

Decreased Response of Human Milk Leukocytes to Chemoattractant Peptides¹

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ABSTRACT. To test the hypothesis that neutrophils and macrophages in human milk may not defend by classical inflammatory mechanisms, experiments were conducted to ascertain whether adherence, orientation, and directed motility of these leukocytes would be enhanced by exposure to chemoattractant peptides including N-formyl-L-methionyl-L-phenylalanine and N-formyl-L-methionyl-L-leucyl-L-phenylalanine and C5a generated from zymosan activated human serum. Adherence and spatial orientation were tested on coverglasses and in Zigmond chambers, and chemotaxis was examined by Boyden chambers and a subagarose technique. Whereas, the adherence, orientation, and directed movement of adult peripheral blood neutrophils and monocytes were significantly enhanced by those chemotactic agents, human milk leukocytes failed to respond. The failure of the response of human milk leukocytes was not due to alterations in maternal peripheral blood leukocytes but appeared to be due partially to inhibitors in human milk. The experiments suggest that human milk leukocytes may be modified in the mammary gland to protect by noninflammatory mechanisms. (*Pediatr Res* 20: 373-377, 1986)

Abbreviations

HMLs, human milk leukocytes
PBLs, peripheral blood leukocytes
HBSS, Hanks' balanced salt solution
MEM, minimal essential media
F-Met-Leu-Phe, N-formyl-L-methionyl-L-leucyl-L-phenylalanine
F-Met-Phe, N-formyl-L-methionyl-L-phenylalanine
ZAS, zymosan activated human serum

There is considerable evidence that breast-fed infants, particularly in underdeveloped countries, are protected against gastrointestinal pathogens (for review, see References 1-3). The manner in which this protection occurs is not known despite a host of descriptive studies of the immunologic system in human milk. Epidemiologic investigations suggest, however, that the protection may not depend upon the induction of inflammation by host resistance factors in human milk. For example, when infants from rural Central America received human milk natu-

rally contaminated with bacterial pathogens such as *Shigella* (4, 5), they failed to display clinical features of gastroenteritis including abdominal pain, vomiting, diarrhea, or gross melena.

This seemed paradoxical since neutrophils and macrophages that cause inflammatory reactions are found in human milk. These leukocytes in human milk, however, display unusual morphology (6-14) including many lipid-filled vacuoles, milk fat globules, and casein micelles. Because of those unusual features and the suggestive clinical evidence that certain enteric pathogens may not evoke an inflammatory response in breast-fed infants, we hypothesized that HMLs may not protect the recipient by mechanisms that are expected from PBLs. For instance, if they fail to recognize or respond to chemoattractant agents, then they may be unable to adhere to or migrate into the intestinal mucosa of the infant. Consequently, they would not generate a local inflammatory response that would injure those tissues. To test that hypothesis, we first examined whether HMLs respond to chemotactic peptides by increasing their adherence, orientation, and directed movement.

MATERIALS AND METHODS

Subject selection; collection-preparation of leukocytes. The use of human subjects in this research was approved by the institutional review board of the medical school. The donors were healthy women age 20-34 yr who had delivered full term healthy infants vaginally 2-3 days before the collections (15). Milk was collected into polypropylene tubes by a low pressure electric pump (Egnell, Inc., Cary, IL). Heparinized venous blood was collected from healthy adult males, healthy nonpregnant adult females, or healthy women in the first two postpartum days.

Human milk leukocytes were prepared by the following procedure. The cells were washed three times in HBSS and resuspended in MEM (Flow Laboratories, McLean, VA) to achieve a final concentration of 1×10^6 cells/ml. Only unfractionated HMLs were used in the experiments. To ascertain whether both neutrophils and macrophages were present, aliquots of preparations were examined for nonspecific esterase positive cells (macrophages) and negative cells (neutrophils) (15, 16). The proportions of neutrophils and macrophages appeared to be typical of early human milk secretions, e.g. 45-55% neutrophils and 40-50% macrophages.

