No increase in endotoxin release during antibiotic killing of meningococci

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Endotoxin is liberated following antibiotic killing of Gram-negative rods, and antibiotics may differ in this respect. Although the amount of filterable endotoxin has also been reported to increase following antibiotic killing of meningococci, it is unknown how this influences the host response. We investigated the influence of three antibiotics on levels of free endotoxin in culture medium and cytokine production in whole blood *ex vivo* during killing of four strains of meningococci. Bacterial killing was significantly more efficient with penicillin or ceftriaxone than with chloramphenicol, and free endotoxin levels were lower after exposure to antibiotics as compared with no treatment (ANOVA, $P < 0.001$). Endotoxin levels were lowest after exposure to chloramphenicol. In three of the four strains exposure to antibiotics resulted in considerably lower cytokine levels (ANOVA, $P < 0.001$), and TNF- α levels were significantly lower after exposure to penicillin or ceftriaxone than after chloramphenicol treatment. Only in the strain that induced the lowest levels of TNF- α were cytokine levels comparable for untreated and treated samples. We conclude that fear of excessive endotoxin release or cytokine production caused by effective antibiotics is not justified in the treatment of meningococcal infections.

Introduction

Endotoxin is considered to be the most important bacterial factor in the pathogenesis of infections caused by Gram-negative rods or meningococci.^{1,2} When Gram-negative rods are exposed to antibiotics, the amount of biologically active endotoxin in culture medium, blood, urine or CSF can increase, as has been shown in many *in vitro*, animal and clinical studies.^{3,4} In several studies this so-called 'endotoxin release' was paralleled by a deterioration of parameters of illness. Significant differences have been recorded between antibiotics in their capacity to induce endotoxin release and subsequent cytokine production during killing of Gram-negative rods *in vitro*.^{4,5} Antibiotic-induced endotoxin release might be especially relevant for meningococcal infections as, in patients with severe meningococcal infections, endotoxin levels are considerably higher than the levels measured in septic

shock caused by Gram-negative rods.^{6,7} During antibiotic killing of meningococci *in vitro* an increase in filterable endotoxin has been reported,^{8,9} and similar findings have been reported in experimental meningococcaemia in mice.¹⁰ However, it is not known to what extent the released endotoxin would affect human host responses.

Therefore, we studied free endotoxin levels in culture medium and cytokine production in whole blood *ex vivo* after exposure of log-phase meningococci to antibiotics that are used to treat meningococcal infection.

Materials and methods

Meningococci

Four strains of *Neisseria meningitidis* (two of serogroup B, two of serogroup C) were used in the experiments. Of each serogroup one strain was originally isolated from the

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blood of a patient with meningococcal septic shock and one strain was isolated from the CSF of a patient with meningitis only. The strains were stored at -70°C and subcultured on chocolate agar at 37°C in 5% CO_2 prior to use. One colony was suspended in 5 mL of RPMI 1640 (Gibco, Life Technologies Ltd, Paisley, UK) and incubated overnight at 37°C in 5% CO_2 . This suspension was diluted ten-fold the next morning in fresh RPMI and incubated at 37°C in 5% CO_2 . When log-phase growth occurred, as indicated by continuously increasing density in a turbidity photometer (Dr Lange, Trübungspotometer LTP4, Düsseldorf, Germany), at a concentration of 2×10^6 – 3.5×10^7 cfu/mL the suspension was diluted 10- to 100-fold in prewarmed RPMI 1640, immediately before the start of the experiments.

Antibiotics

Benzylpenicillin (Brocades Pharma, Leiderdorp, The Netherlands), ceftriaxone (Roche Mydrecht, The Netherlands) and chloramphenicol 0.5% ('Minims' Smith & Nephew, Romford, UK) were used. The MICs for the meningococci of the antibiotics were determined using the Etest (AB Biodisk, Solna, Sweden). The MICs ranged from 0.023 to 0.032 mg/L of benzylpenicillin, the MIC of ceftriaxone was <0.002 mg/L for all isolates and the MICs ranged from 0.75 to 1.5 mg/L of chloramphenicol. Final concentrations used in the experiments were 20 mg/L for penicillin G and ceftriaxone and 10 mg/L for chloramphenicol, which are the serum concentrations to be expected in humans using recommended dosages.^{11,12}

Bacterial killing

Fifty microlitres of the bacterial suspension (containing 1×10^4 – 1.5×10^5 cfu) was added to pyrogen-free tubes (Falcon 2063, Becton–Dickinson, Lincoln Park, NJ, USA) containing 900 μL of prewarmed RPMI 1640, and 50 μL of an antibiotic solution. Control experiments were performed by addition of only 50 μL of bacterial suspension. The tubes were subsequently incubated at 37°C in 5% CO_2 for 4 h.

