The impact of the pre-treatment interval on antimicrobial efficacy in a biological model

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The impact of pre-treatment intervals on the antipseudomonal efficacy of genta-micin, ticarcillin and ceftazidime was studied in an experimental thigh infection model in normal and granulocytopenic mice. Human-equivalent doses were used for simulating human pharmacokinetic profiles of the two study β-lactam drugs. A lethal inoculum of a virulent strain of *Pseudomonas aeruginosa* was injected into the thigh muscle. Treatment was started at various post-infection intervals. Antimicrobial efficacy was assessed by determinations of surviving organisms at the site of infection, and plasma drug concentrations were determined in the same mice. The age of infection had a substantial impact on antipseudomonal efficacy of the three study drugs even though high, brief supra-MIC concentrations of gentamicin and persistent supra-MIC concentrations of the β-lactam drugs were obtained. A pre-treatment interval of six or more hours abolished the bactericidal effect of all three study drugs despite accumulation of the drugs to multiple-MBC plasma concentrations. We believe that the impact of pre-treatment intervals on anti-microbial efficacy is of paramount importance for the interpretation of antimicrobial activity studies in experimental models of infection, although the mechanisms remain to be elucidated.

Introduction

Many different factors are known to affect antimicrobial efficacy *in vivo*. Some of these factors, such as the infecting microorganisms, the site of infection and the intrinsic activity of an antimicrobial agent and its pharmacokinetic behaviour, have been well recognized and investigated in experimental models of infection (Drusano, 1991). On the other hand, the age of the infection (the pre-treatment interval) and the density of the infecting organisms have hardly been investigated, although their impact on overall success of an antimicrobial treatment can be assumed from clinical observation in such diseases as Gram-negative septicaemia in the granulocytopenic host (Hughes *et al.*, 1990).

In the present experimental work the impact on the efficacy of three well-known antipseudomonal agents (gentamicin, ticarcillin and ceftazidime) against *Pseudomonas aeruginosa* of the pre-treatment interval and the number of bacteria at the site of infection were investigated. The experimental model was an artificial infection in the thighs of normal and granulocytopenic mice. After infection with a standardized inoculum, treatment in different groups of infected mice was started immediately or

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after a delay of 2, 4, 6 or 8 h. Dosage regimens of study drugs were chosen to give pharmacokinetic profiles similar to those seen in humans, where appropriate (Gerber *et al.*, 1986; Gerber, 1991). Therapeutic efficacy was assessed by quantification of the number of bacteria at the site of infection over time and compared with plasma drug concentrations.

Materials and methods

Bacterial strain

All the main experiments were performed with *P. aeruginosa* A10, which was a clinical isolate obtained from Institute of Hygiene and Medical Microbiology, Bern, Switzerland.

Media

Trypticase Soy Agar TSA (BBL, Division of Becton, Dickinson & Co., MD, USA) was used for the determinations of cfu. Plasma drug concentrations were determined on Diagnostic Sensitivity Test Agar (DST, Oxoid Ltd, Basingstoke, Hampshire, UK).

Drugs

All drugs were commercial products: gentamicin (Schering Corp., Kennilworth, NY); ticarcillin (Beecham AG, Bern, Switzerland); ceftazidime (Glaxo Group, Greenford, UK); cyclophosphamide (ASTA Werke AG, Bielefeld, Germany). The drugs were dissolved in distilled water according to the recommendations of the manufacturers. Further dilutions were made in physiological, pyrogen-free saline.

Animals

Female ICR-mice with a mean body weight of 28 g (27–31 g) were obtained from Tierzuchtinstitut des Universitätsspitals der Universität Zürich, Switzerland.

Production of leucopenia

After an adaptation period of two days the mice were injected intraperitoneally with a first dose of cyclophosphamide (Endoxan), 150 mg/kg in 1 mL sterile saline. Two days later a second injection of 100 mg/kg in 1 mL saline was given. This treatment resulted in a severe leucopenia which lasted for at least four days (Gerber *et al.*, 1982).

Determination of plasma drug concentrations

Plasma kinetics were determined in the same groups of mice that were used to study the drug's efficacy. Blood was drawn from the retro-orbital sinus of ether-anaesthetized mice into heparinized microcapillaries (Clay Adams, Dickinson & Co). Plasma drug concentrations were determined the same day by a biological method (Sabath & Anhalt, 1980). *Bacillus subtilis* ATCC 6633 was used as the indicator bacterium for determinations of gentamicin and ticarcillin concentrations, and *Escherichia coli* ATCC 25922 for ceftazidime.

