Pathophysiology of in-vitro induced filaments, spheroplasts and rod-shaped bacteria in neutropenic mice

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ABSTRACT

This study compared the in-vitro properties and in-vivo effects of Escherichia coli filaments, spheroplasts and normal cells in a murine thigh infection model. E. coli was exposed to ceftazidime, meropenem or saline to obtain filaments, spheroplasts or normal bacilli, which were then injected into neutropenic mice. After 24 h, morphology, CFUs, local and circulating endotoxin levels, cytokine levels and mortality were recorded, and correlations between bacterial and host parameters of infection were investigated. Filaments and spheroplasts contained more endotoxin/CFU than controls. Histological studies showed that morphologically altered bacteria changed into rod-shaped cells in the absence of antibiotics. Bacterial spread to the liver was significantly higher in mice challenged with rod-shaped cells, compared with antibiotic-exposed bacteria (p 0.007). Muscle endotoxin levels correlated significantly with circulating interleukin (IL)-6 and tumour necrosis factor (TNF)-α, and both pro-inflammatory cytokines were correlated significantly (p 0.011). Despite a tendency toward higher local and systemic concentrations of endotoxin in the filament group, inflammatory responses and survival did not differ between groups. It was concluded that morphologically altered bacteria contain more endotoxin and can regain a rod shape after withdrawal of antibiotics, while non-antibiotic-exposed bacteria show greater spread to the liver. There was a clear intra-individual relationship between local endotoxin, systemic endotoxin, TNF-α and IL-6 production, but these parameters did not differ among groups.

Keywords  Endotoxin, Escherichia coli, filaments, mouse model, pathophysiological effects, spheroplasts

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INTRODUCTION

Endotoxin, or lipopolysaccharide, released from the bacterial cell wall is the most important mediator in the pathogenesis of Gram-negative sepsis [1–10]. Endotoxin release initiates a complex cascade involving production of endogenous mediators by the immune system of the host. The most important pro-inflammatory mediators are tumour necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 [11]. In low concentrations, these cytokines augment host defence [12–14]. However, exaggerated release, which can be triggered by an overload of endotoxin, can cause pathophysiological effects resulting in shock and death [15–18]. Shenep et al. [19] demonstrated an exaggerated release of endotoxin after bacterial exposure to cell-wall-active antibiotics, but this response was absent when rapidly acting bactericidal agents were used. In addition, other investigators have shown variations in endotoxin-liberating capacity among different β-lactam antibiotics [10,20,21].

Penicillin-binding proteins (PBP s) are enzymes anchored in the cytoplasmic membranes of bacteria. PBPs, particularly PBP1, PBP2 and PBP3, have significant biological functions, including determination of cell shape, phage resistance, induction of capsule synthesis, and regulation
of autolysis [15,22–24]. During exposure of Gram-negative bacteria to low ceftazidime concentrations, PBP3 inhibition occurs, leading to formation of bacterial filaments. It has been shown previously that bacterial filaments are capable of releasing large amounts of endotoxin from their cell walls in vitro [22]. PBP2 inhibition, which occurs during meropenem exposure, leads to formation of spheroplasts [25].

In contrast to filaments, spheroplasts contain lower endotoxin concentrations, comparable to those of rod-shaped cells [24,26]. On the basis of these observations, it is possible that antibiotic-induced filament formation, in contrast to spheroplast formation, may have adverse clinical effects because of excessive endotoxin release. To test this hypothesis, the present study challenged neutropenic mice with filaments, spheroplasts or rod-shaped Escherichia coli cells in a murine thigh muscle model of infection [27–29]. The in-vitro properties of the different inocula were studied with respect to morphology and the endotoxin content/CFU, local growth, local and systemic endotoxin concentrations, morphology and systemic spread. Finally, the mortality rate and the plasma concentrations of the pro-inflammatory cytokines TNF-α and IL-6 were determined, as parameters of both clinical effects and host defence [11,14,17,30].

