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Activated and Memory T Lymphocytes in Human Milk[1,3]

Daniel P. Wirt,[2] Linda T. Adkins, Kimberly H. Palkowetz, Frank C. Schmalstieg, and Armond S. Goldman

The Departments of Pathology (D.P.W., L.T.A.), Pediatrics (K.H.P., F.C.S., A.S.G.), Microbiology (F.C.S., A.S.G.), and Human Biological Chemistry and Genetics (F.C.S., A.S.G.), University of Texas Medical Branch, Galveston, Texas, and the Department of Pathology (D.P.W.), The Children's Memorial Hospital, Chicago, Illinois

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Since activated macrophages and cytokines are found in human milk (HM), a flow cytometry study was conducted to determine whether T cells in HM display phenotypic markers of recent or previous activation. HM was collected during the first 3 d of lactation. The Paint-a-Gate program was used to optimize gating on the lymphocyte population. A mean ± 1 SD of 4 ± 3% of total HM leukocytes were lymphocytes and 96 ± 3% were macrophages and granulocytes (N = 33 subjects). HM lymphocyte populations were further analyzed in five subjects. T cells ($CD3^+$) represented 83 ± 11% and B cells ($CD19^+$) were 6 ± 4% of HM lymphocytes. The mean CD4/CD8 ratio of T cells in HM was 0.88 (range 0.40–1.25). This ratio was significantly decreased compared to the peripheral blood (PB) of control adults ($P < 0.02$) and postpartum women ($P < 0.02$), due mostly to a significant increase in $CD8^+$ $CD3^+$ cells in HM compared to the PB of control adults ($P < 0.002$) and postpartum women ($P < 0.05$). T cells bearing markers of recent activation were significantly increased in HM compared to the PB of control adults: 85 ± 7% of $CD3^+$ cells in HM were $HLA\text{-}DR^+$ (controls, 10 ± 4%; $P < 0.001$), and 15 ± 6% of $CD3^+$ cells in HM were $IL\text{-}2R^+$ (controls, 6 ± 2%; $P < 0.001$). Subpopulations of $CD4^+$ and $CD8^+$ cells in HM defined by the T200 isoforms CD45RA and CD45RO were markedly altered compared to PB, indicating a striking shift from virginal to antigen-primed (memory) T cells in HM and suggesting certain functional capacities for these cells. Virtually all $CD4^+$ cells (99.8 ± 0.4%) and 92 ± 5% of $CD8^+$ cells in HM were $CD45RO^+$ (vs. 71 ± 12% and 50 ± 10%, respectively, in the PB of postpartum women; $P < 0.001$). $CD4^+$ and $CD8^+$ cells expressing CD45RA were correspondingly markedly reduced ($P < 0.001$). This phenotypic pattern of T cells in HM may result from T cell activating substances in HM and/or selective homing of T cells to the breast. Conversely, activated T cells in HM may be responsible for cytokines in HM. These activated and memory T cell populations may be transferred to the infant via breastfeeding.

Key terms: Human milk immunology, T lymphocyte subsets, flow cytometry, markers of activation, leukocyte common antigen isoforms

It has been known for some years that leukocytes are part of the immunologic system in human milk (HM) (42,12). About 3 million leukocytes are found in each milliliter of HM produced during the first few days postpartum, and the principal types of leukocytes found in HM during that period are neutrophils (40–60%), macrophages (30–50%), and lymphocytes (5–9%). T cells comprise 60–73% of HM lymphocytes based on the capacity to form rosettes with sheep erythrocytes [12,34], and the thermostability of these rosettes suggests that HM T cells are activated [34]. The

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[2]Address reprint requests to Dr. Daniel P. Wirt, Department of Pathology, Box 17, The Children's Memorial Hospital, 2300 Children's Plaza, Chicago, IL 60614.

[3]Abbreviations: CD, cluster of differentiation; FITC, fluorescein isothiocyanate; FL, fluorescence; HBSS, Hanks' balanced salt solution; HM, human milk; IL-2R, interleukin-2 receptor; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; TCR, T cell receptor; TNF-α, tumor necrosis factor-α.

morphology and buoyancy of the neutrophils and macrophages are unusual because they are lipid-laden. In addition, the macrophages in HM have certain morphologic features suggesting activation [43,44]. Recent studies indicated that certain functions of HM neutrophils and macrophages are altered compared to their counterparts in human peripheral blood (PB). The adherence, orientation, and directed movement of HM neutrophils and macrophages were significantly less than those of PB neutrophils and monocytes after stimulation with chemoattractants such as N-formylmethionyl peptides and C5a [48]. In contrast, in vitro experiments utilizing a type 1 collagen gel assay demonstrated that the spontaneous motility of HM macrophages was much greater than that of PB mononuclear cells [33].

These types of observations have led to a search for immunomodulating agents in HM. Interleukin-1-like activity was found in HM [45], and more recently substantial concentrations of interleukin-1β were detected in early HM by a competitive radioimmunoassay [30]. A proline-rich protein from sheep colostrum induced and supported resting mouse B cells into and through the cell cycle, similar to the effects of lipopolysaccharide [21]. HM was found to augment the proliferation of and synthesis of immunoglobulin by lipopolysaccharide-stimulated murine B lymphocytes [22]. Proteins from human colostrum had a biphasic effect on T cell growth, inhibiting at high concentrations and enhancing at low concentrations [29]. In addition, an immunosuppressive factor functionally similar to transforming growth factors beta was reported to be present in bovine milk [46].

Recently we reported that chemokinetic agents for monocytes are present in HM and that the increased motility of HM macrophages may be due to these agents [31]. These factors proved to be proteins whose approximate *Mr* as determined by gel filtration were 50–60, 25, and 10–17 kDa [31]. The chemokinetic activities in HM were partially blocked by polyclonal antibodies to human recombinant tumor necrosis factor-α (TNF-α) [31]. Since then, we have found immunochemical evidence for TNF-α in HM [36].

