IN VITRO STUDIES ON THE T-LYMPHOCYTE POPULATION OF HUMAN MILK*

By MICHAEL J. PARMELY, ALAN E. BEER, and RUPERT E. BILLINGHAM

(From the Departments of Cell Biology and Obstetrics and Gynecology, The University of Texas Health Science Center, Southwestern Medical School at Dallas, Dallas, Texas 75235)

It has been recognized for more than half a century that, in addition to occasional mammary gland alveolar or ductal epithelial cell fragments, colostrum and milk consistently contain significant concentrations of viable leukocytes. These include lipid-laden macrophages that can exhibit ameboid and phagocytic activity, polymorphonuclear neutrophils, and lymphocytes of various sizes (1, 2). The overall concentration of these leukocytes is of the same order of magnitude as that seen in peripheral blood, although the predominant cell in milk is the macrophage rather than the neutrophil.

While there has been much speculation concerning the functional significance of milk cells, only recently has suggestive evidence been forthcoming that they may constitute an important post-partum component of the maternal immunologic endowment, fulfilling a protective role within the lumen of the suckling's alimentary canal (3). The observations in rats that nursing can mediate adoptive immunization and, in certain genetic contexts, lead to graft-versus-host disease suggest that the lymphocyte moiety may actually gain access to the neonate's tissues (4).

Various investigators have established the presence of significant proportions of both T and B lymphocytes in human colostrum and milk (5) and have demonstrated their capacity to respond to mitogens in vitro (2, 5), synthesize IgA antibody (6), and display in vitro parameters of the delayed hypersensitivities of the donor (1, 2).

The principal objective of the present study was to establish the capacity of milk lymphocytes to function both as stimulator and responder cells in mixed lymphocyte cultures and to compare them with peripheral blood lymphocytes (PBL)[1] in this regard.

Materials and Methods

Milk Leukocytes. Milk samples were collected from puerperal patients at Parkland Memorial Hospital during the 1st wk after delivery and refrigerated immediately. Milk cells were obtained by twofold dilution of milk with Eagle's minimum essential medium (MEM) and centrifugation at

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[1] *Abbreviations used in this paper:* Con A, Concanavalin A; GALT, gut-associated lymphoid tissue; MEM, minimum essential medium; ML, milk lymphocytes; PBC, peripheral blood cells; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; SI, stimulation index.

400 *g* for 15 min. The sedimented cells were then resuspended and washed three times (200 *g* for 10 min) in MEM, counted, and either cultured directly or passed through glass bead columns to reduce the high proportion of macrophages by virtue of their adherent properties. Glass bead (Cataphote-Ferro, Class IV-A) columns were prepared as described by Shortman et al. (7) with one essential modification: milk cells were resuspended in MEM containing 50% human serum rather than plasma to avoid the occasionally observed clotting of plasma-containing medium by high concentrations of milk cells. Each 10-ml (bed volume) column was loaded with 15–50 × 10^6 cells and incubated for 20 min at 37°C. Nonadherent milk lymphocytes (ML) were then eluted with MEM and washed twice before culturing.

Peripheral Blood Leukocytes. Contaminating erythrocytes were removed from blood by sedimentation with 0.2% (wt/vol) methyl cellulose. Peripheral blood cells (PBC) were then isolated from the supernatant fraction by centrifugation over Hypaque-Ficoll (8), and the resulting mononuclear layer contained lymphocytes and 5–20% monocytes. In certain experiments the lymphocytes were further purified by passage through glass bead columns (7) to obtain adherent cell-depleted PBL. When ML and PBC were cultured from the same donor, they were always collected and processed on the same day.

Lymphocyte Cultures. 200-μl aliquots of 5 × 10^4 ML or PBC were cultured in triplicate in flat-bottom microtiter plates (Falcon Plastics, Oxnard, Calif.). Eagle's MEM containing 15% human plasma, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin was used as a standard culture medium. Mitogen-stimulated cultures were incubated in a humid atmosphere of 5% CO_2 in air for 64 h. During an additional 7–8-h period, 1 μCi [^{3}H]methylthymidine (sp act = 6.7 Ci/mmol, New England Nuclear, Boston, Mass.) was added to each culture to allow incorporation of the isotope into newly synthesized DNA. Antigen-stimulated and mixed lymphocyte cultures were incubated for 7 days, with [^{3}H]thymidine being present during the final 7–8 h. One-way mixed lymphocyte cultures were achieved by mitomycin-C treatment of the stimulating cells (25 μg/2 × 10^6 cells/ml at 37°C for 20 min) which were then used at a concentration of 2 × 10^5 cells per culture. One-way cultures contained 5 × 10^4 responding lymphocytes, while two-way cultures contained 5 × 10^4 lymphocytes of each responding type. Finally, the contents of each culture were recovered by a multiple sample harvester (Mash II, Microbiological Associates, Bethesda, Md.) and deposited on glass fiber filters for liquid scintillation counting. Data were expressed as mean counts per minute (cpm) ± 1 SD or as a stimulation index (SI): SI = (cpm, stimulated culture)/(cpm, control culture). An SI of 2.00 or greater indicated significant stimulation above the control culture value.

