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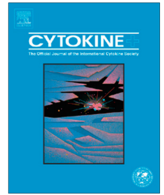

The effect of Indomethacin and Betamethasone on the cytokine response of human neonatal mononuclear cells to gram-positive bacteria

Wolfgang Ernst [a,*], Evelyn Kusi [a], Sara Fill Malfertheiner [a], Edith Reuschel [a], Ludwig Deml [b,c], Birgit Seelbach-Göbel [a]

[a] *Clinic of Gynecology and Obstetrics St. Hedwig, University of Regensburg, Regensburg, Germany*
[b] *Institute of Medical Microbiology, University of Regensburg, Regensburg, Germany*
[c] *Lophius Biosciences GmbH, Josef-Engert Straße 13, 93053 Regensburg, Germany*

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ABSTRACT

Intrauterine infections with gram-positive bacteria pose a serious threat to neonates since they can result in neonatal sepsis, induce a fetal inflammatory response and also cause preterm birth. Despite intensive care, prematurity remains a leading cause of neonatal death, and is often accompanied by a number of morbidities. In order to prevent premature birth, tocolytic agents like Indomethacin are administered. Betamethasone is used to promote lung maturation and prevent respiratory distress syndrome. A combination of both drugs is assumed to prevent premature delivery while simultaneously facilitating lung maturation. This study investigates the effect of Betamethasone, Indomethacin and a combination of both on the cytokine production of neonatal cord blood mononuclear cells (CBMC) after stimulation with lysates of the gram-positive pathogens *Streptococcus agalactiae* and *Enterococcus faecalis*. The aim of the study is to determine the impact of these drugs on the function of the neonatal immune system which should aid clinicians in choosing the optimal therapy in case of preterm birth associated with intrauterine infection. Betamethasone reduced the production of the pro-inflammatory cytokines IL-6, IL-12p40, MIP-1α and TNF and increased the expression of the anti-inflammatory cytokine IL-10, depending on the pathogen used for stimulation. In contrast to Betamethasone, Indomethacin almost exclusively increased IL-10 production. The combination of both drugs decreased the expression of IL-6, IL-12p40, MIP-1α and TNF while increasing IL-10 production, depending on the concentration of Indomethacin and the pathogen used for stimulation. Based on our results, the combination therapy with Indomethacin and Betamethasone has a similar effect on cytokine production as Betamethasone alone, which is generally administered in case of impending preterm birth. However, the combination therapy has the advantage of promoting lung maturation while simultaneously blocking preterm labor effectively.

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1. Introduction

Neonatal sepsis is a very prominent threat with a high mortality rate. The incidence of culture-proven sepsis is approximately 2 per 1000 live births. However, autopsy studies have shown that in more than 60% of neonates, sepsis has not been diagnosed prior to death due to falsely negative blood cultures. Therefore other criteria besides blood cultures are utilized to diagnose neonatal sepsis, leading to an incidence of 2.2–8.6 per live births in developed countries [1,2].

Based on the time of presentation, neonatal sepsis is divided into early onset sepsis (EOS), which occurs within the first 72 h after birth and late onset sepsis (LOS) which presents after 72 h [3]. Not only do EOS and LOS differ in the time of onset, also transmission route, number of incidences, mortality and pathogens vary. EOS is usually transmitted vertically from mother to child via an ascending amnionic infection, and is typically caused by bacteria which are part of the maternal genital flora. The transmission of LOS typically occurs by nosocomial infection or horizontal transfer [2].

Despite intrapartum antibiotic prophylaxis, the gram positive *Streptococcus agalactiae* (group B Streptococcus (GBS)) still remains to be the most frequent bacteria inducing EOS in term infants, and

Abbreviations: EOS, early onset sepsis; LOS, late onset sepsis; GBS, group B Streptococcus, *Streptococcus agalactiae*; EF, *Enterococcus faecalis*; CBMC, cord blood mononuclear cells.

* Corresponding author. Tel.: +49 (0)941 944 8912; fax: +49 (0)941 369 5215.
E-mail address: Wolfgang.Ernst@ukr.de (W. Ernst).

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invasive GBS infection continues to be the leading cause of sepsis, pneumonia and meningitis in neonates [4,5]. Enterococci are the most frequent gram-positive bacteria in vaginal swab cultures in our clinic, and while Enterococci are not yet responsible for a large number of neonatal sepsis cases, there are studies reporting on an increase in incidences of enterococcal bacteremias, with *Enterococcus faecalis* (EF) being the most prominent strain [2,6,7].

Intrauterine infection can not only cause neonatal sepsis, but is also able to induce preterm labor followed by spontaneous preterm birth [8,9] and the so called fetal inflammatory response syndrome (FIRS). Although sepsis poses a considerable threat to newborn infants, prematurity due to preterm birth is a leading cause of neonatal death. And even if the preterm delivery itself is not fatal, infants often suffer from short-term (e.g. respiratory distress, intraventricular hemorrhage), or long-term morbidities (e.g. cerebral palsy, mental retardation) [10] due to sequelae from fetal inflammation. In order to reduce mortality together with other morbidities, drugs that delay or prevent preterm labor are used in the clinic. A very potent member is the non-steroidal anti-inflammatory drug Indomethacin, which inhibits prostaglandin synthesis and thereby delays delivery [11].