We hypothesized that T cells in HM are activated because of the evidence of activated macrophages, immunostimulating substances, and TNF-α in HM, and because of the reported ability of HM T cells to form thermostable rosettes with sheep erythrocytes. To test that hypothesis, we used flow cytometry to determine whether T cells from HM display phenotypes associated with activated or memory T cells. The surface proteins detected were products of class II MHC genes (HLA-DR) [51], interleukin-2 receptor (IL-2R) [6,27,41]; CD45RO (UCHL1), a 180 kDa isoform of the common leukocyte antigen; and CD45RA (Leu-18), a 220/205 kDa isoform of the common leukocyte antigen [1,5,10,11,13,28,39,47,50]. Expression of HLA-DR or IL-2R by T cells is associated with recent activation of these cells. CD45RO is expressed principally on the surface membranes of memory T cells [1,5,10,28,39]. In addition, the isoforms of common leukocyte antigen subdivide $CD4^+$ cells into helper and suppressor-inducer subsets [5,11,39] and subdivide $CD8^+$ cells into cytotoxic and non-cytotoxic subsets [39,50].

MATERIALS AND METHODS

Research Assurances-Donor Selection

The use of these subjects for research was approved by the institutional review board. Informed consent was obtained from each subject before their participation in the study. Donors of HM were healthy women, age 19–35 years, who were 1–3 days postpartum and who were breastfeeding their offspring. HM was collected using a low pressure, electric pump (Egnell, Cary, IL) and placed in polypropylene tubes. Venous blood anticoagulated with EDTA was collected from other (unpaired) women in the first day after delivery ("peripheral blood, postpartum women") or from healthy, non-pregnant, non-lactating adults ("peripheral blood, control adults").

Preparation of Leukocytes

HM samples (33 subjects) were centrifuged at 1,000 × g to separate the cells from lipids, micelles, fat globules, and membranes. The HM cells were then washed in Hanks' balanced salt solution (HBSS) and resuspended to a final concentration of $3–36 \times 10^6$/ml (usually 10×10^6/ml). Fractionation of HM leukocytes by density gradient techniques was not attempted because of the altered buoyant density of the cells. Mononuclear leukocytes were isolated from venous blood by Ficoll-Hypaque density gradient centrifugation [7]. The peripheral blood mononuclear cells (PBMC) were then resuspended in HBSS to achieve a final concentration of $5–10 \times 10^6$ cells/ml. The viability of the PBMC was 95–99% by trypan blue dye exclusion. The viability of HM leukocytes isolated in the manner described above was 90–95% by trypan blue dye exclusion.

Flow Cytometry

The surface phenotypes of unfractionated HM leukocytes and PBMC were examined by multi-color flow cytometry [9,18] with a flow cytometer equipped with a 15mW argon-ion laser tuned to 488 nm (Becton-Dickinson FACScan, Mountain View, CA) and murine monoclonal antibodies (Becton-Dickinson, unless otherwise specified) to human leukocyte cluster of differentiation (CD) antigens. For staining, the HM or PB leukocytes were adjusted to $2–5 \times 10^5$ (usually 5×10^5) in 50 μl HBSS. The antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotin. Unconjugated antibodies were detected with FITC-conjugated goat antibodies [$F(ab')_2$ fragments] to murine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or PE-conjugated rat monoclonal antibody to murine IgM kappa light chains (Becton-Dickinson). Biotinylated antibodies were followed by a PE-

Texas Red conjugate bound to avidin (Duochrome, Becton-Dickinson).

The specificities of the monoclonal antibodies were as follows: CD3 (Leu-4), CD4 (Leu-3a + 3b or Leu-3a), CD8 (Leu-2a), CD16 (Leu-11c), CD19 (Leu-12), CD14 (Leu-M3), CD45 (HLe-1), CD45RA (Leu-18), CD45RO (UCHL1, DAKO, Carpinteria, CA), HLA-DR, CD25 (α-chains of the IL-2R), Leu-8 (SK-11), and T cell receptors (TCR) of the α/β (WT31) and γ/δ (TCRδ1, T Cell Sciences, Cambridge, MA) types.

In analysis of single-color and two-color flow cytometric data, an electronic gate was set on the lymphocyte population based on the forward-angle vs. right-angle light scatter histogram. Quadrant markers in fluorescence histograms were set using matched isotype controls. The Paint-a-Gate program (Becton-Dickinson) was used to optimize gating on lymphocytes, providing an objective means of excluding debris (noncellular events due to particulate matter) and other cells from the lymphocyte gate (Fig. 1). In HM some debris was unavoidably incorporated into the lymphocyte gate (Fig. 1). The percentage of events in the lymphocyte gate due to debris was estimated as the percentage of events negative for CD45 in the corresponding fluorescence histogram of CD45 vs. CD 14 (Fig. 1). The nonfluorescent character of the debris was confirmed in the isotype control histogram, and subsequent data utilizing other fluorescent markers was normalized to exclude these events (Fig. 1). When three-color flow cytometry was performed on PBMC [9], additional monoclonal antibodies conjugated to biotin were used. The three-color data were analyzed using LYSYS software (Becton-Dickinson): (1) lymphocytes were gated based on forward-angle vs. right-angle scatter, (2) a contour graph of FITC-conjugated CD45RA (Leu-18) vs. PE-labeled CD45RO (UCHL1, indirectly detected with PE-conjugated rat antibody to murine IgM kappa light chains) was generated for the total lymphocyte population, and quadrant markers were set so that the percentage of positive cells for each antibody was equivalent to the percentage of positive cells obtained by single-color analysis using each antibody, and (3) a gate was set on Duochrome labeled cells ($CD4^+$ or $CD8^+$) based on the percentage of positive cells obtained by single-color analysis with the same antibody. Using the same quadrant markers determined in step 2, a histogram of FITC-conjugated CD45RA (Leu-18) vs. PE-labeled CD45RO (UCHL1) was generated for the cells in the Duochrome positive gate.

