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Gram-negative bacteria induce proinflammatory cytokine production by monocytes in the absence of lipopolysaccharide (LPS)

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SUMMARY

Tumour necrosis factor-alpha (TNF-α), IL-1α and IL-6 production by human monocytes in response to a clinical strain of the Gram-negative encapsulated bacteria *Neisseria meningitidis* and an isogenic *lpxA*$^-$ strain deficient in LPS was investigated. Wild-type *N. meningitidis* at concentrations between 10^5 and 10^8 organisms/ml and purified LPS induced proinflammatory cytokine production. High levels of these cytokines were also produced in response to the *lpxA*$^-$ strain at 10^7 and 10^8 organisms/ml. The specific LPS antagonist bactericidal/permeability-increasing protein ($rBPI_{21}$) inhibited cytokine production induced by LPS and wild-type bacteria at 10^5 organisms/ml but not at higher concentrations, and not by LPS-deficient bacteria at any concentration. These data show that proinflammatory cytokine production by monocytes in response to *N. meningitidis* does not require the presence of LPS. Therapeutic strategies designed to block LPS alone may not therefore be sufficient for interrupting the inflammatory response in severe meningococcal disease.

Keywords lipopolysaccharide monocytes proinflammatory cytokines *Neisseria meningitidis*

INTRODUCTION

Infections caused by *Neisseria meningitidis* are an important cause of mortality and morbidity world wide [1]. Despite recent advances in intensive care, the mortality for patients presenting with severe meningococcal sepsis remains between 20% and 50%. Those that survive may have extensive tissue injury sometimes requiring amputation and/or skin grafting [2].

The host inflammatory response in meningococcal sepsis is generally believed to be induced by LPS [3,4]. LPS complexed to CD14 and LPS binding protein signals through Toll-like receptors (TLR) to activate NFκB and induce production of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), IL-1 and IL-6 [5,6]. Novel therapeutic interventions in Gram-negative septicaemia have been directed at modulating the effects of LPS [7] but with limited success [8–11]. The reason why LPS antagonists have not been effective is presently unknown. One possibility is that LPS may not be the only or even the main bacterial component responsible for inducing host inflammatory responses.

In the present study, we examined monocyte proinflammatory cytokine production induced by a clinical isolate of serogroup B *N. meningitidis* and an isogenic strain *lpxA*$^-$ that is completely deficient in LPS [12]. Our results show that LPS is not required for cytokine production in response to *N. meningitidis and* that strategies designed to block LPS alone may not be sufficient for interrupting the proinflammatory cytokine response.

H.U. and A.J.W. contributed equally to this work.

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MATERIALS AND METHODS

Bacteria and LPS

Neisseria meningitidis serogroup B (strain H44/76) is a clinical isolate from a case of fatal septicaemia [13]. A viable, LPS-deficient isogenic strain *lpxA*$^-$ derived from H44/76 was constructed by insertional inactivation of the *lpxA* gene required for lipid A biosynthesis [12,14]. Absence of LPS in the strain was confirmed by *Limulus* amoebocyte assay, MoAb binding, and gas chromatography/mass spectrometry. Bacteria were grown on gonococcal agar (Difco, West Mosley, UK) supplemented with Vitox (Oxoid, Basingstoke, UK). Purity of the *lpxA*$^-$ strain was maintained by culturing with 100 μg/ml of kanamycin (Sigma, Poole, UK) [12]. Bacteria were used in stationary phase after culture for 18 h. Suspensions were prepared in RPMI 1640 without phenol red (GIBCO, Paisley, UK) and the optical density (OD) measured at 540 nm. Viability was determined with a modified Miles and Misra technique [15]. An OD of 1 was shown to be equivalent to 10^9 viable organisms per ml. Bacteria were

fixed in 0·5% paraformaldehyde in PBS for 15 min and washed thoroughly in RPMI. Meningococcal LPS from *N. meningitidis* (H44/76) was prepared as previously described [16,17]. The final product contained $<0{\cdot}3\%$ protein and was without detectable nucleic acids.

