

Cytokine Expression in Cord Blood Cells of Children of Healthy and Allergic Mothers

J. HRDÝ^a, P. ZANVIT^a, O. NOVOTNÁ^a, I. KOCOURKOVÁ^b, J. ŽIŽKA^a, L. PROKEŠOVÁ^{a*}

^a*Institute of Immunology and Microbiology, 1st Faculty of Medicine, Charles University in Prague, 128 00 Prague 2, Czech Republic*

^b*Pediatric Department, Institute for the Care of Mother and Child, 147 00 Prague 4, Czech Republic*

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ABSTRACT. To determine some early signs connected with the increased risk of future allergy development, gene expression and production of selected cytokines were tested in children of allergic mothers and compared with newborns of healthy mothers. Expression of IL-1 β , IL-2, IL-4, IL-8, IL-10, IL-13, IFN- γ , TNF- α , TGF- β and EGF was tested in cord blood cells using real-time PCR and production of these cytokines was evaluated in cord sera by ELISA. Gene expression of IL-2, IL-4, IL-8, IFN- γ , IL-1 β , TNF- α and TGF- β was decreased and that of IL-10, IL-13 and EGF increased in children of allergic mothers in comparison with those of healthy mothers. Significant differences in sera of healthy and allergic groups were only in IL-10 and EGF. Different relationship among serum cytokine levels reflects the fact that the cytokines are not produced only by blood cells. Significantly decreased production of EGF in newborns of allergic mothers could negatively influence maturation of mucosal membranes of these children and support thus their easier allergization. Allergic phenotype pointing to the bias to T_H2 response and to possibly impaired intestine maturation was apparent already on the level of cord blood and could serve as a predictive sign of increased allergy risk.

Abbreviations

AM	allergic mothers	CBML	cord blood mononuclear leukocytes	HM	healthy mothers
CBC	cord blood cell(s)	CV	coefficient of variance	RT	real-time (PCR)

Allergic diseases are in the centre of interest of current medicine mainly because of their large and steadily increasing incidence. It is commonly accepted that genetic predisposition is the main risk factor for allergy development and manifestation (Benn *et al.* 2004; Hauer *et al.* 2003; Peden 2005; Prescott *et al.* 2003; Steinke *et al.* 2008; Wang *et al.* 2005). However, the effect of other factors should not be forgotten. Besides environmental impact, mainly prenatal and early postnatal influence of maternal immune system on the development and tuning of offspring's immune reactions has to be kept in mind. A child obtains many biologically active substances from the mother – prenatally *via* placental transfer or amniotic fluid and postnatally by breast-feeding. Already at birth, immunological milieu and immunological reactivity of the newborn could point to the increased risk of future allergy development.

We were able (Žižka *et al.* 2007) to prove a striking difference in the CBC reactivity of newborns of HM and AM in spontaneous and polyclonally stimulated proliferation. Here we should like to reveal the difference in immunological tuning in “healthy” and “allergic” groups of children by testing cytokine expression and production in the cord blood. We were interested mainly in the cytokines involved in the development and promotion of allergy and influencing the production of IgE. Significant differences between children of HM and AM were found already at the time of birth.

MATERIAL AND METHODS

Subjects. HM and AM with physiological pregnancy and children delivered physiologically in full term at the *Institute for the Care of Mother and Child* from October 2005 to April 2006 were included. Diagnostics of allergy in mothers was based on clinical manifestation of allergy persisting for >2 years (allergy against respiratory and food allergens manifested by various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema, *etc.*), monitoring by an allergologist, positive skin prick tests or positive specific IgE and anti-allergic treatment before pregnancy. Father's allergy, known as having smaller impact on children's allergy development (Tadaki *et al.* 2009), was not taken into consideration. The study

*Corresponding author; fax +420 224 968 455, e-mail lprok@lfl.cuni.cz.

was approved by the *Ethical Committee of the Ministry of Public Health* and was carried out with the informed consent of the mothers.

