# Diminished Mammary Gland Lymphocyte Functions Parallel Shifts in Trafficking Patterns during the Postpartum Period

## (44016)

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*Abstract.* **Once activated, lymphocytes can regulate both specific and nonspecific immune responses. Alterations in lymphocyte function may increase the host's vulnerability to bacterial infections such as mastitis. Susceptibility to mastitis as well as diminished leukocyte functional capabilities have been shown to be influenced by lactational stage. Therefore, the present study characterized the phenotypes and functions of several bovine lymphoid populations at two points in the lactational cycle. Mononuclear cells were isolated from peripheral blood, supramammary lymph nodes, and mammary parenchyma of mid-lactating and postpartum dairy cows. The phenotypic composition, proliferative ability, cytokine secretion, and cytotoxic activity of isolated leukocytes were assessed with respect to lactational stage and tissue source. Lower percentages of T lymphocytes were consistent with diminished mitogen-stimulated proliferation and spontaneous cytotoxic activity by lymphocytes isolated from postpartum compared with mid-lactating animals. Stimulation with interleukin-2 did not enhance the cytotoxic activity or proliferative ability of lymphocytes isolated postpartum to similar levels observed for those isolated from mid-lactating animals. These data indicate that certain diminished lymphocyte functions observed during the postpartum period may result from shifts in leukocyte trafficking patterns.**
[P.S.E.B.M. 1996, Vol 212]

Mastitis, an inflammation of the mammary gland, occurs in approximately one-third of all lactating women during a single lactation course (1). The disease is a potentially serious illness in lactating women and most often has been correlated with primiparity, improper nursing technique, and incomplete emptying of the breast (2). It has been suggested that stress and fatigue may predispose a woman to mastitis by lowering or inhibiting postpartum maternal immune responses (2, 3). Mastitis episodes occur most often during the first 3 months postpartum, with a peak in the first 2 weeks (1, 3). Similarly, increased susceptibility to mastitic infections also has been associated with the postpartum period (4) and to diminished mammary gland defense mechanisms in the bovine (5).

Although they are not identical, the compositional and functional aspects of mammary gland immunity appear to be quite similar in the human and bovine mammary glands. Unlike the mammary glands of rats and mice, which contains predominantly more $CD4^+$ than $CD8^+$ lymphocytes (6, 7), milk secretions of both humans and bovines contain greater proportions of $CD8^+$ than $CD4^+$ lymphocytes (8, 9). Additionally, the low CD4:CD8 ratio of human and bovine mammary secretions directly contrasts with the ratio observed in the blood (8, 10). In the porcine mammary

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Received November 28, 1995. [P.S.E.B.M. 1996, Vol 212]
Accepted March 14, 1996.

This research is funded in part by grants from the USDA National Competitive Research Initiative Grants Program (93-37204-9206). This work supports the efforts of the Center for Mastitis Research, College of Agricultural Sciences, The Pennsylvania State University.

0037-9727/96/2123-0271$10.50/0

gland, $CD8^+$ lymphocytes are the predominant T lymphocyte; however, they are also the predominant phenotype observed in the peripheral blood (11, 12). Moreover, under normal conditions the principal phagocyte in porcine milk is the neutrophil (11), whereas in both cows (13) and women (14) the macrophage is the most prevalent phagocyte. Therefore, when comparing the lacteal and blood leukocyte phenotypes and trafficking patterns of these species, it appears that the bovine is the most similar to the human. As such, the bovine mammary gland provides the best model to study the human leukocyte response to mastitic infections.

Mammary tissues are heavily infiltrated with lymphocytes during all stages of lactation (15). The predominance of these cells in the mammary gland suggests that lymphocytes play a significant role in host defense against disease. There is data to suggest that mammary gland lymphocytes are capable of broad range of effector functions including cytotoxic, suppressor, and antibacterial functions (16, 17). However, several studies have shown that the functional capabilities of bovine lymphocytes are diminished during the postpartum period (18, 19). Isolated lymphoid cells have reduced proliferative responses to antigenic and mitogenic stimulation (18, 20), as well as a reduced capacity to produce antibodies and secrete cytokines (19, 21). Other research has suggested that the reduction in lymphocyte effector functions during the postpartum period may contribute to delayed host immune responsiveness during the early stages of pathogenesis (22). However, the mechanisms responsible for decreased functional activity of mammary lymphocytes and the relation to increased susceptibility to infection remain to be elucidated.

