Differential Distributions in Tissues and Efficacies of Aztreonam and Ceftazidime and In Vivo Bacterial Morphological Changes following Treatment

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Aztreonam, a monocyclic β-lactam antibiotic, has a broad spectrum of activity in vitro against aerobic gram-negative organisms (4, 5, 9, 14). Microbiological and clinical efficacies of aztreonam have been widely evaluated for urinary tract, lower respiratory tract, and intra-abdominal infections (18), as well as septicemia (5). It is active against Pseudomonas spp., Serratia spp., and indole-positive Proteus spp. (6, 7, 15).

This investigation was undertaken to compare the pharmacodynamics of aztreonam and ceftazidime in fibrin clots infected with Pseudomonas aeruginosa, Enterobacter cloacae, and Serratia marcescens. Differential distributions and efficacies between the surfaces and the cores of the infected fibrin clots were determined. To better evaluate the in vivo activities of both drugs, we also used light microscopy and electron microscopy (EM) to survey the morphological changes that the bacteria underwent during therapy.

MATERIALS AND METHODS

Bacterial strains. The three strains used for our experiments were E. cloacae ATCC 13047, P. aeruginosa ATCC 27853, and S. marcescens ATCC 29021. The MICs and MBCs of aztreonam and ceftazidime against these strains were determined according to National Committee for Clinical Laboratory Standards recommendations (12). Possible effects from the inoculum were also investigated in vitro with inocula of 10², 10⁶, and 10⁷ CFU/ml.

Preparation of noninfected and infected fibrin clots. As previously described (2, 16), a sterile solution of 3% bovine fibrinogen (Sigma Chemical Co., St. Louis, Mo.) was supplemented with 5% Mueller-Hinton broth (sterile or infected with a mean initial inoculum of 10⁷ CFU/ml for the kinetic studies and 10⁶ CFU/ml for the EM studies). Two-milliliter aliquots were distributed into siliconized test tubes (13 by 100 mm), and 0.1 ml of bovine thrombin (250 U/ml; Parke-Davis and Co., Detroit, Mich.) was added to each tube. After a 1-h incubation at 37°C, the clots were gently removed, washed in sterile water, and inserted subcutaneously in rabbits. The concentration of protein in fibrin clots reached a maximum of 15% of the protein content in serum.

Rabbit model. As published earlier (1, 2, 16), New Zealand White female rabbits (weights, 1.8 to 2.2 kg) were given an intramuscular injection of 20 mg of chlorpromazine per kg of body weight. Both flanks were shaved and swabbed with a commercially available iodine solution. The skin was anesthetized with 2% lignocaine, and a commercial antiseptic iodine solution was used to prepare the site for injection. The skin was anesthetized with 2% lignocaine, and a commercial antiseptic iodine solution was used to prepare the site for injection.

TABLE 1. MICs and MBCs of aztreonam and ceftazidime

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inoculum (CFU/ml)</th>
<th>Aztreonam</th>
<th>Ceftazidime</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
</tr>
<tr>
<td>P. aeruginosa ATCC 27853</td>
<td>10⁰</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>E. cloacae ATCC 13047</td>
<td>10⁰</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>S. marcescens ATCC 29021</td>
<td>10⁰</td>
<td>0.06</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>10⁷</td>
<td>0.25</td>
<td>0.25</td>
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</tbody>
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Determinations of drug concentrations in sera and fibrin clots. Blood samples were centrifuged, and sera were collected. Core and periphery samples were weighed and homogenized in 1 volume of trypsin solution (Difco). As stated before (10), dissolved clots and serum samples were bioassayed by a conventional agar diffusion method with Escherichia coli MB3904 as the assay organism in Difco tryptic soy agar. Standard solutions were prepared by diluting known amounts of antibiotic in blank rabbit serum or in trypsinated clots. The concentration of trypsin used to digest the clots did not in any way influence the bioassay and bacterial numeration.

Mean limits of detection and linearity for serum assays were, respectively, 0.13 ± 0.02 μg/ml and 0.995 ± 0.002 for aztreonam and 0.33 ± 0.05 μg/ml and 0.993 ± 0.002 for ceftazidime. Aztreonam interassay variation was 1.3% for the high concentration (50 μg/ml) and 2.2% for the low concentration (0.5 μg/ml). Ceftazidime interassay variation was 1.0% for the high concentration (50 μg/ml) and 2.0% for the low concentration (0.5 μg/ml). The mean limits of detection and linearity for clot assays were, respectively, 0.19 ± 0.01 μg/ml and 0.992 ± 0.006 for ceftazidime.

