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Contributions of \textit{Neisseria meningitidis} LPS and non-LPS to proinflammatory cytokine response

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Abstract: To determine the relative contribution of lipopolysaccharide (LPS) and non-LPS components of \textit{Neisseria meningitidis} to the pathogenesis of meningococcal sepsis, this study quantitatively compared cytokine induction by isolated LPS, wild-type serogroup B meningococci (strain H44/76), and LPS-deficient mutant meningococci (strain H44/76[pLAK33]). Stimulation of human peripheral-blood mononuclear cells with wild-type and LPS-deficient meningococci showed that non-LPS components of meningococci are responsible for a substantial part of tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-1\(\beta\) production and virtually all interferon (IFN)-\(\gamma\) production. Based on tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of LPS in proteinase K-treated lysates of \textit{N. meningitidis} H44/76, a quantitative comparison was made between the cytokine-inducing capacity of isolated and purified LPS and LPS-containing meningococci. At concentrations of \(\geq 10^7\) bacteria/mL, intact bacteria were more potent cytokine inducers than equivalent amounts of isolated LPS, and cytokine induction by non-LPS components was additive to that by LPS. Experiments with mice showed that non-LPS components of meningococci were able to induce cytokine production and mortality. The principal conclusion is that non-LPS parts of \textit{N. meningitidis} may play a role in the pathogenesis of meningococcal sepsis by inducing substantial TNF-\(\alpha\), IL-1\(\beta\), and IFN-\(\gamma\) production. \textit{J. Leukoc. Biol.} 70: 283–288; 2001.

**Key Words:** LPS-deficient meningococci · meningococcal sepsis · outer membrane · 2-keto-3-deoxy-octanate · TSDS-PAGE

INTRODUCTION

It is generally accepted that the induction of cytokine synthesis and the subsequent pathophysiological events during Gram-negative septic shock are primarily elicited by the lipopolysaccharide (LPS) component (endotoxin) of the bacterial outer membrane [1]. Purified LPS is able to induce a proinflammatory cytokine pattern and a clinical condition that resembles, at a first glance, the symptoms encountered in Gram-negative septic shock. The LPS-molecule is composed of a lipid-A part harboring its toxic properties, a saccharide part, and one or more molecules of 2-keto-3-deoxy-octanate (KDO) connecting the lipid-A and the saccharide part [2].

Anti-LPS strategies explored so far have failed to ameliorate the clinical course of Gram-negative sepsis [3–6], which raises the question whether LPS is the sole toxic element in Gram-negative sepsis [7].

Fulminant meningococcal sepsis is considered the prototypical human gram-negative septic shock, characterized by extremely high endotoxin and cytokine concentrations [8–11]. Thus, the causative bacterium \textit{Neisseria meningitidis} is a suitable subject for the study of cytokine induction by gram-negative bacteria. Recently, a viable \textit{N. meningitidis} mutant was constructed that is devoid of LPS but still contains all other outer membrane constituents [12]. This mutant has made it possible to assess the cytokine-inducing potency of the non-LPS parts of this gram-negative bacterium in a quantitative fashion.

The principal aim of the present study is to determine the contribution of LPS and non-LPS components of \textit{N. meningitidis} to the pathogenesis of meningococcal sepsis. Therefore, we compared quantitatively the cytokine production induced by meningococcal LPS, wild-type meningococci, and LPS-deficient meningococci.

