Tumor Necrosis Factor-α in Human Milk[1]

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ABSTRACT. We previously demonstrated that certain biologic activities in human milk were partially blocked by antibodies directed against human tumor necrosis factor-α (TNF-α). In this study, immunochemical methods were used to verify the presence of TNF-α in human milk obtained during the first few days of lactation. Gel filtration revealed the presence of TNF-α by RIA in molecular weight fractions between 80 and 195 kD. TNF-α could not be detected consistently by conventional Western blotting or cytotoxic assays. Although immunoreactive bands were detected by a Western blot-^{125}I protein A technique in TNF-α-positive fractions from gel filtration, those bands proved to be nonspecific. TNF-α in milk was reliably quantified by the competitive RIA. Those studies revealed that the concentrations of TNF-α in milk were 620 ± 183 pg/mL. Although RNA to TNF-α was detected in milk leukocytes by Northern blotting, little TNF-α was found in those cells before or after stimulation with N-formyl-*l*-methionyl-*l*-leucyl-*l*-phenylalanine or 4β-phorbol-12β-myristate-13α-acetate. The origin of this cytokine in human milk remains unclear. Nevertheless, this study suggests that TNF-α is present in early human milk in sufficient quantities to exert possible biologic effects upon the mammary gland of the mother or the immune system of the infant. (*Pediatr Res* 31: 29–33, 1992)

Abbreviations

TNF-α, tumor necrosis factor-α
rhTNF-α, human recombinant TNF-α
PMA, 4β-phorbol-12β-myristate-13α-acetate
FMLP, N-formyl-*l*-methionyl-*l*-leucyl-*l*-phenylalanine

It is widely accepted that much of the protective effect of human milk (1) is due to a direct-acting antimicrobial system that consists of humoral and cellular components (2–6). The leukocytes are of particular interest because they include not only neutrophils and lymphocytes but also macrophages (4–6) that appear morphologically to be activated (4, 5). The results of recent studies (7) are consistent with those previous observations. In those investigations, human milk macrophages moved faster than human peripheral blood mononuclear leukocytes in a three-dimensional, relatively adherence-independent assay. It was subsequently demonstrated that the increased motility of those macrophages was due in large part to either TNF-α or TNF-α-

inducing factors in human milk. When blood mononuclear cells were incubated in whole human colostrum or its whey protein fraction, the motility of the blood monocytes increased to that of milk macrophages (8). The activity was abrogated by trypsin and considerably decreased by polyclonal antibodies to rhTNF-α. Three peaks of chemokinetic activity, corresponding to 50–55, 25, and 10–17 kD, were demonstrated by gel filtration chromatography. The chemokinetic activity peaks in human milk were blocked by antibodies to rhTNF-α. In addition, the studies suggested that some of the limited cytotoxic activity of human milk against murine L-929 cells was blocked by antibodies to rhTNF-α (8).

In view of the pleiotropic effects of TNF-α upon the immunologic system (9–19), it was important to verify whether TNF-α or TNF-α-inducing agents are present in human milk in sufficient quantities to potentially affect the recipient infant. Indeed, the presence of sufficient amounts of those agents would suggest that human milk might affect the maturation of the defense systems of the infant. We investigated this question by examining human milk for TNF-α by immunochemical methods. Furthermore, we sought to determine whether the leukocytes in human milk are a source of this cytokine by examining them for TNF-α and for its RNA.

MATERIALS AND METHODS

Subject selection. This study was approved by the Institutional Review Board for Human Research. All donors voluntarily consented to participate in the study. They were healthy women between 18 and 35 y of age who planned to breast-feed and had delivered mature, healthy infants after full-term pregnancies. Milk was collected using a low-pressure electric breast pump (Egnell, Inc., Cary, IL) 24–48 h after delivery. The specimens were centrifuged at 680 × g for 10 min, lipids and cells were removed, and the aqueous layer was collected and stored in polypropylene containers at −80°C until it was analyzed.