Bacterial inocula and bacterial challenge

Starting from several colonies of a pure culture of *P. aeruginosa* A10 a broth culture (Mueller-Hinton broth, supplemented with calcium and magnesium, as described by Stratton & Reller, 1977) was inoculated and serially diluted. After overnight incubation at 37°C the culture was chosen that came closest to an optical density of 0.3 (read at 580 nm), which corresponded to approximately 10^8 cfu/mL. The culture was centrifuged (2000 g for 10 min), washed in physiological saline and resuspended to result in 10^8 cfu/mL. An inoculum of 0·1 mL was injected into one thigh of ether-anaesthetized mice. In each individual experiment the challenge inoculum was quantitated by cfu determinations.

Quantification of cfu in the thigh of infected animals

At various time intervals after bacterial challenge and after onset of treatment three to four mice were killed. The infected thigh was dissected and homogenized on ice in a Polytron tissue homogenizer (Kinematica, Luzern, Switzerland) in 4 mL ice-cold saline supplemented with 8·5 g/L NaCl, 50 mg/L Ca^{++}, 20 mg/L Mg^{++} and 1 mL β-lactamase (Watman Biochemicals, Maidstone, Kent, UK). Aliquots of serially diluted mouse-thigh homogenates were plated on agar plates and cfu counts were determined after incubation for 18–24 h.

Antimicrobial chemotherapy and determinations of plasma pharmacokinetics

All drug injections were made in aliquots of 0·1 mL subcutaneously. Treatment was started immediately after bacterial challenge (0 h) or 1, 2, 4, 6, 8 or 10 h later. Gentamicin was given as a single, human-equivalent dose of 32 mg/kg (see Discussion for the definition of 'human-equivalent'). Ticarcillin and ceftazidime were given in fractionated decreasing doses such that a human-like pharmacokinetic profile could be obtained (Gerber *et al.*, 1986, 1991; Gerber, 1991). Thus, the total dose of ticarcillin (1000 mg/kg) was given over 6 h in fractionated doses, every 15 min, as follows: 225, 120, 3 × 80, 3 × 52, 3 × 34, 3 × 22, 3 × 14, 3 × 9, 3 × 6 and 4 mg/kg. The total dose of ceftazidime (250 mg/kg) was injected in decreasing fractional doses every 20 min for a total of 8 h as follows: 56, 25, 30, 3 × 20, 3 × 13, 3 × 8·5, 3 × 5, 3 × 3·5, 3 × 2·25, 3 × 1·5 and 1 mg/kg. Untreated control mice were injected with equal total amounts of sterile saline.

Results

Antimicrobial susceptibility of P. aeruginosa A10

In standardized diffusion tests *P. aeruginosa* A10 was susceptible to the three study drugs gentamicin, ticarcillin and ceftazidime. MICs (and MBCs) were 8 (16) mg/L of gentamicin, 16 (> 256) mg/L of ticarcillin and 1 (8) mg/L of ceftazidime.

