Bactericidal Effects of Ticarcillin-Clavulanic Acid against *Legionella pneumophila* Pneumonia in Immunocompromised Weanling Rats

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A model of acute *Legionella pneumophila* pneumonia in neutropenic weanling rats was developed as a means of assessing the efficacies in vivo of the β-lactams ticarcillin, ticarcillin-clavulanic acid, and clavulanic acid, agents active against the organism in vitro. Weanling rats were dosed with cyclophosphamide 3 days before and immediately prior to infection by intrabronchial intubation with *L. pneumophila*. The bacteria persisted in the lungs of untreated animals at high counts (5.0 to 7.0 log_{10} CFU/g of lung tissue) for up to 168 h after infection, and the histological characteristics of the infection were similar to those of the disease in humans. Transmission electron micrography revealed the presence of *L. pneumophila* multiplying within alveolar macrophages. Therapy with ticarcillin was ineffective in reducing the bacterial numbers in the lung tissue, whereas ticarcillin-clavulanic acid and clavulanic acid were active, producing bactericidal effects similar to those of erythromycin. The ticarcillin-clavulanic acid combination was significantly more efficacious (P < 0.01) than corresponding doses of clavulanic acid alone. Synergistic activity between ticarcillin and clavulanic acid against *L. pneumophila* has been demonstrated in vivo, and the combination showed activity similar to that of erythromycin.

The β-lactamase inhibitor clavulanic acid displays only a low order of antibacterial activity against most pathogenic bacteria, but an exception is *Legionella pneumophila*, which is susceptible to low concentrations of the compound (16). In addition, synergy has been demonstrated between β-lactams and clavulanic acid in vitro (8, 10), and both the inhibitor and the combination of amoxicillin and clavulanic acid have been shown to produce bactericidal effects against intracellular *L. pneumophila* in tissue culture studies in vitro (17) and in immunocompetent rats (13).

These findings suggested that studies with antibiotics against experimental *L. pneumophila* infections were warranted, but initial studies with clavulanic acid combinations showed that the conventional guinea pig model of legionellosis was unsuitable for this purpose because of the sensitivity of this animal species to β-lactam antibiotics. For this reason, we have developed an experimental model of *L. pneumophila* pneumonia with neutropenic weanling rats, suitable for the evaluation of β-lactams and other agents.

The studies reported here were designed to investigate the activity of clavulanic acid and the combination of ticarcillin plus clavulanic acid against *L. pneumophila* in vitro and in the neutropenic rat model of legionellosis.

**MATERIALS AND METHODS**

**Antimicrobial agents.** Erythromycin lactobionate (Erythroxin; Abbott Laboratories, Queensborough, Kent, England) was a commercial preparation. Ticarcillin disodium and potassium clavulanate were supplied by SmithKline Beecham Pharmaceuticals, Woking, Sussex, England.

**Microorganisms.** *L. pneumophila* 1624, serogroup 1, a β-lactamase-producing clinical isolate (supplied by Julian Dennis, Porton Down, Wiltshire, England) was used in tissue culture studies in vitro (16) and in vivo studies with immunocompetent rats (13). A control strain, *L. pneumo-

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philae NCTC 11192, was included in the serial dilution tests for comparative purposes.

**In vitro susceptibility tests.** MICs were determined by serial dilution of the compounds into buffered charcoal-yeast extract agar (Oxoid) (1). Inocula were prepared by harvesting growth of *L. pneumophila* on agar plates after 72 h of incubation into 1 ml of Mueller-Hinton broth (BBL) and adjusting the turbidity to that of a 0.5 McFarland barium sulphate turbidity standard. Volumes (0.003 ml) were inoculated onto each plate, resulting in an inoculum of approximately 5.5 log_{10} CFU per spot. The MIC was determined after 48 h of incubation at 37°C as the lowest concentration to completely inhibit growth of the cultures. The interactions between ticarcillin and clavulanic acid against *L. pneumophila* were measured by the agar dilution checkerboard method, and synergy between the two agents was defined as a fourfold increase in the antibacterial activity of each component.

