Differential Role of Lipooligosaccharide of *Neisseria meningitidis* in Virulence and Inflammatory Response during Respiratory Infection in Mice

Maria Leticia Zarantonelli,1* Michel Huerre,2 Muhamed-Kheir Taha,1 and Jean-Michel Alonso1

*Unité des Neisseria*1 and *Unité de Recherche et d’Expertise Histotechnologie et Pathologie*,2
Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France

Received 24 April 2006/Returned for modification 7 June 2006/Accepted 7 July 2006

Meningococcal lipooligosaccharide (LOS) induces a strong proinflammatory response in humans during meningococcal infection. We analyzed the role of LOS in the inflammatory response and virulence during the early infectious process in a mouse model of meningococcal respiratory challenge. An *lpxA* mutant strain (serogroup B) devoid of LOS (strain Z0204) could not persist in the lungs and did not invade the blood. The persistence in the lungs and invasion of the bloodstream by a *rfaD* mutant expressing truncated LOS with only lipid A and 3-deoxy-d-manno-2-octulosonic acid molecules (strain Z0401) was intermediate between those of the wild-type and Z0204 strains. Both LOS mutants induced acute pneumonia with the presence of infiltrating polymorphonuclear leukocytes in lungs. Although tumor necrosis factor alpha production was reduced in mice infected with the mutant of devoid LOS, both LOS mutants induced production of other proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and the murine IL-8 homolog KC. Together, these results suggest that meningococcal LOS plays a role during the early infectious and invasive process, and they further confirm that other, nonlipooligosaccharide components of *Neisseria meningitidis* may significantly contribute to the inflammatory reaction of the host.

*Neisseria meningitidis* is a gram-negative bacterium that lives in the human nasopharynx, and it is commonly found among the commensal flora of about 10% of asymptomatic carriers. However, this bacterium can provoke severe systemic infections such as septicemia (with or without shock) and meningitis. Endotoxin is a major structural component of the outer membranes of gram-negative bacteria. Endotoxin, and particularly lipid A, potently induces the inflammatory response during meningococcal infection. The severity of meningococcal disease is thought to be linked to the degree of the inflammatory response induced during invasive infection (14, 25). Lipid A anchors the inner core, comprising two 3-deoxy-d-manno-2-octulosonic acid (KDO) and heptose (Hep) residues of meningococcal lipooligosaccharide (LOS) in the outer membrane (9). Meningococcal lipid A has a symmetrical structure. The *lpxA* gene adds the O-linked 3-OH (C12-3-OH) fatty acyl chains to positions 3 and 3′ of the glucosamine disaccharide (19). Another gene, *lpxD*, adds the N-linked 3-OH (C14-3-OH) to positions 2 and 2′. Two others genes, *lpxL1* and *lpxL2*, are involved in lipid A acylxoyacylation and may add a C12 chain to the N-linked C14-3-OH (24). The *rfaD* gene encodes the ADP-t-glycero-d-mannoheptose-6-epimerase, which is responsible for the biosynthesis of the lipooligosaccharide precursor ADP-t-glycero-d-mannoheptose. The *rfaD* mutant produces LOS with only lipid A and the KDO molecules (27). Meningococcal *lpxA* mutants are devoid of LOS but are viable (18, 27) despite the (KDO)2-lipid A structure being required for cell viability in other bacterial species (5). However, *lpxA* mutants seem to be heterodiploid for the *lpxA* gene, as they harbor both the wild-type and the insertionally inactivated *lpxA* alleles. An LOS− phenotype can result from the negative transdominance of the inactivated *lpxA* allele (27).

*N. meningitidis* LOS seems to induce a proinflammatory cytokine response either independently (17) or through the CD14/Toll-like receptor 4 (TLR4) pathway (28). This CD14/TLR4 activation requires the KDO moiety. Meningococcal lipid A expressed by KDO-deficient meningococci is much less biologically active than (KDO)2-containing meningococcal LOS (28). The impact on inflammatory responses of different meningococcal LOSs has been analyzed under ex vivo conditions using cell lines or peripheral blood mononuclear cells (3, 23). It was shown that *lpxA* mutants may be less toxic due to a reduced capacity to induce tumor necrosis factor alpha (TNF-α), although they were still able to induce a significant inflammatory response (23).

