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Interleukin-10 in Human Milk

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ABSTRACT

The concentrations of immunoreactive IL-10 in the aqueous fraction of 20 specimens of human milk obtained during the first 80 h of lactation and stored at −60°C ranged from 66 to 9301 pg/mL (mean ± SD, 3304 ± 3127 pg/mL). IL-10 was present also in the lipid layer of milk. Gel filtration revealed that IL-10 was located in a high molecular weight fraction, where certain other cytokines in human milk have been found. In addition, immunoreactive IL-10 in milk increased after treatment with sodium taurocholate. Bioactive IL-10 was demonstrated by the finding that human milk inhibited [^{3}H]thymidine uptake by human blood lymphocytes and that inhibition was partly overcome by concomitant incubation with antibodies to human IL-10. IL-10 mRNA but no protein product was found in cultured human mammary epithelial cells. Some IL-10 was associated with preparations of human milk leukocytes, but the data did not suggest that the cells were producing the cytokine. Bioactive IL-10 in a possible protected compartment suggests that IL-10 in human milk may have immunomodulating, antiinflammatory effects on the alimentary tract of the recipient infant. (***Pediatr Res* 37: 444–449, 1995**)

Abbreviations

TNF-α, tumor necrosis factor-α
TGF-β2, transforming growth factor-β2
PCR, polymerase chain reaction

There is recent evidence that the immune system in human milk is composed not only of direct-acting antimicrobial agents and antiinflammatory factors, but also of immunoregulators (1). The immunoregulators in early human milk secretions include the proinflammatory cytokines TNF-α (concentrations, ~620 ± 183 pg/mL) (2), IL-1β (concentrations, ~1130 ± 478 pg/mL) (3), IL-6 (concentrations, ~151 ± 89 pg/mL) (4, 5), and IL-8 (concentrations, ~3684 ± 2910 pg/mL) (6). The patterns of the concentrations of these cytokines in human milk suggested that the the levels were physiologically significant. It was ascertained that the production of TNF-α by human milk leukocytes could not be augmented by the addition of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine or 4β-phorbol-12β-myristate-13α-acetate to those cells in culture (2). Thus, the low production of IL-1 (7) and TNF-α (2) by leukocytes in human milk further suggested that human milk contains factors that regulate synthesis of proinflammatory cytokines.

Because of the above evidence and the antiinflammatory character of the protection afforded by human milk, we hypothesized that human milk contains antiinflammatory cytokines. One antiinflammatory cytokine, TGF-β2, had already been found in human milk (6, 8, 9), but it seemed likely that others would be present. The most likely candidate was IL-10 for the following reasons. IL-10, an 18-kD protein homodimer that is a member of the α-helix family of hematopoietic cytokines (10), has an array of antiinflammatory properties (11, 12). These include the ability to inhibit the production of IL-1, IL-2, IL-6, IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, macrophage migration inhibitory factor, and interferon-γ (13–20), many of which are proinflammatory cytokines. Therefore, we undertook an investigation to determine whether IL-10 was present in human milk in sufficient quantities to regulate the production of proinflammatory cytokines and to display other immunoregulatory properties attributed to this agent. This study became possible because of the recent availability of reagents to quantify human IL-10.

METHODS

Subject selection—human research assurances. The use of human subjects was approved by the Institutional Review Board for Human Research of The University of Texas Medical Branch at Galveston. Informed consent was obtained from each participant in the investigation. The 20 subjects recruited for this study were healthy adult women who delivered normal newborns after full-term pregnancies.

Specimen collections. One milk specimen was obtained from each of the 20 subjects during the first 80 h of lactation (mean postpartum time, 39 h; range, 12–80 h). Human milk was collected into sterile polypropylene containers by a low-

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pressure electric pump (Egnell, Inc., Irvine, IL). Each specimen was centrifuged at 680 × *g* for 10 min to obtain an aqueous fraction, a lipid layer, and a cell preparation, the latter of which had the majority of lipids, milk fat globules, membranes, and micelles removed. The aqueous fraction of milk was examined immediately or was stored at −60°C for a few days until analyses were performed.

