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Antenatal Betamethasone Changes Cord Blood Monocyte Responses to Endotoxin in Preterm Lambs

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ABSTRACT

Corticosteroids are routinely administered to women at risk for preterm delivery to induce fetal lung maturation. Antenatal corticosteroids have immunomodulatory effects on fetal immune cells that are poorly understood. We hypothesized that maternal betamethasone would alter in fetal monocytes both the initiation of inflammation in response to pro-inflammatory stimuli and the resolution of inflammation by phagocytosis of apoptotic neutrophils. Preterm lambs at 124 d gestation were delivered 15 h, 1 d, 2 d, or 7 d after 0.5 mg/kg maternal intramuscular betamethasone. Monocytes from cord blood were isolated and cultured and results were compared with monocytes from preterm lambs exposed to maternal saline or monocytes from adult sheep. Phagocytosis of *Escherichia coli* was not changed, however, phagocytosis of apoptotic neutrophils was low in fetal monocytes but increased after 7 d exposure to maternal betamethasone to the level found in adult monocytes. Hydrogen peroxide production after endotoxin stimulus was significantly reduced to 7.1 ± 2.2 μmol at 5 h, 8.7 ± 2.9 μmol at 24 h, and 4.1 ± 1.9 μmol at 48 h *versus* 16.4 ± 3.6 μmol in control animals; at 7 d, the hydrogen peroxide production increased to 74.3 ± 19.7 μmol ($p < 0.05$, per 10^6 monocytes). IL-6 production was reduced at 15 h after maternal betamethasone but at no other time point. Maternal betamethasone initially suppressed several fetal monocyte functions, however, at 7 d, measurements of initiation and resolution of inflammation were increased to levels similar to monocytes from adult sheep. The time-dependent changes in maternal betamethasone modulation of the responses of fetal monocytes may influence immune function of the preterm lamb after delivery. **(*Pediatr Res* 55: 764–768, 2004)**

Abbreviations

BPD, bronchopulmonary dysplasia
GA, gestational age

Corticosteroids are potent modulators of fetal development, and antenatal corticosteroids are routinely used to decrease morbidity and mortality after preterm birth by inducing lung maturation (1). However, corticosteroids also are potent acute suppressors of immune/inflammatory responses (2). Fetal plasma corticosteroid levels are about 30% of the maternal levels after antenatal treatment with betamethasone (3). Fetal plasma corticosteroid concentrations decline rapidly within 4 h after maternal treatment with betamethasone (4). The effects of corticosteroids and their metabolites on fetal immune cells *in vivo* are poorly understood. *In vitro* exposure of fetal immune cells to corticosteroids acutely alters functions such as cell proliferation, apoptosis, phagocytosis, production of colony-stimulating factors, and anti-inflammatory cytokine production (5–9). We have previously used cord blood–derived monocytes to study agents *in vitro* that are frequently used in the treatment of pulmonary inflammation in preterm infants (10). Similar suppressive effects on fetal immune cell function also occur after maternal corticosteroid treatments *in vivo*, and examples are decreased granulocyte chemokines and decreased lymphocyte numbers (11, 12). In contrast, Kavelaars *et al.* (11), also found enhanced natural killer cell activity in cord blood after antenatal corticosteroid exposure. The few evaluations of immune cell responses after maternal corticosteroid treatments did not control for the time interval from corticosteroid exposure to delivery. Because corticosteroids cause both immunosuppression and maturation, we hypothesized that the responses of fetal cord monocytes would change with time after maternal corticosteroid treatment. An initial suppression might be followed by a functional change that could represent reprogramming that might alter subsequent immune responses.

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Therefore, we exposed fetal sheep to a maternal corticosteroid treatment for time intervals from 15 h to 7 d. We evaluated fetal blood monocytes for phagocytosis of *Escherichia coli*, responses to endotoxin exposure by hydrogen peroxide production, and IL-6 production as the marker for fetal inflammatory response syndrome (13). The resolution of inflammation was tested by phagocytosis of apoptotic neutrophils.