Becton-Dickinson CaliBRITE beads were run prior to analysis to monitor instrument performance and to set detector levels for the forward and right-angle light scatter and the fluorescence 1 (FL1) and fluorescence 2 (FL2) channels. For two and three-color analyses, compensation settings (FL1–%FL2 = 0.7 ± 0.1, FL2–%FL1 = 26.4 ± 1.9, FL2–%FL3 = 26.0 ± 4.3, FL3–%FL2 = 51.1 ± 9.8) and the fluorescence 3 (FL3) detector (620 ± 17 volts) were optimized for each subject. The compensation settings represented the percentage of the FL(x) signal amplitude subtracted from the FL(y) signal amplitude to compensate for the spectral overlap of fluorescent dyes (FL1 = fluorescein, FL2 = phycoerythrin, FL3 = phycoerythrin-Texas red conjugate).

Data Presentation-Statistical Analysis

The data are presented as the mean ± S.D. The two-tailed Student's t test (unpaired) was used to compare the means of the population samples. Means were considered to be significantly different when the p value was < 0.05.

RESULTS

Lymphoid and Non-lymphoid Populations in HM

Lymphocytes were found to comprise 4 ± 3% (mean ± 1 S.D.) of the total leukocytes in HM, and granulocytes and macrophages represented 96 ± 3% (N = 33 subjects). The lymphocyte subpopulations were further analyzed in 5 subjects (except as noted below). The percentage of T cells ($CD3^+$) in HM lymphocytes (Table 1, Fig. 2) was significantly greater than that in the PB of control adults ($P < 0.05$), but was not significantly different from the percentage in the PB of postpartum women ($P > 0.2$). In addition, in 2 subjects the type of TCR expressed by HM T cells was determined: 77 ± 16% of HM lymphocytes expressed TCR α/β and 6 ± 6% expressed TCR γ/δ. The percentage of B cells ($CD19^+$) in HM lymphocytes (Table 1, Fig. 2) was significantly less than that in both the PB of control adults ($P < 0.01$) and the PB of postpartum women ($P < 0.05$). A small minority population of $CD16^+$ natural killer cells could be identified in most samples, but could not be accurately quantitated (data not shown). In HM the ratio of the helper-inducer ($CD4^+$ $CD3^+$) and suppressor-cytotoxic ($CD8^+$ $CD3^+$) T cell subsets was found to be significantly decreased compared to the ratio in the PB of control adults and postpartum women (Table 1, Fig. 2). In HM the $CD4^+$ $CD3^+$/$CD8^+$ $CD3^+$ ratio was 0.88 ± 0.35 (range 0.40–1.25), compared to 2.06 ± 0.83 in the PB of control adults ($P < 0.02$) and 1.70 ± 0.45 in the PB of postpartum women ($P < 0.02$). The decreased ratio was mostly due to a significant increase in the percentage of $CD8^+$ $CD3^+$ cells in HM lymphocytes compared to the PB of control adults ($P < 0.002$) and postpartum women ($P < 0.05$). The percentage of $CD4^+CD3^+$ cells in HM lymphocytes was not significantly different than that in the PB of control adults ($P > 0.05$) or postpartum women ($P > 0.2$). No significant difference was found between the PB of control adults and postpartum women in the $CD3^+$, $CD4^+CD3^\bullet$, $CD8^+CD3^+$, or $CD19^+$ lymphocyte populations or in the $CD4^+CD3^+$/$CD8^+CD3^+$ ratio ($P > 0.2$–0.5).

Phenotypic Evidence for Activated and Memory T Cells in Human Milk

In HM the percentage of $CD3^+$ T cells expressing HLA-DR and CD25 (IL-2R) was significantly increased