Immunoquantitation of IL-10. IL-10 in the human milk preparations was quantified by a sandwich ELISA method that used capture rat MAb (IgG1 isotype; clone, JES3-9D7) to human recombinant IL-10 and detector biotinylated rat MAb (IgG2a isotype; clone, JES3-12G8) to human recombinant IL-10 (3 μg/mL of each antibody preparation; Pharmigen, San Diego, CA). The reactions were developed with streptavidin-horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) and carried out in microtiter plates (Immunol-2 plates; Dynatech, Chantilly, VA). The reaction product was detected with *O*-phenylenediamine dihydrochloride (Sigma Chemical Co.). Half-maximal optical absorbance (492–540 nm) readings were used as end points. Standard plots for converting the optical absorbance readings to concentrations of IL-10 were constructed by measuring known concentrations of human recombinant IL-10 (R & D Systems, Inc., Minneapolis, MN). This was accomplished by plotting the log of the standard concentrations of recombinant human IL-10 (pg/mL) against the log of the optical absorbance readings.

The sensitivity of the measurement was 80 pg/mL. Values between 80 and 1200 pg/mL of the protein were detected by the assay. The coefficient of variation of the measurements was ~1–2%. The validity of the assay for quantifying IL-10 in human milk was tested by the following experiment. A human milk preparation was divided into two aliquots. Two hundred picograms of human recombinant IL-10 (R & D Systems, Inc.) were added to one aliquot. IL-10 was then quantified by the previously described ELISA. In addition to the endogenous IL-10 in the preparation, over 98% of the exogenous IL-10 was detected.

To further validate the method for quantifying IL-10 in human milk, experiments were carried out to determine the effects of two different storage temperatures upon the detection of IL-10 in human milk. In those experiments, milk samples were divided into two aliquots. The first was maintained at −60°C for 10 d. The second was stored −60°C for 72 h and then was thawed and maintained at 4°C for an additional 72 h. The second preparation was split into two fractions, one that was maintained at 4°C and the other that was stored at −60°C for 3 d more.

Physical distribution of IL-10 in human milk. Because of reports that TNF-α and some IL-6 in human milk are present in a high molecular weight fraction of human milk (2, 4), the physical distribution of IL-10 in human milk was examined in two ways. The first approach was to examine whether IL-10 was present in the lipid layer that was obtained by centrifuging fresh whole milk at 680 × *g* for 10 min. The second approach was to determine whether treatment of human milk with an artificial detergent, polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co.), and a bile salt, sodium taurocholate, leads to the release of additional immunoreactive human IL-10. That was accomplished by adding polyoxyethylene sorbitan monolaurate (0.025%) to whole fresh milk or sodium taurocholate (12 mM) to either the aqueous or lipid fraction of three specimens of human milk and incubating the mixture for 30 min at room temperature. Controls for this experiment were aliquots of untreated whole milk or untreated fractions of the milk obtained by centrifugation.

The third approach was fractionation of milk by column chromatography. Defatted, acellular human milk was applied to a Sephacryl S-200 gel filtration column (Pharmacia Biotechnol Inc., Piscataway, NJ). The molecular mass standards were blue dextran (250 kD), BSA (68 kD), carbonic anhydrase (29 kD), and cytochrome *c* (12.4 kD). Each eluted fraction was assayed for IL-10 by the previously described ELISA.

Bioactivity of IL-10 in human milk. The bioactivity of IL-10 in human milk was examined by determining whether human milk inhibited the uptake of [^{3}H]thymidine by human peripheral blood mononuclear leukocytes (>90% T cells by immunofluorescent flow cytometry) that were unstimulated or stimulated with antibodies to human CD3 (OKT3, 0.025 μg/mL), and whether that inhibition could be partially blocked by the addition of specific murine MAb to human recombinant IL-10 (19F1) (1:2000 dilution) (hybridoma from the American Type Culture Collection) (15). Human recombinant IL-10 (2 ng/mL) was used as a positive control in the experiments. About 2 × 10^5 mononuclear leukocytes were used in each microtiter well. In those preparations stimulated with antibodies to CD3, the wells were precoated overnight with antibodies before blood mononuclear leukocytes were added. The cells were cultured for 72 h at 37°C in 7% CO_2. Four hours before harvest, the cells were pulsed with 1.0 μCi of [^{3}H]thymidine. The uptake of [^{3}H]thymidine by the cells was then quantified by scintillation spectroscopy.

