Presence of Macrophage Migration Inhibitory Factor in Human Milk: Evidence in the Aqueous Phase and Milk Fat Globules

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ABSTRACT

Human milk is a source of bioactive substances regulating the development and activity of the newborn immune system. Human milk has been found to contain a number of cytokines, including interleukins, growth factors, and colony stimulating factors. In the present study, we assessed 10 specimens of human milk for the presence of macrophage migration inhibitory factor (MIF), a cytokine recently described in several human reproductive organs and tissues. Using biochemical as well as immunologic techniques, we showed that MIF is abundantly present in human milk, mostly distributed in the lipid layer and in the aqueous phase. Fractionation of the lipid layer showed that MIF is highly concentrated inside milk fat globules. In view of its proinflammatory features, we speculate that milk MIF may protect the newborn against infection and play a role in preserving the functionality of the lactating mammary gland. Furthermore, the localization of MIF in lipid globules suggests a possible strategy for the protection of milk cytokines from the gastric barrier. (*Pediatr Res* 51: 619–624, 2002)

Abbreviations
MIF, macrophage migration inhibitory factor
MFG, milk fat globules
MFGM, milk fat globule membranes
TBS, Tris-buffered saline

Human milk is a complex biologic fluid that supplies nutritional and protective factors to the breast-fed infant. It contains a wealth of immunologically active components involved in the innate and acquired immune response against infectious agents and antigenic stimuli (1). Indeed, a number of cytokines have been described in human milk in the past 20 y (2). These include proinflammatory (tumor necrosis factor-α, IL-1β, IL-6, IL-8, IL-12, and γ-interferon) (3–6) and antiinflammatory cytokines (transforming growth factor-β, IL-10) (7, 8), and, as recently reported, colony-stimulating factors (granulocyte colony-stimulating factor, macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor) (9–11). Taken together, these data strongly suggest that most cytokines are provided to the newborn, potentially affecting the development and functions of the immune system.

MIF is a cytokine originally identified as a factor released by activated T lymphocytes capable of inhibiting the random migration of macrophages *in vitro* (12). Later, it became evident that besides T cells, monocytes and macrophages produce MIF that is released in response to proinflammatory stimuli, such as microbial toxins, mitogens, specific antigens, or cytokines (tumor necrosis factor-α and γ-interferon), and to physiologic concentration of glucocorticoids (13–15). Emerging evidence demonstrates MIF expression by a number of human cells other than lymphocytes and macrophages. For instance, MIF protein and mRNA have been detected in the differentiating cells of eye lens, as well as in the human cornea, skin, and kidney (16–19). Previous data from our laboratory have demonstrated MIF expression in human reproductive organs and tissues, including the mammary gland (20–22). Furthermore, MIF has been reported to be secreted by a variety of cell types such as pituitary, adipocytes, testis, epithelial prostatic, and endometrial cells (23–26).

In the study reported herein we tested the hypothesis that MIF is present in human milk. We speculated that in view of its proinflammatory features and action on macrophages, milk MIF may affect the development of the newborn immune system and may play a role in preserving the functionality of the lactating mammary gland.

METHODS

Sample collection. Peripheral blood (3 mL) and milk samples (20 mL) were collected from 10 healthy women (ages 25–33 y) who had delivered normal full-term infants by spontaneous active labor. In particular, no subjects had signs of uterine infection or breast inflammatory diseases. Samples, obtained on postpartum d 5, were collected in sterile polypropylene tubes, and processed within 1 h. The study was approved by the Clinical Investigations Committee at the University of Siena, and informed consent was obtained from each subject.

Sample processing. Blood was centrifuged at $3,000 \times g$ for 30 min at 4°C, and serum aliquots were stored at -80°C until assayed. An aliquot of breast milk was processed for two-dimensional electrophoresis, SDS-PAGE, and immunoblotting. The remainder was fractionated according to the protocol of Basch *et al.* (27). Briefly, milk was centrifuged at $5000 \times g$ for 15 min at 25°C to obtain a pellet containing milk cells, an aqueous phase, and a floating lipid layer, containing MFG. The lipid layer was gently suspended in 10 mM PBS (pH 7.4), and stored at 5°C overnight. It was then churned with a Turrax (IKA Labortechnik, Staufen, Germany) at a medium speed until fat and sera were separated. After a brief warming at 40°C, specimens were centrifuged at $100,000 \times g$ for 1 h at 37°C to obtain a pellet containing crude MFGM, an aqueous phase (MFG aqueous phase), and a lipid phase. Aliquots of lipid layer were centrifuged at $700 \times g$ onto glass slides using a cytospin centrifuge and immediately fixed and used for immunohistochemistry.

