

Stimulated cytokine production correlates in umbilical arterial and venous blood at delivery

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ABSTRACT. *Background.* Umbilical venous blood is easy to obtain after delivery, and thus has been commonly used in many studies for cytokine analysis. Our aim was to evaluate whether or not induced cytokine production differs after stimulation in umbilical artery and vein whole blood samples, using two different stimulation protocols. The effect of such stimulation on fetal and maternal blood was also evaluated. *Methods.* – Blood samples from umbilical artery (UA) and vein (UV), and from the mother were collected from 23 women after delivery at term. Concentrations of cytokines (IL-4, IFN- γ , IL-6 and TNF- α) were measured in plasma and whole blood after PMA/ConA and PMA/ionomycin stimulation. *Results.* Both in maternal and in fetal samples, cytokine concentrations in unstimulated plasma samples were lower than in stimulated samples, except for IL-4 after PMA/ConA stimulation. UA and UV showed similar, average cytokine levels after stimulation and the correlations were high ($r=0.68-0.95$). Cytokine concentrations were clearly higher in umbilical blood than in maternal blood after stimulation, but not in plasma. Correlations between maternal and umbilical samples after stimulation were generally low ($r<0.41$). IFN- γ was not detectable in unstimulated plasma samples. The production of IL-4 and IFN- γ was more intense after PMA/ionomycin stimulation than after PMA/ConA stimulation. *Interpretation of the results.* – Concentrations of the cytokines examined are similar in blood from the UA and UV. For IL-4 and IFN- γ , the stimulant used has a significant effect on the level of cytokine expression, and interestingly, the effect is more pronounced on the fetal than on the maternal side.

Keywords: birth, blood, cytokine, umbilical cord

INTRODUCTION

Umbilical venous blood is simple to obtain after delivery, and thus has been commonly used for various cytokine analyses [1-12]. Umbilical arterial blood, however, is more rarely used for cytokine analyses [13-16], possibly owing to the contractility of the arteries soon after delivery, leading to less easily available blood. In addition, various maternal, fetal and placental factors may influence the concentrations of the different cytokines. To our knowledge, there have been no comparative studies in which cytokine levels or production (other than those of IL-6) have been studied in the umbilical artery (UA) versus the umbilical vein (UV) [14, 15, 17]. Thus, the effect of sampling site on cytokine concentrations is currently not known.

Concentrations of cord blood cytokines, such as IL-4, IL-6, IL-10, IFN- γ and TNF- α have been studied in regard to clinical prognosis and diseases developing later in childhood [3, 5, 6, 9, 18, 19]. In most clinical studies, cytokines in plasma or serum have been directly measured by immunoassay methods [3, 16, 20]. Basal and/or stimulated cytokine levels have also been determined in different cytokine-producing cells such as polymorphonuclear leu-

kocytes, lymphocytes, peripheral mononuclear, endothelial and smooth muscle cells, and in whole blood [1, 2, 6, 10, 20]. Furthermore, many different stimulants of cytokine production are commonly used in experimental studies *ex vivo*, for example phorbol myristate acetate (PMA), concavalin A (ConA), ionomycin, phytohemagglutinin or bacterial products such as lipopolysaccharides (LPS) and *Staphylococcus aureus* Cowan (SAC-1) [1, 2, 10-12, 20]. The stimulants affect various cytokine-producing cells differently, leading to dissimilar cytokine profiles. Thus, it is obvious that the effects of the stimulants used and the blood collection method need to be considered when comparing and evaluating the results of cytokine analyses in different studies.

In this study, we compared the effects of two umbilical blood collection strategies and two different combinations of stimulants: (I) PMA and ConA and (II) PMA and ionomycin, on induced cytokine production in order to evaluate their impact on the results of cytokine analyses at delivery. Both Th1-type IFN- γ and Th2-type IL-4, as well as proinflammatory IL-6 and TNF- α were studied. In addition, the correlations of cytokine responses between maternal and umbilical samples were studied.