Source of IL-10 in human milk. Two potential sources of IL-10 in human milk were tested. The first were cell preparations (>95% leukocytes) obtained from milk specimens by centrifuging the specimens at 680 × *g* for 10 min and washing them three times in Hanks' balanced salt solution. These preparations contained not only cells but also high-density milk fat globules and other particulate material. Cell viability as determined by trypan blue exclusion was ~95%. The number of cells in the preparations ranged between 9 and 20 × 10^6/mL. Aliquots of unfractionated cells were sonicated or incubated in RPMI 1640 for 4 or 24 h in 7% CO_2 at 37°C. The sonicated cells were centrifuged at 1000 × *g* for 3–5 min to remove particulate materials; supernatants from the incubated cells were separated by centrifugation at 680 × *g* for 3–5 min. Supernatants from sonicated cells and the supernatants from the cultured milk leukocytes were then assayed for IL-10 by the previously described ELISA.

A spontaneously immortalized human mammary gland cell line (MCF-10) derived from an adult woman with fibrocystic disease of the breast (American Type Culture Collection) (21, 22) was examined for the presence of IL-10 mRNA and for its ability to produce the cytokine. The cells are SV40-free, not tumorigenic in nude mice, and positive for sialomucins, keratins, and surface protein antigens found on normal human mammary gland epithelial cells. In contrast, they have no

features of myoepithelial or stromal cells (21, 22). The reported diploid pattern with a balanced chromosome 1–3-9 translocation (21, 22) was confirmed in the Cytogenetics Laboratory of this institution. These nonneoplastic cells have also been found to produce IL-6 and IL-8 (6).

The mammary epithelial cells were cultured on 24-well plastic plates (Costar Delta Packaging Corporation, Cambridge, MA) in the presence of insulin (Sigma Chemical Co.) (10 mg/mL), hydrocortisone (Sigma Chemical Co.) (500 ng/mL), and epithelial growth factor (Sigma Chemical Co.) (20 ng/mL) with or without an extracellular matrix (a mixture of enactin, collagen IV, and laminin; E-C-L Cell Attachment Matrix, lot no. 11645, Upstate Biotechnology Inc., Lake Placid, NY) until they reached confluency. Adherent cells were washed gently and fresh culture media was added. The number of cells in each well was not determined, because the experiments were not designed to examine the quantitative relationships between the number of cells and the amount of IL-10 that was produced, but the number of cells, as estimated from other experimental observations in our laboratory, was between 3–5 $\times 10^5$ cells/well. After 24 h of additional culture at 37°C in 7% CO_2, supernatant fluids and sonicated cells were harvested and IL-10 quantified by the previously described ELISA.

Cultured mammary gland epithelial cells were also examined for the expression of the mRNA for human IL-10 by the following reverse-transcriptase PCR and Southern blot methods. Total RNA was isolated from 5×10^6 MCF-10 cells by the acid-guanidinium method (23). One microgram of total RNA was reverse transcribed using random hexamers as the primer. Twenty microliters of each sample were then subjected to PCR using IL-10 primers (see below) in 100-μL volumes with PCR buffer ($MgCl_2$, 2 mM; KCl, 50 mM; and Tris-HCl, 10 mM), and *Taq* DNA polymerase in a thermal cycler (Perkin-Elmer, Norwalk, CT). Samples were denatured at 90°C for 45 s, annealed at 60°C for 45 s, and extended at 70°C for 120 s, for 30 cycles. PCR products were then size-fractionated on 8% acrylamide gels. Subsequently, gels were denatured for 1 h at room temperature in 0.5 M NaOH and 1.5 M NaCl and transferred to nylon membranes. Membranes were prehybridized in 5 × SSPE, 0.01% SDS, 1× Denhardt's solution and 200 μg/mL of salmon sperm DNA (Sigma Chemical Co.) for 2 h at 40°C.