Leukocyte preparations. Cells harvested from colostrum by the above technique were resuspended in 3 mL of RPMI 1640 and counted by direct microscopy. Cytocentrifuge preparations of the cells were stained for nonspecific esterase to identify macrophages (20). The remaining cells were divided into three tubes. One was placed on ice; the others were incubated with 5 μg/mL of PMA (Sigma Chemical Co., St. Louis, MO) or 10^{-8} M FMLP (Sigma Chemical Co.) at 37°C for 4 h. The cells were lysed by sonication and centrifuged for 10 min at 680 × g to remove intact cells and nuclei. The lysates were stored at −80°C until they were analyzed.

Venous blood mononuclear cells from healthy adults were separated from other leukocytes by Ficoll-Hypaque density gradient centrifugation. These mononuclear cells were then treated in the same fashion as the milk cells.

Preparation of whey proteins. After the milk specimens were centrifuged for 10 min at 680 × g, the lipid layer and the cells were removed and the aqueous phase was frozen at −80°C until it was analyzed. After thawing, the pH of the specimen was

adjusted to 4.6 with 30% acetic acid and the precipitated caseins were removed by centrifugation (27 000 × g for 10 min). Then, the pH of the fluid phase was raised to 7.2 with 10% sodium hydroxide, and the proteins were precipitated by adding an equal volume of saturated ammonium sulfate (Sigma Chemical Co.). The proteins were resolubilized in PBS (pH 7.2) and dialyzed overnight in PBS. The concentration of total protein in each preparation was quantified by the Bradford dye-binding method (21), the Lowry method (22), and the Pierce bicinchoninic acid assay. Human albumin (50 mg/mL) and human Ig (Cohn's fraction II) (30 mg/mL) were used as standards to compare the results obtained from the above methods with those found by Kjeldahl's method (23).

Gel filtration. In certain experiments, defatted, acellular colostrum or the whey protein fraction was chromatographed on a Sephacryl S-200 gel filtration column (Pharmacia LKB Biotechnology, Piscataway, NJ) (8). The resultant fractions were concentrated and analyzed by competitive RIA and Western blotting.

Immunologic assays for human TNF-α. Conventional Western blotting was performed as follows. Samples were first electrophoresed on a discontinuous 10% polyacrylamide gel under reducing conditions (24) and then electroblotted onto nitrocellulose (25). Thereafter, the nitrocellulose preparations were blocked and incubated first with rabbit antibodies to rhTNF-α (1:200) (Genzyme, Boston, MA) and then with goat antibodies to rabbit IgG conjugated with biotin (1:5000) (Calbiochem, San Diego, CA). These strips were incubated with streptavidin conjugated with horseradish peroxidase (1:5000) (Calbiochem) and developed with 60 mg of 4-chloro-1-napthol (Bio-Rad Laboratories, Richmond, CA) dissolved in 20 mL of methanol added to 100 mL of distilled water and 100 μL of 30% hydrogen peroxide. The sensitivity of the assay was about 200 ng/mL.

Because the sensitivity of the above procedure was not sufficient for the detection of TNF-α in human milk, an alternative approach was used to augment the sensitivity of the assay. ^{125}I-labeled staphylococcal protein A (ICN Biomedicals, Inc., Costa Mesa, CA) was substituted for the biotin-avidin-labeled antibodies (26). After a 1- to 4-h exposure, radiolabeled bands were visualized by autoradiography. One control in this procedure was the inclusion of serum from a rabbit hyperimmunized against concanavalin A. In that control experiment, after the nitrocellulose preparations were blocked, the rabbit hyperimmune serum was used instead of the antibodies to TNF-α.

