

Breast milk cell supernatants from atopic donors stimulate cord blood IgE secretion *in vitro*

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Summary

Breast milk cell culture supernatants from atopic mothers (nineteen) were compared by ELISA with normal breast-feeding controls (twenty-one) for regulation of cord blood lymphocyte IgA and IgE secretion *in vitro*. A minority of atopic (five out of nineteen) and normal (three out of twenty-one) cell supernatants stimulated cord blood lymphocyte IgA release to the same extent. The others were inactive. Stimulation was not related to breast milk cells donor atopic history or cord blood lymphocyte atopic heredity.

In contrast, 70% of atopic milk cell supernatants stimulated cord blood lymphocyte cultures to form IgE ($\bar{x} \pm \text{s.d.} = 2070 \pm 2240$ pg/culture) while stimulatory supernatants (24%) from normal donors resulted in less lymphocyte IgE release ($\bar{x} \pm \text{s.d.} = 680 \pm 490$ pg/culture) ($p < 0.001$). These differences did not correlate with breast milk cell supernatant IgE concentrations, cord blood donor serum IgE levels or atopic heredity.

Introduction

Data from many laboratories and clinical studies have indicated the influence of maternal immune status and breast feeding on specific offspring humoral and cellular responses (Hale, Hanna & Hansen, 1976; Uphoff, 1977; Head and Beer, 1979; Mohr, 1973; Juto *et al.*, 1982). Sufficient evidence of the immunoregulatory potential of breast milk cells also exist to warrant consideration of their effects on the development of neonatal immune responses that might be involved in atopic disease.

The work of Katz and his colleagues (Katz 1980) showing that cultured human lymphoid cells elaborate separable factors capable of enhancing or suppressing mouse IgE production and the study of Pittard & Bill (1979) indicating that the products of cultured breast milk cells may induce the *in vitro* differentiation of IgA-producing cord blood lymphocytes (CBL), provide illustrations of how such regulation might be accomplished. It seems reasonable to consider that the development of atopy in an

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infant might partly depend upon the balance of immunologically active factors and regulators in the milk of its mother rather than pursuing the perhaps misleading 'Breast vs Bottle Debate' (Fergusson *et al.*, 1981; Juto *et al.*, 1982; Atherton, 1982; Fergusson, 1982).

Because some workers have long maintained that raised IgE and transient IgA deficiency are both associated with childhood atopy (Taylor *et al.*, 1973; Soothill *et al.*, 1976) we compared the abilities of milk cell culture products from atopic or control donors to influence the *in vitro* production of IgA and IgE by cord blood cells employing the culture system described by Pittard & Bill (1979). Two questions were posed: (i) Are breast milk cells capable of influencing the development and secretion of IgA- and IgE-producing CBL? and (ii) Do atopic mothers differ from control mothers in this capacity?

In this paper we report that human breast milk cells contain and release products, in culture, that influence both CBL IgA and IgE production *in vitro* and that CBL IgE synthesis is significantly enhanced by factors obtained from the milk cells of atopic mothers compared with controls.

Materials and methods

Breast milk cell supernatants

Breast milk cells were obtained within 4 days *post partum* from two groups of mothers: (i) those with clear clinical histories and laboratory evidence of IgE-associated skin, respiratory, mucosal, gut or insect sting reactivity to identifiable allergens who were otherwise healthy ($n=19$), (ii) healthy controls in whom there was no indication whatever of food or environment intolerances, skin, gut or respiratory complaints, headaches or reactions to insect stings or bites that could be attributed to IgE-associated atopic reactivity ($n=21$).