Pharmacokinetics and statistical analyses. Pharmacokinetic analysis was done with the pharmacokinetic program Anaphar (School of Pharmacy, Laval University). A one-compartment model was chosen for both sera and clots. The area under the concentration-time curve (AUC) was obtained by the method of successive trapezoidal approximations from time zero to 24 h. The AUC from 0 to infinity was calculated by adding the portion C24h/Ke (K, being the slope of the elimination phase) (13, 16). Statistics were performed by obtaining an analysis of variance with repeated measures and a one-way analysis of variance. Statistics for evaluating efficacy data were obtained by correlation testing. (Type I error was set at 5%).

In vivo efficacy. The efficacies of aztreonam and ceftazidime were evaluated by analysis of the bacterial contents of infected clots (cores and peripheries) at each time interval as previously described (1, 2, 17). Control and peripheral numerations of bacteria were also done and are shown as whole-clot numeration with the killing curves. Appropriate dilutions of trypsinated parts of the clots were inoculated on agar followed by incubation at 37°C for 24 h. Since 0.025-ml samples were diluted from 0.1- to 1,000-fold and were spread over a 15- by 100-mm agar plate, drug carryover was not a problem.

Light microscopy and EM. Two sections of each clot were kept and prepared for light microscopy and EM. The bacterial morphological changes in the periphery or in the core of a clot could then be observed and monitored at different times after the injection of the drugs. These morphological studies were done with P. aeruginosa and E. cloacae.

Optical microscopy. Microscopy was done with P. aeruginosa, E. cloacae, and S. marcescens. A small piece of either the periphery or the core of a clot was compressed between two slides, heated to fix the specimen, and finally gram stained. Observations were made at a magnification of ×1,000 with a Nikon Labophot-2.

Transmission EM. The peripheries and cores of the fibrin clots were cut in cubes of 1 mm³ in a 2.5% glutaraldehyde-1.5% formaldehyde solution in 0.1 M phosphate buffer at pH 6.8, to which 0.03% CaCl₂ and 0.03% picric acid were added, and chilled overnight at 4°C. The samples were rinsed in 0.1 M phosphate buffer containing 4% sucrose at pH 7.4 and postfixed in a solution of 1% osmium tetroxide for 1 h at room temperature before being stained in 1% aqueous uranyl acetate for 45 min. The dehydration was performed in increasing grades of ethanol, and the infiltration was performed in Epon. Light-golden, ultrathin sections were obtained with a Reichert Ultracut S, placed on a copper grid, and subjected to 2% uranyl acetate for 45 min at room temperature and stained in 1% aqueous uranyl acetate for 45 min at room temperature before being stained in 1% aqueous uranyl acetate for 45 min. The dehydration was performed in increasing grades of ethanol, and the infiltration was performed in Epon. Light-golden, ultrathin sections were obtained with a Reichert Ultracut S, placed on a copper grid, and subjected to 2% uranyl acetate and lead citrate to produce contrast. Samples were observed with a JEOL 1010 EM (Sokol Ltd, Montreal, Quebec, Canada), and the micrographs were taken at ×5,000, ×10,000, ×15,000 and ×50,000 magnifications.

![FIG. 1. Serum and clot (periphery and core) concentrations of aztreonam (A) and ceftazidime (B) over 24 h (means [four rabbits] ± standard deviations [bars]).](http://aac.asm.org/Downloaded from http://aac.asm.org on June 22, 2017 by guest)
RESULTS

In vitro susceptibility studies and in vivo evaluation of antibiotic resistance. The MICs and MBCs, with inoculum effects, are shown in Table 1. Both agents had nearly similar in vitro activities against the three bacterial strains. As we increased the inoculum size to $10^7$ CFU/ml, there were sharp increases in the MICs and MBCs, which reached, with the exception of those of ceftazidime against *E. cloacae*, values of more than 32 $\mu$g/ml. The MICs and MBCs of pathogens extracted from fibrin clots were done at each sampling time with animals given 100 mg of either aztreonam or ceftazidime/kg. We could not detect any in vivo development of resistance with either agent.

