colorimeter; then, the appropriate dilutions were made; and finally, the bacterial density was checked by quantitative subculturing onto TSH agar plates. Preliminary studies showed that this inoculum induces a reproducible and fatal systemic infection.

**Establishment of therapy.** Therapy was conducted in two stages. In the first series of experiments, the antibiotics were administered by a single injection at hour 24 postinfection, which corresponds approximately to the midexponential phase of *Y. pestis* growth in the spleen. The following drugs were injected subcutaneously (except for doxycycline, which was given intraperitoneally) at the indicated doses: amoxicillin, 200 mg/kg of body weight; cefotaxime, 200 mg/kg; ceftriaxone, 40 mg/kg; streptomycin, 30 mg/kg; gentamicin, 5 mg/kg; ofloxacin, 5 mg/kg; and doxycycline, 50 mg/kg. In a second series of experiments, the same modalities were used, but ceftriaxone, ofloxacin, and doxycycline were administered two times daily and all other drugs were administered three times daily for 2 days. Finally, because of the efficacy of amoxicillin at high doses when administered i.v., we decided to estimate the effectiveness of this antibiotic when administered by a stomach tube at a moderate dose of 50 mg/kg four times daily for 2 days. All these dosages were administered by taking into account the results of pharmacokinetic assays of the drugs in infected mice (see Table 2). In all the experiments, untreated control animals received sterile physiological saline.

**Assessment of infection and therapy.** At 8 and 24 h postinfection, the control mice (n = 5) were killed to check the exponential growth of *Y. pestis* and to count the number of bacteria in the spleen at the time of the initiation of therapy. Thereafter, five animals from each group of mice, treated or untreated, were sacrificed at 8 and 24 h after the initiation of therapy for the first series of experiments and at 8, 24, and 56 h after the initiation of therapy for the second series of experiments. The spleens of the mice were aseptically removed and were homogenized in 1.5 ml of sterile physiological saline. The whole homogenate of each spleen removed at 8 h postinfection was plated onto TSH agar and was incubated for 48 h at 28°C to determine the number of viable bacteria. At the next time points, serial 10-fold dilutions of the homogenates were made and 150-μl samples were plated in the same manner. Therefore, except for the 8-h postinfection time point, the detection limit for bacterial counts in spleen homogenates was 10 CFU. Finally, for each antibiotic, five mice were observed until day 15 in order to estimate the mortality rate.

**Pathology.** Histologic examinations of the spleens of infected mice obtained at 24, 32, and 48 h postinfection were done. At each time point three mice were killed by cervical dislocation, and the spleens were placed in a solution of Boin de Hollande. The sections stained by the Mann-Dominici method were examined.

**Detection of antibiotic-resistant bacteria.** To determine whether the bacteria recovered from the spleens at 48 h in the first experiment might have been resistant mutants selected by the antibiotics, the MICs of the drugs were determined by using a sample of those bacteria.

**Detection of carryover effect.** Strain 6/69M was seeded into TSH agar, then aliquots of spleen homogenate were removed from treated mice at 32 and 48 h and poured into 0.1-ml wells bored into the agar, and lastly, the plates were incubated for 48 h at 28°C.

**Statistical methods.** Using the Student t test, we calculated the geometric mean and the confidence interval of the mean with a 5% risk of error from the data obtained for each group of five mice at each time point. The in vivo growth curves illustrate these results (see Fig. 1 to 3). The significance of the difference between the results obtained from control and treated mice was evaluated at each time point.
TABLE 1. In vitro activities of seven antibiotics against *Y. pestis* 6/69M and *Y. pestis* 18 strains from different countries

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/liter)</th>
<th>MBC (mg/liter)</th>
<th>50%</th>
<th>90%</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.12-0.5</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25-1</td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.06-0.12</td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.5</td>
<td>4</td>
<td>0.5</td>
<td>0.25-1</td>
<td></td>
</tr>
</tbody>
</table>

*50% and 90%, MICs for 50 and 90% of *Y. pestis* isolates tested.

RESULTS

In vitro susceptibility tests. The results of in vitro susceptibility tests are summarized in Table 1. Taking into account the MICs, all the drugs were active against the 19 *Y. pestis* isolates. Amoxicillin, streptomycin, and doxycycline were found to have MICs in the ranges published in the most important in vitro studies of the 277 strains from Madagascar (25). For strain 6/69M, aminoglycosides and ofloxacin appeared to be bactericidal at 6 h, and beta-lactams appeared to be bactericidal at 24 h. Only doxycycline was bacteriostatic.

