Antibiotic-induced Bacterial Toxin Release – Inhibition by Protein Synthesis Inhibitors

BY

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**Abstract**


Toxic products, such as endotoxin from the gram-negative and exotoxin from the gram-positive bacteria, are the most important initiators of the inflammatory host response in sepsis. In addition to antibacterial treatment, numerous attempts have been made to interfere with the exaggerated proinflammatory cascade initiated by the toxins. As most antibiotic and anti-inflammatory agents have shown no clear efficacy, an attractive alternative has been to prevent or minimise their release. Therefore, it was of interest to further study the antibiotic-induced release of toxins after exposure to antibiotics used for the treatment of the most severe infections, especially if protein synthesis inhibitors could reduce the release induced by PBP 3-specific β-lactam antibiotics.

There were significant reductions in endotoxin release from gram-negative bacteria when the combination of the PBP 3-specific β-lactam antibiotic, cefuroxime, and the protein synthesis inhibitor, tobramycin, was compared with cefuroxime alone. Increasing doses of tobramycin reduced endotoxin release and increased the killing rate. In a kinetic *in vitro* model the endotoxin release from *E.coli* was higher after the second dose of cefuroxime. Nevertheless, it was reduced after addition of tobramycin.

No binding of tobramycin to endotoxin was observed, either *in vivo* or *in vitro*. In a porcine sepsis model, a possible anti-inflammatory effect of cefazidime and tobramycin, expressed as late cytokine inhibition, was seen.

The protein synthesis inhibitor, clindamycin, released less streptococcal pyrogenic exotoxin A (SpeA) from a group A streptococcus strain than penicillin, and addition of clindamycin to penicillin resulted in less toxin production than penicillin alone. The SpeA production was dependent on the bacterial number at the start of treatment. Higher doses of penicillin also led to less SpeA.

The choice of antibiotic class and dose may be important in the severely ill septic patient in whom an additional toxin release could be deleterious. A combination of a β-lactam antibiotic and a protein synthesis inhibitor seems beneficial but further investigations are needed.

**Keywords:** Endotoxin, LPS, exotoxin, SpeA, penicillin-binding protein, aminoglycosides, clindamycin, β-lactam antibiotics, severe sepsis, septic shock

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


Contents

Introduction and background ........................................................................................................ 7
Pathogenesis of severe sepsis and septic shock .......................................................................... 7
Endotoxins .................................................................................................................................... 9
Exotoxins .................................................................................................................................... 11
Endotoxin neutralisation and immunomodulation as treatment strategies ................................ 11
Release of toxin after exposure to antibiotics ............................................................................ 12
Antibiotic-induced release of endotoxin from gram-negative bacteria .................................... 12
Release of exotoxin from gram-positive bacteria ....................................................................... 13
Effects of antibiotics on the immune system .............................................................................. 14

Aims of the study ......................................................................................................................... 15

Materials and methods ................................................................................................................. 16
Bacterial cultures ......................................................................................................................... 16
Media ......................................................................................................................................... 16
Antibiotics .................................................................................................................................. 16
Determination of minimum inhibitory concentrations (MIC) .................................................... 17
Determination of bacterial killing by viable count ....................................................................... 17
In vitro kinetic model and experimental procedure ..................................................................... 18
Morphology .................................................................................................................................. 18
Determination of endotoxin in the in vitro experiments ............................................................. 19
Determination of possible mechanisms for a tobramycin-caused reduction in cefuroxime-induced endotoxin release ................................................................. 19
Determination of SpeA concentration ......................................................................................... 19
Animals, anaesthesia and preparatory procedures .................................................................... 20
Administration of endotoxin and antibiotics .............................................................................. 21
Measurements ............................................................................................................................. 22
Determination of plasma endotoxin, TNF-α and IL-6 concentrations ........................................ 22
Calculations and statistical methods ............................................................................................ 22

Results and discussion .................................................................................................................. 24
MIC values .................................................................................................................................. 24
Killing rates ................................................................................................................................. 24
Antibiotic-induced endotoxin release ....................................................................................... 25
Introduction and background

Sepsis is defined as a condition with fever, tachycardia, tachypnoe and leukocytosis or leukopenia caused by an infection (4). If sepsis leads to organ dysfunction, hypoperfusion or hypotension, it is classified as severe sepsis. When the hypotension persists despite adequate fluid resuscitation and hypoperfusion or organ dysfunction is present the term septic shock is used (4).

Bacteria are the most common agents causing septic shock but fungi, mainly *Candida albicans*, as well as parasites and viruses may be responsible in rare cases (101). Among bacterial agents *E. coli*, group B streptococci and *Listeria* predominate in the neonates whereas pneumococci, meningococci and *S. aureus* are the most common causative agents in children and younger adults (86). Septic shock in elderly patients is more frequently caused by gram-negative bacteria from the gastrointestinal, urinary and respiratory tracts. Since the 1980s there has been a resurgence of severe group A streptococcal (GAS) infections with rapid progression to toxic shock in patients of all ages, also including otherwise healthy young persons (41,88).

Despite adequate antibiotic treatment and optimal intensive care efforts, severe infections caused by gram-negative, as well as gram-positive bacteria still often proceed to shock and multiorgan failure (48,92). Thus, in studies performed on patients with sepsis and septic shock the mortality rate has ranged from 30 to 80 %, depending on severity, duration, underlying conditions and on the definitions used (58,70,80,83).

Since the beginning of the antibiotic era, experimental studies and discussions have been pursued to find the reasons for and to prevent therapeutic failures in severe sepsis where antibiotic treatment even has been suggested by some authors to be responsible for the aggravating conditions. (25,69,85).

Pathogenesis of severe sepsis and septic shock

It is well known that bacterial products, like endotoxin from the gram-negative and exotoxins from the gram-positive bacteria, in different ways
initiate the inflammatory host response in sepsis and that this may further progress to severe sepsis and septic shock (19,43,84). With a high virulence or number of bacteria in the circulation or locally, the proinflammatory reaction thus initiated cannot be inhibited by the anti-inflammatory systems that occur somewhat later. Besides bacterial properties, the degree of response in the host also depends on individual factors like underlying conditions, medications and probably also genetic factors.

The initial response to toxin release includes activation of the macrophages and monocytes to produce IL-1, TNF and other cytokines and, through a complicated interaction with the activated coagulation, fibrinolytic, kallikrein-kinin and complement systems, an activation of the neutrophil granulocytes takes place. Through adhesion of the neutrophils to the endothelium in multiple organs there is an increased permeability that might lead to a leakage from the blood vessels. Simultaneous activation of the coagulation and anti-coagulation may lead to imbalance resulting in microthrombosis or, occasionally, bleeding (86, Fig.1).

![Figure 1. Initial activation of inflammatory response.](image)

In septic shock, a general vasodilatation, caused by released substances, such as TNF, PAF, bradykinins, prostaglandins and nitrous oxide, takes place.
This, together with tissue oedema and microthrombosis, leads to a lowered oxygen uptake by different organs. Hypovolaemia occurs because of the vasodilatation, as well as dehydration, and the increased capillary leakage. Initially, the circulation is hyperdynamic with a high cardiac output and a low vascular resistance. If the hypovolaemia is not corrected, the cardiac output decreases and a hypodynamic septic shock develops.

A serious inflammatory reaction may also lead to development of acute respiratory distress syndrome (ARDS) in the lungs which further lowers the oxygen delivery to the organs. Decreased oxygen delivery and organ uptake causes anaerobic metabolism and metabolic acidosis which, together with the generalised inflammation results in multiple organ dysfunction.

Endotoxins

The term endotoxin refers to the biological activity of the lipopolysaccharide molecule (LPS) in the cell wall of the gram-negative bacteria. The LPS molecule consists of 3 parts: the outer polysaccharide hydrophilic O-side chain, a centre core oligosaccharide and an inner hydrophobic lipid A portion which is the mediator of toxicity (43,58). It has been shown in animals and humans that administration of purified endotoxin can induce the same pathophysiological effects as those in gram-negative sepsis (68).

Endotoxin release from the bacterial cell wall occurs mainly as a consequence of death and lysis of the cell and of growth by cell division (46). When the endotoxin is released and appears as free endotoxin, its biological activity increases considerably as compared with the bacterial cell-bound endotoxin (57).

