

## *Original articles*

# Human milk stimulates B cell function

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**SUMMARY** The effect of human milk on B cell function was studied by using murine spleen cells stimulated with suboptimal doses of lipopolysaccharide. Cell free, defatted, filtered colostrum as well as mature breast milk showed an enhancing effect on B cell proliferation and generation of antibody secretion, but this was not seen with formula milk. The activity was heat sensitive and resisted overnight dialysis. It is suggested that this could represent an important immunological mechanism explaining the anti-infectious properties of breast milk.

There have been reports that feeding with human rather than formula milk reduces morbidity and mortality from infectious diseases, especially in developing countries.<sup>1 2</sup> This protective effect has, however, been questioned and described as marginal in recent studies in some industrialised countries.<sup>3</sup> The beneficial effect seen in developing countries may be due to better hygiene associated with breastfeeding rather than any anti-infectious properties in breast milk.

The basic immunological mechanisms underlying the possible beneficial effects of breastfeeding are only partially understood. Milk is rich in leucocytes, of which the milk macrophage is predominant,<sup>4</sup> and these show in vitro biological effects similar to their counterparts in the blood.<sup>4 5 11</sup>

Several soluble anti-infectious components are present in milk. The non-antigen specific include lysozyme, lactoferrin, and a non-immunoglobulin high molecular weight fraction.<sup>6 7</sup> A non-specific antiviral activity has also been described in milk.<sup>8</sup> The antigen specific activity resides mainly in secretory IgA, and specific antibodies to enteric bacteria and viruses have been shown.<sup>9</sup>

The effect of milk and milk cells on immune responses have been studied little and are difficult to interpret. It has been shown that cultured human milk cells secrete a factor into the culture medium that increases IgA synthesis of cultured adult mononuclear cells.<sup>10</sup> Milk present in cultures exerts a strong inhibitory effect on lymphocyte proliferation.<sup>11</sup> A similar effect was recorded when phythemagglutinin stimulated lymphocyte proliferation was assayed in whole blood cultures of infants and was

related to the type of feeding. Breast fed infants had significantly lower lymphocyte responsiveness than those fed cows' milk.<sup>12</sup>

The present study shows that human milk enhances murine B cell function, measured as proliferation, and antibody synthesis in response to a T cell independent B cell mitogen.

## **Materials and methods**

**Milk donors.** Colostrum was obtained two to seven days post partum from 10 healthy women delivered at the Department of Obstetrics, University Hospital of Umeå. In addition, milk was received from two donors 66 and 100 days respectively after delivery. The milk was expressed by means of an electric pump and was refrigerated at 4°C immediately after collection.

**Processing of milks.** All milk samples were processed at the laboratory within four hours of collection. They were centrifuged at 2800 g for 30 minutes. The fat layer and cell deposit were discarded. The aqueous phase was collected and diluted 1/5 in RPMI 1640 medium supplemented with 10 µg gentamicin/ml, 1 mmol L-glutamine, and 5% pooled heat treated (56°, 30 minutes) human serum. The diluted milks were filtered (0.45 µ) and stored at -20°C in small aliquots until use.

**Spleen cell cultures.** Spleens were removed from Swiss mice by aseptic technique. One spleen was used in each experiment. The spleen cells were squeezed out from the organ, filtered through sterile

gauze and washed three times in RPMI 1640 with 10 µg gentamicin/ml. The cells were adjusted to a concentration of  $1 \times 10^6$ /ml in RPMI 1640 supplemented with 10 µg gentamicin, 1 mmol L-glutamine, and  $10^{-5}$  M 2-mercaptoethanol.

**Assay of B cell proliferation.** Cultures were set up in triplicate in microtissue culture plates (Falcon, Oxward, USA) with  $1 \times 10^5$  cells/well. Milk was added to 50 µl portions at various concentrations. Lipopolysaccharide (LPS, *Escherichia coli* 055:B5, Difco, Detroit, USA) was added in 50 µl portions giving a final non-mitogenic suboptimal concentration of 0.5 µg/ml. The final concentration of filtered, heat treated human serum was 2.5% in each culture. The cultures were incubated for three days at 37°C in humidified air with 5% CO<sub>2</sub>. For the last 18 hours of incubation, 0.5 µCi <sup>3</sup>H-thymidin (specific activity 25 Ci/mmol/l, Amersham, England) was present in each culture. The cultures were collected onto glass fibre filter strips and the incorporated radioactivity was measured by liquid spectrophotometry.

