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Lymphokine Production by Human Milk Lymphocytes

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To assess the functional capability of human milk lymphocytes, we studied phytohemagglutinin-induced lymphokine production by breast milk and, for comparison, peripheral blood lymphocyte cultures. Two lymphokines, lymphocyte-derived chemotactic factor (LDCF) and immune interferon, were assayed in supernatants of milk and blood lymphocyte cultures obtained from women 2 to 6 days postpartum. Eleven parallel milk and blood samples were studied for LDCF production. In nine experiments, both milk and blood lymphocytes produced LDCF. In the two other experiments, milk cells did not produce LDCF. In 10 milk cultures studied, all produced interferon activity. Acid and heat lability characteristics were typical of immune interferon. These results further characterize milk lymphocytes as immunologically competent and possibly important effector cells in neonatal immunity.

Several investigators have examined the cells present in human milk, but the role of these cells in protecting neonates from infection must be better established (9, 20-23, 26, 27). Mohr (18), Ogra et al. (21), and Schlessinger and Covelli (26) have published data suggesting a transfer of cellular immunity to purified protein derivative antigen from mothers to infants via human breast milk. If milk cells are capable of mediating such a transfer, it is important to characterize possible mechanisms. One proposed mechanism (21) is production of immunological mediators by human milk cells which are subsequently absorbed by sucklings.

We have studied lymphokine production by human breast milk cells in order to assess the functional ability of the milk lymphocytes to produce immunological mediators. Two lymphokines, lymphocyte-derived chemotactic factor (LDCF) and immune interferon, were assayed in the supernatants of mitogen-stimulated, Ficoll-Hypaque-separated milk lymphocyte cultures. Peripheral blood specimens were obtained simultaneously and also examined for lymphokine production.

MATERIALS AND METHODS

Heparinized human peripheral blood (10 to 15 U/ml) was obtained by venipuncture, and human breast milk was obtained by using an Egnell electric breast pump.

Cell preparation. Women 2 to 6 days postpartum were studied. Blood and milk lymphocytes were prepared by Ficoll-Hypaque differential centrifugation (4). Milk was first centrifuged at 1,600 rpm (400 × *g*) for 15 min. The fatty top layer and supernatant were removed. The cell pellet was suspended in calcium- and magnesium-free Hanks balanced salt solution (HBSS) and recentrifuged at 1,500 rpm for 10 min to remove additional fat. The pellet was resuspended in calcium- and magnesium-free HBSS and underlayered with lymphocyte-separating medium (Litton Bionetics). Cells were then centrifuged at 1,000 rpm for 35 min, and the mononuclear fraction was removed.

Mononuclear cells were washed three times with HBSS. A wet-preparation differential was done by using toluidene blue stain, and viability was assessed by using trypan blue exclusion (13). In addition, for breast cell preparations, a supervital stain (8) was used to differentiate phagocytic and nonphagocytic cells and confirm toluidene blue stain results. T-cell content was determined by a modification of the sheep erythrocyte rosette method of Perper et al. (24). Lymphocytes, 0.5×10^6 in 0.25 ml of HBSS, were incubated with 0.25 ml of 1% sheep erythrocytes at 37°C for 15 min. Erythrocytes were pretreated with 2-aminoethylisothiouronium bromide hydrobromide by the method of Saxon et al. (25). Cells were centrifuged at 200 × *g* for 5 min and incubated at 4°C overnight. The pellet was gently suspended, mixed with toluidene blue stain (7), and counted for percentage of rosetting lymphocytes.

Lymphocyte cultures. For LDCF studies, 10^6 lymphocytes/ml were suspended in RPMI 1640 (GIBCO Laboratories) with additional L-glutamine, an antibiotic/antimycotic preparation (GIBCO), and gentamicin (10 μg/ml). For interferon studies, 5×10^5 lymphocytes/ml were suspended in the same medium with added 5% pooled human AB sera (GIBCO). Lymphocytes were incubated with phytohemagglutinin (PHA) for 48 h in LDCF studies and for 72 h in interferon studies. Cultures were incubated at 37°C in humidified 5% CO_2. After the incubation period, PHA (same concentration) was also added to control nonstimulated cultures. The cultures were centrifuged at 400 × *g* for 15 min, and supernatants were aspirated and frozen at −70°C.