However, there has yet to be a global analysis of the role of LOS in the inflammatory response during infection. In particular, the induction of the local response in the respiratory tract, the route of entry of *N. meningitidis*, has not been addressed. This study aimed to analyze the impact of the absence of LOS on the inflammatory response and on virulence during the early infectious process, using a mouse model of meningococcal respiratory challenge (1).

**MATERIALS AND METHODS**

Strains of *N. meningitidis* used and culture conditions. *N. meningitidis* LNP4194/12 is a serogroup B clinical isolate with a B:15:P1-7 phenotype and an L3,7,9 immunotype. It belongs to the ST32 (ET-5) clonal complex. Strain Z0204 is a viable isogenic mutant that is completely devoid of LOS, constructed by insertional inactivation of the *lpxA* gene. This mutant is heterodiploid for *lpxA*/*lpxA::aph-3*. The absence of LOS was confirmed as previously described (27). Strain Z0401 is an isogenic mutant with a truncated LOS, constructed by

---

1* Corresponding author. Mailing address: Unité des Neisseria, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France. Phone: 33 1 45 68 89 58. Fax: 33 1 41 60 30 34. E-mail: lzaranto@pasteur.fr.
Cell morphology of LOS mutants. We compared the morphologies of the two LOS mutants, Z0204 (lpxA::aph-3′ heterodiploid, completely devoid of LOS) and Z0401 (rfaD::aph-3′, expressing truncated LOS), by transmission electron microscopy to evaluate the influence of the rfaD and lpxA mutations on the cell structure of *N. meningitidis*. The rfaD mutant exhibited a morphology similar to that of the wild-type strain, with a typical diploccocal shape and a normal septation. By contrast, the lpxA mutant devoid of LOS showed segments of the outer membrane detached from the bacterial bodies, with large and empty detached fragments being observed. In several cases, two separated bacteria remained linked, sharing a detached membrane. However, it was possible to distinguish the inner and outer membranes (Fig. 1A).

Effect of LOS alteration on meningococcal virulence. We investigated the role of LOS mutations in meningococcal virulence by comparing the kinetics of in vivo growth/survival of the wild-type strain LNP14912 with those of both LOS mutants in the mouse model of dual IAV-*N. meningitidis* infection (1). The bacterial loads in the lungs and blood were scored 3 h and 24 h after intranasal bacterial challenge with 5 × 10^7 CFU. As Z0204 is a heterodiploid *lpxA::aph-3′* mutant, CFU were counted on GCB medium supplemented or not with 100 μg/ml of kanamycin. We observed a considerable decrease in CFU counts, of at least three orders of magnitude, in the lungs at 3 h for the mutant devoid of LOS. At 24 h, this mutant was completely cleared from the lungs (Fig. 1B). CFU counts in lungs infected with strain Z0401 (expressing truncated LOS) were found to be intermediate between those in lungs infected with the wild-type and Z0204 strains. We detected no bacteria for either LOS mutant (Fig. 1B). Viable bacteria (2 × 10^5 CFU) from the lungs of one mouse infected with mutant Z0204 (heterodiploid *lpxA::aph-3′*) were recovered only on a GCB plate, whereas no colonies grew in kanamycin-supplemented GCB plates. When passaged on kanamycin-supplemented GCB plates, these colonies were sensitive to kanamycin, suggesting that the bacteria had lost the *aph-3′* gene. Such bacteria may correspond to *lpxA::lpxA* revertants. PCR analysis showed these bacteria to be *lpxA::lpxA* revertants (named Z0204 revertant). We detected no PCR product when DNA from this revertant was amplified using oligonucleotides binding to the *aph-3′* gene (data not shown). We analyzed the cellular fatty acid composition of this revertant strain by gas chromatography. Hydroxylated fatty acids 3-OH-C12:0 and 3-OH-C14:0 derived exclusively from lipid A were observed.

Histological characterization of acute meningococcal infection in mice. The alveolar architecture of lungs similar to those of the parent LNP14912 strain, suggesting a recovery of virulence after loss of the inactivated *lpxA* allele.