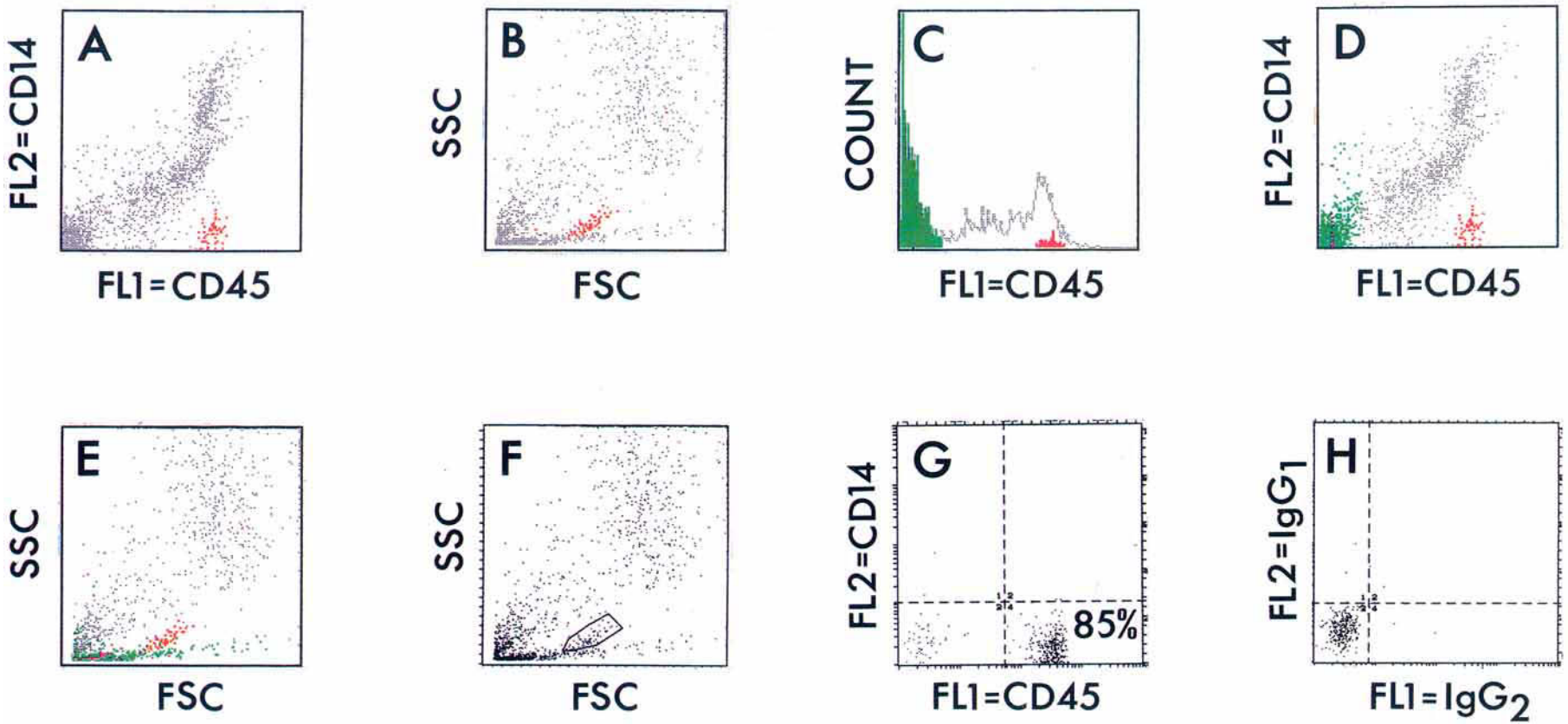

FIG. 1. Setting an electronic gate on the lymphocyte population in HM. In **A,** the lymphocyte population was identified by its characteristic staining pattern, strongly positive for CD45 (HLe-1) and negative for CD14 (Leu-M3), and these events (dots) were colored red using the Paint-a-Gate program (FL1 = fluorescence channel 1, FL2 = fluorescence channel 2). The lymphocyte population could then be positively identified in the Forward Scatter (FSC) vs. Side Scatter (SSC) histogram (**B**) as a cluster of red dots. In **C** and **D,** events negative for CD45 are shaded green, and in **E** these events are seen to have the characteristic light scatter properties of debris (non-cellular particulate matter). The uncolored dots in D and E represent granulocytes and monocytes/macrophages. Note that some overlap of debris and lymphocytes occurs in E, which was used as a guide to draw the boundaries of the gate in the FSC vs. SSC histogram (**F**) used for subsequent analysis of fluorescent markers. Debris incorporated in the gate appears in **G** as events negative for CD45 (15%). In the isotype control histogram (**H**) the nonfluorescent character of this debris is confirmed. In subsequent histograms utilizing fluorescent markers, the percentage of events attributable to debris was subtracted from the third quadrant and the events in each quadrant normalized to a sum of 100%.

TABLE 1
Lymphocyte Subpopulations in Human Milk and Peripheral Blood Determined by Flow Cytometry[a]

Lymphocyte subpopulations[b]	Human milk[c]	Peripheral blood (control adults)[d]	Peripheral blood (postpartum)[e]
Percent of Total Lymphocytes			
$CD3^+$	83 ± 11	74 ± 5	75 ± 7
$CD4^+CD3^+$	36 ± 13	46 ± 8	44 ± 6
$CD8^+CD3^+$	43 ± 12	24 ± 6	27 ± 4
$[CD4^+CD3^+/CD8^+CD3^+]$[f]	[0.88 ± 0.35]	[2.06 ± 0.83]	[1.70 ± 0.45]
$CD19^+$	6 ± 4	12 ± 3	14 ± 5
Expressed as: Percent of $CD3^+$ Lymphocytes			
$HLA\text{-}DR^+$	85 ± 7	10 ± 4	ND
$CD25^+$ ($IL\text{-}2R^+$)	15 ± 6	6 ± 2	ND
Expressed as: Percent of $CD4^+$ Lymphocytes			
$CD45RA^+$	3 ± 3	50 ± 14 [N = 10]	
		(57 ± 7[g]) [N = 13]	62 ± 10[g]
$CD45RO^+$	99.8 ± 0.4	75 ± 7 [N = 7]	
		(82 ± 10[g])	71 ± 12[g]
Expressed as: Percent of $CD8^+$ Lymphocytes			
$CD45RA^+$	24 ± 10	82 ± 3 [N = 8]	80 ± 8
		(75 ± 12[g]) [N = 13]	
$CD45RO^+$	92 ± 5	58 ± 14[g]	50 ± 10[g]

[a]Expressed as mean percent ± 1 S.D. (except for [f]).
[b]Expressed as percentage of the underlined population in the subheadings.
[c]N = 5.
[d]N = 12, except as noted.
[e]N = 6.
[f]Ratio of $CD4^+$ $CD3^+$ to $CD8^+$ $CD3^+$ lymphocytes.
[g]Three color methodology; two color methodology, all others.
ND = not done.

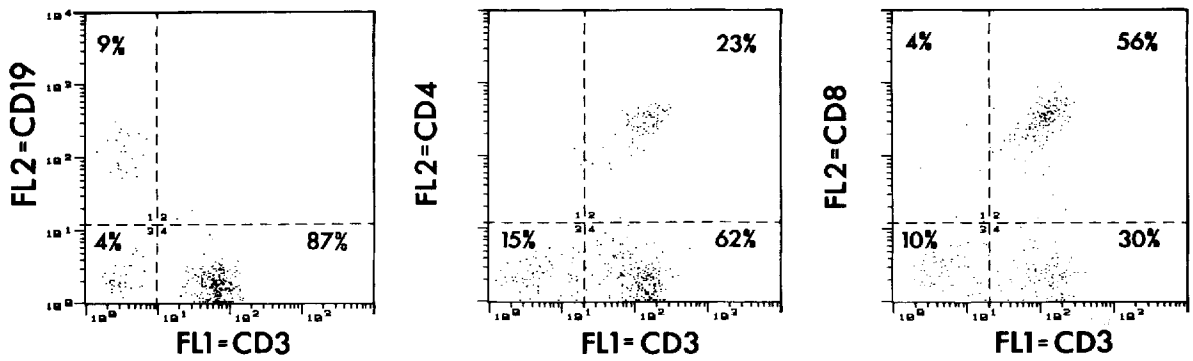

FIG. 2. Two color flow cytometry histograms demonstrating B cells ($CD19^+$, 9%), total T cells ($CD3^+$, 87%), helper/inducer T cells ($CD4^+$ $CD3^+$, 23%), and suppressor/cytotoxic T cells ($CD8^+$ $CD3^+$, 56%) in a sample of HM. The mean percentage of $CD8^+$ $CD3^+$ cells in HM was signficantly greater than in the PB of control adults ($P < 0.002$) or postpartum women ($P < 0.05$).