**MATERIALS AND METHODS**

**Mice**

Female outbred Swiss mice (aged 6–8 weeks, weight 20–30 g) were used for the experiments. Mice were bred by Harlan (Horst, The Netherlands) and arrived at the Central Animal Laboratory 2 weeks before the start of the experiments. Mice were held in a specific pathogen-free unit in filter-top cages with beds of sterilised sawdust. Food (Hope Farms, Woerden, The Netherlands) and acidified water were supplied ad libitum. The temperature was kept at 20–24°C, with a relative humidity of 50–60%, 15 air changes/h, and light and dark periods of 14 and 10 h, respectively. All experiments were approved by the Ethical Committee on Animal Experiments of the University Medical Centre Nijmegen, The Netherlands.

**Induction of neutropenia and anaesthesia**

On day minus 4, all mice received cyclophosphamide (Endoxan–ASTA; Dagra, Diemen, The Netherlands) 150 mg/kg subcutaneously to induce neutropenia. On day minus 1, a second dose of 100 mg/kg was given. Leukocyte counts and differentiation confirmed a neutropenia of <0.1 x 10⁶ leukocytes/L in each of the eight mice examined. During injections, the mice were anaesthetised with ether.

**Microorganisms**

E. coli strain ATCC 25922 was used for all experiments. The MICs of ceftazidime and meropenem for this strain were 0.25 and 0.0025 mg/L, respectively, as determined by Etest and broth microdilution methods [31,32]. Bacteria were stored at −80°C and subcultured on blood agar 2 days before the experiment. Subsequently, two colonies were inoculated into 10 mL of Mueller–Hinton broth and incubated overnight to obtain a culture containing 5 x 10⁶ CFU/mL; this was diluted in triplicate with Mueller–Hinton broth in pyrogen-free glass test tubes to a concentration of 1 x 10⁶ CFU/mL. After mixing, either ceftazidime, meropenem or saline was added (see below); this was followed by incubation for 4 h to induce filaments, spheroplasts or normal-shaped bacilli, respectively. Before injection, bacterial suspensions were mixed, diluted 1:10, washed twice and resuspended in saline at 37°C.

**Antibiotics**

Ceftazidime (Glaxo SmithKline, Zeist, The Netherlands) and meropenem (AstraZeneca, Zoetermeer, The Netherlands) were used at concentrations of 0.125 and 0.0125 mg/L, respectively (0.5 x MIC, for both drugs) to induce in-vitro morphological changes with a minimum effect on bacterial growth. An aliquot (25 μL) of either a ceftazidime 0.05 mg/mL solution or a meropenem 0.005 mg/mL solution was added to a test tube containing 10 mL of E. coli suspension. After discarding the same volume from the test solution, a ceftazidime concentration of 0.125 mg/L or a meropenem concentration of 0.0125 mg/mL was obtained. In a control tube, 25 μL of the bacterial suspension was discarded and replaced with 25 μL of saline.

**Blood and tissue collection**

Blood specimens were collected from the retro-orbital plexus by eye extraction and were stored in sterile pyrogen-free Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA). These tubes contained 10 μL of a 5000 U/mL pyrogen-free heparin solution (Leo Pharmaceutical Products, Weesp, The Netherlands). The tubes were placed immediately on ice and then centrifuged for 5 min to obtain plasma samples. Undiluted plasma was collected in Eppendorf tubes and frozen at −80°C until the cytokine levels were determined. Plasma (0.5 mL) was aspirated in a sterile, pyrogen-free syringe (Monoject; Kendall, Ballymoney, Northern Ireland), diluted 1:10 in saline, and stored in pyrogen-free Falcon tubes at −80°C until limulus amoebocyte assays could be performed. Liver, spleen and thigh muscles were removed aseptically, immersed in saline, weighed, and then homogenised in a tissue grinder. Serial dilutions of homogenised tissues were plated on sheep blood agar. After overnight incubation at 37°C, the number of CFU/g tissue was calculated.

**Endotoxin assay**

Endotoxin concentrations in plasma and muscle were determined by a chromogenic limulus amoebocyte assay (KabiVi-trum, Amsterdam, The Netherlands), according to the manufacturer’s instructions, at the Laboratory of Haematology, University Hospital Groningen, The Netherlands.
TNF-α and IL-6

Circulating TNF-α was detected by a radioimmunoassay developed and validated locally, and IL-6 concentrations were determined by ELISA (Biosource International, Camarillo, CA, USA). The detection limits of the two assays were 40 pg/mL and 30 pg/mL, respectively.