MATERIALS AND METHODS

The wild-type serogroup B \textit{N. meningitidis} H44/76 strain was isolated from a patient with invasive meningococcal disease [13]. Meningococcal strain H44/76[pLAK33] is a viable isogenic mutant, completely devoid of LPS in its outer membrane. This mutant was constructed by insertional inactivation of the lpxA gene, essential for the first committed step in biosynthesis of LPS [12, 14]. The absence of LPS in this strain was confirmed by tricine sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (TSDS-PAGE) with silver staining of LPS [15, 16], whole-cell enzyme-linked immunosorbent assay (ELISA) with LPS-specific monoclonal antibodies, and gas-chromatographic/mass-spectrometric detection of LPS-specific 3-OH fatty acids [17]. Furthermore, absence of LPS activity in the pLAK33 batch suspension was confirmed by nonreactivity in the Limulus amebocyte lysate assay. Heat-killed (1 h, 56°C) bacteria washed in phosphate-buffered saline (PBS) were used in all experi-
The molecular mass of meningococcal strain H44/76 LPS is 4,044 Da [20]; one molecule of LPS contains two molecules of KDO (molecular mass, 238 Da). LPS was isolated by the phenol/water extraction method as described by Westphal and Jann [21]. After isolation, LPS was treated with protease K (Sigma-Aldrich Co.) and with DNase and RNase (Roche Diagnostics) for additional purification, recovered by ultracentrifugation, freeze-dried, and dissolved in sterile PBS. The amount of protein contamination in this purified LPS solution was determined by bicinchoninic acid assay. DNA and RNA contamination was determined by spectrophotometry using a Genequant RNA/DNA calculator (Pharmacia Biotech AB, Uppsala, Sweden).

The KDO content of the purified H44/76 LPS solution and of the bacterial suspensions was measured spectrophotometrically by the method described by Weissbach and Hurwitz [22].

Fig. 1. TSDS-PAGE silver staining of 0.1 μg (lane 1), 0.19 μg (lane 2), 0.39 μg (lane 3), and 0.77 μg (lane 4) of purified H44/76 LPS and LPS in protease K-treated lysates of 5 × 10⁸ (lane 5) and 2.5 × 10⁹ (lane 6) N. meningitidis H44/76 bacteria. Insert: Standard curve as calculated by linear regression for contour density (OD x mm²) of the purified LPS samples. Contour density of the lysates corresponds with 0.7 μg of LPS in sample 5 and 0.34 μg of LPS in sample 6.

RESULTS
Quantification of LPS in N. meningitidis H44/76 bacteria
To enable a quantitative comparison between cytokine induction by isolated LPS and H44/76 meningococci, the purity of N. meningitidis H44/76 LPS was analyzed, and the amount of LPS in N. meningitidis H44/76 bacteria was determined.

Protein contamination in the purified H44/76 LPS was determined by the method described by Weissbach and Hurwitz [22]. TSDS-PAGE silver staining of LPS was used for quantification of LPS in N. meningitidis H44/76. Cell lysates of H44/76 meningococci were made by suspending 1.35 × 10¹¹ bacteria in 500 μL of SDS-buffer (7.5% glycerol, 1.25 M Tris/HCl, 1.5% SDS) and incubating this for 5 min at 100°C. Proteins in this suspension were digested by incubation with protease K (0.5 mg/mL) for 4 h at 56°C [23]. Serial dilutions of purified H44/76 LPS were used as a standard. After silver staining, the polycrylamide gel was analyzed by densitometry.

Bleeding for the isolation of human peripheral blood mononuclear cells (PBMCs) was drawn in 10-mL EDTA-anticoagulated tubes (Vacutainer System; Beckton Dickinson, Rutherford, NJ) from healthy volunteers. PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia Biotech AB). The cells from the interphase were aspirated, washed three times in sterile PBS, and resuspended in culture medium RPMI 1640 (Dutch modification; Flows Lab, Irvine, Scotland) supplemented with L-glutamine (2 mmol), pyruvate (1 mmol), and gentamycin (50 mg/mL) and 5% freshly pooled human serum. PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia Biotech AB). The cells from the interphase were aspirated, washed three times in sterile PBS, and resuspended in culture medium RPMI 1640 (Dutch modification; Flows Lab, Irvine, Scotland) supplemented with L-glutamine (2 mmol), pyruvate (1 mmol), and gentamycin (50 mg/mL) and 5% freshly pooled human serum. PBMCs were incubated with various stimuli in 200-μL 96-well plates (Greiner BV, Alphen a/d Rijn, The Netherlands) at 37°C and 5% CO₂ for 4 and 24 h. The supernatant was obtained by centrifugation and stored at −20°C until the cytokine assay was performed.