Recent studies suggest that the composition of lymphocyte subpopulations may vary during the lactation cycle and that such variation may correlate with susceptibility to new intramammary infections (17,22). However, there is little information available that directly links shifts in leukocyte trafficking patterns with reduced effector cell functions during the postpartum period. Therefore, in the present study, we evaluated the composition of leukocyte subsets isolated from different tissue compartments of mid-lactation and postpartum dairy cattle, including the peripheral blood, supramammary lymph nodes, and mammary parenchymal tissues. Several essential lymphoid effector functions were assayed and compared with shifts in lymphocyte subpopulations.

## Materials and Methods

**Reagents.** Collagenase, hyaluronidase, DNAse, concanavalin A (Con A), phytohemagglutinin (PHA), pokeweed mitogen (PWM), antibiotics/antimycotics (aa), L-glutamine (Lg), and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were obtained from Sigma (St. Louis, MO). Additionally, minimal essential medium (MEM), RPMI 1640, Hanks' balanced salt solution (HBSS), methyl-α-*D*-mannopyranoside, and sodium dodecyl sulfate (SDS) were purchased from Sigma. The 2-mercaptoethanol and *NN*-dimethyl formamide were obtained from Fisher (Pittsburgh, PA). Fetal bovine serum was purchased from Hyclone (Logan, UT). Ficoll-paque (1.077 g/ml) and percoll (1.055 and 1.078 g/ml) were acquired from Pharmacia (Uppsala, Sweden). Maden Darby bovine kidney and K-562 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). All monoclonal antibodies for flow cytometric analysis were purchased from Veterinary Medicine Research and Development Inc. (Pullman, WA). Fluorescein isothiocyanate–labelled $F(ab)_2$ goat anti-mouse IgG was purchased from Becton Dickinson (Mountain View, CA). $^3H$-[methyl]thymidine ($^3HTdr$) was purchased from Amersham (Arlington Heights, IL). Recombinant human interleukin-2 (IL-2) and α-interferon (IFN) were acquired from Boehringer Mannheim (Indianapolis, IN) and Genzyme (Cambridge, MA), respectively. Anthony Castro, at The Pennsylvania State University, kindly provided the vesicular stomatitis virus (New Jersey strain).

**Media/Buffers.** The digest medium used to isolate mammary parenchyma cells contained MEM with collagenase (100 mg/ml), hyaluronidase (100 mg/ml), and DNAse (40 U/ml). Isolated leukocytes were washed with HBSS, pH 7.3, prior to use. For flow cytometric analyses, isolated leukocytes were maintained in phosphate-buffered saline (PBS), pH 7.3, containing 2% fetal bovine serum (FBS). Primary and secondary antibodies for flow cytometry were diluted in the same medium. Lymphocyte proliferation assays were performed in RPMI 1640, 10% FBS, and 0.01 m*M* 2-mercaptoethanol. For stimulation of cytokine production, RPMI 1640, 10% FBS, and 0.05 m*M* 2-mercaptoethanol were used. Mitogens used in lymphocyte proliferation and cytokine production assays were diluted with RPMI 1640. To analyze IL-2 production, IL-2–dependent bovine T lymphocytes were resuspended in RPMI 1640 containing 5% FBS, 0.05 m*M* 2-mercaptoethanol, and 10 mg/ml methyl-α-*D*-mannopyranoside. Maden Darby bovine kidney cells used in determination of IFN activity were cultured in MEM supplemented with 10% FBS. The extraction buffer used in the IFN assay contained 12.5% SDS diluted in 50% *NN*-dimethyl formamide. Known standards for the IL-2 and IFN assays were diluted in RPMI and MEM, respectively. Isolated lymphocytes and K-562 cells were resuspended in RPMI 1640 supplemented with 5% FBS for analysis of lymphocyte cytotoxicity. All media containing RPMI 1640 and

MEM was supplemented with 1% antibiotic/antimycotic and 1% L-glutamine. Media with RPMI 1640 also contained 25 m*M* HEPES.