MATERIALS AND METHODS

Twenty-three healthy women were consecutively enrolled into the study during a three-month period between March and June 2002 at the Department of Obstetrics and Gynecology, Kuopio University Hospital. All women had singleton gestations with term pregnancy (median 40 weeks of gestation; range 38–41) and the median maternal age on admission was 28 (range 20–43) years. In total, 21 (91%) delivered vaginally and 2 underwent cesarean section after prolonged labor. The median duration of labor before delivery was 10 hours (range five–20 hours) and the duration between rupture of the membranes and delivery was less than 24 hours in every subject. All these women gave birth to healthy newborns, with a median birth weight of 3440 (range 3120–4560) grams and Apgar scores of at least seven at five minutes of age. The infants were observed during their hospital stay up until discharge. None of them had verified congenital infection and the median hospital stay after birth was four (range one–eight) days. Informed consent was obtained from all subjects, according to the protocol approved by the Committee for Ethical issues in Human Research at the University of Kuopio.

Venous blood samples (10 mL) were obtained from a peripheral vein of the parturient at a median of 18 (range four–138) minutes and UA and UV blood samples (both 5 mL) were collected after cord clamping at a median of eight (range one–20) minutes after delivery of the child. A green-top, Vacutainer (Becton Dickinson, Rutherford, NJ, USA) blood collecting system was used to collect both the peripheral venous as well as umbilical cord blood. The samples were stored initially at +4 °C until processed further within 24 hours.

Heparinized blood (500 µL) was added to 1.5 mL of RPMI cell culture medium supplemented with heat-inactivated fetal bovine serum (10%), L-glutamine (1%) and penicillin-streptomycin antibiotic mixture (1%), all from Gibco, Paisley, UK. The cells were stimulated either with phorbol 12-myristate 13-acetate (PMA; Sigma, MO, USA) (15 ng/mL) and concavalin A (ConA; Sigma, MO, USA) (10 µg/mL), or with PMA (15 ng/mL) and ionomycin (1 µg/mL) for 24 h at 37 °C, in an environment of 5% carbon dioxide in humidified air. After incubation, the cell

cultures were centrifuged at 380 × g for 10 minutes, and the supernatant was stored in polypropylene tubes at -80 °C for later cytokine analysis. Unstimulated production of cytokines was measured after 24 hours in individual cell cultures and these served as controls. The remaining blood was centrifuged at 1800 × g for 10 minutes and the plasma was stored in polypropylene tubes at -80 °C for later cytokine analysis.

Cytokine levels (IL-4, IFN-γ, TNF-α and IL-6) in the supernatant and in the plasma were measured using enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems (Minneapolis, MN, USA). Assays were performed according to the manufacturer's instructions. The calibration standards ranged from 8.2 to 2000 pg/mL for IL-4, 31.3 to 1000 pg/mL for IFN-γ, 15.6 to 500 pg/mL for IL-6 and 31.3 to 1000 pg/mL for TNF-α. If the cytokine concentration detected exceeded the upper limit, the sample was diluted 1:5 or 1:10 and re-analyzed. Values between 0 and the lowest standard were reported as such. The number of cytokine measurements ranged from 13 to 22 owing to technical reasons, i.e., insufficient amount of sample.

Cytokine concentrations within the same parturient were investigated by Wilcoxon's matched pairs signed rank test. Spearman's nonparametric correlation was used to examine the relationships between different cytokine levels in maternal vein, UA and UV. If the cytokine concentrations detected were mostly lower than the lowest calibration standard, values were reported, but correlation analysis was not performed. Statistical significance was set at $p=0.05$.

RESULTS

Table 1 shows the median values of plasma cytokine levels, the values after stimulation (PMA/ConA and PMA/ionomycin) and the numbers of samples with cytokine concentrations above the lowest standard. In general, plasma cytokine levels were lower than in whole blood after stimulation. In regard to basal plasma levels, those of IL-4 were significantly higher than after PMA/ConA stimulation (mother: 7.5 pg/mL versus 0 pg/mL; $p<0.001$,

Table 1
Maternal and umbilical blood plasma concentrations and cytokine production after PMA and ConA, and PMA and ionomycin stimulation

