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Lactoferrin Inhibits Prostaglandin E_2 Secretion by Breast Milk Macrophages

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ABSTRACT. The interaction between human breast milk macrophages and lactoferrin (LF) in its native form was studied in *in vitro* culture. Competitive inhibition-binding studies with ^{125}I-LF and unlabeled LF showed that a specific receptor for LF was present on breast milk macrophages. LF at concentrations of 10^{-6}–10^{-9} M resulted in a dose-dependent inhibition of prostaglandin E_2 secretion by breast milk macrophages (control −45 ± 7; LF 10^{-6} M −9 ± 1 ng/ml/10^6 cells). This inhibitory effect was also observed when the macrophages were stimulated with Concanavalin A (LF 10^{-6} M −80 ± 5; 10^{-9} M −45 ± 8 %inhibition of prostaglandin E_2 secretion by Concanavalin A stimulated macrophages). Lactalbumin and lactoglobulin had no effect. Similar concentrations of LF had no effect on lysozyme production. We also demonstrated that human milk macrophages are capable of eliciting an oxidative burst as measured by superoxide or hydrogen peroxide production when stimulated by phorbol myristate acetate in *in vitro* culture. (basal superoxide −1.4 ± 0.3; phorbol myristate acetate 28.8 ± 3.5 nmol/1 × 10^6 cells/90 min; basal hydrogen peroxide 11.7 ± 4.6; phorbol myristate acetate −57.5 ± 2.3 nmol/mg protein/90 min). LF had no effect on the oxidative burst. These results suggest that interaction of aqueous and cellular components of breast milk may occur and result in varied physiological effects. (*Pediatr Res* 21: 54–57, 1987)

Abbreviations

LF, lactoferrin
PGE_2, prostaglandin E_2
Con A, Concanavalin A
PMA, phorbol myristate acetate
H_2O_2, hydrogen peroxide
O_2^-, superoxide
PBS, phosphate-buffered saline
BSS, balanced salt solution
CSF, colony-stimulating factor

Numerous beneficial factors have been found in breast milk including the bifidus factor, which enhances growth of *Lactobacillus bifidus* and inhibits colonisation of the intestine by coliforms; several proteins with antiinfective properties, such as secretory IgA, lactoperoxidase, LF, lysozyme, interferon, and large numbers of viable leukocytes mainly macrophages and lymphocytes. All these components are transferred from mother to infant and probably serve as a protective mechanism against various infections until the infant's immune defences mature. The regulation of the production of these factors in breast milk and the interaction of these various components has hardly been studied.

Several of the functions typical of mononuclear phagocytes have also been reported in breast milk macrophages. They are capable of phagocytosis and killing of bacteria and fungi (1) and they modulate lymphocyte reactivity (2). These cells also produce complement (3), lysozyme and prostaglandins (4), and they store and release IgA (5).

Lactoferrin, an iron-binding protein with antibacterial properties, is known to be present in the secondary granules of neutrophils (6), in plasma (7, 8), and in various secretions (9). Recent studies have shown that there are specific lactoferrin receptors on mouse peritoneal macrophages (10) and the human monocyte cell membrane (11, 12). There is now considerable evidence to indicate that lactoferrin regulates several biological functions of mononuclear phagocytes (13). Human colostrum is unusually rich in unsaturated lactoferrin containing up to 7 mg/ml and this protein is not extensively digested by the gastrointestinal tract of the infant (14). Therefore, we have studied the effect of human milk lactoferrin on some of the biological activities of breast milk macrophages *in vitro*.

MATERIALS AND METHODS

The samples of human milk were obtained from nursing mothers during the 1st wk postpartum. They were collected by hand expression into sterile containers kept at room temperature, and cultured within 1 h.

Macrophage monolayers. Milk, 7.5 ml, was layered onto 2.5 ml Ficoll-Hypaque and centrifuged at 400 × *g* for 25 min. The fat layer was removed and the mononuclear cell layer was washed three times in PBS and resuspended at a concentration of 1 × 10^6 cells per ml in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated, millipore-filtered fetal calf serum (complete medium). Of this cell suspension 0.5 ml was pipetted into each well of tissue culture plates (16-mm diameter, Linbro Chemical Co., New Haven, CT). Adherence of macrophages was facilitated by gentle rocking at room temperature for 45 min. The nonadherent cells were removed by vigorous washing. The resultant macrophage monolayers were cultured in complete medium at 37° C in a humidified atmosphere of 5% CO_2 in air.