As an alternative approach to determine the amount of LPS in the LPS-containing H44/76 strain, the amount of KDO detected in the LPS-deficient pLAK33 suspension was subtracted from that in the H44/76 suspension. Because the only difference between these isogenic strains is the presence of LPS, the difference in KDO content reflects LPS-associated KDO. In this way, it was calculated that 7 × 10⁵ H44/76 bacteria contain approximately 1 ng of LPS, a result rather similar to that obtained by TSDS-PAGE analysis.

Quantitative comparison of cytokine induction
The dose-response relationship of meningococcal LPS, wild-type N. meningitidis H44/76, and LPS-deficient N. meningiti-
dis pLAK33 for cytokine production by PBMCs after 24 h is shown in Figure 2. In this figure the x-axis was calibrated for LPS together with the number of H44/76 bacteria that contained an equivalent amount of LPS, based on the above-presented TSDS-PAGE results. Therefore, the effect of all three stimuli could be compared quantitatively.

It can be seen that at concentrations higher than 10^5 bacteria/mL, LPS-containing meningococci were more potent inducers of TNF-α and IL-1β than equivalent amounts of isolated LPS (P<0.05). Below these concentrations, LPS was a more potent inducer of TNF-α and IL-1β production. LPS-deficient pLAK33 meningococci were able to induce substantial amounts of TNF-α, IL-1β, and IFN-γ production. For TNF-α and IL-1β, approximately 10-fold-higher amounts of LPS-deficient bacteria were required to induce the same level of cytokine production as that in wild-type bacteria. Of interest, significant IFN-γ production occurred after stimulation with both the wild-type and the LPS-deficient meningococci, but only minute amounts of IFN-γ were produced after stimulation with isolated LPS (P<0.05). Experiments (n=8) performed with 4 h of incubation showed a similar pattern for TNF-α and IL-1β (data not shown).

To assess whether the IFN-γ production induced by LPS-containing or LPS-deficient meningococci is secondary to the TNF-α or IL-1β production, a series of induction experiments with 6 × 10^6/mL of H44/76 and pLAK33 bacteria was performed with cells from 30 donors. In these experiments, the TNF-α and IL-1β production appeared to be correlated (r=0.061 (P<0.01) and r=0.70 (P<0.001) for wild-type and LPS-deficient meningococci, respectively). However, IFN-γ production did not correlate with the TNF-α or IL-1β production values for r between −0.12 and 0.22 (P=not significant). In addition, it could be seen that IFN-γ production showed a striking interindividual variety [range, 56–7,800 pg/mL (median, 555 pg/mL) and 40–6,800 pg/mL (median, 660 pg/mL) for H44/76 and pLAK33 bacteria, respectively]. Thus, IFN-γ is probably not produced in response to TNF-α or IL-1β, and the individual response in IFN-γ production after stimulation with meningococci differs considerably.

The time course for TNF-α and IL-1β production by PBMCs showed a similar pattern for meningococcal LPS and both meningococcal strains. In brief, TNF-α became detectable after 2 h, reached a maximum after 8 h, and declined thereafter whereas IL-1β was detectable after 4 h and reached a plateau phase after 12 h (data not shown).

The relative contribution of non-LPS structures to the total induction of TNF-α and IL-1β induction by N. meningitidis was assessed in a series of combination experiments. In these experiments, meningococci were supplemented with approximately the same amount of LPS as present in the wild-type strain. Figure 3 shows the results of this experiment for IL-1β after 24 h of incubation. It can be seen that IL-1β production induced by LPS-deficient bacteria was approximately half of that induced by LPS-containing bacteria, whereas after addition of LPS, the IL-1β induction by LPS-deficient bacteria was restored to the level of LPS-containing bacteria. Results for TNF-α and for experiments (n=5) with 4 h of incubation showed a similar pattern (data not shown). This additive effect of LPS to the non-LPS-induced cytokine synthesis suggests that both stimuli may use different pathways for the induction of TNF-α and IL-1β.

