Effects of breast milk from allergic and non-allergic mothers on mitogen- and allergen-induced cytokine production

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Abstract

Breast-milk contains several components that provide specific immunity and affect the maturation of the infant's immune system.

The aim of this study was to analyse the effects of breast milk, on mitogen and allergen induced cytokine production from cord blood mononuclear cells (CBMC), and if those effects differ between allergic and non-allergic mothers.

The cells were incubated for 96h with phytohaemagglutinin, ovalbumin or cat dander in the presence of various dilutions of colostrum.

Colostrum inhibited both mitogen and cat induced IFN-γ and mitogen induced IL-4 production. The inhibition on IFN-γ production was to some extent caused by TGF-β, since the effect was modified when an anti-TGF-β antibody was added to the cultures. In contrast, colostrum enhanced allergen induced production of the Th2-like cytokines IL-5 and IL-13, and this was accompanied with increased production of IL-10. No differences were found between allergic and non-allergic mothers.

The inhibitory effect of breast milk on IFN-γ production, which was partly due to the high levels of TGF-β, together with the enhancing effect on IL-10 secretion, confirm that breast milk is anti-inflammatory. Although the production of IL-5 and IL-13 was enhanced by colostrum, this was accompanied with an increased production of IL-10. Together with the high levels of TGF-β in breast milk and inhibitory effect of colostrum on IL-4 production, this suggests a possible mechanism whereby breast-feeding may protect against the development of allergy. Despite differences in the composition of breast milk between allergic and non-allergic mothers, the effects of

breast milk on cytokine production from CBMC were independent of the atopic status of the mothers.

Key words: human milk, cord blood mononuclear cells, cytokine production, allergy

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Abbreviations

CBL	Cord blood lymphocytes
CBMC	Cord blood mononuclear cells
PBMC	Peripheral blood mononuclear cells
Th	T-helper
PHA	Phytohaemagglutinin

Introduction

The immune system of a new-born baby is not fully developed and it is influenced by maternal immunity, both transplacentally and via the breast-milk. Human milk contains numerous components protecting the infant against infections, e.g. antibodies, viable lymphocytes, the iron-chelating protein lactoferrin, lysozyme and oligosaccharides (1). Moreover, several cytokines and chemokines are present in human milk (2). These factors might influence the maturation of the infant's immune system.

It has been proposed that high concentrations of human milk proteins suppress, while low concentrations enhance, T lymphocyte proliferation (3). The suppressive effect of breast milk on T lymphocyte proliferation has been suggested to be due to inhibitory effect of milk on the interleukin-2 (IL-2) production from T-cells (4). Furthermore, human milk might stimulate cytokine production, e.g. IL-1, IL-3 and IL-6 from peripheral blood mononuclear cells (PBMC) (5). These studies have been performed using PBMC from adult donors (3-5) and cells from different cell lines (4) however. Furthermore, the effects on antigen specific responses have not been examined. It has also been suggested that breast-milk cell supernatants can stimulate cord blood lymphocytes (CBL) to produce IgE (6) and IgA (6, 7) antibodies.

Breast-feeding has been proposed to provide protection against otitis media (8), diarrhoea (9), acute respiratory infections (10) and invasive *Haemophilus influenzae* type b infections (11), even years after ceasing to breast-feed. Breast-fed babies may also have a reduced incidence of conditions with auto- or dysregulated immunity such as Crohn's disease (12) and insulin-dependent diabetes mellitus (13). Moreover, they have enhanced cell-mediated (14) and humoral (15) immunity after vaccination.