Mitogens and Antigens. Each of the following were diluted in MEM and added to cultures in 20-μl vol: phytohemagglutinin (PHA, Burroughs Wellcome & Co., Tuckahoe, N. Y., lot K9036); Concanavalin A (Con A, Sigma Chemical Co., St. Louis, Mo., lot 105C:7220); *Candida albicans* (Hollister-Stier Laboratories Inc., Spokane, Wash., lot C8149601); *Escherichia coli* K1 (kindly donated by Dr. George McCracken). The optimum dose of each stimulant was determined by titration using cells from sensitive donors.

Results

ML Responses to Mitogenic Stimulation. Human milk samples were collected between the 2nd and 7th post-partum day and were examined for their cellular composition. 30 samples contained an average of 1.3 × 10^6 nucleated cells per ml of which 11% were lymphocytes (range = 2–23%). The remaining cells consisted primarily of large, lipid-laden macrophages. Before performing mixed lymphocyte cultures we decided to examine the responses of milk cells stimulated by PHA and Con A to provide a base-line measure of their in vitro reactivity. When unfractionated milk cells were cultured directly in the presence of PHA, a modest though significant mitogenic response was often observed (Table I). Much more dramatic responses were incited, however, when the lymphocytes were first purified by a single passage through glass bead columns to reduce the proportion of macrophages. This technique gave preparations

TABLE I
The Effect of ML Purification on Responses to PHA

Experiment*	Cell purity (%)	Culture	Thymidine incorporation	
			cpm	SI‡
1	Unpurified milk cells (7% lymphocytes)	MC§	247 ± 169	
		MC + 1 μl PHA	2,953 ± 209	10.7
	Purified lymphocytes (46% lymphocytes)	ML	52 ± 43	
		ML + 1 μl PHA	5,251 ± 224	101.0
2	Unpurified milk cells (8% lymphocytes)	MC	7,917 ± 3,589	
		MC + 1 μl PHA	12,682 ± 3,870	1.6
	Purified lymphocytes (38% lymphocytes)	ML	12 ± 7	
		ML + 1 μl PHA	10,572 ± 1,323	881.0

* Experiments 1 and 2 were performed with cells from two different donors.
‡ SI = (cpm, stimulated culture)/(cpm, control culture).
§ MC, milk cells.

containing 35–80% lymphocytes although macrophages were still present. Because suboptimal and supraoptimal milk cell concentrations were found to result in depressed mitogenesis, both ML and PBC cultures contained 5×10^4 lymphocytes per microtiter well.

To assess the relative responsiveness of ML and PBC both cell types were isolated from nine donors, and each preparation was stimulated with varying doses of PHA from 0.06 to 8.0 μl per culture. All preparations gave unimodal dose-response curves over this mitogen range. Representative data presented in Table II show that the total thymidine uptake by stimulated PBC cultures was always greater than that for ML cultures. However, the degree of stimulation seen with ML (as reflected by the stimulation index, SI) was often equivalent to (Exp. 1) or greater than (Exp. 2) that of autologous PBC's. Similar results were also observed for ML and PBC cultures stimulated by Con A. Overall, the data suggested either that mitogen-reactive lymphocytes were under represented in milk compared to their concentration in peripheral blood, or that they were present in sufficient numbers but incapable of responding to the stimuli.

T-Lymphocyte Responses to Antigenic Challenge. With the aid of the one-way mixed lymphocyte culture system (9), milk cells were examined for their ability to respond to foreign histocompatibility antigens by proliferation in vitro. Table III shows the responses of purified ML to allogeneic mitomycin-treated milk cells (Exp. 1) or to peripheral blood leukocytes (PBC) (Exp. 2). Although good proliferative responses were seen, the reactivities of ML were somewhat muted when compared to those of PBC from the same donors (Exp. 3). As with PHA-stimulated cultures, this depressed response was more apparent when total thymidine incorporation by the two populations was compared.