Gel electrophoresis and immunoblotting. SDS-PAGE was performed according to Laemmli (28). Electrophoresis was performed on 16% polyacrylamide gels. Protein concentration was estimated using the method of Bradford (29). Two-dimensional electrophoresis was carried out on milk samples as described by Görg *et al.* (30) and modified by Bjellqvist *et al.* (31). An aliquot of 34 μL of milk was mixed with 17 μL of a solution containing SDS 10% (wt/vol) and dithioerythritol 2.3% (wt/vol). The sample was heated to 95°C for 5 min and then diluted to 500 μL with a solution containing 8 M urea, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonic acid 4% (vol/vol), 40 mM Trizma base, 65 mM dithioerythritol, and a trace amount of bromophenol blue (denaturing solution). The isoelectric focusing (first dimension) was carried out on a commercial sigmoidal immobilized pH gradient (IPG), from pH 3 to 10 (NL18-cm long IPG strips; Pharmacia, Uppsala, Sweden). The second dimension was performed on 9 to 16% polyacrylamide gradient gel. The gel was stained with ammoniacal silver nitrate as described (32). Electrophoretic images were digitized using the Molecular Dynamics Amersham Biosciences, Uppsala, Sweden computing densitometer (4000×5000 pixels; 12 bits/pixel), and analyzed with the Melanie II (Bio-Rad Laboratories, Milan, Italy) software on a SUN SPARC station 10; gel calibration was carried out on the basis of internal standards. The Expasy World Wide Web molecular biology server of the Geneva University Hospital and of the University of Geneva was applied to predict M_r and pI for human MIF. Protein electroblotting was carried out

following the procedure of Towbin *et al.* (33). Goat polyclonal antibody against human MIF (R&D Systems, Abingdon, U.K.) was used at 1:1000 working dilution. For detection, a chemiluminescence kit (Amersham Pharmacia Biotech International, Little Chalfont, U.K.) was used according to the manufacturer's instructions.

MIF ELISA. Samples of blood serum and the aqueous phases obtained by the centrifugation of milk (milk aqueous phase) and by processing the lipid layer (MFG aqueous phase) were assayed using a quantitative colorimetric sandwich ELISA. Briefly, 96-well ELISA plates were coated with 100 μL/well of anti-human MIF MAb (R&D Systems; 2.0 μg/mL) and incubated overnight at room temperature. The plates were washed three times with washing solution [10 mM PBS (pH 7.4), 0.05% (vol/vol) Tween 20], blocked by adding 300 μL of blocking solution [10 mM PBS (pH 7.4), 1% (wt/vol) BSA, and 5% (wt/vol) sucrose], and incubated at room temperature for 1.5 h. After washing three times, the samples and the standard, appropriately diluted in TBS-BSA [20 mM Tris-HCl, 150 mM NaCl, (pH 7.3), 0.1% (wt/vol) BSA, 0.05% (vol/vol) Tween 20], were added in duplicate (100 μL/well) and incubated for 2 h at room temperature. Plates were then washed three times, and 100 μL of biotinylated goat anti-human MIF antibody (R&D Systems; 200 ng/mL) was added to each well and incubated for 2 h at room temperature. Plates were washed again, and streptavidin–horseradish peroxidase (Zymed, San Francisco, CA, U.S.A.) was added to each well and incubated for 20 min at room temperature. The plates were washed, and 3,3',5,5'-tetramethylbenzidine (Zymed) was added. After 20 min, the reaction was stopped by adding H_2SO_4. Absorbance was measured at 450 nm using an ELISA SR 400 microplate reader (Sclavo, Siena, Italy). Sensitivity limit was 18 pg/mL. Intra- and interassay coefficients of variation (CV%) were 3.86 $\pm$ 0.95% and 9.14 $\pm$ 0.47%, respectively.

