

Cytokine expression in the colostral cells of healthy and allergic mothers

Jiří Hrdý · Olga Novotná · Ingrid Kocourková ·
Ludmila Prokešová

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Abstract There is no doubt about the beneficial effect of breastfeeding on the newborn's immune system. It is not fully elucidated what the differences are between the colostrum/milk of healthy and allergic mothers and how beneficial breastfeeding by an allergic mother is. The gene expression of selected cytokines was tested in cells isolated from colostrum of healthy and allergic mothers using quantitative real-time PCR. Allergic phenotype was evident in colostral cells of allergic mothers: gene expressions of IL-4, IL-13 and EGF were increased and those of IFN- γ decreased in comparison with colostral cells of healthy mothers. The allergic phenotype of the colostral cells of allergic mothers supporting the bias to a Th2 type response was found. It remains a question if a small number of these cells could influence the immature newborn immune system.

Introduction

There is no doubt about the usefulness of breastfeeding. Maternal milk includes not only an optimal nutrient composition but also many immunologically active components, with colostrum (early milk) being richer in these constituents than mature milk. Colostrum includes factors of both humoral and cellular immunity—cytokines, antibodies, various types of leukocytes, typically 40–50 % macrophages, 40–50 %

polymorphonuclear neutrophils and 5–10 % lymphocytes, as well as a low number of epithelial cells (Wirt et al. 1992). Up to now, colostrum/milk humoral immunologic factors have been investigated by many authors (e.g. Islam et al. 2006; Zizka et al. 2007). The differences in the concentrations of cytokines and other humoral factors in the milk of healthy and allergic mothers have been described (e.g. Zizka et al. 2007); however, a detailed comparison of milk cells is lacking. The survival of colostral cells in the intestines of offspring and their transfer to the blood circulation were detected in animal models, and even indirectly also in humans (Hanson 2000; Reber et al. 2008; Zhou et al. 2000). The newborn organism is immature both in antibody formation and cellular immunity. This lack of antibodies is compensated by maternal immunoglobulin G, transferred transplacentally and by the colostral/milk IgA supply. Colostral lymphocytes are represented mainly by activated CD45RO⁺ cells (Bertotto et al. 1997). The possible effect of these maternal cells on the newborn immune system remains to be elucidated. Children of allergic mothers are at an increased risk of allergy development. The role of the genetic background is further supported by the close coexistence of child and mother during gestation and in early postnatal life mainly when a child is breastfed. Therefore, the immunological characteristics of the milk of allergic mothers are of interest. In the present study, we tested the possible differences in cytokine gene expression in the colostral cells of healthy and allergic mothers.

Materials and methods

Subjects

Mothers were divided into two groups, according to their allergy status. The diagnostics of allergy in mothers was

J. Hrdý · O. Novotná · L. Prokešová (✉)
Institute of Immunology and Microbiology,
First Faculty of Medicine, Charles University in Prague,
Studnickova 7,
128 00 Prague 2, Czech Republic
e-mail: lprok@lf1.cuni.cz

I. Kocourková
Institute for the Care of Mother and Child,
Prague, Czech Republic

based on the clinical manifestation of allergy persisting for longer than 24 months (allergy to the respiratory and food allergens; manifested with various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema, etc.), monitoring by an allergologist, positive skin prick tests or positive specific IgE antibodies and anti-allergic treatment before pregnancy. Detailed anamnestic data of mothers are given in Table 1. “Allergic mothers” were otherwise healthy. For data analysis were included 9 healthy mothers and 11 allergic mothers. Though a larger number of mothers were tested, not all samples either contained sufficient RNA concentration for analysis of all cytokines or passed strict criteria for RNA purity and quality ($A_{260/230}$, $A_{260/280}$). The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child, Prague, Czech Republic, and was carried out with the written informed consent of the mothers.

Colostrum

Colostrum from healthy and allergic mothers was obtained at day 3 after delivery. Colostrum was diluted 1:1 in minimal essential medium with 10 % fetal calf serum, and cells were obtained after centrifugation ($500\times g$) and three washings with the same medium.