PBLs were prepared from venous blood by sedimentation with 6% dextran (17). Remaining erythrocytes were lysed with hypotonic saline. No further cellular fractionation was carried out. The blood leukocytes were washed three times in HBSS and resuspended in MEM to achieve a final concentration of 1×10^6 cells/ml.

Adherence-orientation of cells. The abilities of HMLs or PBLs to adhere and orient in the presence of chemoattractant peptides were measured by two methods. The concentrations of HMLs

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or PBLs suspended in MEM containing 10% fetal calf serum (Biofluids, Rockville, MD) were adjusted to 1×10^6 cells/ml. One hundred μl of each suspension of HMLs or PBLs were mixed with 100 μl of a 10^{-9} M solution of F-Met-Leu-Phe (Sigma Chemical Co., St. Louis, MO) and examined immediately on coverglasses by phase contrast microscopy (630 \times).

Orientation of the leukocytes to F-Met-Leu-Phe (10^{-9} M concentration) was evaluated by the use of *in vitro* chambers as described by Zigmond (18, 19). Approximately 100 μl of an unfractionated washed suspension containing $1-2 \times 10^6$ cells/ml were streaked across the center of a glass coverslip. After the cells settled on the surface of the coverslip and the excess MEM drained off, the coverslip was inverted over the chamber slide and fixed in place by brass clips. One well was filled with MEM; immediately thereafter, the other was filled with the F-Met-Leu-Phe. The slide was then examined by phase microscopy (630 \times).

Adherence was quantitated microscopically by counting the number of adherent and nonadherent cells in 10 random fields. Similarly, the degree of orientation of the leukocytes in Zigmond chambers was assessed by counting the number of cells in 10 high power fields on each slide which remained rounded or became polarized toward the chemotactic gradient. The results were expressed as a percentage of oriented cells.

Chemotaxis under agarose. Leukocyte migration under agarose was performed as reported by Nelson *et al.* (20, 21). Three different chemotaxins were utilized in these experiments. F-Met-Phe (Sigma; 10^{-6} – 10^{-9} M) and F-Met-Leu-Phe (10^{-6} – 10^{-9} M) were prepared in MEM. A preparation of C5a was made by reacting 5 mg of zymosan (Sigma) with 20% normal human serum in MEM. The optimal concentrations of the chemoattractant agents used in these experiments were determined in prior experiments with PBLs (data not shown) and were similar or identical to the optimal doses of these chemoattractants found in previously reported studies. The possibility that early milk (colostrum) contained preformed chemotactic factors was tested by using whole colostrum, a 50% concentration of whole colostrum, a 10% concentration of the aqueous phase of colostrum or a preparation of washed colostrum leukocytes as potential chemotaxins for PBLs. The concentrations of responding cells, *i.e.* PBLs or HMLs, were adjusted to $0.4-1.0 \times 10^6$ cells/10 μl in MEM. Ten μl of the chemotaxin were placed into the outer wells and 10 μl of the responding cells (PBLs or HMLs) in the inner wells. The chemotactic preparations were incubated in a CO₂ incubator at 37° C for 2 and 20 h. Cellular migration was then assessed microscopically by measuring the distance from the edge of the cell well to the leading front of cells facing toward or 180° away from the chemotaxin well. The measurements were checked in a blinded fashion by two of the investigators. Their observations agreed with those of the observer who knew the source of the leukocytes.

Chemotaxis in Boyden chambers. The Boyden chamber method was modified from the original technique (22) in the following ways. Cellulose nitrate filters (3 μ pore size for PBLs; 5 μ pore size for HMLs; Millipore Filter Corp, Bedford, MA) were used. The concentration of either HMLs or PBLs was adjusted to 1×10^6 /ml in MEM and 1 ml of each cell preparation was placed in the upper chamber. F-Met-Leu-Phe (10^{-9} M in MEM) or ZAS was placed into the lower chamber until the fluid levels of the two chambers were equal. The chambers were capped and incubated in a CO₂ incubator for 3 h. Migration was assessed by a modification of the leading edge method reported by Zigmond and Hirsch (23). The measurements were checked in a blinded fashion by two of the investigators. Their observations agreed with those of the observer who knew the source of the leukocytes.