**Assay of murine Ig synthesis.** The culture conditions in experiments in which Ig synthesis was determined were the same as for the proliferation experiments except that the incubation time was four days. The antibody content in the supernatants was assayed by an ELISA technique. Immunoplates (NUNC, Århus, Denmark) were coated overnight at 4°C with rabbit antimouse Ig (Z 259, DAKO, Copenhagen) diluted 1/1000 in carbonate buffer, pH 9.5. After washing in distilled water the plates were saturated with 0.5% bovine serum albumin diluted in phosphate buffered saline (PBS) with 0.05% Tween 20 (PBS-Tween) for 60 minutes at room temperature. After washing the cell free culture supernatants were added in duplicate diluted 1/500 in PBS-Tween. The plates were incubated for two hours at room temperature. After washing peroxidase conjugated rabbit antimouse Ig (P260, DAKO, Copenhagen) diluted 1/1000 in PBS-Tween was incubated for one hour at room temperature on the plates. After a final washing step o-phenylenediamine and H<sub>2</sub>O<sub>2</sub> were added. The reaction was stopped after 10 minutes with 1N HCl. The optical densities were then read immediately in a Titertek Multiscan photometer. Results are given as optical density values. A mouse serum was included as positive control.

## Results

**Effect on B cell proliferation.** The effect of colostrum obtained from five different donors on spleen cell proliferation in response to lipopolysaccharide is

shown in three separate experiments in Fig. 1. As can be seen there was a significant increase in all three experiments, when 5% defatted, cell free and filtered colostrum was present in the cultures. Milk without mitogen did not stimulate the proliferation significantly. Mature milks obtained at 66 and 100 days of lactation also exerted a stimulatory effect of the same magnitude as colostrum (Table).

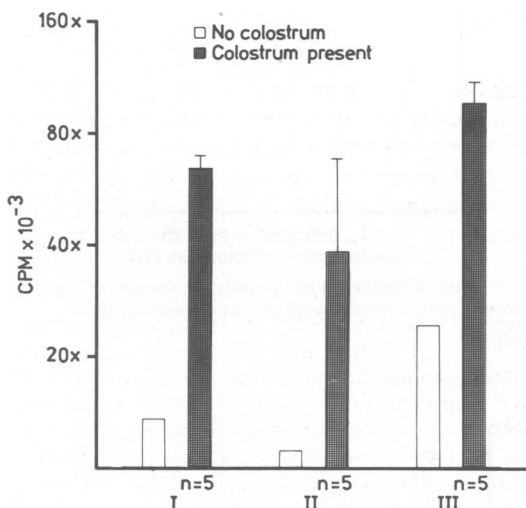


Fig. 1 Spleen cell proliferation in response to lipopolysaccharide (0.5 µg/ml) in the absence and presence of 5% colostrum in culture.

In the three separate experiments (indicated by Roman numerals) the same milks from five different donors were used. The bars indicate counts per minute (CPM) +1 SD for the five different cultures. The proliferation of cultures with medium only was  $794 \pm 159$  CPM and with medium and 5% colostrum  $1244 \pm 303$  CPM.

Table The effect of heat treated and dialysed colostrum, mature breast milks, and formula milk on lipopolysaccharide (LPS) induced (0.5 µg/ml) spleen cell proliferation. The figures indicate counts per minute of triplicate cultures for three different experiments

	Counts per minute		
	Exp 1	Exp 2	Exp 3
LPS only (0.5 µg/ml)	880	5 025	28 449
<i>Additives (5% v/v)</i>			
Colostrum, untreated	40 197	41 483	98 994
Heat treated colostrum (56°C, 10 min)	9 802	138	1 229
Dialysed colostrum	72 800	ND	80 621
Mature milks, 66 days post partum	55 331	41 065	125 829
100 days post partum	55 174	41 580	119 605
Formula milk	416	403	5 329

ND=not done.

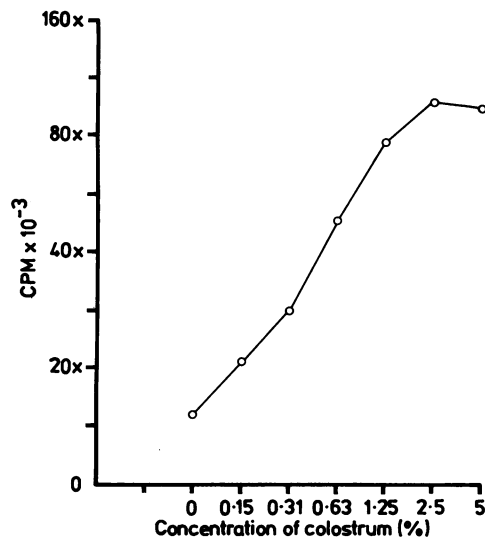


Fig. 2 Dose dependency of lipopolysaccharide (0.5 µg/ml) spleen cell proliferation by different concentrations of colostrum.

CPM=counts per minute.