Stimulation of monocytes

Monocytes were stimulated with *N. meningitidis* H44/76, the *lpxA*$^-$ LPS-deficient strain or purified meningococcal LPS at the indicated concentrations in a modified whole blood culture technique described previously [18]. Brefeldin A (Sigma) was added at 10 μg/ml to prevent cytokine secretion. Cultures were incubated on a rocking platform for 4 h at 37°C before analysis for intracellular cytokine production. Where indicated, 10 μg/ml of the LPS antagonist $rBPI_{21}$ (recombinant bactericidal/permeability-increasing protein; a gift from Xoma, Berkeley, CA) were also added.

Intracellular determination of monocyte cytokine production

After stimulation, 200-μl aliquots of the blood cultures were dispensed into 5-ml Falcon tubes (cat. no. 2054) and stained with CD14–FITC MoAb (TUK4) (Dako, Ely, UK). Erythrocytes were then lysed with FACS lysing buffer (Becton Dickinson, Oxford, UK), and the cells fixed in 250 μl of 4% paraformaldehyde for 15 min, and permeabilized with 50 μl of Leucoperm (Serotec Ltd, Oxford, UK) at room temperature. Monocytes were identified by gating on CD14. Granulocytes were excluded by forward and right angle scatter. Intracellular cytokines were detected with PE-conjugated MoAbs to TNF-α, IL-6 or IL-1α (Becton Dickinson) and analysed with a FACScalibur using Cellquest software (Becton Dickinson). Statistical comparisons were made by paired *t*-test using Sigmaplot.

RESULTS AND DISCUSSION

Monocyte proinflammatory cytokine production in response to wild-type N. meningitidis *bacteria and the LPS-deficient* lpxA$^-$ *strain*

Intracellular TNF-α production by CD14$^+$ monocytes was compared following activation with wild-type *N. meningitidis* and the isogenic *lpxA*$^-$ strain added at concentrations between 10^6 and 10^8 organisms/ml. The results of a typical experiment from more than six are given in Fig. 1. TNF-α was produced in response to wild-type bacteria added at concentrations between 10^6 and 10^8 organisms per ml in a dose-dependent manner. Equivalent high levels of TNF-α production were induced by the *lpxA*$^-$ strain at 10^8/ml. TNF-α was also induced but at lower levels with 10^7 and 10^6 *lpxA*$^-$ per ml. This result clearly shows that *N. meningitidis* can induce proinflammatory cytokine production by monocytes in the absence of LPS.

We next compared the ability of wild-type and *lpxA*$^-$ bacteria to induce IL-1α, IL-6 and TNF-α in the presence and absence of the LPS antagonist $rBPI_{21}$ (Fig. 2). At 10^8 organisms/ml, the wild-type and *lpxA*$^-$ bacteria induced equivalent high levels of all three cytokines. Significant cytokine production ($P < 0{\cdot}05$) was also obtained with 10^7 organisms/ml, although the response to wild-type bacteria was higher than to the *lpxA*$^-$ strain. Below this concentration, the response to wild-type bacteria was still easily detectable but the response to the *lpxA*$^-$ strain was very low or absent. LPS at concentrations >1 ng/ml also induced cytokine production.

