Comparison of 2 Antibiotics That Inhibit Protein Synthesis for the Treatment of Infection with Yersinia pestis Delivered by Aerosol in a Mouse Model of Pneumonic Plague

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Introduction. Intentional release of Yersinia pestis will likely be propagated by aerosol exposure. We explored the effects of neutropenia on the outcome of doxycycline and gentamicin therapy.

Methods. Female BALB/c mice were exposed to 20 LD50 of Y. pestis CO92 by aerosol. Treatments were saline (negative control), levofloxacin at 15 mg/kg every 12 h (positive control), doxycycline at 40 mg/kg every 6 h, and gentamicin at 12 mg/kg every 6 h, 24 mg/kg every 12 h, and 48 mg/kg every 24 h in cohorts of normal and neutropenic mice for 5 days.

Results. Control mice died. Positive control mice (levofloxacin) had 100% survivorship in both neutropenic and nonneutropenic groups. Doxycycline treatment in the presence of granulocytes yielded 90% survivorship; all neutropenic mice died after the termination of treatment (P < .001). For gentamicin, survivorship of mice receiving drug every 24, 12, and 6 h was, respectively, 80%, 80%, and 90% for normal mice and 80%, 100%, and 70% for neutropenic mice. No significant differences were seen in the neutropenia versus normal mouse comparison or by schedule.

Conclusions. Doxycycline behaves in vivo as a bacteriostatic drug, requiring an intact immune system for clearance of the infection after aerosol challenge with Y. pestis. Gentamicin is bactericidal, even when given on a daily schedule. Neutropenia did not significantly affect survivorship.

Yersinia pestis is a pathogen highly likely to be used in incidents of bioterrorism and biowarfare. The syndrome resulting from intentional release of this agent will be different from the naturally occurring disease in that the well-known buboes will not form. Rather, because the release will likely be in the form of an aerosol, clinicians will see the less-well-known syndrome of pneumonic plague [1]. In contrast to the clinical syndromes seen with anthrax, human-to-human transmission through droplets is to be expected with pneumonic plague [2]. Indeed, the mortality seen with untreated pneumonic plague is very high and is expected to be in the range of 80%–100% [1]. Time of therapy initiation is particularly critical, with mortality rising if therapy is delayed longer than 24 h [1].

The standard of therapy for Y. pestis infection has been streptomycin [1–3]. This antibiotic is now exceedingly difficult to obtain and can be ototoxic; therefore, it is important to evaluate other antibiotics for the treatment of infection with this pathogen. Gentamicin is an aminoglycoside antibiotic that is widely available, and clinicians have considerable experience with its use. Once-daily therapy for the usual clinical gram-negative infections has become standard, to limit the nephrotoxicity seen with the use of this agent [4]. Evaluation of gentamicin use is therefore critical.

Gentamicin is a protein synthesis inhibitor and is
bactericidal [5]. Doxycycline is also an inhibitor of bacterial protein synthesis but is only bacteriostatic [6]. Doxycycline has broad-spectrum activity and is intended for the treatment of both *Y. pestis* and *Bacillus anthracis* infection. It is orally bioavailable, inexpensive, and less toxic than gentamicin. The question then arises as to which of these treatments is to be regarded as the therapy of choice. Also, because one is a bactericidal agent and the other a bacteriostatic agent, there is a question concerning the need for host defenses and the effect on survivorship if granulocytes are removed. Finally, previous data from our group [7] indicated that the fluoroquinolone levofloxacin has excellent bactericidal activity against *Y. pestis*. We thus chose this agent as a positive control for comparison with both gentamicin and doxycycline therapies. We evaluated these agents in a previously described mouse model of pneumonic plague [8].

**MATERIALS AND METHODS**

**Mice.** Female BALB/c mice (20 g) were obtained from the National Cancer Institute and were used for all experiments. The mice had free access to food and water throughout the course of the study. When death was imminent, mice were humanely killed. The time of death was recorded as the time of antibiotic administration (5 days) and at least twice daily thereafter out to 21 days after therapy initiation.

**Preparation of the *Y. pestis* challenge strain for aerosolization.** *Y. pestis* CO92 (provided by T. Quan, Centers for Disease Control and Prevention) was originally isolated in 1992 from a person with a fatal case of pneumonic plague [10]. The LD<sub>50</sub> in mice for this strain is 2.3 × 10<sup>4</sup> cfu inhaled when administered as an aerosol (nose only) [8].