At the start of the culture, the monolayers were treated with various concentrations of human milk lactoferrin (Sigma Chemical Co., St. Louis MO). The purity of lactoferrin was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis which showed a single band of molecular weight ~80,000. This preparation which is prepared from human milk is about 20% iron saturated. Controls included macrophage monolayers without additions and cultures treated with lactalbumin and β-lactoglobulin (Sigma). Addition of Con A (Sigma) to the mac-

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rophage monolayers served as the positive control. Supernatants were harvested after 48 h and stored at −20° C until assayed for PGE_2 and lysozyme.

Binding assay. LF was labeled with ^{125}I by the chloramine T method (15) to a specific activity of 170 μCi/μg. Breast milk mononuclear cells were suspended at concentration of 2×10^6 cells/ml in RPMI-1640 supplemented with 2% fetal calf serum. Of this cell suspension 0.5 ml was transferred to each plastic tube. Inhibition of ^{125}I-lactoferrin (1×10^{-10} M) binding was tested by adding increasing concentrations of unlabeled lactoferrin to the cell suspension. Each concentration was tested in duplicate samples. After incubation at 37° C for 60 min, the cells were separated by centrifugation at $600 \times g$ for 10 min. The supernatants were decanted and the pellets were washed twice with cold buffer; the washes were added to the supernatants. Parallel incubations were performed with cell-free tubes to correct for nonspecific binding of ^{125}I-lactoferrin to the tube. The ^{125}I-activity of the samples were counted in a γ counter.

Prostaglandin assay. The concentration of PGE_2 in supernatants was determined by radioimmunoassay (16). Formed complexes were separated from free radioactive PGE_2 by dextran-coated charcoal. Specific antiserum which shows no cross-reactivity with F prostaglandins was used (Miles Yeda, Kiryat-Weizman, Rehovot, Israel).

Lysozyme assay. Lysozyme concentration was measured by determining the initial rate of lysis of a suspension of micrococcus lysodeikticus (Sigma) in 1 M acetate buffer pH 6.2 using a spectrophotometer fitted with a recorder. Egg white lysozyme (Sigma) was used as a standard (17).

Assay for O_2^- production. O_2^- anion was measured by the reduction of ferricytochrome C (18). For this assay immediately after adherence, the macrophage monolayers were covered with a 80 μM solution of ferricytochrome C (type III, Sigma) in phenol red-free BSS. Human milk lactoferrin diluted in PBS was added directly to the wells in the presence or absence of PMA. Controls included macrophages in cytochrome C-containing BSS without stimulants and only cytochrome C solution without macrophages. These were incubated for 90 min at 37° C in humidified air with 5% CO_2. After incubation, the cell-free supernatants were transfered to tubes in ice, centrifuged for 5 min at $2000 \times g$ at 4° C, and the optical density was measured at 550 nm against blanks consisting of cytochrome C solution from wells without macrophages. Superoxide anion production was quantitated using the formula:

$$E_{550\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$$

In all experiments at least duplicate cultures were used for both control and stimulated cells.

Hydrogen peroxide assay. A microassay method was used as described (19). Monolayers that had been cultured in 96 well flat bottom tissue trays were incubated in the presence of a phenol red solution (100 μl/well) which contained 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM of dextrose, 0.56 mM (0.2 g/liter) phenol red and 19 U/ml of horseradish peroxidase.

The cultures were incubated for 60 min and the reaction stopped by the addition of 10 μl 1 N NaOH per well. The optical density of each sample was determined with the use of a microelisa reader model MR 580 (Dynatech Laboratories, Alexandria, VA) fitted with a wave length filter of 600 nm. Protein concentrations of the monocyte monolayer were determined in replicate wells by the Lowry method. Standard curves were established using dilutions of a H_2O_2 solution of known molarity as described.

