Relevance of Serum Protein Binding of Cefoxitin and Cefazolin to Their Activities against Klebsiella pneumoniae Pneumonia in Rats

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An experimental Klebsiella pneumoniae pneumonia in rats was used to study the effect of protein binding of cefoxitin and cefazolin on their therapeutic activity. Both cefalosporins were similar with respect to their antimicrobial activity with K. pneumoniae in vitro, but they differed in their degree of protein binding, being 34% and 89% for cefoxitin in uninfected rats and 24 and 71 to 83%, respectively, in infected rats. Various doses of these agents were administered by continuous infusion, which started 5 h after bacterial inoculation and continued for 65 h. Antimicrobial response was evaluated with respect to the numbers of bacteria recovered from lung and blood at the end of treatment. An inhibitory effect of protein binding on the in vivo antimicrobial activity was demonstrated. Cefoxitin was therapeutically effective at a constant plasma level that reached the MIC. To obtain a similar effect with cefazolin, the plasma level of that drug had to be increased to a concentration more than three times the MIC.

Binding of antimicrobial agents to serum proteins and tissues affects their distribution, elimination rate, and microbiological activity (10, 22, 24, 32, 33). Only the free, unbound fraction can pass into the extravascular compartment or be excreted by the kidneys. Protein-bound antibiotic has no antibacterial activity. In spite of extensive coverage of this subject in the literature, the clinical significance of this phenomenon is still controversial. The effect of serum protein binding on the rate at which antibiotics diffuse out of the vascular compartment into the tissues has been studied in various animal models (6, 15, 21, 29, 31). The actual concentration of active antibiotic at the site of infection is also determined by tissue binding and the severity of the infection (6, 10, 14). Binding varies from tissue to tissue. In severe infections with hypoproteinemia, the percentage of unbound antibiotic in serum is increased (8, 28). There has been limited experimental work on the relevance of protein binding of antibiotics to therapeutic efficacy (17–19, 25). As will be discussed below, these studies have also yielded somewhat contradictory results.

In the work reported here, we compared the therapeutic activity of cefoxitin and cefazolin in rats with experimental pneumonia induced by Klebsiella pneumoniae. Both cephalosporins are similar with respect to their microbiological activity against K. pneumoniae in vitro but differ in their degree of protein binding in uninfected and infected rats. Because the half-life of the antibiotic in plasma may be as important as the protein binding in determining the degree of extravascular tissue distribution, the cephalosporins were administered in various doses by way of continuous infusion.

MATERIALS AND METHODS

Animals. Female R strain albino rats (specific pathogen free; 14 to 18 weeks old; weight, 185 to 215 g; bred at the REP-Institutes TNO, Rijswijk, The Netherlands) were used in all experiments.

Bacteria. A K. pneumoniae strain (capsular serotype 2) was used in these experiments. Inocula were prepared as described previously (2).

Antimicrobial susceptibility tests. Cefoxitin was obtained from Merck Sharpe & Dohme, The Netherlands, and cefazolin was obtained from Eli Lilly, S.A., St. Cloud, France. The MICs of the respective drugs were defined as the lowest concentrations that suppressed visible growth after incubation of an inoculum of 10⁶ CFU/ml for 18 h at 37°C in tubes containing 4 ml of Todd-Hewitt broth (Oxoid Ltd., London, England) (3). Logarithmic-phase cultures of bacteria were used. The MBCs were defined as the lowest concentrations that killed 99.95% of the original inoculum (3). MBCs were determined by spreading subculture volumes of 0.01 ml onto Iso-Sensitest agar (Oxoid) plates. The concentrations of the serial dilutions decreased by steps of 0.4 μg/ml in a range extending from 6.4 to 0.4 μg/ml. The effect of the two drugs on the short-term growth in vitro of the K. pneumoniae strain was determined as follows. A stationary-phase culture which had been incubated for 16 h at 37°C was diluted in Iso-Sensitest broth (Oxoid) to a concentration of 5 × 10⁶ CFU/ml. After reincubation for 2 h at 37°C the antibiotic was added at concentrations of 3.2 and 6.4 μg/ml at time zero. The numbers of viable organisms were then determined at regular intervals over a 4-h period by plate counts on Iso-Sensitest agar. Before plating, antibiotic was inactivated by the addition of beta-lactamase (Whatman Biochemicals Ltd., Miles Laboratories). In a second series of experiments the effect of rat serum (50 or 90%) on the in vitro killing of K. pneumoniae by cefoxitin or cefazolin at a concentration of 6.4 μg/ml was determined. Serum and antibiotic were preincubated at 37°C during 1 hour. The capacity of the K. pneumoniae strain to produce