compared to the PB of control adults (Table 1, Fig. 3; $P < 0.001$). Significant changes in the expression of the T200 isoforms CD45RA and CD45RO were also found in the $CD4^+$ and $CD8^+$ T cell subpopulations. The percentages of $CD4^+$ T cells and $CD8^+$ T cells in HM that were positive for CD45RO were increased, whereas the percentages that were positive for CD45RA were decreased, compared to both the PB of control adults and postpartum women (Table 1, Fig. 4; $P < 0.001$). No significant difference was found between the PB of control adults and postpartum women in any of the subsets of $CD4^+$ or $CD8^+$ cells defined by CD45RA or CD45RO, regarldess of whether two or three color methodology was used ($P > 0.1$–0.5; except $P > 0.05$ for one pair of values determined by three color flow cytometry, the percentage of $CD4^+$ lymphocytes bearing CD45RO). In addition, within the group of control adults (PB, control adults), no significant difference

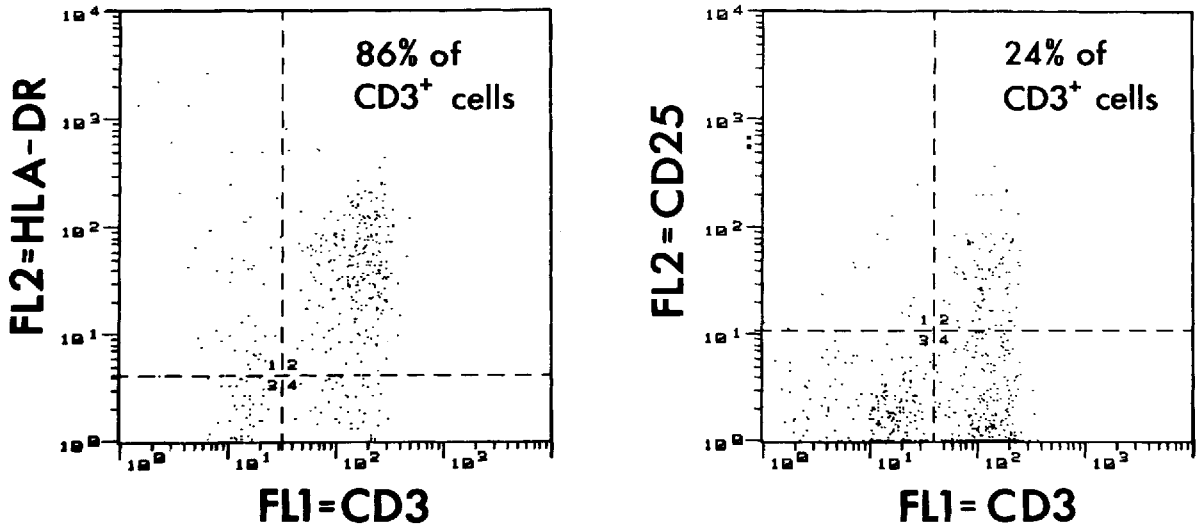

FIG. 3. Flow cytometry histograms demonstrating increased HLA-DR and IL-2R expression on HM T cells. The mean percentage of $CD3^+$ cells bearing HLA-DR or IL-2R was significantly increased compared to PB from control adults ($P < 0.001$).

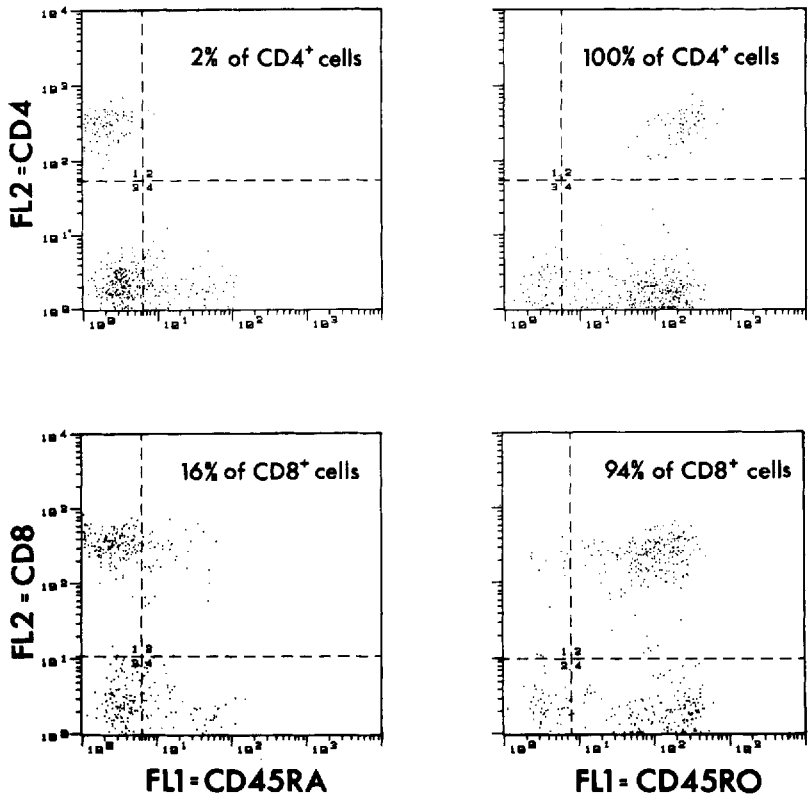

FIG. 4. Flow cytometry histograms demonstrating increased CD45RO expression and concomitantly decreased CD45RA expression on both the $CD4^+$ and $CD8^+$ subsets of T cells in the same sample of HM. When the mean values in HM were compared to those in PB of control adults (non-pregnant, non-lactating) and postpartum women, the differences were highly significant ($P < 0.001$).

was found between these subset values determined by two color vs. three color methodology ($P > 0.1$). The percentages of $CD4^+$ T cells and $CD8^+$ T cells in HM that were positive for Leu-8 were significantly decreased compared to the PB of control adults ($P < 0.003$, data not shown).