Monocyte chemotaxis assay for LDCF. Lymphocyte culture supernatants were assayed for LDCF activity by the method of Altman et al. (1) and Snyderman et al. (28). Human monocytes were obtained from peripheral blood by Ficoll-Hypaque differential centrifugation. These monocytes, 0.2 ml of 1.5×10^6 monocytes/ml, were placed on one side of a 5-μm Nuclepore filter in a chemotaxis chamber (Bio-Rad Laboratories). Test supernatant (0.2 ml) was placed on the other side of the filter. The chemotaxis chambers were incubated for 90 min at 37°C in 5% humidified CO_2. The filters were removed, fixed with ethanol, and stained with hematoxylin and Wright stain. For each filter, the number of monocytes migrating through the filter toward test supernatants was counted under oil immersion in 20 fields. The mean number of migrating monocytes per oil field was determined. Filters were tested in triplicate.

Chemotactic activity was defined as the number of monocytes migrating toward stimulated supernatants minus the number of monocytes migrating toward nonstimulated control supernatants. Student's t test (10) was used to compare monocyte migration toward stimulated supernatants with migration toward control supernatants.

Interferon assay. Lymphocyte culture supernatants were assayed in duplicate for interferon activity, using a modification of the micro dye uptake method (2, 5, 6). The challenge virus, encephalomyocarditis virus, was large plaque purified, propagated in CCL-1 cells from the American Type Culture Collection (ATCC), and stored at −70°C. Confluent human skin trisomy 21 fibroblast monolayers (ATCC CCL-54) were incubated overnight with 0.1 ml of maintenance medium (2% fetal bovine serum–1% L-glutamine) per well of the microtiter plate. Sample supernatants were serially diluted 1:3 in these microtiter plates. After overnight incubation, media were removed and cultures were infected with 10 plaque-forming units of virus per cell. New media were added to each well, and monolayers were incubated for 24 h at 37°C in 5% CO_2 until 100% cytopathic effect was noted in unprotected virus control cultures. Cells were then fixed in methanol, stained with 1% aqueous crystal violet, and read under ×10 microscopy. Viral cytopathic effect was scored from 0 to 4+. Interferon titers were read as the reciprocal of the dilution giving 50% protection of the cultures from destruction by encephalomyocarditis virus (2+ cytopathic effect). An international leukocyte interferon standard from the National Institutes of Health was run simultaneously.

Acid lability of interferon was determined by acidifying the specimen to pH 2 for 24 h with 1 N HCl and then returning the pH to the neutral range with 1 N NaOH before assay. Heat lability was determined by heating at 56°C for 1 h.

RESULTS

Cellular composition of lymphocyte cultures. Table 1 summarizes the cellular composition of lymphocyte cultures. Milk lymphocyte cultures contained fewer rosetting lymphocytes but more polymorphonuclear cells and macrophages than blood lymphocyte cultures.

Dose response. PHA dose response was determined. A PHA concentration of 0.83 μg/ml usually stimulated lymphokine production in both interferon and LDCF studies. Since the PHA dose for maximal response varied, lymphocytes were incubated with additional concentrations of PHA when cell yield permitted. PHA concentrations ranging from 0.02 to 6.8 μg/ml stimulated lymphokine production.

Preliminary experiments determined 48 h as an optimal time to assay supernatants for LDCF. Interferon supernatants were obtained after 72 h of incubation with the exception of experiment 20, for which 48-h supernatants were used. Seventy-two-hour supernatants were used for comparison with the previously published work of Lawton et al. (16).

LDCF production. Parallel milk and blood lymphocyte cultures from 11 women were stimulated with PHA. Forty-eight-hour supernatants were examined for monocyte chemotactic activity. Preliminary experiments determined optimal dilutions for assaying supernatants. Milk supernatants were assayed undiluted and at a 1:3 dilution; blood supernatants were assayed at a 1:3 dilution only. In specimens from nine women, both blood and milk lymphocyte cultures produced LDCF (Table 2). In experiment 1, milk lymphocytes did not produce LDCF when stimulated with PHA at 0.83 μg/ml, although the corresponding blood lymphocytes did produce LDCF. Limited cell yield did not permit stimulating milk lymphocytes at ad-

TABLE 1. *Cell composition for LDCF and interferon production*

Product	% Rosetting lymphocytes	% Viability	Differential		
			Polymorphonuclear cells	Lymphocytes	Monocytes/macrophages
LCDF					
Blood	63 ± 13 (30–83)[a]	98 ± 1	1 ± 2	76 ± 5	23 ± 4
Milk	23 ± 8 (8–45)	93 ± 4	31 ± 19	33 ± 15	36 ± 18
Interferon					
Blood	59 ± 11 (38–77)	97 ± 2	2 ± 5	79 ± 5	19 ± 5.0
Milk	31 ± 13 (8–52)	90 ± 7	15 ± 16	34 ± 14	51 ± 20.0

[a] Numbers in parentheses are ranges.