Once released, endotoxin binds to several receptors and carrier proteins, the most important being the LPS binding protein (LBP) (99, Fig.2).
Figure 2. Release of free endotoxin from gram-negative bacteria.

The LBP-endotoxin complex interacts with the CD14-receptor on the macrophage where a Toll-like receptor-4 (TLR-4) transduces the endotoxin signal to the cell which responds by cytokine production. LBP is crucial for the interaction with the CD14 receptor and it has been demonstrated that TNF-production is reduced 1000-fold in LBP-depleted plasma (64). Besides binding to the CD14 receptor, endotoxin may also activate the macrophage via other receptors, such as the β2 integrin CD11/CD18 receptor (45). Membrane bound CD14 may be released from the cell surface and this soluble CD14 (sCD14) may bind to the LBP-endotoxin complex. LBP is freed and the endotoxin-sCD14 complex may then activate cells that do not express CD14, such as endothelial cells (29).

The effect of endotoxin may be inhibited by binding to a LBP-like protein, the bactericidal/permeability increasing protein (BPI) which is synthesised by the neutrophil granulocytes upon endotoxin stimulation (93). Already at the onset of endotoxin release, LPS can bind to specific antibodies that bind to the O-chain and represent the result of previous endotoxin exposures (28,35). In addition, low- and high-density lipoproteins and apolipoprotein A have been shown to inhibit endotoxin-induced cytokine release (26).
Exotoxins

Gram-positive bacteria like staphylococci and streptococci produce exotoxins with varying biological activities that can be released without bacterial growth or lysis. (92). As an example, in severe group A streptococcal infections the pyrogenic exotoxins together with the phagocytosis-inhibiting M-proteins on the streptococcal surface are the most important virulence factors. In similarity to endotoxin, the exotoxins stimulate monocytes and macrophages to release TNF and other cytokines (37). In addition, the exotoxins have superantigenic properties, meaning that they are mitogenic for certain T-cell subsets but do not require processing by antigen presenting cells (52). Consequently, release of excessive amounts of inflammatory cytokines, such as TNF-β, IL-1 and IL-6 can take place. Exotoxins of major importance are the superantigens streptococcal pyrogenic exotoxin A (speA), B , C and F (72).

Endotoxin neutralisation and immunomodulation as treatment strategies

The cornerstones in the treatment of severe sepsis and septic shock include antibiotics and, if necessary, surgery for the killing and removal of causative bacteria, fluid replacement for reversal of hypovolemia, oxygen therapy for the treatment of hypoxia and support of deteriorating vital organs. Despite this, mortality is still high and therefore numerous attempts have been made to interfere with the proinflammatory cascades in severe sepsis and septic shock.

As endotoxin is the major activator in severe gram-negative sepsis, several clinical trials have studied the effect of polyclonal or monoclonal antibodies directed against the inner biological part of the LPS molecule (36,102,103). Positive effects, especially in the most severely sick patients, were seen but in the confirmatory trials all-cause mortality was not significantly affected. Other studies have demonstrated a binding and neutralization of LPS after addition of recombinant BPI that shows higher affinity for endotoxin than LBP (30,98). In a clinical study in patients with meningococcemia, known to have high endotoxin concentrations, there was a trend towards an improved clinical outcome in the group that was treated with rBPI but no statistically significant effects could be demonstrated (59).

Polyclonal intravenous immunoglobulin (IVIG) has been shown to neutralise
streptococcal toxins (73). However, clinical experience is limited to one retrospective comparative study demonstrating a beneficial effect on mortality in patients with streptococcal toxic shock syndrome (50) and one small prospective study showing a significant decrease in sepsis-related organ failure (20). In patients with severe sepsis or septic shock caused by other bacteria, no beneficial effects have been demonstrated.

Clinical trials with antibodies or other molecules antagonising the effect of several proinflammatory cytokines, such as TNF-α, IL-1β, platelet activating factor, or coagulation factors, have shown positive results in some subgroups but no significant effect in all-cause mortality (1,2,3,16,22,74,81,97) and therefore these products have not been licensed for clinical use. Recently, beneficial effects on mortality have been demonstrated for corticosteroids (6) and activated protein C (10). However, with these treatments there are always difficulties in establishing the optimal dose because, if doses are too high, the beneficial effect may proceed into an adverse one leading to immune paralysis or bleeding.

Release of toxin after exposure to antibiotics

Above their primary bactericidal and bacteriostatic activity, antibiotics have been shown to exert other effects that may be of importance for treatment of infections. One of these is the impact on the liberation of bacterial toxins.

Antibiotic-induced release of endotoxin from gram-negative bacteria

A number of *in vitro* and *in vivo* studies have shown an increase in endotoxin release after exposure to different antibiotics (5,18,23,44,77,85).

In general, bactericidal antibiotics liberate initially more endotoxin than bacteriostatic antibiotics and antibiotics active on the cell wall, such as penicillins and cephalosporins, release more than antibiotics with other modes of action, such as protein synthesis inhibitors. However, there are wide variations between different antibiotics and even among the β-lactam antibiotics, there are great differences in the propensity to free endotoxin (11,24,85,95).

Penicillin-binding proteins (PBP) are enzymes that are located in the bacterial cell wall and responsible for the cell wall synthesis. They are also the
primary targets for the β-lactam antibiotics and, depending on the affinity to these PBPs, varying amounts of endotoxin are liberated from the gram-negative bacteria at exposure to these antibiotics (78). Beta-lactam antibiotics with affinity for PBP 1, lead to rapid killing without additional release of endotoxin whereas antibiotics with selective affinity for PBP 2, lead to conversion of the bacteria to round cells, spheroplasts, with loss of viability but without cell wall destruction and excessive endotoxin release. Binding to PBP 3, causes selective inhibition of septation and continuing bacterial elongation with formation of long filaments and a subsequent increased endotoxin production.

Thus, release of high amounts of endotoxin is mainly associated with PBP 3 binding. Cefuroxime, cefotaxime, piperacillin and aztreonam bind to PBP 3 and are associated with antibiotic-induced endotoxin release (24,78). Cefazidime, at high concentrations, binds to PBP 1 and the carbapenems to PBP 2 (24,49,78). At lower concentrations, cefazidime and meropenem bind predominantly to PBP3 resulting in higher release of endotoxin at lower than at higher doses (49,71,78,94). Simultaneous inhibition of PBP 1a and 3, that may be caused by cefazidime at mid-concentration levels, has also been demonstrated to result in formation of spheroplasts. These findings have been explained as the sum of inhibitory effects (39).

Among the bactericidal antibiotics, aminoglycosides inhibit protein synthesis by binding to 16S rRNA (32), which results in rapid killing without excessive endotoxin release (8,24,27). Limited data also suggest that aminoglycosides may even reduce the endotoxin liberation induced by β-lactam antibiotics (24). The quinolones inhibit protein synthesis via inhibition of bacterial DNA synthesis (42). Despite this mode of action, quinolones have in some studies been associated with a substantial endotoxin release, whereas in others this has not been observed (58,78).

Antibiotic-induced endotoxin release has been shown to be biologically active and to induce a macrophage TNF response in vitro (23). In animal models, there is strong evidence that soluble endotoxin released from gram-negative bacteria by antibiotics contributes to pathogenesis and mortality in experimental sepsis (70). However, the clinical significance of antibiotic-induced endotoxin release has still not been established.

Release of exotoxin from gram-positive bacteria

Data on exotoxin release from gram-positive bacteria are more limited than those on endotoxin release from gram-negative bacteria but recently it has
been shown the liberation of streptococcal toxin may be dependent on the antibiotic treatment (17,62,89). It has been demonstrated in vitro that liberation of SpeA is lower after treatment with protein synthesis inhibitors, like clindamycin and linezolid, than the β-lactam antibiotic penicillin (17,62). The proposed mechanism has been inhibition of toxin production. In severe group A streptococcal myositis in mice, these results have been supported and in the experiments it has been shown that clindamycin significantly increases survival compared with penicillin treatment (91). Inhibition of toxin synthesis as well as inoculum independency and a long post-antibiotic effect of clindamycin were suggested as explanations for the result.