Effects of colostrum upon PBLs. A series of experiments was conducted to test if colostrum inhibited the response of HMLs to chemoattractants. Fresh, whole human colostrum and a cell-free preparation of colostrum (aqueous phase) were used in these experiments. The aqueous phase was prepared by centrifuging

human colostrum repeatedly until the preparation was cell-free by microscopic examination. The effect of the aqueous phase upon the adherence and orientation of human PBLs was assessed in the following manner. Peripheral blood leukocytes ($1-2 \times 10^6$ /ml) suspended in the aqueous phase of colostrum were incubated for 1 h at 22° C and then placed on coverglasses which were mounted on depression slides. One hundred μl of this suspension (*i.e.* $1-2 \times 10^5$ cells) were mixed with a 10^{-9} M solution of F-Met-Leu-Phe and examined for adherence and orientation as previously described. The remaining PBLs suspended in the aqueous phase of colostrum were washed twice, resuspended in MEM containing 10% fetal calf serum and incubated for another hour at room temperature. Following this incubation, 100 μl of the cells were again tested for adherence and orientation as previously described.

The effect of colostrum upon the motility of human PBLs was examined. PBLs were preincubated in whole colostrum or the aqueous phase of colostrum for 60 min at 22° C and either washed with HBSS or not washed. The movement of PBLs toward F-Met-Leu-Phe was then quantified as previously described and compared to the movement of PBLs not exposed to human colostrum.

Statistical analysis. An unpaired Student's *t* test was used to compare the responses of HMLs and PBLs to chemoattractants, whereas a paired Student's *t* test was used to test the effect of colostrum upon the response of PBLs to N-formylmethionyl peptides.

RESULTS

Adherence. HMLs and PBLs from two groups each consisting of five individuals, were used in these experiments. Few fresh HMLs adhered to the coverglasses of the Zigmond chamber in the absence or presence of F-Met-Leu-Phe (Figs. 1A and 2). Those HMLs that were adherent remained in a round configuration and did not spread (Fig. 1A). In contrast, many PBLs suspended in MEM were adherent (Figs. 1B and 2) and spread quickly following stimulation with F-Met-Leu-Phe (Fig. 1B).

Orientation. HMLs and PBLs from two groups, each consisting of five individuals, were used in these experiments. HMLs failed to orient toward chemotactic gradients of N-formylmethionyl peptides or ZAS (Figs. 2 and 3A), whereas the majority of adherent PBLs oriented toward the chemotactic gradient (Figs. 2 and 3B). The lack of orientation of HMLs toward chemotaxins was also observed with nonadherent HMLs in Zigmond chambers and when they were tested by the subagarose technique (see next section).

Chemotaxis. There were remarkable differences between the migration of human PBLs and HMLs in response to chemotactic agents. In six separate experiments utilizing the subagarose method, virtually no movement of HMLs was found in the first 2 h, whereas PBLs consistently displayed a chemotactic response within 2 h (Fig. 4). By 24 h, random movement of some human milk lymphocytes and macrophages was observed (data not presented), but no chemotactic response was found. Neither whole colostrum, a 50% concentration of whole colostrum, a 10% concentration of aqueous colostrum, nor colostrum cells were found to be chemotactic. Those data were therefore pooled for presentation (Fig. 4).

In the Boyden chamber experiments, virtually no neutrophils or macrophages in human milk moved in response to chemotactic factors (Table 1), although some human milk lymphocytes did so (data not presented). In addition, no directed movement of HMLs could be detected when substantially higher or lower doses of these chemoattractant agents were used (data not presented).

Behavior of maternal leukocytes. Because of the failure of HMLs to respond to chemoattractants by increased adherence and directed movement, we questioned whether PBLs from women in the first 2 postpartum days would be similarly affected.