To determine whether this relationship also existed for responses to soluble antigens, milk and blood donors were selected for their sensitivities to certain bacterial or mycotic antigens. As seen in Table IV, five of six *Candida albicans*-positive patients failed to show antigen-induced blastogenesis by their milk

TABLE II
Comparison of Mitogenic Responses of Cultured Lymphocytes from Peripheral Blood and Milk to PHA and Con A

Experiment*	Mitogen	Thymidine incorporation			
		Blood lymphocytes (PBC)		Milk lymphocytes (ML)	
		cpm	SI	cpm	SI
1	–	438 ± 303		159 ± 142	
	0.5 μl PHA	14,726 ± 3,559	33.6	5,823 ± 1,084	36.6
	1.0 μl PHA	33,835 ± 2,629	77.2	6,815 ± 1,430	42.9
2	–	203 ± 51		15 ± 12	
	0.25 μl PHA	35,706 ± 4,471	176.0	7,397 ± 2,001	493.0
	0.5 μl PHA	46,930 ± 4,659	231.0	7,839 ± 3,512	523.0
	1.0 μl PHA	53,097 ± 6,778	261.0	3,699 ± 1,702	247.0
3	–	711 ± 61		50 ± 7	
	0.25 μl PHA	32,055 ± 692	45.1	823 ± 56	16.5
	0.5 μl PHA	37,148 ± 434	52.2	1,025 ± 37	20.5
	1.0 μl PHA	49,130 ± 1,490	69.1	1,204 ± 21	24.1
4	–	1,263 ± 831		34 ± 31	
	2.5 μg Con A	9,966 ± 654	7.9	342 ± 243	10.1
	10.0 μg Con A	31,944 ± 1,500	25.3	4,312 ± 1,253	126.8
	40.0 μg Con A	77,924 ± 4,665	61.7	8,630 ± 125	253.8

* Each experiment represents the responses of PBC and ML from a single donor.

lymphocytes, while PBC from the same donors responded to the antigen. In the sixth patient, proliferation was observed with both cell populations. Thus, although in vitro proliferative responses to this soluble antigen were easily demonstrable with blood lymphocytes, they were absent, in most cases, from lymphocytes obtained from milk.

Analysis of ML Hyporesponsiveness. Several explanations might account for the observed hyporesponsiveness of milk lymphocytes, and these were tested by the following experiments.

(a) Uncharacterized cellular or acellular material endogenous to milk or evoked by cellular fractionation could have prevented in vitro blastogenesis in a manner analogous to the inhibitory influence of platelets in mixed lymphocyte cultures (10). To test this hypothesis we cocultured ML and PBC from the same donor and stimulated the mixture with PHA. In Fig. 1 it can be seen that the addition of 5×10^4 ML to various doses of PBC added to the overall responses, but did not alter the slope of the dose-response curve. This indicated that there was nothing inherent in the ML preparation that prevented lymphoproliferation in an otherwise reactive PBC population. The data also argued against the elaboration of nonspecific toxic factors by milk cells and indicated an inherent ML hyporesponsiveness at the cellular level.

(b) To ascertain whether this hyporesponsiveness was mediated by soluble suppressive factors present in the mammary exosecretion, PBC were pretreated with cell-free autologous milk "plasma" and then exposed to various stimuli in

TABLE III
Mixed Lymphocyte Culture Reactions of Peripheral Blood and Milk Lymphocytes

Experiment*	Cell type		Culture	Thymidine incorporation	
	Responder	Stimulator		cpm	SI
1	ML	MC	A + A_m‡	507 ± 79	
	ML	MC	A + B_m	8,156 ± 2,734	16.09
	ML	MC	C + C_m	48 ± 29	
	ML	MC	C + D_m	1,168 ± 1,257	24.34
2	ML	MC	A + A_m	1,094 ± 394	
	ML	PBC	A + B_m	23,528 ± 3,062	21.51
	ML	MC	C + C_m	17 ± 1	
	ML	PBC	C + D_m	11,038 ± 5,223	649.29
3	PBL	PBC	A + A_m	432 ± 148	
	PBL	PBC	A + B_m	35,663 ± 3,437	82.55
	ML	PBC	A + A_m	140 ± 204	
	ML	PBC	A + B_m	8,308 ± 6,444	59.34
	PBL	PBC	B + B_m	571 ± 191	
	PBL	PBC	B + A_m	17,757 ± 2,740	31.10
	ML	PBC	B + B_m	424 ± 225	
	ML	PBC	B + A_m	4,046 ± 1,569	9.54
4	ML	ML	A + A	419 ± 70	
	ML	ML	B + B	855 ± 364	
	ML	ML	A + B	15,529 ± 1,491	24.38
	ML	ML	C + C	148 ± 14	
	ML	ML	D + D	1,058 ± 346	
	ML	ML	C + D	14,238 ± 3,096	23.61
	ML	ML	E + E	116 ± 30	
	ML	ML	F + F	87 ± 50	
	ML	ML	E + F	6,381 ± 2,177	63.18
5	ML	ML	A + A_m	813 ± 143	
	ML	PBL	A + B_m	5,393 ± 636	6.63