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beta-lactamases that inactivate cefoxitin or cefazolin was investigated by the method described by van de Klundert et al. (J. M. van de Klundert, M. Van Gestel, E. Van Doorn, and R. P. Mouton. Proc. 13th Int. Congr. Chemother. p. 1-4, 1983).

**Pneumonia.** Experimental pneumonia was produced as described previously (2). In brief, rats were anesthetized with Hypnorm (Duphar B.V., Amsterdam, The Netherlands) and pentobarbital (Abbott Laboratories, North Chicago, Ill.). The left main stem bronchus was intubated, and the left lobe of the lung was inoculated with 0.02 ml of a saline suspension of *K. pneumoniae* containing 8 x 10^6 (6 x 10^6 to 10 x 10^6) CFU. After bacterial inoculation the necrotic antagonists Nalorphine bromide and Pentetrazolm (Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands) were injected. Infection developed within 24 h and increased in severity afterwards. Untreated infections terminated fatally within 5 to 6 days.

**Antimicrobial treatment.** Cefoxitin or cefazolin was administered as a continuous infusion over a period of 65 h. Doses of 5.25 mg of cefoxitin per kg per h or 0.68, 1.59, or 2.31 mg of cefazolin per kg per h were administered to noninfected and infected rats. Treatment was started 5 h after bacterial inoculation. Because intravenous infusion techniques did not function properly over such a long period, an alternative technique, developed by Thonus et al. (27), was used. In brief, 6 weeks prior to its use a tissue cage constructed from perforated Teflon (length, 40 mm; outer diameter, 7 mm; inner diameter, 5 mm; 30% perforation) connected with 50 mm of polyethylene tube was implanted subcutaneously. Rats were anesthetized prior to treatment with antibiotics. The distal portion of the polyethylene tube was recovered via an incision in the neck of the animal and connected with a polyethylene tube and a swivel to a 12 ml syringe. This syringe was placed on a Varifluxor pump (Breda Scientific, Breda, The Netherlands). The cephalosporin at the required concentration was infused at a constant rate of 0.116 ml/h. By this technique prolonged steady-state levels were reached within 3 h after the start of the infusion.

**Therapeutic results.** Response to antimicrobial treatment was evaluated with respect to the numbers of bacteria in the lung (left lobe) and in blood and pleural fluid at the time of sacrifice of the rats, i.e., 70 h after bacterial inoculation. Samples of blood and pleural fluid were obtained for culture. Pleural exudate was obtained by washing the chest cavity with physiological saline (0.5 ml). After macroscopic examination, the left lung was removed and homogenized in 20 ml of physiological saline containing beta-lactamase (Vir'tis homogenizer; 30 s at 10,000 rpm). Serial 10-fold dilutions of homogenates and blood in saline were prepared, and 0.2-ml volumes of each dilution were spread on blood agar plates. The numbers of viable organisms in the residual homogenate of the left lung were measured by the pour-plate method.

**Statistics.** The Wilcoxon rank-sum test was used to estimate whether results of quantitative cultures of the left lung in antibiotic-treated rats or untreated rats differed significantly from each other.