**RESULTS**

**Cell morphology of LOS mutants.** We compared the morphologies of the two LOS mutants, Z0204 (*lpxA::aph-3′* heterodiploid, completely devoid of LOS) and Z0401 (*rfaD::aph-3′*, expressing truncated LOS), by transmission electron microscopy to evaluate the influence of the *rfaD* and *lpxA* mutations on the cell structure of *N. meningitidis*. The *rfaD* mutant exhibited a morphology similar to that of the wild-type strain, with a typical diploccocal shape and a normal septation. By contrast, the *lpxA* mutant devoid of LOS showed segments of the outer membrane detached from the bacterial bodies, with large and empty detached fragments being observed. In several cases, two separated bacteria remained linked, sharing a detached membrane. However, it was possible to distinguish the inner and outer membranes (Fig. 1A).

**Effect of LOS alteration on meningococcal virulence.** We investigated the role of LOS mutations in meningococcal virulence by comparing the kinetics of in vivo growth/survival of the wild-type strain LNP14912 with those of both LOS mutants in the mouse model of dual IAV-*N. meningitidis* infection (1). The bacterial loads in the lungs and blood were scored 3 h and 24 h after intranasal bacterial challenge with 5 × 10^7 CFU. As Z0204 is a heterodiploid *lpxA::aph-3′* mutant, CFU were counted on GCB medium supplemented or not with 100 μg/ml of kanamycin. We observed a considerable decrease in CFU counts, of at least three orders of magnitude, in the lungs at 3 h for the mutant devoid of LOS. At 24 h, this mutant was completely cleared from the lungs (Fig. 1B). CFU counts in lungs infected with strain Z0401 (expressing truncated LOS) were found to be intermediate between those in lungs infected with the wild-type and Z0204 strains. We detected no bacteria for either LOS mutant (Fig. 1B). Viable bacteria (2 × 10^5 CFU) from the lungs of one mouse infected with mutant Z0204 (heterodiploid *lpxA::aph-3′*) were recovered only on a GCB plate, whereas no colonies grew in kanamycin-supplemented GCB plates. When passaged on kanamycin-supplemented GCB plates, these colonies were sensitive to kanamycin, suggesting that the bacteria had lost the *aph-3′* gene. Such bacteria may correspond to *lpxA::lpxA* revertants. PCR analysis showed these bacteria to be *lpxA::lpxA* revertants (named Z0204 revertant). We detected no PCR product when DNA from this revertant was amplified using oligonucleotides binding to the *aph-3′* gene (data not shown). We analyzed the cellular fatty acid composition of this revertant strain by gas chromatography. Hydroxylated fatty acids 3-OH-C12:0 and 3-OH-C14:0 derived exclusively from lipid A were observed (Fig. 1C) indicating that the Z0204 revertant strain had recovered the LOS+ phenotype. We analyzed the virulence of this revertant by intranasal challenge of IAV-infected mice and found that the Z0204 revertant had recovered the virulent phenotype (Fig. 1D). It showed meningococcal infection kinetics in lungs and blood similar to those of the parent LNP14912 strain, suggesting a recovery of virulence after loss of the inactivated *lpxA* allele.
the alveoli, indicating that the mice had recovered from viral pneumonia (Fig. 2A). The microscopic examinations of lung sections 3 h after bacterial challenge with strain LNP14912 revealed an intense inflammation of the alveolar septa (Fig. 2B), including focal recruitment of polymorphonuclear cells within the alveoli. We also observed a similar and intense inflammatory reaction when mice were infected with LOS mutants. Both isogenic LOS mutants were able to induce acute pneumonia with the presence of infiltrating polymorphonuclear leukocytes (Fig. 2, compare panels C and D with panel B). Moreover, immunohistochemistry using a rabbit polyclonal serum directed against the serogroup B meningococcal capsule revealed bacteria in the lungs.

Cytokine and chemokine production in the lungs during acute experimental meningococcal infection. We have previously reported that meningococcal IAV-N. meningitidis-infected mice showed a local cytokine response in the lungs. Therefore, we quantitatively studied the local production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in lung homogenates 3 h after bacterial challenge to assess the role of meningococcal LOS in inducing the inflammatory response during acute experimental infection. As a control, we evaluated cytokine production in the lungs of mice 7 days after IAV infection. After infection, all three strains, including the mutant devoid of LOS, increased the production of IL-1β and IL-6 versus that by control mice. Moreover, the levels of induction of these two cytokines by the two mutants were not significantly different from that by the wild-type strain (P = 0.164 and P = 0.140 for IL-1β and IL-6, respectively, for the mutant Z0204) (Fig. 3). As expected, the mutant devoid of LOS (Zi0204) induced a lower production of TNF-α than the wild-type strain (P = 0.023). The TNF-α levels induced by the mutant devoid of LOS were similar to those observed for the control mice. The deep-rough mutant (Z0401) induced lower production of TNF-α than the wild-type strain, although this difference was not significant (P = 0.073) (Fig. 3).