	IL-4 (pg/mL) median, range, n	Above LS % (n/n) ^a	IFN-γ (pg/mL) median, range, n	Above LS % (n/n) ^a	IL-6 (pg/mL) median, range, n	Above LS % (n/n) [*]	TNF-α (pg/mL) median, range, N	Above LS % (n/n) [*]
Plasma levels								
Mother	7.5 (0-547) 20	50% (10/20)	0 (0-1) 20	0% (0/20)	8 (0-536) 22	27% (6/22)	20 (0-1153) 22	41% (9/22)
Umbilical vein	2.3 (0-356) 21	33% (7/21)	0 (0) 21	0% (0/21)	0 (0-87) 21	19% (4/21)	12 (0-986) 19	42% (8/19)
Umbilical artery	1.3 (0-74) 13	38% (5/13)	0 (0) 13	0% (0/13)	0 (0-41) 13	23% (3/13)	25 (0-977) 15	47% (7/15)
PMA/ConA stimulation								
Mother	0 (0-5) 19§	0% (0/19)	45 (0-704) 20†	75% (15/20)	473 (0.25-743) 19	89% (17/19)	249 (0-910) 19	89% (17/19)
Umbilical vein	0.85 (0-11) 20†	5% (1/20)	84 (0-521) 20†	85% (17/20)	723 (26-779) 19§	100% (19/19)	1112 (26-1229) 19	95% (18/19)
Umbilical artery	0.20 (0-8) 20‡	0% (0/20)	87 (0-1522) 20‡	95% (19/20)	723 (60-832) 19	100% (19/19)	1136 (23-1334) 19	95% (18/19)
PMA/ionomycin stimulation								
Mother	2.3 (0-11) 17§	12% (2/17)	314 (0-1018) 18†	89% (16/18)	141 (18-717) 17	100% (17/17)	224 (77-826) 17	100% (17/17)
Umbilical vein	11 (3-35) 19†	74% (14/19)	902 (0-1696) 19†	95% (18/19)	543 (55-784) 18§	100% (18/18)	792 (85-1243) 18	100% (18/18)
Umbilical artery	13 (0-24) 16‡	63% (10/16)	1147 (0-1809) 16‡	94% (15/16)	678 (20-806) 15	100% (15/15)	984 (153-1364) 15	100% (15/15)

Significant differences between PMA/ConA, and PMA/ionomycin stimulation with p-values: † $p<0.001$, ‡ $p<0.01$, § $p<0.05$.

^a % (n/n) of samples over lowest standard (LS) limit.

Table 2
Correlations (r) between cytokine production in maternal and umbilical arterial and venous whole blood samples

	IL-4	IFN- γ	IL-6	TNF- α
PMA/ConA stimulation				
UA-UV	^a	0.71 [†]	0.90 [†]	0.75 [†]
Mother-UA	^a	0.14	0.15	0.29
Mother-UV	^a	0.15	0.31	0.32
PMA/ionomycin stimulation				
UA-UV	0.68 [†]	0.80 [†]	0.95 [†]	0.91 [†]
Mother-UA	^a	0.21	0.59 [‡]	0.35
Mother-UV	^a	0.38	0.41	0.18

UA umbilical artery, UV umbilical vein. [†] p<0.01; [‡] p<0.05.

^a Correlations not calculated owing to low number of samples over the lowest standard.

UV: 2.3 pg/mL versus 0.85 pg/mL; p<0.04 and UA: 1.3 pg/ml versus 0.2 pg/mL p<0.06). In contrast to IL-4, IFN- γ was not detectable in unstimulated plasma samples. The levels of IL-4 and IFN- γ in paired samples were significantly higher after PMA/ionomycin than after PMA/ConA stimulation (table 1). The concentration of IL-4 was above the lower standard in 0–5% of whole blood samples after PMA/ConA stimulation compared with 12% (maternal levels) to 74% (umbilical vein levels) after PMA/ionomycin stimulation. In contrast, there was a tendency towards higher levels of plasma IL-6 and TNF- α after PMA/ConA stimulation compared with PMA/ionomycin stimulation, but the difference between the stimulants was statistically significant only as regards UV IL-6 production (table 1).

Correlation analysis was not possible in plasma samples and the levels of IL-4 in whole blood samples after PMA/ConA stimulation, owing to low cytokine concentrations. In the rest of the samples, significant correlation in the levels of cytokines produced after the same stimulants, was noted between UA and UV samples (r=0.68–0.95) (table 2). Scatterplots of UA versus UV cytokines after different stimulations are shown in figure 1 and figure 2. The data were clustered as regards IL-6 and TNF- α levels after PMA/ConA stimulation (figure 1).

