The Motility of Human Milk Macrophages in Collagen Gels¹

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ABSTRACT. The hypothesis that the diminished motility of human milk leukocytes is due to a decrease in adherence was tested by using a collagen gel system in which leukocyte movement is less dependent on adherence. Unfractionated human milk leukocytes (HML) or fractionated peripheral blood leukocytes were placed on collagen gels in microwells and the leading edge of migration was determined by inverted phase microscopy. The mean rates of invasion of HML, blood neutrophils, and mononuclear blood leukocytes were 14, 240, and <1 μ /h, respectively ($p < 0.01$). We then examined the identity of motile HML by immunoperoxidase techniques using antibodies to selected cell markers. Motile HML were positive for a specific macrophage marker (cathepsin B) and a neutrophil and monocyte marker (Mac-1) but were negative for specific neutrophil (cathepsin G) or lymphocyte markers (CD3 and CD5). The directed motility of these cells was not enhanced by exposure to the chemoattractant, N-formyl-L-methionyl-L-phenylalanine, but was inhibited by a T cell lectin, phytohemagglutinin. The movement was actin dependent but was not dependent on calcium or Mac-1 surface glycoproteins. Thus, the diminished motility of milk neutrophils does not appear to be due to decreased adherence per se, and those HML that are motile are macrophages. This suggests a dichotomy for the function of HML. Neutrophils may be relegated to the lumen of the alimentary tract, whereas macrophages may penetrate into mucosal sites for host defense. (*Pediatr Res* 23: 449–452, 1988)

Abbreviations

HML, human milk leukocyte(s)
PBL, peripheral blood leukocyte(s)
F-Met-Phe, N-formyl-L-methionyl-L-phenylalanine
PHA, phytohemagglutinin

Recent investigations have demonstrated that the motility of neutrophils in human milk is greatly decreased (1–3), and that their adherence, orientation, or directed movements are not increased after exposure to chemoattractant agents (3). In one study (3), preliminary evidence was found that some mononuclear cells in human milk moved spontaneously in a two-dimensional system, e.g. under agarose. Those findings were in keeping

with earlier observations that lymphocytes and macrophages from human milk could migrate on glass (4–6).

Herein, we extended the previous investigations by posing two new questions. The first was whether the decreased motility of human milk neutrophils was incidental to defective adherence. The design of those experiments was based on the fact that motility in two-dimensional systems involves adherence-dependent, traction events, whereas movement in three-dimensional models such as collagen is largely adherence-independent and involves hydraulic or pushing motions (7). We thus exploited this difference to examine the adherence-independent motility of HML in a type I collagen gel matrix. These experiments also allowed us to address the second question, the identity of the types of motile mononuclear HML and to subsequently quantitate the movement of those cells, to determine whether their movement was dependent on surface glycoproteins that mediate cellular adherence or on actin filaments that are necessary for the propulsion of other cells, and to ascertain whether their movement could be modulated by other agents.

METHODS

Donor selection and specimen preparations. The study was approved by the Institutional Review Board for Human Research. Venous blood was collected from healthy adult volunteers. PBL were subjected to sedimentation with 6% dextran and then to Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradients (7) to obtain neutrophils ($>95\%$ pure) and mononuclear cells (75% pure). Unfractionated HML were prepared in the following manner. Human colostrum collected from healthy women in the first 3 days after delivery (8) was diluted 1:4 in RPMI-1640 (Whittaker M.A. Bioproducts, Walkersville, MD) and centrifuged at $100 \times g$ for 5 min. The cellular pellets were then resuspended in RPMI 1640 to achieve a final concentration of 1×10^7 leukocytes/ml. Viability of the cells was determined by trypan blue exclusion. Macrophages were detected by the presence of nonspecific esterase and neutrophils by myeloperoxidase (10). The relative frequencies of HML in the preparations (neutrophils 40 to 60%; macrophages 35 to 55%; lymphocytes 5 to 8%) were similar to that found in previously (3).

Preparation of collagen gels. Type I collagen was prepared from stripped rat tendons that were solubilized in cold 3% (v/v) acetic acid for 2 days (11). Clear supernatant preparations obtained by centrifugation were mixed with 20% (w/v) NaCl. The precipitated type I collagen was pelleted by centrifugation at $3000 \times g$ for 1 h, washed and dissolved in 3% acetic acid (final concentration 1.2 mg/ml). The solution was dialyzed against distilled H₂O that was adjusted to pH 4.0 with HCl. Eight ml of the collagen solution was mixed with 1 ml of a 4% (w/v) solution of sodium bicarbonate and 1 ml of a 10-fold concentrated solution of minimal essential medium (Eagle modified, McLean Flow Laboratories, Inc., McLean, VA) to restore the pH to 7.4

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and the ionic strength to physiologic levels. In certain experiments, one of the following was added to some collagen preparations before gelling: decomplexed or fresh human serum (10%), acellular human colostrum (10%), monoclonal antibodies to Mac-1 or its β -subunit (13), or F-Met-Phe (Sigma) (40 μ g F-Met-Phe/ml of collagen solution).

