

Effect of breast milk of healthy and allergic mothers on *in vitro* stimulation of cord blood lymphocytes

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Maternal milk has beneficial effects on the development and function of the newborn's immune system. Whether the milk of allergic mother has the same effects as the milk of healthy mothers is not yet quite clear. To contribute to the characterization of its immunomodulatory action, we tested the effect of milk of healthy and allergic mothers on the proliferation and immunoglobulin formation in cultures of cord blood mononuclear leucocytes (CBML) of newborns of healthy and allergic mothers. CBML proliferation was tested by ³H-thymidine incorporation, IgM, IgG and IgA production by reverse ELISPOT. CBML response was examined in unstimulated cultures and after stimulation with polyclonal activators in the presence or absence of colostrum or milk. The cells of children of allergic mothers have a significantly higher proliferative activity than those of children of healthy mothers.

Maternal colostrum/milk in high doses markedly suppresses cell proliferation after stimulation with polyclonal activators, whereas lower milk doses in the cultures have no such effect and exert a rather stimulatory action. Immunoglobulin production by cord blood lymphocytes is also different in the two groups of children. Low basal immunoglobulin formation is increased after stimulation with a strong polyclonal activator of B cells – *Bacillus firmus*, CBML of children of allergic mothers produce more IgA than those of children of healthy mothers. The stimulated production of all immunoglobulin classes in cells of children of healthy mothers is still enhanced by colostrum/milk. Children of allergic mothers show a markedly increased production of only IgM and IgA. The effect of healthy and allergic colostrum and milk on cell proliferation and immunoglobulin production is similar. The lymphocytes of children of allergic mothers differ from the lymphocytes of children of healthy mothers in their proliferative activity and the ability to form immunoglobulin already at birth.

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Breast feeding represents not only an optimum food for infants in the early postnatal period, but also affects the development of the newborn's immune system. Colostrum and milk contain a number of cytokines, growth factors, antibodies and other soluble components regulating the immune response, and several cell

types (macrophages, neutrophils, lymphocytes, epithelial cells) that take part in immune responses (1–3).

We can assume that the immunoregulatory components of colostrum and milk act not only locally in the gastrointestinal tract of the newborn, but can also cross the mucosal barrier and affect

both mucosal and systemic immunity because macromolecular substances are less degraded in the immature newborn's intestine; the possibility that intact proteins pass through the intestinal wall without losing their biological activity was repeatedly proved (4–6). The passage of cytokines with preserved biological activity was proved in animal models (7). The intestine of newborns has been found to contain live cells from the milk (8) and their passage through the intestinal wall was documented in sheep and calves (9, 10) and in baboons (11); it has been indirectly implicated also in humans (transfer of reactivity to tuberculin from the mother to the newborn; 12, 13). General consensus also exists about the beneficial effect of breast feeding on the infants; breast feeding is stated to lower the probability of later allergies, infections and some other pathological conditions (14, 15). This holds undoubtedly for the breast feeding of an infant by a healthy mother while there is some doubt as to the effect of breast feeding by allergic mother. Children of allergic mothers have an enhanced genetic predisposition for the development of allergy. Certain differences in the composition of milk of healthy and allergic mothers have been described (16–19); a question can be raised if the milk of an allergic mother could not affect the development of the newborn's immunity in a way different from that exerted by the milk of a healthy mother. Since an adverse effect of allergic milk has not been unambiguously documented, while the positive benefit of breast feeding is clear, allergic mother are not recommended to reduce the breast feeding (14). In this study, we investigated and compared the effect of soluble components of colostrum and milk of healthy and allergic mothers on the stimulation of cord blood mononuclear leucocytes (CBML) collected from children of both healthy and allergic mothers in order to contribute to solving the question whether the milk of allergic mothers affect the immune system of the newborn in a way different from that exhibited by the milk of healthy mothers.