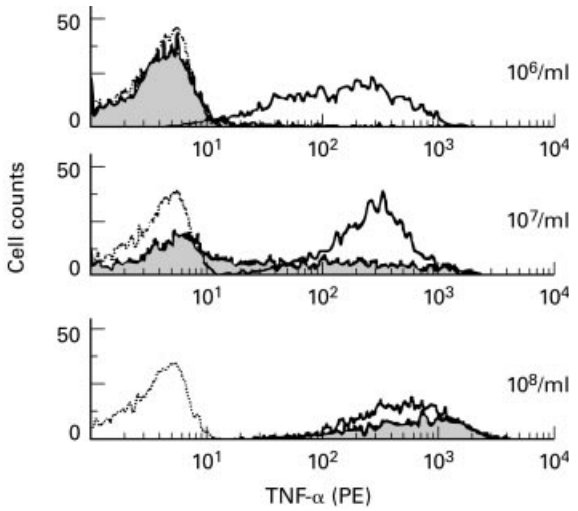

Fig. 1. Tumour necrosis factor-alpha (TNF-α) production by monocytes in response to wild-type *Neisseria meningitidis and* the LPS-deficient *lpxA*$^-$ strain. Blood was cultured with the bacteria at a concentration of 10^6-10^8 organisms/ml for 4 h in the presence of Brefeldin A and the cells then surface stained for CD14, permeabilized and stained intracellularly for TNF-α. The results are expressed as FACS histograms using Cellquest software. Dotted line, unstimulated; solid line, wild-type *N. meningitidis*; grey fill, *lpxA*$^-$ LPS-deficient *N. meningitidis.*

Addition of $rBPI_{21}$ to the cultures inhibited cytokine production induced by LPS at concentrations up to 100 ng/ml ($P < 0{\cdot}05$), confirming its activity as an LPS antagonist. Cytokine production induced with wild-type *N. meningitidis* bacteria at 10^5 organisms/ml was also significantly inhibited in all six experiments, typically to about 50% of control levels ($P < 0{\cdot}05$). At higher concentrations of bacteria, $rBPI_{21}$ did not inhibit cytokine production. Furthermore, it did not inhibit the response to the *lpxA*$^-$ strain at any concentration of bacteria tested. It is possible that the concentration of $rBPI_{21}$ used in these experiments was not high enough to inhibit LPS present on the bacteria above 10^6/ml. Quantification of the LPS content in *N. meningitidis* based on spectrophotometric analysis of the LPS-specific sugar 2-keto-3-deoxyoctonic acid (KDO) showed that there are approximately $1{\cdot}5 \times 10^5$ LPS molecules (mol. wt 4044) per *N. meningitidis* (H44/76) bacterium. LPS at 100 ng/ml is therefore equivalent to about 10^8 bacteria. As $rBPI_{21}$ significantly inhibited responses to LPS at 100 ng/ml, the failure to inhibit cytokine production induced by 10^6-10^8 organisms/ml cannot be explained by the presence of high concentrations (>100 ng/ml) of LPS. It is possible that $rBPI_{21}$ does not completely inhibit cell-associated LPS, in which case the response with higher concentrations of bacteria ($>10^5$/ml) may have been due in part to LPS together with other bacterial components. This may explain the inability of *lpxA*$^-$ bacteria to stimulate significantly at concentrations $<10^7$/ml. Nevertheless, our experiments show unequivocally that *N. meningitidis* can activate monocytes to produce proinflammatory cytokines in the absence of LPS. Various components of Gram-negative bacteria such as pili, outer membrane proteins, and opacity factors have been shown to activate NFκB and AP1/cjun in epithelial cells and may contribute to activation by the lpxA-strain [19–21]. Our results suggest that LPS may not have an exclusive role in monocyte activation by intact bacteria at concentrations $>10^5$/ml. The concentrations of bacteria present