**Measurement of antibiotic concentrations in serum.** Blood specimens were taken from the heart of noninfected and infected rats, and serum was separated. Cefoxitin or cefazolin in serum was measured via a modified high-pressure liquid chromatographic method (2). We used the high-pressure liquid chromatographic method because standard curves cannot be prepared in fluid with protein concentrations equal to those of the unknown samples of antibiotic-treated, infected rats (23). Separation was carried out by reverse-phase partitioning on a column of with an internal diameter of 4.6 mm and a length of 15 cm packed with Lichrosorb RP-8 (Merck) with a 5-μm particle size. The eluent was a 1:1 mixture of 0.02 M sodium acetate-15% methanol. Column elution was carried out with a flow of 1.4 ml/min at a pressure of 230 atm (23,306 kPa). A Spectra Physics 3500 B high-pressure liquid chromatograph with an SP 770 variable UV detector was used. The eluent was monitored at 256 nm. Standards of known cefoxitin or cefazolin content were made up in pooled rat serum to give concentrations ranging from 0.1 to 10 μg/ml. Volumes of 100 μl of serum were mixed with 400 μl of methanol and centrifuged for 5 min at 4,000 x g; 300 μl of the supernatant was evaporated to dryness. The residue was dissolved in 400 μl of water, and 100 μl was injected directly onto the column. Protein binding of cefoxitin and cefazolin was measured by means of the ultrafiltration technique with EMIT Free Level System I filters (Syva, Palo Alto, Calif.; Merck Darmstadt, Amsterdam, The Netherlands). For determination, 300 μl of serum was used. Centrifugation through the filter was performed in an angle-head rotor at 2,500 rpm for 25 min at 22°C. The filter was exhibited greater than 99% protein retention. Filter binding of cefoxitin or cefazolin was negligible. Both total and free concentrations of antibiotic were measured, and the average protein binding (+ standard deviation) was calculated.

**Measurements of albumin concentrations in serum.** Albu min concentrations in serum were estimated by single radial immunodiffusion as described by Mancini et al. (16). Goat anti-rat/Alb and purified rat serum albumin were purchased from Nordic Immunology (Tilburg, The Netherlands).
result of the difference in protein binding, the body clearance of total drug differed markedly, being $1.90 \pm 0.20$ liter/kg h for cefoxitin and cefazolin, respectively, whereas for both cephalosporins the clearance of unbound drug was not significantly different. An increase in the continuous infusion dose of cefazolin to 1.59 and 2.31 mg/kg per h resulted in steady-state total serum levels of $5.63 \pm 0.75$ and $8.34 \pm 1.19$ µg/ml, respectively, whereas steady-state concentrations of unbound drug were $0.45 \pm 0.06$ and $0.92 \pm 0.13$ µg/ml. Serum protein binding of cefazolin decreased slightly with these increasing serum concentrations. In general, the levels of cephalosporin in serum of infected rats were slightly but not significantly different from those in uninfected rats treated with the same dose. However, infected rats treated with 0.68 mg of cefazolin per kg per h had a serum level of $2.31 \pm 0.27$ µg/ml which was significantly lower ($P = 0.0002$) than the serum level of $3.34 \pm 0.25$ µg/ml in uninfected recipients of the same dose.

Serum protein binding of both cephalosporins was decreased significantly ($P < 0.05$) in infected as compared with uninfected rats (Table 1). For cefazolin the differences in protein binding declined with increasing doses. The concentrations of albumin in the sera of infected rats treated with 5.25 mg of cefoxitin per kg per h or 0.68 mg of cefazolin per kg per h were $21.8 \pm 0.86$ and $23.8 \pm 1.35$ mg/ml, respectively, being significantly lower ($P = 0.009$) as compared with the values for uninfected rats, which had albumin concentrations in serum of $36.3 \pm 0.38$ mg/ml. As a result of infection, body clearance of total drug was unchanged for cefazolin and slightly decreased for cefoxitin; however, clearance of free drug was significantly decreased ($P < 0.05$) to 58 and 42% for cefoxitin and cefazolin, respectively (Table 1).