TNF-α was measured with competitive RIA procedures (Amersham, Arlington Heights, IL; Genzyme). Standards or specimens of human milk were incubated with specific rabbit antibodies to human TNF-α at 37°C overnight. Then, 11 nCi of ^{125}I-labeled rhTNF-α were added, and the preparations were held at 4°C overnight. Afterwards, polyclonal antibodies (donkey) to rabbit Ig attached to magnetic beads were added, and the mixtures were kept at room temperature for 10 min. The mixtures were centrifuged, and all supernatant fluids were aspirated while the beads were fixed by magnetic or centrifugal force to the bottom of the tube. The pellets were counted in a gamma counter. The sensitivity of the competitive RIA used to detect rhTNF-α was about 100 pg/mL. The amounts of TNF-α in the specimens of human milk were determined from a standard curve generated from concentrations of rhTNF-α ranging from 0 to 2000 pg/mL.

Cytotoxic assay for TNF-α. Cytotoxic activity in milk due to TNF-α was sought by assays using murine L-929 cells plated at 2 × 10⁴ cells/well for 24 h in RPMI 1640 with 2% FCS. Milk was defatted, and the cells, particles, and membranes were removed by centrifugation at 1000 × g for 10 min. After passage through 0.22-μm filters, various dilutions of the milk were placed in the wells with actinomycin D (5 μg/mL) and incubated for 20 h. Supernatants were removed, and 0.2% crystal violet in PBS was added to detect adherent, viable cells. After 2 min, excess dye was washed from the wells, and the remaining cells fixed with 50% ethanol. The plates were read at 600 nm (EIA Reader;

Bio-Tek Instruments, Inc., Burlington, VT). Controls included rhTNF-α (0–10⁵ pg/mL) in culture medium alone and in dilutions of milk. In addition, polyclonal antibodies to human TNF-α were added to certain dilutions of milk in every experiment.

Detection of RNA to TNF-α. The presence of RNA to TNF-α in human milk macrophages was tested by Northern blotting. The procedure was as follows (27). Unfractionated cells from human milk, 10 to 30% of which were macrophages as shown by microscopy and nonspecific esterase staining, were used. Cytoplasmic RNA for Northern blots was extracted by lysis with a buffer solution containing guanidine isothiocyanate (4.0 M) and gradient centrifugation with a buffer solution containing cesium chloride (5.7 M). The material was resuspended, precipitated with ethanol, centrifuged, washed, and then resuspended in H₂O. The RNA was denatured in a loading buffer containing formamide and formaldehyde. The concentration of RNA was estimated spectrophotometrically at 260 nm. Approximately 8 μg of RNA in 20 μL of the formamide/formaldehyde buffer solution was placed on a horizontal agarose (1.4%) gel. Then the material was electrophoresed at 40 V overnight. After electrophoresis, the RNA was transferred to nitrocellulose membranes. A cDNA probe for human TNF-α (0.8-kb *Eco*RI fragment maintained in plasmid pSP64; Genentech, Inc., So. San Francisco, CA) was labeled with ³²P 2'-deoxycytidine 5'-triphosphate random primer and Klenow fragment (Multiprime; Amersham). Prehybridization and hybridization buffer was 50% formamide, 5 × Denhardt's, 5 × SSPE (sodium chloride, sodium phosphate, EDTA), 0.1% SDS, and 250 μg/mL carrier DNA. Prehybridization was accomplished at 42°C for 4 h, and hybridization was performed at 42°C overnight. The resultant preparations then were washed four times for 15 min at room temperature in a solution containing 2 × SSPE and 0.1% SDS. Radiolabeled bands were visualized by autoradiography.

Data presentation and statistical analysis. Concentrations of TNF-α were expressed as pg per mg of total protein, per mL of milk, or per 10⁶ leukocytes. Data were presented as the mean ± SD. The relationships between certain parameters were examined by linear regression analysis, and the significance of the relationships was tested by the *t* test.