**Animals.** Weanling male rats (60 to 80 g), CD strain, were supplied by Charles River UK Ltd., Manston, Kent, England.

**Preparation of infective inoculum.** *L. pneumophila* 1624 was grown on buffered charcoal-yeast extract agar for three days at 37°C, and the growth was resuspended in Mueller-Hinton broth. The suspension was standardized by using a nephelometer and diluted 1:1,000 to yield a count of 4.0 to 5.0 log_{10} CFU/ml.

**Cyclophosphamide treatment.** Rats were dosed intraperitoneally with cyclophosphamide (Endoxana; Boehringer Ingelheim Ltd., Bracknell, Berkshire, England) at 50 mg/kg of body weight 3 days before and immediately prior to infection, and leukocyte (WBC) counts were taken daily during the experimental period to monitor the level of neutropenia in the cyclophosphamide-treated animals in comparison with that in normal rats. Groups of five treated and five non-treated rats were sacrificed daily from days 0 to 7, and blood samples were taken from the posterior vena cavae. For each rat, the trachea was exposed and the lungs were lavaged twice with 10 ml of sterile 0.9% phosphate-buffered saline. WBC counts from the blood and lavage fluid were

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determined by using a Coulter Counter (Coulter Electronics Ltd., Luton, Bedfordshire, England). A number of studies indicated, that the third dose (50 mg/kg) of cyclophosphamide was administered at the end of the therapy period, 96 h after infection.

**Infection of rats by intrabronchial instillation.** Rats were anesthetized with fentanyl fluaniinone (Hypnorm [0.01 ml/kg]; Janssen Pharmaceuticals Ltd., Grove, Oxfordshire, England) and diazepam (Valium [0.5 mg/kg]; Roche Products Ltd., Welwyn Garden City, Hertfordshire, England). The animals were placed on their backs and infected by nonsurgical intrabronchial intubation with a metal cannula, through which was passed a plastic intravenous cannula, allowing intrabronchial instillation of a 0.05-ml volume of the inoculum (4.0 to 5.0 log_{10} CFU). The method of infection used in these studies was that reported by Smith (12), which was previously used to initiate a mixed *Streptococcus pneumoniae*-Staphylococcus aureus respiratory infection in rats (14) and has since been used for the production of *L. pneumophila* pneumonia in this species (13).

Initial experiments involved groups of 18 animals infected with *L. pneumophila* 1624 and treated from 6 h to 4 days postinfection, with six animals from each group taken at intervals up to 7 days for assessment of therapy. Subsequently, groups of 10 infected animals were treated for 4 days, and lung samples were taken at 4 or 10 days postinfection. Subcutaneous 0.5-ml doses of ticarcillin, clavulanic acid, and ticarcillin-clavulanic acid were given four times daily, and doses of erythromycin were given three times daily.

Lungs were removed aseptically and homogenized in 2 ml of Mueller-Hinton broth in a Colworth stomacher (A. J. Seward and Co. Ltd., London, England) for 2 min. The homogenates were serially diluted and plated onto buffered charcoal-yeast extract agar plates containing BIMA selective supplement (Oxoid) (1) and incubated at 37°C for 3 days for enumeration.

**Histology and transmission electron microscopy.** Specimens for histology were obtained at each time point and fixed in buffered formal saline before being processed to wax blocks by conventional means. Hematoxylin and eosin sections were prepared. In order to determine the distribution of *L. pneumophila* in these sections, indirect immunogold labeling and silver enhancement were used. Briefly, dewaxed sections were labelled with primary monoclonal mouse immunoglobulin G antibody to *L. pneumophila* (Sera Lab Ltd., Crawley Down, Sussex, England). A secondary gold probe (goat anti-mouse immunoglobulin G–1 nm gold; Janssen, Beerse, Belgium) was then applied and amplified by silver intensification with commercial reagents (Biocell Silver Enhancer; Biocell Research Laboratories, Cardiff, Wales, United Kingdom). The sections were hematoxylin and eosin stained before examination by ordinary bright-field and epipolarizing optics by using a Zeiss Axiosplan microscope and a 63× oil immersion objective.