Methods

Cord blood cells and maternal milk

Two groups of mothers and children according to mothers' clinical statement were used as donors: (i) Healthy individuals (H) – non-allergic mothers and newborn children of non-allergic mothers. (ii) Allergic individuals (A) – allergic mothers and newborns of allergic mothers. Cord blood was obtained from 17 children of healthy mothers and 16 children of allergic mothers.

Colostrum was given by 14 healthy and nine allergic mothers, milk by seven healthy and 10 allergic mothers. All samples were obtained after informed agreement of the mothers.

Diagnostics of allergy in mothers was based on clinical manifestation of allergy persisting for more than 24 months (allergy against respiratory and food allergens manifested by various individual combinations of high fever, conjunctivitis, bronchitis, asthma, eczema, etc.), monitoring by an allergologist, positive skin prick tests or positive specific IgE antibodies and antiallergic treatment before pregnancy.

Colostrum and milk. Colostrum and milk samples were collected on the third day and in the sixth month after delivery, respectively. Mothers expressed their breast milk manually into sterile polypropylene tubes and stored it at 4°C for 1–2 h; the fat layer and cell pellet were then removed by centrifugation (8000 g, 25 min) and soluble fraction was stored in aliquots at –20°C until processed. The effect of colostrum/milk on *in vitro* stimulation of CBMC was tested. CBMC could not be cultivated with the colostrum/milk of donor's own mother because it was not possible to obtain sufficient amount of colostrum at term and mature milk was taken 6 months after delivery. In each experiment, CBML were treated in parallel by both healthy and allergic colostrum/milk and one colostrum/milk sample was used for various CBML cultures.

Cord blood cells. Cord blood samples were collected in sterile heparinized tubes (10 U heparin/ml) immediately after delivery with the usual procedures including careful cleaning of the cord and strict puncture of umbilical vein to avoid maternal contamination. Cord blood mononuclear leucocytes were isolated on Ficoll-Paque, washed three times in MEM medium, incubated for 60 min to liberate cell-bound immunoglobulin (37°C, 5% CO₂), and again washed three times in MEM medium. Isolated CBML were used for testing proliferation and immunoglobulin formation *in vitro*.

Cell cultures

Cell cultures were performed in RPMI-1640 medium supplemented with L-glutamine (2 mM), HEPES – Sigma (0.002 M; Sigma-Aldrich, Munich, Germany), gentamicin (40 mg/l) and human transferrin (20 mg/l). The medium was further supplemented with 5% fetal calf serum (FCS) for proliferation assay or with 10% FCS

and 2-mercaptoethanol (25 μ M) for immunoglobulin formation. Cultivation proceeded at 37°C in humid atmosphere with 5% CO₂.

Proliferation assay. Cord blood mononuclear leucocytes were cultured in triplicate (2×10^5 cells per culture) in 96-well polystyrene plates. Cells were cultured alone (controls), with various doses of colostrum/milk (HC, HM – colostrums and milk of healthy mothers; AC, AM – colostrums and milk of allergic mothers), with polyclonal activators – Phytohaemagglutinin Sigma (PHA; 10 μ g/ml culture), Concanavalin A Sigma (ConA; 5 μ g/ml culture), heat-inactivated *Escherichia coli* O86 – EC (10^9 bacteria/ml culture), Nocardia Delipidated Cell Mitogen (NDCM; 20, 21; 100 μ g/ml culture) kindly donated by Prof. H. Tlaskalová, and formaldehyde-inactivated *Bacillus firmus* CCM 2212 (BF; 22, 23; 100 μ g/ml culture) and with polyclonal activators + colostrum/milk. The cultures were incubated for 48 h. After 48 h, the cells were pulsed with 1 μ Ci per well of ³H-thymidine and 18 h later harvested on glass fibre Filtermat A (Wallac, Turku, Finland) by Harvester 96 Mach 3 (Tomtec, Orange, CT, USA) and counted in 1450 MicroBeta (Wallac) using melt-on scintillator 1450-441 Meltilex A (Wallac). Results are expressed as counts per minute (cpm).