DISCUSSION

In this investigation, we demonstrated by flow cytometry that lymphocytes comprise about 4% of the total leukocytes in HM produced early in lactation. In addition, we demonstrated that about 83% of the lymphocytes in HM were T cells as shown by the presence of CD3. These findings are similar to those in previous investigations that utilized flow cytometry or fluorescence microscopy to identify the major populations of lymphocytes in HM. There has been controversy, however, concerning the proportions in $CD4^+$ and $CD8^+$ T cells in HM. Richie et al. reported that the CD4/CD8 ratio was decreased in HM compared to PB [35], whereas Keller et al. found the ratio in HM to be similar to that in PB [24]. The reason for the discrepancy in these studies is unclear, although the use of fractionated leukocytes and flow cytometry [35] or unfractionated leukocytes and fluorescence microscopy [24] may have accounted for some of the difference. In our investigation (using unfractionated cells and flow cytometry), the frequency of $CD4^+$ T cells in HM was not significantly altered compared to PB, but the frequency of $CD8^+$ T cells was increased, resulting in a significant decrease in the CD4/CD8 ratio. This change was not due to a shift in $CD8^+$ T cells in the PB of women during the initial postpartum period. The cause(s) and effect(s) of this increase in the $CD8^+$ T cell subset in HM are not known. However, it is interesting to note that in the normal buccal mucosa about 41% of all T cells were located in the epithelium, the CD4/CD8 ratio was 0.48 in the epithelium and 0.74 in the papillary layer, and numerous Langerhans cells (antigen processing/presenting cells for T cells) were present in the epithelium [49]. In the human small bowel, most intraepithelial lymphocytes were T cells expressing CD8 and TCR α/β [8]. In our study, the great majority of T cells in HM expressed TCR α/β. Thus the oral and intestinal epithelia may have a selective affinity for $CD8^+$ T cells over $CD4^+$ cells. The epithelia of the digestive and upper respiratory tracts may provide a favorable environment for maternal T cells transferred to infants in HM.

Our studies also revealed that significantly more T cells in HM expressed phenotypic markers of recent or previous activation than T cells in the PB of control adults or postpartum women. Expression of HLA-DR or CD25 (IL-2R) by HM T cells indicated recent activation [40,51]. About 85% of T cells in HM were found to be $HLA\text{-}DR^+$, whereas about 10% were positive in the PB of control adults, a highly significant difference. Compared to the PB of control adults, a significantly increased number of T cells in HM expressed high affinity IL-2 receptors [6,27,41] (as detected by anti-CD25), which appear rapidly on T cells after activation but disappear after removal of the inducing stimulus. The presence of CD45RO on HM T cells indicated previous activation. Virtually all of the $CD4^+$ (99.8%) and most of the $CD8^+$ T cells (92%) in HM were $CD45RO^+$, significantly increased compared to the PB of control adults and postpartum women. A significant decrease in CD45RA expression was concomitantly noted in both of these subpopulations in HM. These findings are of interest since the maturation of PB T cells from the virginal, unprimed state to antigen-primed or memory T cells is associated with alternative mRNA splicing that leads to a change from the $CD45RA^+$ $CD45RO^-$ to the $CD45RA^-$ $CD45RO^+$ phenotype [1,5,10,11,13, 28,39,47]. The differential expression of CD45RA and CD45RO reflects the differentiation of T cells along this pathway. Although it is generally accepted that this change is unidirectional, that paradigm has recently been challenged [2]. In addition, $CD4^+$ $CD45RA^-$ $CD45RO^+$ cells are known to provide help for antibody production (helper cell function) or to induce suppression of antigen-specific antibody production (antigen-specific suppressor-inducer function) [11,39]. In contrast, $CD4^+$ $CD45RA^+$ $CD45RO^-$ cells induce suppression of polyclonal antibody production [11,39]. Within the $CD8^+$ population, cytotoxic function is associated with the $CD45RA^-$ phenotype [39,50]. Finally, the predominance of $CD45RA^-$ $CD45RO^+$ T cells in HM is consistent with the more limited mitogenic response of HM T cells to phytohemagglutinin [4,32] and their capacity to produce interferon-Γ [4,15,23], features that have been attributed to $CD45RO^+$ T cells [14,38,39].

These findings regarding CD45RA and CD45RO expression on HM T cells are consistent with those recently reported by Bertotto et al. [4]. They separated T cells from HM and paired PB samples using magnetic beads coated with anti-CD4 and anti-CD8, and then performed single color flow cytometry. In histograms from a single sample, said to be representative of eight samples, Bertotto et al. (4) qualitatively demonstrated a marked increase in the numbers of HM T cells expressing CD45RO (UCHL1), CDw29 (4B4) and CD11a (LFA-1), and a marked decrease in cells expressing CD45RA (2H4), compared to PB T cells. In addition, they found that expression of CD54 (ICAM-1) and CD2 (T11) by HM T cells was increased compared to PB T cells. However, in contrast to our study, unfractionated HM cells were not examined, expression of HLA-DR and IL-2R by HM T cells was not examined, the helper-inducer and suppressor-cytotoxic subset status of HM T cells was not determined, the subsets of helper-inducer and suppressor-cytotoxic T cells defined by CD45RA and CD45RO were not determined, and none of the populations defined phenotypically in their study were quantitated. Bertotto et al. [3] have also recently reported that the proportion of TCR γ/δ cells in HM is twofold greater than in PB.

The results of our investigations led to several questions regarding the genesis and biological effects of the activated and memory T cell populations in HM. First, we considered whether these findings were related to alterations in these T cell subsets in the PB of lactating women compared to the PB of non-pregnant, non-lac-

tating adults. This was not found to be the case for the subsets of $CD4^+$ and $CD8^+$ T cells defined by CD45RA and CD45RO. We then considered whether the activation was due to factors that are produced at or specifically secreted by the mammary gland. Since TNF-α and possibly other cytokines are present in HM [36] and since TNF-α and other cytokines stimulate T cells [16], it may be that they play a role in the activation of T cells in HM. It is also possible that these activated T cells produce cytokines, including the TNF-α in HM. Activated normal human T cells are known to produce TNF-α [26]. Specific homing of activated and memory T lymphocytes to the breast is another possibility. Further efforts will be required to determine which of these propositions is/are correct.