Differential pharmacokinetics of aztreonam and ceftazidime in serum and in the peripheries or cores of the fibrin clots. Figure 1 shows the comparative concentrations in serum and their standard deviations of aztreonam and ceftazidime after a 100-mg/kg dose. Peak levels of ceftazidime in serum (153.9 ± 3.9 $\mu$g/ml) were higher than those of aztreonam (88.4 ± 6.2 $\mu$g/ml), but levels were identical after 2 h. Levels of 3.7 ± 0.8 $\mu$g/ml for ceftazidime and 0.2 $\mu$g/ml for aztreonam were detectable at 8 h. We could not detect any aztreonam in serum at 24 h, while the level of ceftazidime at that time was 0.7 $\mu$g/ml. The comparative concentrations of both agents in the peripheries and the cores of fibrin clots are also shown in Fig. 1. In the first 2 h, the concentrations of aztreonam in the peripheries were significantly higher than in the cores of the fibrin clots ($P < 0.05$). Thereafter, up to 24 h, the levels were not significantly different. At 24 h, the fibrin clot concentration of aztreonam was 1.0 $\mu$g/g while it was 1.2 $\mu$g/g for ceftazidime. Whether the clots were infected with either *P. aeruginosa*, *E. cloacae*, or *S. marcescens*, the concentrations of both agents were not significantly different.

The pharmacokinetic evaluation of aztreonam and ceftazidime in sera and clots is shown in Table 2. In serum, times to peak concentrations were identical for both drugs. The drugs, especially aztreonam, were cleared much more rapidly in serum than in the clots. Aztreonam’s half-life in the cores of the clots was up to six times that in serum. With ceftazidime, it was twice as long. The AUC of aztreonam was significantly higher ($P < 0.01$) in the peripheries and the cores of fibrin clots than in serum.

For each individual agent over a period of 24 h, the AUC in the peripheries was not significantly different ($P = 0.6579$) than that in the cores of fibrin clots. There was no significant difference ($P = 0.6603$) in the penetration ratios, as determined by dividing the AUC in clots by the AUC in serum for each individual antibiotic, when we compared the values for the cores to those for the peripheries of the clots, but the penetration ratio of aztreonam in the cores of the clots (2.58) was significantly higher than that of ceftazidime (1.65) ($P < 0.01$).

In vivo bactericidal activity. The comparative in vivo efficacies of aztreonam and ceftazidime against *P. aeruginosa*, *E. cloacae*, and *S. marcescens* are shown in Fig. 2, 3, and 4. The results are expressed as the mean log differences between the number of CFU at baseline and those recovered at different times following a single injection of aztreonam or ceftazidime in the fibrin clots. There was no significant difference between the in vivo activity of each individual agent in the cores and that in the peripheries of the clots, although there was a slightly higher CFU number (1 log) in the cores.

*P. aeruginosa*. There was no difference between the reductions in the numbers of CFU of *P. aeruginosa* (Fig. 2) following the injections of both agents, but with ceftazidime, there was a much more rapid 4-log reduction in CFU/g. Both agents maintained their activity up to 24 h after the single dose but could not sterilize the clots. It is interesting that even though the whole-clot levels of aztreonam were below the MIC of *P. aeruginosa* 8 h after the injection, the activity was maintained for 24 h.

*E. cloacae*. There was a fast 5-log reduction in CFU up to 8 h with ceftazidime, while aztreonam showed less activity up to 8 h, with a 2-log reduction, which was maintained up to 24 h.
As with *P. aeruginosa*, the initial reduction in CFU was more rapid with ceftazidime, but after 8 h there was a significant regrowth of *E. cloacae*. At 24 h, aztreonam was 1 log more active than ceftazidime (Fig. 3).

*S. marcescens*. Ceftazidime demonstrated a 2.5-log reduction in colony counts up to 24 h after the beginning of injection. Aztreonam induced a 0.8-log reduction in CFU and also maintained its activity for 24 h (Fig. 4).

**Sequential morphological changes following treatment with aztreonam or ceftazidime.** (i) *P. aeruginosa*. At the peripheries of the clots, an observation made at a magnification of ×1,000 showed no real difference between the two antibiotics. As early as 0.5 h after bolus injection, it was possible to observe both an elongation of and an increase in the diameters of the bacteria, culminating in spaghetti-like bacteria after 4 h of treatment (Fig. 5). Likewise, on an electron micrograph the same phenomenon can be observed except that, first, *P. aeruginosa* cells become longer and thinner after 8 h of treatment with ceftazidime while cells are more irregular when exposed to aztreonam (Fig. 6G and H) and, second, *Pseudomonas aeruginosa* cells are thicker and more irregular after 24 h of aztreonam treatment. This was not observed after ceftazidime administration (Fig. 6I and J).