RESULTS

LF binding to breast milk mononuclear cells. The affinity of LF binding to adherent mononuclear blood cells has been shown to be 100-fold lower than to cells in suspension (20). Therefore, we chose to measure binding of LF to its receptor on the mononuclear human milk cells in suspension rather than to an adherent macrophage monolayer. The vast majority of the cells in this mononuclear cell suspension are macrophages but we have not taken into account the possibility that some lactoferrin binding may have occurred to the small fraction of lymphocytes in this mononuclear suspension. Binding of increasing concentrations of ^{125}I-lactoferrin to breast milk mononuclear cells in suspension was shown consistently by the increasing amount of radioactivity found in the cell fraction. This binding was shown to be specific as addition of increasing concentrations of unlabeled lactoferrin resulted in increasing inhibition of tracer ^{25}I-lactoferrin binding (Fig. 1*A*). A Scatchard plot was constructed from this data (Fig. 1*B*).

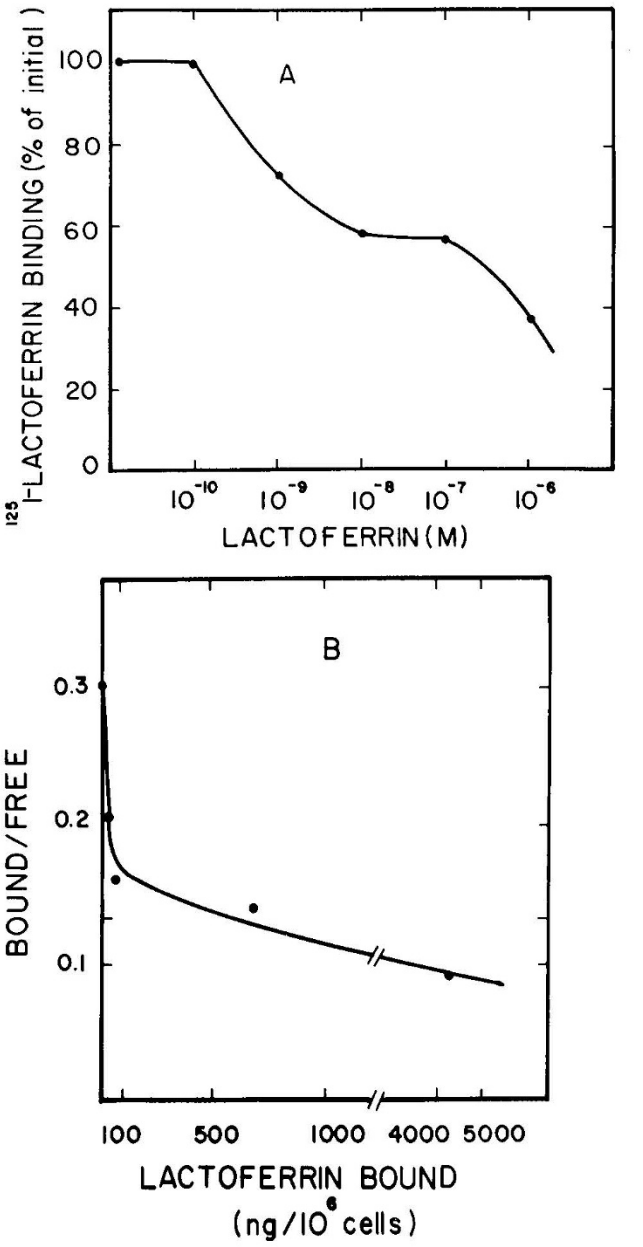

Fig. 1. Competition-inhibition curve (*A*) and Scatchard analysis (*B*) of ^{125}I-LF binding to mononuclear human milk cells. Sixty-min incubation at 37° C of cell suspension (2×10^6 cells/ml) was performed with tracer amounts of ^{125}I-LF (1×10^{-10} M) and increasing concentrations of unlabeled LF. Data plotted are the means of two separate experiments each done in duplicate.