Cytokine induction by outer membrane complexes

To determine whether the non-LPS components responsible for cytokine induction reside in the outer membrane, cytokine induction by OMCs isolated from the wild-type H44/76 meningococci and the LPS-deficient pLAK33 meningococci was assessed. Figure 4 shows the results for IL-1β after 24 h of incubation. It appeared that the wild-type H44/76 OMCs were potent inducers of cytokine synthesis, whereas the cytokine-inducing capacity of pLAK33 OMCs was 1,000- to 10,000-fold less than that of H44/76 OMCs. Results for TNF-α and for
Cytokine induction and lethality in mice

In mice we assessed whether the observed cytokine induction by non-LPS components of meningococci coincides with the capacity to provoke disease. Results in Table 1 indicate that LPS-deficient meningococci were able to induce cytokine production in murine peritoneal macrophages in vitro. Table 2 demonstrates that LPS-deficient meningococci were able to induce lethality in galactosamine-sensitized mice in vivo. The dose needed for LPS-deficient meningococci to induce mortality was approximately 100-fold higher than for LPS-containing meningococci.

DISCUSSION

The principal finding of the present study is that LPS is not the sole cytokine-inducing element of N. meningitidis. Using a meningococcal mutant completely devoid of LPS, it was shown that non-LPS components of this bacterium were responsible for a substantial part of the TNF-α and IL-1β production, that non-LPS components induced IFN-γ, and that non-LPS parts could provoke disease. Based on TSDS-PAGE analysis of LPS in proteinase K-treated cell lysates of N. meningitidis H44/76, a quantitative comparison between the cytokine-inducing capacity of LPS and that of LPS-containing bacteria became possible. It was shown that concentrations of >107/mL of bacteria were more potent inducers of cytokine synthesis than equivalent amounts of isolated LPS and that cytokine induction by non-LPS components was additive to that by LPS. Furthermore, it was demonstrated that the non-LPS components responsible for the cytokine induction do not reside in sarcosyl-extracted outer membrane complexes.

Non-LPS components of bacteria can induce cytokine production [7], as has been shown by experiments with gram-positive bacteria [27-31] and with various isolated elements of these bacteria like peptidoglycan [32-34], lipopeptides [28], lipoproteins [28], lipoteichoic acid [35], and capsular polysaccharides [36]. However, so far studies trying to assess quantitatively the contribution of these non-LPS structures to cytokine induction by gram-negative bacteria were hampered by the inevitable copresence of LPS, outflanking the cytokine induction by non-LPS structures. To circumvent this problem we used a meningococcal mutant that is entirely deficient of LPS. With this strain we demonstrated that approximately half the amount of TNF-α and IL-1β induced by meningococci in human PBMCs or murine peritoneal macrophages was elicited by non-LPS structures. Similarly, LPS-deficient meningococci could cause lethal disease in galactosamine-pretreated mice, albeit the dosages of LPS-deficient meningococci required to induce mortality were approximately 100-fold higher than for wild-type meningococci.

TNF-α, IL-1β, and IFN-γ are pivotal mediators in the pathogenesis of Gram-negative septic shock. After infusion of LPS in human volunteers or primates, TNF-α and IL-1β appear within 1 to 2 h [37], but no IFN-γ seems to appear [38]. However, after the infusion of whole bacteria, IFN-γ is induced and can be detected in the circulatory system after 6–8 h [38, 39]. During meningococcal infections, IFN-γ is increased in plasma or cerebrospinal fluid and correlates with the severity of disease [40, 41]. In our in vitro study, meningococcal LPS stimulated only minimal IFN-γ production, whereas both LPS-containing and LPS-deficient bacteria induced significant amounts of IFN-γ. In addition, IFN-γ was not produced in response to TNF-α or IL-1β, which indicates that non-LPS components of meningococci were primarily responsible for IFN-γ induction. Because the primary source of IFN-γ is the lymphocyte and marked differences occur between individuals, it is tempting to speculate that certain non-LPS components of meningococci can act as superantigens.

In this study we used the widely employed KDO assay to determine the amount of LPS in the H44/76 LPS preparation [22, 42, 43]. With this method (0.8/0.92) × 100% (i.e., 85%)

<table>
<thead>
<tr>
<th>Stimulus (dosage)</th>
<th>mTNF-α (ng/mL)</th>
<th>mIL-1α (ng/mL)</th>
</tr>
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<tbody>
<tr>
<td>N. meningitidis H44/76 (2×10⁹/mL)</td>
<td>1.85 ± 0.39</td>
<td>4.67 ± 1.09</td>
</tr>
<tr>
<td>(2×10⁹/mL)</td>
<td>0.86 ± 0.54</td>
<td>1.96 ± 0.77</td>
</tr>
</tbody>
</table>

Mean data ± SE are presented [n=4 for H44/76 meningococci; n=5 for H44/76[pLAK33] meningococci].