Specimens for transmission electron microscopy were excised as 1-mm³ pieces and were fixed in 2.5% glutaraldehyde buffered with 100 mM sodium cacodylate, pH 7.2. To improve subsequent immunolabelling, osmium postfixation was avoided. Samples were dehydrated via an ethanol series and infiltrated with LR White acrylic resin (London Resin Co., Woking, Surrey, England), which was cured at 52°C for 16 h. Indirect immunogold labelling and staining of ultrathin
RESULTS

In vitro susceptibility. Agar dilution studies showed that the test strains of \textit{L. pneumophila} were susceptible to ticarcillin (MIC, 4 to 8 \( \mu \)g/ml), clavulanic acid (MIC, 0.25 \( \mu \)g/ml), and erythromycin (MIC, 0.5 \( \mu \)g/ml). Combinations of ticarcillin plus clavulanic acid resulted in MICs of 1 + 0.03 \( \mu \)g/ml for \textit{L. pneumophila} 11192 and 2 + 0.03 \( \mu \)g/ml for \textit{L. pneumophila} 1624, demonstrating synergy (\( \Sigma \) fractional inhibitory concentration, 0.38).

Histology and transmission electron microscopy. At 6 h postinfection, there was no evidence of an inflammatory response, but by 48 h postinfection, considerable consolidation of the lung tissue had occurred. Polymorphonuclear WBCs and macrophages typified the inflammatory tissue (Fig. 1). Immunogold-silver labelling demonstrated that clusters of \textit{L. pneumophila} were consistently associated with these cell types. The intense signal provided by this technique facilitated close observation at high power (950×), and this demonstrated the presence of the bacteria adjacent to host cell nuclei (Fig. 2). By 96 h postinfection, large contiguous regions of inflammation were evident and fibrin deposits were common. Bacterial clusters remained commonly associated with phagocytic cell types. The intracellular nature of the pathogen was confirmed by transmission electron microscopy. Sections of polymorphs and macrophages revealed bacteria within dilated cytoplasmic vesicles at 48 and 96 h after infection (Fig. 3).

Neutropenia. Figure 4 shows the WBC counts from the blood and alveolar lavage fluid from normal rats and rats treated with cyclophosphamide as described previously. Cyclophosphamide reduced the number of WBCs from 3.8 log\(_{10}\) cells per mm\(^3\) (normal rats) to 3.3 log\(_{10}\) cells per mm\(^3\) for the first four days of the infection, after which time the WBC counts returned to normal. There was no difference in the numbers of WBCs recovered from the lung lavage fluid of untreated and cyclophosphamide-treated animals.

Distribution in rats. The concentrations of ticarcillin and clavulanic acid measured in the plasma and lung homogenates of rats given a single subcutaneous dose (200 + 40 mg/kg) of the ticarcillin-clavulanic acid combination are shown in Table 1. The peak plasma concentration of ticarcillin measured in rats at 15 min was of the same order as that obtained after a 3.0-g infusion of ticarcillin in humans, but thereafter the plasma concentrations and consequently the
elimination half-life in rats ($t_{1/2}$, 15.6 min) were much lower than those in humans ($t_{1/2}$, 52.5 min). In the case of clavulanic acid, the plasma concentrations in rats were higher initially than those in humans, but the compound was eliminated more rapidly from the rats, and clavulanic acid was not detectable 2 h after dosing. For this reason, the dosing schedule was designed to produce in the infected rats areas under the concentration curves of ticarcillin and clavulanic acid similar to those in humans (7). The concentration profiles of ticarcillin and clavulanic acid in lung homogenates were similar to those in rat plasma, but the homogenate concentrations were on average approximately one quarter of the corresponding values in plasma.

**Therapy.** In neutropenic rats, *L. pneumophila* 1624 produced a severe, nonfatal pneumonia that persisted for up to 7 days after infection in the lungs but that did not produce bacteremia. The infection generally reached a peak by 48 to 96 h, with nontreated animals showing signs of weight loss, dyspnea, nasal discharge, and ocular discharge with hemoporphyrin-stained exudate. Postmortems involving macroscopic examination of the lungs revealed inflammation and marked consolidation of lung tissue. After 96 h, the WBC counts returned to normal, resulting in a natural resolution of the infection. Administration of a third dose of cyclophosphamide at 96 h resulted in a prolongation of the infection, the bacterial lung counts of *L. pneumophila* remaining at about $6 \times \log_{10}$ CFU/g of lung tissue until 168 h before the infection began to resolve.

