Human Milk Cell Migration and Production of Monocyte Chemotactic Factor: Lack of Activity

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ABSTRACT. The lymphocytes of human breast milk have been previously shown to be immunologically competent while the monocytes have been described as actively motile in some reports but not in another. Cells were separated from milk samples collected by lactating women 2 to 9 days postpartum. Milk cell migration and production of the lymphokine, monocyte chemotactic factor, by milk cells were assayed using membrane filters. Milk cells were poorly motile although prior culturing improved their migratory ability. Cell-free milk was chemotactic for blood monocytes. Chemotactic factor production was detected in only two of 16 phytohemagglutinin-stimulated milk cell cultures. (*Pediatr Res* 19: 996-999, 1985)

Abbreviations

PHA, phytohemagglutinin
HBSS, Hanks' balanced salt solution
MCF, monocyte chemotactic factor

Human milk contains a large number of immunologically active components (1). Soluble factors include secretory component, immunoglobulins, and complement. Cells identified in colostrum and milk are predominantly mononuclear phagocytes with small proportions of lymphocytes and neutrophils. Functional studies of these cells were first described by Smith and Goldman (2). They found that the mononuclear phagocytes, comprising about 80% of the milk leucocytes, ranged in size up to 30 μm diameter, with a prominent nucleolus and cytoplasm containing many fat globules. They adhered to and spread on glass, extending a cytoplasmic veil, and were described as exhibiting ameboid movements. Adherence and spreading on glass also have been described by Lascelles *et al.* (3), Mohr *et al.* (4) and Pitt (5). From these descriptions and the work of Mohr *et al.* (4), who also studied capillary tube migration, it has been assumed that milk mononuclear phagocytes are actively motile. Pitt (6), however, found that they were not motile in capillary tubes or under agarose. Milk cells are actively phagocytic and microbicidal (2-4, 7, 8). Milk mononuclear cells react with the commercially available monoclonal antibodies OKMI and OKIa and present antigens to T-cell blasts (9).

The lymphocytes, which comprise between 5 and 35% of the total milk cells (10-13) also exhibit *in vitro* activity. They proliferate in response to stimulation by PHA, antigens, and allogeneic cells (2, 10, 11, 14-16) and produce the lymphokines, migration inhibition factor (4), interferon (17-19), and MCF (19).

It has been postulated that these cellular activities are of benefit to the breast-fed neonate. Thus, the described transmission of

cellular immunity to purified protein derivative via breast milk may possibly be mediated by lymphokine transfer (20, 21).

In view of the contrasting findings regarding milk cell motility and the indication that lymphokine is produced by milk cells, we decided to investigate milk cell migration and production of monocyte chemotactic factor using Millipore membrane filters to assay migration.

METHODS

Samples of breast milk were collected from healthy lactating women 2-9 days after delivery. The milk was defatted by diluting 3 fold in phosphate-buffered saline and centrifuging 400 $\times$ g/15 min. The fat layer was aspirated off, and the tubes were inverted and drained for a few minutes to remove more fat from the walls of the tubes. The cell buttons were washed three times in phosphate-buffered saline.

Cell migration was assayed using methods previously described (22). Cells were resuspended at 5×10^6 cells/ml in HBSS (Commonwealth Serum Laboratories, Melbourne, Australia), pH 7.4, containing 2% bovine serum albumin and dispensed in 0.1 ml volumes into chemotactic chambers. These were constructed by sawing off tuberculin syringes to 18 mm length and attaching 8 μm pore size cellulose ester filters (SCWP, Millipore Corp., Bedford, MA) to the bottom of each chamber using a nontoxic adhesive. Each chamber was suspended in a 2 ml autoanalyzer cup containing 0.4 ml of test solution and then incubated for 2.5 h in a humidified atmosphere of 5% CO_2 in air. All assays were performed in triplicate.

Migration was quantified after fixing and staining the filters by measurement of the leading front of migrating cells, taken as the furthest distance through the filter with at least three cells in focus. Each filter was read in quadruplicate and the migration in μm was expressed as the mean of the determinations made on each of the three filters.