**Cell Preparation.** Lymphocytes were isolated from the blood, supramammary lymph nodes, and uninfected mammary tissue of four mid-lactating (>150 days into lactation) and four postpartum dairy cows in their second to fourth lactations. All cells from postpartum animals were obtained within 3 days of calving. Parenchyma and lymph node samples were taken at an U.S.D.A. certified abattoir according to approved animal care protocols. Mononuclear cells were isolated from mammary parenchyma by first mincing the tissue with a Waring blender (New Hartford, CT) and then digesting the tissue in media for 15 min at 37°C (16). Next, the suspension was passed consecutively through 250- and 90-μm mesh tissue sieves. Mammary parenchymal mononuclear cells were purified further using a discontinuous Percoll gradient (16). Lymph node mononuclear cells were isolated by mincing the organ and rinsing the minced tissue through a 90-μm mesh tissue sieve. Isolated lymph node mononuclear cells then were purified using a Ficoll-Paque density gradient. Peripheral blood mononuclear cells were isolated from citrated venous blood samples (15). Isolated cells then were purified using a Ficoll-Paque density gradient. Any red blood cell contamination of the mononuclear cell pellet was eliminated by water lysis. Following mononuclear cell purification from each tissue source, the isolated cells were washed three times in HBSS and resuspended in the appropriate medium.

For evaluation of lymphocyte cytotoxic activity, aliquots of isolated cells were added at $5 \times 10^6$/ml in a 75-cm$^2$ tissue culture flask and allowed to incubate for 1–2 hr at 37°C, 5% $CO_2$ to remove adherent macrophages. The resulting nonadherent cells were confirmed to be $\geq$97% lymphocytes by flow cytometric analysis. These cells then were aliquotted into 12-well culture plates and incubated with or without recombinant human IL-2 (100 U/ml) for 48 hr.

**Flow Cytometric Analysis.** Flow cytometry was performed as previously described (15) to characterize the phenotype of isolated cell populations from peripheral blood, lymph node, and mammary parenchyma tissue. Isolated leukocytes were stained with monoclonal antibodies raised in mice that were specific for bovine leukocyte surface antigens. All monoclonal antibodies were originally at a stock concentration of 1 mg/ml and diluted: CD2 (Pan-T lymphocyte, BAQ95A at 1:100), CD4 (T-helper, CACT83B at 1:160), CD8 (T-cytotoxic/suppressor, CACT80C at 1:400), surface IgM (B-cell, BAQ44A at 1:100), WC1 (γδ T lymphocyte, B7A1 at 1:200), WC2 (non-T lymphocyte; non–B lymphocyte, CACTB6A at 1:100), CD5 (thymocytes; T lymphocyte; B lymphocyte, CACT105A at 1:100), CD11 (leukocyte function–associated antigen 1, BAQ153A at 1:100), major histocompatibility complex (MHC) class II (TH14B at 1:200), and granulocytes-monocytes (DH59B at 1:100). Controls to detect nonspecific labeling of fluorescein isothiocyanate–labeled F(ab)$_2$ goat anti-mouse IgG were run in parallel. Analyses were performed with an Coulter EPICS PS 753 (Coulter Electronics, Miami, FL) and immunofluorescence histograms were expressed as percentage of positive-staining cells.

**Lymphocyte Proliferation.** The ability of isolated peripheral blood, lymph node, and mammary parenchymal mononuclear cells to proliferate in response to various mitogenic stimuli was examined. Briefly, isolated cells ($1 \times 10^5$/100 μl) were resuspended in proliferation media and added to a 96-well round bottom plate. Triplicate sets of wells were stimulated with Con A (0.5 μg/well); PHA (1.0 μg/well); PWM (0.1 μg/well); and recombinant human IL-2 (12.5 U/well). A triplicate set of wells containing only cells and media served as controls. After incubation for 48 hr, 0.4 μCi of $^3$HTdr was added to all wells. Following an additional 18 hr of incubation, the cells were harvested with a semi-automatic cell harvester (Skatron, Sterling, VA) and $^3$HTdr incorporation into cellular DNA measured with a liquid scintillation counter. Results are expressed as mean corrected counts per minute (stimulated cpm − control cpm). All control cultures had approximately $\leq$1000 cpm.

**Cytokine Production.** The ability of isolated peripheral blood, lymph node, and mammary parenchymal mononuclear cells to produce cytokines *in vitro* was examined following stimulation with Con A. Mononuclear cells ($2.5 \times 10^6$/ml) were incubated with media in a 24-well plate. Duplicate wells were stimulated with either Con A (5 μg/ml) or RPMI 1640 without Con A (control). Supernatants were collected after incubation at 37°C for 24, 48, and 72 hr, and stored at −70°C until analyzed.