RESULTS

TNF-α in whole milk and whey proteins of human milk. When whole milk or the whey protein fraction was subjected to gel filtration on Sephacryl S-200, TNF-α was detected by RIA only in higher molecular weight fractions (Fig. 1). Three immunoreactive bands were detected by SDS-PAGE-Western blotting in each specimen of acellular human milk, the whey protein fraction, or the 80- to 195-kD gel filtration fractions when the preparations were first reacted with antibodies to human TNF-α and then with ^{125}I-labeled staphylococcal protein A as the final detector, but those bands proved to be nonspecific because they were also detected in the control experiments where hyperimmune rabbit serum to concanavalin A was used.

Before quantifying TNF-α in specimens of human milk by RIA, experiments were performed to test whether human milk contained substances that interfered with the measurement of TNF-α. TNF-α was quantified in selected specimens of human milk before and after the addition of known quantities of rhTNF-α. The observed measurements of total TNF-α were essentially identical to the calculated amounts of TNF-α in those preparations (data not shown). TNF-α was found in all specimens of milk examined by RIA. The concentrations of TNF-α in human milk were 25 ± 7 pg/mg of total protein or 620 ± 183 pg/mL of milk (Fig. 2).

In contrast to the RIA, the cytotoxicity assay was not appropriate for quantifying TNF-α in human milk, because human milk significantly disrupted the adherent layer of L-929 cells. Some of the cytotoxicity may have been due to residual lipids in the preparations. However, in about half of the milk specimens,
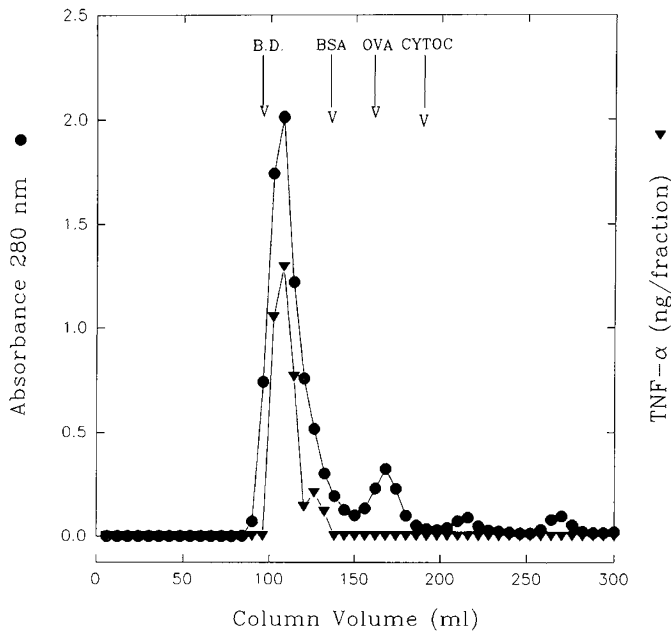
Fig. 1. Gel filtration of human milk on a Sephacryl S-200 column. Total protein (●) and TNF-α (▼) were quantified. Two mL of human colostrum were applied to the column. Proteins were eluted from the column with a standard borate buffer. Molecular weight markers were blue dextran (*B.D.*; M_r, 2000 kD), BSA (M_r, 68 kD), ovalbumin (*OVA*; M_r, 43 kD), bovine cytochrome *c* (*CYTOC*; M_r, 12.4 kD). TNF-α was detected in molecular weight fractions between 80 and 195 kD.
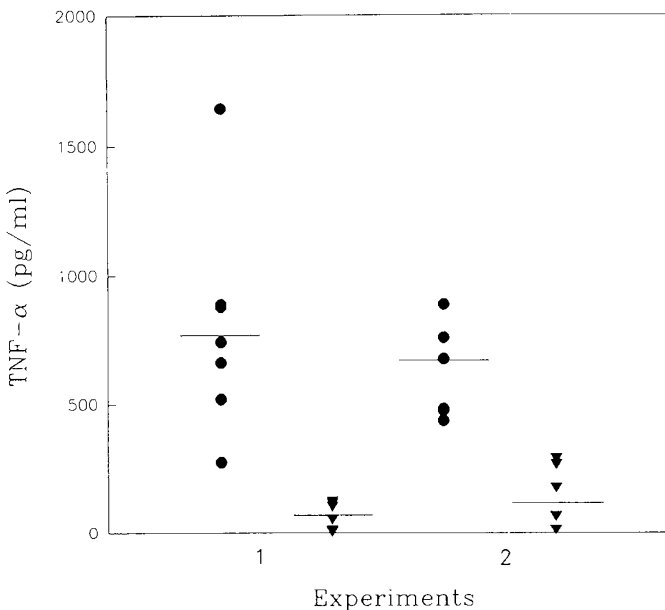