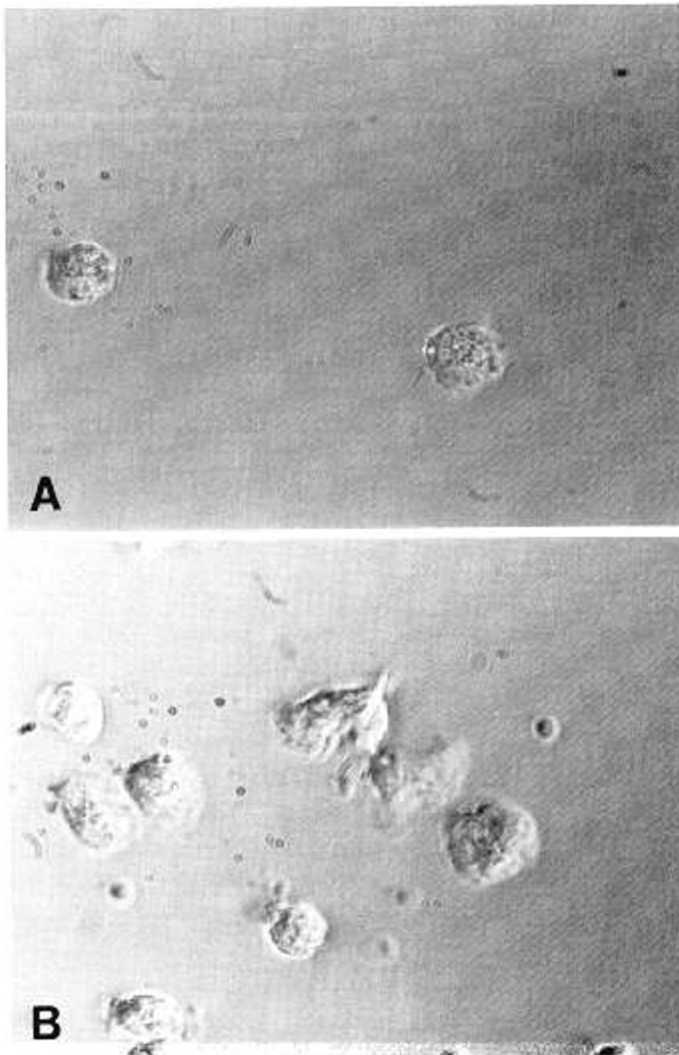


Fig. 1. Representative microphotograph (1000x) of HMLs (A) and PBLs (B), exposed to F-Met-Leu-Phe. Few HMLs were adhered to the coverglasses, whereas many adherent PBLs were found.

