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Complement Activation and Complement-Dependent Inflammation by Neisseria meningitidis Are Independent of Lipopolysaccharide

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Received 28 November 2003/Returned for modification 21 January 2004/Accepted 19 February 2004

Fulminant meningococcal sepsis has been termed the prototypical lipopolysaccharide (LPS)-mediated gram-negative septic shock. Systemic inflammation by activated complement and cytokines is important in the pathogenesis of this disease. We investigated the involvement of meningococcal LPS in complement activation, complement-dependent inflammatory effects, and cytokine or chemokine production. Whole blood anticoagulated with lepirudin was stimulated with wild-type Neisseria meningitidis H44/76 (LPS⁺), LPS-deficient N. meningitidis H44/76ΔpxA (LPS⁻), or purified meningococcal LPS (NmLPS) at concentrations that were relevant to meningococcal sepsis. Complement activation products, chemokines, and cytokines were measured by enzyme-linked immunosorbent assays, and granulocyte CR3 (CD11b/CD18) upregulation and oxidative burst were measured by flow cytometry. The LPS⁺ and LPS⁻ N. meningitidis strains both activated complement effectively and to comparable extents. Purified NmLPS, used at a concentration matched to the amount present in whole bacteria, did not induce any complement activation. Both CR3 upregulation and oxidative burst were also induced, independent of LPS. Interleukin-1β (IL-1β), tumor necrosis factor alpha, and macrophage inflammatory protein 1α production was predominantly dependent on LPS, in contrast to IL-8 production, which was also markedly induced by the LPS⁻ meningococci. In this whole blood model of meningococcal sepsis, complement activation and the immediate complement-dependent inflammatory effects of CR3 upregulation and oxidative burst occurred independent of LPS.

Invasive disease caused by the gram-negative bacterium Neisseria meningitidis is a life-threatening infection in children and young adults. Meningococcal septic shock (or fulminant meningococcal sepsis [FMS]), the prototype of overwhelming gram-negative sepsis, is feared especially because it is able to cause devastating disease with a high case fatality rate. The pathogenic mechanisms leading to FMS are thought to be the uncontrolled growth of meningococci in the circulation, resulting in a massive activation of diverse inflammatory systems, such as the complement system, the cytokine network, and the coagulation cascade (6, 35).

In FMS, high lipopolysaccharide (LPS) levels are correlated with an increased bacterial load, high levels of pro- and anti-inflammatory cytokines, increased complement activation, the induction of intravascular coagulation, and a poor outcome. Therefore, meningococcal LPS is regarded as the principal bacterial pathogenic element during FMS (3).

The complement cascade plays a dual role in the pathogenesis of meningococcal infections. A complement deficiency predisposes some individuals to meningococcal infection (10, 17, 28), but on the other hand, with FMS, extensive complement activation is correlated with severe disease and a poor outcome (4, 16). Thus, the complement system plays an important role in the first line of defense against meningococcal infection, whereas massive activation of the complement system contributes to the development of shock.

Complement is activated on the meningococcal surface by one or more of the three initial complement-activating pathways, i.e., the classical, the lectin, and the alternative pathways (Fig. 1). After the activation of complement factor 3 (C3) by any of these pathways, a C5 convertase is formed, which cleaves the pivotal C5 molecule into C5a and C5b. C5b is the initial molecule in the formation of the terminal C5b-9 complement complex (TCC). Membrane-associated TCC, also designated the C5b-9 membrane attack complex, leads to lysis of the bacterium, whereas C5a is a potent anaphylatoxin (24). Experimentally, the role of the complement system in inflammation by N. meningitidis and Escherichia coli was recently given more emphasis by our observation that granulocyte CR3 (CD11b/CD18) upregulation and the formation of reactive oxygen species in human whole blood, key elements in the induction of vascular damage during sepsis, are highly dependent on C5a (22, 29).