Cord blood from children of HM and AM was collected immediately after delivery with puncture of umbilical vein after careful cleaning of the cord to avoid maternal blood contamination. For RNA isolation, 2.5 mL blood was placed into Paxgene Blood RNA Tubes (*PreAnalytiX*) containing RNA stabilization solution preventing RNA degradation. Choice of 10 cord blood samples of children of both HM and AM for RNA isolation was at random, there were no selection criteria. Cord blood serum was obtained for cytokine detection.

RNA isolation. Whole cord blood RNA was isolated from CBC using Paxgene Blood RNA kit (*PreAnalytiX*) according to the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1.5 % agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance A_{260} and A_{280} . The purity was in the range 1.8–2.1. The total RNA concentration was estimated spectrophotometrically (A_{260}) assuming that 44 µg of RNA per mL equals one absorbance unit. RNA was stored in aliquots at -20°C until used for reverse transcription.

Quantitative RT PCR. Isolated mRNA was converted to cDNA using reverse transcription reagents (*Applied Biosystems*) according to the manufacturer's instructions.

Every 25 µL reaction mix for RT PCR was made with 12.5 µL TaqMan Universal PCR master mix (*Applied Biosystems*), 100 ng of cDNA diluted in 5 µL RNAase free water, 6.25 µL RNAase free water and 1.25 µL specific TaqMan probes for mentioned genes (all *Applied Biosystems*). Eukaryotic 18S rRNA (4319413E; *Applied Biosystems*) was used as an endogenous control (housekeeping gene). Each reaction was run in duplicate.

PCR reaction was run on the 7300 RT PCR system (*Applied Biosystems*) using standard conditions. Cycling parameters were: initial denaturation (10 min, 95°C), 40 cycles consisting of denaturation (15 s, 95°C) and annealing–extension (1 min, 60°C). Fluorescence was acquired after each extension step. The efficiency of reactions was very similar to that of endogenous control. A no-template control contained water instead of cDNA. Expression of all genes was normalized to cDNA of endogenous control loading for each sample and used as an internal standard.

The relative quantity of mRNA was given as $2^{-\Delta\Delta\text{ct}}$, $\Delta\Delta\text{ct}$ being calculated as follows:

$$\begin{aligned}\Delta\Delta\text{ct} &= \Delta\text{ct}_a - \Delta\text{ct}_h \\ \Delta\text{ct} &= \text{ct}_{\text{gi}} - \text{ct}_{\text{hg}}\end{aligned}$$

where Δct_a is allergic individual, Δct_h healthy individual, calibrator, ct_{gi} gene of interest, and ct_{hg} house-keeping gene (endogenous control).

The threshold cycle (ct) is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. mRNA from cord blood from 10 children of HM was used as calibrator (its value was set = 1). The gene expressions in CBC from 10 children of AM were expressed as relative values referred to the value of the calibrator (evaluated by SDS Software; *Applied Biosystems*).

ELISA. IL-2, IL-4, IL-8, IL-10, IL-13, INF- γ , TNF- α , IL-1 β , TGF- β_1 and EGF of 54 children of HM and 55 children of AM were quantified by ELISA using primary antibody–detection antibody pairs from *R&D Systems*. Analysis was carried out according to the manufacturer's recommendation. The assays were optimized by testing various dilutions of tested samples and antibodies. Samples were assayed in duplicates and the CV differs; lower CV (typically 0–0.12 %) is in assays with high concentration of cytokines detected (TGF- β , INF- γ , IL-13) and CV is higher (0–0.26 %) in assays with lower cytokine concentration (IL-4, IL-8, IL-10, EGF, TNF- α). Detection limit for IL-4, IL-8, EGF, INF- γ and TNF- α was 10 pg/mL, for IL-2, IL-10, IL-13 and TGF- β 30 pg/mL and for IL-1 β 250 pg/mL; cytokine values were read from calibration curves. Results are expressed as medians and 25–75 % percentiles. All cytokine concentrations (Table I) are appended with numbers of samples in which concentration was upper detection limit.

Statistics. Comparison of the gene expression of cytokines and cytokine concentrations of the two groups (children of HM and of AM) was examined by non-parametric Mann–Whitney test, with $p \leq 0.05$ being considered significant.