METHODS

Animals. The animal component of the study was performed in Western Australia, as approved by the animal care and use committees of Cincinnati Children's Hospital Medical Center (Cincinnati, OH, U.S.A.) and the Western Australian Department of Agriculture. Lung development in sheep at 124 d GA is in the saccular phase corresponding to human fetuses at 26–29 wk of gestation (14). A single dose of 0.5 mg/kg betamethasone induces lung maturation in preterm sheep (15). Injections were given at different gestational ages to deliver all animals at 124 d GA. Date-bred Merino ewes with singleton fetuses were randomly assigned in groups of five to nine animals. A single dose of maternal betamethasone (0.5 mg/kg intramuscularly) (Celestone Chronodose, Schering-Plough Pty. Ltd., Baulkham Hills, NSW, Australia) was given 15 h, 1 d, 2 d, or 7 d before preterm delivery at 124 d GA. Control animals were concurrently given 0.9% saline injections at 15 h, 1 d, 2 d, or 7 d before delivery at 124 d GA.

Cell isolation and culture. Cord blood was collected from an umbilical artery from each fetus at the time of cesarean section. Jugular venous blood from healthy adult ewes also was collected ($n = 10$). Complete white blood counts were performed on umbilical cord blood samples. Fetal plasma cortisol concentrations were determined with a commercial RIA (Valeant Pharmaceuticals International, Costa Mesa, CA, U.S.A.). The blood was diluted with PBS and layered onto a Percoll gradient (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.) (16). After centrifugation for 40 min at 400 $\times$ *g* at 20°C, the mononuclear cells and granulocytes were recovered, washed twice with PBS at 4°C, centrifuged for 5 min at 400 $\times$ *g* at 4°C, and resuspended in culture media (Dulbecco's modified minimal media) supplemented with 10% heat-inactivated FCS (Sigma Chemical, St. Louis, MO, U.S.A.). Cells were counted using trypan blue to evaluate viability and then plated in culture dishes. After incubation at 37°C for 2 h, nonadherent cells were removed and plates were washed twice with PBS. To estimate the number of monocytes, cells were scraped from the culture dishes, and differential cell counts were performed on cytospin preparations stained with DiffQuick (Baxter Healthcare Corp., McGraw Park, IL, U.S.A.). The adherent cell population was 90 $\pm$ 3% monocytes for all treatment and control groups. Monocytes in other culture dishes were cultured overnight, and the experiments were done the next day after about 16 h in culture.

***Phagocytosis of* E. coli.** After overnight incubation, monocytes were washed with PBS and new culture media was added. Monocytes were exposed to FITC-labeled *E. coli* (Sigma Chemical) for 6 h. The monocytes were resuspended, washed three times with PBS, and divided into two equal aliquots. The total bound and ingested bacteria were measured by flow cytometry. Trypan blue (1.25 mg/mL) was added to one aliquot to measure ingested bacteria only. Trypan blue quenches fluorescence of FITC-labeled *E. coli* that are surface bound but not ingested (17).

Hydrogen peroxide production and IL-6 concentration. After overnight incubation, monocytes were washed with PBS and new culture media was added. After 6 h incubation with endotoxin (100 ng/mL, *E. coli*, serotype 055:B5, Sigma Chemical) [a dose that induces a robust response in preterm monocytes (10)], monocytes were scraped from the culture dish, counted, and assessed for viability with trypan blue. Production of hydrogen peroxide by 1×10^6 monocytes was measured with an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H_2O_2-560 assay, OXIS International, Portland, OR, U.S.A.). Control samples were exposed to saline instead of endotoxin and were included in all experiments (10). Concentrations of IL-6 were determined in the supernatant of endotoxin-stimulated monocytes by an ovine-specific ELISA as previously described (18).

Induction of apoptosis and phagocytosis of apoptotic neutrophils. Neutrophils were isolated from adult animals. Contaminating erythrocytes were removed by osmotic lysis (16). Apoptosis in neutrophils was induced by 10 min of UV radiation followed by 3 h of culture (19). Apoptosis and necrosis were quantified by flow cytometry. Apoptotic cells were stained with fluorescent-labeled annexin V. Necrotic cells were stained with proprium iodide and the relative cell populations counted by flow cytometry (20). After overnight incubation, monocytes were washed with PBS and new culture media was added. Phagocytosis of apoptotic neutrophils was tested by adding 2×10^6/mL apoptotic neutrophils to the adherent monocytes in culture dishes (19). The monocytes and apoptotic neutrophils were incubated for 6 h at 37°C and washed with ice-cold PBS containing 0.5 mM EDTA. Cells in the culture dishes were fixed and stained with hematoxylin and eosin. Phagocytosis was evaluated by counting apoptotic vesicles in 300 monocytes per well (10).