Effects of antibiotics on the immune system

In a considerable number of in vitro and ex vivo experiments, antibiotics have also been found to modulate host inflammatory mechanisms (31,38,51). Graded doses of various antibiotics have been tested for their ability to affect functions such as chemotaxis, phagocytosis as well as bacterial killing with the generation of reactive oxygen molecules. This oxidative stress induced by polymorphonuclear activation during septic shock has been shown to trigger an inflammatory response with later cytokine production (55). Several studies have demonstrated that certain β-lactam antibiotics and aminoglycosides provide protection from oxygen radicals with subsequent reduction of epithelial damage (15,76). While immune modulation in these in vitro and ex vivo experiments appear to be marked, in vivo data are limited and clinically decisive effects have not been demonstrated (38).
Aims of the study

As the endo- and exotoxins are primary important mediators of septic shock and since antitoxic and most anti-inflammatory agents have shown no clear efficacy, an attractive alternative seems to be prevention or minimisation of their release in order to diminish the effects on the inflammatory response. Therefore, it was of interest to further study the antibiotic-induced release of toxins after exposure to antibiotics used for the treatment of the most severe infections, severe sepsis and septic shock and especially if protein synthesis inhibitors could reduce the release induced by PBP 3-specific β-lactam antibiotics.

The aims were:

- To investigate the inhibitory effects of varying doses of a protein synthesis inhibitor, tobramycin, on endotoxin release in vitro from various gram-negative bacteria induced by a PBP 3-specific β-lactam antibiotic, cefuroxime.

- To study the relationship between endotoxin release and bacterial killing.

- To study if tobramycin binds unspecifically to endotoxin in vitro or in vivo or affects the binding of the LBP-endotoxin complex to the macrophages in vivo.

- To study the effects on endotoxin release by repeated cefuroxime doses in an in vitro pharmacokinetic model simulating the human concentration time-profile.

- To study if tobramycin or ceftazidime can reduce the cytokine and physiological response to endotoxin in vivo.

- To investigate the release of SpeA from a GAS strain after exposure to a β-lactam antibiotic, penicillin G, a protein synthesis inhibitor, clindamycin or a combination of the two and to relate the toxin release to the initial bacterial concentration, the antibiotic doses and the killing rate.
Materials and methods

Bacterial cultures

Clinical isolates of *Escherichia coli*, B 049-3036 (I,IV), B 940-3012 (II), B 942-1035 (II) and *Klebsiella pneumoniae* B 541-1003 (II) obtained from the Department of Clinical Microbiology, Uppsala, Sweden, were used. In addition, a clinical isolate of *Streptococcus pyogenes* group A, 8004 (V) obtained from Mount Sinai Hospital, Toronto, Canada was studied.

The reference strains of *Escherichia coli* CCUG 12620* (II), *Salmonella enteritidis* CCUG 35852 (II) and *Neisseria meningitidis* CCUG 15117 (II) purchased from the Department of Clinical Microbiology, Gothenburg, Sweden were used, as well as the reference strain of *Escherichia coli* ATCC 25922 (IV).

* The CCUG number was discovered to be wrong in the lab. storage. The strain should have been denominated CCUG 17620 which is identical with ATCC 25922.

Media (I, II, IV, V)

The GAS strain (V) was cultured in Todd-Hewitt broth supplemented with 1.5% yeast extract. The *Neisseria meningitidis* (II) was grown in a serum containing broth (Fildes and horse serum) made from pyrogen-free water and all the other gram-negative strains were cultured in Brain Heart Infusion (BHI) which was also made with pyrogen-free water.

Antibiotics

The antibiotics were obtained as reference powders with known potencies. Cefuroxime and ceftazidime were purchased from Glaxo Wellcome AB,
Gothenburg, Sweden, bensylpenicillin from Astra Läkemedel AB, Södertälje, Sweden, clindamycin from Pharmacia Upjohn, Kalamazoo, Michigan, USA and tobramycin from Eli Lilly Sweden AB, Stockholm, Sweden.

**Determination of minimum inhibitory concentrations (MIC)**

MICs were determined in triplicate by two-fold microdilution in broth with an inoculum of approximately $10^5$ cfu of the test strain per ml according to NCCL’s standard (I), by E-test (Biodisk, Solna, Sweden) (II, IV), and the microdilution method (V).

**Determination of bacterial killing by viable count (I, II, V)**

Strains in exponential growth phase were diluted and transferred into pyrogen-free glass vials to obtain a starting inoculum of approximately $10^6$ cfu/ml (I, II). The bacteria were then exposed to 0.1 (I), 2 (I, II), 10 (I, II) and 50 (I, II) x MIC of cefuroxime, tobramycin or a combination of the two and further incubated at 35°C. In each experiment, one glass vial served as a control. Samples for viable counts were drawn and diluted in phosphate buffered saline (PBS) immediately before and after addition of antibiotics and at 1, 2, 6, 24 h (I) and 2, 4 h (II). Dilutions of *N. meningitidis* were seeded on chocolate agar plates and the remaining gram-negative strains on blood-agar plates.

The GAS strain (V) in a log phase culture of approximately $10^9$ cfu/ml was centrifuged and diluted to around 7.0 log$_{10}$ cfu/ml in the low and 8.6 log$_{10}$ cfu/ml in the high inoculum experiment. The strain was then exposed to 2 and 1000xMIC of bensylpenicillin, 2 and 32xMIC of clindamycin and combinations of the two at 2xMIC of both and 1000xMIC and 32xMIC, respectively. These concentrations were chosen to mimic low and high concentrations in the clinical setting. One glass vial without antibiotic served as a control. Samples for viable counts were drawn immediately before and after addition of antibiotics, and, after further incubation at 37°C, at 3, 6, and 24 h. Dilutions were spread on blood-agar plates.
In vitro kinetic model and experimental procedure (IV)

The in vitro kinetic model has been described in detail elsewhere (61). It consists of a spinner flask with a total volume of 110 ml with a filter membrane (0.45μm) fitted in between the upper and bottom part, impeding elimination of bacteria. A magnetic stirrer ensures homogeneous mixing of the culture and prevents membrane pore blockage. In one of the side arms of the culture vessel, a silicon membrane is inserted to enable repeated sampling. A thin plastic tubing from a vessel containing fresh medium is connected to the other arm. The medium is drawn from the flask at a constant rate by a pump (type P-500, Pharmacia Biotech. Norden, Sollentuna, Sweden), while fresh, sterile medium is sucked into the flask at the same rate by the negative pressure built up inside the culture vessel. The flow-rate of the pump was adjusted to obtain the desired half-life of the drug. The apparatus was placed in a thermostatic room at 37°C during the experiments.

The bacteria were added to the antibiotic suspension immediately before it was transferred to the culture vessel at time zero, resulting in a starting inoculum of 5x10^6 cfu/ml. The initial cefuroxime concentration in the vessel was 60 μg/ml. The half-time of 1.5 h for cefuroxime was chosen in order to simulate the human pharmacokinetics. The same dose was placed into the vessel after about 12 h when the inoculum had reached a level approximating that of the starting inoculum. Six experiments were performed with each strain.

In an additional experiment, the same initial dose was given, but at the time of the second dose, a combination of cefuroxime and tobramycin to result in final concentrations of 60 μg/ml and 20 μg/ml, respectively, was given. Two experiments with each strain were performed.

Samples for viable counts were drawn before addition of antibiotics and at 2 h, 4 h, 14 h and 16 h. After dilution in PBS, the samples were seeded on Columbia agar plates and incubated at 35°C.

Morphology (IV)

The morphology of E. coli was examined with scanning electron microscopy. For this purpose a separate experiment was performed with the ATCC 25922 strain given the same antibiotic concentrations as those used in the preceding experiments and with samples drawn before the antibiotic doses
and at 1 h and 2 h after each dose. The examinations were made with a LEO Gemini 1530 scanning electron microscope at an accelerating voltage of 2kV.

**Determination of endotoxin in the *in vitro* experiments (I, II, IV)**

For all endotoxin assays endotoxin-free glass tubes and vials, preheated at 180° for 4 h, were used. Sterile, pyrogen-free plastic syringes and pipettes were used, and to analyse the free endotoxin, non-pyrogenic filters of 0.45 μm were employed. Samples for endotoxin analysis were drawn at the same time points as those for viable counts. The samples were kept on ice, diluted in pyrogen-free water, filtered and frozen at –20° pending analysis.