Membranes were subsequently hybridized with a ^{32}P-3′-end-labeled complementary oligonucleotide probe, specific for a sequence internal to the primers used in the amplification (see below). As a positive control, a human IL-10 cDNA fragmentation (Clontech, Palo Alto, CA) was included. After hybridization for 14 h at 40°C, filters were washed in 5 × SSPE and 0.01% SDS and exposed to Kodak XAR-5 films for 24 h. Primers and probe used were as follows: IL-10 sense primer 5′-AAGCTGAGAACCAAGACCCAGAGACATCAAGGCG, IL-10 antisense primer AGCTATCCCAGAGCCCCAGATCCGATTTTGG, IL-10 oligo-probe AGCAGGTGAAGAATGCCTTT, β-actin sense primer 5′-GTGGGGCGCCCCAGGCACCA, β-actin antisense primer GTCCTTAATGTCACGCACGATTTC (Clontech).

Statistical methods. Group data were presented as means ± SD. Differences in the concentrations of IL-10 in milk specimens collected during the first 24 h and thereafter were tested by a nonparametric method based on ranks (24).

RESULTS

Concentrations of IL-10 in human milk. The concentrations of IL-10 in 20 specimens of human milk (one measurement from each subject) collected during the first 80 h of lactation were 3304 ± 3127 pg/mL (Fig. 1). As shown by a rank order nonparametric analysis, IL-10 concentrations were higher during the first 24 h postpartum ($p < 0.05$).

Effect of storage temperatures on quantitation of IL-10 in human milk. In paired experiments, concentrations of IL-10 in milk stored at −60°C, thawed, and then stored at 4°C for 72 h increased by 2% in one specimen and fell by 8, 27, and 30%, respectively, from three specimens stored at −60°C for 10 d. When aliquots kept at 4°C for 72 h were placed at −60°C for 72 h, detectable levels of IL-10 increased to values found in the original frozen preparations.

Physical distribution of IL-10 in human milk. IL-10 was detected in the lipid layer of two specimens of fresh human milk that were tested (116 and 1155 pg/mL, respectively). After treatment of one specimen of whole milk with polyoxyethylene sorbitan monolaurate, the concentration of immunologically detectable IL-10 rose from 930 to 2553 pg/mL. No further studies with that artificial detergent were pursued, because we were more interested in examining the effects of one of the bile salt that occurs in small intestine, sodium taurocholate. Sodium taurocholate caused an increase in the amount of immunoreactive IL-10 from both the aqueous and the lipid phase of human milk of the three specimens that were tested. In the first specimen, the amount in the aqueous phase rose from 236 pg to 441 pg/mL and the amount in the lipid layer increased from 161 to 312 pg/mL. Only the aqueous phase was examined in the second specimen. No IL-10 was detected before treatment; 250 pg/mL was found after treat-

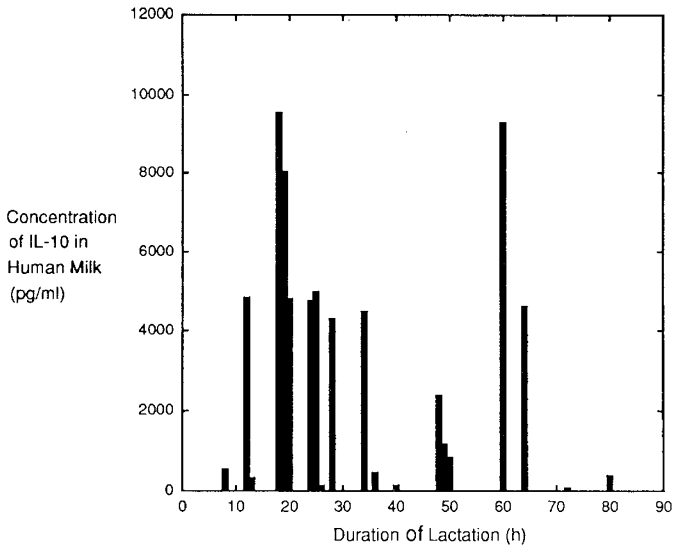

Figure 1. Concentrations of IL-10 in the aqueous phase of 20 specimens of human milk determined by a sandwich ELISA. The concentrations of IL-10 were highest in the first 24 h of lactation (*i.e.* postpartum period) ($p < 0.05$).

ment. In the third specimen, the lipid layer was tested; the concentration of immunologically detectable IL-10 rose from 1155 to 2618 pg/mL.

Further evidence for possible compartmentalization of IL-10 in human milk was found in the gel filtration experiments. In those studies, IL-10 was detected principally in a high molecular mass fraction (~80 kD) of human milk (Fig. 2).