The median levels of TNF- α after PMA/ConA stimulation were significantly higher in 14 out of 19 (74%) UA samples compared with simultaneous, paired UV samples (p<0.05). A similar but nonsignificant association was also

detected after PMA/ionomycin stimulation; TNF- α levels were higher in 10 out of 15 (67%) UA samples when compared with paired UV samples (p<0.08). The levels of other cytokines in UA versus UV blood were of the same magnitude and no significant differences were detected.

The levels of stimulated IL-4 and IFN- γ were significantly higher in umbilical blood samples compared with simultaneous maternal blood samples after PMA/ionomycin, but not after PMA/ConA stimulation. When umbilical IL-4 concentrations were compared with those in maternal peripheral vein blood, significantly higher levels were noted in 10 out of 14 (71%) UA samples and in 16 out of 17 (94%) UV samples (UA: p<0.005 and UV: p<0.0001). Similarly, 12 out of 15 (80%) and 15 out of 18 (83%) cases had significantly higher levels of stimulated IFN- γ in UA and UV compared with maternal peripheral venous blood (UA: p<0.006 and UV: p<0.004). In addition, both stimulants significantly increased the concentrations of IL-6 and TNF- α in umbilical cord blood versus maternal blood (IL-6: PMA/ConA stimulation, UV 18/19 pairs, p<0.0001 and UA 18/19 pairs, p<0.0001; PMA/ionomycin stimulation, UV 14/17 pairs, p<0.003 and UA 13/14 pairs, p<0.002; TNF- α : PMA/ConA stimulation, UV 18/19 pairs, p<0.0001 and UA 18/19 pairs, p<0.0001; PMA/ionomycin: UV 14/17 pairs, p<0.002 and UA 14/14 pairs, p<0.0001). In contrast, the levels of IL-6 and TNF- α in UA and UV plasma were significantly lower than in simultaneous maternal plasma (IL-6: UV 19/20 pairs;

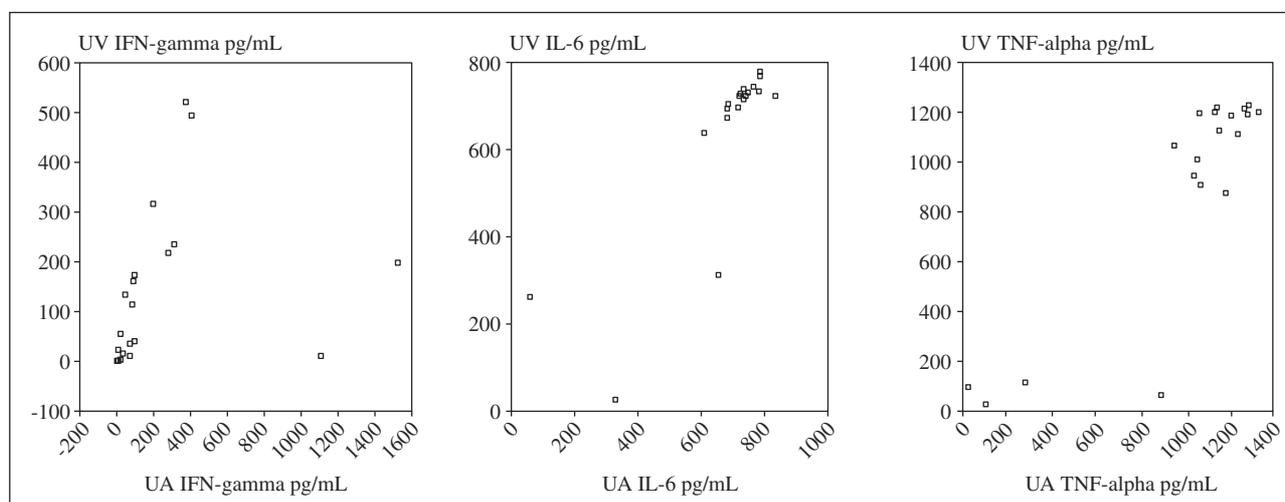


Figure 1

Scatterplots of IFN- γ , IL-6 and TNF- α production in umbilical arterial (UA) versus venous (UV) blood samples after PMA/ConA stimulation.