Fig. 2. Concentrations of TNF-α in the aqueous phase (●) and in total leukocytes (▼) from human milk determined by two separate RIA experiments (experiments 1 and 2). The aqueous phase and the leukocytes were derived from the same specimens of human milk. There was no correlation between the concentrations of the cytokine found in the aqueous phase and those found in the leukocytes from human milk.

a portion of the cytotoxic activity was blocked by antibodies to human TNF-α (data not shown).

TNF-α in human milk leukocytes. Based on the number of macrophages in the pellet and the assumption that the TNF-α was principally in those cells, the amount of TNF-α found by RIA in those lysates ranged from 12 to 720 pg/10^6 macrophages (mean ± SD, 275 ± 282 pg/10^6 macrophages) (Fig. 3). TNF-α in blood mononuclear cells increased 616 ± 539% and 434 ±
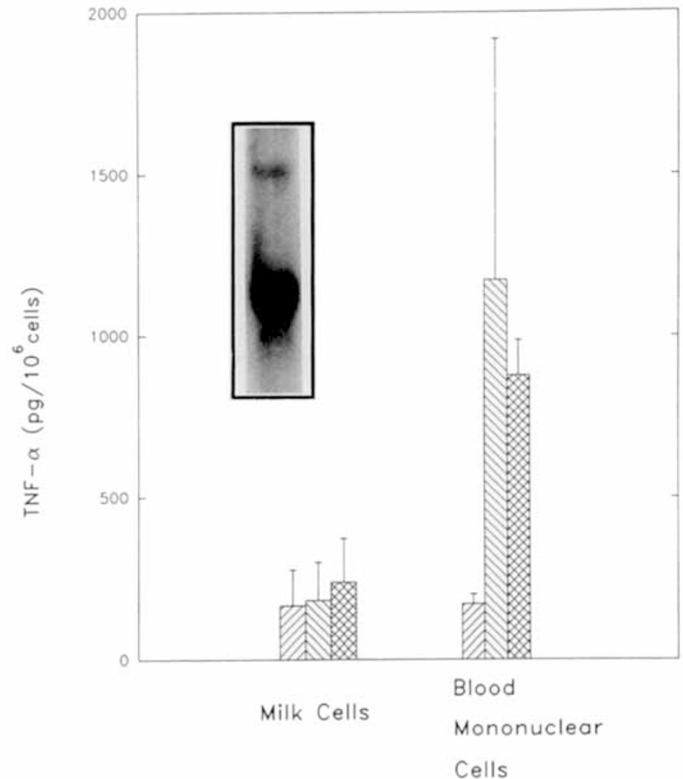

Fig. 3. The production of TNF-α by human milk leukocytes or blood mononuclear cells unstimulated (▨) or stimulated with FMLP (▨) or PMA (▨). The *bars* represent 1 SD. Little TNF-α was found in unstimulated human milk leukocytes, despite the presence of 28s and 18s RNA to TNF-α (*inset*, a representative Northern blot). In addition, there was only a small rise in the production of TNF-α from stimulated milk leukocytes as compared to blood mononuclear cells.

134% after exposure to FMLP and PMA, respectively (Fig. 3). In contrast, the amount of TNF-α in human milk leukocytes was only slightly increased after incubating them with FMLP (increment, 7 ± 16%) or PMA (increment, 60 ± 76%) (Fig. 3).