As will be described later, it was not practicable to use the leading front method to detect milk cell migration as so few cells entered the filters. Hence to assess migration, cells in focus were counted at successive depths within the filters. Ten fields ($\times 500$) were counted at each level in each of the three filters and the mean number of migrating cells determined.

Polycarbonate membranes (Nuclepore, N500 Pleasanton, CA) (5 μm pore size) were also used for the detection of milk and blood cell migration. These were attached to chemotactic chambers in the same manner as the cellulose ester filters. Incubation was for 90 min. Migration was assessed by counting cells which had migrated through the filter and reached the bottom surface. Ten fields ($\times 1250$) were counted on each of triplicate filters.

To assess random migration, HBSS was added to the autoanalyzer cups. To assess stimulated migration, 5 mg/ml casein in HBSS was used as a chemotactic stimulus. To detect MCF, control and PHA-stimulated cell culture supernatants were placed below the filters.

Cells were cultured for MCF production as previously de-

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scribed (23). Washed Ficoll-Hypaque separated peripheral blood mononuclear cells or milk cells prepared as described above were resuspended at 3×10^6 cells/ml in medium 199 containing 0.5% pooled male human serum and antibiotics (penicillin 50 units/ml and streptomycin 50 $\mu\text{g}/\text{ml}$) for culture. Each 1 ml culture was dispensed as 5×0.2 ml aliquots in microtiter trays and incubated for 48 h at 37°C in a humidified 5% CO_2 in air atmosphere. Control cultures had no addition; stimulated cultures contained 1 $\mu\text{g}/\text{ml}$ PHA (purified, Wellcome Laboratories, Kent, England). The supernatants were harvested by gentle aspiration and each group of five pooled before storage at 4°C .

Monocytes harvested by Ficoll-Hypaque separation from peripheral blood were used to detect MCF in the supernatants (23). The migration of monocytes toward control and stimulated culture supernatants was determined by the leading front method and the significance of any difference between the migration obtained toward each pair of control and stimulated supernatants was assessed by the Mann-Whitney U test. PHA-stimulated culture supernatants were considered positive if they promoted significantly enhanced monocyte migration ($p < 0.05$) as compared to control supernatants.

When testing milk cell culture supernatants, pretested positive supernatants produced by PHA-stimulation of blood mononuclear cell cultures were also run concurrently as a control. These cells were obtained either from peripheral blood of healthy adult volunteers or from cord blood obtained at delivery. Cord blood mononuclear cells are active in both production and detection of MCF (24).

RESULTS

Milk cell preparation. The yield of milk cells varied widely (0.11 to 4×10^6 cells per ml milk from 40 samples collected from 37 subjects). Ficoll-Hypaque centrifugation proved unsatisfactory due to large cell losses. Although this step was omitted, polymorphonuclear cell migration into the filters was detected in only two of 15 assays of samples from different subjects.

Milk cell migration in Millipore filters. Determination of cell migration by the leading front method is based on measuring the distance traveled into the filter by a "leading front" of three cells in focus. This was found not to be practicable in the milk cell experiments since so few cells migrated into the filter that very few microscopic fields contained three cells. To enable a comparison between milk and blood cells to be made, the number of cells migrating into the filters was compared by counting cells in focus at successive depths within the filters. Ten fields were counted at each level in each of three filters.

The migration of milk cells collected from five subjects on days 4, 5, 6, 7, and 9 postpartum is compared with cord blood cell migration in Figures 1 (HBSS) and 2 (casein). The lack of motility of milk cells can be clearly seen. In HBSS, an average total of 52 milk cells migrated into the filter compared with 1182 cord blood cells and in response to casein 52 milk cells compared with 1182 cord blood cells migrated into the filter.

By the leading front method, migration of the milk cells was less than $5 \mu\text{m}$ in the presence of both HBSS and casein whereas the migration of the cord blood cells was $31 \mu\text{m}$ in HBSS and $85 \mu\text{m}$ in casein.