**Analysis of IL-2 and IFN Activity.** The level of IL-2–like activity present in cytokine production supernatants was determined by measuring $^3$HTdr incorporation by IL-2–dependent bovine T lymphocytes (23). The IL-2–dependent lymphocytes ($1 \times 10^4$/100 μl) were incubated with 100 μl of supernatant collected from Con A–stimulated cultures. Following a 24-hr incubation period, 0.4 μCi of $^3$HTdr was added to all wells and cultured for an additional 18 hr. The cells were harvested with a semi-automatic harvester (Skatron) and the amount of $^3$HTdr incorporated into cellular DNA was quantified by liquid scintillation as described for the lymphocyte proliferation assay. The level of IL-2–like activity present in the supernatants was expressed as a stimulation index (SI): stimulated culture cpm/unstimulated culture cpm.

The analysis of IFN production was determined through modification of a colorimetric antiviral assay (24). Briefly, Maden Darby bovine kidney cells (ATCC# CCL22; 1 × $10^4$/100 μl) were loaded into a 96-well flat-bottom plate and allowed to incubate for 24 hr at 37°C, 5% $CO_2$. The media then was removed and replaced with fresh media. Serial dilutions of the standard and sample supernatants were added to triplicate sets of wells. Following an additional 24-hr incubation, the supernatants were removed and 150 μl of vesicular stomatitis virus (1:1000) was added to each well and incubated for an additional 24 hr. At this time, 25 μl of MTT (5 μg/ml) was added to each well. After a 2-hr incubation, 100 μl of extraction buffer was added to each well and incubated overnight. The concentration of live cells was determined through colorimetric analysis with a microplate reader (Bio-Rad, Melville, NY) at 595 nm. The determination of antiviral activity was extrapolated from a standard curve generated with serial 1:2 dilutions of recombinant human IFN-α.

**Analysis of Lymphocyte Cytotoxicity.** The direct killing of tumor target cells (K-562 cell line, ATCC# CCL243) was evaluated using the Cytotox 96 nonradioactive Assay Kit (Promega, Madison, WI). Briefly, cultured lymphoid cells (effector cells, 1 × $10^6$/100 μl) were added to six wells of a 96-well culture plate. Serial 1:2 dilutions of the effector cells were made to provide effector:target ratios of 100:1 through 3:1. Target cells (5 × $10^3$/50 μl) then were added to each of the experimental wells. Effector cell control wells received 50 μl of RPMI media (total volume in wells was 100 μl). Each ratio was set up in triplicate and the plates were run according to the manufacturer's instructions. Plates were read at 490 nm on the enzyme-linked immunosorbent assay (ELISA) microplate reader (Bio-Rad) and corrected values were calculated according to manufacturers instructions. Percentage cytotoxicity for each effector:target ratio was calculated using the corrected values in the following formula: {[(Experimental − Effector Control) − Spontaneous Target Lysis] / (Detergent Target Lysis − Spontaneous target lysis)}× 100.

**Statistical Analysis.** Data were analyzed by least squares analyses of variance using the general linear models procedure of SAS (SAS Inst., Inc., Cary, NC) to determine differences between proliferative responses, cytokine production, lymphocyte cytotoxicity, and isolated cell leukocyte phenotypes between lactational stages. The statistical model included the effects of cow nested within the stage of lactation, stage of lactation, and mononuclear cell source. Preplanned comparisons of least square means between the two stages of lactation were made by pairwise *t* test from the overall model. For cytotoxicity, the model also included the effect of IL-2 treatment. These means were contrasted between IL-2–stimulated and unstimulated lymphocytes within lactation stage, as well as between lactation stages.

## Results

**Phenotypic Characterization of Isolated Mononuclear Cells.** Within the peripheral blood, T lymphocyte phenotypes ($CD2^+$, $CD4^+$, $CD8^+$, and $CD5^+$) represented a significantly lower ($P < 0.05$) proportion of cells isolated during the postpartum versus the mid-lactation period (Fig. 1A). A concomitant increase in the percentage of blood monocytes/granulocytes was observed at this time. In contrast, the frequency of various leukocyte subpopulations within the supramammary lymph node remained fairly consistent throughout lactation, with only a significant relative reduction ($P < 0.05$) of $CD5^+$ thymocytes/lymphocytes during the postpartum period (Fig. 1B). Similar to the peripheral blood, the mammary tissue exhibited significantly lower ($P < 0.05$) percentages of $CD8^+$ and $CD5^+$ cells postpartum. The mammary tissue also demonstrated a significant relative decrease ($P < 0.05$) in γδ T lymphocytes ($WC1^+$) percentages at this time (Fig. 1C).