For counting of viable cfu in the control experiments, 10- and 100-fold dilutions were made in phosphate-buffered saline (PBS) and 10 μL aliquots of the dilutions were plated on chocolate plates. For counting of viable cfu after exposure to penicillin or ceftriaxone, 100 μL of the suspension was plated on chocolate plates, while for the chloramphenicol-exposed cultures 10 μL was plated. The plates were incubated at 37°C in 5% CO_2 overnight and counting was performed. The experiments were performed in triplicate.

Morphological studies

N. meningitidis group C (approximately 2×10^6 cfu/mL) was incubated in RPMI 1640 in the absence or presence

of the various antibiotics. After 4 h, specimens were taken, put on a slide, and fixed in a cacodylate-buffered (pH 7.4) glutaraldehyde/formaldehyde mixture for one night. The next morning, the slides were thoroughly rinsed in cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried at the critical point with CO_2 . The slides were mounted on small metal plates with conducting carbon cement, and were coated with platinum. The specimens were studied in a Philips SEM 505 electron microscope, using a back-scatter detector (B. Willekens, The Netherlands Ophthalmic Research Institute, Amsterdam, The Netherlands).

Free endotoxin levels

Five hundred microlitres of the bacterial suspension (containing 4×10^4 – 1.4×10^5 cfu) was added to pyrogen-free tubes (Falcon 2059, Becton–Dickinson, Lincoln Park, NJ, USA) containing 9 mL of prewarmed RPMI 1640, and 0.5 mL of one of the antibiotic solutions. Control experiments were performed by addition of 0.5 mL of bacterial suspension and 0.5 mL normal saline. The tubes were subsequently incubated at 37°C in 5% CO_2 .

After 4 h, the tubes were centrifuged at 3300g for 30 min and supernatant was collected, immediately frozen and stored at -20°C until assessment. In pilot experiments bacterial numbers in supernatant were reduced to 0.1% of the numbers before centrifugation (data not shown). All experiments were performed in triplicate.

Whole blood simulations

Blood was collected by venipuncture in a pyrogen-free collecting system. The donor was known to have no bactericidal antibodies against group B or C meningococci (assay modified after Zangwill *et al.*¹³). Anticoagulation was obtained with pyrogen-free heparin (Organon Teknika BV, Boxtel, The Netherlands; final concentration 50 IE/mL blood). Fifty microlitres of the bacterial suspension (containing 6×10^3 – 1.8×10^4 cfu; low inoculum) was added to pyrogen-free tubes (Falcon 2063) containing 900 μL of heparinized whole blood; 50 μL of one of the antibiotic solutions was added. In the control experiments only 50 μL of bacterial suspension or antibiotic solution was added. The tubes were incubated at 37°C in 5% CO_2 for 4 h. Incubations were terminated by centrifugation (1500g for 20 min at 4°C). The plasma was collected, immediately frozen and stored at -20°C until assessment of tumour necrosis factor α (TNF- α).

In order to analyse further the TNF-inducing potency of endotoxin released from bacteria, an aliquot of bacterial suspension of each of the strains (in a concentration ten-fold higher than in the former experiment; high inoculum) was centrifuged at 3300g for 30 min, and supernatant was collected. Fifty microlitres of the bacterial suspension or the supernatant was added to 950 μL of heparinized

whole blood, and incubated for 4 h. All experiments were performed in triplicate.

Assays

Endotoxin assay. The endotoxin content was determined by a chromogenic *Limulus* amoebocyte lysate (LAL) assay (Coatest Endotoxin; Chromogenix AB, Mölndal, Sweden). The method has a detection limit in blood of 0.036 EU/mL. Each sample was assayed in duplicate, and the results were expressed as the means. The antibiotics did not influence endotoxin detection (data not shown).

TNF assay. TNF- α was measured using a sandwich TNF- α ELISA.¹⁴ The detection limit in plasma was 50 pg/mL. In the concentrations used, the antibiotics had no influence on TNF- α detection (data not shown).

Statistical analysis

Values were expressed as means $\pm$ S.D. of three experiments. Data were analysed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. Multiple comparisons between groups were performed by the Newman-Keuls test.

Results

Bacterial killing

In cultures without antibiotics (untreated cultures), bacterial numbers increased maximally three-fold during 4 h of incubation. Treatment of the four strains with penicillin or ceftriaxone resulted in a mean 3.4 log₁₀ and 3.7 log₁₀ decrease of cfu, respectively. Treatment with chloramphenicol yielded a mean 1.6 log₁₀ decrease of cfu (difference among antibiotics: ANOVA: $P = 0.002$; Newman-Keuls: penicillin or ceftriaxone versus chloramphenicol: $P < 0.05$).