The inoculum for aerosol challenge was prepared as described elsewhere [8], and the suspension of *Y. pestis* was diluted to the appropriate aerosol challenge dose. Colonies were counted after serial dilution and plating on sheep blood agar plates (SBAPs). These plates were incubated for 2 days at 35°C.

**Aerosol infection.** Inhaled doses of 20 LD<sub>50</sub> (LD<sub>50</sub> equals 6.8 × 10<sup>4</sup> cfu) of *Y. pestis* were administered to mice by whole-body aerosol. Aerosol was generated using a 3-jet Collison nebulizer [11]. All aerosol procedures were controlled and monitored using the Automated Bioaerosol Exposure system [12] operating with a whole-body rodent-exposure chamber. Integrated air samples were obtained from the chamber during each exposure using an all-glass impinger. Aerosol bacterial concentrations were serially diluted and plated on SBAPs, as described above. The inhaled dose (in colony-forming units per mouse) of *Y. pestis* was estimated using GUYTON’s formula [13].

**MIC determinations.** MIC values were determined using standard techniques recommended by the Clinical and Laboratory Standards Institute [14] in Mueller-Hinton broth at 35°C. End points were determined at both 24 and 48 h.

**Antibiotics.** Gentamicin (Hospira) and doxycycline (Bedford Laboratories) were pharmaceutical grade. Levofloxacin was obtained from Robert Wood Johnson Pharmaceutical Research and Development. All antibiotics or normal saline (placebo) were administered by intraperitoneal injection in a volume of 0.2 mL for 5 days, with different cohorts receiving different drugs (or schedules, in the case of gentamicin) or saline placebo. Initiation of therapy occurred 24 h after *Y. pestis* exposure. Antibiotic doses and schedules were as follows: levofloxacin at 15 mg/kg every 12 h, doxycycline at 40 mg/kg every 6 h, and gentamicin at 12 mg/kg every 6 h, 24 mg/kg every 12 h, or 48 mg/kg every 24 h.

**Induction of neutropenia.** Mice were rendered neutropenic by injection of 2 doses of cyclophosphamide intraperitoneally 4 days (150 mg/kg) and 1 day (100 mg/kg) before challenge.

**Assessment of efficacy.** Cohort size for statistical evaluation was 10 mice. Mortality was assessed and recorded every 6 h during antibiotic administration (5 days) and at least twice daily thereafter out to 21 days after therapy initiation.

**Murine pharmacokinetics.** Single doses of a range of 3 dose sizes of doxycycline, levofloxacin, and gentamicin were administered intraperitoneally to naive BALB/c mice. Mice were humanely killed in groups of 3 at 7 time points over 12 h. Blood was obtained by cardiac puncture, separated into serum, and assayed for drug concentrations as indicated below. All samples for a specific drug were analyzed simultaneously using the non-parametric adaptive grid population computer program of Leary et al. [15]. Weighting was as the inverse of the observation variance. Point estimates of the mean parameter values were used to calculate the area under the curve for 24 h at steady state (AUC<sub>0–24h</sub>) for each drug.

**Drug assays.** Levofloxacin and doxycycline concentrations in serum were determined according to a modified bioassay, using Staphylococcus aureus ATCC 29213 as the indicator organism, and compared with a standard curve for that antibiotic in control mouse serum [16]. All samples were assayed in triplicate. The lower limit of detection for the assays was 0.25 µg/mL for levofloxacin and 0.5 µg/mL for doxycycline. All pharmacokinetic experiments were performed on the same day. Gentamicin levels were assayed by fluorescent antibody assay, using the TDXFLx instrument (Abbott Laboratories) [17].

**Statistical analysis.** Control mice had an intact immune system.
Table 1. Area under the curve for 24 h at steady state (AUC₀–₂₄ss) and AUC₀–₂₄ss :MIC ratios for mice receiving doxycycline, gentamicin, or levofloxacin.