Breast-feeding has also been proposed to enhance IgA antibody synthesis (16), even if this is controversial (17). Finally, a long lasting protection of breast-feeding against the development of respiratory allergy has been suggested (18), even if the protective effect of breast-feeding against development of allergy and atopy is also controversial (19). Possibly, some of these controversies could be due to individual differences in the composition of breast milk. We have recently shown that allergic mothers have higher levels of IL-4 (20), RANTES and IL-8 (21) in their breast milk than non-allergic mothers. These qualitative differences might affect immune-modulating mechanisms of the breast-milk. In line with this, it has been reported that among children, whose mothers had low IgE levels, breast-feeding was associated with lower levels of total IgE at 6 years of age compared with those who were never breast-fed (22). In contrast, breast-feeding was associated with higher IgE levels in children whose mothers had high IgE levels. Furthermore, in a study by Allardyce et al, it was shown that breast milk cell supernatants from allergic mothers stimulated higher IgE production by CBL compared to breast milk from non-allergic mothers (6).

The aim of this study was to analyse the effects of breast milk, on mitogen and allergen induced cytokine production from cord blood mononuclear cells (CBMC), and if those effects differ between allergic and non-allergic mothers.

Material and Methods

Collection and processing of colostrum samples

Colostrum from 22 mothers was collected in sterile plastic tubes using a manual breast-pump within four days after delivery. The samples were immediately frozen and stored at –20°C until analysis. The mothers received verbal information about

the aim and design of the study. Sixteen of these mothers participate with their children in a prospective study regarding development of allergy, and an experienced allergy research nurse recorded ongoing or previous typical allergic symptom, i.e. rhinoconjunctivitis, bronchial asthma, and flexural, itchy dermatitis in 8 of these mothers. Their allergic status was confirmed with a screening test for IgE antibodies to a panel of inhalant allergens (Magic Lite SQ Allergy Screen, ALK, Hørsholm, Denmark). The breast milk from these 16 mothers was used to investigate possible differences in the effect of breast milk from allergic and non-allergic mothers.

The breast milk fatty layer and cellular elements were removed by centrifugation at 680 g for 10 min at 4ºC, followed by a second centrifugation of the supernatants at 10 000 g for 30 min at 4ºC. The resulting milk whey was then added to cell cultures as described below. The effect of freezing and centrifugation on the cytokine composition in breast-milk has been evaluated (20). Centrifugation before or after freezing or different centrifugation protocols did not affect the levels of different cytokines. In samples centrifuged after freezing 88% (median value) (83-96%, ranges) of the IL-10, 89% (78-109%) of the IL-13, 83 (81-97) of the IL-4, 110% (83-125%) of the IFN-γ and 107% (98-112%) of the TGF-β found in samples centrifuged before freezing could be detected. Interleukin-5 could not be detected in the samples used for evaluating the effects of centrifugation before and after freezing.

Cell cultures

Cord blood samples from 30 new-borns were collected in tubes with preservative-free heparin (Beckton Dickinson, Stockholm, Sweden). The families of these infants were randomly invited to participate in this study at the maternity clinic. These infants do not participate in any of our prospective studies and therefore the atopic

status or any other clinical information of these babies and their parents are unknown. Cord blood mononuclear cells were isolated on Ficoll Paque density gradient (Pharmacia Biotech, Sollentuna, Sweden), and washed three times in RPMI-1640 (Life Technologies AB, Täby, Sweden) containing 2% foetal calf serum (Life Technologies AB). They were then cryopreserved by standard methodology in 10% DMSO (Sigma-Aldrich, Stockholm, Sweden), 50% foetal calf serum and 40% RPMI-1640. After thawing, the cells were resuspended at 2×10^6 viable cells/mL AIM-V serum-free medium (Life Technologies) supplemented with 20μM mercaptoethanol (Sigma-Aldrich). One half mL aliquots (1×10^6 cells) were cultured at 37ºC with 5% CO_2 with medium alone, 2 μg/mL phytohemagglutinin (PHA) (Sigma-Aldrich), 10 000 SQU/mL Aquagen cat extract (ALK) or 100 μg/mL ovalbumin purified grade IV (Sigma-Aldrich) in sterile tubes. One half mL of AIM-V medium or colostrum undiluted or diluted 1/2.5, 1/5 or 1/10 in AIM-V medium (giving the final concentration 1/2, 1/5, 1/10 and 1/20) were added to the cell cultures giving a final volume of 1 mL/culture. Separate cultures were performed with monoclonal antibodies to human IL-4 receptor, 2 μg/mL, (clone 25463.111, R&D Systems, Abingdon, UK), enabling measurement of IL-4 (23). Cells from eight children were cultured as described above but in the presence of colostrum from both allergic and non-allergic mothers in separate cultures. To study the effect of neutralising TGF-β in breast-milk, four separate cultures were performed with monoclonal antibodies to human TGF-β1 and –β2, 10 μg/mL, (clone 1D11, R&D Systems). According to information from the manufacturer, 0.1 μg/ml of this antibody almost completely neutralise the activity of 250 pg/mL of TGF-β. We have reported earlier that the median concentrations of TGF-β1 and –β2 in colostrum are 325 and 1131 pg/mL, respectively (20). Therefore, 10 μg/mL of the anti-TGF-β antibody should be enough to neutralise the TGF-β present in the colostrum in the cell cultures. After 96 h, the