**Therapeutic efficacy of cefoxitin and cefazolin in experimental pneumonia.** Responses of *K. pneumoniae* pneumonia to cephalosporin treatment are summarized in Table 2. Therapy was started 5 h after bacterial inoculation and continued for 65 h. At 5 h after inoculation (group F) the numbers of CFU in the left lung of untreated rats had increased up to sevenfold; a median number of $5 \times 10^5$ was cultured from the left lung; blood and pleural fluid were sterile. At 70 h (group E) a median number of $10^{10}$ CFU was cultured from the left lung; all rats had bacteria in the blood and pleural fluid. A steady-state total serum concentration of cefoxitin at the MIC and MBC level (group A) resulted in decreased numbers of bacteria cultured from the left lung, which was significantly different from that of untreated controls ($P = 0.0003$). A median number of $4 \times 10^4$ CFU was cultured from the left lung. Only 1 of 10 rats had bacteria in the blood. Cefazolin at a continuous total serum concentration at the MIC and MBC level (group B) did not result in a therapeutic effect. Numbers of bacteria in lung, blood, and pleural fluid were not significantly different ($P = 0.199$) from those in untreated rats. In addition, the numbers of bacteria in the lungs of these cefazolin-treated rats were significantly different ($P = 0.0002$) from those treated with cefoxitin (group A). With an increase in the cefazolin dose, producing a total serum concentration of 5.69 µg/ml (group C), the numbers of bacteria in the lung were slightly decreased but still significantly different ($P = 0.005$) from those in the cefoxitin-treated rats (group A). Treatment of rats with cefazolin at a total level of 11.53 µg/ml in serum (group D) produced a...
therapeutic response similar ($P = 0.212$) to that attained in rats with a steady-state level of 4.17 μg of cefoxitin per ml (group A).

**DISCUSSION**

The effects of serum protein binding on extravascular penetration of antibiotics have been studied with contradictory results in various settings in animals and humans (4, 5, 7, 15, 21, 29, 30, 31, 33, 34). The discrepancies may be explained by differences in the mode and duration of antibiotic administration as shown by Peterson and co-workers, who compared continuous infusions and single-dose treatments (12, 21, 22, 29). In addition they demonstrated that the extent of binding in the extravascular fluid is also an important factor (21). Antibiotics vary in the capacity to bind to different types of tissues and with the degree and severity of infection (6, 9, 10, 14). Moreover hypoalbuminemia associated with severe infections may account for an increase in the percentage of unbound drug (8, 28). Therefore, estimation of concentrations of biologically active antibiotic in the tissues is complicated. The relevance of protein binding of antibiotics to therapeutic efficacy can only be established in experimental infections. For that reason, we studied the effect of protein binding on the therapeutic efficacy of cefoxitin and cefazolin in K. pneumoniae pneumonia in rats. Neither cephalosporin was susceptible to beta-lactamases of K. pneumoniae. The antibacterial activity of both nonprotein-bound cephalosporins against K. pneumoniae in vitro was similar at the concentrations tested. An inhibitory effect of serum protein binding on the in vitro killing of the K. pneumoniae strain was demonstrated for the highly protein-bound cefazolin, but was not observed for cefoxitin with low protein binding. No microbiologically active metabolites were recovered, which is in accordance with other reports (1, 20, 26). However, the protein binding of the cephalospori-

**TABLE 1.** Steady-state serum concentrations, serum protein binding, and total body clearance of cefoxitin and cefazolin in uninfected rats and rats with K. pneumoniae pneumonia