Immunohistochemistry. Immunohistochemistry of MIF in lipid layer preparations was performed using the streptavidin-biotin method as described (21). Lipid layer samples centrifuged onto glass slides were rehydrated and washed in TBS [20 mM Tris-HCl, 150 mM NaCl (pH 7.6)]. Antigen retrieval was performed by incubating slides in 10 mM sodium citrate buffer, pH 6.0, in a microwave oven at 750 W for 5 min. Slides were preincubated with normal rabbit serum to prevent nonspecific binding, and incubated overnight at 4°C with the anti-human MIF goat polyclonal antibody diluted 1:300 in TBS. Slides were then washed three times with TBS for 5 min, and incubated with a rabbit anti-goat antibody labeled with biotin (Dako, Copenhagen, Denmark) at the dilution of 1:500 for 30 min. The reaction was revealed using the streptavidin-biotin complex (Dako). Slides were mounted and examined under a light microscope. For each case, a negative control was obtained by replacing the specific antibody with nonimmune serum immunoglobulins at the same concentration as the primary antibody. As positive control, the reaction was performed on first-trimester human trophoblasts (21).

Data analysis. MIF concentrations in milk and serum samples are expressed as median [interquartile range, 25th–75th percentiles]. The paired Wilcoxon test was used for comparison. The relationship between milk and serum MIF concentra-

tions and between milk aqueous phase and MFG aqueous phase MIF concentrations were assessed using univariate linear regression. A two-sided $p < 0.05$ was considered to be statistically significant.

RESULTS

The presence of MIF in human milk and in milk fractions was first evaluated by immunoblotting. Samples of milk were run on SDS-PAGE and transferred to nitrocellulose. The blot was exposed to the anti-human MIF antibody, and bands were visualized by chemiluminescence. A single band with an apparent molecular weight of 12 kD was observed in the samples analyzed (Fig. 1). When immunoblotting was used after two-dimensional gel electrophoresis, two immunoreactive spots of a molecular weight of 12 kD and pI of 6.98 and 7.8 were observed (Fig. 2), with the latter being the most represented and the nearest to the theoretical pI value of 8.24 predicted from MIF sequence in Swiss-Prot database.

The distribution of MIF in different fractions of milk was then assessed. To maintain the same protein ratio of the unprocessed milk, the pellet, the aqueous phase, and the lipid layer obtained by centrifugation were readjusted to the initial sample volume. As shown in Figure 3, MIF was present in the milk lipid layer (lane 3) and milk aqueous phase (lane 4). In the samples examined, the intensity of the band was always stronger in the lipid layer compartment. Pellet MIF did not appear to contribute to the immunoreactive band of the total milk, possibly because the pellet is only a small part of the milk volume. In the fractions obtained by processing the lipid layer, MFGM, and MFG aqueous and fat phases, an intense band corresponding in size to MIF was observed only in the MFG aqueous phase (lane 7). The localization of MIF in the milk lipid layer was then examined by immunohistochemistry. Figure 4 shows an image of an unprocessed milk lipid layer stained with the anti-MIF antibody. Immunoreactivity is con-
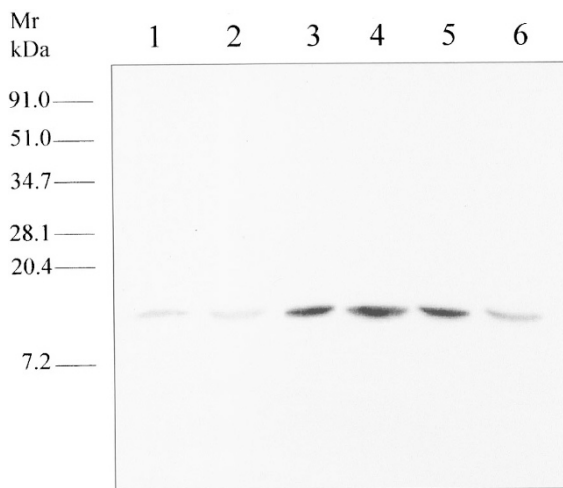