Migration of milk cells after culture. To investigate whether components of milk were inhibiting milk cell migration despite washing, milk cells were prepared as above and cultured at 1×10^6 cells/ml in medium 199 for 24 h. The cells were harvested by aspiration and scraping, washed twice, and added to chemotactic chambers for migration assay. Table 1 shows that three of four milk cell preparations had enhanced random migration (determined in the presence of HBSS) after culturing.

The cultured samples also had enhanced chemotactic migration when casein was used as a chemotactic stimulus. The number of cells migrating was greatly increased, enabling measurement of migration by the leading front method to be made

Fig. 1. Cell migration into Millipore filters in the presence of HBSS. The total migrating cell count in ten microscopic fields at each depth within triplicate filters is indicated. Solid bar, milk cells (mean of five samples from five subjects). Open bar, cord blood mononuclear cells (one sample).

Fig. 2. Casein-stimulated cell migration into Millipore filters. Casein (5 mg/ml in HBSS) was placed below the filters. Migrating cells were counted in ten microscopic fields within each of three filters at the depths indicated in μm . Solid bar, milk cells (mean of five samples from five subjects). Open bar, cord blood mononuclear cells (one sample).

(samples 1 and 2). When the chemotactic peptide, f-Met-leu-phe, was used, however, no enhancement was seen (samples 3 and 4, Table 1).

Milk cell migration through polycarbonate membranes. Polycarbonate membranes ($5 \mu\text{m}$ pore size) have been commonly used in assays of human monocyte migration (25, 26). Migration of milk and cord blood cell preparations through these $10\text{-}\mu\text{m}$ thick filters was compared. Migration is assessed by counting cells which have migrated through the filter and reached the lower surface after 90 min incubation. Ten oil immersion fields were counted in each of triplicate filters. Random migration of milk cells was extremely low (0.8 ± 0.4 cells in 10 fields, mean

Table 1. Milk cell migration after culture

Sample no.	HBSS (average cell count on 10 fields*)		Casein (migration distance in $\mu\text{m}\dagger$)	
	Before culture	After culture	Before culture	After culture
1*	20	96	nd	37 μm
2	46	521	<5 μm	19 μm
Chemotactic peptide f-Met-leu-phe (10^{-8} M) (average cell count in 10 fields)				
3	10	10	13	11
4	10	30	24	6

* Cells were counted in triplicate filters for each determination. In sample 1, counting was at a depth of 10 μm since there were too many cells (>100 per field) at 5 μm . In the remainder, cells were counted at 5 μm .

† Enhanced migration by the cultured cell samples in the presence of casein enabled leading front determinations of migration.

Table 2. Effect of milk on blood monocyte migration*

Concentration of milk above filter (%)	Concentration of milk below filter (%)			
	0	1	10	20
0	38	52	70	63
1	40	45	52	55
10	47	49	46	52
20	51	48	48	51

* The milk sample used was collected on day 2 and diluted in HBSS. Monocytes were separated from cord blood. Monocyte migration (in μm) is expressed as the mean of four determinations in each of three filters.

$\pm$ SD; $n = 4$) compared to cord blood monocytes (11 ± 4 ; $n = 2$). When casein was present, milk cell migration was again very poor (2.8 ± 4.6 ; $n = 4$) compared to cord monocytes (217 ± 144 ; $n = 2$).

Effect of milk on blood monocyte migration. To test whether milk inhibits cell migration, a possible explanation for the lack of milk cell migratory activity, a checkerboard assay was set up in which a range of concentrations of defatted, cell free milk was used above and below the Millipore filters with a monocyte preparation from cord blood. Migration was determined as shown in Table 2. When equal concentrations of milk were present on each side of the filter, monocyte migration, shown in the diagonal from upper left to lower right, increased slightly over the concentration range 1 to 20%. This increase in migration, however, was not significant by the Mann Whitney U test ($p > 0.05$), thus indicating little or no chemokinetic activity. Monocyte migration obtained in response to a positive gradient, represented by those results on the right of the diagonal, was slightly, but significantly increased ($p < 0.05$, Mann Whitney U test) over the migration in the presence of a negative gradient, represented by the results on the left of the diagonal. This indicates that milk exerts a chemotactic, but little or no chemokinetic, effect on blood monocytes.