Data analysis. Results are presented as mean $\pm$ SEM. Comparisons between betamethasone-treated groups and untreated controls were by ANOVA with Student-Newman-Keuls tests used for posthoc analyses. Results also were compared with monocytes from healthy sheep. Statistical significance was accepted at $p < 0.05$.

RESULTS

Monocyte counts in complete white blood counts and plasma cortisol concentrations. Maternal betamethasone did not change the number of monocytes in umbilical cord blood in preterm lambs (Table 1). The fetal plasma cortisol concentrations were not different after maternal treatment with betamethasone (Table 1).

Phagocytosis. Although phagocytosis of *E. coli* by blood monocytes from adult sheep tended to be higher than for fetal sheep at 124 d gestation, the difference was not significant (Fig. 1*A*). Maternal betamethasone treatments had no effect on the

Table 1. *Number of monocytes in cord blood and cortisol concentration in cord plasma*

		Time after maternal betamethasone treatment			
	Control	15 h	1 d	2 d	7 d
Monocytes (per μL)	60 ± 15	35 ± 25	95 ± 35	120 ± 40	70 ± 20
Cortisol (μg/dL)	0.5 ± 0.2	0.7 ± 0.3	0.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.2

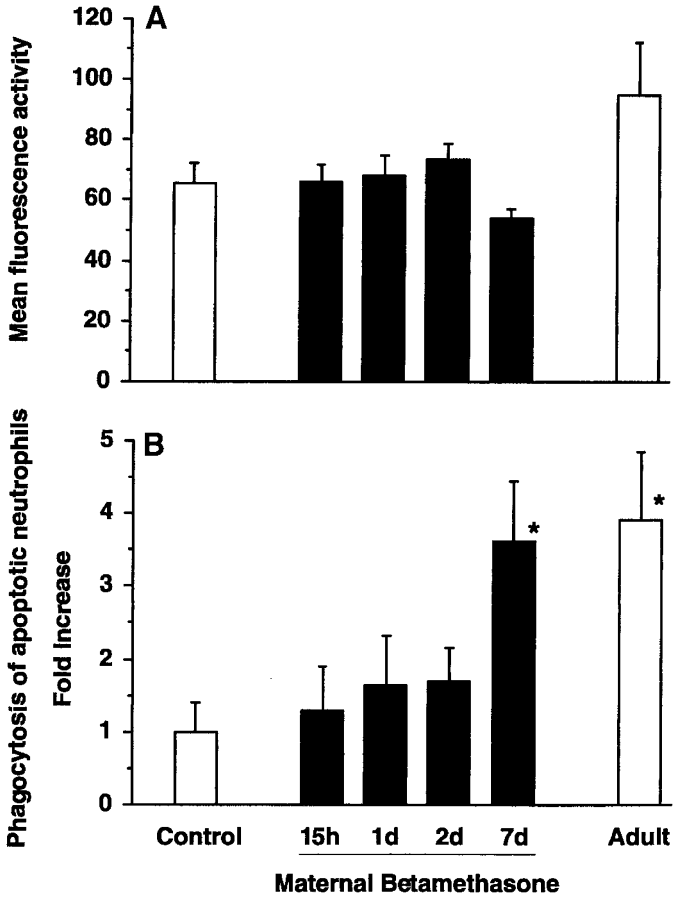

Figure 1. (*A*) Phagocytosis of fluorescent-labeled bacteria was not different between monocytes from preterm lambs (n = 9) and monocytes from adult sheep (n = 10). Phagocytosis was not changed by exposure to maternal betamethasone. The number of measurements were at 15 h n = 5, at 1 d n = 6, at 2 d n = 6, and 7 d n = 6. (*B*) Phagocytosis of apoptotic neutrophils was quantified by counting apoptotic vesicles in monocytes. Results are shown as fold increase over control animals, which were set to 1. The low phagocytosis in monocytes from preterm lambs increased 7 d after maternal betamethasone to a value similar to the phagocytosis of monocytes from adult sheep ($p < 0.05$ *vs* control).

phagocytosis of *E. coli* by monocytes from the preterm lamb. However, phagocytosis of apoptotic neutrophils was increased in blood monocytes from adult sheep relative to the preterm lamb (Fig. 1*B*). The maternal betamethasone treatment did not change apoptotic neutrophil phagocytosis at 15 h, 1 d, or 2 d. However, phagocytosis increased 3-fold to be similar to the value in the adult monocytes 7 d after maternal betamethasone treatment.