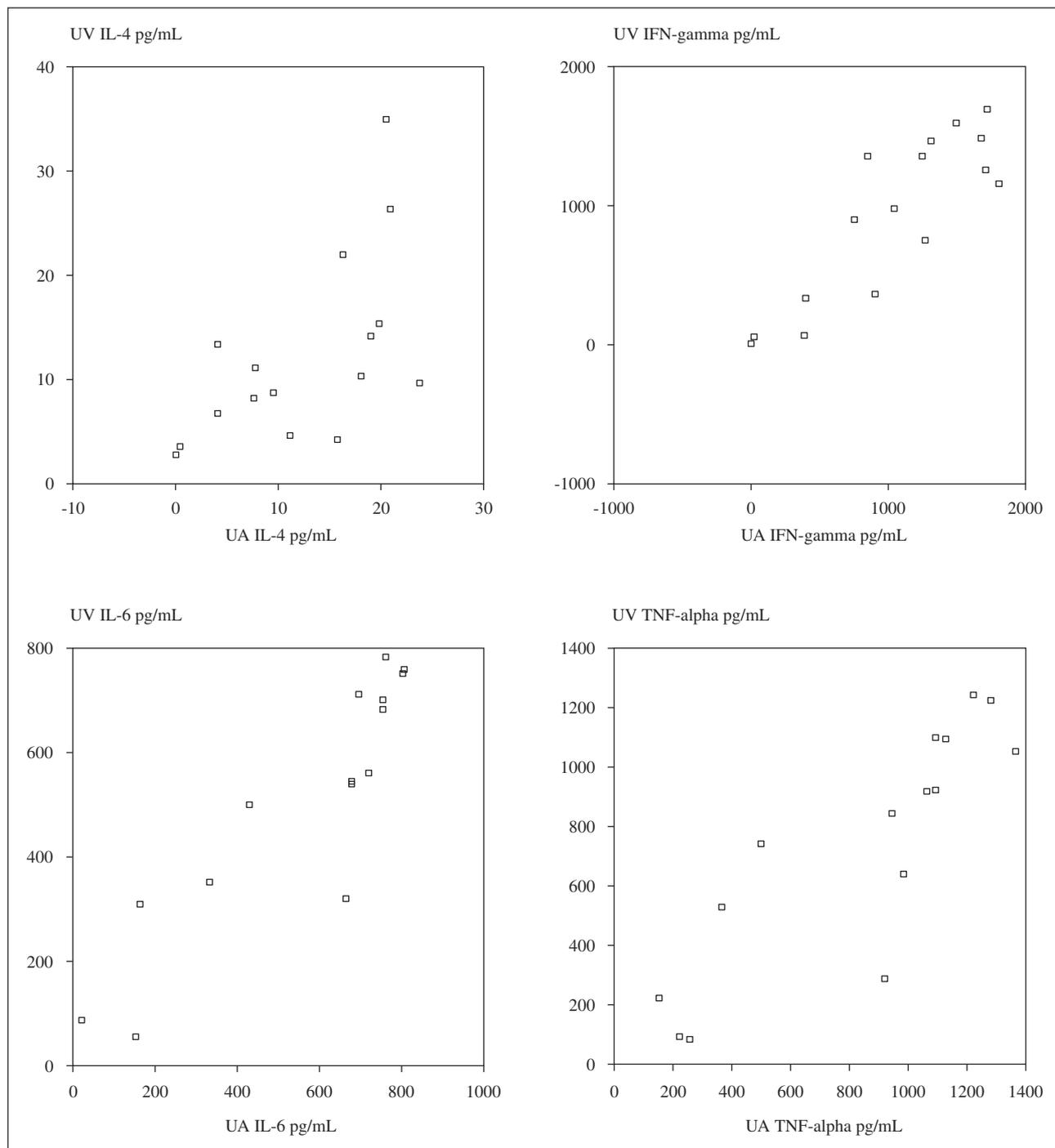


Figure 2

Scatterplots of IL-4, IFN- γ , IL-6 and TNF- α production in umbilical arterial (UA) versus venous (UV) blood samples after PMA/ionomycin stimulation.

$p < 0.0001$ and UA 11/12 pairs; $p < 0.003$. TNF- α : UV 9/19 pairs; $p < 0.03$ and UA 9/15 pairs; $p < 0.03$).