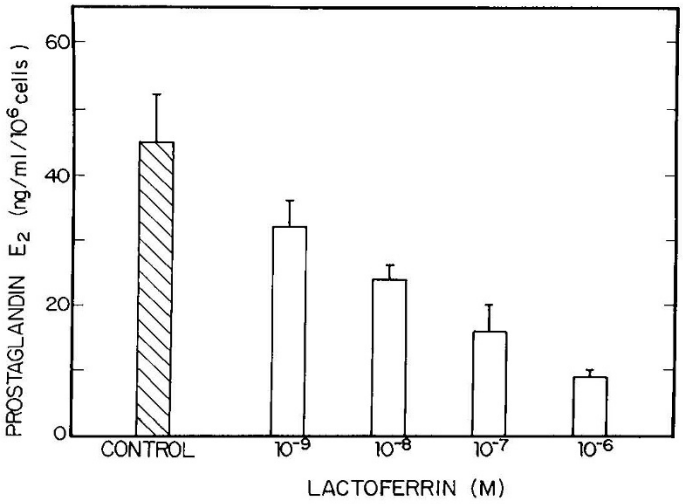

Fig. 2. PGE_2 production by macrophages in culture in the presence of LF at various concentrations after 48 h in culture. Each *point* represents mean ± SD of duplicate cultures from a representative experiment.

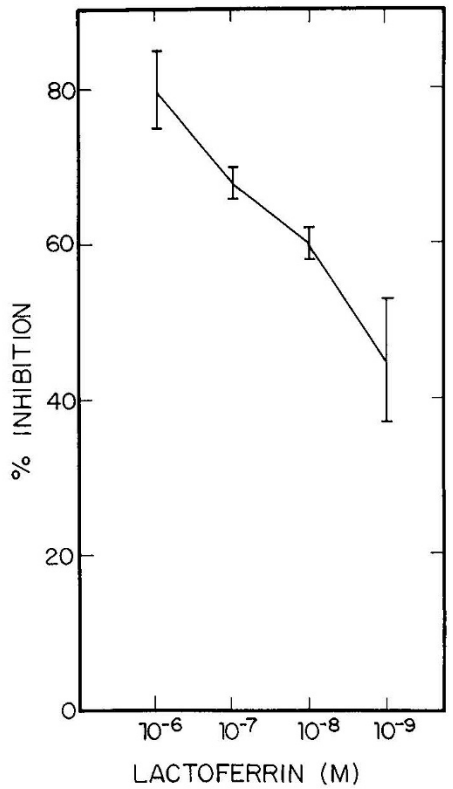

Fig. 3. Inhibition of PGE_2 secretion by macrophages in culture by various concentrations of LF in the presence of Con A (10 μg/ml). Each *point* represents mean ± SD of duplicate cultures from two separate experiments.

PGE_2 secretion by human milk macrophages. As we have shown in a previous study (4) milk macrophages secreted PGE_2 in culture and addition of Con A enhanced their PGE_2 production. Addition of human milk lactoferrin at concentrations of 10^{-6}–10^{-9} M resulted in a dose-dependent inhibition of PGE_2 production (Fig. 2). Dose-dependent suppression of PGE_2 secretion by lactoferrin was also observed in the presence of Con A (Fig. 3).

The same concentrations of lactoferrin did not influence the assay of PGE_2. Addition of lactalbumin (10^{-6} M) or lactoglobulin (10^{-6} M) to the culture medium did not significantly alter PGE_2 production compared to that of the control cultures either in the presence or absence of Con A (results not shown).

Lysozyme secretion by human milk macrophages. The secretion of lysozyme by milk macrophages did not differ significantly in the presence of various milk proteins when compared to that of unstimulated cells (Table 1). The concentration of lysozyme in the extracellular medium progressively increased during the incubation period up to 96 h (results not shown).

Oxidative burst of human milk macrophages. Human milk macrophages produced small amounts of O_2^- and H_2O_2 in culture. Macrophages incubated with PMA responded with the release of increased amounts of O_2^- and H_2O_2. Human milk lactoferrin had no effect on either O_2^- or H_2O_2 production in the presence or absence of PMA (Tables 2 and 3).