Growth of P. aeruginosa A10 in normal and granulocytopenic mice

In comparative growth curves in normal and granulocytopenic mice, the clinical isolate of *P. aeruginosa* (A10) proved to be more virulent than the conventionally used laboratory strain ATCC 27853. The generation time of this bacterium in granulocyto-
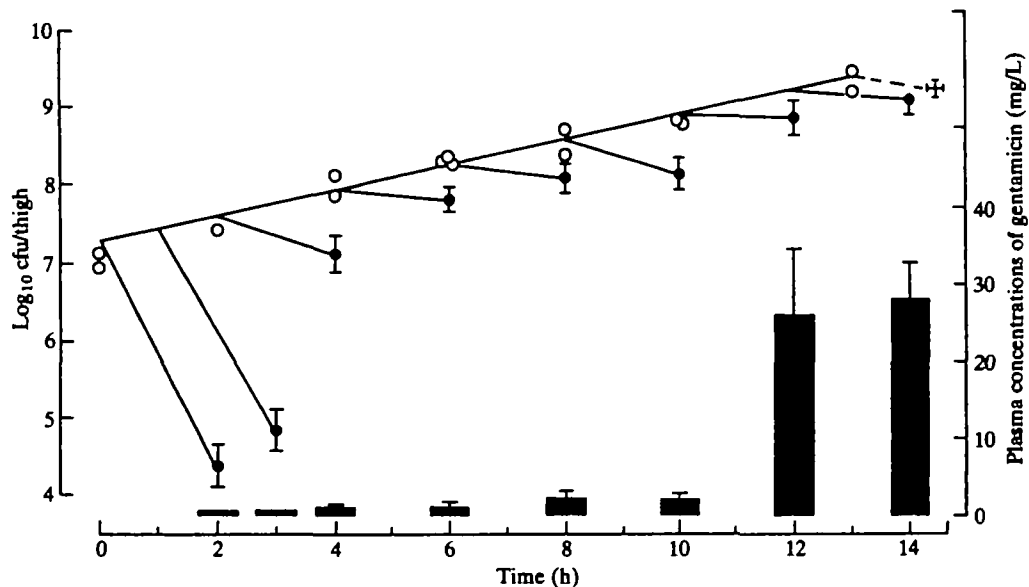
Figure 1. Bactericidal effect of a single dose of gentamicin (32 mg/kg) on *P. aeruginosa* A10 in normal mice after various pre-treatment intervals. ●, Geometric mean of cfu/thigh ± 1 sp in four mice as obtained 2 h after the gentamicin injection; ○, control saline injections; ■, mean gentamicin plasma concentration (± 1 sp) 2 h after the gentamicin injection. The impact of the pre-treatment interval on bactericidal efficacy was highly significant and so was the gentamicin accumulation in mice after a pre-treatment interval of > 10 h.

penic mice (0·98 h) was significantly shorter than that in normal, non-granulocytopenic mice (1·86 h).

Pharmacokinetics of the study drugs in plasma

In previous experiments the plasma half-life of gentamicin in mice was found to be 20 min (Gerber *et al.*, 1986). In the experiments described below, this proved to be similar in freshly infected gentamicin-treated mice but not in septicaemic mice. For ticarcillin and ceftazidime a human-like pharmacokinetic profile was simulated, since in previous experiments these drugs had been shown to be poorly effective when given as a single bolus injection (Gerber, 1991; Gerber *et al.*, 1991). By fractionation of the total dose of ticarcillin (1000 mg/kg given over 6 h), plasma peak concentrations of 185 ± 28 mg/L and a plasma half-life of 88 min were obtained. This kinetic profile resulted in supra-MIC concentrations of ticarcillin for *P. aeruginosa* A10 for 6 h. The human-adapted pharmacokinetic profile of ceftazidime showed a plasma peak concentration of 108 ± 17 mg/L (after 20 min) followed by a half-life of 131 min. Supra-MIC concentrations of ceftazidime for *P. aeruginosa* A10 persisted for a total of 8 h. Thus, plasma peak concentrations and half-lives of the two β-lactam antibiotics corresponded to values usually seen in healthy human volunteers.

Efficacy of gentamicin impact of the pre-treatment interval

The impact of the pre-treatment interval on the efficacy of gentamicin is shown in Figures 1 and 2 for normal and granulocytopenic mice, respectively. In the thigh of
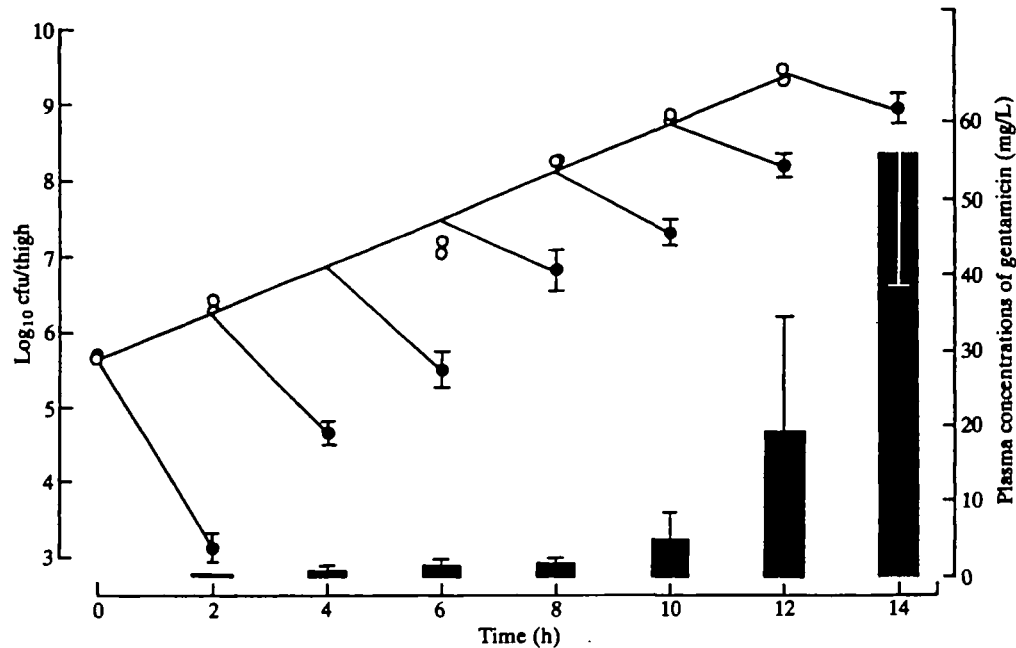
Figure 2. Impact of the pre-treatment interval on the antimicrobial efficacy of a single dose of gentamicin (32 mg/kg) on *P. aeruginosa* A10 in granulocytopenic mice. See Figure 1 for the key to the symbols.