Each specimen was expressed into a sterile siliconized glass container. The whole milk was diluted 1:1 with medium (RPMI 1640) and the cells separated by centrifugation (300 *g* for 10 min at room temperature). The isolated cells were resuspended, counted with a haemocytometer and adjusted to 2×10^6 macrophages per ml of complete medium (RPMI 1640 containing glutamine, penicillin, streptomycin and 10% fetal calf serum) following morphological differentiation of stained dried smears and non-specific esterase staining (Yam, Li & Crosby, 1971). Two ml aliquotes of the cells were incubated for 7 days at 37°C in a humid 5% CO₂ environment then centrifuged (400 *g* for 10 min at room temperature) and the milk cell supernatant withdrawn for storage at -20°C before testing. Preculture, cell contents, supernatants were obtained from culture replicate samples by centrifugation (1000 *g* for 15 min at 22°C) after freezing and thawing ($\times 3$) in a solid CO₂/methanol bath.

Cord blood lymphocyte (CBL) cultures

Cord blood lymphocytes were isolated from Ficoll/Hypaque density gradient sedimentation (Boyum, 1968) from 30 ml of heparinized (preservative-free) blood drawn from nine separate placentas. Cell identification and counting were performed as before and viability was assessed by trypan-blue exclusion (McLimans *et al.*, 1957). The atopic status of each of the CBL donor's parents was assessed by interview as well as medical records whenever possible.

CBL cultures were prepared in triplicate sets of (i) CBL alone, (ii) CBL plus

preculture BMC supernatant, or (iii) CBL plus BMC culture supernatant. Each culture tube (Falcon, 2063, Oxnard) contained 2×10^6 CBL suspended in 2 ml of complete medium. To each tube a 100- μ l aliquot of BMC supernatant (or control medium) was added. In each of the nine CBL culture sets BMC supernatants from several donors were used as available. The CBL control and test cultures were incubated for 7 days at 37°C in a humid 5% CO₂ atmosphere prior to harvest of the culture supernatants after centrifugation (400 g for 10 min at 22°C).

Measurement of IgA and IgE

The amounts of IgA and IgE in BMC supernatants, cord blood sera and CBL culture media were measured by enzyme-linked immunosorbent assays (ELISA) performed in ninety-six-well polystyrene micro-ELISA plates. For IgE assays the wells were filled with 200 μ l of goat anti-human ϵ -chain-specific affinity purified antibody (Tago, Biotek, Auckland, New Zealand, lot 04-05-01) at 400 ng/ml. Similarly purified goat anti-human α -chain-specific antibody (Tago, lot 01-04-01/02) was used at 800 ng/ml as primary coating antibody for IgA determinations. After overnight incubation (22°C) the wells were washed ($\times 3$) with physiological phosphate buffered saline containing 0.5% Tween 20 (PBS-Tween) before the addition of 250 μ l of 10% fetal calf serum (FCS) in sodium bicarbonate buffer (0.1 M, pH 9.6) to block subsequent non-specific binding. After washing with PBS-Tween ($\times 3$) 200 μ l of the samples were added to individual wells following dilution, when necessary, in 5% FCS-PBS-Tween and incubation for 2 hr at 22°C in a humid chamber.

The wells were again washed ($\times 3$) and 200 μ l of either horseradish peroxidase conjugated goat anti-human ϵ chain (Tago, lot 94-01-01) or α chain (Tago, lot 91-02-01) affinity purified secondary labelling antibodies were added to each well (diluted 1/3000 with 5% FCS-PBS-Tween) and incubated for 2 hr as before. After washing ($\times 3$) 200 μ l of ortho-phenylenediamine (OPD) (Sigma, St Louis, U.S.A., No-P3888) substrate (40 mg OPD in 100 ml phosphate-citrate buffer, pH 5.0 with addition of 40 μ l 30% H₂O₂) were added to each well. Following development in the dark the reaction was stopped with addition of 50 μ l of 2.5 M H₂SO₄ and colour read at 492 nm (Titertek Multiscan). For each ELISA carried out ten-point IgA and IgE standard curves (0.5–512 ng/ml) were run in parallel with samples on each plate. Generally, each culture sample was assayed once but triplicate cultures were performed for each data point as the variability between culture tubes (coefficient of variation, 13.5%, range 4.6–21%) exceeded that of the ELISA technique (coefficient of variation 4.2%, range 1.3–8.0%). The IgE standard used was that employed in the Phadebas IgE PRIST assay (Pharmacia Diagnostics AB, Uppsala, Sweden). The IgA standard was supplied by Hoechst, Behring Diagnostics (No. OTRB 07, Auckland, New Zealand).