Recent in vitro studies suggested that components of N. meningitidis other than LPS may also contribute to the inflammatory response of the host. Using a meningococcal mutant that was deficient for LPS in the outer membrane, we and others have shown that proinflammatory cytokines can be induced independent of LPS in cultured human cells (27, 30, 34). In addition, we showed that purified meningococcal LPS (NmLPS) is a poor activator of complement, whereas experiments with outer membrane vesicles that were depleted of or completely lacking LPS suggested that non-LPS components in...
Cocci were grown overnight on Kellogg’s to mostly short-chain, saturated fatty acids (31). LPS-decreased the outer membrane phospholipid composition, with a switch in proteins by the LPS-decreased protein expression. The model is based on anticoagulation with lepirudin, a recombinant hirudin which is a highly specific thrombin inhibitor that minimally influences complement activation or cytokine production. Whole blood from healthy, adult volunteers was collected in polypropylene tubes containing lepirudin (50-μg/ml final concentration). Informed consent was obtained from all donors prior to the experiments, and the human experimentation guidelines of the local ethics committee were followed in conducting the research. Immediately after being drawn, the whole blood (1 ml) was incubated with the stimulants (0.1 ml).

For the determination of granulocyte CR3 (CD11b/CD18) expression and oxidative burst, samples were incubated for 10 min at 37°C in a water bath, followed by immediate processing for flow cytometry.

For the detection of complement activation products, samples were incubated for 1 h at 37°C in a Coulter mixer (Coulter, Luton, England). Complement activation was stopped by the addition of EDTA to a final concentration of 20 mM, after which samples were centrifuged at 1,400 × g for 10 min. The plasma was collected and stored at −70°C until samples were assayed in one batch.

Samples for the measurement of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-8, and macrophage inflammatory protein 1α (MIP-1α) were processed as described for complement activation, but the incubation time was increased to 2 h.

Oxidative burst. Oxidative burst was measured by using a commercially available Burst-test kit (Orpegen Pharma, Heidelberg, Germany). After incubation as described above, samples were treated according to the manufacturer’s instructions and assayed by flow cytometry. Granulocytes were identified in a forwardscatter-side scatter plot by their typical patterns.

CR3 (CD11b/CD18) expression. After being incubated as described above, cells were washed once in PBS and fixed with 0.5% (vol/vol) paraformaldehyde in an equal volume of PBS for 4 min at 37°C. Cells were stained with an anti-CD11b-PE or isotype control (γ2a) (Becton Dickinson) antibody for 15 min at room temperature. An anti-CD14-FITC (Becton Dickinson) antibody and the nuclear dye LDS-751 (Molecular Probes Inc, Eugene, Ore.) were used to discriminate granulocytes in washed whole blood.

Enzyme immunoassays. Complement activation was measured in plasma by enzyme immunoassays based on MAbS that were highly specific for the activation products; the native, nonactivated components did not interfere in the assays. Activation of the three initial pathways was selectively detected.

(i) Clrs-C1inh complexes. Activation of the classical complement pathway was determined as described previously (12). Clrs-C1 inhibitor (Clrs-C1inh) complexes were measured by using MAb Kok-12, specific for a neoepitope exposed only when Clrinh is complex with its substrates. In brief, microtiter plates were coated with the Kok-12 antibody, the antibody was reacted with plasma or controls, and the complex was detected with a cocktail of anti-C1r and anti-C1s antibodies. Human serum activated with heparin was used as a standard and was defined to contain 1,000 arbitrary units (AU)/ml.

(ii) C4bc. Activation of the classical and lectin pathways was determined by an assay using a MAb specific for a neoepitope that is exposed in activated C4 (42). The C4bc assay detects the sum of the activation products C4b, iC4b, and C4c. The same standard was used as for the Clrs-C1s assay, defined to contain 1,000 AU/ml. Both the MAb for this assay and Kok-12 were a kind gift from C. E. Hack, Department of Immunopathology, Sanquin Research, Amsterdam, The Netherlands.