Bacterial morphology

Scanning electron microscopy revealed that after treatment with penicillin or ceftriaxone the remaining meningococci had a wrinkled appearance; the characteristic diplococcal configuration could no longer be seen. After chloramphenicol treatment some small particles could be seen, possibly bacterial remnants, but meningococci that had a normal appearance were also present in large numbers (Figure 1).

Free endotoxin levels

The free endotoxin levels in RPMI 1640 after the addition of bacterial suspension at time (t) = 0 h ranged from 1.63 $\pm$ 0.04 EU/mL to 13.1 $\pm$ 0.97 EU/mL (Figure 2). After correction for the bacterial inoculum (range: 4×10^3 – 1.4

$\times 10^4$ cfu/mL), endotoxin levels ranged from 4.08 to 9.36 EU/ 10^4 cfu.

Free endotoxin levels in the supernatant after 4 h of incubation were significantly lower in the presence of antibiotics than in the absence of antibiotics (Figure 2). Among the antibiotic-treated groups endotoxin levels were highest after treatment with penicillin and lowest after treatment with chloramphenicol. However, this difference reached statistical significance only for strain 2 (Newman-Keuls test, $P < 0.05$).

TNF- α production in whole blood

TNF- α level was below the detection limit after 4 h of incubation when no microorganisms, or only antibiotics were added to whole blood. After addition of the high inoculum of meningococci without antibiotics the TNF- α concentration measured after 4 h of incubation ranged from 4126 $\pm$ 491 pg/mL to 12,355 $\pm$ 1518 pg/mL (Table I). A remarkable and reproducible strain-dependent effect on TNF- α -induction was observed, and the difference between the four strains in TNF- α level induced without treatment was highly statistically significant (ANOVA: $P < 0.001$). The strain-dependent effect on TNF- α induction was also observed 4 h after adding supernatant instead of bacterial suspension to whole blood (Table I). From the supernatant only 1×10^2 – 1.4×10^3 cfu/mL could be cultured, i.e. $\leq 0.1\%$ from the bacterial suspension, indicating that 50 μ L of supernatant contained only 5–70 cfu. This bacterial inoculum induced no measurable TNF- α production in the whole blood stimulation model used (data not shown). Hence, unbound ('free') endotoxin was responsible for the TNF- α production after the addition of supernatant.

TNF- α levels after 4 h of incubation were significantly lower in the presence of antibiotics when compared with untreated samples for three of the four strains (Table II). In addition, in these strains treatment with penicillin and ceftriaxone resulted in significantly lower TNF- α levels when compared with chloramphenicol treatment. In the fourth strain, which induced the least TNF- α , no differences were found in TNF- α levels between treated or untreated cultures.

Discussion

The primary aim of this study was to investigate the influence of antibiotics on endotoxin concentration in culture medium and cytokine production in whole blood *ex vivo* during exposure of log-phase meningococci to antibiotics.

Bacterial killing was more efficient with penicillin or ceftriaxone than with chloramphenicol. For all strains free endotoxin levels were significantly lower after exposure to antibiotics as compared with no treatment, and free endotoxin levels were lowest after exposure to chloramphenicol. With the exception of the strain inducing the lowest

Figure 1. Scanning electron micrographs (magnification, $\times 10,000$) of *N. meningitidis* group C after 4 h of incubation without antibiotics (a), or in the presence of (b) penicillin (20 mg/L), (c) ceftriaxone (20 mg/L) or (d) chloramphenicol (10 mg/L).

levels of TNF- α , exposure to antibiotics resulted in considerably lower cytokine levels as compared with no treatment, and significantly lower TNF- α levels were recorded after exposure to penicillin and ceftriaxone as compared with chloramphenicol treatment. These results suggest that in our experiments the efficacy of killing of meningococci was a more important determinant of cytokine induction than the level of free endotoxin.

In earlier studies in which meningococci were exposed to penicillin *in vitro* for 1–2 h, an increase in filterable endotoxin was measured,^{8,9} whereas total endotoxin level was comparable to the level in untreated cultures.⁸ This increase in free endotoxin level did not occur after treatment with chloramphenicol.^{8,9} After 6 h of treatment, free endotoxin levels were comparable for penicillin-treated, chloramphenicol-treated or untreated cultures.⁸ In mice with an experimental meningococcal infection a decrease in bacterial numbers and total endotoxin concentration, but an increase in filterable endotoxin, was found after penicillin treatment compared with no treatment.¹⁰ We found a lower free endotoxin concentration in treated cultures. This difference with former studies may be explained by differences in the technique used to separate free from total endotoxin. We used a centrifugation

method, whereas in the other studies a filtration method (through a 0.45 μm Millipore filter) was used, and it is possible that endotoxin is released from the bacteria during this filtration technique.³

Total endotoxin levels have been serially assessed after initiation of antibiotic treatment in only one human study.⁷ A decrease in total endotoxin levels was observed in all patients, but in this study data were collected from the surviving patients only. In addition, a decrease in the total endotoxin level does not differentiate between the effects of the reduction in bacterial numbers and changes in endotoxin release by the bacteria during killing.⁵ Nevertheless, these observations in humans are in accordance with our results.