Figure 1. Representative Western blot profile of specimens of human milk, each from a different subject. Twenty micrograms of total protein were resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to an anti-MIF antibody. The positions of molecular weight markers are indicated. *Lanes 1–6*, specimens of human milk.
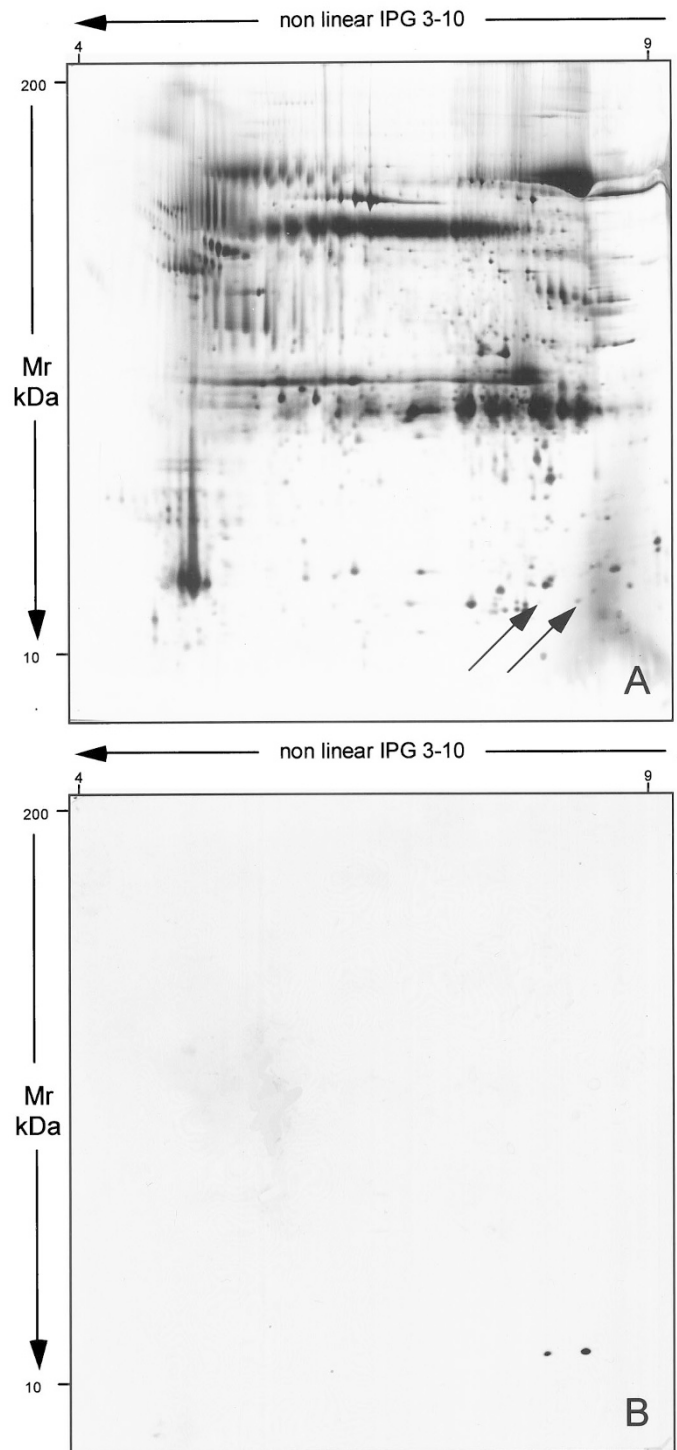

Figure 2. Identification of MIF in human milk by two-dimensional PAGE and Western blot. *A*, 45 μg of milk proteins was separated on a nonlinear immobilized pH gradient (IPG) gel strip (pH 3–10) for the first dimension. Acrylamide gradient gel (9–16.5%) was run as second dimension and the gel stained with silver. *B*, 90 μg of proteins from the same sample was separated as above and transferred to a nitrocellulose membrane. The polyclonal anti-human MIF antibody identified in the blot as two isoforms, indicated with *arrows* in the top panel.

sistently found as a rim beneath the plasma membrane of the fat globule.