Analyses of endotoxin were performed in duplicate with the chromogenic limulus amoebocyte (LAL) assay (Bio Whittaker, Walkersville, MD, USA).

**Determination of possible mechanisms for a tobramycin-caused reduction in cefuroxime-induced endotoxin release (I)**

Tobramycin at concentrations of 0 (control), 10, 50 and 100xMIC was added to BHI spiked with endotoxin from *E. coli* O55:B5 to concentrations of 500 and 2000 EU/mL, respectively. Samples for endotoxin analysis were taken immediately before addition of tobramycin and after 0.5 h, 2 h, and 4 h of incubation at 35°C.

In another experiment, tobramycin at similar concentrations as above was added to bacteria pre-exposed to cefuroxime 10xMIC for 2 h. Samples for endotoxin analyses were taken before and after addition of tobramycin and the reduction in the cefuroxime-induced endotoxin release was analyzed and compared with the control after an additional 2 h.

**Determination of SpeA concentration (V)**

Samples for SpeA analysis were drawn concomitantly with those for viable
counts. At each time point, centrifugation was performed and 5 ml of the supernatants were transferred to Falcon tubes containing 15 ml of ethanol and kept at −20°C pending analysis.

The levels of the SpeA present in the concentrated bacterial culture supernatants were quantified by a competitive ELISA described in detail elsewhere (100).

Animals, anaesthesia and preparatory procedures (III)

The study included 13 domestic breed piglets with a median weight of 24 kg. The animals were 12-14 weeks old and without evidence of illness. Furthermore, the animals had to have a PaO2 >10 kPa (75 mmHg) in arterial blood and a mean pulmonary arterial pressure (MPAP) of <2.7 kPa (20 mmHg) at baseline 20 minutes after completed preparatory procedure (see below). The Ethics committee of Uppsala University, Sweden, approved the experiment. The piglets were treated according to the European Convention on Animal Care.

Anaesthesia was induced with the mixture of 6 mg/kg tilétamin-zolazepam (Zoletil forte vet™, Boehringer Ingelheim Vetmedica, Ingelheim, Germany), mixed with 2.2 mg/kg of xylazin (Rompun Vet™, Bayer, Leverkusen, Germany) and atropine 0.04 mg/kg (Atropin™ NM Pharma, Stockholm, Sweden). Anaesthesia was maintained by a continuous infusion of sodium pentobarbital 8 mg/kg per hour (Pentobarbitalnatrium™, Apoteket, Umeå, Sweden), pancuronium bromide 0.26 mg/kg per hour (Pavulon™, Organon, Oss, the Netherlands) and morphine 0.48mg /kg per hour (Morphine™, Pharmacia, Uppsala, Sweden) dissolved in a 2.5% glucose-electrolyte solution. During the experiment, sodium chloride infusion was given resulting in a total fluid administration rate of 30 mL/kg per hour.

A bolus dose of 20 mg morphine was given intravenously before the performance of a tracheotomy, which was done in order to secure a free airway during the experiment and to administer nitrous oxide (N2O) during surgical preparation. The animals were artificially ventilated throughout the experimental procedure (Servo 900CTM, Siemens Elema, Stockholm, Sweden). During catheter insertions, 30% oxygen in N2O was given. The gas mixture was switched to 30% oxygen in air during the rest of the experiment. The ventilation after preparation was set to yield a PaCO2 between 5.0 and 5.5 kPa. The respiratory rate was 25 per min and the inspiratory-expiratory ratio was 1:3. Ventilator settings were then kept constant throughout the experi-
ment. Atelectasis was prevented by placing the piglets into prone position after preparatory procedures and by maintaining a positive end expiratory pressure of 5 cm H\textsubscript{2}O. A central venous line as well as a 7 F Swan-Ganz catheter equipped with thermistor were inserted through the internal jugular vein into the superior caval vein and the pulmonary artery, respectively. In addition, a cervical artery was catheterised with an arterial line used for blood sampling and continuous measurement of the arterial blood pressure. A minor vesicotomy was performed for the urinary catheter and a heating pad (Operatherm 200W\textsuperscript{TM}, KanMed, Bromma, Sweden) was used to keep the animals at a constant body temperature.

Administration of endotoxin and antibiotics (III)

As the preparation was completed, at least 20 minutes of stabilisation time passed before baseline values were registered and baseline blood samples were taken prior to administration of antibiotics and endotoxin. All animals were subjected to endotoxin infusion (Escherichia Coli O111:B4; Sigma Chemical, St. Louis, MO, USA) with an initial infusion rate of 4 \( \mu \text{g/kg per hour} \). After 30 minutes the infusion rate was reduced to 1 \( \mu \text{g/kg per hour} \) which was continued during the rest of the experiment. At least a two-fold increase in MPAP (mm Hg) was taken as a sign of severe endotoxemic shock.

If the mean arterial pressure (MAP) decreased to the level of the MPAP during the first hour of the experiment, a single dose of 0.2 mg adrenaline was given intravenously. Values were registered hourly after baseline for six hours after which all surviving piglets were killed by an intravenously injected overdose of potassium chloride.

The animals were randomised by the sealed envelope method to receive either tobramycin, ceftazidime or saline solution. Tobramycin was given as an iv infusion of 140 mg in 100 ml saline solution for 20 minutes, starting 10 minutes before initiation of endotoxin infusion. Ceftazidime was injected as an iv bolus dose of 1 g in 10 ml saline 5 minutes before the start of endotoxin infusion and followed by 90 ml of saline for a total infusion time of 10 minutes. In the control group, an infusion of 100 ml saline was initiated 5 minutes before the start of endotoxin administration and given for 10 minutes.
Measurements (III)

MAP and MPAP were continuously monitored through the arterial and the Swan-Ganz catheter, respectively. The central line was used for injection of 10 mL cold saline for determining the cardiac output (CO). CO was expressed as cardiac index (CI) and body surface area was calculated as weight$^{0.67} \times 0.112$. CI was assessed hourly by the thermodilution method using the thermistor in the Swan Ganz catheter. The average value of at least three serial measurements was registered. Heart rate (HR) was continuously monitored. Blood samples for analysis of endotoxin, TNF-α and IL-6 were drawn immediately before addition of antibiotics and hourly until the end of the experiment. At the same time points, arterial blood gas and base deficit were analyzed (ABL™ 300, Radiometer, Brønhøj, Denmark).

Determination of plasma endotoxin, TNF-α and IL-6 concentrations (III)

Blood samples for analysis of plasma endotoxin, TNF-α, and IL-6 concentrations were drawn immediately before addition of antibiotics and hourly until the end of the experiment at 6 h. Endotoxin-free, heparinized tubes were used for the endotoxin samples and EDTA tubes for the TNF-α and IL-6 samples. After centrifugation the supernatants were transferred to endotoxin-free tubes and subsequently kept at −70°C until analysis.

Analyses of endotoxin were performed in duplicate with the limulus amoebocyte lysate assay (Coatest™ Plasma ChromoLAL; Charles River Endosafe, Charleston, SC, USA). Commercial sandwich enzyme-linked immunosorbent assays were used for detection of IL-6 (Quantikine™ porcine IL-6, P6000; R&D Systems, Minneapolis, MN, USA) and TNF-α (KSC3012; Biosource International, Nivelles, Belgium).

Calculations and statistical methods

Reduction in cfu at various time points was calculated by subtraction of the remaining number of cfu from that at time zero (I, II, IV, V) and from that at the time for the second antibiotic dose (IV). The number of cfu at time zero was calculated as the mean of the values obtained immediately before and after addition of antibiotics (I, II, V). The endotoxin and SpeA release was
calculated similarly (I, II, IV,V). The bacterial killing rate in papers I and II was calculated as the time needed for the antibiotic to kill $2 \log_{10}$ cfu and in the same studies analysis of variance (ANOVA) with repeated measurements was used to compare propensities to release endotoxin and bacterial killing rates. Regression analysis was used to determine the relationship between the number of killed bacteria and the increase in endotoxin (I,II). In papers I and II, the propensity to release endotoxin was expressed as endotoxin release per killed bacterium, whereas in paper IV, in which the variation in the viable count was greater, this was expressed as the $\log_{10}$ endotoxin release per $\log_{10}$ killed bacteria.