RNA isolation

Total intracellular RNA was isolated by an RNeasy Minikit (Qiagen) according to manufacturer 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 at 260 and 280 nm. RNA purity ranged from 1.9 to 2.3. The total RNA concentration was estimated by spectrophotometric measurements at 260 nm, assuming that $44\text{ }\mu\text{g/mL}$ RNA corresponds to one absorbance unit. RNA was stored in aliquots at $-20\text{ }^{\circ}\text{C}$ until used for reverse transcription.

Quantitative real-time polymerase chain reaction

Isolated mRNA was converted to cDNA using reverse transcription reagents (Applied Biosystems), according to manufacturer instructions. A reaction mix for real-time PCR was made with TaqMan Universal PCR master mix, RNase free water and Assays on Demand gene expression products for interleukins (IL) IL-2, IL-4, IL-8, IL-10, IL-13, interferon gamma (IFN- γ), transforming growth factor 1 (TGF- β 1) and epidermal growth factor (EGF), all Applied Biosystems. Eukaryotic 18S ribosomal RNA (Applied Biosystems) was used as an endogenous control. All reactions were run in duplicate. The efficiency of reactions for all assays was very similar to the efficiency of the endogenous control.

PCR reactions were carried out on the 7300 real-time PCR system (Applied Biosystems) using standard conditions as described previously (Hrdy et al. 2010). Gene expression of cytokines in the colostrum cells of 11 allergic mothers was expressed relatively to the mean of the gene expression of cytokines in the colostrum cells of nine healthy mothers, using GenEx software (MultiD).

Statistics

Differences between groups were evaluated using the non-parametric Mann–Whitney test, due to data not being normally distributed and because of the low number of individuals tested. Statistical significance was set at $p\leq 0.05$.

Results

Cytokine gene expression in colostrum cells of 9 healthy and 11 allergic mothers was compared by quantitative real-time polymerase chain reaction. Increased levels of expression of IL-4, IL-13 and EGF and a decreased level of IFN- γ were detected in the colostrum cells of allergic mothers in comparison with those of healthy mothers (Fig. 1.). Differences in the expression of IL-2, IL-8 and regulatory cytokines IL-10 and TGF- β were negligible.

Although the differences detected are not significant (IL-2, $p=0.37$; IL-4, $p=0.15$; IL-8, $p=0.42$; IL-10, $p=0.47$; IL-13, $p=0.18$; IFN- γ , $p=0.18$; TGF- β , $p=0.32$) with the exception of a border significance of difference in EGF expression ($p=0.04$), a tendency is quite evident, especially in the case of IL-4, IL-13, IFN- γ and EGF. The low significance is mainly due to high individual variability. The expression of Th2 cytokines supporting allergy development is increased in the colostrum cells of allergic mothers. Because of a low number of mRNA copies of IL-4, this cytokine was detected in only three of nine samples in the healthy group and 6 of 11 in the allergic group (Table 2); therefore, its very low expression does not allow a reliable comparison. However, the increased expression of the other Th2 cytokine tested, IL-13, is relatively convincing. Furthermore, proportional representation of individual cell types was tested in our colostrum samples, and the result corresponded with available published data. No substantial differences were found in the ratio of different types of leukocytes (macrophages, NK, CD4 and CD8 T lymphocytes) estimated by flow cytometry between healthy and allergic mothers (data not shown).