To determine the contribution of circulating MIF to the milk cytokine level, MIF concentrations were measured in a total of
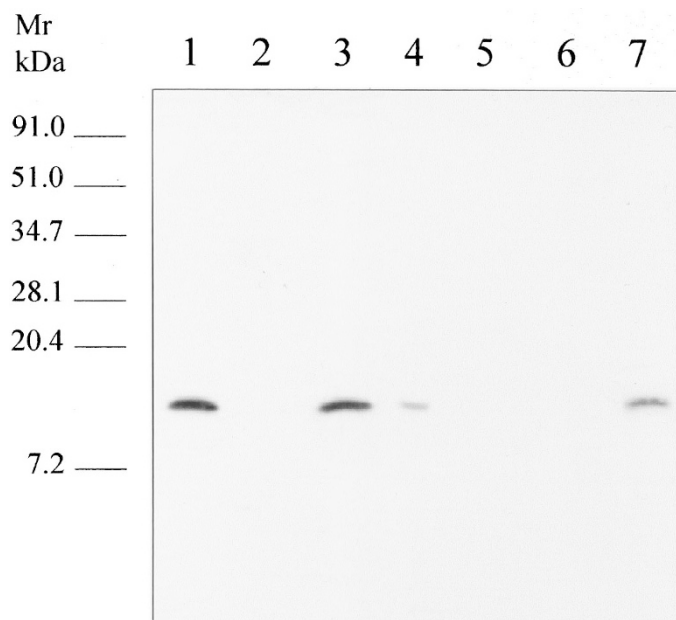
Figure 3. Western blot analysis of fractions of human milk. Proteins were resolved on a 16% SDS-PAGE, transferred to nitrocellulose, and analyzed with the anti-human MIF antibody. The positions of molecular weight markers are indicated. Samples of unprocessed milk (*lane 1*), pellet (*lane 2*), lipid layer (*lane 3*), aqueous phase (*lane 4*), MFGM (*lane 5*), lipid phase (*lane 6*), and MFG aqueous phase (*lane 7*) are shown.
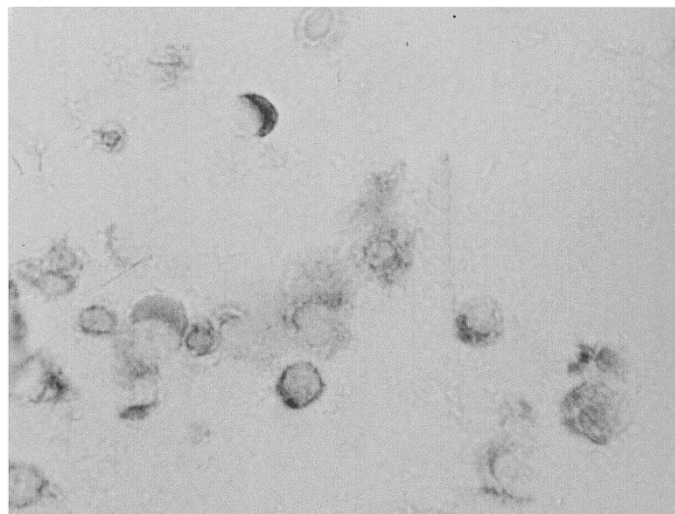

Figure 4. Immunolocalization of MIF in MFG. Milk lipid layer samples centrifuged onto glass slides were analyzed by immunohistochemistry using the streptavidin-biotin method (original magnification, ×1000).

10 milk and blood serum samples. As shown in Figure 5, MIF concentration in milk aqueous phase, expressed as nanograms per milliliter, was found to be approximately 7.5-fold higher than serum (38.29; interquartile range, 25.32–55.69; value range, 9.72–79.51 *versus* 5.13; interquartile range, 4.78–5.40; value range, 4.02–5.78; $T = 55.0$; $p < 0.001$). No significant correlation was found between milk aqueous phase and serum MIF concentrations ($r^2 = 0.0041$; $p = 0.86$).