(iii) C3bBbP complexes. Activation of the alternative pathway was detected by measuring the alternative convertase C3bBb-properdin (C3bBnP), as recently described (22). The capture antibody used was an antiproperdin antibody, and the detection antibody was a polyclonal anti-C3 antibody. The standard was normal human serum activated with zymosan and defined to contain 1,000 AU/ml.

(iv) TCC. Activation of the terminal pathway was quantified by using MAb aE11, specific for C9 incorporated in the fluid-phase SC5b-9 complex, as described previously (23). The TCC assay was modified with a biotinylated anti-C9 antibody used as the detection antibody. Human serum activated with zymosan was used as the standard.
were considered statistically significant.

Total complement activation (Fig. 1).

C5bBbP (classical and lectin pathways), whereas soluble TCC, the C4bc (classical and lectin pathways) and C3bBbP (alternative pathway), and TCC (final common pathway) after the stimulation of lepirudin-treated human whole blood for 1 h with LPS\(^+\) (diamonds) or LPS\(^-\) (squares) \(N. meningitidis\) or purified NmLPS (circles). The x axes are calibrated for the numbers of \(N. meningitidis\) cells as well as the amounts of LmLPS that are present in the bacteria, according to SDS-PAGE analysis. Medians and upper quartiles are presented from four separate experiments. *, \(P < 0.05\); **, \(P < 0.01\), by two-way ANOVA. Dashed lines indicate baseline complement activation.

An overview of the complement activation markers measured is illustrated in Fig. 1.

Cytokines and chemokines. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturers’ instructions to quantify TNF-\(\alpha\), IL-1\(\beta\), IL-8 (Duo Set ELISA development system [BioSource International, Cammarillo, Calif.]) for all three, and MIP-1\(a\) (MIP-1\(a\) Cyto- screen [Biosource International, Camarillo, Calif.]). The lower limits of detection were 8 pg/ml for TNF-\(\alpha\), IL-1\(\beta\), and IL-8 and 2 pg/ml for MIP-1\(a\).

LAL assay. Quantiﬁcation of the biological activity of LPS in the bacterial strains was performed as described previously (5). \(E. coli\) O55:B5 LPS was used as a reference. The lower limit of detection was 0.25 endotoxin units/ml.

Statistics. A two-way analysis of variance (ANOVA) for repeated measures was used to test the data for statistically signiﬁcant differences. \(P\) values of <0.05 were considered statistically signiﬁcant.

RESULTS

Complement activation by LPS\(^+\) and LPS\(^-\) \(N. meningitidis\) or puriﬁed NmLPS. Assays for C1rs-C1inh (classical pathway), C4bc (classical and lectin pathways) and C3bBbP (alternative pathway) complexes were used to determine the initial complement activation pathways, whereas soluble TCC, the final product of the terminal pathway, was used as an indicator of total complement activation (Fig. 1).

The amount of LPS per \(10^6\) \(N. meningitidis\) cells was estimated by SDS-PAGE analysis; this showed that \(10^6\) bacteria contained approximately 5 ng of LPS. In Fig. 2 and 3, the x axes are calibrated for the numbers of LPS\(^+\) \(N. meningitidis\) cells as well as the amounts of LPS that are present in the bacteria. In this way, the activities of the bacteria and puriﬁed LPS can be compared quantitatively.

The levels of the markers of initial pathway activation, C1rs-C1inh, C4bc, and C3bBbP, as well as that of TCC from the terminal pathway, were all increased from the baseline after stimulation with LPS\(^+\) or LPS\(^-\) \(N. meningitidis\) (Fig. 2). The activation of initial complement activation products was seen at concentrations of \(10^6\) meningococci/ml or higher, which correspond to approximately 5 ng of NmLPS or more/ml. In contrast, puriﬁed NmLPS did not induce complement activation at concentrations up to 1,000 ng/ml. The combination of puriﬁed NmLPS and LPS\(^-\) \(N. meningitidis\) induced complement activation to a similar extent as LPS\(^+\) or LPS\(^-\) \(N. meningitidis\) (not shown). These data indicate that complement activation by \(N. meningitidis\) in human whole blood occurs independently of the LPS outer membrane component.