untreated control mice pseudomonas grew log-linearly. In contrast, in normal mice, an initial inoculum of 10^6 organisms replicated very unreliably (not shown), and for this reason experiments using non-granulocytopenic mice were started with an inoculum of 10^7 cfu per thigh. Thigh swelling occurred in normal and granulocytopenic mice approximately 3 to 4 h after bacterial challenge. Untreated, non-granulocytopenic control mice died within 14–24 h after bacterial challenge. In contrast, untreated granulocytopenic animals clearly had an overwhelming infection resulting in early septicaemia, shock and death within 14 to 16 h.

When gentamicin treatment was started at the time of bacterial challenge, a pronounced bactericidal effect was obtained in normal and in granulocytopenic animals. More than 99% of the bacteria died within 2 h. However, increasing the pre-treatment interval resulted in a substantial reduction in bacterial killing. A pre-treatment interval of more than 4 h in normal mice and more than 6 h in granulocytopenic mice almost completely abrogated the bactericidal effect. This abrogation of antimicrobial efficacy was most impressive when it was correlated with the plasma drug concentrations. In fact, probably owing to shock, the drug almost failed to be eliminated in the very ill animals. As a consequence of abolished drug elimination, plasma gentamicin concentrations of more than 20 mg/L persisted in animals with treatment onset later than 8 h after bacterial challenge. Paradoxically, such supra-MBC concentrations of gentamicin had a very poor antipseudomonal effect.

Efficacy of ticarcillin: impact of the pre-treatment interval

Experiments with ticarcillin were similar to those performed with gentamicin with the one exception that the drug was given in such a manner that a human-like pharmaco-
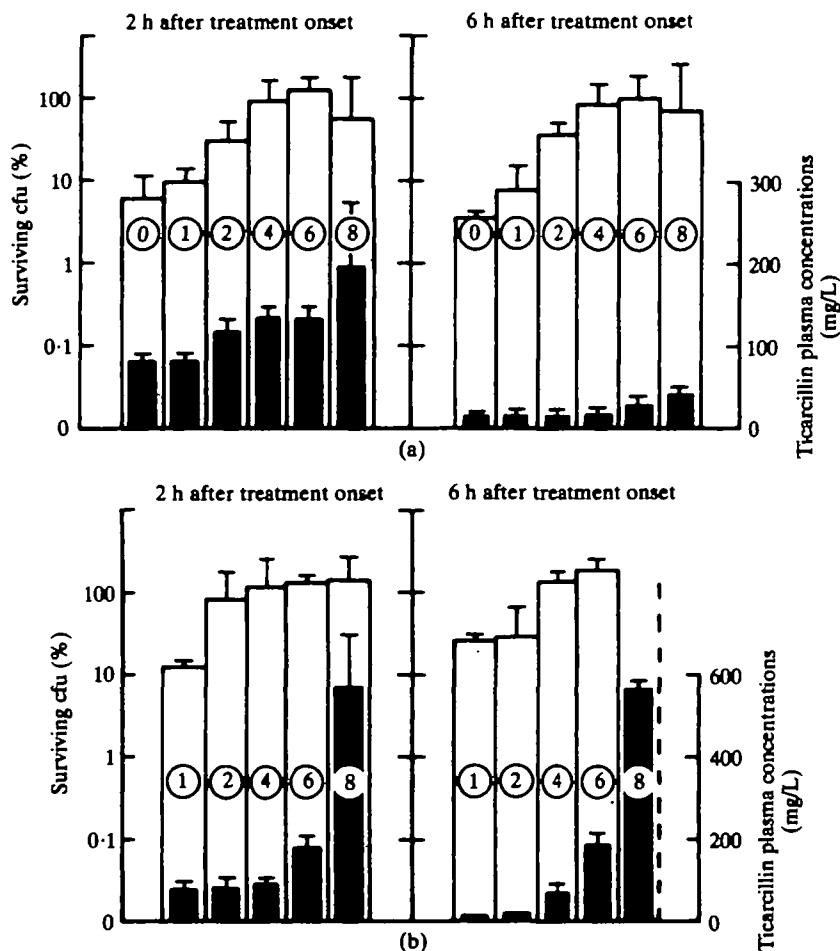
Figure 3. Impact of the pre-treatment interval on antipseudomonal efficacy of a human-simulated single dose of ticarcillin in normal (a) and granulocytopenic (b) mice. □, Surviving bacteria (% of the pre-treatment cfu value per thigh (±1 SD)). Circled numbers within the bars represent the pre-treatment interval (h). The left panels represent the numbers surviving after 2 h of treatment; the right panels those surviving after 6 h of treatment. ■, Ticarcillin plasma concentrations determined at 2 h (left panels) and 6 h (right panels). See text for comment.