Bioactivity of IL-10 in human milk. In three experiments, the inhibition of [^{3}H]thymidine incorporation by human blood T cells exposed to human milk was similar to that found with T cells treated with human recombinant IL-10 (see Fig. 3 for representative data). Human milk inhibited the uptake of [^{3}H]thymidine by human peripheral blood mononuclear leukocytes that were or were not stimulated with antibodies to CD3, and that the inhibition in both situations was partially blocked by the addition of neutralizing antibodies to human IL-10 (Fig. 3).

Source of IL-10 in human milk. Although low levels of IL-10 were associated with the human milk leukocyte preparations (data not shown), the data do not suggest that IL-10 is produced by those cells. That is consistent with the reported inability to detect IL-10 in human milk leukocytes by immunofluorescence (25).

Constitutive expression of mRNA for IL-10 by the spontaneously immortalized human mammary gland cell line was demonstrated by Southern blotting (Fig. 4). The DNA product obtained from PCR reactions using primers for IL-10 hybridized with a probe specific for human IL-10, and the size of the PCR product was 328 bp (Fig. 4). However, only small amounts of IL-10 were detected in human mammary gland epithelial cells cultured on plastic (intracellular concentrations, 24 ± 7 pg/mL of sonicated cells; extracellular concentrations, 20 ± 1 pg/mL of supernatant) or from cells cultured on an extracellular matrix (data similar to that shown above, but not included).

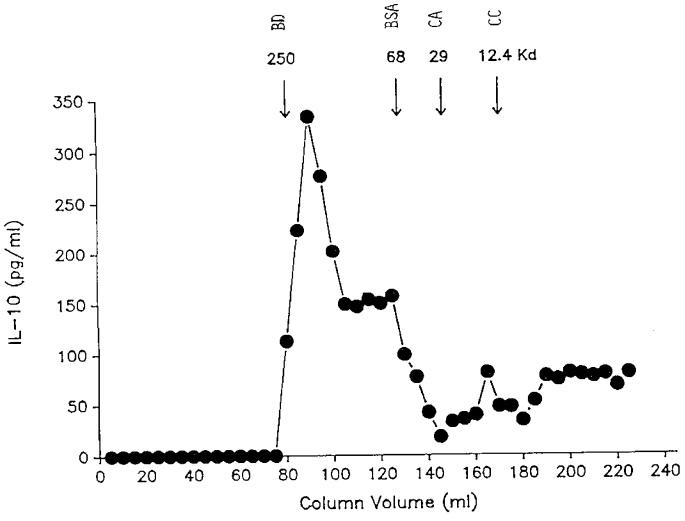

Figure 2. Gel filtration of human milk on a Sephacryl S-200 column. Defatted, acellular milk was applied to the column. Proteins were eluted from the column with a standard borate buffer. Molecular mass markers were blue dextran (*BD*, 250 kD), BSA (68 kD), carbonic anhydrase (*CA*, 29 kD), cytochrome *c* (*CC*, 12.4 kD). IL-10 was detected in a molecular mass fraction >80 kD.