samples were centrifuged at 2000 g for 5 min, the supernatants were aspirated and stored at -20°C, and the viability of the cells was controlled with trypan blue exclusion.

Cord blood mononuclear cells from four children were stimulated with PHA and cat allergen in the presence of colostrum from their own mothers and colostrum from an "irrelevant" mother. The effect of colostrum on antigen induced cytokine secretion were similar irrespective if the mother and child were "paired" or "unpaired" (data not shown).

ELISA for detection of TGF-β1, IL-4, IL-5, IL-10, IL-13 and IFN-γ

The levels of TGF-β1 (R&D Systems) in breast milk and IL-4, IL-10, IL-13 and IFN-γ (CLB Pelikine Compact™, Research Diagnostics Inc., Flandern, NJ) in breast milk and cell-supernatants were determined by commercially available ELISA kits as described by the manufacturer. The levels of IFN-γ in supernatants from cultures where colostrum from both allergic and non-allergic mothers was used and IL-5 in all breast milk samples and cell-supernatants were determined by an in-house ELISAs as described elsewhere (23). Briefly, Costar 3690 plates (Life Technologies AB) were coated with 50 μL/well of 2 μg/mL monoclonal mouse anti-human IFN-γ (clone 2571811, R&D systems) or 0.25 μg/mL monoclonal rat anti-human IL-5 (clone TRFK5, Pharmingen, Becton-Dickinson). Free plastic spaces were blocked with 100 μL/well of a blocking solution (CLB). Recombinant human IFN-γ (R&D systems) and IL-5 (PharMingen, Becton-Dickinson) diluted two-fold (range 12.5-2000 and 3.1-200 pg/mL, respectively) in AIM-V medium (Life Technologies) was used as a standards. 50 μL/well of standards, samples, or for controls, AIM-V medium (Life Technologies) only, were added to the plates. For detection, 50 μL/well of biotinylated goat anti-

human IFN-γ (0.2 μg/mL) (R&D systems) or rat anti-human IL-5 (1.0 μg/mL) (PharMingen) antibodies were used followed by 50 μL/well of streptavidin conjugated polyhorseradish peroxidase (CLB) diluted 1/10 000 in dilution buffer (CLB). Tetramethylbenzidine (Sigma-Aldrich) was used as substrate, 50 μL/well, and the reaction was stopped by adding 50 μL/well of 1.8 M sulfuric acid. The sensitivity limit for quantitative determinations were 6.2 pg/mL for IL-4, 3.1 pg/mL for IL-5, 4.6 pg/mL for IL-10, 2 pg/mL for IL-13, 25 pg/mL for both IFN-γ assays and 62.5 pg/mL for TGF-β1. Those samples that had a value below the limit for quantitative determinations were assigned a value corresponding to half of that value. Background responses (cultures with medium only) from mitogen or allergen induced cytokine concentrations, and the cytokine levels in the breast milk samples (median values and ranges (pg/ml) for IL-4 (3.5, 3.1-106), IL-5 (not detectable), IL-10 (2.3, 2.3-89), IL-13 (4.4, 1-40) and IFN-γ (12.5, 12.5-734)) were subtracted from the responses from cultures with breast milk. The responses were expressed in pg/mL, or as percent of the responses from cultures without breast milk.

