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Meconium-induced release of cytokines is mediated by the TRL4/MD-2 complex in a CD14-dependent manner

Bodil Salvesen[a,b,*], Jørgen Stenvik[c], Carlo Rossetti[d], Ola D. Saugstad[b], Terje Espevik[c], Tom E. Mollnes[a]

[a] Institute of Immunology, University of Oslo and Rikshospitalet University Hospital, Oslo, Norway
[b] Department of Pediatric Research, Medical Faculty, University of Oslo and Rikshospitalet University Hospital, Oslo, Norway
[c] Institute of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway
[d] Department of Biotechnology and Molecular Sciences, University of Insubria, Via Dunant 3, 21100 Varese, Italy

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ABSTRACT

Objective: Meconium, the first intestinal discharge of the newborn, contains material accumulated during fetal life. Meconium activates complement and CD14 and may induce a systemic inflammatory response. Toll-like receptors are classical pattern-recognition receptors recognizing both exogenous and host-derived ligands. The cyanobacterial product CyP is a potent LPS antagonist binding to the TLR4/MD-2 complex. The aim of the present study was to investigate the role of the CD14/TLR4/MD-2 complex in meconium-induced inflammation.

Methods: Whole blood from six donors was preincubated with anti-CD14 or CyP. Meconium was added and the samples were incubated for 4 h. Twenty-seven inflammatory mediators were measured in a Bio-plex Array Reader. Human embryonic kidney cells transfected with plasmids containing NF-κB dependent luciferase reporter, human MD-2, TLR4, TLR2 and/or CD14, were incubated with meconium or LPS for 18 h. Luciferase activity in cytoplasmic extracts was measured using a Luciferase Assay System kit.

Results: Meconium induced formation of a broad panel of inflammatory mediators. CyP and anti-CD14 significantly ($p < 0.001$) inhibited meconium-induced formation of (a) proinflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ) by 60–80% and 72–94%, respectively, (b) anti-inflammatory cytokines (IL-10, IL-1Ra) by 58–59% and 50–65%, respectively, (c) chemokines (IL-8, MCP-1, MIP-1α, MIP-1β, eotaxin, IP-10) by 43–77% and 57–87%, respectively, and (d) growth factors (G-CSF, GM-CSF, basic FGF, PDGFbb) by 53–71% and 40–78%, respectively, with no statistical significant difference between Cyp and anti-CD14. The inflammatory response could only partly be explained by LPS, suggesting that endogenous components of meconium contribute to the inflammatory response. Meconium activated NF-κB dose-dependently in cells expressing TLR4/MD-2 together with CD14, while no effect was seen in cells expressing TLR4/MD-2 alone or in TLR2/CD14 transfected cells.

Conclusions: The results indicate that the CD14-dependent meconium-induced inflammatory reaction is mediated through the TLR4/MD2 complex. These data may have implications for future therapeutic strategies for meconium aspiration syndrome.

1. Introduction

Meconium, the first intestinal discharge, represents material accumulated during fetal life, like secretions from salivary and intestinal glands like mucus, bile and bile acids, fatty acids and steroids from the gut, cellular debris, lanugo hair, components from vernix caseosa, amniotic fluid, and blood (Antonowicz and Shwachman, 1979). Meconium aspiration syndrome (MAS), a serious disease in newborns including aspiration of meconium into the lungs, has a complex, not well-defined pathophysiology (Cleary and Wiswell, 1998; Dargaville and Copnell, 2006). The complement system and Toll-like receptors (TLR), including the CD14-associated TLR4/MD-2 complex, are two important components of the innate immune system, which both act upstream and partly indepen-

Abbreviations: ANOVA, analysis of variance; basic FGF, fibroblast growth factor; CI, confidence interval; CyP, cyanobacterial product; DAMPs, damage/danger-associated molecular patterns; GM-CSF, granulocyte macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; HEK cells, human embryonic kidney cells; IFN-γ, interferon-gamma; IgG, immunoglobulin G; IL, interleukin; IP-10, interferon-γ-inducible protein 10; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAS, meconium aspiration syndrome; MCP-1, monocyte chemotactic protein-1; MD-2, myeloid differentiation protein-2; MIP-1α, macrophage inhibitory protein-1α; NF-κB, nuclear factor kappa-light-chain enhancer of activated B-cells; PAMPs, pathogen-associated molecular patterns; PRR, pathogen recognition receptor; PBS, phosphated buffered saline; RANTES, regulated upon activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factor-alpha; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

* Corresponding author at: Department of Pediatric Research, Barneklinikken, Rikshospitalet University hospital, 0027 Oslo, Norway. Tel.: +47 23074507.
E-mail address: bodil.salvesen@rr-research.no (B. Salvesen).

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dently (Uthaisangsook et al., 2002). We have earlier shown that meconium is an important activator of complement (Castellheim et al., 2004; Mollnes et al., 2008) as well as of the CD14-mediated inflammatory reaction reflected by synthesis of a broad spectrum of cytokines, chemokines and growth factors in human whole blood and in cord blood (Salvesen et al., 2008). Inhibition of either CD14 or the complement system reduced the meconium-induced formation of most inflammatory mediators, but the combined inhibition of both systems had a remarkable effect indicating a synergistic effect of inhibition of both these two main branches of innate immunity.

The innate immune system recognizes highly conserved structures present on large numbers of microorganisms referred to as pathogen-associated molecular patterns (PAMPs). These exogenous ligands include bacterial lipopolysaccharide (LPS), peptidoglycan, lipotheichoic acids, mannan, zymosan, double stranded RNA, bacterial DNA and glucans (Janeway and Medzhitov, 2002). Host-derived immunostimulators, i.e. endogenous ligands, like heparan sulphate, hyaluronan, heat-shock proteins, surfactant proteins, uric acid, fibronectin, β-defensin and cardiolipin may also signal through the same receptors as PAMPs (Levy et al., 2006; Gay and Gangloff, 2007), and was originally termed damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004; Matzinger, 1994). Recent years DAMP has alternatively been used as abbreviation for "danger-associated molecular patterns", covering the general phenomenon of danger signaling of both exogenous and endogenous ligands.