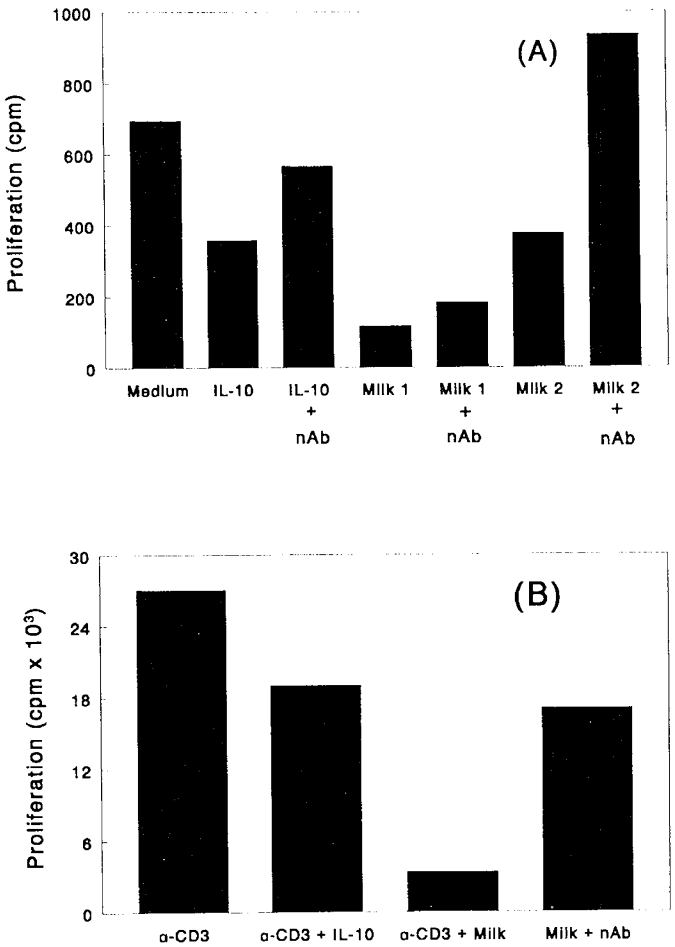

Figure 3. Bioactivity of IL-10 in human milk. The bioactivity of IL-10 in human milk was examined by determining whether human milk inhibited the uptake of [^{3}H]thymidine by human peripheral blood mononuclear leukocytes, and whether that inhibition was partially blocked by the addition of neutralizing antibodies (*nAb*) against recombinant human IL-10. Representative data from cells cultured in the absence (*A*) or presence (*B*) of antibodies to CD3 (*α-CD3*) are shown. The uptake of [^{3}H]thymidine (proliferation) is expressed on the *y* axis as cpm.

DISCUSSION

In this study, physiologically significant concentrations of immunoreactive IL-10 were found in the aqueous phase of early human milk secretions. Indeed, the concentrations of IL-10 in the aqueous phase of human milk were greater than other immunochemically measured cytokines in milk, except for IL-8 (6). IL-10 concentrations were highest at 12–24 h of lactation. If ~120 mL of milk are produced during the first few days postpartum (26), then ~350 ng of IL-10 would be secreted per day.

To further interpret the quantitative measurements, the physical state of IL-10 in human milk was investigated. Evidence was found that some IL-10 was present in a nonaqueous phase as well as the aqueous phase of human milk. IL-10 was demonstrated in the lipid fraction of human milk, increased amounts of IL-10 were detected after the aqueous or lipid phase of human milk was treated with sodium taurocholate, and IL-10 was detected in a high molecular mass column chromatography fraction (>80 kD) where human recombinant

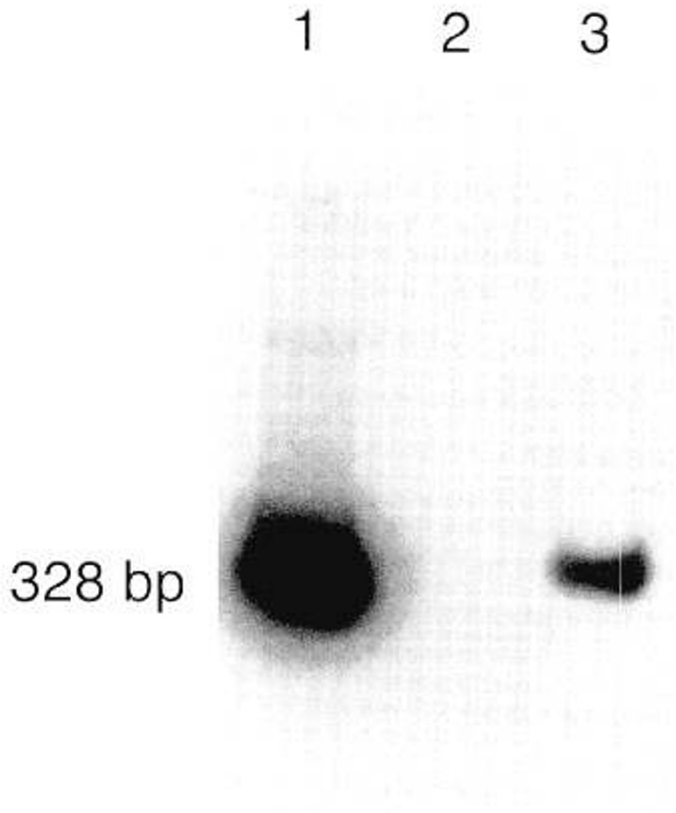

Figure 4. Demonstration by Southern blotting of constitutive expression of mRNA for IL-10 by spontaneously immortalized human mammary gland cells. *Lane 1* is a positive control IL-10 cDNA fragment, *lane 2* is a negative control (water), and *lane 3* is the PCR product from mammary epithelial cells. The sizes of the PCR product from the positive control and the mammary gland epithelial cells were identical (328 bp).