Data in Fig. 5 show the counts of *L. pneumophila* 1624 in the lungs of infected animals after therapy with ticarcillin, ticarcillin-clavulanic acid, clavulanic acid, and erythromycin. In untreated rats, the bacterial numbers fell from an initial count of $5.7 \times \log_{10}$ CFU/g of lung tissue immediately after infection to $2.9 \times \log_{10}$ CFU/g of lung tissue at 6 h, after which time the counts increased to a maximum of $8.0 \times \log_{10}$ CFU/g of lung tissue at 96 h. After this time, the bacterial numbers fell as the organisms were cleared from the body after the increase in WBCs to normal values. This pattern was common in all studies in which the animals were treated with cyclophosphamide twice before infection. Ticarcillin suppressed the bacterial numbers during the first 48 h but thereafter the counts increased rapidly, and at 96 h after infection there was no significant difference ($P > 0.1$) in the counts of the ticarcillin-treated group and the untreated control animals. Ticarcillin-clavulanic acid and erythromycin produced similar effects, suppressing bacterial growth for the duration of the test. Clavulanic acid at the dose administered (40 mg/kg) was less effective than ticarcillin-clavulanic acid, the difference being significant ($P < 0.01$) at 96 h.

The results of three studies are summarized in Table 2, which reports the mean counts of *L. pneumophila* 1624 96 h
after infection, the time of maximum counts in infected animals prior to the recovery of WBCs to normal values. The data show that ticarcillin at the doses administered (200 and 600 mg/kg) was ineffective and that the bacterial counts were not significantly different from those in the untreated animals. Ticarcillin-clavulanic acid (200 + 10 mg/kg) was no more efficacious than ticarcillin alone, but all other combinations containing 20, 40, or 100 mg of clavulanic acid per kg were significantly more active (P < 0.01) than ticarcillin alone. Likewise, in most cases, the ticarcillin-clavulanic acid combinations containing 20 or 40 mg of clavulanic acid per kg were significantly more effective (P < 0.01) than the corresponding doses of clavulanic acid and were as effective as erythromycin in reducing the bacterial lung counts.

In the study whose results are depicted in Fig. 6, the rats were treated at 96 h with a third dose of cyclophosphamide to prolong the neutropenia and thereby delay the rate of clearance of *L. pneumophila* from the lungs of infected animals. At 96 h, bacterial counts from the lungs of rats dosed with ticarcillin-clavulanic acid or erythromycin were significantly lower than those from the nontreated controls (P < 0.01). However, by 168 h, 3 days after cessation of dosing, and up to 240 h postinfection, bacterial numbers present in the erythromycin-treated group were higher than those in the ticarcillin-clavulanic acid-treated group, although some regrowth of the organism was seen in all treated groups after the end of the dosing period.

**DISCUSSION**

It is widely recognized that β-lactam antibiotics may demonstrate high levels of antibacterial activity against *L. pneumophila* in serial dilution tests in vitro (8, 10). However,
TABLE 2. Counts of *L. pneumophila* 1624 from rat lung tissue 96 h after infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Study 1</th>
<th>Study 2</th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>6.1 ± 1.5</td>
<td>6.6 ± 1.1</td>
<td>7.4 ± 1.3</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>200</td>
<td>5.3 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.9 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ticarcillin + clavulanic acid</td>
<td>200 + 10</td>
<td>5.0 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200 + 100</td>
<td>2.4 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600 + 20</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6 ± 0.8&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>3.1 ± 1.1&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600 + 40</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 0.8&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>2.4 ± 1.1&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clavulanic acid</td>
<td>10</td>
<td>5.9 ± 1.0</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.1 ± 1.1</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.0 ± 1.3</td>
<td>4.0 ± 1.0</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.2 ± 1.3</td>
<td>2.4 ± 0.7</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.9 ± 0.8</td>
<td>2.1 ± 0.8</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Mean counts from groups of 10 animals. NT, not tested.
<sup>b</sup> Not significantly different (*P* > 0.1) from untreated controls.
<sup>c</sup> Significantly more effective (*P* < 0.01) than ticarcillin.
<sup>d</sup> Significantly more effective (*P* < 0.01) than corresponding dose of clavulanic acid.

the significance of this activity is often disregarded on the grounds that β-lactams penetrate mammalian cells to a limited extent and would not be effective in vivo against *L. pneumophila*, which multiplies within phagocytic cells (4, 6).