We have considered the potential role of these activated and memory T cells from HM in the recipient infant. Some T cells in HM may survive passage through the stomach, especially prior to the development of high gastric acidity after the first several days of life. These HM T cells may interact with the epithelia of the alimentary or upper respiratory tracts or even establish residency within those mucosal sites. Many T lymphocytes in the epithelium [8,20] and most T lymphocytes in the lamina propria [19,20,40] of the intestinal tract and in the pulmonary epithelium [37] are $CD45RA^-$ $CD45RO^+$, as are the T cells in HM.

It is provocative that very few $CD45RO^+$ T lymphocytes are present at birth [17,25] and the number of these cells do not reach adult values until about 16 years of age [17]. It is possible that the paucity of memory T cells in the newborn period and early infancy is compensated for, in part, by the transfer of maternal $CD45RO^+$ T lymphocytes in HM. In the report by Hayward et al. [17], it was unclear whether the infants that were investigated were breastfed or not. Since that study population was drawn from infants and children hospitalized for elective surgical procedures, few may have been breastfed. It will be important to determine whether breastfeeding speeds the development of $CD45RO^+$ T lymphocytes by comparing the numbers of these cells in completely breastfed and non-breastfed infants.

Finally, it is likely that the agents responsible for the activation of HM T cells are present in HM. Thus, we have begun experiments to determine whether the cell free portion of HM activates human blood T cells and whether the phenotypic features of those cells correspond to HM T cells.

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LITERATURE CITED

1. Akbar AN, Terry L, Timms A, Beverley PCL, Janossy G: Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. J Immunol 140:2171–2178, 1988.
2. Bell EB, Sparshott SM: Interconversion of CD45R subsets of CD4 T cells in vivo. Nature 348:163–166, 1990.
3. Bertotto A, Castellucci G, Fabietti G, Scalise F, Vaccaro R: Lymphocytes bearing the T cell receptor γδ in human breast milk. Arch Dis Child 65:1274–1275, 1990.
4. Bertotto A, Gerli R, Fabietti G, Crupi S, Arcangeli C, Scalise F, Vaccaro R: Human breast milk T lymphocytes display the phenotype and functional characteristics of memory T cells. Eur J Immunol 20:1877–1880, 1990.
5. Beverley PCL: Human T cell subsets. Immunol Lett 14:263–267, 1987.
6. Bich-Thuy LT, Dukovich M, Peffer NJ, Fauci AS, Kehrl JH, Greene WC: Direct activation of human resting T cells by IL 2: The role of an IL 2 receptor distinct from the Tac protein. J Immunol 139:1550–1556, 1987.
7. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest 219 (suppl 97):77–89, 1968.
8. Brandtzaeg P, Bosnes V, Halstensen TS, Scott H, Sollid LM, Valnes KN: T lymphocytes in human gut epithelium preferentially express the α/β antigen receptor and are often CD45/UCHL1-positive. Scand J Immunol 30:123–128, 1989.
9. Brooks EG, Schmalstieg FC, Wirt DP, Rosenblatt HM, Adkins LT, Lookingbill DP, Rudloff HE, Rakusan TA, Goldman AS: A novel X-linked combined immunodeficiency disease. J Clin Invest 86:1623–1631, 1990.
10. Byrne JA, Butler JL, Cooper MD: Differential activation requirements for virgin and memory T cells. J Immunol 141:3249–3257, 1988.
11. Clement LT, Yamashita N, Martin AM: The functionally distinct subpopulations of human $CD4^+$ helper/inducer T lymphocytes defined by anti-CD45R antibodies derive sequentially from a differentiation pathway that is regulated by activation-dependent postthymic differentiation. J Immunol 141:1464–1470, 1988.
12. Crago SS, Prince SJ, Pretlow TG, McGhee JR, Mestecky J: Human colostral cells. I. Separation and characterization. Clin Exp Immunol 38:585–597, 1979.
13. Deans JP, Boyd AW, Pilarski LM: Transitions from high to low molecular weight isoforms of CD45 (T200) involve a rapid activation of alternate mRNA splicing and slow turnover of surface CD45R. J Immunol 143:1233–1238, 1989.
14. Dohlsten M, Hedlund G, Sjogren H-O, Carlsson R: Two subsets of $CD4^+$ T helper cells differing in kinetics and capacities to produce interleukin 2 and interferon-γ can be defined by the Leu-18 and UCHL1 monoclonal antibodies. Eur J Immunol 18:1173–1178, 1988.
15. Emodi G, Just M: Interferon production by lymphocytes in human milk. Scand J Immunol 3:157–160, 1974.
16. Hackett RJ, Davis LS, Lipsky PE: Comparative effects of tumor necrosis factor-α and IL-1β on mitogen-induced T cell activation. J Immunol 140:2639–2644, 1988.
17. Hayward AR, Lee J, Beverley PCL: Ontogeny of expression of UCHL1 antigen on $TcR\text{-}1^+$ (CD4/8) and $TcR\delta^+$ T cells. Eur J Immunol 19:771–773, 1989.
18. Jackson AL, Warner NL: Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes. In: Manual of Clinical Laboratory Immunology, 3rd ed. Rose NR, Friedman H, Fahey JL (eds). American Society for Microbiology, Washington, DC, 1986, pp 226–235.
19. James SP, Fiocchi C, Graeff AS, Strober W: Phenotypic analysis of lamina propria lymphocytes. Predominance of helper-inducer and cytolytic T-cell phenotypes and deficiency of suppressor-inducer phenotypes in Crohn's disease and control patients. Gastroenterology 91:1483–1489, 1986.
20. Janossy G, Bofill M, Rowe D, Muir J, Beverley PCL: The tissue distribution of T lymphocytes expressing different CD45 polypeptides. Immunology 66:517–525, 1989.
21. Julius MH, Janusz M, Lisowski J: A colostral protein that induces the growth and differentiation of resting B lymphocytes. J Immunol 140:1366–1371, 1988.