DISCUSSION

In this study we found that human milk LF inhibited PGE_2 production by breast milk macrophages *in vitro* in a dose-dependent manner. Similar findings have been reported following the addition of iron saturated lactoferrin to murine peritoneal macrophages (13). We observed the same effect when breast milk macrophages were stimulated by Con A. In addition lactoferrin had no effect on lysozyme production. We have also demonstrated the ability of human milk macrophages to produce O_2^- anion and hydrogen peroxide in culture and we found no evidence that native human milk LF influences the oxidative burst of breast milk macrophages. Thus, LF derived from human milk

Table 1. *Lysozyme secretion by breast milk macrophages in the presence of the various milk proteins**

Addition	Lysozyme (μg/ml/10^6 cells)
None	3.6 ± 0.4
Lactalbumin (10^{-6} M)	3.6 ± 0.4
Lactoglobulin (10^{-6} M)	3.3 ± 0.8
LF (10^{-6} M)	3.4 ± 0.7
LF (10^{-7} M)	3.1 ± 0.5
LF (10^{-8} M)	3.2 ± 0.5

* Results are expressed as mean ± SD. They were derived from cumulative data of at least duplicate cultures from three separate experiments.

Table 2. *O_2^- production by human milk macrophages in culture**

Addition	nmol O_2^-/1 × 10^6 cells/90 min
None	1.4 ± 0.3
PMA (20 nM)	28.8 ± 3.5
LF (10^{-6} M)	1.3 ± 0.3
LF (10^{-6} M) + PMA (20 nM)	28.7 ± 3.4

* Results are expressed as mean ± SD; they were derived from cumulative data of at least duplicate cultures from three separate experiments.

Table 3. *Hydrogen peroxide production by human milk macrophages in culture**

Addition	nmol H_2O_2/1 × 10^6 cells/90 min
None	11.7 ± 4.6
PMA (20 nM)	57.5 ± 2.3
LF (10^{-6} M)	14.2 ± 2.6
LF (10^{-7} M)	10.9 ± 1.7
LF (10^{-6} M) + PMA (20 nM)	45.4 ± 9.1
LF (10^{-7} M) + PMA (20 nM)	64.6 ± 11.0

* Results are expressed as mean ± SD ($n = 6$); they were derived from a representative experiment.

appeared to selectively alter only one of the macrophage activities we studied.

We were also able to show that ^{125}I-LF binds to human milk mononuclear cells. Inhibition of this binding by increasing concentrations of unlabeled LF indicated the presence of a specific receptor. This demonstration of LF receptors on breast milk macrophages is consistent with the previous findings of other investigators who demonstrated the presence of specific LF receptors on both mouse peritoneal macrophages (10) and human blood monocytes (11, 12, 20).

Mature bovine milk contains 20–200 μg/ml lactoferrin (21) while its concentration in human milk is not less than 1 mg/ml (22) and plasma concentration of LF is only about 1 μg/ml (8). Since cells of the macrophage lineage have clearly defined LF receptors on their plasma membrane, it seemed important to study the interaction of LF with the breast milk macrophage, the major cellular component of human milk. It is also of interest that the level of LF in breast milk diminishes with lactation along with concomitant decreasing amounts of macrophages (22).

Recent studies indicate that LF may act as a feedback inhibitor of granulopoesis by suppressing CSF production from monocytes and macrophages (13). *In vitro*, in addition to inhibition of granulopoesis, LF reduces the primary antibody response (23) and is involved in complement activation (24). LF has also been shown to enhance hydroxyl radical production by neutrophils (25) and stimulate phagocytosis of certain parasites by murine macrophages and human monocytes (26). We found no evidence that native human milk LF influences superoxide and hydrogen peroxide production by breast milk macrophages. Therefore, the role of lactoferrin regarding phagocytosis and microbiocidal activity of breast milk macrophages remains unclear and requires further investigation.

Human milk contains prostaglandins (27); although their sources are uncertain, milk macrophages are probably one of them (4). Prostaglandins affect various physiological functions of the gastrointestinal tract including gastric secretion (28) and release of brush border enzymes (29). They also exert a potent secretory effect on the intestinal epithelium (28) and increase intestinal motility (31). Interestingly, specific LF receptors have also been demonstrated in human intestinal epithelial cells (32). Our study demonstrated that LF inhibits PGE_2 synthesis by breast milk macrophages and therefore may regulate some of the possible effects of milk prostaglandins on the gastrointestinal tract of the infant.

The clinical significance of our findings for the breast-fed infant is still to be determined. However, it is likely that the biological role of human milk LF is more complex than is known and in addition to its antimicrobial action it may influence immune responses and functions of the hematopoetic and gastrointestinal systems of the infant.

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