RNA for TNF-α. Northern blots of colostral leukocytes revealed 28s and 18s RNA to TNF-α. A representative example of the study is shown as an *inset* in Fig. 3.

Linear regression analyses. There was a positive correlation between TNF-α in the fluid phase of human milk and the total number of cells in human milk ($r^2 = 0.59$, $p < 0.01$) (Fig. 4). Little correlation, however, was found between the amount of TNF-α in the fluid phase and the number of macrophages from the same milk specimens ($r^2 = 0.24$) (Fig. 4).

DISCUSSION

This is one of the first immunochemical demonstrations of a cytokine in human milk and the first report of substantial amounts of TNF-α in human milk. A number of other studies have reported effects of mammalian milks on the growth and development of T and B lymphocytes that were attributed to the possible presence of cytokines (28–32). However, no studies were performed to determine if cytokines were present. Biologic activity in human milk consistent with IL-1 was previously reported (33), but its presence was not tested by other means. More recently, Munoz *et al.* (34) found considerable amounts of IL-1β (mean ± SD, 1130 ± 478 pg/mL) by a RIA in human milk collected at 3–4 d of lactation from Chilean women. In a previous study from our laboratory, chemokinetic and cytotoxic activities in human milk were partially abrogated by specific antibodies to rnTNF-α, but the presence of the cytokine in human milk was not investigated immunochemically (8).

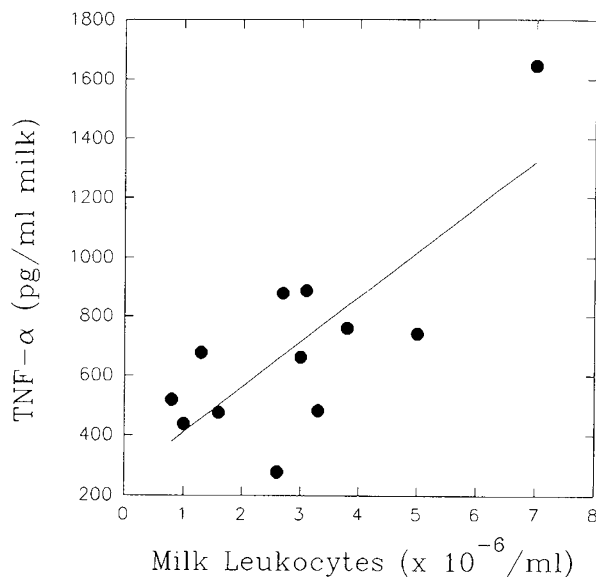In this study, immunochemical evidence was found for TNF-

Fig. 4. Correlation between the concentrations of TNF-α and the numbers of leukocytes in human milk. TNF-α and the total number of leukocytes were quantified in the same specimens. The correlation between the concentrations of TNF-α and total leukocytes in human milk was significant ($r^2 = 0.59, p < 0.01$).

α in human milk. First, proteins that were detected with RIA to TNF-α were found in a high molecular weight fraction (80–195 kD) of human milk obtained by gel filtration. Because the M_r of known molecular forms of TNF-α [(monomeric (35), transmembrane (36), and trimeric (37)] are 17, 26, and 51 kD, respectively, the experiments suggested that TNF-α in human milk was either bound to carrier proteins or that it was compartmentalized. We attempted to further understand the molecular moieties of TNF-α in human milk by subjecting this high molecular weight fraction of human milk to SDS-PAGE under reducing conditions and then to Western blotting modified by the use of ^{125}I-labeled staphylococcal protein A as the final detector. Several molecular moieties that appeared to be immunoreactive with polyclonal antibodies to human TNF-α were found, but those bands proved to be nonspecific because they were also detected with the hyperimmune rabbit serum to concanavalin A.