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Dose (mg/kg/h)</th>
<th>Steady-state level (μg/ml) of:</th>
<th>Protein binding (%)</th>
<th>Clearance (liter/kg h) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total drug Unbound drug</td>
<td></td>
<td>Total drug Unbound drug</td>
</tr>
<tr>
<td>Uninfected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>5.25</td>
<td>2.83 ± 0.49 1.85 ± 0.31</td>
<td>34 ± 6.9</td>
<td>1.90 ± 0.27 2.96 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>0.68</td>
<td>3.34 ± 0.25 0.23 ± 0.02</td>
<td>93 ± 1.6</td>
<td>0.20 ± 0.02 2.92 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>1.59</td>
<td>5.63 ± 0.75 0.45 ± 0.06</td>
<td>92 ± 0.9</td>
<td>0.29 ± 0.04 3.58 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>2.31</td>
<td>8.34 ± 1.19 0.92 ± 0.13</td>
<td>89 ± 0.5</td>
<td>0.28 ± 0.04 2.57 ± 0.36</td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>5.25</td>
<td>4.17 ± 0.74 3.17 ± 0.64</td>
<td>24 ± 6.5</td>
<td>1.29 ± 0.22 1.71 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>0.68</td>
<td>2.31 ± 0.27 0.65 ± 0.22</td>
<td>71 ± 10</td>
<td>0.30 ± 0.04 1.19 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>1.59</td>
<td>5.69 ± 1.26 1.19 ± 0.19</td>
<td>79 ± 3.7</td>
<td>0.29 ± 0.06 1.38 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>2.31</td>
<td>11.53 ± 3.09 1.95 ± 0.41</td>
<td>83 ± 1.8</td>
<td>0.21 ± 0.05 1.22 ± 0.20</td>
</tr>
</tbody>
</table>

* Each value represents the mean of 10 rats ± standard deviation.

* Dose/area under the curve. Area under the curve was based on total or unbound serum concentration.

**FIG. 3.** Effect of cefoxitin (6.4 μg/ml) on the growth of K. pneumoniae in broth with 90% serum (A), broth with 50% serum (B), and broth without serum (C). Control growth was in broth with 90% serum (D) and broth without serum (E). Pooled serum from 50 rats was used.

**FIG. 4.** Concentrations of antibiotic in serum at various intervals after administration of different doses in continuous infusion (infusion rate, 0.116 ml/h) to infected rats. Each point represents the mean of five rats ± standard deviation. Group A, cefoxitin (5.25 mg/kg per h); group B, cefoxitin (0.68 mg/kg per h); group C, cefoxitin (1.59 mg/kg per h); group D, cefoxitin (2.31 mg/kg per h).
TABLE 2. Efficacy of cefoxitin or cefazolin regimens after administration of various doses in continuous infusion of K. pneumoniae

<table>
<thead>
<tr>
<th></th>
<th>Bacterial recovery in the following*</th>
<th>Blood (log_{10} CFU/ml of blood)</th>
<th>Pleural fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B C D E F</td>
<td>A B C D E F</td>
<td>A B C D E F</td>
</tr>
<tr>
<td>4.16</td>
<td>9.26 4.38 3.26 7.05 5.00</td>
<td>0 0 0 0 1.7 0</td>
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</tr>
<tr>
<td>4.34</td>
<td>9.43 4.71 3.27 9.60 5.26</td>
<td>0 0 0 0 3.5 0</td>
<td></td>
</tr>
<tr>
<td>4.37</td>
<td>9.72 8.95 3.87 9.79 5.60</td>
<td>0 3.0 0 5.9 0</td>
<td></td>
</tr>
<tr>
<td>4.43</td>
<td>9.88 9.68 3.96 10.00 5.61</td>
<td>1.9 2.0 0 4.3 0</td>
<td></td>
</tr>
<tr>
<td>4.62</td>
<td>9.64 9.73 4.26 10.11 5.64</td>
<td>2.5 2.3 0 4.5 0</td>
<td></td>
</tr>
<tr>
<td>4.66</td>
<td>10.04 9.77 4.57 10.21 5.68</td>
<td>3.2 3.9 0 3.6 0</td>
<td></td>
</tr>
<tr>
<td>5.17</td>
<td>10.05 9.80 4.68 10.40 5.72</td>
<td>2.7 2.9 0 5.5 0</td>
<td></td>
</tr>
<tr>
<td>6.45</td>
<td>10.08 9.86 5.28 10.52 5.72</td>
<td>2.1 2.9 0 ND 0</td>
<td></td>
</tr>
<tr>
<td>8.19</td>
<td>10.15 9.90 7.06 10.52 5.72</td>
<td>3.6 2.5 0 ND 0</td>
<td></td>
</tr>
<tr>
<td>9.12</td>
<td>10.34 10.23 7.69 10.52 5.88</td>
<td>2.3 3.9 3.5 0 ND 0</td>
<td></td>
</tr>
</tbody>
</table>

* Infusion rate (0.116 ml/h) was started 5 h after inoculation of the left lung with 8 x 10^8 CFU of K. pneumoniae. Bacterial recovery in lung, blood, and pleural fluid 70 h after inoculation is indicated.