Immunoglobulin formation. CBML were cultured in duplicate in polystyrene tubes (10^6 cells in 1 ml culture) in supplemented RPMI-1640 medium as described above. Cells were cultured alone (controls), with colostrum/milk in a final dilution of 1:100, with polyclonal activators of B lymphocytes (EC, BF), with Pokeweed mitogen Sigma (PWM; 1 μ g/ml) and with polyclonal activators + colostrum/milk. Immunoglobulin-producing cells were detected by reverse ELISPOT (24). After 6-day cultivation, the cells were washed. 96-well ELISPOT plates with nitrocellulose bottom (Millipore MultiScreen_{HTS}; Millipore, Bedford, MA, USA) were coated overnight with 100 μ l of goat antihuman immunoglobulin antibodies (anti- μ , anti- γ or anti- α antibodies; Immunotech, Birmingham, UK) in 0.1 M sodium carbonate buffer pH 9.6, washed and blocked 30 min with cultivation medium containing 10% FCS. Then, washed cells were applied (10^5 cells per well) in triplicate and cultivated overnight. On the next day, the cells were discarded and the plates were processed in a similar way as in the ELISA method but with 3-amino-9-ethylcarbazole (AEC) as substrate. Spots were counted by binocular stereomicroscope (Motic, Wetzlar, Germany). Results are expressed as the number

of spots (corresponding to immunoglobulin-producing cells)/ 10^6 cells.

Statistics

The data are presented as mean $\pm$ s.e.m. Differences between groups were evaluated using non-parametric Mann–Whitney *U*-test. A probability level $p \leq 0.05$ was considered significant.

Results

Proliferation of cord blood mononuclear leucocytes

The processes to be studied included spontaneous proliferation of cells alone, proliferation of cells cultured with different dilutions of colostrum/milk, cells stimulated with polyclonal activators and cells cultivated with polyclonal activators + colostrum/milk. The cultures were supplied with milk undiluted and in four 10-fold dilutions so that the resulting milk dilutions in the cultures were from 1:5 to 1:50,000. Unstimulated cord blood cells in culture exhibit a very low spontaneous activity and its change caused by colostrum/milk is inconspicuous and difficult to evaluate. We therefore stimulated the cells in culture by polyclonal activators and studied the effect of milk on this activation.

Proliferation of unstimulated cells and cells after stimulation with polyclonal activators. Cord blood cells of children of allergic mothers, when cultivated in a culture without stimulation (spontaneous proliferation), incorporate significantly more ³H-thymidine than cells of children of healthy mothers. After stimulation with polyclonal activators, the cells of children of allergic mothers also proliferate much more intensively than cells of children of healthy mothers. This points to a higher reactivity of lymphocytes from children with a higher risk of allergic affliction (Table 1).

Effect of colostrum and milk on proliferation. Cultivation of cord blood cells from children of healthy and allergic mothers with colostrum or milk does not cause statistically significant change of the proliferation, although there is a perceptible tendency of higher milk doses to lower the proliferation while lower doses increase it.

No statistically significant differences were found between the effect of colostrum of healthy and allergic mothers. However, the proliferation is clearly affected in the presence of polyclonal activators. The presence of colostrum/milk in the culture in a final dilution of 1:5 very markedly

Table 1. *In vitro* proliferation of CBML after stimulation with polyclonal activators tested by ³H-thymidine incorporation

Stimulator	Proliferation (cpm)				p-Value
	CBML(H)		CBML(A)		
	cpm	s.e.m.	cpm	s.e.m.	
0 (control)	3619	2045	7267	3304	0.0026116
PHA	140,384	40,075	251,467	69,113	0.0000017
ConA	103,810	48,042	209,278	60,761	0.0000015
BF	9962	4480	25,571	8863	0.0000001
EC	4846	2493	14,190	7972	0.0000204
NDCM	17,185	15,198	50,688	21,940	0.0000072

Comparison of children of healthy and allergic mothers.

CBML, cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; PHA, phytohaemagglutinin; ConA, concanavalin A; BF, *B. firmus*; EC, *Escherichia coli*; NDCM, Nocardia Delipidated Cell Mitogen; cpm, counts per minute; p-Value, significance of differences between CBML of children of healthy and allergic mothers.