RESULTS

Cytokine expression. For the comparison of cytokine expression in non-stimulated CBC of newborns of HM and AM tested by quantitative RT PCR see Fig. 1. Significant differences were detected in the expression of the majority of cytokines tested – expression of IL-2, IL-4, IL-8, INF- γ , IL-1 β , TNF- α and

TGF- β being significantly lower and that of IL-10, IL-13 and EGF higher at children of AM. IL-13 and IL-10 increase is quite evident in allergic group but non-significantly because of large individual variability.

Table I. Cytokine concentrations in cord blood sera from children of HM and AM (ELISA, pg/mL)

Cytokine ^a	HM			AM			<i>p</i>
	median	25–75 %	<i>n</i> ^b	median	25–75 %	<i>n</i> ^b	
IL-4	221	169–367	23	248	172–545	36	0.6635
IL-8	580	412–805	43	649	436–1 202	43	0.3206
IL-10	306	210–464	20	321	197–448	43	0.8943
IL-13	943	289–2 609	29	2 011	556–3 521	43	0.1666
INF- γ	1086	614–2 178	20	486	195–854	22	0.0355
TGF- β	70 008	39 911–103 040	42	42 356	24 512–88 889	54	0.1358
EGF	1498	743–2 539	40	1 304	701–1 945	44	0.0312

^aConcentration of IL-1 β , IL-2 and TNF- α were below the detection limit.

^bNumber of samples in which concentration was above the detection limit (total number was 54 in healthy group and 55 in allergic group).

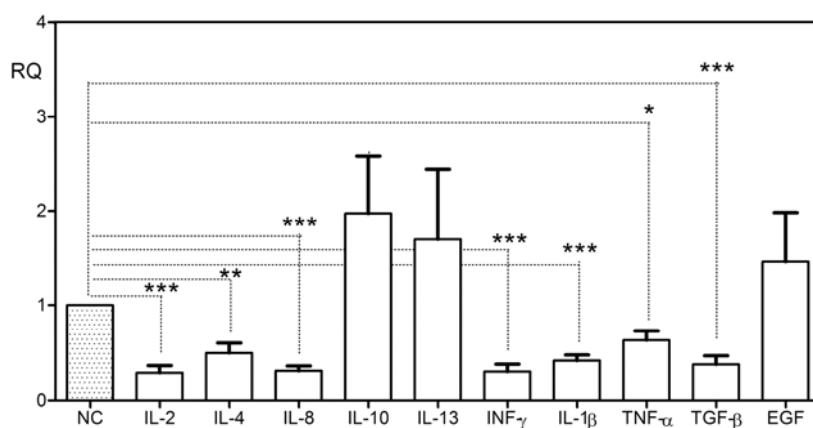


Fig. 1. Gene expression of cytokines in CBC from children of healthy and allergic mothers measured by quantitative RT PCR; means of relative values (relative quantification, RQ) of cytokine mRNA determined in CBC from 10 children of allergic mothers; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; NC – normal control (mRNA sample of 10 children of HM corresponding to RQ = 1).

Cytokine concentration. For cytokine concentrations detected by ELISA see Table I. These results are not exactly comparable with the gene expression because they reflect the cytokine production by various cells and tissues, not only production by blood cells. As concerns IL-10, IL-13, INF- γ and TGF- β , the trend of differences between their levels in both groups corresponds to that revealed by testing cytokine expression in CBC but, except for INF- γ , differences are not significant. An inverse relationship between cytokines of children of HM and AM was in IL-4, IL-8 and EGF. When comparing EGF by RT PCR, no significant differences were proved, while its serum levels were significantly lower in children of AM. The slight increase in IL-4 and IL-8 concentration in children of AM was not significant. IL-2, IL-1 β and TNF- α values were on the border of detection sensitivity in serum.