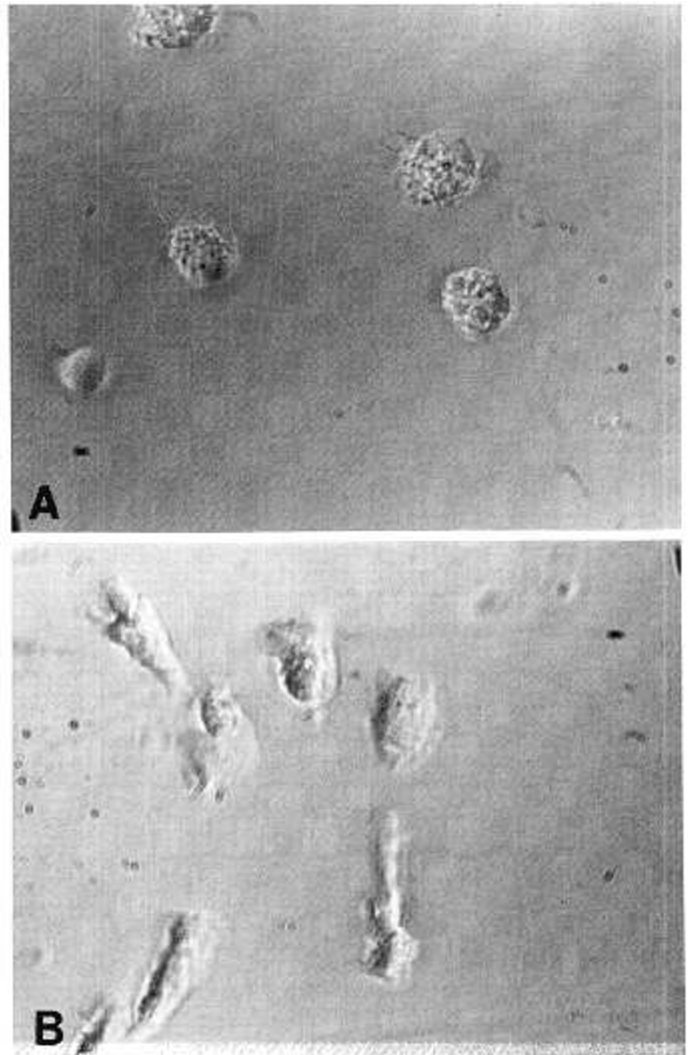


Fig. 3. Representative photomicrographs (1000x) of HMLs (A) and PBLs (B) exposed to a chemotactic gradient of F-Met-Leu-Phe. The source of the chemotaxin is at the top of each microscopic field. Virtually all PBLs oriented toward the gradient, whereas the HMLs did not.

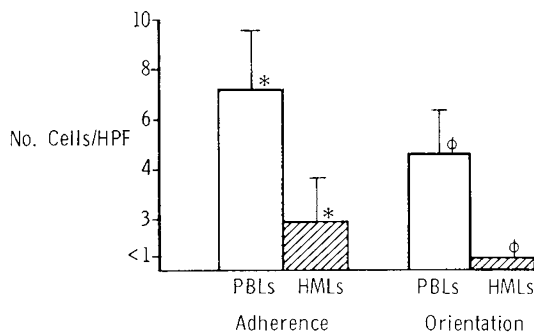


Fig. 2. Adherence and orientation of PBLs and HMLs after exposure to F-Met-Leu-Phe (10^{-9} M). Data from five separate experiments are presented as the mean \pm SD of leukocytes adherent to coverglasses after exposure to F-Met-Leu-Phe and cells oriented toward the chemoattractant. The abilities of HMLs to adhere and orient in response to F-Met-Leu-Phe were significantly decreased (symbols indicate $p < 0.001$).

The adherence and directed movement of maternal PBLs following exposure to F-Met-Leu-Phe or ZAS were, however, indistinguishable from nonmaternal adult neutrophils (Table 2). In addition, the random movements of maternal and nonmaternal adult blood neutrophils were similar (data not shown).

Effects of colostrum on PBLs. The effect of human colostrum

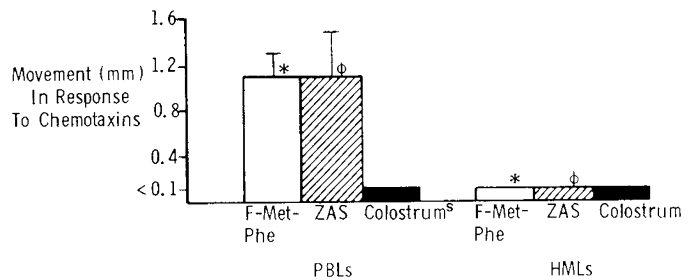


Fig. 4. Leukocyte chemotaxis under agarose. Data from six separate experiments are presented as the distance of migration after 2 h of incubation of the cells toward the well containing the chemotaxin (mm; mean \pm SD). The chemotactic responses of HMLs to F-Met-Phe and ZAS were reduced significantly ($p < 0.001$). There were no differences in the migration of PBLs and HMLs 180° away from the chemotaxins (e.g. < 0.1 mm; no data are presented).^S Data were similar for cells and fluid phase. * φ The responses of HMLs to both chemotaxins were decreased ($p < 0.001$).