A serious and frequent complication of preterm birth and also a prominent cause of neonatal death is the respiratory distress syndrome. Preterm infants suffer from respiratory distress and failure due to lack of surfactants and incomplete lung development. In order to induce surfactant production, and hence prevent respiratory distress syndrome, and also reduce intraventricular hemorrhage, corticosteroids, especially Betamethasone are administered. They are given to the mother twice, 24 h apart, prior to preterm birth [12]. Both Betamethasone and Indomethacin readily cross the placenta and can therefore affect fetal leukocytes.

The present study investigates the effect of the drugs Indomethacin, Betamethasone or a combination of both on the cytokine production of the fetal and neonatal immune system. This should give new insights, how these therapies influence the neonatal production of important immunomodulatory cytokines and therefore affect the immune response against the gram-positive bacteria GBS and EF. The goal of this study is to help clinicians choose the best suited therapy for imminent preterm birth associated with intrauterine infection.

2. Material and methods

2.1. Subjects

Umbilical cord blood samples were taken from ten healthy term newborns which were delivered spontaneously, and received no medication prior to delivery. The blood was drawn from the umbilical cord immediately after birth, and processed within 12 h. Participants were recruited between March 2010 and May 2010. The study was conducted in accordance with the World Medical Association Declaration of Helsinki and approved by the ethics committee of the University of Regensburg (file reference 06/098, Amendment 09/26/2008).

2.2. Purification of cord blood mononuclear cells

Cord blood was diluted 1:2 with phosphate buffered saline (Lonza, Verviers, Belgium), and subsequently layered on Pancoll (Pan Biotech, Aidenbach, Germany) to separate cord blood mononuclear cells (CBMC) by density gradient centrifugation (600 rcf; 30 min; 20 °C). The buffy coat, containing the CBMC was collected and washed with PBS. To remove contaminating erythroblasts, erylysis was performed using BD Pharm Lyse (BD, Franklin Lakes, USA) according to manufacturer's manual. Purified CBMC were resuspended in RPMI 1640 medium with L-Glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat-inactivated human AB serum.

2.3. In vitro stimulation of CBMC

For the in vitro stimulation, 2.6×10^5 cells (1×10^6 cells/ml) were preincubated with either 80 ng/ml Betamethasone (Celestan; essex pharma GmbH, Munich, Germany), different concentrations (0.3; 1; 3 μg/ml) of Indomethacin (Confortid; Alpharma, Copenhagen, Denmark) or a combination of both in RPMI 1640 medium with L-Glutamine supplemented with 10% human AB serum containing ampicillin. After 1 h, cells were stimulated by adding 0.1 μg lysate of either *S. agalactiae* or *E. faecalis*. Cells without stimulation and cells without preincubation with drugs served as negative controls. The culture conditions were 37 °C and a humidified atmosphere containing 5% CO_2. After 24 h, cultured cells were centrifuged (300 rcf; 10 min; 4 °C) and supernatants were stored at −80 °C for cytokine measurement. The stimulation of CBMC of each cord blood sample was performed in quadruplicates.

2.4. Cytokine detection

To determine the concentration of the pro-inflammatory cytokines TNF, IL-1β, IL-6, IL-12p40 the chemokines IL-8, MIP-1α and MIP-1β and the anti-inflammatory cytokine IL-10 produced by the CBMC, the supernatants were analyzed using multiplex bead-based Luminex assays (Invitrogen Corporation, Carlsbad, USA) in combination with the Luminex System (Luminex corporation, Austin, USA). The analysis was performed by the Multimetrix GmbH (Regensburg, Germany).

2.5. Statistical analysis

The statistical analysis was conducted using Graph Pad Prism 5 (Graph Pad software, La Jolla, USA). *P*-values were calculated using a paired student's *t*-test. Differences were considered to be statistically significant when the value of p was below 0.05. Statistical differences were denoted as follows: $^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$. In the results the mean and the standard error of the mean (±SEM) of individual cytokines, without (lysate alone) and with treatment (lysate in combination with drug/s), was depicted in brackets.

3. Results

Treatment with Betamethasone (80 ng/ml) reduced the concentrations of the cytokines MIP-1α (EF: 4928 pg/ml ± 672 to 4270 pg/ml ± 644 and GBS: 6283 pg/ml ± 633 to 4743 pg/ml ± 471) and TNF (EF: 1052 pg/ml ± 146 to 852 pg/ml ± 130 and GBS: 814 pg/ml ± 79 to 633 pg/ml ± 67) in the cell culture supernatant of cord blood mononuclear cells (CBMC) stimulated 24 h with both GBS and EF (Fig. 1). However, differences between the two pathogens used for stimulation could also be detected. Betamethasone treatment resulted in increased levels of IL-10 (76 pg/ml ± 10 to 92 pg/ml ± 11) in the supernatant, when CBMC were incubated with EF. When GBS was used as a stimulating agent, Betamethasone reduced the concentration of IL-6 (3356 pg/ml ± 696 to 2686 pg/ml ± 498) and IL-12p40 (1075 pg/ml ± 107 to 808 pg/ml ± 106) in the cell culture supernatant. Incubation with Betamethasone did not affect the levels of IL-1β, MIP-1β, MCP and IL-8 (data not shown).

Similar to the Betamethasone, treatment with 0.3 μg/ml Indomethacin had a diverging effect on cytokine levels produced

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