Statistics

As the results were not normally distributed, comparisons between paired groups were analysed using non-parametric Wilcoxon Signed rank test and correlations were analysed with Spearman's rank order correlation coefficient test. A probability level of <5% was considered to be statistically significant. Calculations were performed with a statistical package StatView 5.0 for PC (SAS Institute Inc., Cary, North Carolina, USA).

Ethics

The Regional Ethics Committee for Human Research at the University Hospital of Linköping approved the study.

Results

The cord blood cells were viable after 96 h in culture in the presence of colostrum diluted 1/5, 1/10 and 1/20 and they even tended to proliferate. In three separate experiments, including both unstimulated, as well as allergen and mitogen stimulated cells, the median number of cells after 96 h in culture without colostrum was $1.1x10^6$ and in cultures with colostrum diluted 1/5 $1.7x10^6$, 1/10 $1.5x10^6$ and 1/20 $1.7x10^6$. The cells died in cultures with higher concentrations of colostrum.

Colostrum inhibited PHA induced IFN-γ production in a dose dependent way (fig 1a). This was also true for cultures stimulated with cat allergen (fig 1b). Furthermore, colostrum inhibited PHA induced IL-4 production in all but two cell-cultures (fig 1c) but not IL-10, IL-5 or IL-13 production (data not shown). In contrast, cat (fig 2) induced IL-10, IL-5 and IL-13 production was enhanced by colostrum. Similar results were observed for ovalbumin induced and spontaneous secretion of IL-10, IL-5 and IL-13 production (data not shown). The stimulatory effect was most pronounced at 1/10 and 1/20 dilutions. No IL-4 production was observed in response to cat and ovalbumin stimulation, and ovalbumin did not induce IFN-γ production (data not shown). No spontaneous secretion of IFN-γ and IL-4 was observed in absence or in presence of colostrum.

The effects of colostrum on different cytokines were not statistically significantly correlated, except for the increase of cat allergen induced IL-5 and IL-13 production in the presence of colostrum diluted 1/10 (rho=0.73, $p<0.001$).

The effect of breast-milk on PHA (table 1) and allergen (table 2 and 3) induced cytokine production was similar when using colostrum from allergic and non-allergic mothers. In table 1and 2 the results for PHA and cat induced cytokine production in absence and presence of colostrum diluted 1/10 are shown. The results were similar for ovalbumin induced cytokine production and when using different concentrations of colostrum (data not shown). Neither spontaneous secretion was affected differently by colostrum from allergic and non-allergic mothers.

Addition of an anti-TGF-β antibody blocked the inhibitory effect of colostrum on PHA induced IFN-γ production. In cell cultures with colostrum, diluted 1/10 or 1/5, the median PHA induced IFN-γ production was 25 and 22%, respectively, of the production in control cultures, *i e* PHA stimulated cells with no colostrum. The corresponding figures were 97 and 110% in cultures with colostrum plus the anti-TGF-β antibody. The figures are based on four separate experiments. Furthermore, there was a negative correlation between TGF-β1 levels in colostrum and IFN-γ production from CBMC in the presence of colostrum. This indicates a dose dependent relationship between TGF-β levels in breast milk and inhibitory effect on IFN-γ production. In figure 3, the results from cultures with colostrum diluted 1/5 are presented. The most pronounced effect of breast milk on PHA induced IFN-γ production was observed using this dilution. The results were similar, however, using colostrum diluted 1/10 and 1/20 (rho=-0.46, p=0.11 and rho=-0.62, and p=0.06, respectively).