Effects of changes in morphology

Changes in the length of filaments and the shape of spheroplasts during the in-vitro part of the study were determined following Gram’s stain. In-vivo morphology after 24 h was assessed in haematoxylin–eosin-stained histological sections of muscle, liver and spleen, using light microscopy with magnifications of ×400 and ×1000. The effects of centrifugation, dilution, and the washing and mixing steps on morphology during the preparation of inocula were checked by examining Gram’s stains of cells at several time-points during the procedure. Bacterial counts, endotoxin content and the relationship between these parameters in inocula were also studied. In addition, initial test suspensions containing $10^5$ and $10^6$ CFU/inoculum of E. coli bacilli (eight mice for each inoculum size), and $10^5$ and $10^6$ CFU/inoculum of E. coli filaments (four mice for each inoculum size) were used to define the inoculum size required to obtain mice with a severe sepsis and 100% survival after 24 h. The effectiveness of cyclophosphamide for the induction of neutropenia was checked by measuring leukocyte counts and differentiation using light microscopy of blood smears from eight mice.

Sepsis and mortality studies

On each test day, 12 neutropenic mice were randomised between inoculation into the left thigh muscle with 0.1 mL of filament solution, 0.1 mL of spheroplast solution, or 0.1 mL of control solution. In each experiment, four mice per group were investigated. The mice were kept in cages for 24 h following inoculation, and then anaesthetised with ether and killed. Immediately thereafter, blood was collected from the retro-orbital plexus for endotoxin and cytokine assays. Liver, spleen and thigh muscles were removed surgically, homogenised and plated on agar to determine bacterial counts. Parts of the homogenates of thigh muscles were stored in pyrogen-free tubes for endotoxin assays. The experiments were performed in duplicate on two separate days.

In a separate experiment, mortality was investigated in 12 mice. Mice were randomised with bacterial filaments, spheroplasts or rod-shaped cells in thigh muscles, and then kept in cages and observed until death occurred.

Statistical analysis

Statistical analysis was performed using the SPSS v.10.0 package for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism v.3.0 (GraphPad Software Inc., San Diego, CA, USA). Levene’s test for equality of variances was used to determine whether parameters were distributed normally. Differences in viable counts, survival, endotoxin concentrations and cytokine concentrations were analysed by multivariate analysis (ANOVA) for normally distributed parameters, followed, when appropriate, by post-hoc t-tests. Parameters without a normal distribution were analysed with the non-parametric Kruskal–Wallis test, followed by the Mann–Whitney test to investigate differences among groups. Correlations among parameters with a normal distribution were determined with the Pearson correlation test. For non-normally distributed parameters, the non-parametric Spearman’s rank correlation test was applied. Statistical tests were considered significant at $p < 0.05$.

RESULTS

Comparison of filaments, spheroplasts and normal E. coli cells in vitro

Incubation with ceftazidime resulted in the formation of filaments, whereas exposure to meropenem resulted in 100% formation of spheroplasts. Morphological studies demonstrated filaments with a length of four to eight cells after the centrifugation, dilution and mixing procedures. The morphology of the spheroplasts and controls did not change during these procedures.

Table 1 summarises the viable counts, endotoxin concentrations and ratios of endotoxin/CFU for filaments, spheroplasts and controls, respectively. From these data, it was calculated that filaments and spheroplasts contain 13.49-fold and 5.36-fold, respectively, more endotoxin/CFU than normal cells. Assuming that the characteristics of filaments are equivalent to those of rod-shaped cells, it was expected that the amount of endotoxin/CFU ($\times 1000$) would vary between 9.52 and 19.04 ($4 \times 2.38$ and $8 \times 2.38$, respectively), after taking into account the fact that the length of one filament was the length of between four and eight normal cells; however, the actual value was up to 3.4-fold higher than expected.

Survival studies

When eight neutropenic mice were challenged intra-muscularly with $1 \times 10^6$ CFU of normal E. coli, only one mouse survived for 24 h (a survival rate of 12.5%). Challenge of eight mice...
with an inoculum of $10^5$ CFU of normal *E. coli* resulted in 100% survival after 24 h. Similarly, challenge of four mice with either $10^4$ or $10^5$ CFU of *E. coli* filaments resulted in 100% survival after 24 h. On the basis of these findings, a maximum inoculum of $10^5$ CFU/mouse was chosen for controls in further experiments.