Cellular penetration into collagen gels. Unstimulated movement was tested in the following manner. A total of 50 μ l of a fresh solution of collagen was placed in each well of flat bottom, 96-well microtiter plates (Becton Dickinson, Oxnard, CA) and allowed to gel by incubating the plates at 37°C for 15 min. Unfractionated HML or fractionated PBL (2×10^5 cells/25 μ l RPMI 1640) were placed on the surface of each gel and were then incubated at 37°C. The leading edge of migrating cells was determined at 1–24 h by direct microscopy (12) (Diavert, Inverted Phase Microscope, Leitz, West Germany).

Phenotypic characterization of motile human milk leukocytes. In these experiments the types of HML that invaded the collagen gels were ascertained after 24 h of incubation. Gels were stained for myeloperoxidase (10) to identify neutrophils and for nonspecific esterase (10) to identify macrophages and then examined microscopically. Immunocytologic examinations were carried out in the following manner. Gels were quick-frozen in precooled isopentane and embedded in O.C.T. Compound (Tissue-Tek Miles Scientific, Naperville, IL). After the top portion of the gel was discarded, it was vertically sliced into serial 4- μ sections. Sections were air dried for 60 min and fixed in cold acetone (–20°C) for 10 min. After rehydration, they were incubated with the following primary antibodies: sheep anticathepsin B (14) and anticathepsin G (15) (ICN Immunobiologicals, Lisle, IL), and mouse monoclonal anti-OKT3 (CD3), anti-Leu1 (CD5), anti-Leu7 (Becton Dickinson, Mountainview, CA), (16, 17) (Ortho Diagnostic System Inc., Raritan, NJ), and anti-Mac-1 (prepared in our laboratory). The preparations were then exposed accordingly to either biotinylated rabbit anti-sheep IgG (Vector Labs., Burlingame, CA) or biotinylated sheep anti-mouse IgG (Cooper Biomedicals, Westchester, PA) (1:80). Finally, the reaction with Streptavidin biotinylated peroxidase complex (Amersham, Arlington Heights, IL) (1:500) was visualized by incubating with diaminobenzadine and hydrogen peroxide.

Modulation of the motility of human milk leukocytes. Certain requirements for the motility of HML and the effects of certain modulators on these cells were tested. The need for actin filament was examined by exposing these cells to 1 mM cytochalasin B (Sigma). The necessity for calcium was tested by treating the cells with a 10 mM solution of EDTA (Sigma) for 20 min before they were placed on collagen. Inasmuch as human milk macrophages display Mac-1 on their surfaces (unpublished data), the need for this protein for motility in this system was assessed by reacting the cells with mouse monoclonal antibodies to Mac-1 prepared in our laboratory or with monoclonal antibodies to its β -subunit (13) for 20 min before their movement was tested and by adding the same monoclonal antibodies to the collagen solution before gelling. The possible inhibitory effect of serum was examined by incubating HML in 10% fresh human serum overnight at 21°C on a horizontal shaker. The influence of PHA (Difco Corp., Detroit, MI) on HML was tested with concentrations of PHA ranging from 10 times to 0.001 of the optimal mitogenic dose (20 μ l/100 μ l RPMI 1640). The effects of F-Met-Phe on the motility of colostrum cells were determined by employing collagen gels prepared with F-Met-Phe as described above. Controls for these experiments consisted of aliquots of untreated cells placed on unmodified collagen gels.

Effects of acellular human colostrum. The effects of acellular human colostrum on the motility of human blood mononuclear leukocytes were measured by incubating them in previously stored (–80°C) acellular milk (10%) (3) for 15 min. Motility was determined 4 h after the cells were applied to the gel. Controls for these experiments were peripheral blood mononuclear leukocytes incubated in RPMI 1640.

Statistical analysis. Data concerning the leading edge of cell migration were expressed as the means and 95% confidence intervals. Differences between the degree of movement of HML and neutrophils or mononuclear leukocytes in human blood were tested by an unpaired Student's *t* test, whereas the effects of modulating agents (human serum, acellular human colostrum, cytochalasin B, EDTA, anti-Mac-1, anti- β -subunit, PHA, or F-Met-Phe) on leukocytes were tested by a paired Student's *t* test.

RESULTS

Invasion of leukocytes into collagen gels. As previously reported (18), peripheral blood neutrophils rapidly invaded the collagen gels (240 μ /h), whereas few peripheral blood mononuclear cells penetrated the gels during the period of observation (Fig. 1). The rate of penetration of HML into the gels (13 μ /h) was much slower than peripheral blood neutrophils ($p < 0.001$), but more rapid than the blood mononuclear cells ($p < 0.001$) (Fig. 1).

Identification of motile HML. The identity of motile HML was sought in specimens obtained from six individuals. In each case, although neutrophils and lymphocytes as well as macrophages were found on the surface of the gels, the only HML which invaded the collagen matrix displayed markers for macrophages (nonspecific esterase, cathepsin B), but not for neutrophils or lymphocytes (Table 1).