Mean values of 12 experiments with CBML(H) and 11 experiments with CBML(A).

decreases the stimulation with polyclonal activators. The decrease was observed in some cases even at a colostrum/milk dilution of 1:50. Lower dilutions of milk and colostrum tend to increase the cell stimulation but the differences are not significant. However, even in this case no significant difference was shown between the effect of colostrum/milk of healthy and allergic mothers (Fig. 1). The significance of differences between the stimulation with polyclonal activator alone and with polyclonal activator together with maternal colostrum/milk at 1:5 final dilution is given in Table 2.

Ig formation by cord blood lymphocytes

Production of immunoglobulin of individual classes by CBML was detected by reverse ELISPOT.

Ig formation by unstimulated lymphocytes and after stimulation with polyclonal activators. Spontaneous production of immunoglobulins of all classes by unstimulated cord blood cells is minimal and only isolated spots can be detected. In our experimental set up, EC does not stimulate immunoglobulin formation and PWM increases the number of IgM-producing cells only in children of healthy mothers. The most potent stimulator of all was *B. firmus*, which markedly increases the production of IgM in both healthy and allergic group ($p = 0.005$ and $p = 0.001$, respectively), does not affect significantly IgG formation and perceptibly increases IgA formation in the healthy group ($p = 0.002$), and still more conspicuously, in the allergic group ($p = 0.00004$). The difference in IgA stimulation between children of healthy and allergic mothers is significant at $p = 0.0043$ (Table 3).

Effect of colostrum/milk on Ig formation. Cultivation of cord blood cells from children of both healthy and allergic mothers with colostrum alone (healthy or allergic) does not perceptibly affect the basal formation of immunoglobulin. When using colostrum/milk together with a strong stimulator – *B. firmus* – all individual analyses of cells of children of healthy mothers show pronounced increase in the number of cells producing immunoglobulin of all classes while only IgM formation is markedly increased in children of allergic mothers. The results are highly convincing although they are not significant in all cells/milk combinations in view of the large individual variability and low number of examined samples. The changes have the same trend in all individual cases. There is no difference between the action of colostrum of healthy and allergic mothers (Fig. 2). In stimulations with *E. coli* and PWM the numbers of immunoglobulin-producing cells are very low and the effect of colostrum is therefore difficult to assess. When stimulating the cells from children of allergic mothers, both normal and allergic colostrum causes a less marked increase of the effect of polyclonal activators than in the stimulation of cells from children of healthy mothers. 6-month milk affects immunoglobulin formation in the same way as colostrum but the effect is smaller.

Discussion

The direction of the perinatal development of the immune system is partially determined by the character of interactions of newborn's cells with regulatory factors acquired from the mother by transplacental transfer or through the maternal milk, or prenatally through the amniotic fluid. Several groups have described the differences,