upon the adherence and directed movement of PBLs following exposure to F-Met-Leu-Phe are presented in Table 3. Five separate experiments were performed. The glass adherence of PBLs incubated in the aqueous phase of colostrum was greatly inhib-

Table 1. *Leukocyte chemotaxis in Boyden chambers**

Cells	Chemotaxins	Distance of leading edge (μ)	No. cells HPF at leading edge
PBLs	None	21	>100†
	F-Met-Leu-Phe	21	>100
	ZAS	21	>100
HMLs	None	5 \pm 5	<1
	F-Met-Leu-Phe	6 \pm 6	1 \pm 1
	ZAS	5 \pm 5	1 \pm 1

* Data from four separate experiments are expressed as the mean \pm SD of the maximal distance (μ) of migration (leading edge) of the cells and the number of cells per HPF at the leading edge. The depth of each filter was 21 μ . In the case of PBLs, only the mean distance (21 μ) was therefore presented since the leading edge was the surface of the filter facing the chemotactic chamber in each case. The chemotactic responses of HMLs to F-Met-Leu-Phe and ZAS were greatly decreased ($p < 0.001$).

† Because the incubations were increased to 3 h to more completely test the movement of HMLs, the degree of random movement did not permit a true assessment of directed movement of the PBLs. Nevertheless, the inhibition of movement of HMLs was evident.

Table 2. *Comparison of adherence to glass and chemotaxis under agarose of nonmaternal adult and maternal PBLs**

PBLs	Adherence	Chemotaxis (mm)	
	No. cells/HPF	F-Met-Leu-Phe	ZAS
Nonmaternal	7.2 \pm 2.3	1.1 \pm 0.2	1.2 \pm 0.3
Maternal	14.0 \pm 0.3	0.9 \pm 0.1	1.8 \pm 0.3

* The mean \pm SD of data from four separate experiments are presented. No differences were found between nonmaternal and maternal blood leukocytes (principally neutrophils).

Table 3. *Effects of colostrum on glass adherence and movement of PBLs after exposure to F-Met-Leu-Phe**

Experimental conditions		Adherence	Chemotaxis (mm)
Media	Washing	No. cells/HPF	F-Met-Leu-Phe
Fetal calf serum-RPMI	—	7.8 \pm 2.7	1.5 \pm 0.1
Human colostrum	—	Not done	0.7 \pm 0.1
	+	Not done	0.6 \pm 0.6
Aqueous phase	—	1.5 \pm 0.8	1.5 \pm 0.4
	+	10.0 \pm 2.8	1.0 \pm 0.6

* Directed movement of adult (nonmaternal) PBLs under agarose was examined. The mean \pm SD of data from five separate experiments are presented. The inhibition of adherence induced by colostrum was reversed by washing ($p < 0.001$).

ited, but the inhibition was reversed by washing the colostrum-treated cells and reincubating them in tissue culture media containing fetal calf serum. The Zigmond chamber observations did not indicate that the orientation of adherent PBLs was inhibited by colostrum, but no data were presented because comparatively few adherent cells were found. No inhibition of chemotaxis was found with the aqueous phase. The effect of whole colostrum upon the chemotaxis of PBLs varied from complete inhibition with two specimens to marginal inhibition with three samples (Table 3).

DISCUSSION

In keeping with their unusual morphology, it appears from this study that certain functions of HMLs may be altered. Even though there are limitations in extrapolating from *in vitro* obser-

vations to the *in vivo* state, it is clear that the neutrophils and macrophages from human milk do not respond to chemotactic agents that consistently affect their counterparts in peripheral blood. In that respect, the adherence, orientation, and directed movement of HMLs in response to zymosan-activated complement components (24) or N-formylmethionyl peptides (25) were significantly decreased. These findings are in keeping with those of Khan *et al.* (26) who reported a limited chemotactic response of human colostrum leukocytes to bacterial products in Boyden chambers. However, our studies were more complete since we used filters with a larger pore size to accommodate the comparatively large human milk leukocytes, a second method was used to quantitate chemotaxis (the subagarose technique), chemoattractants of human as well as bacterial origin were used, and adherence and orientation were directly examined. Recently, Hawes and Jones (27) reported that cells from human milk did not migrate through 5 or 8 μ m pore size filters in a random manner or in response to casein. No other chemoattractant agents or chemotactic assays were used and adherence and orientation of HMLs were not examined. Nevertheless, their data are consistent with the findings from the present study.