kinetic profile was simulated. A bactericidal effect was observed after 2 or 6 h of treatment provided that ticarcillin had been started earlier than 2 to 4 h after bacterial challenge (Figure 3). In contrast, a merely bacteriostatic effect was obtained whenever the onset of ticarcillin treatment was further delayed. In both normal and granulocytopenic mice, the maximal antibacterial effect of ticarcillin treatment was obtained after 2 h despite the fact that supra-MIC ticarcillin plasma concentrations were maintained for four additional hours. Thus, a long pre-treatment interval clearly abrogated any bactericidal effect of ticarcillin.

Drug accumulation also proved to be dependent on the duration of the pre-treatment interval (Figure 3). Drug accumulation in granulocytopenic mice was significantly greater than in normal mice. As in gentamicin-treated mice, this phenomenon was probably due to persistent shock in granulocytopenic mice. However, despite the
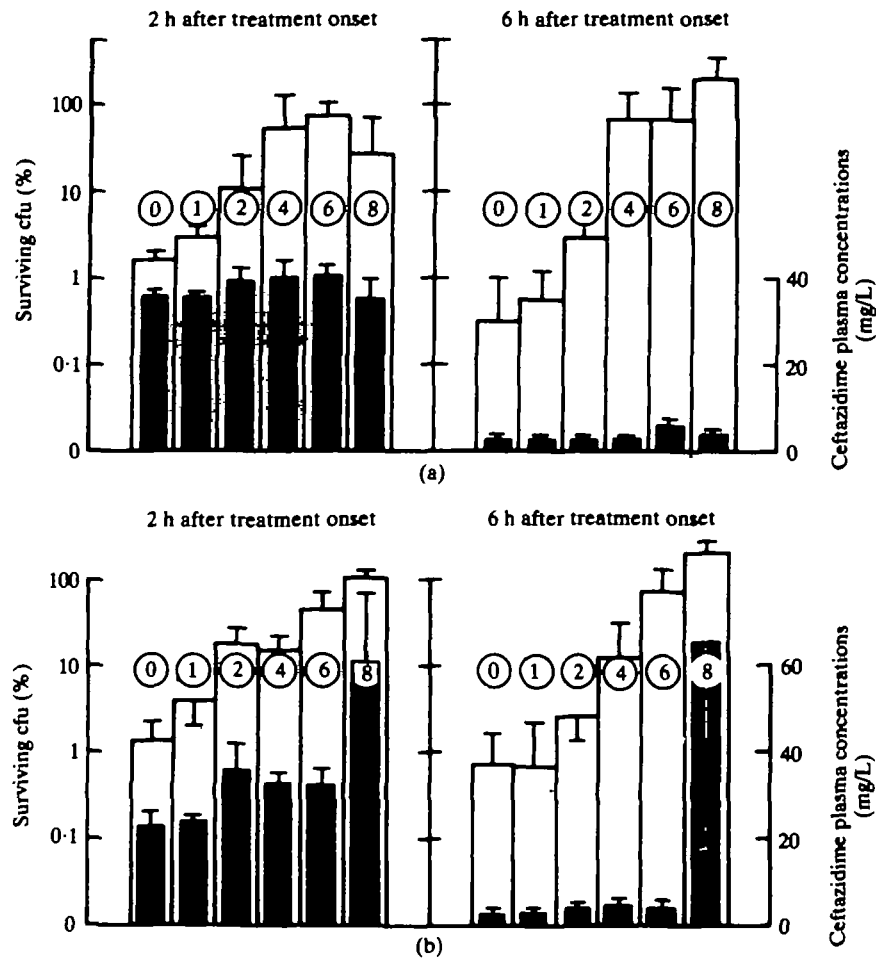
Figure 4. Impact of pre-treatment interval on the antibacterial efficacy of ceftazidime in normal (a) and granulocytopenic (b) mice. Effect (□) and drug concentration (■) as obtained with human-adapted dosing. Left and right panels show the results obtained 2 and 8 h after treatment onset, respectively. Circled numbers represent the pre-treatment interval (age of the infection) which had a significant impact on the efficacy of ceftazidime.