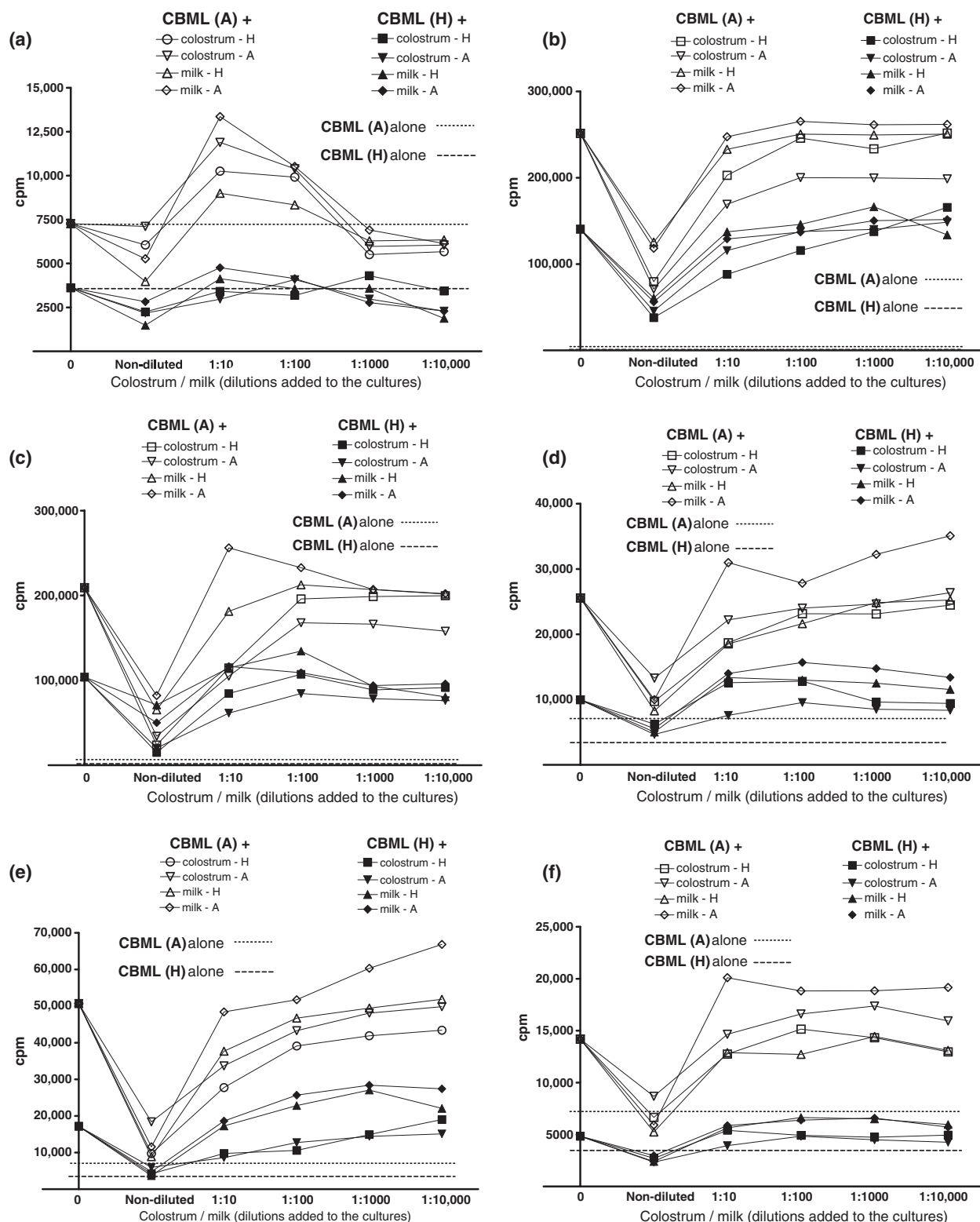


Fig. 1. Effect of various concentrations of maternal colostrum/milk on *in vitro* proliferation of cord blood mononuclear leucocytes stimulated by polyclonal activators. (a) Non-stimulated controls cultivated with various concentrations of milk. (b) Stimulation with phytohaemagglutinin. (c) Stimulation with concanavalin. (d) Stimulation with *Bacillus firmus*. (e) Stimulation with *Nocardia Delipidated Cell Mitogen*. (f) Stimulation with *Escherichia coli*. CBML: cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; colostrum/milk H, A: colostrum/milk of healthy, allergic mothers, respectively; dashed line: proliferation of non-stimulated CBML of children of healthy mothers; dotted line: proliferation of non-stimulated CBML of children of allergic mothers. Each curve represents mean of seven experiments.