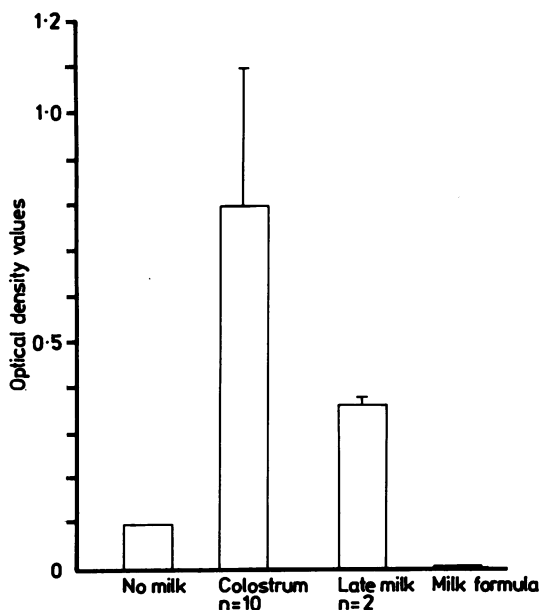


Fig. 3 Immunoglobulin secretion in supernatants of cultured spleen cells in response to lipopolysaccharide (0.5 µg/ml) in the presence of 5% colostrum, mature (late) milk, or milk formula, respectively.

The antibody content is expressed as optical density values at 500 fold dilution of culture supernatants. The values of cultures with medium only was 0.02 and of cultures with medium and 5% milk 0.007±0.01. The vertical bars indicate +1 SD.

There was a dose related dependency of proliferation to the milk concentration present in the culture (Fig. 2). Optimal responses were found in the range of 1 to 5% colostrum in culture. An effect was seen in concentrations as low as 0.1%.

The activity was heat labile. Heating at 56°C for only 10 minutes reduced almost all the activity in one experiment and it was completely lost in two other experiments. The activity resisted extensive overnight dialysis with phosphate buffered saline (Table). Formula milk did not stimulate proliferation (Table).

**Effect of Ig synthesis.** Lipopolysaccharide-induced Ig synthesis assayed by ELISA was higher in all cultures with 5% colostrum obtained from 10 different donors and in cultures with two samples of mature milks (Fig. 3). The antibody content was lower in cultures with mature milks than early milks. Formula milk had no stimulatory effect on antibody synthesis (Fig. 3).

## Discussion

In the present study data are presented showing that human milk contains an activity that enhances murine spleen B cell function measured as cell proliferation and antibody synthesis in response to lipopolysaccharide, a T cell independent B cell mitogen.<sup>13</sup> Early as well as mature milks were active. In contrast, formula milk was completely inactive.

The activity was not dialysable as it remained after overnight dialysis. It was heat labile, as heating at 56°C for 10 minutes stopped almost all the activity. These physical properties indicate that the activity resides in a heat labile protein.

Human milk contains a well defined growth stimulating activity.<sup>14</sup> This is epidermal growth factor, which is a polypeptide (MW 6000D) promoting growth and differentiation of epidermis, pulmonary epithelium, and gastrointestinal mucosa. The mitogenic activity could not be stopped totally by antiserum to epidermal growth factor and furthermore it was found that milk supported growth of a cell line devoid of receptors to this factor.<sup>14</sup> This means that there must be additional cell growth promoting activities besides epidermal growth factor in breast milk.

To the best of my knowledge no soluble activity in milk affecting the growth or differentiation of the B cell, or both, has been shown. Recent studies of B cell function have shown that soluble products influence both the growth and differentiation of the B cell. One of the first described was a monokine.<sup>15</sup> It is a soluble monocyte product essential for both

proliferation and generation of immunoglobulin production. It was inhibited by antisera against interleukin 1.<sup>15</sup> This strongly supports the necessity of the presence of interleukin 1 for B cell activation. The purpose of interleukin 1 would be to prime the B cell to appropriate T cell signals. Milk is rich in macrophages, so it is reasonable to suggest that the mediator of the activity might be a macrophage product similar to interleukin 1. This is also supported by the *in vitro* biological effects that are comparable with those of interleukin 1 on B cell function.

Another soluble factor affecting the B cell is the so called B cell growth stimulating factor, which according to recent data selectively induces proliferation but not maturation of the B cell.<sup>16 17</sup> Maturation into Ig synthesis would in turn be induced by a new molecule, B cell maturation factor.<sup>16 17</sup> Whether the activity shown in milk is similar to any of the known B cell stimulatory factors or represents a new one is still unknown.

The newborn infant has an altered immune reactivity. In coculture experiments with adult lymphocytes cord blood lymphocytes have been found to exert a profound inhibitory activity on proliferation and Ig synthesis.<sup>18</sup> The mode of Ig production is restricted:<sup>19</sup> considerable amounts of IgM class antibodies are synthesised, whereas IgG and IgA synthesis is virtually absent. This restriction is probably due to a primary immaturity of the B cell itself, but there also seems to be a lack of appropriate T helper cell signals.<sup>19</sup> The finding of a B cell stimulatory activity in milk fits well in this context of immaturity of the newborn's B cell function. It is therefore tempting to suggest that breast milk provides the baby with this activity in order to relieve the immune responses of his immature hypo-responsive B cells. The activity seems to act directly on the B cell and not by interacting through T helper cells. This conclusion is supported by the fact that lipopolysaccharide, the stimulating agent used in the study, is a T cell independent B cell selective mitogen.<sup>13</sup> This activity together with secretory IgA may represent a major immunological mechanism explaining how breast milk compared with artificial feeding has considerable anti-infectious properties, thereby supporting the numerous clinical observations of this effect.

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