To compare the differences in killing rates and SpeA release between antibiotic regimens, a repeated measures ANOVA was performed (V). A Spearman rank correlation test was employed to test relationships between SpeA release, inoculum size and killing rate.

In the in vivo study (III), the TNF-$\alpha$ response at 1 h and the IL-6 response at 2 h were compared in the primary analyses of the immediate initial cytokine response. Since there was a variation in the immediate cytokine response, the reduction in the concentrations of the TNF-$\alpha$ and IL-6 from 1 h and 2 h, respectively, to 6 h, as well as the concentrations at 6 h, were considered as late cytokine responses. In order to be able to detect a difference in the initial log concentration of the cytokines of at least 20 % with an $\alpha$-error of 0.05, a $\beta$-error of 0.2, a power of 0.8, and a calculated interindividual variation of 14 %, eight evaluable animals should be included in each treatment group. In this exploratory trial, an interim analysis was planned when there were at least four animals in each group that had survived for 6 h. The distribution of the logarithm of the initial cytokine response approximated normal distribution in this model and was therefore analysed by an unpaired t-test. Other differences between the treatment groups in this study were calculated by the non-parametric Mann-Whitney U-test.

P-values <0.01 were considered significant in study I due to multiple statistical analyses, in the remaining studies p-values <0.05 were significant. Results were expressed as mean $\pm$ SE or median $\pm$ range.
Results and discussion

MIC values (I II, IV, V)

The MICs for the gram-negative strains are shown in Table 1. The difference in MIC-values for the B049 strain against cefuroxime in papers I (MIC=8 mg/L) and V (MIC=2 mg/L) may be explained by differences in methods and a method error of plus one step.

Table 1. MICs against cefuroxime and tobramycin for the gram-negative strains.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Cefuroxime</th>
<th>Tobramycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli B 049-3036 (I)</td>
<td>8,0</td>
<td>0,25</td>
</tr>
<tr>
<td>E. coli B 049-3036 (IV)</td>
<td>2,0</td>
<td>0,5</td>
</tr>
<tr>
<td>E. coli CCUG 17620 (II)</td>
<td>2,0</td>
<td>1,5</td>
</tr>
<tr>
<td>E. coli ATCC 25922 (IV)</td>
<td>4,0</td>
<td>1,0</td>
</tr>
<tr>
<td>E. coli B 940-3012 (II)</td>
<td>2,0</td>
<td>1,0</td>
</tr>
<tr>
<td>E. coli B 942-1035 (II)</td>
<td>1,5</td>
<td>1,0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae B 541-1003 (II)</td>
<td>2,0</td>
<td>1,0</td>
</tr>
<tr>
<td>Salmonella enteritidis CCUG 35852 (II)</td>
<td>8,0</td>
<td>1,0</td>
</tr>
<tr>
<td>Neisseria meningitidis CCUG 15117 (II)</td>
<td>0,05</td>
<td>2,0</td>
</tr>
</tbody>
</table>

The group A streptococcus strain used was highly sensitive to penicillin and clindamycin, with MIC values of 0.008 mg/L and 0.125 mg/L against penicillin G and clindamycin, respectively.

Killing rates (I, II)

The killing rate was expressed as the time needed for 2 log10 killing. In all the tested bacteria, including the four E. coli strains, the Salmonella enteriti-
dis, Klebsiella pneumoniae and N. meningitidis strains, there was an increased killing rate after exposure to tobramycin and cefuroxime in combination with tobramycin as compared with cefuroxime alone (p<0.001). At higher concentrations within the same treatment, the killing rate was significantly greater for all three regimens. The concentration-dependency of the killing rate was greater for tobramycin and tobramycin plus cefuroxime than for cefuroxime alone, which was confirmed by using interaction terms in the multivariate analysis (p<0.01).

Antibiotic-induced endotoxin release (I, II)

The PBP-3 specific cefuroxime led to a considerably greater endotoxin release than tobramycin or the combination of the two at all concentration levels, even if there was a large species variation (Table 2).
Table 2. Increase in endotoxin release after 2 and 4 hours. EU/ml ± SE.

<table>
<thead>
<tr>
<th></th>
<th>E.coli CCUG</th>
<th>E.coli B940</th>
<th>E.coli B942</th>
<th>Salmonella</th>
<th>Klebsiella</th>
<th>Neisseria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2h</td>
<td>4h</td>
<td>2h</td>
<td>4h</td>
<td>2h</td>
<td>4h</td>
</tr>
<tr>
<td><strong>Cefuroxime</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2xMIC</td>
<td>141±80</td>
<td>3006±1484</td>
<td>843±272</td>
<td>2699±534</td>
<td>791±296</td>
<td>656±376</td>
</tr>
<tr>
<td>10xMIC</td>
<td>414±83</td>
<td>4186±2470</td>
<td>452±181</td>
<td>1038±283</td>
<td>1064±62</td>
<td>1421±145</td>
</tr>
<tr>
<td>50xMIC</td>
<td>292±50</td>
<td>5027±3602</td>
<td>520±217</td>
<td>1312±478</td>
<td>1247±45</td>
<td>182±34</td>
</tr>
<tr>
<td><strong>Tobramycin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2xMIC</td>
<td>140±81</td>
<td>250±13</td>
<td>242±31</td>
<td>199±61</td>
<td>200±51</td>
<td>300±69</td>
</tr>
<tr>
<td>10xMIC</td>
<td>197±58</td>
<td>197±34</td>
<td>142±15</td>
<td>122±45</td>
<td>122±45</td>
<td>21±10</td>
</tr>
<tr>
<td>50xMIC</td>
<td>134±26</td>
<td>139±65</td>
<td>144±22</td>
<td>168±78</td>
<td>156±10</td>
<td>160±37</td>
</tr>
<tr>
<td><strong>Cefuroxime +</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2xMIC</td>
<td>160±11</td>
<td>202±29</td>
<td>216±32</td>
<td>238±43</td>
<td>199±21</td>
<td>262±28</td>
</tr>
<tr>
<td>Tobramycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10xMIC</td>
<td>130±29</td>
<td>193±37</td>
<td>184±37</td>
<td>218±49</td>
<td>157±25</td>
<td>204±50</td>
</tr>
<tr>
<td>50xMIC</td>
<td>97±18</td>
<td>142±11</td>
<td>194±49</td>
<td>238±81</td>
<td>205±17</td>
<td>215±6</td>
</tr>
</tbody>
</table>
Most of the endotoxin was liberated within 2 h after exposure to tobramycin or the combination, whereas an important additional increase was seen between 2 and 4 h from most of the strains exposed to cefuroxime alone (I,II). The prolonged and high endotoxin liberation by cefuroxime can be explained by the PBP 3-associated filamentous elongation of the bacteria with continuing endotoxin production and liberation (24,78).

Endotoxin release in relation to the number of killed bacteria (I, II)

For the *E. coli* B049-3036, investigated in paper I, there was a linear relationship between the endotoxin release and the number of killed bacteria during the first 4 h for each antibiotic regimen at each dosage level, with $r^2$ values 0.61–0.96. This indicated a proportionality, and that the propensity to release endotoxin could be expressed as endotoxin per killed bacterium.

At 4 h, there was a significant difference between the antibiotics, with the highest propensity to release endotoxin for cefuroxime, lower for tobramycin and the lowest for the combination of the two (p<0.001) (Fig.3).

![Figure 3. Endotoxin release per killed bacterium 4h after exposure to antibiotics at 2xMIC, 10xMIC, and 50xMIC.](image)
With increasing doses, there was a significant reduction (p<0.001) in endotoxin release per killed bacterium at 10xMIC and 50xMIC for tobramycin and the combination and at 50xMIC also for cefuroxime alone. It seems reasonable to assume that the reduction in the propensity to release endotoxin by cefuroxime at the highest doses is due to changes in PBP-affinity, as has earlier been shown for ceftazidime (78). Thus, the affinity for PBP 3 decreases in favour of PBP 1, resulting in bacterial lysis without filament formation and with less endotoxin release. When the bacterial killing rate was correlated to the propensity to release endotoxin in bacteria exposed to tobramycin or the combination of tobramycin and cefuroxime, a significant negative correlation was found.