Table 2. Effect of non-diluted colostrum and milk (final dilution in culture = 1:5) on *in vitro* proliferation of CBML stimulated by polyclonal activators

Stimulator	Statistical significance (p-value)							
	CBML(H)				CBML(A)			
	Colostrum(H)	Colostrum(A)	Milk(H)	Milk(A)	Colostrum(H)	Colostrum(A)	Milk(H)	Milk(A)
PHA	0.000009	0.000003	0.006120	0.001032	0.000003	0.000006	0.005821	0.000049
ConA	0.000003	0.000002	0.415916	0.002174	0.000002	0.000002	0.000011	0.000007
BF	0.112838	0.017339	0.027936	0.005546	0.000106	0.000073	0.000002	0.000005
EC	0.054754	0.009555	0.008591	0.078014	0.011916	0.050505	0.003497	0.001280
NDCM	0.000287	0.011489	0.000374	0.000912	0.000007	0.000314	0.000001	0.000002

Statistically significant differences between proliferation of cells stimulated with polyclonal activator alone and with activator + colostrum/milk.

CBML, cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; PHA, phytohaemagglutinin; ConA, concanavalin A; BF, *Bacillus firmus*; EC, *Escherichia coli*; NDCM, Nocardia Delipidated Cell Mitogen.

Mean values of seven experiments in each combination [CBML(H) + col(H); CBML(H) + col(A); CBML(H) + milk(H); CBML(H) + milk(A); CBML(A) + col(H); CBML(A) + col(A); CBML(A) + milk(H); CBML(A) + milk(A)].

Table 3. Immunoglobulin-forming cells detected by reverse ELISPOT after *in vitro* stimulation of CBML by polyclonal activators

Stimulator	Spots/10 ⁶ CBML								
	IgM			IgG			IgA		
	CBML(H)	CBML(A)	p-Value	CBML(H)	CBML(A)	p-Value	CBML(H)	CBML(A)	p-Value
O (control)	2.50	3.08	0.8818	12.22	1.58	0.4264	14.25	5.50	0.1919
EC	1.83	1.00	0.4255	27.78	1.75	0.6827	3.25	3.50	0.5395
BF	205.75	353.08	0.1939	26.22	22.50	0.8307	86.75	220.33	0.0043
PWM	37.25	1.42	0.0136	14.89	3.92	0.5129	5.83	7.83	0.7490

CBML, cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; EC, *Escherichia coli*; BF, *Bacillus firmus*; PWM, pokeweed mitogen; p-value, statistical significance of differences between CBML of children of healthy and allergic mothers.

Mean values of five experiments with CBML(H) and five experiments with CBML(A). Immunoglobulin-producing cell estimation in each experiment was carried out in triplicate.

though not always unequivocal, in the composition of the milk of healthy and allergic mothers (16–19). The question therefore arose whether the milk of allergic mothers does not enhance the risk of a later development of allergic afflictions in children of allergic mothers (although genetic factors are known to play a decisive role).

We tried to contribute to the characterization and comparison of immunomodulatory activity of milk of healthy and allergic mothers by following the effect of milk on *in vitro* activation of cord blood cells. These experiments cannot reflect precisely the *in vivo* situation, but we do not see presently any other possibility how to address this problem. As in all other experiments, *in vitro* tests are not equivalent to *in vivo* situation and we were not able to obtain a sufficient amount of newborn cells in another way than from the cord blood. Besides, the effect of maternal milk on the proliferation of normal mononuclear leucocytes from peripheral blood and cord blood and on the production of cytokines by these cells was studied also by others (25–28). Differences between CBML of

children of healthy and allergic parents have previously been followed, albeit without testing the effect of milk (29, 30). We studied the stimulation of CBML from children of healthy and allergic mothers and its change under the action of colostrum and mature milk of healthy or allergic mothers, with the use of different combinations of cell and milk samples. CBML is a heterogeneous population of cells – this is similar to the *in vivo* situation where co-operation of various cell types is necessary for induction of the majority of immune reactions. Like in peripheral blood cells (27, 28), we showed in cord blood cells a lowering of proliferation by high concentrations of colostrum and milk. According to the literature, maternal milk has an immunosuppressive activity and helps thereby to induce oral tolerance to a number of food antigens and environmental antigens in breast-fed children (31–33). It lowers the probability of induction of IgE antibody response by these antigens in genetically predisposed individuals. However, we do not assume that milk, after being diluted in the gastrointestinal tract and