In subsequent experiments, we investigated the cause of the lack of response of HMLs to those agents. To begin with, we tested whether PBLs from women in the immediate postpartum period were similarly inhibited. The adherence and directed movement of maternal blood leukocytes (principally neutrophils) following exposure to the chemotactic agents increased significantly and those findings were in keeping with the thesis that those functions are necessary for blood leukocytes to traverse the mammary gland epithelium and hence become constituents of human milk.

Because of the decreased ability of HMLs to respond to chemoattractants, we questioned whether the cells were inhibited by constituents in human milk. In addition to fresh whole colostrum, an aqueous phase of colostrum prepared by low speed centrifugation was tested. The lipid phase per se was not used because the aggregated fat globules and other particles might have created an artificial physical barrier to the adherence or movement of the tested leukocytes. The nonaqueous phase was tested indirectly, however, by comparing the effects of fresh whole colostrum with the aqueous phase. The studies demonstrated that the adherence of HMLs was reversibly inhibited by the aqueous phase of milk, as we had suspected from a previous study (15). Chemotaxis of PBLs was not inhibited by the aqueous phase. In contrast, chemotaxis was inhibited by certain specimens of whole colostrum, presumably by the lipid phase. It did not appear that the decreased movement of the cells was due to chemotactic deactivation (28) since no preformed chemotactic activity was discernable. Those results were similar to those reported by Bjorksten *et al.* (29). Our results, however, may conflict with those of Hawes and Jones (27) who reported a slight but significant chemoattractant effect of human milk in their two chamber assays. Evidently, the effect of colostrum upon leukocytes is complex and the analysis of these effects is beyond the scope of this study. Nevertheless, our study suggest that certain factors in milk may be responsible in part for the failure of HMLs to respond to chemoattractants.

It is known that peripheral blood neutrophils and monocytes have discrete receptors for N-formylmethionyl peptides and C5a (25, 30–32) and that binding of these ligands to the receptors activates a train of events which lead to an enhancement of adherence, polarization, and directed movement. In that regard, a number of reports indicate that there are subpopulations of human neutrophils in respect to certain functional activities including the response to chemotactic agents (33–35). Moreover, it recently was demonstrated that subpopulations of blood neutrophils that respond most actively to chemotaxins bind more of the chemoattractant (F-Met-Leu-Phe), exhibit a depolarization of their membranes, and are able to displace the chemoattractant ligands from receptors more readily than subpopulations that

respond less well to those agents (35). Thus, it is possible that HMLs may represent special subpopulations of PBLs. In addition, it is possible that surface membrane adherence glycoproteins (36, 37) or the cytoskeleton may be altered in HMLs. Studies of ligand binding, adherence proteins, and the cytoskeleton may therefore help to identify the mechanism of the diminished response of HMLs to chemoattractants.

Regardless of the exact change in these HMLs, it is likely that HMLs are modified when they pass through the mammary gland. Apparently, leukocytes in human milk are adapted not to adhere to epithelium of the ducts of the mammary gland and are thus free to be transferred to the infant via breast-feeding. Based upon these and previous studies, we postulate that HMLs may defend the nursing infant without injuring the mucosa of the alimentary tract of the recipient. The limited response of these cells to chemotactic agents also suggests that the action of these cells is relegated to the surface or the lumen of the alimentary tract. Such an hypothesis is particularly appealing in view of the recent report that human milk macrophages stimulated *in vitro* produce toxic oxygen compounds (38) which potentially could damage the epithelium of the aeroalimentary tract. Further studies will be required, however, to ascertain their *in vivo* fate and functions of HMLs in the recipient infant.

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