Granulocyte CR3 upregulation and oxidative burst by LPS\(^+\) and LPS\(^-\) \(N. meningitidis\) or puriﬁed NmLPS. Recently, the CR3 upregulation and oxidative burst induced by \(N. meningitidis\) in granulocytes were shown to be complement dependent (29). Therefore, we investigated the role of LPS in these processes.

Both LPS\(^+\) and LPS\(^-\) \(N. meningitidis\) at concentrations of \(10^6\) ml induced marked granulocyte CR3 upregulation and oxidative burst. The addition of puriﬁed NmLPS to LPS-deﬁcient \(N. meningitidis\) did not inﬂuence the CR3 upregulation or oxidative burst (not shown). Notably, puriﬁed NmLPS did not induce signiﬁcant CR3 upregulation or oxidative burst at concentrations up to 1,000 ng/ml (Fig. 3). These results indicate that the upregulation of CR3 expression and the oxidative burst in whole blood granulocytes after stimulation with \(N. meningitidis\) are not dependent on NmLPS.

Cytokine and chemokine induction by LPS\(^+\) and LPS\(^-\) \(N. meningitidis\). Cytokine and chemokine induction in a human whole blood model of meningococcal sepsis was assessed after

![Fig. 2. Complement activation by LPS\(^+\) and LPS\(^-\) \(N. meningitidis\) and by puriﬁed NmLPS. The results are for the formation of C1rs-C1inh (classical pathway), C4bc (classical and lectin pathways), C3bBbP (alternative pathway), and TCC (final common pathway) after the stimulation of lepirudin-treated human whole blood for 1 h with LPS\(^+\) (diamonds) or LPS\(^-\) (squares) \(N. meningitidis\) or puriﬁed NmLPS (circles). The x axes are calibrated for the numbers of \(N. meningitidis\) cells as well as the amounts of NmLPS that are present in the bacteria, according to SDS-PAGE analysis. Medians and upper quartiles are presented from four separate experiments. *, \(P < 0.05\); **, \(P < 0.01\), by two-way ANOVA. Dashed lines indicate baseline complement activation.](image1)

![Fig. 3. Granulocyte CR3 upregulation and oxidative burst by LPS\(^+\) and LPS\(^-\) \(N. meningitidis\) and by puriﬁed NmLPS. The results are for CR3 (CD11b/CD18) upregulation and oxidative burst in human granulocytes after the stimulation of lepirudin-treated human whole blood for 10 min with LPS\(^+\) (diamonds) or LPS\(^-\) (squares) \(N. meningitidis\) or puriﬁed NmLPS (circles). The x axes are calibrated for the numbers of LPS\(^+\) \(N. meningitidis\) cells as well as the amounts of NmLPS that are present in the bacteria, according to SDS-PAGE analysis. One representative of three separately performed experiments is shown. Dashed lines indicate baseline CR3 expression or oxidative burst.](image2)
flament activation and the complement-dependent in the absence of LPS (Fig. 4), suggesting that these mediators can also be induced in *N. meningitidis*. The results are for the production of IL-1β, TNF-α, IL-8, and MIP-1α after the stimulation of lepirudin-treated human whole blood for 2 h with LPS⁺ (diamonds) or LPS⁻ (squares) *N. meningitidis*. Mean cytokine or chemokine production levels from three separate experiments are presented. *, *P < 0.05, by two-way ANOVA.

2 h of incubation with LPS⁺ or LPS⁻ *N. meningitidis* (Fig. 4). No cytokine or chemokine production was seen in this whole blood model in the absence of a stimulus. LPS⁺ *N. meningitidis* was a potent inducer of IL-1β, TNF-α, IL-8, and MIP-1α production, as it was able to induce cytokine and chemokine production at concentrations higher than 10⁴ meningococci/ml. IL-1β, TNF-α, IL-8, and MIP-1α production by LPS⁺ meningococci was significantly reduced compared to that by LPS⁻ *N. meningitidis* (*P < 0.05 for all cytokines and chemokines measured*). However, marked IL-8, and to a lesser extent, TNF-α and MIP-1α production was also induced by LPS⁻ *N. meningitidis*, suggesting that these mediators can also be induced in the absence of LPS (Fig. 4).