Both the blood and the lymph node had significantly higher ($P < 0.05$) CD4:CD8 ratios than mammary tissue during both the mid-lactation and postpartum periods (Fig. 2). Following parturition, the ratio of CD4:CD8 lymphocytes in the peripheral blood significantly increased ($P < 0.05$), even though there were decreased proportions of $CD4^+$ and $CD8^+$ cells. In contrast, only a slight change of the CD4:CD8 ratio with respect to lactational stage was observed within the lymph node and mammary parenchyma. However, a significant decrease in the CD4:CD8 ratio from the blood, lymph node, and mammary tissue was observed postpartum (Fig. 2).

**Lymphocyte Proliferation.** The proliferative response of isolated peripheral blood, supramammary lymph node, and mammary parenchyma of mid-lactating and postpartum periods are summarized in Table I. In general, lymphocyte proliferative responses to all mitogens were reduced in postpartum cows when compared to mid-lactating ones, regardless of mononuclear cell source. Each source isolated postpartum demonstrated a significant reduction ($P < 0.05$) in T lymphocyte proliferation following PHA stimulation. However, only peripheral blood and mammary parenchyma T lymphocytes isolated from postpartum animals experienced significantly decreased ($P < 0.05$) proliferative responses to IL-2. Significantly lower ($P < 0.001$) proliferation of mammary parenchyma T lymphocytes also was observed following stimulation with Con A during this time. Although not significant, B lymphocyte proliferative responses to PWM were 1.5- to 2-fold lower postpartum when compared with responses of mid-lactating animals.