* The donors in each experiment were different and are designated by capital letters.
‡ A_m designates mitomycin-treated cells from donor A.

vitro. Cell-free milk was prepared by centrifugation (450 *g* for 15 min) and prewarmed to 37°C. Autologous PBC were then added to a final concentration of 10^5 lymphocytes/ml and incubated at 37°C for 30 min. The cells were then washed three times in medium, resuspended at the appropriate concentration, and cultured under standard conditions. Whereas milk treatment of PBC caused a 30–35% reduction in their ability to respond to PHA (Table V), it did not affect specific antigen-induced proliferation. Therefore, it seemed unlikely that the hyporesponsiveness of milk lymphocytes could be entirely attributed to the

TABLE IV
Stimulation of Cultured Lymphocytes by Candida albicans

Patient*	Antigen	Thymidine incorporation			
		Blood lymphocytes (PBC)		Milk lymphocytes (ML)	
		cpm	SI	cpm	SI
M. W.	–	121 ± 71		539 ± 106	
	C. albicans‡	3,792 ± 1,241	31.34	516 ± 202	0.96
B. M.	–	243 ± 113		261 ± 332	
	C. albicans	2,028 ± 1,844	8.45	170 ± 49	0.65
K. U.	–	586 ± 330		191 ± 58	
	C. albicans	7,814 ± 5,284	13.33	244 ± 37	1.28
S. T.	–	113 ± 44		14 ± 3	
	C. albicans	483 ± 144	4.28	12 ± 1	0.88
C. J.	–	184 ± 129		36 ± 11	
	C. albicans	2,620 ± 661	14.24	61 ± 36	1.69
M. Y.	–	686 ± 129		111 ± 36	
	C. albicans	3,437 ± 1,695	5.01	3,123 ± 883	28.13

* 6/11 patients tested were sensitive to the antigen.
‡ 1 μl *C. albicans* per culture.

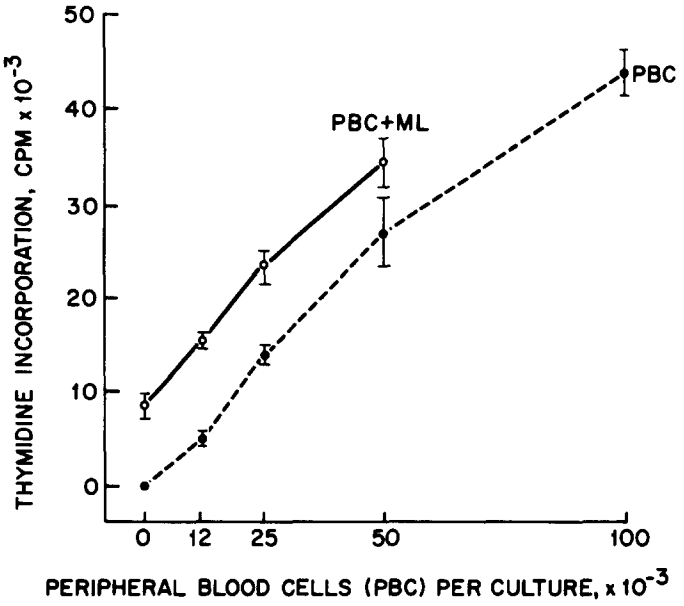

FIG. 1. The effect of coculturing ML with autologous PBC stimulated by PHA. Various numbers of PBC were either cultured alone (PBC) or with 5 × 10^4 ML from the same donor (PBC + ML). Each culture was stimulated by 1 μl PHA, and the results are plotted as mean cpm ± 1 SD.

TABLE V
The Influence of Milk Pretreatment of PBC on Their Responses to PHA and Candida albicans*

Patient	Lymphocyte pretreatment	Culture	Thymidine incorporation		Reduction in SI
			cpm	SI	
					%
A	None	A	271 ± 186		
		A + PHA	25,543 ± 2,876	94.25	
	Milk	A	273 ± 26		
		A + PHA	17,995 ± 2,315	65.91	30.0
B	None	B	763 ± 367		
		B + *C. albicans*	3,358 ± 1,659	4.40	
	Milk	B	871 ± 576		
		B + *C. albicans*	3,728 ± 2,432	4.28	2.7
C	None	C	272 ± 57		
		C + PHA	9,398 ± 1,305	34.55	
		C + *C. albicans*	3,358 ± 1,817	12.35	
	Milk	C	290 ± 94		
		C + PHA	6,509 ± 624	22.44	35.0
		C + *C. albicans*	3,728 ± 2,664	12.86	−4.1

* For treatment with cell-free milk, see text.

action of soluble milk factors, particularly with reference to antigen stimulation.