persisting supra-MIC drug levels in granulocytopenic (as opposed to normal) mice, antimicrobial efficacy was still poor. This was most evident in granulocytopenic animals with a pre-treatment interval of 8 h. These animals all died despite a highly significant accumulation of ticarcillin (Figure 3(b)).

Antibacterial efficacy of ceftazidime: impact of the pre-treatment interval

Ceftazidime was the second β-lactam drug studied. Like ticarcillin, it was administered in fractionated doses such that a human-like pharmacokinetic profile was obtained. A total dose of 250 mg/kg of ceftazidime proved to be significantly bactericidal in normal as well as in granulocytopenic mice (Figure 4). Again, however, a highly significant correlation between the duration of pre-treatment interval and antimicrobial efficacy was found. If antimicrobial therapy with ceftazidime had not been started by 4 to 6 h

after bacterial challenge, a merely bacteriostatic effect was seen during the 8 h treatment. When the onset of treatment in leucopenic mice was delayed for 8 h after bacterial challenge (i.e. when the bacteria had grown to 10^8 cfu/mL) no cidal effect whatsoever could be obtained despite the fact that the drug accumulated in these mice up to 60 mg/L (i.e. to 60 times and eight times the MIC and MBC, respectively).

Discussion

Our investigations have clearly demonstrated the impact on antimicrobial efficacy of a period between bacterial challenge and onset of treatment with gentamicin, ticarcillin or ceftazidime. The impact of the pre-treatment interval (IPTI) correlated with the duration of this interval. The results of our study are in accord with the clinical truism that successful antimicrobial therapy varies with the severity of the bacterial infection. This clinical experience is most evident in granulocytopenic patients (Love, Schimpff & Schiffer, 1980; Hughes *et al.*, 1990).

Our studies were performed with a single treatment course of 6 to 8 h duration; drug doses were used which may look very high. It must be emphasized however, that (with the exception of gentamicin) drug levels in the plasma of the study animals reflected those normally seen under clinical conditions. By using the method of fractionating dosing we could approximate human pharmacokinetic profiles of the two β-lactam drugs. We have previously shown that the therapeutic effect of β-lactam antibiotics depends not only on the total amount of injected drug, but equally on the 'shape' of the area under the plasma concentration versus time-curve (Gerber *et al.*, 1986, 1991; Gerber, 1991). In those experiments we had found in mice the human-equivalent dose and defined it as the amount of drug that was needed to simulate the human pharmacokinetic profile.

In the present study we did not simulate the human pharmacokinetic profile of gentamicin since earlier studies had shown that a single does of 32 mg/kg injected into mice corresponded to the human-equivalent dose, as this amount of drug was needed to simulate in a mouse the human pharmacokinetic profile. The shape of the AUC of gentamicin was not relevant to the efficacy of this particular drug (Gerber *et al.*, 1986).

Our present study demonstrates that a pre-treatment interval can completely abolish the bactericidal effect of potentially bactericidal antibiotics. Moreover, if a very long pre-treatment interval was chosen, the animals became so severely ill that the study drug was not eliminated and hence accumulated to potentially toxic concentrations. Nevertheless, these extremely high drug concentrations proved to be less bactericidal than the very much lower drug concentrations seen after early treatment onset. Thus, for overall therapeutic efficacy the severity of the infection seems to be more important than the heights of attainable plasma levels or the overall AUC of the therapeutic agent.