Modulation of motility. Cytochalasin B inhibited the migration of human milk macrophages ($p < 0.001$), whereas the rate of migration of EDTA-, anti-Mac-1-, or anti- β -subunit-treated HML were similar to the rate of spontaneous motility of untreated cells (15.5, 13.8, 14, and 13 μ /h, respectively) (Fig. 2). The motility of human milk macrophages was reduced significantly after exposure to PHA ($p < 0.001$). Also the inhibition was directly proportional to the amount of PHA that was used (range 0.01–10 times the optimal mitogenic dose; data not shown). The rates of migration of HML in control and F-Met-Phe-stimulated preparations were similar (13.0 and 12.5 μ /h, respectively) (Fig. 2). Finally, the motility of peripheral blood mononuclear cells was unaffected by acellular human colostrum ($< 1 \mu$ /h, $p > 0.05$) and the motility of HML was not affected by human serum (12.0 μ /h, $p > 0.05$; data not shown).

DISCUSSION

The results of these experiments and previous studies (1–4) indicate that the types of human milk leukocytes that migrate spontaneously or in response to stimulating agents are quite different from those of peripheral blood. Blood neutrophils display spontaneous movement in *in vitro* systems and their movements are enhanced by chemoattractant agents (19, 20). In contrast, neutrophils from human milk do not move spontaneously or in response to chemoattractants in either two-dimensional (1–3) or, as in the present study, three-dimensional systems. The cause of the decrease in motility of neutrophils from human milk is unclear. We previously reported that the motility of peripheral blood neutrophils in a two dimensional system was not decreased by incubating them in human colostrum (3). In addition, the rates of movement of blood neutrophils obtained early in lactation and from nonpregnant, nonlactating adults were indistinguishable. Because movement into collagen gels by human blood neutrophils does not appear to be dependent on adherence (18), the failure of human milk neutrophils to invade the matrix may be due to a different mechanism such as a lack of deformability as previously suggested (3).

Motile leukocytes in human milk, as shown by cytochemical and immunochemical markers, proved to be macrophages. The rate of movement of these cells was less than peripheral blood neutrophils, but much greater than blood mononuclear cells. In these studies, we found that their motility was actin-dependent as demonstrated by the response to an inhibitor of actin polym-

Fig. 1. The rates of invasion of peripheral blood neutrophils (▽), HML (●), and blood mononuclear leukocytes (○) into collagen gels. Data from 28 experiments are displayed, but not all data points are shown because of overlapping values. First order regression lines are also included. The rates of movement of HML were slower than peripheral blood neutrophils ($p < 0.001$), but greater than blood mononuclear leukocytes ($p < 0.001$).

Table 1. Immunologic-cytochemical markers of motile HML invading collagen gels

Cell markers	Type of leukocytes			
	Motile HML	Monocytes macrophages	Neutrophils	Lymphocytes
Nonspecific esterase	+	+	—	—
Cathepsin B	+	+	—	—
Mac-1	+	+	+	—
Myeloperoxidase	—	—	+	—
Cathepsin G	—	—	+	—
CD5, CD3, Leu7	—	—	—	+

erization (cytochalasin B), but was calcium-independent since a calcium chelator (EDTA) did not alter their motility. Furthermore, the motility of human milk macrophages in the collagen matrix was found to be similar to blood neutrophils in that it was not dependent upon adherence mediated by Mac-1 or its β -subunit. Finally, it is of interest that the motility of milk macrophages was greatly inhibited by PHA. Although the genesis of this effect was not explored, it is possible that the inhibition was caused by production of lymphokines, such as macrophage migration inhibition factor from milk lymphocytes (21).

Our studies failed to elucidate why the motility of human milk macrophages is much greater than that of blood mononuclear cells because human serum did not inhibit the movement of milk macrophages and human colostrum did not augment the motility of blood mononuclear leukocytes. It is important to understand, however, that the *in vitro* conditions of these experiments, *e.g.* the duration of exposure, concentration of reagents, physiologic state of the cells, may not have been approximated *in vivo* conditions.

Regardless of the mechanisms that are responsible for the altered motility of HML, a dichotomy of function of HML based on the presence or absence of motility is suggested. The action of human milk neutrophils may be relegated to the lumen of the alimentary tract, whereas the macrophages may interact with the epithelium of the aeroalimentary tract of the recipient infant. It is not clear, however, how these cells would function in those circumstances.

Note added in proof. Since this paper was accepted, we have found that the motility of blood monocytes is enhanced by incubating them in human milk for 3 h.

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Fig. 2. The effect of modulating agents on the motility of human milk macrophages. Data are presented as the mean and 95% confidence interval of five to 25 observations for each experiment. Since there were no differences between the results of the anti-Mac-1 and the anti- β -subunit studies, only the results of the anti-Mac-1 experiments were presented. The rate of movement of the cells was significantly decreased after treatment with cytochalasin B ($p < 0.001$) or PHA ($p < 0.001$).

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