(c) The presence of a functional population of adherent cells has been shown to be essential to in vitro blastogenic responses to soluble or cell-bound antigens (11, 12). Therefore it was conceivable, though unlikely, that reduced ML responses may have resulted from a deficiency of adherent cells in the final preparation. Since depletion of adherent cells can abrogate mixed lymphocyte culture proliferation (12), two-way mixed lymphocyte culture reactivity between allogeneic ML preparations would indicate the presence of contaminating macrophages. This was in fact the case (Table III, Exp. 4); column-purified ML responded to similarly prepared allogeneic ML. Moreover, ML also responded to allogeneic adherent cell-depleted PBL in a one-way culture (Table III, Exp. 5), further indicating the presence of functional macrophages in the ML preparation.

This hypothesis was also tested with a soluble antigen. If reduced ML responses were due to the absence of adherent cells, one might predict the reconstitution of these reponses by a monocyte-rich source. Therefore, either glass bead column-purified ML or PBL were added to cultures of autologous PBC, as a source of adherent cells, and stimulated by *C. albicans* (Fig. 2). The addition of 5×10^4 purified PBL increased the PBC response in a manner consistent with the addition of more antigen-reactive lymphocytes. These additional PBL were evidently capable of collaborating with the existing adherent cells from the PBC preparation. By contrast, ML from the same antigen-

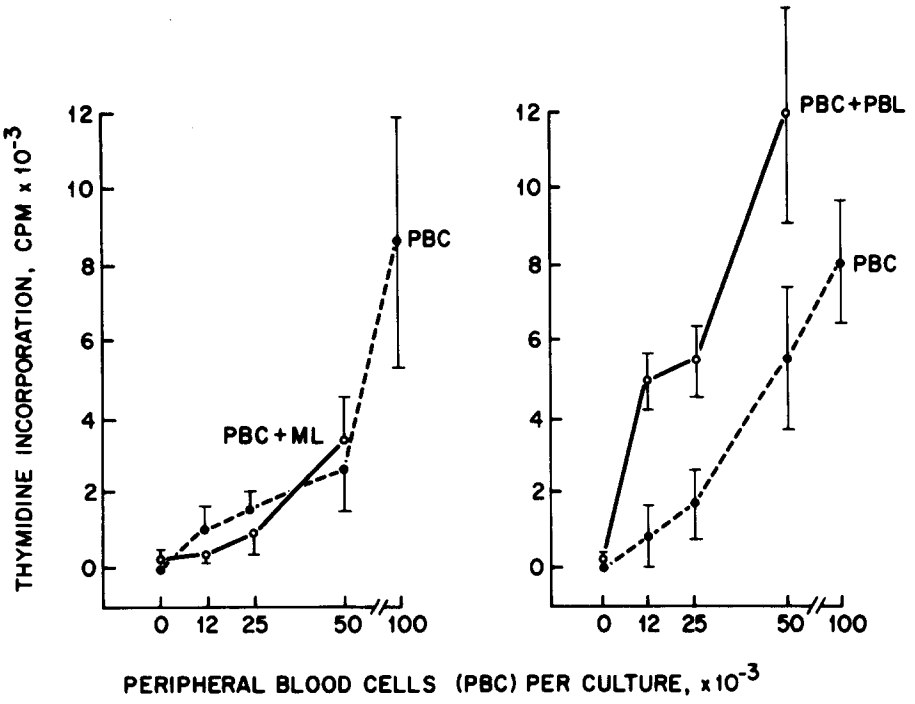

FIG. 2. The effect of coculturing PBL or ML with adherent cell-rich PBC stimulated by *C. albicans*. Various numbers of PBC were either cultured alone (PBC) or with 5×10^4 ML or PBL from the same donor (PBC + PBL; PBC + ML). Each culture was then stimulated with 2 μl *C. albicans* antigen, and the results are plotted as mean cpm ± 1 SD.

sensitive donor did not augment the PBC response even though sufficient adherent cell function was present. This afforded further evidence that the ML population lacked *C. albicans*-reactive lymphocytes. An additional conclusion was indicated by the ML data. Because the response to antigen by PBC was not reduced by the addition of 5×10^4 ML, it seemed unlikely that either specific or nonspecific suppressor cells were present in the ML preparation.