The intention with paper II was to see if the demonstrated reduction in the propensity to release endotoxin by tobramycin in one strain was applicable to other *E. coli-*strains and to other gram-negative species. Moreover, the proportionality between endotoxin release and number of killed bacteria, observed in our previous study, was studied over the wider range of bacteria often seen in clinical practice.

After 4 h of antimicrobial exposure, large species variations in the propensity to release endotoxin were demonstrated. After exposure to cefuroxime, the *Klebsiella* strain liberated 6.3 – 9.9 x 10^{-3} EU/bacterium, whereas the *N. meningitidis* strain released considerably less, 0.1 - 0.4 x 10^{-3} EU/bacterium. Even among the *E. coli* strains, there was a difference in cefuroxime-induced liberation, the B 940 strain releasing only half of that of the B 942 strain. Corresponding variations were seen after exposure to tobramycin or the combination of cefuroxime and tobramycin, but these were of smaller magnitude. The propensity to release endotoxin upon antimicrobial exposure seems to be a strain-specific characteristic, since there was a significant correlation between the release caused by tobramycin and that by cefuroxime (p<0.05 at each concentration level). The endotoxin released by the *N. meningitidis* strain was remarkably low in comparison with that of the other strains, especially when the exposure to cefuroxime was compared. The findings are in agreement with the results from an *ex vivo* study by Prins et al. (79). It may also be the result of the large intraspecies variation that has earlier been described for meningococci (5). In addition, PBP 1, 2 and 3 have all been found in the meningococcal cell wall and varying affinities to β-lactam antibiotics have been described for all of them (67,75,82).

In the endotoxin release per killed bacterium after 4 h, there was a significant treatment effect in the *E.coli* CCUG strain (p<0.001), the B940-3012 strain (p<0.001), the B942-1035 strain (p<0.001), the *Klebsiella* strain (p<0.001), and the *Salmonella* strain (p<0.01). Paired comparisons demon-
strated highly significant differences between cefuroxime and the combination of tobramycin and cefuroxime. The reduction was 96%, 93%, 97%, 86%, and 85%, respectively, from that caused by cefuroxime alone. Similarly, the difference between cefuroxime and tobramycin alone was large and strongly significant. For the *N. meningitidis* strain, these differences did not reach statistical significance but a similar trend was seen with a reduction of 52% when tobramycin was added to cefuroxime. In none of these strains there was a statistical difference between the combination and tobramycin alone.

After 4 h, there was a significant dose-dependent reduction in endotoxin release per killed bacterium in the *Salmonella*, the *E. coli* CCUG, the *E. coli* B942, and the *Klebsiella* strains (p<0.05 for each strain). The reductions in tobramycin-induced endotoxin release from 2xMIC to 50xMIC were 67%, 48%, 46% and 29%, respectively. Corresponding values for the combination were 44%, 33%, 16%, and 41%, respectively. In the *N. meningitidis* strain, a similar trend was demonstrated (p=0.05), with a mean reduction of 71% and 29% by tobramycin and the combination, respectively. No dose-dependency at all was seen in the *E. coli* B940 strain.

There was no significant dose-dependency in the cefuroxime-induced release in any of the strains.

Despite large variations in antibiotic-induced endotoxin release, there was in all strains a considerable reduction in the cefuroxime-induced endotoxin release per killed bacterium when tobramycin was given concomitantly. This indicates that the effect observed in the previously investigated *E. coli* strain seems to be applicable to many other gram-negative bacteria. Similarly, dose-dependency with a reduction in endotoxin release at higher tobramycin concentrations was seen in all strains with the exception of the *E. coli* B940 strain. This response constitutes another advantage besides the increase in bacterial killing rate and the reduction in toxicity associated with the once daily high-dose regimen of aminoglycosides.

In a separate experiment, the endotoxin release of *K. pneumoniae* with different starting inoculae (10³, 10⁵ and 10⁷ cfu/ml) after exposure to 2x MIC of cefuroxime, tobramycin or a combination of cefuroxime and tobramycin, was studied in relation to the number of killed bacteria (Fig 4).
Figure 4. *Klebsiella pneumoniae*. Correlation between endotoxin release and number of killed bacteria after 4h at starting inoculae of $10^3$, $10^5$ and $10^7$ cfu/ml, respectively.

The correlations between logarithmic values of endotoxin release and number of killed bacteria were highly significant for cefuroxime ($r=0.99$; $p<0.001$), tobramycin ($r=0.93$; $p<0.001$), and the combination ($r=0.99$; $p<0.001$). The slopes of the regression lines did not differ significantly, the mean value being $0.75\pm0.04$. This implies that there is a considerably higher release at higher bacterial concentrations but per killed bacterium the endotoxin release is somewhat lower. The reason for this is not known but it may be speculated that a high environmental endotoxin concentration may lead to formation of endotoxin aggregates and a reduction in the biological activity. The magnitude of the slope demonstrates that within 1 log$_{10}$ the deviation from the straight line leads to only a small systematic error when expressing the endotoxin release in terms of release per killed bacterium and in comparison with the effect on endotoxin release due to variations in inoculum size, this error is negligible.

Possible mechanisms for reduction in endotoxin release by tobramycin (I,III)

The initial hypothesis to explain the reduction in endotoxin release by the aminoglycosides was by an unspecific binding similar to that demonstrated
for polymyxin B (47,78,85). However, in paper I it was shown that tobramycin at 2.5, 12.5 and 25mg/L added to BHI spiked with endotoxin did not reduce the endotoxin activity in the solution as compared with the control.

In a separate experiment with cefuroxime at a concentration of 10xMIC in a bacterial solution, it was demonstrated that if the addition of tobramycin was delayed for 2 h, no reduction in the cefuroxime-induced endotoxin release took place, neither immediately nor after 4 h. Thus, neutralisation can be excluded as the principal explanation of the reduction in cefuroxime-induced endotoxin release.

The absence of in vitro neutralisation indicates that there is no aminoglycoside binding that affects the biological activity in the limulus amoebocyte lysate assay but, on the other hand, a tobramycin binding that in vivo neutralises the endotoxin effect cannot be excluded. Inhibition of the binding of endotoxin to LBP, the most important carrier protein in the body which is crucial for the attachment to the CD14 receptor on the macrophages, represents one possible mode of action. Another might be that binding with LBP is unaffected, whereas the tobramycin-endotoxin-LBP complex has a reduced affinity for the CD14 receptor or a lower potential to elicit a signal via TLR 4 for a subsequent TNF-α- and IL-6- production.

In the porcine in vivo model (III), the primary endpoints were therefore to analyse the immediate TNF-α and IL-6 responses after 1 h and 2 h, respectively, in relation to the endotoxin concentration. These time points were chosen since cytokine values obtained later might be influenced by an aminoglycoside-induced protection against oxidative injury, which has been demonstrated in vitro (15). The plasma TNF-α levels reached peak values 1 h after the start of endotoxin infusion with a median value of 7265 ng/L (range 2492 – 10625 ng/L). There were no significant differences in peak TNF-α values between the treatment groups. TNF-α concentration decreased to baseline levels within 3 h in most animals.

Plasma IL-6 levels peaked after 2 h or 3 h with a median maximum IL-6 value of 2372 ng/L (range 1544–7138 ng/L). There were no significant differences in IL-6 concentration at 2 h between treatment groups (Fig. 5).
Figure 5. Plasma IL-6 concentrations 2-6 hours after start of endotoxin infusion (n=4 in each group).

These results indicate that there is no *in vivo* neutralisation of endotoxin-induced cytokine production and consequently that there is no interaction with the binding to LBP or the CD14 receptor.

In order to maximise a possible neutralising effect, the tobramycin dose chosen was in the highest range of that used clinically. Nonetheless, neither tobramycin nor ceftazidime had any endotoxin neutralising effect in the LAL assay in comparison with that caused by saline. These results are thus in agreement with the *in vitro* results described in paper I.