Counter to this, we have reported that clavulanic acid and the combination of amoxicillin plus clavulanic acid produced potent bactericidal effects in vitro against *L. pneumophila* growing within human fetal lung fibroblast cells (17). In subsequent studies in our laboratory (unpublished data), clavulanic acid, amoxicillin-clavulanic acid, and ticarcillin-clavulanic acid were active against intracellular *L. pneumophila* in the tissue culture system, whereas amoxicillin, ticarcillin, and other β-lactams—ampicillin, cefoxitin, ceftazidime, and sulbactam—which were active in vitro were ineffective in tissue culture.

The model of nonfatal acute pneumonia produced in rats after intrabronchial inoculation of *L. pneumophila* has been described as resembling, in some respects, the disease in...
humans (18), and we have confirmed this with weanling rats, which we have found to be more susceptible to respiratory infection than adult rats (12). However, with immunocompetent rats, which are comparatively resistant to infection with L. pneumophila, a large inoculum is required to produce an infection, and the organism is cleared relatively rapidly from the animal, presenting a transient infection (13). For this reason, we have attempted to enhance the severity of the infection by rendering the animals neutropenic prior to infection with L. pneumophila: a similar approach has been reported recently by Skerrett, Schmidt, and Martin (11), who used corticosteroids to induce immunosuppression before infection with L. pneumophila. Corticosteroids are an accepted major risk factor, and although neutropenia per se is not, it is generally associated with the many kinds of immunosuppression that predispose humans to legionella infection.

In the studies reported here, L. pneumophila 1624 produced a severe nonfatal pneumonia that persisted in the lungs for up to 4 days after intrabronchial instillation of a small infective inoculum but did not result in bacteremia. Microscopic examination of lung tissue revealed that the organism was residing within polymorphonuclear WBCs and alveolar macrophages at the time of infection, and the histological characteristics of the infection were similar to those of the disease in guinea pigs and humans (3, 5, 9). The bacteria persisted at high numbers in the respiratory tract of the neutropenic rats longer than in immunocompetent animals, and the disease was very reproducible, making the model suitable for the evaluation of antibacterial agents, especially β-lactams.

Therapy of the L. pneumophila respiratory infection with clavulanic acid and with ticarcillin-clavulanic acid resulted in significant reductions in the bacterial numbers in lung tissue. The bactericidal effects observed with effective doses of these agents producing serum concentrations of ticarcillin and clavulanic acid similar to those attained in humans were of the same order as those produced by erythromycin, the control antibiotic. Of interest, in the studies in which a third dose of cyclophosphamide was administered at 96 h, the end of therapy, the bacterial lung counts measured between 96 and 240 h increased to a greater extent in the animals treated with erythromycin than did those in the group treated with ticarcillin-clavulanic acid. In all studies, ticarcillin was ineffective, and the lung counts of ticarcillin-treated animals were similar to those of untreated controls. This suggests that ticarcillin did not penetrate the infected cells sufficiently or was inactivated too rapidly to achieve inhibitory concentrations.

Synergistic effects between ticarcillin and clavulanic acid were observed with checkerboard in vitro tests. Clavulanic acid inhibits the β-lactamase produced by L. pneumophila (2, 8), but it is not established whether this is responsible for the synergy or whether the interaction between two antibacterial agents binding to different penicillin-binding proteins is responsible (15). In vivo, the combination of ticarcillin plus clavulanic acid was significantly more effective than clavulanic acid alone.

The results of these experimental infection studies show that the model of acute L. pneumophila pneumonia in weanling rats is suitable for the assessment of the efficacy of β-lactam antibiotics. The findings that ticarcillin-clavulanic acid was as efficacious as erythromycin in reducing the bacterial lung counts of infected animals suggests that this broad-spectrum antibiotic combination is worthy of clinical trial for the treatment of respiratory infections due to L. pneumophila.

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