Specific ML Responses to an Enteric Antigen. From these findings it seemed reasonable to conclude that although lymphocytes in human milk can undergo proliferation in response to certain antigens (e.g., foreign histocompatibility antigens), they are devoid of proliferative reactivity to others (e.g., *C. albicans*). Furthermore, the "defect" appears to reside at the cellular level and may be due to the absence from the population of specific antigen-reactive lymphocyte clones. Such cellular compartmentalization finds a precedent in mammary secretory immunity, in that specific clones of IgA-secreting plasma cells often populate secretory organs like the breast while ignoring peripheral lymphoid tissues such as the spleen (13, 14). The demonstration that this local immune system may derive its initial sensitization from the mucosal layer of the gut (13–15) and involves T as well as B lymphocytes (16, 17) led us to investigate the responses of human ML to an antigen from an enteric microorganism. Sensitivity to the K1 antigen of *E. coli* is common in post-partum women (44% carry the

TABLE VI
Stimulation of Cultured Lymphocytes by E. coli K1

Patient*	Antigen	Thymidine incorporation			
		Blood lymphocytes (PBC)		Milk lymphocytes (ML)	
		cpm	SI	cpm	SI
M. A.	–	1,890 ± 304		67 ± 27	
	E. coli K1‡	1,905 ± 692	1.01	501 ± 124	7.48
C. T.	–	492 ± 427		1,601 ± 188	
	E. coli K1	345 ± 146	0.70	15,147 ± 174	9.46
C. J.	–	184 ± 129		36 ± 11	
	E. coli K1	232 ± 65	1.26	2,818 ± 4,410	78.28
K. U.	–	586 ± 330		191 ± 58	
	E. coli K1	73 ± 5	0.12	35,410 ± 4,306	185.39

* 4/9 patients tested were sensitive to the antigen.
‡ 2.0 μl K1 per culture.

organism), manifesting itself primarily as high-titered colostral and copro antibody with an absence of specific antibody in the serum (McCracken, G. H. Personal communication). To determine whether this kind of compartmentalization exists for T-lymphocyte function, we examined the in vitro responses of PBC and ML from post-partum patients to the K1 antigen (Table VI). In the four sensitive patients tested, no responses were observed in peripheral blood cultures, whereas their ML showed dramatic antigen-induced proliferation after 7 days in vitro. In five additional patients tested neither blood nor ML responded.

Discussion

Besides confirming previous reports that human ML, like those of peripheral blood, proliferate in vitro in the presence of mitogens (2, 5), we have shown that these cells can respond to foreign transplantation antigens on allogeneic blood or milk leukocytes and are capable of serving as stimulating cells in mixed lymphocyte cultures. While ML were as viable both at the onset and conclusion of culture as blood lymphocytes from the same donors, these two cell populations were found to differ in several important ways: (*a*) Lymphocytes from milk were hyporesponsive to PHA and Con A, as revealed by the finding that thymidine incorporation by milk lymphocyte cultures was always less than that of cultures of autologous blood lymphocytes. (*b*) ML responded to histocompatibility antigens present either on allogeneic blood leukocytes or milk cells, but again their responses were significantly inferior to those of blood lymphocytes from the same donors. (*c*) In nearly all cases, lymphocytes obtained from the milk of *C. albicans*-sensitive donors showed no lymphoproliferation when cultured with the antigen, while blood lymphocytes from the same donors gave significant responses. (*d*) The reverse relationship applied to responses to the K1 antigen of

E. coli; ML from four of nine patients responded to the antigen, but in no case was proliferation observed in blood lymphocytes from these patients.

Although Diaz-Jouanen and Williams (5) have suggested that human cell-free milk, when present in relatively large amounts throughout the entire culture period, will inhibit PHA-induced proliferation, our data indicate that this effect is not evident if the lymphocytes are first washed free of soluble milk components.

Conceivably, mitogen and antigen-reactive lymphocyte clones may have been inadvertently removed during fractionation that involved passage of the cells through glass bead columns. However, two findings make this interpretation implausible. ML from patient M. Y. (Table IV) were prepared in this manner and responded just as well as her blood lymphocytes to antigenic challenge by *C. albicans*. Furthermore, ML isolated on another occasion by centrifugation over a bovine serum albumin gradient (18) were as hyporesponsive to PHA as ML prepared by glass bead columns (data not shown).