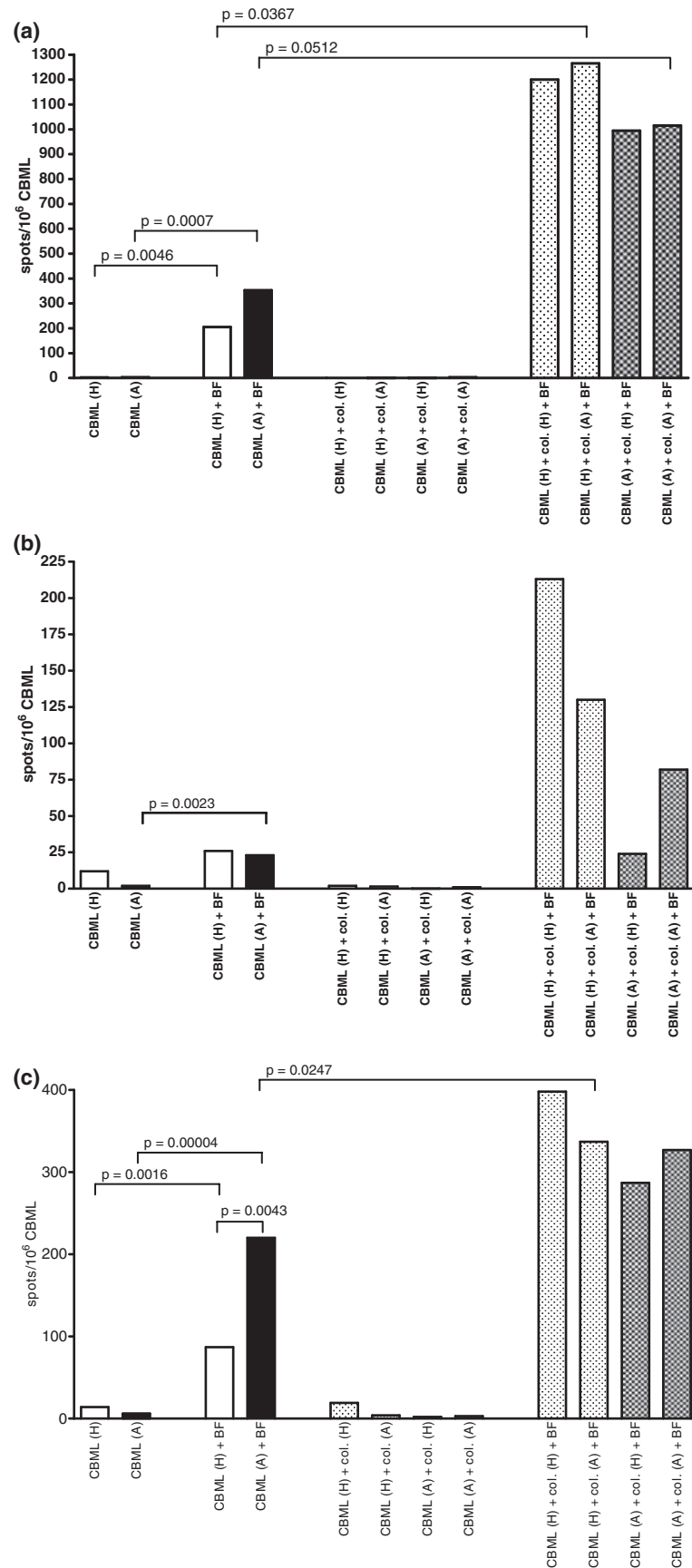


Fig. 2. Effect of maternal colostrum on the number of IgM-, IgG- and IgA-forming cells in cultures of cord blood mononuclear leucocytes stimulated by *Bacillus firmus* (measured by reverse ELISPOT). (a) Effect of maternal colostrum on the number of IgM-forming cells. (b) Effect of maternal colostrum on the number of IgG-forming cells. (c) Effect of maternal colostrum on the number of IgA-forming cells. CBML: cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; col: colostrum of healthy (H) and allergic mothers (A); BF: *B. firmus*. Mean values of four experiments in each combination [CBML(H) + col(H); CBML(H) + col(A); CBML(A) + col(H); CBML(A) + col(A)].