It should be noted that the present results differ from the effects of antibiotic killing of *Escherichia coli*. When four equally efficacious antibiotics were compared in the same in-vitro model, ceftazidime or ciprofloxacin induced approximately 50% more TNF- α , while imipenem and gentamicin induced approximately 50% less TNF- α than did no treatment.⁵ This difference between the antibiotics was explained by the observation that ceftazidime and ciprofloxacin treatment resulted in filament formation, leading to an increase in release of free endotoxin and an

Endotoxin release by meningococci

Figure 2. Free endotoxin levels after 4 h of incubation of meningococci in RPMI 1640. No antibiotics were added ($\square$), or penicillin (hatched), ceftriaxone ($\blacksquare$), or chloramphenicol (cross-hatched) were added at $t = 0$ h. Groups were compared by one-way ANOVA. Multiple comparisons between groups were performed by the Newman-Keuls test. (a) $P < 0.05$ (Newman-Keuls) for untreated versus treated; (b) $P < 0.05$ (Newman-Keuls) for untreated versus treated and for penicillin or ceftriaxone versus chloramphenicol.

Table I. TNF- α level (pg/mL) after 4 h of incubation of meningococci, or supernatant from the bacterial suspension only, in whole blood (means $\pm$ S.D. of three experiments)

Strain	Serogroup/site of isolation	Bacterial suspension	Supernatant only
1	B/blood	12,355 $\pm$ 1518	4659 $\pm$ 513
2	B/CSF	4126 $\pm$ 491	1862 $\pm$ 285
3	C/CSF	10,691 $\pm$ 152	3170 $\pm$ 112
4	C/blood	4456 $\pm$ 437	1952 $\pm$ 272

Table II. TNF α -level (pg/mL) after 4 h of incubation of meningococci in whole blood, in the absence or presence of antibiotics (mean $\pm$ S.D. of three experiments)

Strain	No treatment	Penicillin	Ceftriaxone	Chloramphenicol	ANOVA
1	4354 $\pm$ 235	1239 $\pm$ 58	1786 $\pm$ 56	2490 $\pm$ 12	<0.001 ^a
2	747 $\pm$ 39	76 $\pm$ 32	123 $\pm$ 24	240 $\pm$ 45	<0.001 ^b
3	3762 $\pm$ 724	499 $\pm$ 60	643 $\pm$ 217	1466 $\pm$ 178	<0.001 ^c
4	348 $\pm$ 59	361 $\pm$ 33	414 $\pm$ 21	369 $\pm$ 38	0.28

^a $P < 0.05$ (Newman-Keuls): untreated versus treated; chloramphenicol versus penicillin and ceftriaxone; ceftriaxone versus penicillin.

^b $P < 0.05$ (Newman-Keuls): untreated versus treated; chloramphenicol versus penicillin and ceftriaxone.

^c $P < 0.05$ (Newman-Keuls): untreated versus treated; comparing treated groups only: chloramphenicol versus penicillin and ceftriaxone.

increase in bacterial biomass. No such phenomenon was observed in meningococci.

It is well known that meningococcal strains show great variation in the amount of endotoxin liberated spontaneously *in vitro*, and isolates from patients have higher endotoxin-liberating activities than strains isolated from carriers.^{15–17} We established that there are significant differences in TNF- α -inducing potential between the four strains. These differences were not related to serogroup or site of isolation of the meningococci. When supernatant (containing only 'free' endotoxin) was added instead of bacterial suspension, similar differences in TNF induction occurred. This suggests that a difference in spontaneous endotoxin liberation is at least in part responsible for the differences between the four strains in TNF- α -inducing potential. The free endotoxin levels measured by the LAL assay did not differ significantly for the four strains. However, it has been established that the LAL assay is not a reliable method for predicting differences between neisserial strains in host responses.¹⁸

The well-known range in endotoxin and cytokine concentrations found in patients with meningococcal disease^{7,19} is thus determined not only by individual endotoxin responsiveness of the host and bacterial numbers, but possibly, also by the properties of the bacterial strain involved.

In conclusion, fear of endotoxin release caused by effective antibiotics is not justified in the treatment of severe meningococcal infections. On the contrary, effective bacterial killing must be the main goal of therapy in order to reduce the production of pro-inflammatory cytokines.

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