Production of MCF by milk cells. Milk cells separated from 10 samples collected from nine subjects on days 2, 4, 5, 6, 7, and 8 postpartum were cultured with PHA. The culture supernatants were tested for MCF using blood monocytes. Only one of the 10 cultures was positive. Half of these cultures were set up in duplicate and tested on separate occasions with different indica-

tor monocytes but remained negative. Additional milk cells collected on days 2, 4, 6, and 8 from another four women were cultured for 72 h. One of six was positive.

DISCUSSION

Human milk cells displayed poor migratory activity and production of PHA-induced monocyte chemotactic factor.

The poor migratory ability found in two types of filter assays is in agreement with results using the capillary tube and agarose migration assays briefly reported by Pitt (6). The motility previously attributed to milk mononuclear cells was based on visual observations during incubation on glass slides (2) in which cell spreading may have been equated with locomotion. In fact, spreading of blood monocytes is accompanied by a loss of locomotion (27). In another study, the large colostrals cells were described as displaying "very occasional, extremely sluggish movement" (3).

Not only did the milk cells exhibit a very low random mobility into the filters in the absence of any stimulant but no increase in migration was seen when the chemoattractants casein and the peptide f-Met-leu-phe were used. Nor was there any stimulation of milk cell migration in the presence of serum or monocyte chemotactic factor (results not shown). It is possible that the milk cells become deactivated by the continual presence of chemoattractants in milk and are not able to respond even after their removal by washing. After 24 h in culture, random and casein-stimulated migration was noticeably increased, implying that deactivation may be a contributing factor to the poor mobility of freshly isolated cells. Since migration is observed after culture, it seems less likely that the poor migration of milk cells is simply due to the absence of motile cells. The chemotactic property of milk was confirmed by the checkerboard experiment and is presumably due to its casein content, a known chemotactic factor for human monocytes (28). The migratory activity of the milk cells on polycarbonate filters may be compared with published results for human alveolar macrophages (29). Average cell counts obtained were 5.7 and 16.9 cells undergoing random and stimulated migration, respectively (20 oil fields were counted per filter). Thus, migratory ability of alveolar cells was much lower than that of blood monocytes but that of milk cells was even less.

In contrast to the low activity of milk macrophages, milk neutrophils are capable of both random and stimulated migration (30). Milk neutrophil migration was not extensively assessed in this study since only two milk samples were found to contain neutrophils. In these samples migration was less than that of blood neutrophils, in agreement with Khan *et al.* (30).

Monocyte chemotactic factor was detected in only two of 16 PHA-stimulated milk cell cultures. By comparison, MCF was detected in 70% of PHA-stimulated peripheral blood mononuclear cell cultures using the leading front method to determine migration of indicator monocytes (23). In contrast to the present study, nine of 11 milk lymphocyte cultures produced MCF using polycarbonate filters to detect migration (19). The low incidence of MCF detection in unseparated milk cell cultures may have been due to the lower proportion of lymphocytes present. Peripheral blood mononuclear cell preparations contain 70–80% lymphocytes while milk cell preparations contain 5–35% (10–13). However, MCF could still be detected in blood mononuclear cell preparations diluted 30-fold (23). The apparent lack of activity of the milk cell cultures, when MCF was assayed using Millipore membranes, may be due to a relative hyporesponsiveness of milk cells compared to blood lymphocytes which was not detected by the polycarbonate filter assay. Hyporesponsiveness of milk lymphocytes to mitogen stimulation also has been seen in transformation assays (10, 15, 16).

These studies indicate that milk cells have both an impaired migratory activity and ability to produce the lymphokine, mon-

ocyte chemotactic factor, in comparison to blood mononuclear cells.

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