Endotoxin-stimulated H_2O_2 and IL-6 production. Monocyte activation was assessed after exposure of monocytes in culture to endotoxin. Blood monocytes from adult sheep produced about three times more H_2O_2 in response to endotoxin than did the monocytes from the preterm lambs (Fig. 2).

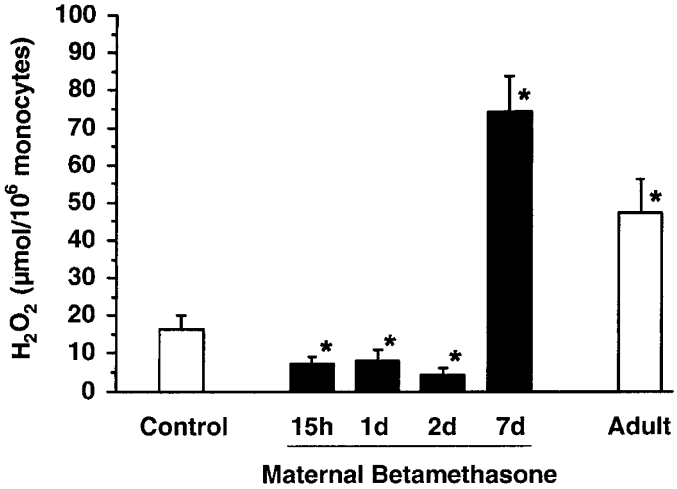

Figure 2. Production of hydrogen peroxide after stimulation with 100 ng/mL endotoxin. Production of hydrogen peroxide was lower in monocytes from preterm animals than in monocytes from adult sheep. Exposure to maternal betamethasone decreased production of hydrogen peroxide at 15 h, 1 d, and 2d. At 7 d after maternal betamethasone exposure, hydrogen peroxide production was increased in monocytes from preterm animals and not different from monocytes from adult sheep ($p < 0.05$ *vs* control).

Maternal betamethasone treatment suppressed H_2O_2 production at 15 h, 1 d, and 2 d. However, 7 d after the maternal treatment, the H_2O_2 production was similar to that of adult monocytes. IL-6 secretion by the cultured monocytes was about 2.5 times higher for adult monocytes than for preterm monocytes (Fig. 3). Maternal betamethasone treatment initially

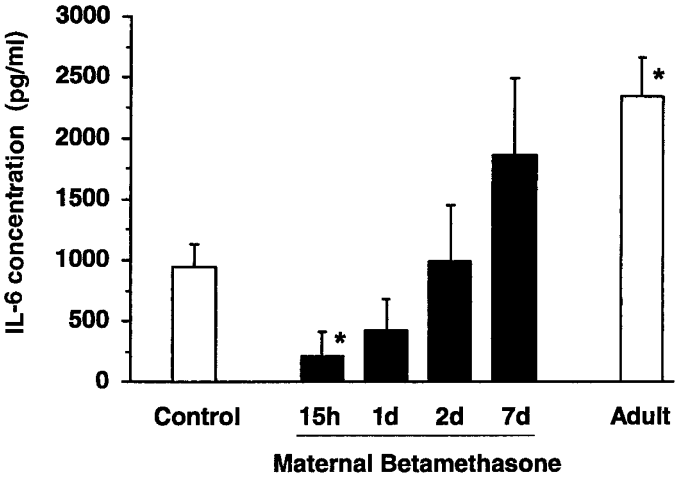

Figure 3. Concentrations of IL-6 in culture media after stimulation with endotoxin. Concentrations were determined by ELISA. IL-6 concentrations were lower in monocytes from preterm sheep than in monocytes from adult sheep. Exposure to maternal betamethasone reduced IL-6 concentrations after 15 h ($p < 0.05$ *vs* control).

suppressed IL-6 after a 15 h exposure, and IL-6 was qualitatively increased to a value similar to the adult cell response at 7 d.