In the cores, the samples examined by light microscopy revealed no detectable difference between the condition at time zero and that at 24 h after the ceftazidime bolus. In contrast, 4 h after administration of aztreonam, the bacteria became longer. Thereafter, there was no noticeable difference from the control (Fig. 5). A similar observation was made from an electron micrograph: a slight elongation and a volume increase were seen with aztreonam, while ceftazidime had no apparent effect (Fig. 6O and P). We have also observed with both drugs (Fig. 7), in a limited number of bacteria located in the peripheries of the clots, major abnormalities in the structure of the cell wall: undulations, thickening, irregularities, and breaks.

Of major interest, even in the absence of treatment, is the fact that the morphologies of *P. aeruginosa* were different in the core and in the periphery. This was not observed with the other bacteria.

(ii) *E. cloacae*. At low magnification, we have noticed elongated *E. cloacae*. We observed wool ball-shaped microcolonies with very long, twisted strands of bacteria (Fig. 8). No difference was noticed between morphological alterations either in the peripheries or the central cores of the fibrin clots infected with *E. cloacae*. The sequence in the development of the morphological alterations observed in *E. cloacae* (Fig. 8) after treatment with either aztreonam or ceftazidime was as follows. At 0 h, there was no difference in morphology between bacteria in the control and those in the periphery or the core of a treated, infected clot: colonies of rod-shaped bacteria were seen. At 1 h, the number of thin, stretched bacteria increased: each colony had 2 to 4 spaghetti-like bacteria. At 6 h, wool ball-shaped colonies were abundant, with most bacteria being elongated.

On electron micrographs at 0.5 h, we observed in the peripheries of clots an elongation of some bacteria at the edges of the colonies treated with aztreonam (Fig. 9). At 2, 4, and 6 h on electron micrographs, the noticeable bacterial alterations increased, but in the cores the changes were slower to appear and lagged behind the changes in the peripheries until the

*FIG. 4.* In vivo killing curve of aztreonam (four rabbits) or ceftazidime (four rabbits) against *S. marcescens*. Δ, change in.

*FIG. 5.* Optic micrographs of *P. aeruginosa* observed in the peripheries and the cores of fibrin clots inserted subcutaneously in rabbits following treatment with aztreonam, ceftazidime, or no drugs. Note in the peripheries the elongation of bacteria following treatment with each agent. In the cores, a slight difference exists between growth after the administration of each drug and the limited growth in the control. All pictures reflect a magnification of ×500. No change in morphology was evident in the cores after treatment with ceftazidime, while bacteria were longer at 4 h following treatment with aztreonam.
sampling at 6 h. After 6 h, there was less difference between the levels of alterations in the peripheries and cores. There was a major difference between the two treatments, as we could observe many more spheroblasts following treatment with ceftazidime. The spheroblasts and destroyed bacteria were present within 2 h in the animals treated with 100 mg/kg. In previous experiments, using a lower dose of 50 mg/kg, we did not see spheroblasts but only the elongation of bacteria (data not shown).

**DISCUSSION**

Our model allows for the evaluation of the pharmacodynamic interactions between antibiotics and bacteria embedded in fibrin clots. Fibrin, which participates in tissue repair and in the inflammatory process, creates a protective environment for bacteria which favors the survival of pathogens. Moreover, fibrin clots are extremely hard to penetrate and the amount of an antibiotic that reaches the clots varies from one antibiotic to
the next and may be affected by several factors, including the route of administration of antibiotics and protein binding (1, 16). Both aztreonam and ceftazidime do not bind avidly to serum (50 versus 35%) (7, 10), explaining in part their good diffusion into fibrin clots. The in vitro activity of ceftazidime against \textit{P. aeruginosa} was better than that of aztreonam, but both agents had similar in vitro activities against \textit{E. cloacae} and \textit{S. marcescens} at an inoculum of $10^5$ CFU/ml. At a higher inoculum, the MIC of aztreonam against \textit{E. cloacae} was greater than that of ceftazidime.