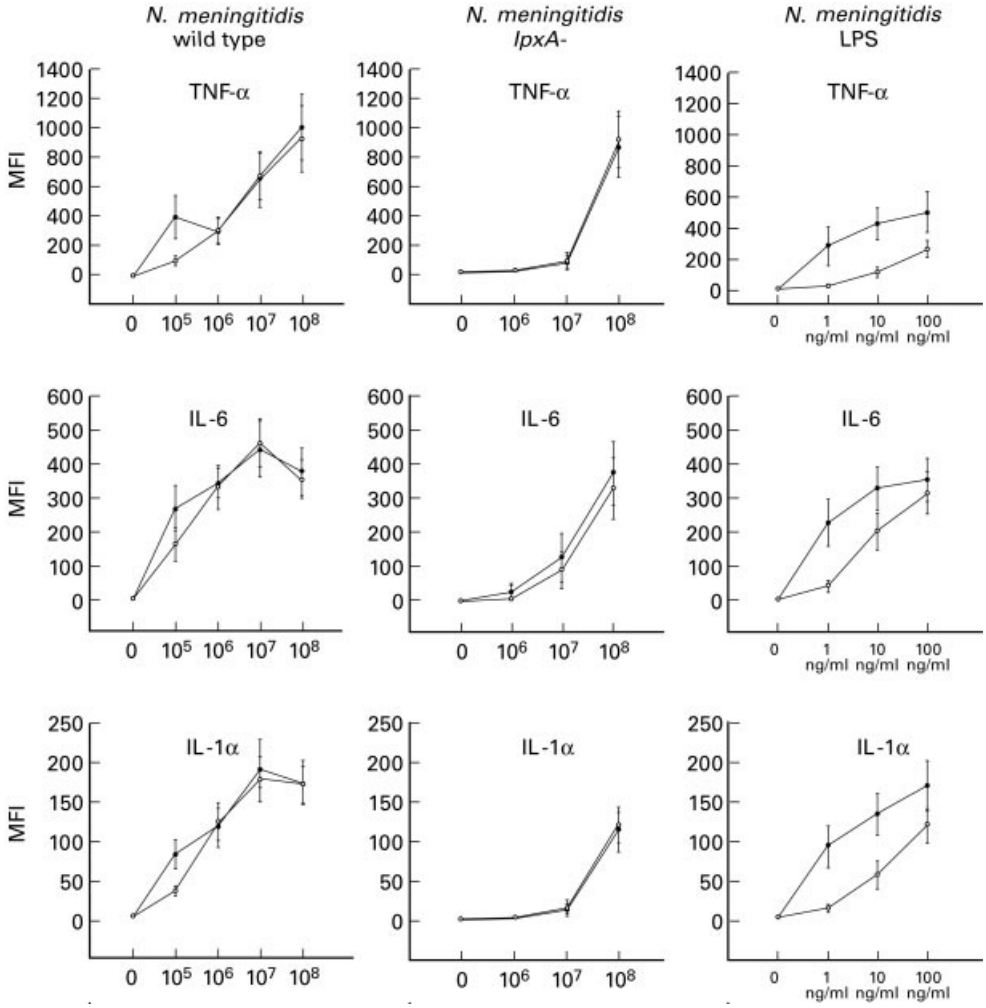

Fig. 2. Cytokine production by monocytes activated with wild-type *Neisseria meningitidis*, *lpxA*$^-$ LPS-deficient strain and purified meningococcal LPS. Blood was cultured with the bacteria at a range of concentrations from 10^5 to 10^8 organisms/ml or with purified LPS at concentrations of 1, 10 and 100 ng/ml for 4 h in the presence of Brefeldin A. The cells were then surface stained for CD14, permeabilized and stained intracellularly for IL-1α, IL-6 and tumour necrosis factor-alpha (TNF-α). The results were determined as median fluorescence intensity (MFI) from histograms as shown in Fig. 1 and plotted as the mean $\pm$ s.e.m. from six separate experiments. ●, Response without bactericidal/permeability-increasing protein ($rBPI_{21}$); ○, response obtained in the presence of 10 μg/ml of $rBPI_{21}$.

in septicaemic patients have been estimated at between 10^3 and $> 10^5$/ml [22,23], but more recent estimates using quantitative polymerase chain reaction have indicated much higher concentrations of $\geq 10^7$/ml (R. Borrow, personal communication). This bacterial load is significantly higher than previously estimated by quantitative blood cultures.

Our data indicate that bacteria at this concentration can induce an inflammatory response in the absence of LPS. Therapeutic measures aimed at blocking endotoxin may not be as effective as originally hoped [9,10]. Additive/synergistic effects of LPS and non-LPS pathways may explain our observation that wild-type bacteria frequently induce higher TNF-α levels than high concentrations of LPS and should be considered in meningococcal disease. Prompt administration of anti-microbial therapy in addition to anti-LPS therapy may be critical for avoiding catastrophic activation of host inflammatory processes induced by both LPS and non-LPS activated pathways. Future strategies for treating this disease should be directed against both LPS and non-LPS pathways.

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