An alternative hypothesis to endotoxin neutralisation may be that the mode of action of tobramycin is responsible for the reduced release of endotoxin. The significant negative correlation between the bacterial killing rates of tobramycin and the combination of tobramycin and cefuroxime, on the one hand, and the endotoxin release on the other, supports this hypothesis. Failure of tobramycin to reduce cefuroxime-induced endotoxin release if added after a 2 h pre-exposure to cefuroxime, indicates that initial mechanisms probably are the most important. Previously, Kusser et al. have shown that aminoglycosides are able to inhibit the synthesis of endotoxin (53). Our results strongly support that such a mechanism is quantitatively more important in comparison with neutralization for the reduction in endotoxin release.
Possible anti-inflammatory effects by antibiotics (III)

The inflammatory damage to endothelial cells and other tissue cells mediated by oxygen radicals induces cytokine production (55,76). In several in vitro experiments, it has been demonstrated that ceftazidime and some of the other β-lactam antibiotics inactivate hypochlorous acid (HOCl) (21) and, in the case of ceftazidime, also singlet oxygen (66). In clinically relevant concentrations, ceftazidime has also been shown to partially protect endothelial cells from the oxidative stress of activated neutrophils in vitro (55). The documentation for aminoglycosides is less extensive but both tobramycin and gentamicin have been shown to protect lung epithelial cells against myeloperoxidase-dependant oxidant injury by binding to anionic cell surfaces and neutralising HOCl (15). These findings led to the hypothesis that the anti-inflammatory effects might affect the cytokine response during the last phase of our porcine model. At the end of the experiment, at 6 h, there was a treatment effect expressed as a significantly greater reduction in IL-6 by ceftazidime as well as tobramycin (p<0.05) and a lower absolute IL-6 level in the ceftazidime group as compared with placebo (p<0.05). Since there are in vitro data that ceftazidime neither directly affects IL-6 production (66) nor neutrophil function (34), this result may have been caused by its antioxidative properties. For tobramycin, in vitro data indicate that neutrophil function and oxidative metabolism might be influenced but these results have not been reproduced in vivo (34). Whether our findings are due to antioxidative properties of the antibiotics or to other mechanisms cannot be concluded from data presented, but the result indicates that in vitro reported anti-inflammatory properties may be demonstrable in an animal model.

Effect of repeated dosing on endotoxin release and morphology (IV)

In an in vitro kinetic model, simulating the human concentration time-profile, the endotoxin release from two E. coli strains after exposure to two repeated doses of cefuroxime was investigated. In addition, it was studied whether the combination with tobramycin at the second dose resulted in a reduction similar to that previously shown when combined with the first dose.
Figure 6. Bacterial killing rate (mean ± SE) and endotoxin release (geometric mean ± antilog of the SE of the logarithmic values) after the first and second dose of cefuroxime in the ATCC strain (a) and the B049 strain (b). Note that a considerably lower number of bacteria were killed after the second dose.
There was a large variation in endotoxin concentrations, mainly due to the variation in the number of killed bacteria. Median endotoxin release after correction for elimination via the outflow of the pump was 2 h and 4 h after the first dose 1720 and 12600 EU/ml, respectively, for the ATCC strain and 8570 and 21100 EU/ml, respectively, for the B049 strain. Corresponding values after the second dose were 102000 and 124000 EU/ml for the ATCC strain ($p<0.01$) and 128000 and 149000 EU/ml (NS) for the B049 strain, respectively. The geometric mean of the endotoxin release is shown in Fig. 6.

When the endotoxin release was related to the number of killed bacteria, there was a marked reduction in the variation in both strains. The increase in the tendency to release endotoxin after the second dose in comparison with that after the first was highly significant ($p<0.001$ for both strains). The difference between the doses ranged from 0.15 to 0.26 log$_{10}$ EU/log$_{10}$ number of killed bacteria. This implies that the difference between the doses will be greater at higher numbers of killed bacteria and this will, in effective treatment, covariate with the bacterial concentration at the time of the dose. The time course was also significantly different, with an earlier release after the second dose ($p<0.001$ for both strains).

One possible mechanism behind the higher endotoxin release after the second dose of cefuroxime might be the continued release from remaining filaments caused by the first dose. This hypothesis is supported by Jackson and Kropp, who found an increased endotoxin release at sub-MIC concentrations of several β-lactam antibiotics (48). Our electron microscopy findings just before the second dose with a few elongated forms may also be in agreement with this. However, at that time, the endotoxin concentration was relatively low, indicating that even if there may be some sustained release, the contribution of this to the total release after the second must be limited. A change in PBP affinity with a higher binding to PBP 3 than to PBP 1 represents another possibility, but the presence of spheroplasts together with filaments after the second dose does not favour this hypothesis. Thus, the mechanism is not clear but it might be speculated that there is an enduring antibiotic effect on cell wall synthesis after the first dose resulting in a quicker and more extensive bacterial elongation after the second dose.

After addition of tobramycin to the second dose of cefuroxime, the propensity to release endotoxin was significantly reduced in comparison with that after cefuroxime alone ($p<0.001$), even if the bacteria had previously been exposed to cefuroxime. In addition, the filamentation was less pronounced. The effect of a repeated combined aminoglycoside β-lactam antibiotic treatment could not be studied, because, at the clinical concentrations used all
bacteria were killed already after the first dose at the inoculum sizes possible to use in our in vitro kinetic model.

Exotoxin release (V)

The purpose was to investigate the amount and time course of SpeA liberation from a GAS strain, at different starting inoculae, after exposure to different doses of penicillin, clindamycin or a combination of the two, and to relate the release to the bacterial concentration and the killing rate.

The starting inoculum sizes in the low and high inoculum experiments were $7.0 \pm 0.1$ and $8.6 \pm 0.2 \log_{10}$ cfu/ml, respectively. At the low, as well as the high initial inoculum, a considerably higher killing rate was seen for penicillin than for the combination of penicillin and clindamycin ($p<0.01$ and $p<0.001$, respectively), but there was no difference in bacterial killing rate between low and high antibiotic doses within any of the treatment regimens. A similar result was seen in the high inoculum experiment when penicillin was compared with clindamycin alone ($p<0.01$), whereas in the low inoculum experiment, only a trend in the similar direction was observed ($p=0.09$).

In contrast to antibiotic-induced endotoxin liberation from gram-negative bacteria which, after exposure to β-lactam antibiotics, initially leads to higher endotoxin concentration than in the control (24), all treatment regimens in the present study resulted in a SpeA release, which was many times lower than that of the control. In the high inoculum experiments (Fig.7), there was a large variation in the SpeA production, from $39\pm9$ ng/ml for low dose penicillin to $-2\pm3$ ng/ml for the high dose of the combination. The toxin release was significantly higher at low than at high concentrations of penicillin ($p<0.05$). This dose-dependency was also seen for the combination ($p<0.05$) but not for clindamycin alone. Since there was no difference in killing rate between the two doses this cannot explain the result. For certain β-lactam antibiotics, such as ceftazidime or cefuroxime, binding to different *E.coli* PBPs has been demonstrated at varying concentrations, which results in a higher endotoxin release after low than after high doses (71). It may be speculated that one possible mechanism is that penicillin has a similar dose-dependent varying affinity to different PBPs. This would imply a different binding pattern at low and high concentrations, which then may lead to a divergent effect on cellular activity prior to lysis. Varying clindamycin concentrations did not influence the SpeA release but addition of clindamycin could not abolish the dose-effect of penicillin in the combination.
Figure 7. SpeA production in the high inoculum experiment, at low (a) and high (b) antibiotic doses, µg/L ± SE.
In the low inoculum experiments, the highest release of SpeA was seen at low penicillin concentration. However, the release was $>1 \log_{10}$ lower than that seen in the high inoculum experiment and no statistically significant differences between treatments could be demonstrated.

Clindamycin alone released less SpeA than penicillin, which, however, was significant only at low concentrations ($p<0.01$). When clindamycin was added to penicillin, there was a significant reduction in SpeA release both at low and high concentrations ($p<0.01$ for both comparisons) which is in accordance with an in vitro study by Coyle et al. (17). The most probable explanation of this is that clindamycin, as a protein synthesis inhibitor, reduces the production of toxins or proteins responsible for their release to the environment (52,89).