The data reflecting CBL IgA and IgE *in vitro* release were normally distributed and comparisons of grouped data means $\pm$ s.d., presented in the text and tables, were carried out using Student's *t*-test.

Results

Stimulation of CBL IgA by milk cell supernatants

Comparisons of breast milk cell IgA content at the beginning of culture and release into the supernatant after incubation indicated no significant differences ($P < 0.05$)

Table 1. IgA release from CBL cultured with BMC supernatants from atopic and normal donors

Milk cell donors stimulatory/total	Stimulatory BMC supernatants n/total tests		CBL IgA* ng/culture (mean $\pm$ s.d.)
Atopic 5/19 (26%)	freeze/thaw	4/59	310 $\pm$ 280
	after culture	7/59	300 $\pm$ 250
Normal 3/21 (14%)	freeze/thaw	4/59	490 $\pm$ 688
	after culture	4/59	975 $\pm$ 1030

* $P > 0.05$ in all comparisons.

between normal and atopic mothers. Secreted IgA after 7 days of culture exceeded total IgA milk cell contents at the outset of incubation in thirteen of nineteen atopic donors (mean gain of 69 ± 83 μ g/ml) and in fifteen of twenty one normal donors (mean gain 42 ± 54 μ g/ml).

Cord blood serum IgA levels did not exceed 5 mg/100 ml and did not correspond to the atopic heredity of the donor neonate. After 7 days incubation the median viability for CBL alone and CBL plus 100- μ l milk cell supernatants exceeded 80% in both cases. Low-level IgA synthesis was detected in seven out of ninety-three control CBL cultures (4.6 ± 4.8 ng/culture). The results presented in Table 1 indicate that with the addition of 100 μ l of 7-day milk cell culture supernatants from atopic donors increased IgA synthesis was detectable in seven of fifty-nine cultures (300 ± 250 ng/culture) and significantly exceeded ($P < 0.001$) that measured at the onset of culture. Of fifty-nine CBL cultures incubated with 100 μ l of atopic donors' breast milk cell contents obtained by freeze/thawing prior to incubation, four were stimulated to synthesize 310 ± 280 ng of IgA per culture. Overall, atopic donor milk cell supernatants (before and after culture) stimulated five of the nine cord blood donors' cells to produce IgA in culture. These stimulatory supernatants represented milk cell products obtained from five of the nineteen atopic donors. Eight out of fifty-nine CBL cultures produced IgA in the presence of 100 μ l of normal donors' milk cell supernatants (four obtained before = 490 ± 688 ng/culture; four obtained after 7 days of culture 975 ± 1030 ng/culture). These IgA-inducing cell supernatants were derived from three of the twenty-one normal milk donors. Responsive CBL cultures represented four of the nine cord blood donors. In summary, CBL from five of nine donors responded to at least one breast milk cell supernatant by producing IgA *in vitro*. Neither the ability to produce IgA, or the response to either atopic or normal milk cell supernatants, was related to the atopic heredity of the CBL donor or the IgA concentration of the cord blood serum. Moreover, the ability of milk cell supernatants to stimulate CBL IgA synthesis was not related to the atopic status of the breast milk donor nor the IgA contents or *in vitro* release of the milk cells.