Pharmacokinetics. The early concentrations of each of the seven antibiotics measured in the sera of the mice at 30 min (or 60 min for doxycycline) were of the same order as those found in the sera of humans (14, 18) at approximately the same time following administration of the usual doses (Table 2). The pharmacokinetic characteristics of the drugs in mice infected with *Y. pestis*, especially the short elimination half-lives, were in accordance with previously reported data from other experimental infection models in mice (4, 10, 12, 13, 29, 32).

Antibiotic efficacy against experimental plague. The efficacies of the antibiotics against experimental plague were quantified by comparing the bacterial counts in the spleens of treated mice and untreated control mice. *Y. pestis* 6/69M was highly virulent for mice at the dose we selected for i.v. injection. As illustrated in Fig. 1 to 3, a reproducible exponential kinetic growth was observed in the spleens (and livers [data not shown]) of the control mice starting at 8 h postinfection. This infection procedure produced septicemia and secondary lung localization at 24 h postinfection (data not shown). The deaths of the animals occurred between 54 and 72 h postinfection.

In the first series of experiments performed with a single antibiotic injection, all the antibiotics reduced by at least 1 log_{10} CFU the mean of the bacterial counts in the mouse spleens 8 h after they were administered (Fig. 1). This in vivo bactericidal activity was particularly marked with ceftriaxone, streptomycin, and ofloxacin, which induced the greatest reduction (>3 log_{10}). However, no drug injected once was able to eliminate *Y. pestis* from the mice, and regrowth was observed at 48 h postinfection. There was a correlation between this phenomenon and the high mortality rate for each antibiotic (four or five of five mice) in this series of experiments, and the regrowth can be explained by the short half-lives of the drugs in sera and tissues. Only doxycycline, which possesses the longest half-life of the drugs tested, stabilized the bacterial population between 32 and 48 h postinfection.

In the second series of experiments carried out with repeated injections over 48 h, the efficacies of all the drugs were confirmed (Fig. 2). At 80 h postinfection, which corresponds to either 16 or 20 h after the last antibiotic administration, all the treatments reduced the number of *Y. pestis* bacteria to the limit of detection. In this series of experiments, as in the first series, ceftriaxone, streptomycin, and ofloxacin were the most rapidly effective drugs, reducing bacterial counts below the limit of detection at between 32 and 48 h postinfection. The survival of mice (four or five of five) corroborated these results.

Finally, amoxicillin was administered per os at a dose of 50 mg/kg; this dose achieves about the same peak level in serum as oral administration of 1 g of amoxicillin to humans does (Table 2): it was as effective as the preceding dosage of amoxicillin administered subcutaneously at a dose of 200 mg/kg (Fig. 3), and mouse survival was 100% (n = 10).

Histologic results. No damage was observed in mouse spleens at 24 h postinfection. At 32 h postinfection, a moderate congestion in the marginal zone with an increasing number of polymorphonuclear neutrophils was observed. Finally, at 48 h postinfection, *Y. pestis* caused abscesses with areas of necrosis containing scattered polymorphonuclear neutrophils.

Detection of antibiotic-resistant mutants. No antibiotic-resistant mutants that might have been selected by therapy in the first series of experiments were detected.

Detection of antibiotic amounts in spleens. No bacteriostatic effect of the spleen homogenates was observed. Moreover, the numbers of CFU were in multiples of 10 in each bacterial count. Therefore, we can conclude that the remaining concentrations of antibiotics in tissues did not have much of an influence on our results.

DISCUSSION

In the present study, we used a reproducible mouse model of systemic fatal plague caused by a highly virulent strain of *Y. pestis*. To infect the animals, the nonnatural i.v. route of
infection was chosen because of its highly reproducible results, as was found in previous studies with Y. enterocolitica and Y. pseudotuberculosis (19, 26, 28). This necessary property was not observed with Y. pestis injected subcutaneously either in our preliminary work (data not shown) or in another previously reported study (33). The experimental model described here, which mimicked the pathogenesis of septicemic plague in humans, enabled us to estimate antibiotic activities against the clinical form of the disease with the worst prognosis.