Additional experiments indicated that ML hyporesponsiveness cannot be attributed to suppressor cells or to a paucity of nonspecific auxiliary cells, such as macrophages. Since antigen-induced proliferation by human lymphocytes in vitro has been shown to measure thymus-derived (T) lymphocyte function (19, 20), the most likely interpretation is that ML hyporeactivity depends on the paucity of certain subpopulations of T lymphocytes in the milk which are abundant in blood. This is best exemplified by the experiments with *C. albicans* in which lymphocytes obtained from milk lack the responses given by blood lymphocytes. Similarly, differences in the responses of the two populations to the K1 antigen suggest that human mammary exosecretion contains a selected population of immunocompetent T lymphocytes. This is to say, the breast may be a source of local T-lymphocyte immunity.

Local immunity, as described for antibody production, depends in part upon induction by a particular route followed by the compartmentalization of B lymphocytes and their progeny. The intestinal mucosa provides a well-studied example. Here, sensitization by intraluminal antigens leads to the local accumulation of specific B-lymphocyte clones within the lamina propria and a marked deficiency of the same cells in peripheral lymphoid tissue (14, 16, 21). Functionally this results in high IgA antibody concentrations along the intestinal tract but little or no antibody in the blood. Evidence also exists that immunization across the gut mucosa is an important factor in the induction of colostral humoral immunity (13, 15), and that this may be accomplished by the population of mammary tissue with B lymphocytes derived from the gut-associated lymphoid tissue (GALT) (6). Oral immunization can also lead to T-cell immunity in GALT (e.g., Peyer's patches) as exemplified by the induction of helper T cells for antibody production (22). Thus, in mammary tissue lymphocyte clones of GALT origin may accumulate in such a way that the cells in its secretion express a restricted repertoire of "gut-derived" responses (e.g., to *E. coli* K1) and lack some responses found in blood lymphocyte populations (e.g., to *C. albicans*). By contrast, peripheral lymphocytes would be deficient in responses originating in the gut and demonstrable in milk.

While lymphocytes in milk appear not to represent the total immunological experience of the mother, they may contain reactive clones beneficial for the

suckling. For example, *E. coli* of the K1 serotype is the predominant etiological agent in neonatal meningitis, accounting for 84% of the *E. coli* meningitis cases in one study (23). These organisms, which have been shown to colonize the intestinal tract of approximately 44% of post-partum women (24), invariably lead to the appearance of high-titered antibody of the IgA class in their colostrum (McCracken, G. H. Personal communication). Thus, intestinal colonization of the mother by an organism that is pathogenic for the newborn leads to the accumulation of anti-K1 lymphocytes and their products in her mammary tissue, which in turn makes them available for the suckling. In this example the direct role played by T lymphocytes is unclear, and protection may be due primarily to the products of B-lymphocyte clones, i.e., colostral antibody (24). However, Mohr (25) has reported that tuberculin-sensitive mothers can adoptively immunize their infants through breast feeding, and that the immunity may be long lasting. Similarly, in laboratory rats, T-lymphocyte reactivity to skin allografts can be transferred by nursing naive neonates on allogeneic "donors" (4). In both cases neonatal responses suggest the direct transmission of viable T lymphocytes from mother to suckling via milk and their incorporation in the recipient's tissues. We are currently extending the present model to investigate these examples.

Summary

Human milk lymphocytes (ML) can be partially purified and propagated in vitro as a means of assessing their immunological function. When exposed to a variety of stimuli known to activate T lymphocytes, ML respond in a unique manner that indicates a selected population of immunocompetent cells. ML are hyporesponsive to nonspecific mitogens and respond in a reduced manner to histocompatibility antigens on allogeneic cells. In most cases, they are completely unresponsive to *C. albicans* although blood lymphocytes from the same patients respond to the antigen. The K1 capsular antigen of *E. coli* induces significant proliferation in lymphocytes obtained from milk, but fails to stimulate blood lymphocytes. This dichotomy of reactivity does not appear to result from suppressive factors or cells in milk or insufficient adherent cell function. Rather it appears to reflect the accumulation of particular lymphocyte clones in the breast and the local nature of mammary tissue immunity at the T-lymphocyte level.

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References

1. Mohr, J. A., R. Leu, and W. Mabry. 1970. Colostral leukocytes. *J. Surg. Oncol.* 2:163.
2. Smith, C. W., and A. S. Goldman. 1968. The cells of human colostrum. I. In vitro studies of morphology and function. *Pediatr. Res.* 2:103.
3. Barlow, B., T. V. Santulli, W. C. Heird, J. Pitt, W. A. Blanc, and J. W. Schullinger.