Discussion

To the best of our knowledge, there have been only three studies evaluating the gene expression of cytokines in

Table 1 Detailed anamnestic characteristics of mothers and their children

Mothers	Age of mother	Smoker	Health condition (diagnosis) excepting allergy	Allergy	Medication	Number of pregnancies	Abortions	Course of pregnancy	Course of delivery	Sex of child	Body mass (g)	Length (cm)
A1	37	No		Acylpyrin, beta-lactam antibiotics		2nd	0		Cesarean section after delivery induction	Female	3,470	51
A2	27	No		Pollen, dust, food allergens		1st	0		Forceps delivery after induction	Male	3,960	50
A3	29	Yes		Metals		1st	1×		Cesarean section	Male	4,020	52
A4	34	No		Metals		1st	0		Spontaneous	Female	2,900	48
A5	32	No		Pollen, dust		1st	0		Cesarean section	Female	2,540	44
A6	29	No		Asthma bronchiale—pollen, grass, dust, mites	Symbicort, Zyrtec	1st	0		Cesarean section	Male	3,380	50
A7	35	No	<i>Streptococcus</i> group B+	Ajatin, beta-lactam antibiotics		1st	0		Cesarean section	Female	3,780	48
A8	29	No		Atopic dermatitis		1st	0		Cesarean section	Male	3,710	51
A9	30	No		Dust, pollen, mites		1st	0		Cesarean section	Female	3,620	50
A10	30	No		Pollen, dust		1st	0		Cesarean section	Male	3,210	48
A11	32	No	<i>Streptococcus</i> group B+	Asthma bronchiale—pollen, mould	Zyrtec	1st	0		Cesarean section	Male	3,550	50
H1	32	No		None		1st	0		Cesarean section	Female	3,490	40
H2	39	No		None		1st	0	Pregnancy diabetes mellitus	Cesarean section	Male	2,600	48
H3	27	No		None		1st	0		Cesarean section	Male	3,630	51
H4	38	No	<i>Streptococcus</i> group B+, vaginal mycosis	None		1st	0		Cesarean section	Male	3,490	50
H5	30	No	Recurrent colpitis	None		1st	0		Cesarean section	Male	3,830	51
H6	31	No		None		2nd	1×	Pregnancy diabetes mellitus	Cesarean section	Male	3,340	50
H7	38	No		None		2nd	1×	Pregnancy diabetes mellitus	Spontaneous	Male	3,420	51
H8	28	No		None		1st	0		Spontaneous	Female	3,030	50
H9	33	No		None		3rd	1×	Pregnancy diabetes mellitus	Cesarean section	Female	3,450	48

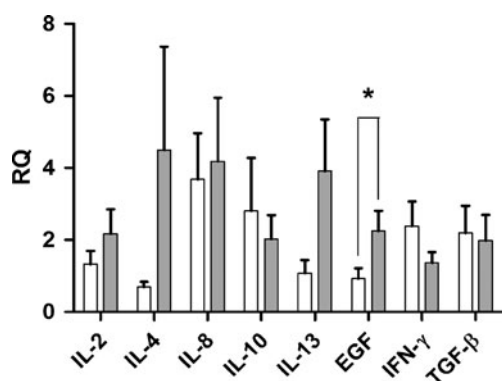


Fig. 1 Gene expression of cytokines in colostrals cells of healthy (open columns) and allergic mothers (filled columns). *EGF* epidermal growth factor, *TGF* transforming growth factor, *RQ* relative quantification

colostral cells, and unfortunately, none related to the allergy status of the mother (Srivastava et al. 1996; Hashira et al. 2002; Nagatomo et al. 2004). The present study is the first report comparing the gene expression of cytokines between colostrals cells of healthy and allergic mothers. The gene expression of the following cytokines was chosen: IL-2 and IFN-gamma, as representatives of Th1 cytokines; IL-4 and IL-13, as representatives of Th2 cytokines; IL-10 and TGF-beta, as regulatory cytokines; IL-8 as an inflammatory cytokine

Table 2 Individual levels of gene expression of cytokines in colostrals cells of allergic and healthy mothers; values are expressed by relative quantification