<table>
<thead>
<tr>
<th>Drug and dosage</th>
<th>AUC₀–₂₄ss</th>
<th>MIC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline, 160 mg/kg/day</td>
<td>214</td>
<td>428</td>
</tr>
<tr>
<td>Gentamicin, 48 mg/kg/day</td>
<td>53</td>
<td>107</td>
</tr>
<tr>
<td>Levofloxacin, 30 mg/kg/day</td>
<td>30</td>
<td>922</td>
</tr>
</tbody>
</table>

system and received treatment with saline. Normal mice had an intact immune system and received treatment with drug. Neutropenic mice received cyclophosphamide to decrease their granulocyte count as well as drug treatment. Groups were compared by hypothesis. The first hypothesis was that neutropenia would have an impact on survivorship for doxycycline. The second was that neutropenia would have an impact on survivorship for gentamicin. The third was that the schedule of administration would have an impact on survivorship for gentamicin. The fourth was that there would be a difference in survivorship (nonneutropenic cohorts, most effective schedule) among levofloxacin, doxycycline, and gentamicin therapies. Finally, we hypothesized that all of the regimens would differ from the saline control. All analyses were performed using a stratified Kaplan-Meier analysis with a log-rank test as implemented in SYSTAT for Windows (version 11.0; Systat Software).

RESULTS

MIC values. The *Y. pestis* CO92 MIC for doxycycline was 0.5 mg/L at 24 and 48 h; the MBC was >64.0 mg/L at both time points. For gentamicin, the MIC was 0.5 mg/L at 24 and 48 h, and MBCs were 1–2 mg/L on multiple occasions (n = 4). For levofloxacin, these values were a MIC of 0.03 mg/L for both time points and an MBC of 0.125 mg/L for both time points.

Drug pharmacokinetics. The AUC₀–₂₄ss and the AUC₀–₂₄ss :MIC ratio for each agent are displayed in table 1.

Regimen efficacy: doxycycline efficacy as influenced by neutropenia. The efficacy of 40 mg/kg doxycycline administered intraperitoneally every 6 h is shown in figure 1. There is a highly significant difference in time to death among all 3 groups (control mice, doxycycline-treated normal mice, and doxycycline-treated neutropenic mice) (P < .001). When the subanalysis was performed comparing normal mice with neutropenic mice, there was again a highly significant difference (P < .001), which remained significant even after Bonferroni adjustment for α decay. Although there was a significant difference in survivorship between doxycycline-treated neutropenic mice and control mice, it was observed that the doxycycline-treated neutropenic mice appeared to be sicker than the doxycycline-treated normal mice, even during the 5 days of therapy, when none of the mice died.

Regimen efficacy: gentamicin efficacy as influenced by neutropenia. Gentamicin was tested at the same daily dose but with 3 different dosing schedules (12 mg/kg every 6 h, 24 mg/kg every 12 h, and 48 mg/kg every 24 h). The results are presented as a comparison between normal and neutropenic mice by schedule in figure 2A–2C. We point out that the control mice are the same as those displayed in figure 1, as all groups were studied contemporaneously. In figure 2A, the 6-h dosing schedule yielded 90% and 70% survivorship for normal and neutropenic mice, respectively. Both results differed significantly from those for control mice. The difference between normal and neutropenic mice was not significant before the Bonferroni adjustment.

For the 12-h regimen, there was 80% survivorship for the normal mice and 100% survivorship for the neutropenic mice. Again, both groups differed significantly from the control group (P < .001) but did not differ from each other before the Bonferroni adjustment.

For the daily regimen, both normal and neutropenic groups had 80% survivorship. As above, there were differences from control survivorship (P < .001) but no difference between normal and neutropenic survivorship. To address the hypothesis most robustly, we collapsed all active gentamicin-treatment groups by regimen and looked for differences between normal and neutropenic mice. First, however, schedule was explicitly...
Regimen efficacy: levofloxacin efficacy as influenced by neutropenia. Levofloxacin completely protected both normal and neutropenic mice out to the termination of the experiment on day 21 (figure 3). For both, there was a highly significant difference relative to control mice. There were no differences between normal and neutropenic mice.

Normal mouse survivorship in relation to doxycycline, gentamicin, and levofloxacin. When all regimen groups were compared with the control group, a highly significant effect on survivorship was observed ($P < .001$). When the control group was removed, there were no significant differences noted among the regimens ($P = .6$).

**DISCUSSION**

There is a critical need for evaluating new drugs and dosing schedules for the treatment of *Y. pestis* infection. Streptomycin has been the standard antibiotic for infection caused by *Y. pestis*. However, streptomycin is toxic, needs to be given parenterally, and is not widely available. For this reason, it is imperative to identify new antibiotics for the treatment of plague, particularly after an intentional release, which would lead to the less-well-known syndrome of pneumonic plague, a form that causes very high (nearly 100%) mortality.