DISCUSSION

Many authors have already dealt with cytokines in cord blood in relation to allergy but they focused mainly on INF- γ , IL-4 and IL-13 (Chung *et al.* 2007; Schaub *et al.* 2005; Silberer *et al.* 2008) though many different cytokines are involved in allergy development and other ones have anti-allergic effect. The cytokines tested by us are supposed to have some relationship to the induction and regulation of allergic reaction: T_H1 (IL-2, INF- γ) and T_H2 (IL-4, IL-13) cytokines which inhibit or support allergy development, respectively (Chung *et al.* 2007), IL-10 and TGF- β promoting isotype switch towards IgA (Fayette *et al.* 1997) which

is believed to act against allergy development; IL-4 and IL-13 being important for IgE production (Fayette *et al.* 2007; Graves *et al.* 2000; Tanaka *et al.* 2001); IL-10 and TGF- β mediating the establishment of peripheral tolerance hampering the rise of allergy (Jutel *et al.* 2003); EGF and TGF- β as growth and differentiation factors of great importance for intestine maturation (McGee *et al.* 1992; Nair *et al.* 2008), the failure of which can cause increased intestinal permeability leading to easier access of allergens to the immune system resulting in allergy induction; IL-1 β , IL-8 and TNF- α act as inflammatory cytokines (Mohammed *et al.* 2007).

Cytokine expression detected by RT PCR revealed significant differences between CBC from children of HM and AM; our results are in accordance with the known increased risk of allergy manifestation in children of AM. Allergic phenotype is evident already on the level of CBC. We observed lower expression of IFN- γ in CBML (as we and many other authors describe on protein level in cord blood; Contreras *et al.* 2003; Kondo *et al.* 1998; Prescott *et al.* 2003) and higher expression of IL-13 and IL-10 – that points to an increased propensity of newborns of AM to T_H2 response. High expression of IL-13 is pro-allergic and was described as a marker of a high risk of future allergy (Ohshima *et al.* 2002). Increased gene expression of IL-13 in CBML of children of AM after *in vitro* stimulation in comparison to healthy ones was also observed by other authors (*e.g.*, van der Veden *et al.* 2001). However, gene expression of IL-4 in CBML of children of HM was significantly higher in comparison with allergic group. This discrepancy was also described by Prescott *et al.* (1998). The tendency to the increased concentration of IL-4 in allergic group is evident only on serum level, which can point to the participation of additional sources of IL-4 influencing serum level of this pro-allergic cytokine. Therefore IL-13 expression and production seems to correlate better with increased propensity of allergy than that of IL-4. Decreased expression and production of TGF- β in these children of allergic group could contribute to the increased risk of allergy development by limiting the induction of peripheral tolerance against allergens, and by impaired maturation of the intestine and possibly by lowering IgA production.

The cytokine expression by CBC is not fully reflected by cytokine concentration in serum. This is understandable because cord serum cytokines originate not only from fetal leukocytes and could also be transferred to the newborn from the mother *via* placenta or by engulfing amniotic fluid whose constituents are partially of maternal and partially of fetal origin. Thus serum cytokines reflect summation of both intrinsic and extrinsic factors characterizing newborn's immunological milieu. In the majority of cytokines tested, the differences between children of HM and AM are similar on mRNA and serum level but serum differences are less significant. A different relationship between both groups on mRNA and serum level is only in the case of IL-4, IL-8 and EGF. mRNA for IL-8 in CBC is lower in children of AM but there is no huge difference on serum level pointing to other sources of IL-8 than leukocytes. There are no significant differences in EGF expression in CBC but there is a significantly higher concentration of EGF in healthy group on serum level. This is quite in keeping with the fact that leukocytes are not the main source of EGF. Decreased total production of EGF in allergic group reflected in decreased serum level could support the induction of allergy by its negative impact on mucosal membrane maturation. Because the differences between cord blood serum cytokines of children of HM and AM are less significant than differences detected in CBC, it may be concluded that the maternal impact on the development of offspring's allergy is asserted mainly on genetic level, the phenotypic products of maternal organism being mostly less important. Differences in gene expression of CBC cytokines and in serum levels of EGF could potentially serve for assessment of future allergy risk. Health condition (allergy development) of tested children will be followed prospectively in the effort to evaluate the prognostic value of data mentioned *above*.

Allergic phenotype pointing to the bias to T_H2 response (decreased gene expression of T_H1 cytokines IL-2, IFN- γ and increased expression of IL-13 in CBC of newborns of AM) and to impaired intestine maturation (decreased concentration of EGF in cord blood sera of children of AM) were apparent already on the level of cord blood and could serve as possible predictive sign of future allergy development.

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