The enhancing effect of breast-milk on IL-5, IL-10 and IL-13 production was even more pronounced with the addition of the anti-TGF-β antibody (data not shown). Interleukin-4 was not **detected** in these cultures.

Discussion

The inhibited IFN-γ and enhanced IL-10 production by CBMC support that breast-milk is anti-inflammatory (1). The pro-inflammatory cytokine IFN-γ is produced by T helper (Th) 1 cells (24), while IL-10 has immunosuppressive properties (25). Possibly, these effects of breast-milk could explain the lower incidence of insulin-dependent diabetes mellitus (13) and Crohn's disease (12) among breast-fed children, as the two conditions are considered to be due to a dysregulation of the immune system resulting in excessive Th1-like immunity.

Interleukin-10 might also have indirect anti-inflammatory properties since it stimulates IgA synthesis together with TGF-β (26). Antigen specific IgA antibodies mediate exclusion of antigens capable of provoking immune inflammatory responses. The IL-10 enhancing capacity of breast-milk, together with the high levels of TGF-β in breast-milk (20), could possibly explain the earlier induction of IgA production seen in breast-fed, as compared to bottle-fed, infants (16). Some of the anti-inflammatory properties of breast-milk might also be due to the high levels of TGF-β. This interpretation is supported by the abrogated inhibition of colostrum when anti-TGF-β was added to the cultures and the negative correlation between TGF-β1 levels in colostrum and IFN-γ production from CBMC in the presence of colostrum.

Breast-milk has also been suggested to inhibit IL-2 production from T-cells (4). This cytokine is important for the induction of IFN-γ production. Unpublished data from our laboratory suggest that the IFN-γ production is partially restored when exogenous IL-2 is added to the cell cultures. The inhibition of IFN-γ production was not due to decreased proliferation or low viability of the cells, since most of the cells

were viable and proliferated in the presence of diluted colostrum. In cultures with colostrum at 1/2 concentrations, however, most of the cells were dead. This might either be due to that high concentration of TGF-β is cytolytic, or to the presence of other cell death inducing agents in human milk (27). As several cell types, including mononuclear cells, are present and viable in breast-milk, however, a low concentration of medium would be the most probable explanation to the low viability in cell-cultures with high concentration of colostrum.

Mitogen induced IL-4 production was decreased in the presence of colostrum supporting the hypothesis that breast-feeding protects against allergy. On the other hand, colostrum increased allergen induced IL-5 and IL-13 production. These two cytokines are also associated with atopy. Colostrum also enhanced allergen induced IL-10 production. Recently it was proposed that anti-inflammatory networks, including e.g. IL-10 and TGF-β, might balance the elevated concentrations of IgE and Th2 cytokines in helminth-infected populations with a low prevalence of allergic disease (28). High levels of TGF-β in breast milk has recently been reported to be associated with a reduced risk for development of IgE mediated cow's milk allergy (29) and to delay the onset of atopic disease (30). Furthermore, IL-10 is an important factor for the induction of tolerance to specific antigens (31). Thus, the increased production of allergy associated cytokines IL-5 and IL-13 may be balanced by an enhanced production of anti-inflammatory cytokines such as IL-10 and the high levels of TGF-β present in milk (20).

Extrapolation of data from *in vitro* studies like ours to what is really happening in the breast-fed baby must of course be done with caution. Lymphocytes from breast-fed infants have been reported to display lower integrin expression and lower

proliferative responses and IFN-γ production, spontaneously and after stimulation with tetanus toxoid and *Candida,* than lymphocytes from bottle-fed infants (32). Moreover, breast-feeding has been proposed to offer long lasting protection against infections (33) and breast-fed babies have enhanced cell-mediated (14, 32) and humoral (15) immunity after vaccinations. One possible explanation to these findings may be that immuno-modulatory factors in breast milk reach and affect the immune system of the baby. Animal studies have shown that cytokines survive and retain their biological activity during the passage of the gastrointestinal tract and that they even may be taken up into the circulation and affect immune functions (34). Human breast milk leukocytes fed to newborn baboons have been shown to be taken up into the infant's circulation (35). In human, the transient tuberculin positivity seen in breast-fed infants born to tuberculin positive mothers, but not in bottle-fed infants or in infants breast-fed by tuberculin negative mothers (36, 37), constitute indirect evidence that functionally active T-cells are taken up from the maternal milk by the infant. Together these studies support that cells and other immunological components in breast milk may survive and may be taken up from the gastrointestinal tract, and then reach and affect the immune system of the infant.