In the main survival experiment, the survival times of infected mice were 42.25 h (SD 9.5, range 28–47 h), 41.25 h (SD 9.5, range 28–47 h) and 42.25 h (SD 11.5, range 24–47 h) for filament-exposed, spheroplast-exposed and control mice, respectively, with 100% mortality after 47 h, irrespective of the group. Thus, at the time when measurements were performed in the sepsis studies, the mice were suffering from a severe sepsis, leading to death within 24 h from that time-point.

**Sepsis studies**

At 24 h after inoculation, bacteria were identified readily in the perivascular and perineural spaces in haematoxylin–eosin-stained thigh muscle sections. However, despite injection of filaments, only normal-shaped Gram-negative bacilli were observed. No bacteria were identified in histological sections of liver and spleen using light microscopy, despite bacterial counts of $10^4$–$10^6$ CFU/g of tissue, perhaps because of the architecture of these tissues.

Fig. 1 shows the bacterial counts in muscle, liver and spleen at 24 h after inoculation with normal bacilli, filaments or spheroplasts. CFUs in liver tissue showed significant differences between groups (p 0.025), with the highest CFUs in liver homogenates from controls. There were also significant differences between spheroplasts and controls (p 0.015), between filaments and controls (p 0.027), and between all antibiotic-exposed bacteria and controls (p 0.007). Although the same trend of lower CFU counts was seen in spleen tissue infected with spheroplasts, the bacterial counts did not differ significantly among groups (p 0.1644). Similarly, no significant differences in viable counts were observed after 24 h in thigh muscles. CFUs in muscle, liver and spleen did not show significant correlations with local or systemic levels of endotoxin, TNF-α or IL-6.

Plasma and muscle endotoxin concentrations are shown in Table 2. Although mean values suggest higher endotoxin concentrations in the plasma and muscle of mice injected with filaments, the differences among groups did not reach significance (p 0.063 for filaments vs. spheroplasts). However, there was considerable variation in the local endotoxin levels, reflected in large standard deviations. Nevertheless, significant correlations could be shown between plasma and muscle endotoxin levels (p 0.014).

Also shown in Table 2 are the mean levels of plasma TNF-α and IL-6. Although these did not differ among groups, there was a significant correlation between muscle endotoxin and plasma IL-6 levels (p 0.025), and between muscle endotoxin and TNF-α levels (p 0.03). There was no significant correlation between plasma endotoxin and IL-6 or TNF-α levels (p 0.074 and p 0.09, respectively). There was a significant correlation between plasma TNF-α and IL-6 levels (p 0.011).

**DISCUSSION**

The in-vitro part of this study demonstrated a higher endotoxin content/CFU in antibiotic-exposed bacteria than in controls. In contrast to previous findings, this study also demonstrated higher endotoxin contents in spheroplasts [24,26]. This could be caused by the induction of spheroplasts with meropenem, which binds to PBP2 and PBP3. In previous studies [24], imipenem, which binds to PBP1 and PBP2, was used to induce spheroplasts. Thus, it is possible that the toxic properties of spheroplasts may differ according to
the inducing antibiotic and its affinity for specific PBPs. The study also revealed that the higher endotoxin content of filaments could not be explained simply by normal bacterial growth without septation. It is not clear whether a higher density of endotoxin molecules in the cell wall, a larger cell surface, or a change in the endotoxin molecule itself, contributed to these higher than expected endotoxin levels. However, the findings correlate with those of Jackson and Kropp [33] for Pseudomonas isolates exposed to sub-MIC concentrations of ceftazidime.