Despite the brief half-lives of the tested antibiotics in mice, the results of the therapeutic scheme could be analyzed; in the first series of experiments, a single injection provided discriminating results on the bactericidal effects of the antibiotics in a confined model; in the second series, repeated doses given over 48 h enabled us to extrapolate the results more easily to humans.

Although treatments are intended to ensure the survival of animals, the aim of the present study was to compare bacterial counts and not mortality rates. Indeed, the treatment failures could have been due to the short treatment times used in the present study (48 h vis-à-vis 10 days in humans). Then, the in vivo bactericidal effect is more informative about the potencies and rapid activities of the drugs. Anyway, we observed that the mortality rates were in agreement with the bacterial counts.

Up to now, penicillins were considered ineffective in the treatment of human plague (3). Experimental infections and clinical reports have demonstrated the inefficacy of benzylpenicillin (24), but the dosage used was very low by contemporary standards (400,000 units daily [14a]). Moreover, benzylpenicillin is primarily active against gram-positive bacteria, and no other beta-lactam antibiotic has been repudiated in a published report. On the other hand, Butler (6) clearly established the effectiveness of ampicillin in a murine model of plague. Moreover, a case of human meningeal plague was successfully treated with ampicillin (3). The results obtained in the present study with amoxicillin, ceftriaxone, and cefotaxime showed that their efficacies were outstanding. Further investigations on beta-lactam antibiotics could well be carried out to confirm or not confirm their efficacies against Y. pestis. Indeed, these drugs could be of use in certain clinical situations, such as against septicemic syndrome in an endemic zone and meningitis in pregnant patients.

Gentamicin used more often than streptomycin, and doxycycline, a lipophilic cycline with an excellent half-life (20 h) and
The growth kinetics of the strains were measured from five mice that were either treated or not treated (control). The error. The horizontal line indicates the limit of detection. SEM (Student t test and standard error of the mean) with a 5% risk of error. The horizontal lines indicate the limits of detection. The antibiotic doses, expressed in milligrams per kilogram, were as follows: amoxicillin, 200; cefotaxime, 200; ceftriaxone, 40; streptomycin, 30; gentamicin, 5; ofloxacin, 5; and doxycycline, 50.

FIG. 2. Effect of repeated antibiotic injections during 48 h on Y. pestis infections in mice. The arrows on the horizontal axes represent the time points of antibiotic injection. The growth kinetics of the strains were measured in the spleens of mice infected i.v. with a standardized inoculum (see the arrows on the vertical axes). Each point represents the geometric mean of bacterial enumeration after homogenization of the spleens from five mice that were either treated or not treated (control). The vertical bars indicate half of the confidence interval of the mean ± SEM (Student t test and standard error of the mean) with a 5% risk of error. The horizontal lines indicate the limits of detection. The antibiotic doses, expressed in milligrams per kilogram, were as follows: amoxicillin, 200; cefotaxime, 200; ceftriaxone, 40; streptomycin, 30; gentamicin, 5; ofloxacin, 5; and doxycycline, 50.

FIG. 3. Effect of amoxicillin administered repeatedly and orally on Y. pestis infections in mice. The arrows on the horizontal axis represent the times of antibiotic administration. The dosage was 50 mg/kg six times daily for 48 h. The growth kinetics of the strains were measured in the spleens of mice infected i.v. with a standardized inoculum (see the arrow on the vertical axis). Each point represents the geometric mean of bacterial enumeration after homogenization of the spleens from five mice that were either treated or not treated (control). The vertical bars indicate half of the confidence interval of the mean ± SEM (Student t test and standard error of the mean) with a 5% risk of error. The horizontal line indicates the limit of detection.

intracellular diffusion, could be used depending on the clinical form of the Y. pestis infection.

Ofloxacin was active in vitro against Y. pestis 6/69M and 18 other strains of Y. pestis from different countries; this drug, like the other fluoroquinolones tested, has a good intracellular diffusion (30, 31). Its rapid bactericidal effect in vivo confirms its previous successes against murine septicemia caused by Y. enterocolitica (28) and Y. pseudotuberculosis (19) and confirms the clinical results for Y. enterocolitica septicemia (11, 15). Finally, we studied only a single virulent strain of Y. pestis; however, the successes of fluoroquinolones against yersiniosis create opportunities for further investigations into plague and fluoroquinolones.

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