We have earlier reported higher levels of IL-4 in breast-milk from allergic, as compared to non-allergic, mothers (20). Interleukin-4 is the most important factor for induction of Th2-cell development and thereby production of Th2-like cytokines (24). Earlier studies have indicated that breast-milk from allergic mothers may induce higher IgE levels than breast-milk from non-allergic mothers (6, 22). No differences on the effect of breast-milk on cytokine production were, however, observed between allergic and non-allergic mothers.

In conclusion, the inhibitory effect of breast milk on IFN-γ production, which was partly due to the high levels of TGF-β, does together with the enhancing effect on IL-10 secretion confirm that breast-milk is anti-inflammatory. Although the allergen induced production of IL-5 and IL-13 was enhanced by colostrum, this was accompanied with an increased production of IL-10. Together with the high levels of TGF-β in breast milk and the inhibitory effect of colostrum on IL-4 production, this suggests a possible mechanism whereby breast-feeding might protect against the development of allergy. Despite differences in the composition of breast milk between allergic and non-allergic mothers, the effects of breast milk on cytokine production from CBMC were independent of the atopic status of the mother.

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Legends to figures

Fig 1. PHA (a) and cat (b) induced IFN-γ and PHA induced IL-4 (c) production from CBMC in the absence or presence of colostrum diluted 1/20, 1/10 or 1/5. The levels are expressed as % of the levels in the controls (PHA stimulated cells with no colostrum=100%). (*=$p<0.05$, ***=$p<0.001$). The number of samples with decreased (⇓) and unchanged (⇔) IFN-γ and IL-4 levels in the presence of colostrum are also given.

Fig 2. Cat induced IL-10 (a), IL-5 (b) and IL-13 (c) production from CBMC in the absence or presence of colostrum diluted 1/20, 1/10 or 1/5. The levels are expressed as % of the levels in the controls (cat stimulated cells with no

colostrum=100%). (*=p<0.05, **=p<0.01, ***=p<0.001). The number of samples with increased (⇑) and unchanged (⇔) IL-10, IL-5 and IL-13 levels in the presence of colostrum are also given.

Fig 3. Correlation between TGF-β1 levels in colostrum and the levels of PHA induced IFN-γ production from CBMC in the presence of colostrum diluted 1/5. The IFN-γ levels are expressed as % of the levels PHA stimulated cells with no colostrum

Table 1
PHA induced cytokine production from CBMC in the absence or presence of colostrum diluted 1/10 from allergic and non-allergic mothers. The concentrations are given in real values (pg/mL) or as % of the levels in the controls (cultures with no colostrum=100%).

Konc col	Allergic		Non-allergic	
	Median (range) (pg/mL)	Median (range) (%)	Median (range) (pg/mL)	Median (range) (%)
IFN-γ				
0	3125 (13-7210)	100	3125 (13-7210)	100
1/10	187 (13-387)	5 (1-59)	161 (13-413)	5 (1-91)
IL-4				
0	22 (7-40)	100	22 (7-40)	100
1/10	4 (3-29)	31 (13-78)	7 (3-18)	38 (8-48)
IL-10				
0	33 (2-85)	100	33 (2-85)	100
1/10	33 (2-136)	159 (5-365)	21 (2-47)	52 (17-314)
IL-5				
0	22 (2-95)	100	22 (2-95)	100
1/10	16 (2-77)	81 (6-131)	25 (2-65)	52 (6-149)
IL-13				
0	390 (1-640)	100	390 (1-640)	100
1/10	96 (5-448)	16 (1-70)	173 (22-413)	35 (4-119)

Table 2
Cat induced cytokine production from CBMC in the absence or presence of colostrum diluted 1/10 from allergic and non-allergic mothers. The concentrations are given in real values (pg/mL) or as % of the levels in the controls (cultures with no colostrum=100%).