To quantify the distribution of MIF in the aqueous and lipid phases of milk, the milk aqueous phase and the MFG aqueous phase, both readjusted to the specimen starting volume, were
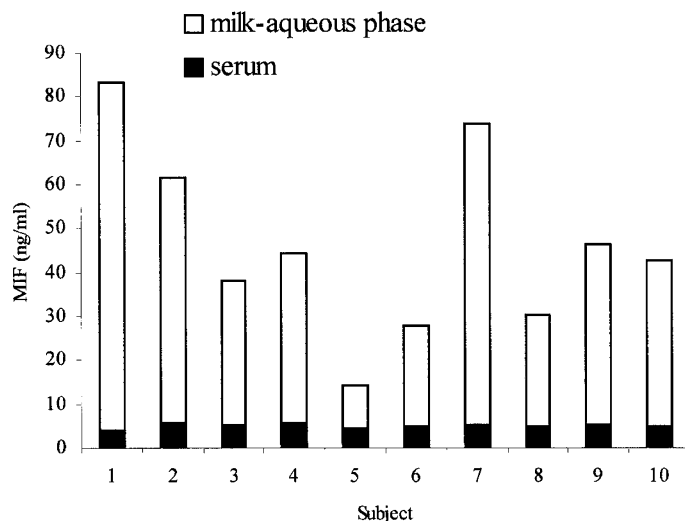

Figure 5. MIF concentration in milk aqueous phase ($n = 10$; *white bars*) and paired blood serum (*black bars*). Samples were assessed by ELISA. Data, expressed as nanograms per milliliter, are reported as the mean of two assays. No significant correlation was found between milk and serum MIF concentrations ($r^2 = 0.0041$; $p = 0.86$).

assayed. MIF concentration, was found to be approximately 2-fold higher in MFG aqueous phase than in the milk aqueous phase in examined samples (73.07; interquartile range, 48.15–146.33; value range, 15.41–231.16 *versus* 35.96; interquartile range, 31.81–55.68; value range, 31.81–55.68; $T = 30.0$; $p = 0.12$). No significant correlation was found between milk and MFG aqueous phase MIF concentrations ($r^2 = 0.54$; $p = 0.088$; Fig. 6).

DISCUSSION

Breast-feeding is a natural means of administration of cytokines to offspring, occurring when the immune system is immature and still developing (34). During this time, the newborn is adjusting to the external environment, establishing a bacterial flora in the oral cavity, gut, and upper respiratory tract. Although direct evidence is not yet available, orally administered cytokines are thought to modulate immune functions through activation of the mucosal and lymphoid cells of the oropharyngeal and gut-associated lymphoid tissue (35, 36). It is still unclear, however, how milk cytokines can avoid inactivation and retain their biologic activity through the gastrointestinal tract. Human milk contains factors limiting proteolysis (37, 38) or increasing the pH of the stomach, thereby protecting cytokines from degradation after exposure to gastric secretion (39). Compartmentalization in different fractions of human milk has also been suggested as an additional mechanism of protection (2). Thus, Garofalo *et al.* (8) showed that IL-10 is present in both the lipid layer and the aqueous phase of human milk. These authors speculated that the lipid phase could protect the cytokine during the transit through of the newborn gastrointestinal tract until it is released by the action of the bile salts in the duodenum. The current study is the first to describe the presence of MIF in human milk. This cytokine was found in high concentrations in the milk aqueous phase and in the lipid layer (compared with serum), in the latter
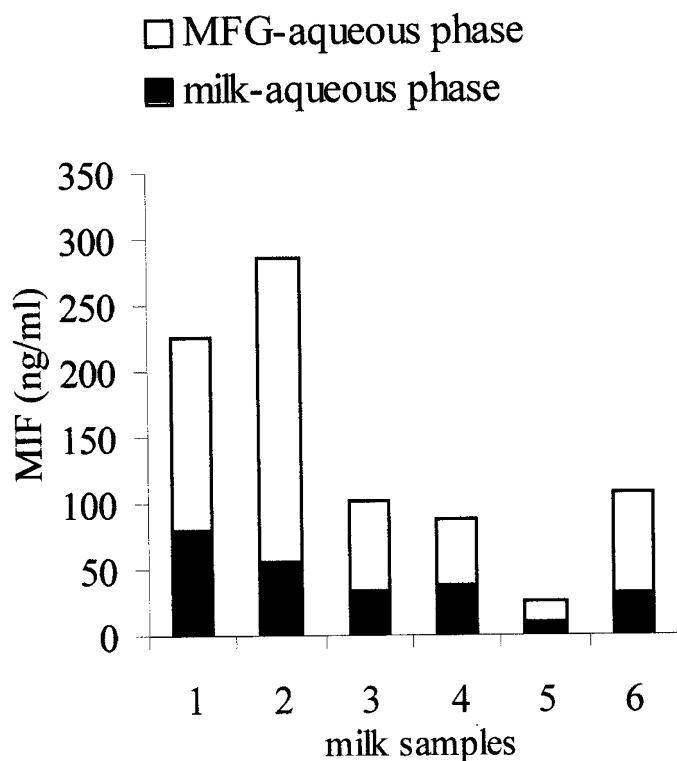
Figure 6. MIF in milk aqueous phase (*n* = 6; *black bars*) and paired MFG aqueous phase (*white* bars). Samples were assessed by ELISA. Data are expressed as nanograms per milliliter of total milk. Values are reported as the mean of two assays. No significant correlation was found between milk and MFG aqueous phase MIF concentrations (r^2 = 0.54; p = 0.088).