Pattern-recognition receptors (PRRs) recognize structural patterns on exogenous as well as endogenous ligands (Medzhitov and Janeway, Jr., 2000). The classical PRRs are the Toll-like receptors (TLRs). TLR4, TLR1, 2, and 6 are found on the plasma membrane of immune cells and recognize lipoproteins and lipoglycans on the surface of microbes. All TLRs except TLR3 signal through the MyD88 pathway leading to activation of the NFκB gene-transcription program and formation of proinflammatory cytokines. TLR4-mediated LPS recognition depend on the protein MD-2, which forms a complex with the ectodomain of TLR4 (Shimazu et al., 1999). The TLR4/MD-2 complex engages with LPS on cell surfaces via LPS-binding protein (LBP) and CD14 (Kawai and Akira, 2009). In addition to being essential for TLR4 function, the CD14 molecule, but not MD-2, is also involved in the function of TLR2 (Muta and Takeshige, 2001; Nilsen et al., 2008) and TLR3 (Lee et al., 2006).

The cyanobacterial product (CyP), a LPS-like molecule derived from the cyanobacterium *Oscillatoria Planktotrix FP1,* is a potent and selective antagonist of bacterial LPS *in vitro* and *in vivo* by its competitive binding to the TLR4/MD-2-complex (Macagno et al., 2006).

The aim of the present study was to investigate the role of the TLR4/MD-2 complex in meconium-induced inflammation by comparing the effect of MD-2 inhibition by CyP with CD14 inhibition in human whole blood and to investigate the involvement of CD14, MD-2, TLR4 and TLR2 in human transfected cell lines. The results unequivocally indicate that the CD14-mediated meconium-induced inflammatory reaction is mediated through the TLR4/MD-2 complex.

2. Methods

2.1. Reagents and equipment

All materials and solutions were endotoxin-free according to the manufacturers. Sterile phosphate-buffered saline (PBS) was Dulbecco (Sigma–Aldrich, Oslo, Norway). Propylene tubes were NUNC cryotubes (Nalgene NUNC, Roskilde, Denmark). Lepirudin (Refludan®) was from Hoechst (Frankfurt am Main, Germany).

2.2. Antibodies and other reagents

Mouse anti-human CD14 (clone18D11) was purchased from Diatec (Oslo, Norway).

2.3. CyP

CyP was purified from the freshwater cyanobacterium *Oscillatoria planktothrix* FP1 by a phenol–guanidium thiocyanate-based method as described previously (Macagno et al., 2006).

2.4. Preparation of meconium and the endotoxin assay

Meconium was collected and processed as previously described (Salvesen et al., 2008). No bacteria were detected after cultivation. The endotoxin or LPS content in meconium was analyzed in a Pyrochrome® *Limulus* Amoebocyte Lysate assay by an endpoint chromogenic method using a diazo-coupling assay kit (Associates of CAPE COD, Inc., Falmouth, MA). Meconium samples were diluted in depyrogenated Pyrotube-D® tubes with LAL Reagent water (both Associates of CAPE COD, Inc., Liverpool, UK). The diluted samples were heated to 75 °C for 10 min, mixed with pyrochrome solved in Glucashield®, a β-glucan inhibiting buffer, and incubated in a 96-well Pyroplate® (both Associates of CAPE COD, Inc., Falmouth, MA) on a dry block incubator. The assay was performed according to instructions from the manufacturer. The LPS content in the meconium was found to be 30 EU/mg meconium, corresponding to 3 ng/mg meconium.

2.5. Experimental set-up

2.5.1. Titration of CyP

To investigate the optimal concentration of CyP needed in the following experiments, human whole blood anticoagulated with lepirudin at 50 μg/mL (Mollnes et al., 2002), was collected from two adult healthy donors and distributed into tubes containing PBS and CyP, the latter diluted 2-fold from 20 to 0.156 μg/mL whole blood. The samples were preincubated for 5 min before adding either PBS or a high dose of meconium (1 mg/mL) (Salvesen et al., 2008). Baseline sample (T0), containing only blood and PBS was processed immediately while the other samples were incubated for 4 h at 37 °C. Negative control samples after 4 h (T4) contained blood and PBS. At the end of the incubation ethylenediaminetetraacetic acid (EDTA) was added to all samples to a final concentration of 20 mM to avoid further complement activation. Then the tubes were centrifuged for 15 min at 1400 × *g* (4 °C). The plasma samples were stored at −70 °C until assayed as described below. Based on these results a CyP concentration of 20 μg/mL was chosen for the subsequent meconium experiments.

2.5.2. Titration of meconium

The effect of CyP on cytokine release over a range of meconium concentration was then investigated. Human whole blood from two adult healthy donors was collected and handled as describe above. The samples were preincubated for 5 min with PBS or CyP 20 μg/mL before adding meconium in increasing concentrations (0.003, 0.01, 0.03, 0.1, 0.3 and 1 mg/mL whole blood). Baseline sample (T0), containing only blood and PBS was processed immediately while the other samples were incubated for 4 h at 37 °C. Negative control samples (T4) contained blood and PBS. At the end of the incubation ethylenediaminetetraacetic acid (EDTA) was added to all samples to a final concentration of 20 mM to avoid further complement activation. Then the tubes were centrifuged for 15 min at 1400 × *g* (4 °C). The plasma samples were stored at −70 °C until assayed as described below. Based on these results two different meconium