Konc col	**Allergic**		**Non-allergic**	
	Median (range) (pg/ml)	Median (range) (%)	Median (range) (pg/ml)	Median (range) (%)
IFN-γ				
0	28 (13-205)	100	28 (13-205)	100
1/10	13 (13-402)	44 (6-403)	13 (13-109)	44 (6-100)
IL-10				
0	8 (2-101)	100	8 (2-101)	100
1/10	12 (2-97)	122 (73-408)	11 (2-71)	127 (37-412)
IL-5				
0	2 (2-40)	100	2 (2-40)	100
1/10	31 (4-31)	1151 (146-4100)	27 (2-162)	574 (66-4713)
IL-13				
0	14 (1-314)	100	14 (1-314)	100
1/10	48 (1-397)	463 (100-3496)	55 (1-219)	325 (29-4770)

Fig 1a

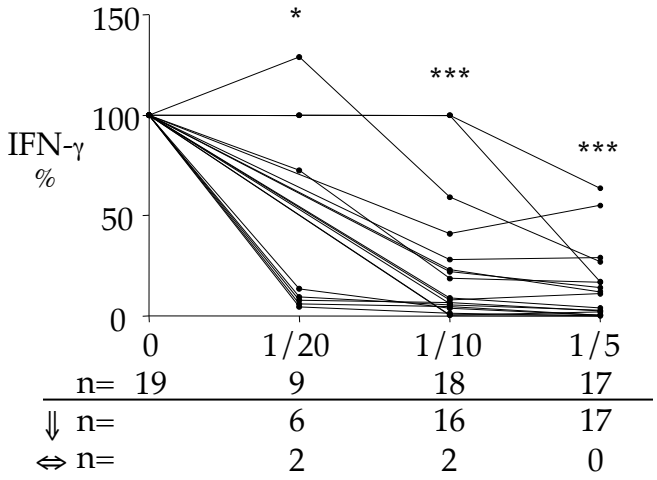

Fig 1b

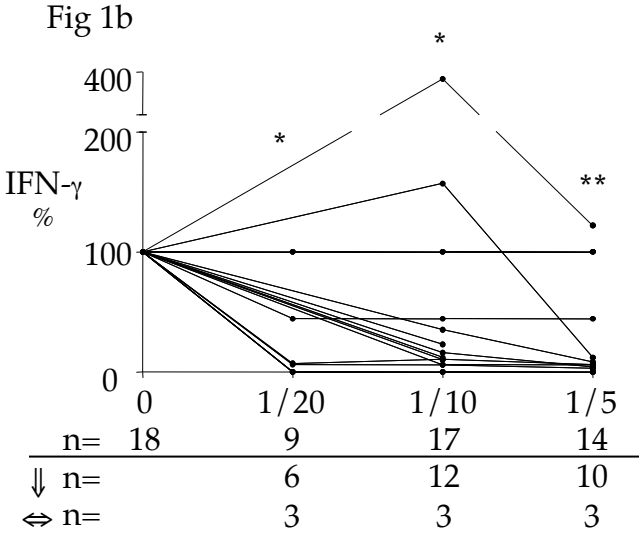

Fig 1c

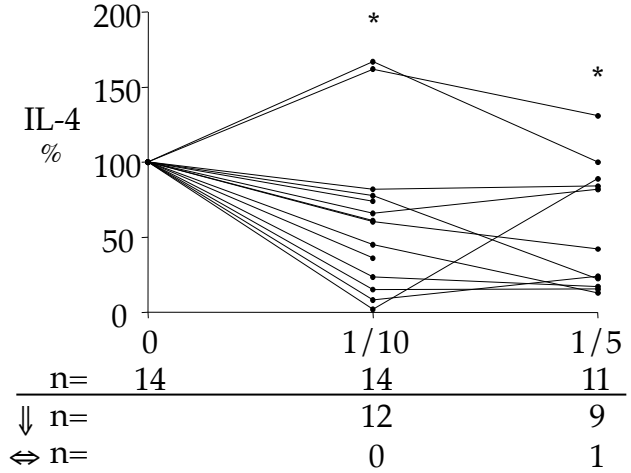

Fig 2a

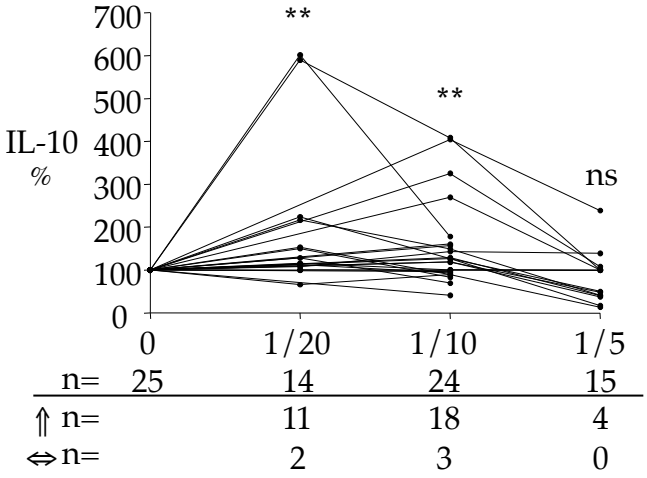

Fig 2b

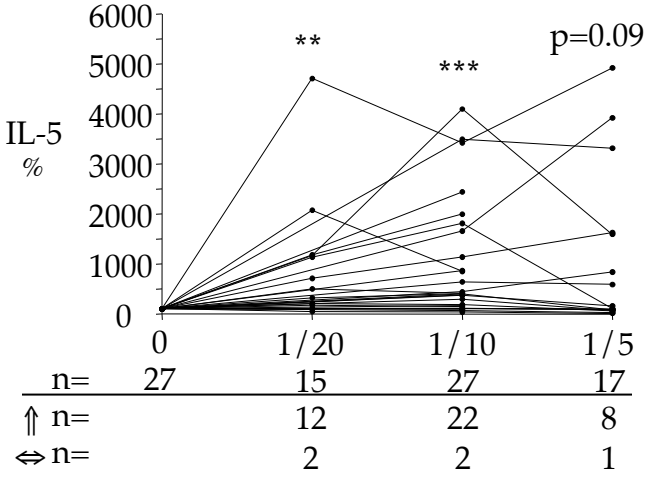

Fig 2c

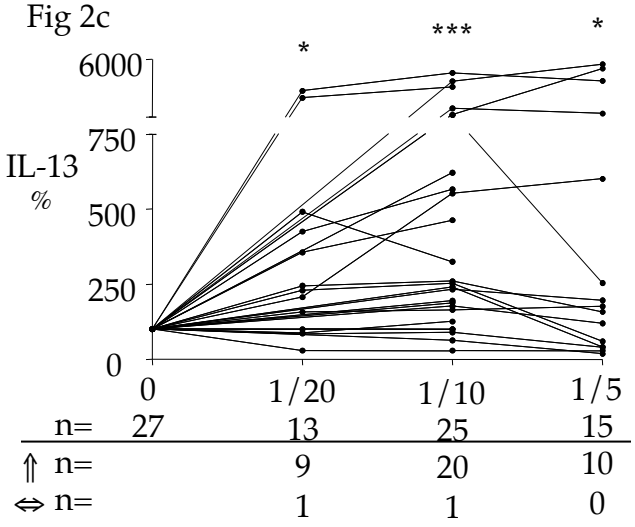

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Fig 3