Stimulation of CBL IgE by milk cell supernatants

Breast milk cell suspension aliquots from nineteen donors contained 4280 ± 3150 pg/ml of IgE prior to culture and released into the culture medium 4210 ± 3530 pg/ml following 7 days of incubation. Corresponding figures relating to breast milk cell

aliquots from twenty-one normal donors were 2890 ± 2200 pg/ml and 3420 ± 2360 respectively. Differences between these mean values were not significant at the $P < 0.10$ level.

Cord blood lymphocyte baseline synthesis of IgE after 7 days' incubation in complete medium was ≤ 500 pg/culture in eight of the nine donors tested (1000 pg culture was attained by a donor without atopic parents). Each of the nine cord blood cell samples investigated was capable of being stimulated to produce greater than baseline amounts of IgE with at least one breast milk cell supernatant. However, some CBL were stimulated by one milk cell supernatant and not by another.

Of the nineteen atopic donor milk cell products tested, thirteen (70%) were stimulatory to one or more CBL cultures. Of the twenty-one normal control milk cell supernatants tested, five (24%) stimulated IgE production when added to at least one CBL sample. When net IgE release in all the stimulated CBL cultures were compared

Table 2. IgE release from CBL cultured with BMC supernatants from atopic and normal donors

Milk cell donors stimulatory/total	Stimulated CBL IgE release pg/culture	
	mean $\pm$ s.d.	range
Atopic 13/19 (70%)	2070 ± 2240	300-7600
Normal 5/21 (24%)	680 ± 490	100-1400

($P < 0.001$)

Table 3. Parenteral atopy, cord serum IgE and CBL IgE release *in vitro*

Blood Cord	Parental Atopy (δ/φ)	Cord Serum IgE (ng/ml)	CBL IgE release (<i>in vitro</i>)			Stimulated CBL cultures $\ddagger$ (%)
			Spon. rel. pg/culture*	max. stim. index % $\dagger$	max. IgE pg/culture	
1	+/+	3.2	< 500	320	1250	30
2	+/+	1.9	500	1260	6300	33
3	+/+	1.9	500	220	1100	14
4	+/-	0.5	< 500	490	1900	67
5	+/-	2.9	< 500	350	1530	20
6	-/-	3.2	< 500	140	350	33
7	-/-	2.9	< 500	1550	7600	100
8	-/-	2.6	1000	260	2600	17
9	-/-	0.4	< 500	280	1400	8

* IgE release from CBL alone.

$\dagger$ Maximum net IgE release/mean CBL spontaneous IgE release.

$\ddagger$ Number of stimulatory BMC supernatants (i.e. net IgE > spon. rel. IgE)/total number of BMC supernatants tested.

atopic BMC supernatants induced an IgE increase of 2070 ± 2240 pg/culture (range 300–7600 pg/culture) while stimulatory milk cell supernatants from normal donors increased IgE release 680 ± 490 pg/culture (range 100–1400 pg/culture). Comparison of these means indicated a highly significant difference ($P < 0.001$). These results are summarized in Table 2.

Taken together, these results suggest that BMC supernatants from atopic donors are more likely to stimulate significantly greater CBL IgE synthesis *in vitro* than are supernatants derived from normal, non-atopic, donors' milk cells. This apparent difference in the immunoregulatory capacities of milk cell products did not correlate with either the IgE concentrations of the individual supernatants added to the CBL cultures or the atopic heredity of the CBL donors. It is also worth noting that the response of CBL to milk cell supernatants did not reflect cord blood sera IgE concentrations regardless of whether one considered (i) the unstimulated baseline IgE synthesized *in vitro*; (ii) the maximal IgE synthesis attainable by a particular CBL sample; or (iii) whether the CBL sample was more or less often stimulated by the various atopic or control milk cell supernatants added (see Table 3).

Discussion

The results suggest that breast milk cells contain, and secrete in culture, factors that may influence the *in vitro* formation of IgA- and IgE-secreting lymphocytes from neonates. It was also found that milk cell supernatants derived from atopic mothers were approximately three times more likely to induce three times as much IgE released from cultured cord blood lymphocytes than were supernatants from normal mothers.