Consequently, we examined doxycycline and gentamicin, both inhibitors of protein synthesis, as potential replacements for streptomycin. Levofloxacin, an agent previously demonstrated by our group to be promising for the treatment of plague [7], was used as the positive control.

Although both doxycycline and gentamicin inhibit protein synthesis, doxycycline is recognized as being bacteriostatic in comparison with the aminoglycoside gentamicin. This implies that the immune system must play a far more important role in the ultimate therapeutic success of doxycycline, although the
bacterial physiological effect of the agents is the same—the inhibition of protein synthesis. Miller’s laboratory has demonstrated that aminoglycosides enter into gram-negative bacterial cells through ion-gated channels [18]. When the transmembrane $\Delta \psi$ declines to a certain level, the ion-gated channel closes, effectively turning the bacterium into a bag containing the drug for a period of time long enough to affect bacterial death. Tetracycline congeners, alternatively, are actively and rapidly effluxed from the cell [19], rendering them unable to kill many bacterial cells, although they do produce a prolonged postantibiotic effect that allows these agents to be given relatively infrequently. It is natural to assume that a short course of therapy would result in better outcomes if the organisms were rapidly killed. The ability of the immune system to participate in this process would be critical.

In comparing the effect of neutropenia on the survivorship of mice for doxycycline versus gentamicin, the outcomes were clear. Doxycycline produced a quite acceptable survivorship when the immune system was left intact (figure 1). This was significantly different from the no-treatment normal control group ($P < .001$). When the granulocytes were removed by cyclophosphamide treatment, however, the mice rapidly died after the termination of therapy, indicating that the bacterial population persisted. Note that the doxycycline-treated neutropenic group ($P < .001$) from the no-treatment normal control group. Death of mice occurred starting on day 9, which was 4 days after the end of therapy. This time course is consistent with the prolonged postantibiotic effect produced by this agent in vivo plus the time necessary to amplify the surviving bacterial population to a size that will result in bacteremia and the ultimate death of the mouse. This indicates that the intact immune system played a central role in the ability of doxycycline to eradicate $Y. pestis$ during therapy (5 days). This addresses the first hypothesis noted above (see the subsection on statistical analysis in Materials and Methods).

Gentamicin, on the other hand, showed no propensity for the outcomes to differ as a function of neutropenia (figure 2A–2C). This strongly implies that gentamicin therapy eradicated the infecting pathogen during drug administration. This is not surprising, given the rapid, concentration-dependent kill generated by gentamicin administration along with the understanding of why this protein synthesis inhibitor is strongly bactericidal, compared with tetracycline congeners. On the basis of this difference, we recommend that doxycycline would be most appropriate to employ in a broad-based postexposure prophylaxis scenario, in which its cost and relative lack of toxicity would dominate the decision process, whereas seriously ill patients (already symptomatic) would benefit from gentamicin therapy, with its rapid bactericidal activity and the resultant sterilizing effect. This addresses the second hypothesis noted above.

There is a question relating to the appropriate schedule of administration for gentamicin. Even in the present mouse system, with its rapid gentamicin half-life (1.06 h), there was absolutely no trend for better effect with shorter dosing interval when every 6, every 12 h, and daily dosing were compared. Consequently, given the significant reduction in gentamicin’s nephrotoxic potential when administered once daily [4], it is recommended that use of this agent for the treatment of plague should be on a daily basis. This addresses the third hypothesis cited above.

When all nonneutropenic regimens were compared, there was a significant treatment effect, but there was no difference across regimens. This addresses the fourth hypothesis indicated above. Consequently, in a clinical scenario of early treatment or postexposure prophylaxis, all of these agents should produce effective therapy, and the decision as to which one to use should be driven first by toxicity profile, second by route of administration (oral vs. parenteral), and third by price. For patients in the later stages of disease—who perhaps have sepsis or extensive plague pneumonia (with lots of potential for aerosol spread)—the choice should be a rapidly bactericidal agent that does not depend as much on the immune system and that produces rapid sterilization, which will decrease markedly the probability of generation of secondary cases. This would narrow the choice to gentamicin versus levofloxacin. Gentamicin is active only parenterally, whereas levofloxacin’s bioavailability [20] allows both parenteral and oral administration. Gentamicin, on the other hand, is considerably less expensive. It would be important to compare and contrast these agents for the treatment of later-stage infection with respect to efficacy as well as the propensity to allow the amplification of resistant subpopulations. In this way, optimal therapy for plague can be recommended for patients.

References

1. Pneumonic Plague Therapy