essentially localized inside the fat globules. Moreover, immunohistochemical analysis of unprocessed lipid layer revealed MIF immunoreactivity inside the MFG, localized in a gap between the core fat and the surrounding membrane. Taken together, these data represent the first direct evidence of cytokine compartmentalization in human milk, thus revealing a novel strategy for the protection of milk cytokines during the transit through the newborn gastrointestinal tract.

Besides being localized inside the MFG, MIF was also consistently found in the milk aqueous phase. This finding supports the existence of an alternative mechanism of release of this cytokine by breast epithelial cells. Several other studies demonstrated that MIF, despite the lack of an NH_2-terminal signal sequence, is actively secreted by a variety of cell types (40). In this context, the observation of Nishino and colleagues (23) that localized MIF to the secretory granules of ACTH- and TSH-secreting cells of the pituitary gland is of relevance. In the epithelial cells of lactating mammary gland, cytoplasmic lipid globules are surrounded by secretory vesicles, which fuse to form large membrane-bound compartments around the fat globule (41). Such secretory vesicle–surrounded fat vacuoles ascend to the secretory apex, and before detachment of the apical portion of the cell occurs, the secretory compartment fuses with the plasma membrane (42). It appears therefore conceivable that after this process, secretory MIF-containing vesicles would be finally released into the glandular lumen. Unlike the lipid layer MIF, which might exert its function on the distal portions of the newborn gastrointestinal tract, the

aqueous phase MIF could mainly target the oropharyngeal lymphoid system. Furthermore, additional biologic roles can be suggested for the soluble cytokine, in consideration of the specific actions exerted by MIF on macrophages (43, 44). It is well known, in fact, that human milk contains an abundant leukocyte population of which macrophages represent a major cellular component (1). These cells, migrating from the maternal circulation into the breast milk (45), are known to protect the breast-fed infant against infectious diseases by virtue of their antimicrobial activity (46). In addition, as proposed for the nonlactating mammary gland (47), macrophages in the lactating breast tissue may play an important role by cleaning up ducts and removing cell debris.

Previous studies described the presence in human milk of cytokines with well-defined functions in promoting monocyte migration and activation (granulocyte colony-stimulating factor, macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor) (9–11). Nevertheless, the mechanisms involved in maintaining macrophage population in the lactating mammary gland are not fully defined. Macrophages are the main target of MIF, which acts on these cells by inhibiting migration and enhancing their phagocytic activity. Hence, it is speculated that milk MIF, by affecting macrophage accumulation and activation, may play an important role in the physiologic activity of the mammary gland. Further studies evaluating the effects of MIF neutralizing antibodies on the migration and phagocytic activity of milk macrophages *in vitro* are needed to confirm this hypothesis.

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