DISCUSSION

Our data demonstrate that there were no significant differences between UA and UV samples in their cytokine-producing capacity. Basal and stimulated levels of IL-4, IFN- γ , IL-6 and TNF- α were of the same magnitude in both samples, and after stimulation there was significant correlation between the concentrations of most cytokines

examined in UA and UV. The current findings markedly expand those in earlier reports showing a significant correlation between IL-6 plasma levels in UA and UV [14, 15, 17]. Such data are not available as regards the basal and stimulated levels of other cytokines. Nevertheless, production of the proinflammatory cytokines IL-6 and TNF- α in both UA and UV was very high after PMA/ConA stimulation. It is known that an estimated correlation between two different variables is valid only within the range of observed data [21]. Thus, some correlation analyses should be regarded with caution, because extrapolation might be misleading owing to missing values [21].

The concentrations of all the cytokines examined, except for TNF- α , were at the same level in both UA and UV. The induced production of TNF- α was higher in 74% of UA samples compared with paired UV samples after PMA/ConA stimulation. This finding may reflect the importance of fetal regulation in the complex network of cytokine production at delivery. However, this association needs to be confirmed in future studies as a result of the low number of samples and minor differences in cytokine concentrations.

In line with earlier reports, the concentrations of cytokines examined were constantly at a lower level in plasma compared with whole blood cultures. The only exception was plasma levels of IL-4, which were higher than those measured after 24-hour stimulation of whole blood with PMA/ConA. This may be a result of the inability of PMA/ConA to stimulate IL-4 production in whole blood cultures. Thus, normal degradation of IL-4 during the time of stimulation leads to decreased detection of this cytokine. It is also possible that whole blood cultures contain suppressor factors or antagonists, such as prostaglandins, which inhibit mitogenesis or accelerate destruction of specific cytokines [22].

We found that the cytokines examined were constantly expressed in greater quantities in umbilical blood after stimulation compared with paired maternal whole blood samples, but the concentration changes were unclear in plasma samples. It is known that neonatal umbilical cord blood lymphocytes, compared with cells isolated from adult peripheral blood, reveal differences in phenotypic features [23, 24], secretion of cytokines following stimulation [25], and the response pattern to other stimulants such as hormones and antibodies [26, 27]. The hormonal milieu in intrauterine life and during labor, however, clearly has an effect as can be observed when comparing umbilical neonatal blood samples with blood of adult nonpregnant controls, and might affect results obtained. To avoid these hormonal differences resulting from pregnancy and labor, in some studies, cytokine production has been evaluated in umbilical and simultaneously sampled maternal blood, using mononuclear cell cultures [2, 28]. In these studies, the cellular reactivity of umbilical cord mononuclear cells was higher after ConA stimulation compared with that of maternal cells [2, 28]. Secretion of IFN- γ and the T lymphocyte proliferative response was significantly lower after PHA and SL-O stimulation [2, 28]. Our results demonstrate the well-developed capacity of umbilical whole blood to synthesize both Th1- and Th2-type cytokines in response to mitogenic stimulation *ex vivo*. Whole blood culture represents a more physiological method to study cell function compared with specific cell cultures, since it contains all blood components. It is also faster and cheaper compared with specific cell cultures.

The present results show that both stimulants significantly affected the levels of Th1-type (IFN- γ) and Th2-type (IL-4) cytokines, as well as the production of proinflammatory cytokines (TNF- α and IL-6). The production of IL-4 and IFN- γ induced by PMA/ionomycin was profoundly increased in peripartum maternal and umbilical whole blood cultures compared with responses triggered by PMA/ConA stimulation. This is in line with earlier reports suggesting that PMA/ionomycin is a more potent stimulant of both IL-4 and IFN- γ production in peripheral

blood T cells compared with other polyclonal stimulants such as PHA, ConA alone and PMA alone [29]. The precise function of these stimulants in the production of cytokines is not yet known. In addition, a variety of other cell types including amnion, chorion and decidual cells are also involved in the production of cytokines in gestational tissues, and thus they might affect cytokine levels in plasma and umbilical whole blood cultures.

The present results have at least three important implications. First, almost identical cytokine results can be obtained in UA and UV samples. Secondly, production of cytokines after mitogenic stimulation is more efficient in umbilical blood than in maternal blood. Thirdly, the stimulant used has significant effects on IL-4 and IFN- γ production. The present results enhance understanding of the fetal cytokine environment at birth.

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