Because human milk did not interfere with the measurement of TNF-α by the competitive RIA, the method was used to quantify TNF-α in human milk. Although there may be multiple forms of TNF-α in human milk, only monomeric rhTNF-α was used as the standard in the measurement of that cytokine. Therefore, the results of this study are a preliminary estimate of the amounts of this protein in human milk. Given that caveat, the mean concentration of TNF-α in early human milk secretions was 620 pg/mL. If an average of 120 mL of colostrum is produced per day in the first 2 d of lactation (38), a breast-fed infant would ingest about 60 ng of TNF-α per day during the first few days of life.

Munoz *et al.* (34) examined human milk collected on the 3rd and 4th d of lactation from Chilean women for TNF-α by an RIA. They found small amounts of that cytokine in human milk as compared with our study (mean $\pm$ SD, 99 $\pm$ 10 pg/mL *versus* 620 $\pm$ 183 pg/mL). We are unable to account for this discrepancy. It is possible, but doubtful, that a difference of a few days between the times of collection would be responsible for the 6-fold difference between the studies. That possibility is currently being investigated in our laboratory.

The observed range of the concentration of TNF-α in the fluid phase of colostrum and the wide variation in the amount of this substance found in milk macrophages suggest that TNF-α in colostrum was not produced by those macrophages once they left the mammary gland. However, the finding of RNA for TNF-

α in milk macrophages suggests that the cells were engaged in the production of TNF-α when they resided in the mammary gland and that the production of TNF-α may have been subsequently inhibited. That idea is consistent with the finding that the production of TNF-α by milk macrophages was not increased after stimulation with agents that enhance the production of that cytokine by peripheral blood monocytes. Thus, there may be agents in human milk that regulate the production of TNF-α. Two of the more likely candidates are n-3 polysaturated fatty acids (39) and IL-6 (40, 41). N-3 polyunsaturated fatty acids are found in human milk (42), but it is unknown whether IL-6 is present.

We questioned whether TNF-α may have autocrine or paracrine effects in the mammary gland. One possibility is that this cytokine may influence the maturation of the epithelium of that organ. That is suggested by *in vitro* observations that TNF-α enhances the up-regulation of the receptor for polymeric Ig (secretory component) on epithelial cells (16). A second potential effect is that the cytokine influences leukocyte trafficking in the mammary gland. That possibility was suggested by the finding in the present study of a high correlation between the concentrations of leukocytes and TNF-α in human milk.

Does TNF-α in human milk affect the recipient infant? It may be argued that this cytokine would be inactivated after ingestion because of the digestive processes in the recipient's alimentary tract. It seems likely, however, that ingested TNF-α may affect tissues in the proximal parts of the alimentary tract that are devoid of endogenously produced proteolytic enzymes. Also, there is no evidence of intragastric protein digestion for the first several days of age (43). Moreover, human milk contains high concentrations of antiproteases, α_1-antichymotrypsin and α_1-antitrypsin, that impede proteolysis (44). Thus, TNF-α in human milk may persist in the recipient long enough to be biologically active.

Very little is known, however, concerning the effects of one of the principal types of TNF-α, the transmembrane form. That form has previously been reported to have little cytotoxic activity (36). This is in keeping with our observations that only about half of the milk specimens studied displayed cytotoxicity that possibly could be attributed to TNF-α.

Will TNF-α in human milk display other functions? Perhaps some insight may be found from reports of the enhancing effects of TNF-α on the immune system. These include monocyte development (9), the production of IL-1 (10) and IL-6 (11–13), the presentation of class I and class II major histocompatibility antigens (14, 15), and the up-regulation of secretory component by epithelial cells (16).

In summary, immunochemical evidence is presented for the presence of TNF-α in human milk during the initial phase of lactation. The concentrations of this cytokine in human milk are high enough to suggest that TNF-α could be physiologically active. The exact molecular forms and compartmentalization of the cytokine and its effects upon the mammary gland of the mother or the immune system of the infant remain to be elucidated.

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