** Treatment groups are as follows. 70 h after inoculation: group A, cefoxitin (5.25 mg/kg per h); group B, cefazolin (0.68 mg/kg per h); group C, cefazolin (1.59 mg/kg per h); group D, cefoxitin (2.31 mg/kg per h); group E, untreated. 5 h after inoculation: group F, untreated.

rins in rat serum differed considerably. Because factors such as concentrations of antibiotic and protein, temperature, rate of binding, and pH affect the extent of protein binding (32), we measured the degree of protein binding of the cephalosporins in both uninfected and infected rats at steady-state serum concentrations reached during the treatment doses. We found a striking difference in the clearance of cefoxitin and cefazolin based on total concentrations in serum. This may explain why the daily dose of cefoxitin needed to obtain a steady-state serum concentration of about 3 μg/ml was substantially greater than that of cefazolin. Kirby and Regamey (13) also have demonstrated that the half-life of cephalosporins in serum increased with increasing degree of protein binding. However this correlation was not observed by Wise et al. (34) and Dudley and Nightingale (11). The elimination rate of unbound cefoxitin or cefazolin in uninfected rats was similar. In infected rats, however, the clearance of unbound drug was significantly decreased for both cephalosporins. The infection with K. pneumoniae resulted in a significant decrease of protein binding for both drugs, with that for cefazolin being dependent on the dose administered. This may be explained by the hypoalbuminemia due to the infection. With most antibiotics binding in serum involves the albumin fraction. At a dose of 0.68 mg of cefazolin per kg per h the percentage of unbound cefazolin increased from 7 to 29% as a result of a 65% decrease in serum albumin concentration. As a result the rate of elimination of cefazolin from serum increased. This could explain why at the same cefoxitin dose the steady-state serum concentration of cefoxitin in infected rats was significantly lower than the serum concentration of uninfected rats. The data illustrate the importance of measuring the degree of protein binding in vivo during the course of infection.

The results on antibiotic treatment demonstrate an inhibitory effect of protein binding on the in vivo antimicrobial activity. Thus, cefoxitin was therapeutically effective at a sustained plasma level that approximated the MIC, whereas a similar response with cefazolin required a plasma concentration three of more times the MIC. Therapeutically effective antibiotic doses relate more closely to the concentrations of the unbound antibiotic than to the concentrations of total drug, even if concentrations in plasma are maintained constant during the period of treatment. However, at the therapeutically active doses the concentration of unbound cefoxitin in serum is still lower than the concentration of unbound cefazolin, which may suggest that the difference in therapeutic activity of both cephalosporins may not be due solely to a difference in their binding to serum proteins. Our data are in agreement with the experimental studies of Merrikin et al. (18) in mice with intraperitoneal Staphylococcus aureus infection, in which isoxazolylpenicillins of similar activity and pharmacology were compared. Therapeutic activity was diminished as a result of protein binding. This finding was also confirmed by Mückter et al. (19) in similar studies with cicaclillin and dicloxacillin. However, in renal infections caused by S. aureus in mice, Mattie et al. (17) have demonstrated that the therapeutic effect of nafillin was superior to that of cloxacillin, which is in contrast with what one would expect on the basis of the protein binding. The difference in efficacy was only observed 18 h after the last antibiotic injection. The conclusion derived from this study should be considered with caution because the model of infection in the renal medulla might not be appropriate for this purpose. Our results differ from those of Sande et al. (16), who have shown that the therapeutic activity of cephalosporins against Streptococcus pneumoniae meningitis in rabbits is not directly related to low protein binding.

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LITERATURE CITED