**DISCUSSION**

The principal finding of the present study was that complement activation and the complement-dependent inflammatory events of CR3 upregulation and oxidative burst are independent of LPS in a whole blood model of meningococcal sepsis. The construction of the LPS-deficient meningococcal mutant H44/76ΔpxA has made it possible to study the relative importance of the LPS moiety of a gram-negative bacterium in diverse inflammatory processes (32). We used LPS-deficient *N. meningitidis* to assess the involvement of meningococcal LPS in complement activation, complement-dependent inflammatory effects, and cytokine or chemokine production in a human whole blood model of meningococcal sepsis. The lack of reactivity of the LPS⁻ strain in a LAL assay confirmed the absence of LPS in the batch used for the experiments. The estimate that 10⁵ LPS⁺ meningococci contained approximately 5 ng of meningococcal LPS was in line with previous estimations (2, 30). *N. meningitidis* is exclusively a human commensal or pathogen, and none of the animal models available accurately simulate FMS. Therefore, we used an in vitro experimental system approaching, as closely as possible, the human in vivo situation (22, 29). The principle of this model is to keep all ambient inflammatory systems intact so that they can be activated and mutually interact but still to avoid coagulation. Because most anticoagulants, such as EDTA, citrate, and heparin, interact with critical steps in the inflammatory network, this model uses the highly specific thrombin inhibitor lepirudin, a recombinant hirudin analogue, as an anticoagulant. CR3 upregulation and oxidative burst were found to be induced to a similar extent by live or heat-inactivated bacteria in this model. Since these processes are highly complement dependent, this indicates that complement activation is also not affected by inactivation of the bacteria. Therefore, heat-inactivated bacteria were used because of practical considerations, as live meningococci constitute a significant health hazard (29).

LPS is present in solution in the form of micellar structures, which is quite distinct from its organization when it is present in the outer membrane. Hypothetically, the physicochemical presentation of LPS could affect the complement-activating ability, favoring membrane-bound LPS as the stronger complement activator. However, the observation that LPS-containing meningococci activate complement to a similar extent as LPS-deficient meningococci suggested that this is not the case, as did the absence of an increase in the complement-activating ability when isolated LPS was added back to the LPS-deficient strain. This is supported by a previous study in which neisserial outer membrane vesicles (OMVs) containing LPS were equipotent to OMVs deficient for LPS (1).

Complement activation by LPS⁺ or LPS⁻ *N. meningitidis* was seen at concentrations of 10⁶/ml or higher. This is a concentration of meningococci that is frequently found during FMS (3, 13); 10⁶ LPS⁺ *N. meningitidis* cells contain approximately 5 ng of LPS. In contrast, complement was not activated by purified LPS at concentrations up to 1,000 ng/ml. This suggests that during meningococcal sepsis, complement activation is not induced by LPS, but by other constituents of *N. meningitidis*.

Members of our laboratory previously reported that the up-regulation of CR3 and the induction of oxidative burst in granulocytes in this whole blood model are dependent on complement activation (22, 29). When complement is activated, C5a is formed, and this anaphylatoxin reacts with the C5a receptor on granulocytes and monocytes to induce the upregulation of CR3. CR3 is the principal receptor involved in the phagocytosis of C3b-coated bacteria, and when upregulated, it will enhance phagocytosis of the bacteria and in this way stimulate the oxidative burst process. Our results indicate that CR3 expression and oxidative burst are mediated by non-LPS components of *N. meningitidis*.