TABLE 2. *LDCF production by milk and blood lymphocyte cultures*

Expt no.	No. of migrating monocytes ± standard error/oil immersion field[a]		
	Milk supernatant		Blood supernatant, 1:3 dilution
	Undiluted	1:3 dilution	
1	NE[b]	0 ± 4	27 ± 4[c]
2	19 ± 6[c]	14 ± 4[c]	24 ± 4[c]
3	26 ± 5[c]	24 ± 5[c]	23 ± 11[c]
4	11 ± 6	8 ± 2[c]	11 ± 2[c]
5	5 ± 4[c]	0 ± 3	9 ± 1[c]
6	20 ± 7[c]	13 ± 5[c]	18 ± 5[c]
7	21 ± 1[c]	18 ± 7[c]	30 ± 8[c]
8	8 ± 5	2 ± 1	7 ± 2[c]
9	42 ± 8[c]	36 ± 5[c]	11 ± 3[c]
10	20 ± 8[c]	7 ± 5	17 ± 3[c]
11	38 ± 4[c]	35 ± 6[c]	7 ± 2[c]

[a] Number of monocytes migrating toward stimulated supernatants minus number of monocytes migrating toward control supernatants. Maximal responses are listed. PHA ranged from 0.03 to 1.7 μg/ml.
[b] Not examined.
[c] Indicates $P \leq 0.05$ by Student's t test.

ditional mitogen concentrations or assaying the T-cell content. In experiment 8, milk lymphocyte production of LDCF activity was not statistically significant ($P \leq 0.05$), although milk lymphocytes were stimulated at PHA concentrations of 0.03, 0.08, 0.83, and 1.7 μg/ml. Parallel blood lymphocyte production of LDCF was statistically significant by Student's t test. Only 8% of the milk lymphocytes were T cells, compared with 30% of the peripheral blood lymphocytes. These were the lowest percentages of T cells for both blood and milk obtained in the study. An inadequate number of T cells possibly explains the lack of LDCF production by milk lymphocytes in this patient.

Interferon production. Milk and blood lymphocyte cultures from 10 women were stimulated with PHA, and 72-h supernatants were assayed for interferon activity. All 10 milk lymphocyte cultures produced interferon activity; 8 of the 10 blood lymphocyte cultures assayed also produced interferon activity. Control culture supernatants produced 0 to 8 IU of interferon activity (Table 3).

Acid and heat lability of interferon produced were examined in five milk supernatants and two blood supernatants. The interferon produced showed acid (pH 2) and heat (56°C for 1 h) lability characteristics of immune interferon (Table 4).

DISCUSSION

Lymphocytes reactive to various mitogens and infectious antigens (17, 20–23) have been found in human milk, but how these lymphocytes can influence neonatal immunity is not yet understood. Using Fisher and Lewis rats and nude mice, previous investigators were able to produce runting, graft-versus-host disease, and variation in skin graft survival with experiments in foster-nursing neonatal rats and nude mice (3, 14). These observations have suggested possible absorption of immunocompetent cells from milk in these animal models, yet to date there is no similar evidence which suggests absorption of immunocompetent cells by human neonates.

Several studies (18, 21, 26) suggest transfer of tuberculin cellular immunity via breast milk to human neonates. If transfer of cellular immunity via milk does occur in human neonates, the role of humoral mediators is a largely unexplored area of considerable importance. As proposed by Ogra et al. (21), the neonatal intestine may be permeable for absorption of immunological mediators that modulate neonatal immunity. The role of macrophages and lymphocytes in the

TABLE 3. *Interferon production*

Expt no.	Interferon (IU)[a]	
	Milk cultures	Blood cultures
12	384	5
13	222	384
14	385	384
15	128	384
16	1,152	56
17	1,158	386
18	669	3
19	386	386
20	168	222
21	384	875

[a] Maximal response is shown. All data listed were obtained at a PHA concentration of 0.03 to 1.25 μg/ml. Control cultures produced 0 to 8 IU.