The tissue pharmacokinetics of both agents were different. Ceftazidime had much greater peak concentrations in serum and fibrin clots than aztreonam, and differential pharmacokinetics in the peripheries and cores of the fibrin clots were observed. In the cores of the clots, the peak levels of both drugs were much lower than those observed in the peripheries and in serum. From 2 h on, the levels in clots were much higher than those in serum and were detectable even 24 h after therapy. Aztreonam's half-life within the peripheries and the cores of the fibrin clots was up to six times higher than that observed in serum, while ceftazidime's half-life in clots was twice that in serum. This resulted in a much greater penetration ratio for aztreonam than for ceftazidime. We have previously shown such differential antibiotic distributions between the peripheries and the cores of fibrin clots (3). Moreover, there was no difference in the tissue pharmacokinetics of both agents whether clots were infected with \textit{P. aeruginosa}, \textit{E. cloacae}, or \textit{S. marcescens}, suggesting that local infection does not disturb the diffusion of these agents in fibrin.

Although the levels of antibiotics observed in the cores were lower than those in the peripheries, they were above the MICs of the pathogens for at least 4 h, thus explaining the apparently similar in vivo activities in both areas. Although the colony counts between peripheries and cores were not statistically different, the log number of CFU was in general 1 log lower in the peripheries than in the cores. Morphological changes, as determined by light microscopy, also revealed that the activities of aztreonam and ceftazidime were less striking in the cores than in the peripheries. While elongated bacteria that were most likely associated with the specific activities of both agents against PBP3 were abundant in the peripheries, no such observations were made within the first 4 h in the cores of fibrin clots infected with \textit{P. aeruginosa} and treated with ceftazidime. In contrast, aztreonam did induce morphological changes in the cores. It is possible that \textit{P. aeruginosa}, a pure aerobic nonfermenter, could not respond to antibiotics as well in the cores of fibrin clots as in the peripheries. The morphology of the bacteria in the control group was different from that of the bacteria in the peripheries of clots, suggesting that this bacterium might be inhibited by a less proper environment. In these experiments, the $O_2$ levels were not determined in the cores of the clots. With \textit{E. cloacae}, the morphological changes were identical in both parts of the fibrin clots.

The EM studies done with \textit{P. aeruginosa} were also quite revealing. They confirmed the fact that the activities of antibiotics against the bacteria located within the cores of the clots were less efficient. Moreover, morphological changes associated with aztreonam seemed different than those of ceftazidime. Along with elongation of bacteria, more bow tie and herniated bacteria were observed with aztreonam. Though both agents selectively affect PBP 3, as manifested by elongated bacteria, they induce, in the peripheries of the clots, thickening of and breaks and detachment in bacterial cell walls.
alterations which are generally associated with antibiotics affecting PBP 1a and 1b.

While both aztreonam and ceftazidime seemed to have resulted in similar morphological changes in *E. cloacae* as determined by light microscopy, EM revealed that ceftazidime was much more bactericidal than aztreonam, as it induced more empty cells (dead cells).

After the administration of ceftazidime, activities against *P. aeruginosa* and *S. marcescens* persisted for 24 h while regrowth of *E. cloacae* was seen within 6 to 8 h. Interestingly enough, aztreonam continued to inhibit *P. aeruginosa* for several hours (≥16 h), even when the concentrations within the peripheries and the cores of the fibrin clots were lower than the MICs against these bacteria. This suggests the possibility of an in vivo postantibiotic effect. In general, the number of CFU/ml or g of infected tissue from a patient is greater than 10⁵ CFU/g. For most infections where host defenses are adequate, the MIC of an antibiotic determined for 10⁵ CFU/ml generally correlates with the antibiotic’s in vivo activity. In our model, where host defenses are limited, the inoculum may influence the activity of the antibiotic.

This pharmacodynamic analysis reveals that fibrin is a protected environment where diffusion of antibiotics varies depending on the agent. Both agents penetrated well the infected fibrin clots, but the penetration ratios and half-lives of aztreonam in the cores and peripheries of the clots were greater than those of ceftazidime. In this model, aztreonam was successful in controlling the growth of *P. aeruginosa* but the response was slower than with ceftazidime. Both *E. cloacae* and *S. marcescens* responded better to ceftazidime. Aztreonam may have induced an in vivo postantibiotic effect against *P. aeruginosa*. Both drugs caused morphological changes compatible with an action on PBP 3 but also on PBP 1a and 1b, for which the drugs have a limited affinity.

As fibrin is an integral component of intravascular thrombi, surgical wounds, and hematomas, which are often infected with the pathogens evaluated in our study, the differential tissue distribution of antibiotics within fibrin and the limited activities...
of the antibiotics that we have observed within the cores of the clots may explain some of the therapeutic failures observed in patients suffering from the above-described infections and possibly the slow therapeutic response seen in bacterial endocarditis.

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REFERENCES