It is commonly accepted that LPS extracted from diverse gram-negative bacteria activates complement readily via classical and alternative pathway-dependent mechanisms (25, 38). In addition, LPS has been shown to be able to upregulate CR3 expression and to prime neutrophils for oxidative burst (8, 9, 19, 21). However, for the experiments presented in these stud-
ies, very high concentrations of LPS were applied (>5 to 10 μg/ml), incubation times of longer than 30 min were employed for granulocyte activation, or isolated cell cultures or plasma were used. In addition, it was recently shown that a contaminating substance of commercially available LPS is, at least in part, responsible for the upregulation of neutrophil responses (20). We claim that the conditions used in our study more closely resemble the in vivo situation than those used in previous studies. In our model, purified NmLPS was tested in whole blood at concentrations that are relevant for the situation encountered during FMS. The relatively short incubation times mimic the function of the complement cascade system, the components of which are present in plasma and able to be immediately activated by an invading pathogen, in this way avoiding the contribution of activation processes secondary to other inflammatory events.

We propose that the observed weak complement-activating property of NmLPS is not specific for this type of LPS, as it has a relatively short saccharide side chain, but that other types of LPS are also weak complement activators at relevant concentrations. Preliminary data from our laboratory suggest that this is the case for Escherichia coli LPS in the same in vitro model. In addition, in primate models, complement is not activated after E. coli LPS infusion, but after whole E. coli cell infusion, marked complement activation occurs within 15 min (15, 37). Also, in a human endotoxemia model, no complement activation is observed, whereas significant cytokine production, endothelial cell activation, and activation of the coagulatory and fibrinolytic systems are found (36a).

The bacterial components that are involved in the activation of complement, the upregulation of CR3 expression, and oxidative burst remain elusive and will be subjected to further investigation. It was previously reported that OMVs isolated from LPS-deficient Neisseria meningitidis induce substantial complement activation (1). This suggests that the non-LPS components that induce complement activation reside in the outer membrane. The lecin pathway seems to be an important player in complement activation by meningococci when they are grown on Kellogg’s medium and studied in the whole blood model (1, 29). Since mannose-binding lecin binding to the bacteria occurs via the outer membrane proteins porin and opacity protein (OpA) independently of LPS (9α), it is reasonable to suggest that the porins and OpA are responsible, at least in part, for the observed complement activation and subsequent CR3 upregulation and oxidative burst.

The proinflammatory cytokines TNF-α and IL-1β are primary cytokine mediators during meningococcal sepsis, whereas IL-8 and MIP-1α are important chemotactic proteins that are important for pathogenesis (14, 36, 39). We found that LPS−− N. meningitidis induced marked IL-8, and to a lesser extent, TNF-α and MIP-1α production in the whole blood model, indicating that bacterial components other than LPS are also proficient at inducing cytokine or chemokine production. However, LPS+ N. meningitidis was more potent in the induction of these proinflammatory mediators, in particular TNF-α and MIP-1α, indicating that LPS is an important factor involved in the regulation of these processes, which is in agreement with previous reports (27, 30, 34). It is tempting to speculate that the observed LPS-independent induction of IL-8, and possibly also TNF-α and MIP-1α, is mediated or potentiated by the activation of complement factor C5a, as has been suggested previously (7, 22, 26).

We conclude that complement activation occurs independently of the LPS moiety of N. meningitidis. Therefore, CR3 upregulation and oxidative burst, previously shown to be highly complement dependent, are also independent of LPS. Cytokine and chemokine production can be induced via LPS-dependent as well as LPS-independent mechanisms.

ACKNOWLEDGMENTS

We thank Anne Pharo, Gunnul Ulvund, Hilde Fure, Karin Bolstad, and Børre Nyland for their excellent technical assistance. Peter van der Ley and Liana Steeghs are thanked for supplying the LPS-deficient meningococcal strain.

Financial support was provided by The Research Council of Norway (International Scholarship Section), Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) grant number 920-03-176, The Norwegian Foundation for Health and Rehabilitation, The Norwegian Council on Cardiovascular Disease, 19. M. Infectious Diseases, and the Research Council of the Riks Hospiutalet University Hospital.

REFERENCES


15. Hangen, D. H., J. H. Stevens, P. S. Satoh, E. W. Hall, P. T. O’Hanley, and...


Editor: J. N. Weiser