TABLE 4. *Heat and acid lability of interferon*[a]

Expt no.	Supernatant	Pretreatment titer	Interferon production	
			Post heat treatment (56°C for 1 h)	Post acid treatment (pH 2 for 24 h)
12	Milk	384	0	0
13	Milk	222	0	NA[b]
15	Milk	128	0	0
	Blood	384	0	0
16	Milk	1,152	0	0
21	Milk	168	0	0
	Blood	74	0	0

[a] All data are presented in international units.
[b] NA, Not assayed.

milk may correlate with the lymphokines or monokines produced.

Although many investigators of human milk lymphocytes have studied blastogenesis (14, 17, 20, 22, 26), milk cell lymphokine studies have been few. Mohr (19) first demonstrated inhibition of leukocyte migration by incubation of purified protein derivative with sensitized milk leukocytes. Emodi and Just (11) demonstrated production of classical type I interferon by milk leukocytes incubated with Newcastle disease virus. Lawton et al. (16) have shown production of interferon by both PHA-induced and Newcastle disease virus-induced milk leukocytes. The PHA-induced interferon produced in that study was acid stable and relatively heat stable. Their observations disagree with our results showing acid and heat lability of the interferon produced. In our study, milk lymphocytes were separated by Ficoll-Hypaque differential centrifugation, whereas in Lawton's study, the total milk cell population was used. Macrophage content of cell cultures was much higher in the Lawton et al. (16) study, and accessory cells are important in determining the type of interferon produced (12). Differences in cell populations studied may explain discrepancies between Lawton's results and ours. Neither study identifies the cell of origin of the interferon produced.

We chose to examine LDCF and interferon production by milk cells in order to assess their functional ability to produce immunological mediators. These studies do not imply that LDCF or immune interferon itself modulates neonatal immunity, but they do provide additional data on the potential role of milk cells in neonatal immunity. Since previous studies have reported hyporesponsiveness of milk cells to the mitogen PHA (15, 20, 22) and possible presence or absence of selected lymphocyte populations in human milk (22), milk lymphokine production was compared with peripheral blood lymphokine production. Although milk cell and blood mononuclear cell preparation differed in terms of T lymphocytes, monocytes/macrophages, and polymorphonuclear cells, the milk cell preparations generally did not have impaired lymphokine production.

In conclusion, our study shows mitogen-stimulated milk cells to be functionally as competent as blood lymphocytes in production of two lymphokines, LDCF and immune interferon. The biological role of these mediators is not clearly defined. However, it appears that LDCF production in the gut of neonates may have direct importance in attracting neonatal monocytes to a mucosal surface pathogen, and thus provide an important component of neonatal defense against infection. Similarly, production of interferon in the neonatal gut may provide significant protection against viral disease. Further studies of human milk cells are needed to establish their role in neonatal immunity.

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LITERATURE CITED

1. **Altman, L. C., R. Snyderman, J. J. Oppenheim, and S. E. Mergenhagen.** 1973. A human mononuclear leukocyte chemotactic factor: characterization, specificity and kinetics of production by homologous leukocytes. J. Immunol. **110**:801–810.
2. **Armstrong, J. A.** 1971. Semi micro, dye binding assay for rabbit interferon. Appl. Microbiol. **21**:723–725.
3. **Beer, A. E., R. E. Billingham, and J. A. Head.** 1974. The immunologic significance of the mammary gland. J. Invest. Dermatol. **63**:65–74.
4. **Boyum, A.** 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. **21**:77–88.
5. **Bryson, Y. J., and L. H. Kronenberg.** 1977. Combined antiviral effects of interferon, adenine arabinoside, hypoxanthine arabinoside, and adenine arabinoside 5'-monophosphate in human fibroblast cultures. Antimicrob. Agents Chemother. **11**:299–306.
6. **Bryson, Y. J., H. S. Winter, S. E. Gard, T. J. Fischer, and E. R. Stiehm.** 1980. Deficiency of immune interferon production by leucocytes of normal newborns. Cell. Immunol. **55**:191–200.
7. **Combs, R. R. A., B. W. Gurner, A. B. Wilson, G. Holm, and B. Lindgren.** 1970. Rosette-formation between human lymphocytes and sheep red blood cells not involving immunoglobulin receptors. Int. Arch. Allergy **39**:658–663.
8. **Conn, H. J., M. A. Darrow, and V. M. Emmel (ed.).** 1960. Staining procedures used by the Biological Stain Commission, 2nd ed., p. 141–143. The Williams & Wilkins Co., Baltimore.
9. **Diaz-Jouanen, E., and C. Williams, Jr.** 1974. T and B lymphocytes in human colostrum. Clin. Immunol. Immunopathol. **3**:248–255.
10. **Documenta Geigy.** 1968. Scientific tables, 6th ed., p. 32–35. Geigy Pharmaceuticals, Summit, N.J.
11. **Emodi, G., and M. Just.** 1974. Interferon production by lymphocytes in human milk. Scand. J. Immunol. **3**:157–160.
12. **Epstein, L. B.** 1979. The comparative biology of immune and classical interferons, p. 464. *In* S. Cohen, E. Pick, and J. Oppenheim (ed.), Biology of the lymphokines. Academic Press, Inc., New York.
13. **Ford, W. L.** 1978. The preparation and labelling of lymphocytes, p. 23.10. *In* D. M. Weir (ed.), Handbook of experimental immunology, vol. 2, Cellular immunology, 3rd ed. Blackwell Scientific Publications, Oxford, England.
14. **Head, J. R., and A. E. Beer.** 1979. *In vivo* and *in vitro* assessment of the immunologic role of leukocytic cells in milk, p. 207–220. *In* P. L. Ogra and D. Dayton (ed.), Immunology of breast milk. Raven Press, New York.