Chemokines play a central role in recruiting and regulating the leukocyte traffic during the acute inflammatory response.
Studies of patients with meningococcal disease have shown that plasma levels of MCP-1, MIP-1α, and IL-8 directly correlate with lipopolysaccharide (LPS) levels, whereas levels of RANTES are negatively correlated (11). Therefore, we quantified in vivo the levels of KC (the functional murine homologous of human IL-8), MIP-1α, RANTES, and MCP-1 production in the lungs 3 h after infection of mice with the wild-type strain and the LOS mutants to determine the importance of meningococcal LOS in inducing chemokines. All three strains induced production of significantly higher levels of all the tested chemokines in the lungs of infected mice than in the lungs of control mice (IAV infected). However, the levels of MIP-1α, RANTES, and MCP-1 induced by the wild-type strain and the rfaD mutant (Z0401) were significantly higher than those induced by the mutant devoid of LOS (Z0204) and by control mice ($P < 0.05$ in all cases). We observed no significant differences between the production of KC in lungs of mice infected with the wild-type or the LOS mutant strains.

We observed a similar pattern of cytokine and chemokine production when experiments were carried out with the same standardized inoculum of heat-inactivated bacteria instead (data not shown).

**DISCUSSION**

LOS is a major virulence factor during meningococcal sepsis and meningitis. The severity of meningococcal disease is directly correlated with the levels of endotoxin in the plasma and cerebrospinal fluid (2). *N. meningitidis* is the first viable gram-negative bacterium harboring an inactivated *lpxA* gene, and thus it completely lacks LOS. We infected mice with a serogroup B *N. meningitidis* mutant devoid of LOS. Our dual IAV-meningococcal mouse model mimics the different steps of infectious meningococcal disease, with a local infection of the respiratory tract that progresses to meningococcemia (1). We have shown that the mutant devoid of LOS (Z0204) and the
FIG. 3. Lung cytokine levels in mice after challenges with meningococcal wild-type strain and the LOS mutants. Cytokine and chemokine levels were measured 3 h after intranasal bacterial infection. IAV-infected mice on day 7 after viral infection were used as controls. Significant decreases in cytokine production induced by the two mutants versus the wild-type strain are indicated with an asterisk (calculated using a two-tailed Student t-test, P < 0.05). Error bars indicate standard deviations.
deep-rough LOS mutant (Z0401) were affected mostly in their ability to invade and persist in the bloodstream. In particular, the Z0204 mutant was very affected in its capacity to persist in the lungs and was unable to invade the blood. This shows that meningococcal LOS plays a major role during the early infectious and invasive process. The presence of the capsule in N. meningitidis is the major element in mediating serum resistance (26). However, LOS sialylation may be important when capsule is downregulated. This occurs during meningococcal infection and the crossing of the epithelium (4, 7). Our results suggest that the lack of LOS renders N. meningitidis more susceptible to in vivo complement-mediated bacteriolysis and opsonophagocytosis, both of which are major mechanisms of the early innate defense against meningococcal infections. This was further confirmed by the observation of the Z0204 revertant strain, which recovered its ability to synthesize LOS and recovered the same virulent phenotype as the parent LNP14912 strain. All these results are consistent with the expression of a complete LOS molecule being crucial for meningococcal survival in situations in which the principal mechanism allowing meningococci to escape the innate immune response, the neisserial capsule, is not present. It is possible that this takes place during meningococcal infection and the crossing of the epithelium (4, 7). Alternatively, the lack of LOS may have an indirect effect on meningococcal virulence through the alteration of bacterial integrity. Indeed, electron microscopy examination of the lpxA mutant showed alteration in the outer membrane (Fig. 1A). In summary, all these data show that the lack of LOS will affect multiple aspects of the bacterial behavior and host-pathogen interaction.