DISCUSSION

We used a sheep model that allowed us to evaluate monocyte responses to antenatal corticosteroid exposures that are similar in dose and duration to the clinical use of maternal corticosteroid treatments. We and others have demonstrated that monocytes and macrophages from preterm subjects have multiple differences in responses from the adult blood monocytes (10, 21–25). The effects of antenatal corticosteroids on human monocytes have not been evaluated in detail, although corticosteroids can acutely modulate inflammatory responses when cord blood monocytes are exposed to corticosteroids in culture (7, 26, 27). As would be anticipated from anti-inflammatory effects, antenatal betamethasone suppressed H_2O_2 production for 2 d and IL-6 production at 15 h. The interesting responses were the augmented phagocytosis of apoptotic neutrophils, the increased H_2O_2, and the trend to increase IL-6 secretion by the monocytes from the 7 d corticosteroid group. These monocytes have responses that are similar to monocytes from the adult animal. With the limited information that we have, it is not clear what these changes in responses are. It is too simplistic to say that the monocytes were initially suppressed and then matured by the corticosteroid because we do not know the fate or persistence of the changed responses. However, at least for an interval of 7 d, a single corticosteroid exposure reprogrammed the responses of the fetal monocytes.

This finding adds to other effects of maternal corticosteroids on fetal immune cells. Several deficits in immune function have been reported after maternal corticosteroid treatment, such as decreased neutrophil chemotaxis (28) and decreased absolute numbers and proliferation of fetal lymphocytes with a reduced production of IL-2 (12, 29). This effect was not limited to the fetus but affected maternal lymphocytes as well (29). However, maternal corticosteroid effects are not exclusively immunosuppressive inasmuch as maternal treatment increased the activity of natural killer cells in preterm infants (11).

The effects of altered monocyte function in the fetus will depend on how other components of the immune/inflammatory system are altered by the antenatal corticosteroid exposure. There are several target organs for adverse immune/inflammatory effects in the preterm infant. The brain may be injured as a consequence of a systemic inflammatory response (30). BPD is associated with chorioamnionitis and fetal infection and antenatal corticosteroids suppress alveolar septation in the developing lung (31). Assuming that blood monocytes populate the fetal lung and differentiate to become alveolar macrophages (10, 32, 33), altering monocyte function to be more pro-inflammatory by increasing the capacity for H_2O_2 and IL-6 production could be detrimental. In a previous study, we used cord blood–derived monocytes to study agents *in vitro* that are used in the treatment of pulmonary disease in preterm infants (10). The phagocytosis of apoptotic cells by monocytes was increased in the presence of both surfactant proteins and phospholipids. As an example of interactive phenomena resulting in the potential for increased lung injury, we reported that fetuses exposed to intra-amniotic endotoxin 30 d before preterm delivery had increased numbers of monocytes in alveolar washes and more pro-inflammatory cytokine mRNA after mechanical ventilation than did control lambs (34). This experiment demonstrates that a fetal exposure can change a postnatal immune/inflammatory response. Antenatal corticosteroids decrease respiratory distress syndrome but do not decrease BPD (1). In the meta-analysis of Crowley (1), the relative risk of BPD after antenatal steroids was 1.38 (95% confidence interval, 0.90–2.11) which was a trend toward increasing the risk of BPD. This unanticipated outcome has been explained by survival of the smaller and more marginal infants at highest risk for BPD. Banks *et al.* observed severe respiratory failure in some preterm infants after repetitive courses of antenatal corticosteroids (35). Perhaps antenatal corticosteroids can augment an inflammatory response to a second stimulus after birth and contribute to the development of BPD in some infants.

This concept of interacting signals amplifying a fetal response also has been demonstrated by the simultaneous exposure of fetal sheep to maternal corticosteroids and intra-amniotic endotoxin. Intra-amniotic endotoxin is a model of the chronic chorioamnionitis that is frequently associated with preterm birth in humans (36). Intra-amniotic endotoxin causes chorioamnionitis and lung inflammation in fetal sheep (15, 18, 37). Maternal corticosteroids suppress the acute inflammation for about 3 d after the intra-amniotic endotoxin, but there is a late inflammatory response that persists for 5–15 d (38). The late inflammation that follows the initial corticosteroid-induced suppression of inflammation may result in part from priming of the immature inflammatory response by corticosteroids.

We found that maternal corticosteroid had a biphasic immunomodulatory effect on blood monocytes in the fetal sheep—suppression of function followed by augmented function. We speculate that this augmented function represents reprogramming that could have long-term effects on subsequent immune responses in fetal and later life.

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