IL-10 would not be anticipated, but where TNF-α and a significant proportion of IL-6 in human milk reside (2, 4). Therefore IL-10 in human milk may be bound to other proteins or compartmentalized in human milk. One possibility is that IL-10 in human milk is a complex of a dimer of the cytokine (37 kD) bound to its receptor (60 kD) (27). Regardless of its precise physical state, the experiments suggest that the total IL-10 in human milk is significantly higher than the amounts measured in the aqueous phase of milk, and that some of it may be released after encountering physiologic concentrations of bile salts (28) such as were used in these experiments.

Does that finding suggest that IL-10 in human milk is secreted in an apocrine fashion from mammary epithelial cells? There is *in vitro* evidence of IL-6 and IL-8 production and secretion by human mammary gland epithelial cells (6, 29). The *in vitro* experimental conditions with those cells do not completely model the *in vivo* environment of mammary gland epithelium. Only small amounts of IL-10 protein were detected in those cell preparations, but the mRNA for the cytokine was constitutively expressed by them. It therefore seems possible that IL-10 is produced *in vivo* by mammary gland epithelial cells. Although no evidence of IL-10 production by the leukocytes in human milk was found, we cannot rule out the possibility that some IL-10 was produced by those cells while they were in the mammary gland.

The sequence of production of cytokines in human milk and the interplay between them is unknown. Inasmuch as IL-10 inhibits the production of IL-1, IL-6, IL-8, and TNF-α (30) and TNF-α stimulates the production of IL-10 (31), IL-10 may be produced sometime after the synthesis of the proinflammatory cytokines. Experimental animal models may be required to examine that question.

This investigation demonstrates that IL-10 in human milk is bioactive. Indeed, those results are in keeping with a previous report that human milk inhibits the production of IL-2 by stimulated human blood T cells (32). In addition, recent experiments in our laboratory suggest that human newborns have a developmental delay in the production of IL-10 by blood leukocytes (unpublished data). Thus, bioactive IL-10 in human milk may be necessary during that period of developmental delay.

How would IL-10 in human milk affect the recipient infant? Several possibilities are suggested from past *in vitro* investigations of the biologic activities of IL-10. IL-10 inhibits the formation of proinflammatory cytokines by monocytes/macrophages (11–15), limits the participation of Th1 cells in delayed hypersensitivity (33–35), attracts $CD8^+$ T cells (36), and enhances the growth and differentiation of and immunoglobulin synthesis by B cells (37–41), including acting as a cofactor with TGF-β to increase IgA1 and IgA2 production (40). Thus, IL-10 in human milk may aid in regulating mucosal defenses and limiting inflammatory reactions in the upper parts of the alimentary and respiratory tracts.

Is ingested IL-10 active past the esophagus? In that regard, there are high concentrations of antiproteases in human milk (42) and low concentrations of H^+ in gastric secretions, a lack of intragastric protein digestion, and poor production of trypsin and chymotrypsin during the first week of life (43). In addition, part of the IL-10 in human milk may be protected by compartmentalization from gastrointestinal digestion.

Our *in vitro* experiments also suggest that compartmentalized IL-10 is released after exposure to bile salts in the duodenum. Consequently, it is likely that the released IL-10 would aid in protecting the intestinal tract. Indeed, the occurrence of a severe enterocolitis in IL-10-deficient mice generated by gene targeting (44) suggests that IL-10 plays an important role in regulating the defenses of the intestinal tract against inflammatory processes associated with enteric infections.

In summary, IL-10 may bridge the immunomodulating and antiinflammatory segments of the defense system in human milk. Both short- and long-term effects of cytokines in human milk upon the mother, and the recipient infant will require further exploration.

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