The development of IgA secretion by IgA-bearing neonatal lymphocytes *in vivo* (Lawton *et al.*, 1972; Brasher & Hartley, 1969) and *in vitro* (Wu *et al.*, 1976; Pittard & Bill, 1979) may be subject to isotype-specific immunoregulation (Kearney & Lawton, 1975; Klinman & Press, 1975). Pittard & Bill (1979) have reported such control (independent of IgG and IgM production) by soluble products obtained from cultured breast milk cells but not by stimulation with pokeweed mitogen.

The proportion of milk cell supernatants demonstrating this immunoregulatory capability in their study was not indicated. And, although our results give some support to those findings they also indicated that such immunoregulation was not a feature of more than a minor proportion of the forty milk cell supernatants studied. It was also noted that the effects were not consistent from one CBL culture to another. These findings might account for the negative results of Crago *et al.*, (1981) who appear to have carried out eight similar tests using an unspecified number of BMC supernatants. We were unable to demonstrate a significant difference in the capacities of atopic and normal BMC supernatants to influence CBL IgA release *in vitro*.

The effects of BMC supernatants on CBL IgE release contrasts with CBL IgA in that (i) a greater proportion of supernatants from atopic (70%) than normal (24%) donors were stimulatory, and (ii) CBL stimulated by atopic BMC supernatants released significantly more IgE in culture than did those stimulated with control supernatants.

Whether these *in vitro* interactions have any relevance *in vivo* is unknown. However, the likelihood of this is increased by current understanding of the transfer of immunoregulators in milk from mother to infant and the specific regulation of IgE responses. For example, immunoregulatory cells (Head & Beer, 1979; Hale *et al.*, 1976; Uphoff, 1977) humoral factors and antigen (Frier, Hosking & Hill, 1981; Bienenstock,

McDermott & Befus, 1979; Hanson *et al.*, 1979) are transferred in active forms in the breast milk. In addition, breast milk cells are known to gain access to the tissues and circulation of the suckling neonate (Head & Beer, 1979) while Jarrett & Halls' (1979) experiments in rats (immunized and non-immunized to specific antigens) show that the passive cutaneous anaphylactic responses of offspring depend not upon the womb from which they came but the breast at which they suckled.

The predominant lymphocytes of breast milk are known to include antigen-reactive and functionally selected T-cell populations (Parmely & Williams, 1979; Head & Beer, 1979). Furthermore, several groups (Strannegård & Strannegård, 1979; Hughs *et al.*, 1982; Björkstén, 1983) have reported T-cell subpopulation abnormalities associated with atopic individuals; most notably a relative deficiency of suppressor T cells which may, in part, explain their greater numbers of IgE-bearing cells and hyperproduction of IgE (Matsumoto *et al.*, 1981).

IgE production *in vivo* (Katz, 1980) and *in vitro* (Zuraw *et al.*, 1981; Saxon & Stevens, 1979) is T dependent and not directly related to the amounts of other immunoglobulin isotypes. Katz and his colleagues also demonstrated (for review see Katz, 1980) that the IgE phenotype of the T-cell contributor determines where the balance occurs between enhancement or suppression of IgE production. Recent work reported by Ishizaka (1983), Katz *et al.* (1983) and Kishimoto *et al.* (1983) indicates that the environment in which antigen-specific T suppressor cells are stimulated appears to determine the differentiation of a further T-cell subtype to produce one or the other of two closely related IgE-binding factors (suppressive or enhancing) that determine the amounts of IgE secreted by stimulated B cells.

The effects of atopic and normal BMC supernatants on CBL IgE production described here may reflect similar interactions. This possibility is currently under investigation since the supplementation of atopic breast milk with an appropriate balance of IgE regulatory factors could provide a feasible means of avoiding the development of atopy in the genetically predisposed neonate.

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