Against Gram-positive cocci the impact on antimicrobial efficacy of both inoculum size and age of infection on experimental therapeutic efficacy has been known since the early days of penicillin (Eagle, 1949; Smith & Wood, 1956). Pneumococci were easily eradicated with small doses of penicillin provided that penicillin treatment was started at the first day of infection and suppuration was absent (Smith & Wood, 1956). The phenomenon was explained by the poor growth rate of the target bacteria in pus, but equally by the poor phagocytic activity of leucocytes in pus. More recently this phenomenon has been termed phenotypic tolerance and its mechanism has been partly

elucidated (Tuomanen, 1986). Phenotypic tolerance in non-growing pneumococci was shown to correlate with the loss of autolysin molecules followed by a progressive change in the cell wall structure, which becomes less susceptible to autolysin-induced hydrolysis (Tuomanen & Tomasz, 1991).

Against Gram-negative bacteria and *P. aeruginosa* in particular abrogation of antimicrobial activity by postponed treatment onset has previously been observed. As early as in 1956, Miles reported that streptomycin exerted a protective effect against *P. aeruginosa* infection only when treatment was started within 3 h. He termed this 3-h interval the decisive period. More recently, Davey, Barza & Stuart (1987, 1988), reported on antipseudomonal chemotherapy in experimental endophthalmitis and in the rat croton oil pouch model. Treatment with ciprofloxacin, gentamicin and imipenem was effective when started after 24 h of infection, but had little or no effect when delayed until 48 h after infection. Similarly, the pre-therapeutic duration of infection reduced the efficacy of ceftazidime and ciprofloxacin in *Klebsiella pneumoniae* pneumonia and septicaemia in leukopenic rats (Roosendaal *et al.*, 1991).

Various factors might explain the effect of the pre-treatment interval on efficacy against Gram-negative bacteria. In previous studies (Davey *et al.*, 1987, 1988) the relative bacterial resistance after delayed treatment onset was attributed to slower growth of the target organisms. This could definitely not be the decisive factor in our model, since treatment efficacy was diminished before the target bacteria came into a lag-phase. Thus, other factors must be involved. It is well known from in-vitro studies that a high density of β-lactamase producing bacteria diminishes the effect of β-lactam antibiotics (inoculum effect). The concentration of bacterial β-lactamases probably correlates with the density of the bacterial inoculum (Sabath *et al.*, 1975; Eng, Smith & Cherubin, 1984; Hall & Opfer, 1984).

Another explanation of the inoculum effect could be that at very high bacterial densities bacterial clusters are formed through which the drug may hardly penetrate, so that the inner bacteria are protected. This mechanism of an inoculum effect may be of particular importance for β-lactam drugs such as ticarcillin and ceftazidime which predominantly bind to penicillin-binding protein 3 resulting in formation of bacterial filaments. Filaments are likely to be formed at the surface of bacterial clusters, and these may further hamper the penetration of the drug into the clusters (Gwynn, Webb & Rolinson, 1981). Preliminary histological observations in our mouse thigh model revealed indeed very dense accumulations of bacteria and the formation of bacterial filaments in β-lactam treated animals.

Other mechanisms are likely to interfere with the activity of aminoglycosides. The biochemical environment at the site of infection could explain, at least in part, the effect of pre-treatment delay on the effectiveness of gentamicin in particular. The antimicrobial activity of aminoglycosides is greatly affected by a low pH and a low redox potential, i.e. factors known to be low at the site of infection (Hays & Mandell, 1974; Verklin & Mandell, 1977). Yet another mechanism could be some bacterial adaptation to the environment *in vivo* which in turn might lead to reduced susceptibility of the target organisms to various antimicrobial agents.

Finally, many different organisms and *P. aeruginosa* in particular produce an extracellular matrix which might well have a protective effect (Nickel *et al.*, 1985). The production of this matrix is reversible and is hence probably due to genetic regulatory events or phenotypical changes (Bryan, 1989).

Our present work contributes to our understandings of the phenomenon of the pre-

treatment interval—a factor of paramount importance for the study of antimicrobial activity *in vivo*.

Acknowledgement

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