1974. An experimental study of acute neonatal enterocolitis – the importance of breast milk. *J. Pediatr. Surg.* 9:587.

4. Beer, A. E., R. E. Billingham, and J. R. Head. 1975. Natural transplantation of leukocytes during suckling. *Transplant. Proc.* 7:399.
5. Diaz-Jouanen, E. P., and R. C. Williams. 1974. T and B lymphocytes in human colostrum. *Clin. Immunol. Immunopathol.* 3:248.
6. Goldblum, R. M., S. Attlstedt, B. Carlsson, L. A. Hanson, U. Jodal, G. Lindin-Janson, and A. Sohl-Akerlund. 1975. Antibody-forming cells in human colostrum after oral immunization. *Nature (Lond.).* 257:797.
7. Shortman, K., N. Williams, H. Jackson, P. Russell, P. Burt, and E. Diener. 1971. The separation of different cell classes from lymphoid organs. IV. The separation of lymphocytes from phagocytes on glass bead columns, and its effect on subpopulations of lymphocytes and antibody-forming cells. *J. Cell Biol.* 48:566.
8. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* 21:1.
9. Bach, F. H., and N. K. Voynow. 1966. One-way stimulation in mixed leukocyte cultures. *Science (Wash. D. C.).* 153:545.
10. Yachnin, S. 1972. The potentiation and inhibition by autologous red cells and platelets of human lymphocyte transformation induced by pokeweed mitogen, concanavalin A, mercuric chloride, antigen, and mixed leukocyte culture. *Clin. Exp. Immunol.* 11:109.
11. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. *J. Immunol.* 101:262.
12. Alter, B. J., and F. H. Bach. 1970. Lymphocyte reactivity in vitro. I. Cellular reconstitution of purified lymphocyte response. *Cell. Immunol.* 1:207.
13. Bohl, R. H., R. K. P. Gupta, M. V. Fernando Oloquin, and L. J. Saif. 1972. Antibody responses in serum, colostrum and milk of swine after infection or vaccination with transmissible gastroenteritis virus. *Infect. Immun.* 6:289.
14. Heremans, J. F., and H. Bazin. 1971. Antibodies induced by local antigenic stimulation of mucosal surfaces. *Ann. N. Y. Acad. Sci.* 190:268.
15. Montgomery, P. C., J. Cohn, and E. T. Lolly. 1974. The induction and characterization of secretory IgA antibodies. *Adv. Exp. Med. Biol.* 45:453.
16. Pierce, N. F., and J. L. Gowans. 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. *J. Exp. Med.* 142:1550.
17. Guy-Grand, D., C. Griscelli, and P. Vasalli. 1974. The gut-associated lymphoid system: nature and properties of large dividing cells. *Eur. J. Immunol.* 4:435.
18. Raidt, D. J., R. I. Mishell, and R. W. Dutton. 1968. Cellular events in the immune response. Analysis and in vitro response of mouse spleen cell populations separated by flotation in albumin gradients. *J. Exp. Med.* 128:681.
19. Geha, R. A., E. Scheenberger, F. S. Rosen, and E. Merler. 1973. Interaction of human thymus-derived and nonthymus-derived lymphocytes in vitro. *J. Exp. Med.* 138:1230.
20. Chess, L., R. P. MacDermott, and S. F. Schlossman. 1974. Immunologic functions of isolated human lymphocyte subpopulations. II. Antigen triggering of T and B cells in vitro. *J. Immunol.* 113:1122.
21. Crabbe, P. A., D. R. Nash, H. Bazin, H. Eyssen, and J. F. Heremans. 1969. Antibodies of the IgA type in intestinal plasma cells of germ-free mice after oral or parenteral immunization with ferritin. *J. Exp. Med.* 130:723.
22. Kagnoff, M. F. 1975. Functional characteristics of Peyer's patch cells. III. Carrier priming of T cells by antigen feeding. *J. Exp. Med.* 142:1425.

23. Robbins, J. B., G. H. McCracken, E. C. Gotschlich, F. Ørskov, I. Ørskov, and L. A. Hanson. 1974. *Escherichia coli* K1 capsular polysaccharide associated with neonatal meningitis. *N. Engl. J. Med.* 290:1216.
24. Sarff, L. D., G. H. McCracken, M. S. Schiffer, M. P. Glode, J. R. Robbins, I. Ørskov, and F. Ørskov. 1975. Epidemiology of *Escherichia coli* K1 in healthy and diseased newborns. *Lancet.* 1:1099.
25. Mohr, J. A. 1973. The possible induction and/or acquisition of cellular hypersensitivity associated with ingestion of colostrum. *J. Pediatr.* 82:1062.