passing through the intestinal wall, could be present in the intercellular space in an amount that would cause significant suppression *in vivo*. Milk in higher dilutions, which could be encountered *in vivo*, has a rather stimulatory effect on proliferation. The low significance of this stimulatory effect is probably due to large individual differences, the overall trend is, however, appreciable in all individual experiments. We found no substantial differences between the action of allergic and healthy milk and our results do not support the apprehension of breast feeding in allergic mothers. It is in keeping with the results of Böttcher et al. (34) who did not find any correlation between cytokine composition of milk of healthy and allergic mothers on one hand and future allergy manifestation in their children on the other hand.

An important result of these experiments was the finding of a significantly higher proliferation activity, both spontaneous and after stimulation with polyclonal activators, of cells from children of allergic mothers. The higher reactivity of cells from these children allows us to assume that these cells could also be more reactive after encounter with allergens. The question remains if these cells cannot be pre-stimulated already during the intrauterine development. It should be useful to follow the development of cell reactivity in postnatal ontogeny but it is difficult to perform it for ethical reasons.

Differentiation of cord blood cells into immunoglobulin-producing cells was tested after cultivation of cells by reverse ELISPOT. The cells of children of both healthy and allergic mothers were again stimulated and the stimulation was affected by the colostrum/milk of healthy and allergic mothers. In view of the above-mentioned low probability of a contact of newborn's lymphocytes with high milk concentrations, we used milk in a final dilution of 1:100 for influencing immunoglobulin production. Spontaneous immunoglobulin formation in cord blood cells is very low and, among the polyclonal activators, it was markedly stimulated only by BF, which has previously been described to exert a strong stimulatory action on B lymphocytes in both humans and mice (22, 23). The present results show that BF stimulates well also IgM and IgA production by cord blood lymphocytes, whereas stimulation of IgG formation is less significant. The effect of milk could best be assessed with a simultaneous stimulation with this activator. In general, colostrum/milk in the above dilution has a stimulatory effect on immunoglobulin formation after polyclonal activation. While the

number of experiments may not be large enough to provide sufficiently massive support for this conclusion, the fact that all possible cells/milk combinations tested [CBML(H) + colostrum(H); CBML(H) + colostrum(A); CBML(H) + milk(H); CBML(H) + milk(A); CBML(A) + colostrum(H); CBML(A) + colostrum(A); CBML(A) + milk(H); CBML(A) + milk(A)] yielded very similar results represent a serious piece of evidence. Like with blast transformation, we found no distinct differences between the action of healthy and allergic colostrum/milk. Milk acts in the same way as colostrum but its effect is less pronounced, probably due to the lower concentration of immunologically active components in mature milk. We again showed differences in the reactivity of cord blood lymphocytes of children of healthy and allergic mothers. IgA production after stimulation with BF is increased much more in children of allergic mothers than in children of healthy mothers. Addition of colostrum to cultures of cells from children of healthy mothers stimulated with BF markedly increases the production of immunoglobulin of all three classes; in cells from children of allergic mothers this increase is lower and is clearly seen only in IgM. It therefore seems that the ability of spontaneous and stimulated IgM formation is comparable in children of healthy and allergic mothers. The higher ability of IgA formation after polyclonal activation of cells of children of allergic mothers could represent a physiological compensatory mechanism in children with a higher risk of allergy. IgA in the intestine contributes to the elimination of antigens and is assumed to reduce thereby the probability of allergy induction (35).

In conclusion, it can be stated that maternal milk in concentrations that are likely to occur *in vivo* has stimulatory rather than inhibitory effects on newborn's lymphocytes. No differences were found in the action of colostrum/milk of healthy and allergic mothers but substantial differences were found in the reactivity of cord blood lymphocytes of children of healthy and allergic mothers.

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