TABLE 2. Mortality at 24 h (%) in Mice Pretreated with Galactosamine after i.v. Injection of Heat-Killed N. meningitidis

<table>
<thead>
<tr>
<th>Strain</th>
<th>10⁷</th>
<th>10⁸</th>
<th>10⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitidis H44/76</td>
<td>60</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>N. meningitidis H44/76[pLAK33]</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
</tbody>
</table>

Each group contained five animals.
of the LPS was detected. Limitations of this assay are conversion of a fraction of KDO during acid hydrolysis to entities inert to the thiobarbituric acid reaction and incomplete hydrolysis, both leading to an underestimation of the amount of KDO. On the other hand, contaminants in the LPS may coextract in the assay, which leads to overestimation of the amount of LPS [44, 45]. The relatively accurate yield of KDO in the present study showed that these limitations of the KDO assay are likely to be of minor importance for determination of H44/76 LPS.

By TSDS-PAGE analysis of LPS in lysates of N. meningitidis H44/76, the amount of LPS in H44/76 bacteria was determined. It appears that 7 × 10^5 bacteria corresponded to approximately 1 ng of LPS, which fits rather well with the results obtained by KDO analysis in H44/76 and pLAK33 bacteria. This estimate is in good accordance with reported estimates of 1 ng of LPS for 10^5 bacteria with Escherichia coli [1, 46–50], taking into account that the MW of meningococcal LPS is 2- to 10-fold lower than that of E. coli LPS. In addition, our estimate compares fairly well to clinical data of Brandtzaeg et al. [51], Mariani-Kurkdjian et al. [52], and Bingen et al. [53], who detected LPS values up to 500 ng/mL and bacterial numbers up to 5 × 10^8 CFU/mL in cerebrospinal fluid during meningococcal meningitis.

Based on the estimate that 7 × 10^5 bacteria correspond to 1 ng of LPS, we could compare the cytokine-inducing potency of isolated LPS with that of meningococci containing the same amount of LPS. It was found that at concentrations below 10^8 bacteria/mL or equivalent amounts of LPS, LPS induced more TNF-α and IL-1β, but at higher concentrations, complete meningococci were more potent. Because at these higher concentrations the TNF-α and IL-1β-inducing capacities of LPS-deficient meningococci increased in a parallel fashion, the higher activity of bacteria at these higher concentrations is likely to have been caused by the non-LPS components of the bacterium.

Fulminant meningococcal sepsis is characterized by high plasma concentrations of endotoxin that range from 0.75 to 170 ng/mL [8, 10, 54–56]. Based on our estimation of 1 ng of LPS per 7 × 10^5 bacteria, the reported range of endotoxin concentrations corresponds to 5 × 10^5 to 1.2 × 10^6 bacteria/mL. We speculate that strategies designed to block LPS-induced cytokine synthesis alone are of limited value, because at this degree of bacteremia, a substantial part of the proinflammatory cytokine response is elicited by non-LPS parts of the meningococcus [56a].

Because several outer membrane proteins of N. meningitidis are known to interact with human cell receptors [57, 58], we examined whether sarcosyl-extracted outer membrane complexes are able to induce cytokine production. LPS-deficient OMCs primarily composed of the major outer membrane proteins PorA, PorB, RmpM, and OpaA did not induce cytokines. Thus, other meningococcal components not retained after sarcosyl extraction, for instance certain lipoproteins, chromosomal DNA, the polysaccharide capsule [59], or the peptidoglycan cell wall [32–34], must have been responsible for the observed cytokine induction by the LPS-deficient mutant. Further research is needed to identify which of the non-LPS components are responsible for cytokine induction and to quantify their relative contribution to the pathogenesis of invasive meningococcal disease.

**ACKNOWLEDGMENTS**

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