The present model showed that filaments were able to regain normal shapes after withdrawal of antibiotics. However, there was a difference in the dissemination pattern of bacteria during the first 24 h of sepsis, even though local bacterial counts in thighs were equal after 24 h. Dissemination of bacteria to the liver occurred significantly more efficiently in the group infected with rod-shaped cells, compared with the groups infected with antibiotic-exposed bacteria. Differences in the systemic spread of morphologically altered microorganisms may be explained by differences in size or changes in the vitality of bacteria, causing inhibition of migration of the larger forms. Chen et al. [34] have described altered motility of Burkholderia pseudomallei following exposure to filament-inducing antibiotics. A second explanation could involve a greater vulnerability of morphologically altered bacteria to host defence mechanisms, e.g., differences in serum bactericidal effect. The fact that no significant difference in spread to spleen tissue was detected might be explained by the overwhelming numbers of CFUs in spleen tissue masking more subtle differences.

Antibiotic-induced morphological changes, and changes in dissemination of E. coli, did not affect parameters of severity of infection and survival, perhaps because the bacteria were able to seaptate again after the withdrawal of the antibiotics, and thus regained the morphological characteristics, and probably the pathogenicity, of controls. Furthermore, the numbers of CFUs in thighs were the same in all groups after 24 h. Alternatively, the infection may be so severe that a relatively low endotoxin content associated with the natural forms of E. coli may already have a maximum effect. In all groups, the animals developed a severe infection within the first 24 h, leading to death within 48 h of the start of the experiment. The relative resistance of mice to endotoxin could also be an explanation; thus, the amounts of endotoxin associated with the various morphological forms might not have contributed to death. Finally, it is also possible that endotoxin is retained in the membranes of filaments, i.e., locally at the site of infection, for a longer period of time, thus exposing the host to toxic molecules to a lesser extent.

The study showed a clear relationship between muscle and plasma endotoxin concentrations, and between local endotoxin concentrations and circulating levels of IL-6 and TNF-α. Although local and circulating endotoxin levels were related significantly, the correlation between plasma endotoxin levels and both cytokines did not reach significance. However, multiple plasma factors, e.g., protein binding, might influence the concentration and clearance of circulating lipopolysaccharide. The relationship between endotoxin and cytokines has been investigated in other models and in human studies [17,35–40]. It will be interesting to focus further studies with the model on the inflammatory responses that occur during the first hours after inoculation, and on the host reactions to milder infections, in order to investigate more subtle differences. Another valuable use of the model might involve a situation in which the presence of antibiotics is maintained for longer, in order to keep the bacteria in their altered shapes.

In conclusion, the in-vitro part of this study confirmed the finding that filaments contain more endotoxin in their cell wall than do controls [41].

Table 2. Mean muscle endotoxin, plasma endotoxin, tumour necrosis factor (TNF)-α and interleukin (IL)-6 levels at 24 h after inoculation of neutropenic mice with different bacterial cell types

<table>
<thead>
<tr>
<th>Cell type (number of mice)</th>
<th>Muscle LPS (U/mL) Mean (SD)</th>
<th>Plasma LPS (U/mL) Mean (SD)</th>
<th>Plasma TNF-α (pg/mL) Mean (SD)</th>
<th>Plasma IL-6 (pg/mL) Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filaments (n = 8)</td>
<td>262 363 (141 592)</td>
<td>91 (32)</td>
<td>288 (477)</td>
<td>18 229 (15 163)</td>
</tr>
<tr>
<td>Spheroplasts (n = 8)</td>
<td>139 670 (109 980)</td>
<td>31 (25)</td>
<td>268 (506)</td>
<td>29 229 (32 378)</td>
</tr>
<tr>
<td>Controls (n = 8)</td>
<td>253 213 (179 257)</td>
<td>66 (62)</td>
<td>120 (124)</td>
<td>37 183 (31 991)</td>
</tr>
</tbody>
</table>

LPS, lipopolysaccharide (endotoxin).
However, in contrast with earlier findings, spheroplasts also contained more endotoxin compared with normal bacilli. Local endotoxin, plasma endotoxin and pro-inflammatory cytokine levels showed a clear correlation in the present model, and endotoxin levels in muscle showed a significant correlation with plasma endotoxin levels. From the present study of overwhelming and rapidly fatal septicemia, it is clear that filaments and spheroplasts are potentially more virulent and pathogenic than rod-shaped cells, and that they are able to regain normal shapes after withdrawal of antibiotic treatment. Bacteria that have been in an altered morphological shape show different dissemination characteristics.

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