Most of the speA was released within the first 3 h. The SpeA production during this period correlated significantly to the number of killed bacteria during the same period ($n=18$, $r=0.50$; $p<0.05$). When analysed separately for each treatment regimen, a positive correlation could be demonstrated after treatment with penicillin and the combination, the latter being significant ($p<0.01$), whereas no similar correlation was seen for clindamycin. Within the limited range of the inoculum size of $0.8 \log_{10}$ cfu/ml, there was a significant positive correlation between this and the speA concentration at time zero ($r=0.54$; $p<0.05$). In contrast, there was a trend towards a negative correlation to the increase during the following hours ($r=-0.42$; NS), being negative for all treatment regimens. Therefore, variation in the initial inoculum size does not explain the correlation between toxin release and number of killed bacteria. The reason for this is not clear, but the killing rate of β-lactam antibiotics has been associated with growth rate and hence the metabolic activity of the bacteria (90), and it may therefore be speculated that an initially higher metabolism also results in an increased toxin production. When a protein synthesis inhibitor, such as clindamycin, shuts this off, the relationship is no longer seen.

In several experimental infections of animals with GAS, clindamycin has turned out to be more effective than penicillin (12,91). Therefore, the combination of penicillin and clindamycin has often been recommended for the treatment of severe GAS infections (41,87). Even if there is a limited reduction in the killing rate, this seems to be outweighed by the reduced inoculum effect (90,91), longer post-antibiotic effect (89), suppressed synthesis of virulence factors, such as M-protein and capsule (12,32), enhanced opsonisation and phagocytosis (32) and decreased toxin release by clindamycin.
Clinical implications

In animal models, there is strong evidence that soluble endotoxin released from gram-negative bacteria by antibiotics contributes to pathogenesis and mortality of the sepsis (70). However, the clinical significance of antibiotic-induced endotoxin release is still not known (78). Anecdotal clinical data from the studies by Dofferhoff et al. and Arditi et al. demonstrate that increases in endotoxin concentration may be associated with clinical deterioration (7,24) and the results from a prospective clinical study in patients with gram-negative urosepsis by Prins et al. suggest that the difference between imipenem and ceftazidime in endotoxin release may also be reflected by differences in the systemic inflammatory response (77). Furthermore, a retrospective study by Mock et al. has demonstrated that mortality was higher in trauma patients treated with endotoxin-releasing PBP 3-specific antibiotics than in those given other antibiotics, which lends some support to a possible clinical significance (69).

On the other hand, there are clinical studies that do not find any differences in the concentrations of endotoxin and cytokines or in clinical outcome between PBP 2-specific imipenem and PBP 3-specific ceftazidime treatment of various gram-negative infections (14,60,63). In a study by Maury et al., in which patients had both a verified gram-negative bacteremia and a severe infection in the form of severe sepsis or septic shock, endotoxin concentration was significantly decreased after one and four hours of antibiotic treatment, suggesting that antibiotic-induced endotoxin release is not a clinically relevant phenomenon (65). However, in this non-comparative study, the total endotoxin concentration was measured, also including bacterial cell-bound endotoxin. As has been shown by Arditi et al. in studies on children with gram-negative meningitis, there is a fall in total endotoxin concentration that parallels the reduction in bacterial count, whereas the free endotoxin level concomitantly increases (7). In another study, Hurley et al. found that free endotoxin concentrations increased when chronically bacteriuric patients were treated with different antibiotics (44) Thus, if measured as free endotoxin, which is the most biologically active form, antibiotic-induced endotoxin liberation has been demonstrated to occur also in patients.
In clinical trials, there are difficulties to prove an effect between treatments, even if there is one, due to the large variations in the endotoxin release. As shown in our study, endotoxin liberation is highly dependent on the number of bacteria which may clinically vary considerably. If the release of endotoxin is related to the number of bacteria, it may be possible to reduce the interindividual variation and consequently increase the chance in clinical trials to detect a difference between treatments. However, even if this variation is reduced, the strain specific propensity to release endotoxin still represents an important confounding factor.

Taken together with the similarity between the responses of human volunteers and animals to administration of endotoxin, the resemblance of this response to the symptoms seen in sepsis and septic shock (96), and the reduction in systemic inflammatory response demonstrated in at least one prospective and controlled study (77), available data suggest that reduction in endotoxin release may be of clinical importance. In patients with uncomplicated gram-negative infections, antibiotic-induced endotoxin release is most probably of limited value. On the other hand, in patients with progressing severe sepsis or who have developed septic shock, an additional dose of endotoxin may be deleterious (58).

Despite a great inter- and intraspecies variation among the gram-negative bacteria, it was possible in our in vitro endotoxin studies to demonstrate significant reductions in endotoxin release when the combination of the PBP 3-specific β-lactam antibiotic, cefuroxime, and the protein synthesis inhibitor, tobramycin, was compared with cefuroxime alone. Furthermore, increasing doses of tobramycin led to a lower endotoxin release and a higher bacterial killing rate. If tobramycin was added as late as 2 h after cefuroxime administration, no inhibitory effect on the endotoxin release was registered.

These findings indicate that it is probably an advantage to add an aminoglycoside to the β-lactam antibiotic for the treatment of severe infections, such as progressing severe sepsis and septic shock, a combination that has been recommended for a long time (101). Besides a broad spectrum and a high killing rate, also the release of endotoxin is diminished. If the treatment is initiated with a PBP 3-specific antibiotic but the clinical condition of the patient deteriorates, an aminoglycoside dose may be given concomitantly or before the second dose. Our data also indicate that it is an advantage to give a high dose of the aminoglycoside. High doses have also been recommended in critically ill patients due to the increase in the volume of distribution (13,40). However, the beneficial effects should be weighed against the nephro- and neurotoxic effects of the aminoglycosides. In a study in critical care patients with a creatinine clearance of >70 ml/min, very high doses
were given for six days without impairment of renal function, provided that the aminoglycoside concentrations were followed (54). In these patients, kidney function was more or less preserved but, a recent study in patients with poor renal function and peritoneal dialysis has demonstrated that aminoglycoside treatment did not affect residual renal function (9). The use of aminoglycosides in a high dose regimen in oliguric or anuric patients or patients who present with a rapidly decreasing renal function needs further investigation. However, taken together, these data indicate that one or a few doses of an aminoglycoside in the initial phase of the treatment of severe sepsis or septic shock may have beneficial effects and that the risk of renal injury, with the exception of the patients with the poorest function, seems to be very low.

In some similarity with the discussion above, there seems to be an advantage to combine penicillin with the protein synthesis inhibitor, clindamycin, for the treatment of severe group A streptococcal infections.

In animal studies clindamycin alone, or in combination with penicillin, has shown beneficial effects in the treatment of invasive group A streptococcal infections (56,91). This is in part due to inhibited expression of different streptococcal virulence factors, such as SpeA (62,90).

The findings in our in vitro study that clindamycin released less SpeA from a GAS strain than penicillin and that the addition of clindamycin to penicillin resulted in less toxin production than treatment with penicillin alone, are in agreement with earlier in vitro and animal experiments. The study also demonstrated that the SpeA concentration was dependent on the number of bacteria prior to antibiotic exposure and the dose of penicillin. In the clinical setting, early treatment seems to be very important and, in addition, combination therapy from the start with high doses of penicillin may offer an advantage.
Conclusions

The *in vitro* release of endotoxin is associated with the number of killed bacteria, the logarithm of the release being proportional to the logarithm of the number of killed bacteria.

The protein synthesis inhibitor, tobramycin, reduces endotoxin release *in vitro* from various gram-negative strains induced by the PBP 3-specific β-lactam antibiotic, cefuroxime. Increasing doses of tobramycin lead to less endotoxin liberation.

There is no unspecific binding between endotoxin and tobramycin *in vitro*. Nor does tobramycin affect the binding of the LBP-endotoxin complex to the macrophages *in vivo*.

The reduction in endotoxin release is associated with the killing rate of tobramycin.

The propensity to liberate endotoxin *in vitro* is higher after the second dose of cefuroxime than after the first, which results in a higher release of endotoxin than expected from the bacterial count. The release of the second dose can be reduced by the addition of tobramycin.

There is a possible anti-inflammatory effect given by ceftazidime and tobramycin expressed as a late cytokine inhibition in porcine endotoxin shock.

The SpeA release from a GAS strain *in vitro* is correlated to the initial number of bacteria and the killing rate. Addition of a protein synthesis inhibitor, clindamycin, reduces the toxin release in comparison with that of the β-lactam antibiotic, penicillin G, alone. The SpeA release is also dependent on the dose of penicillin, with high doses leading to lower SpeA levels as compared with low doses.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine*. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)