Mother	IL-2	IL-4	IL-8	IL-10	IL-13	EGF	IFN- γ	TGF- β
Allergic group								
A1	0.80	0.59	18.85	1.02	13.37	1.14	0.17	0.83
A2	3.09	18.61	9.45	1.68	5.60	0.37	1.42	7.04
A3	7.60	2.29	2.71	1.72	0.09	0.70	2.78	1.31
A4	4.03	0.45	1.23	6.61	0.80	4.07	0.70	0.59
A5	0.66	1.20	0.39	0.28	2.99	4.63	2.20	3.96
A6	0.70	3.80	0.04	1.55	2.63	3.16	1.50	0.52
A7	0.62		0.15	0.18	0.51	0.19	0.25	0.65
A8	0.97		7.28	3.64	0.12	0.48	0.67	0.28
A9	1.13		0.42	0.22	4.62	4.84	3.11	1.10
A10	3.87		5.26	5.15	12.13	3.79	0.98	5.32
A11	0.38		0.25	0.18	0.19	1.29	1.21	0.16
Healthy group								
H1	0.32	0.45	1.02	0.32	0.49	0.34	1.26	0.35
H2	0.70	0.73	10.98	12.60	1.57	0.32	1.21	4.75
H3	1.84	0.92	6.86	3.04	3.13	0.10	4.07	4.64
H4	0.65		0.15	0.22	0.27	2.13	0.17	0.19
H5	4.00		1.15	0.29	0.20	1.02	0.34	0.20
H6	0.95		6.13	7.68	0.25	0.25	5.95	3.54
H7	1.53		5.52	0.56	0.48	0.36	4.27	0.46
H8	0.82		1.27	0.23	2.64	2.58	0.92	5.27
H9	1.09		0.07	0.28	0.64	0.91	3.25	0.32

and EGF and TGF-beta, as mediators important for the development and differentiation of the intestinal epithelium, whose condition influences the permeability of the mucosal barrier for antigens and possible allergens. Increased expression of IL-4 and IL-13 and decreased expression of IFN-gamma point to the allergic phenotype of the colostrals cells of allergic mothers. Increased expression of EGF in the allergic group, although being significant, is not probably of practical importance because the colostrals cells are only minority producers of this growth factor.

In our previous work (Zizka et al. 2007), cytokine concentrations in colostrals were evaluated by ELISA. On a protein level, IL-4 and IL-13 showed similar trends as the gene expression in colostrals cells. IL-2 protein in colostrum was under the level of detection in both groups of mothers; IL-8 and IFN-gamma were not changed in allergic mothers; IL-10 was significantly increased, and TGF-beta and EGF were significantly decreased in the colostrals of the allergic group. The results of different research groups' testing of cytokine concentrations in the colostrum/milk of healthy and allergic mothers are very heterogeneous, with large individual variability (Zizka et al. 2007; Hashira et al. 2002; Rigotti et al. 2006; Peroni et al. 2010). However, it is not possible to compare cytokine gene expression by colostrals cells with cytokine concentration in colostrum because colostrals cells are not the only source of colostrals cytokines.

It is necessary to take into account that a certain portion of colostrals cytokines could be transferred through the intestinal wall at the very beginning of postnatal life and could influence the early development of the immune system. From this point of view, the regulatory cytokines TGF-beta and IL-10 can be of importance (Rigotti et al. 2006; Peroni et al. 2010). As mentioned above, the transfer of maternal colostrals cells to the newborn's circulation has been described in several mammal species, and Reber et al. (2008) proved the positive effect of these cells on the postnatal development of leukocytes in the early postnatal period of calves. Therefore, it is necessary to keep in mind a possible effect of colostrals cells having the allergic phenotype transferred from the allergic mother by breastfeeding. Nevertheless, it is difficult to distinguish between the effect of transferred colostrals cells and the action of soluble colostrals cytokines.

In conclusion, we are able to show in the present work the increased gene expression of Th2 cytokines (IL-4 and IL-13) and decreased expression of IFN-gamma in the colostrals cells of allergic mothers, in comparison with those of healthy ones. Our results point to an allergic phenotype of colostrals cells of allergic mothers with a bias to a Th2-type response. It remains a question if the small number of these cells could influence the immature newborn immune system.

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