22. Juto P: Human milk stimulates B cell function. Arch Dis Child 60:610–613, 1985.
23. Keller MA, Kidd RM, Bryson YJ, Turner JL, Carter J: Lymphokine production by human milk lymphocytes. Infect Immun 32: 632–636, 1981.
24. Keller MA, Faust J, Rolewic LJ, Stewart DD: T cell subsets in human colostrum. J Pediatr Gastroenterol Nutr 5:439–443, 1986.
25. Kingsley G, Pitzalis C, Waugh AP, Panayi GS: Correlation of immunoregulatory function with cell phenotype in cord blood lymphocytes. Clin Exp Immunol 73:40–45, 1988.
26. Kinkhabwala M, Sehajpal P, Skolnik E, Smith D, Sharma VK, Vlassara H, Cerami A, Suthanthiran M: A novel addition to the T cell repertory. Cell surface expression of tumor necrosis factor/cachectin by activated normal human T cells. J Exp Med 171: 941–946, 1990.
27. Lowenthal JW, Greene WC: Contrasting interleukin 2 binding properties of the α (p55) and β (p70) protein subunits of the human high-affinity interleukin 2 receptor. J Exp Med 166:1156–1161, 1987.
28. Merkenschlager M, Terry L, Edwards R, Beverley PCL: Limiting dilution analysis of proliferative responses in human lymphocyte populations defined by the monoclonal antibody UCHL1: implications for differential CD45 expression in T cell memory formation. Eur J Immunol 18:1653–1661, 1988.
29. Mincheva-Nilsson L, Hammarstrom M-L, Juto P, Hammarstrom S: Human milk contains proteins that stimulate and suppress T lymphocyte proliferation. Clin Exp Immunol 79:463–469, 1990.
30. Munoz C, Endres S, van der Meer J, Schlesinger L, Arevalo M, Dinarello C. Interleukin-1 beta in human colostrum. Res Immunol 141:505–513, 1990.
31. Mushtaha AA, Schmalstieg FC, Hughes TK, Rajaraman S, Rudloff HE, Goldman AS: Chemokinetic agents for monocytes in human milk: Possible role of tumor necrosis factor-α. Pediatr Res 25:629–633, 1989.
32. Ogra SS, Ogra PL: 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, 1978.
33. Ozkaragoz F, Rudloff HB, Rajaraman S, Mushtaha AA, Schmalstieg FC, Goldman AS: The motility of human milk macrophages in collagen gels. Pediatr Res 23:449–452, 1988.
34. Richie ER, Steinmetz KD, Meistrich ML, Ramirez I, Hilliard JK: T lymphocytes in colostrum and peripheral blood differ in their capacity to form thermostable E-rosettes. J Immunol 125:2344–2346, 1980.
35. Richie ER, Bass R, Meistrich ML, Dennison DK: Distribution of T lymphocyte subsets in human colostrum. J Immunol 129:1116–1119, 1982.
36. Rudloff HE, Schmalstieg FC Jr, Mushtaha AA, Palkowetz KH, Liu SK, Goldman AS: Tumor necrosis factor-alpha (TNF-α) in human milk. Pediatr Res 31:29–33, 1992.
37. Saltini C, Kirby M, Trapnell BC, Tamura N, Crystal RG: Biased accumulation of T lymphocytes with "memory"-type CD45 leukocyte common antigen gene expression on the epithelial surface of the human lung. J Exp Med 171:1123–1140, 1990.
38. Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S: Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-γ production. J Immunol 140:1401–1407, 1988.
39. Sanders ME, Makgoba MW, Shaw S: Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. Immunol Today 9:195–199, 1988.
40. Schieferdecker HL, Ullrich R, Weiss-Breckwoldt AN, Schwarting R, Stein H, Riecken E-O, Zeitz M: The HML-1 antigen of intestinal lymphocytes is an activation antigen. J Immunol 144:2541–2549, 1990.
41. Sharon M, Siegel JP, Tosato G, Yodoi J, Gerrard TL, Leonard WJ: The human interleukin 2 receptor β chain (p70). Direct identification, partial purification, and patterns of expression on peripheral blood mononuclear cells. J Exp Med 167:1265–1270, 1988.
42. Smith CW, Goldman AS: The cells of human colostrum. I. In vitro studies of morphology and functions. Pediatr Res 2:103–109, 1968.
43. Smith CW, Goldman AS: Interactions of lymphocytes and macrophages from human colostrum: characteristics of the interacting lymphocyte. J Reticuloendothel Soc 8:91–104, 1970.
44. Smith CW, Goldman AS, Yates RD: Interactions of lymphocytes and macrophages from human colostrum. Electron microscopic studies of the interacting lymphocyte. Exp Cell Res 69:409–415, 1971.
45. Soder, O: Isolation of interleukin-1 from human milk. Int Arch Allergy Appl Immunol 83:19–23, 1987.
46. Stoeck M, Ruegg C, Miescher S, Carrel S, Cox D, Von Fliedner V, Alkan S: Comparison of the immunosuppressive properties of milk growth factor and transforming growth factors β1 and β2. J Immunol 143:3258–3265, 1989.
47. Terry LA, Brown MH, Beverley PCL: The monoclonal antibody, UCHL1, recognizes a 180,000 MW component of the human leucocyte-common antigen, CD45. Immunology 64:331–336, 1988.
48. Thorpe LW, Rudloff HE, Powell LC, Goldman AS: Decreased response of human milk leukocytes to chemoattractant peptides. Pediatr Res 20:373–377, 1986.
49. van Loon LAJ, Krieg SR, Davidson CL, Bos JD: Quantification and distribution of lymphocyte subsets and Langerhans cells in normal human oral mucosa and skin. J Oral Pathol Med 18:197–201, 1989.
50. Yamashita N, Clement LT: Phenotypic characterization of the post-thymic differentiation of human alloantigen-specific $CD8^+$ cytotoxic T lymphocytes. J Immunol 143:1518–1523, 1989.
51. Yu DTY, Winchester RJ, Fu SM, Gibofsky A, Ko HS, Kunkel HG. Peripheral blood Ia-positive T cells. Increases in certain diseases and after immunization. J Exp Med 151:91–100, 1980.