Electron microscopy examination (Fig. 1A) and data obtained by whole-cell ELISA (using monoclonal antibodies against two major outer membrane proteins, PorA and PorB) and by gas chromatography analysis of cellular fatty acid composition (data not shown) showed that the rfaD mutant has no detectable alteration in the integrity of the outer membrane. However, this mutant showed reduced virulence and cytokine production. These observations are in accord with a direct role of LOS in meningococcal virulence. Plant et al. recently showed that the meningococcal gmhB mutant, which expresses a truncated LOS molecule similar to that of the rfaD mutant, was avirulent in mice (12).

In the model of dual IAV-N. meningitidis infection of adult mice, N. meningitidis colonizes the lungs and induces inflammatory pneumonia followed by bacteremia. During the virus-induced susceptibility to meningococcal superinfection, a normal polymorphonuclear cell response to bacterial infection is observed, with an intense influx of polymorphonuclear leukocytes and monocytes (1). However, the local inflammatory response in the lungs seems to be LOS independent. Histological examination revealed no differences in lungs from mice infected with a meningococcal strain completely devoid of LOS and the wild-type strain. Both isogenic LOS mutants Z0204 and Z0401 induced an acute alveolitis, with equivalent intense influxes of polymorphonuclear cells.

However, the mutant devoid of LOS, Z0204, induced significantly lower levels of TNF-α than the wild-type strain. This is consistent with many studies of an LOS-free lpxA mutant of the N. meningitidis serogroup B strain H44/76 conducted in a cell model system (8, 16, 21, 22). The mutant devoid of LOS showed an enhanced release of membrane fragments. Membrane-associated proteins and peptidoglycan could be responsible for the proinflammatory response observed for this mutant. The mutant devoid of LOS induced significant levels of IL-1β and IL-6 versus those in control mice. These levels were not significantly different from those induced by the wild-type strain. Moreover, we have recently shown that other meningococcal structures, such as peptidoglycan and pili, are inducers of an inflammatory response through the NF-κB-dependent signaling pathway (6, 20). Peptidoglycan is a major pathogen-associated molecular pattern that is sensed by the cytosolic Nod1/Nod2 proteins (6).

Cytokine production was quantified 3 hours after bacterial challenge. As a difference in bacterial counts in lungs was detected, the experiment was repeated by injecting mice with heat-killed bacteria (the same concentration of 5 × 10⁷ bacteria). Indeed, live and heat-inactivated bacteria (wild type, rfaD mutant, and mutant devoid of LOS) were able to induce similar patterns of cytokine production (data not shown). These results suggest that the induction of cytokine production in lungs does not seem to be dependent on the bacterial growth/survival rate.

We report, for the first time, a differential induction of TNF-α and IL-1β/IL-6 mediated by a meningococcal mutant devoid of LOS. This strongly suggests that meningococcal LOS is a central mediator of TNF-α induction in vivo. However, other non-LPS constituents of N. meningitidis contribute to the in vivo induction of IL-1β and IL-6 (8, 16, 21, 22). Ramphal et al. (15) have recently shown a similar differential induction of TNF-α and IL-6 in a mouse model of Pseudomonas aeruginosa infection. TNF-α production in TLR2/4-deficient mice was severely impaired, whereas IL-6 production was observed, with no change in the neutrophil count in the lungs.

We observed a significant decrease in the production of the chemokines MIP-1α, RANTES, and MCP-1 after challenge with the mutant devoid of LOS. For both LOS mutants, the KC response in vivo was associated with an intense influx of polymorphonuclear leukocytes in lungs (Fig. 2C and D). Together, these findings strongly suggest that non-LPS components of N. meningitidis may contribute significantly to the inflammatory reaction of the host.

Although the proinflammatory action of meningococcal LOS is thought to be mediated by the CD14/TLR4 pathway (28), the cell-activating property of the H44/76 lpxA mutant is thought to be mediated by the CD14/TLR2 receptor pathway (8, 13). However, the differential pattern of cytokine and chemokine production observed in the absence of LOS suggests other unknown signaling pathways for the different proinflammatory cytokines and chemokines.

ACKNOWLEDGMENTS

This work was supported by the Institut Pasteur.

We gratefully acknowledge Jean-Philippe Carlier for excellent assistance with gas chromatography, Huot Khun for histological studies, and Isabelle Bonne for the electron microscopy.

REFERENCES


Editor: J. N. Weiser