15. **Keller, M. A., J. L. Turner, J. A. Stratton, and M. E. Miller.** 1980. Breast milk lymphocyte response to K1 antigen of *Escherichia coli*. Infect. Immun. **271**:903–909.
16. **Lawton, J. W. M., K. F. Shortridge, R. L. C. Wong, and M. H. Ng.** 1979. Interferon synthesis by human colostral leukocytes. Arch. Dis. Child. **54**:127–130.
17. **Meggs, P. D., and A. E. Beer.** 1979. *In vitro* stimulation of human colostral lymphocytes by cytomegalovirus. Am. J. Obstet. Gynecol. **133**:703–707.
18. **Mohr, J. A.** 1973. The possible induction and/or acquisition of cellular hypersensitivity associated with ingestion of colostrum. J. Pediatr. **82**:1062–1064.
19. **Mohr, J. A., R. Leu, and W. Mahry.** 1980. Colostral leukocytes. J. Surg. Oncol. **2**:163–167.
20. **Ogra, S. S., and P. L. Ogra.** 1978. Immunologic aspects of human colostrum and milk. II. Characteristics of lymphocyte reactivity and distribution of E-rosette forming cells at different times after the onset of lactation. J. Pediatr. **92**:550–555.
21. **Ogra, S. S., D. Weintraub, and P. L. Ogra.** 1977. Immunologic aspects of human colostrum and milk. III. Fate and absorption of cellular and soluble components in the gastrointestinal tract of the newborn. J. Immunol. **119**:245–248.
22. **Parmely, M. J., A. E. Beer, and R. E. Billingham.** 1976. *In vitro* studies on the T-lymphocyte population of human milk. J. Exp. Med. **144**:358–370.
23. **Parmely, M. J., D. B. Reath, A. E. Beer, and R. E. Billingham.** 1977. Cellular immune responses of human milk T lymphocytes to certain environmental antigens. Transplant. Proc. **9**:1477–1483.
24. **Perper, R. J., T. W. Zee, and M. W. Mickelson.** 1968. Purification of lymphocytes and platelets by gradient centrifugation. J. Lab. Clin. Med. **72**:842–848.
25. **Saxon, A., J. Feldhaus, and R. A. Robins.** 1976. Single step separation of human T and B cells using AET treated SRBC rosettes. J. Immunol. Methods **12**:285–288.
26. **Schlessinger, J. J., and H. D. Covelli.** 1977. Evidence for transmission of lymphocyte responses to tuberculin by breast feeting. Lancet **ii**:529–532.
27. **Smith, C. W., and A. S. Goldman.** 1968. The cells of human colostrum. I. *In vitro* studies of morphology and functions. Pediatr. Res. **2**:103–109.
28. **Snyderman, R., L. Meadows, and D. B. Amos.** 1977. Characterization of human chemotactic lymphokine production induced by mitogens and mixed leukocyte reactions using a new microassay. Cell. Immunol. **30**: 225–235.