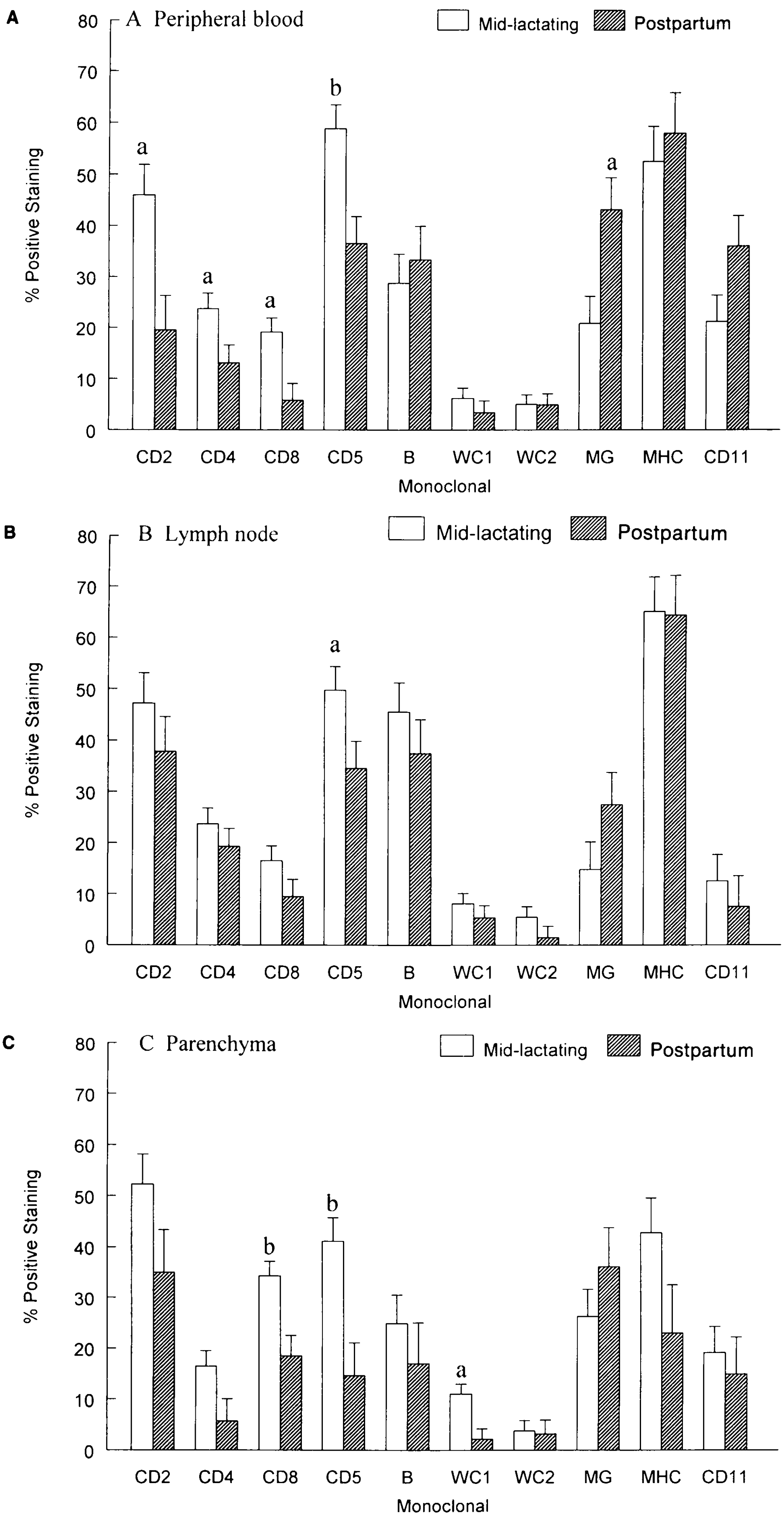

**Figure 1.** Flow cytometric profile of mononuclear cells isolated from bovine blood (A), lymph node (B), and mammary parenchyma tissue (C). Data is expressed as the least square mean ± SEM of positively stained cells. Solid bars represent mid-lactating cows, and hatched bars represent postpartum cows. MG, monocytes/granulocytes, MHC, major histocompatibility complex class II molecules; B, B lymphocytes. [a,b]Significantly different with respect to stage of lactation; $P < 0.05$ and 0.01, respectively.

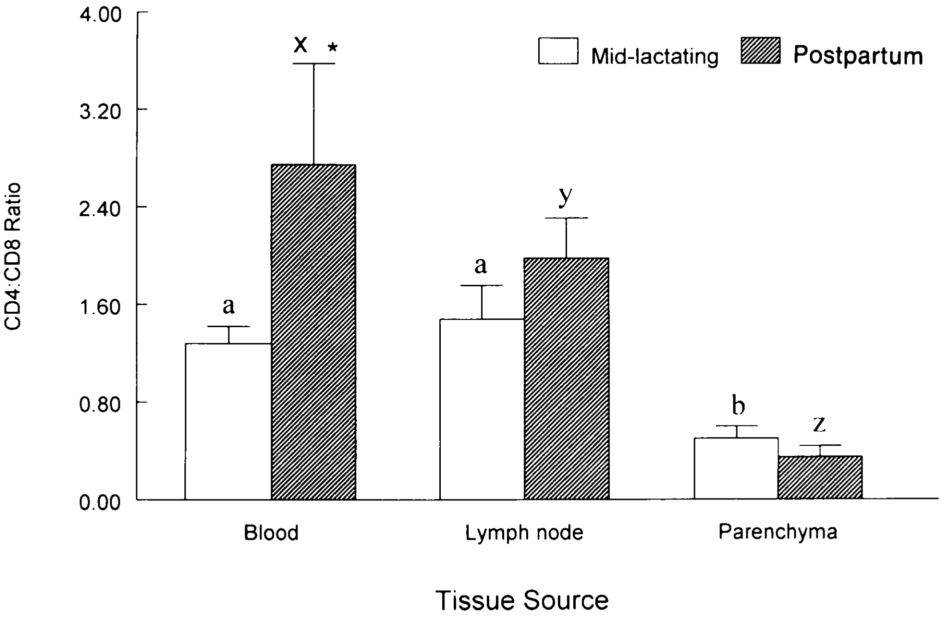

**Figure 2.** Ratio of CD4:CD8 T lymphocyte phenotypes isolated from bovine blood, lymph node, and mammary parenchyma. Data is expressed as least square mean ± SEM of the CD4:CD8 ratio. Solid bars represent mid-lactating cows, and hatched bars represent postpartum cows. *Significantly different with respect to stage of lactation within a tissue location; $P < 0.05$. $^{a,b}$Significantly different with respect to tissue location within the mid-lactation group. $^{x,y,z}$Significantly different with respect to tissue location within the postpartum group.

**IL-2 and IFN Activity.** The expression of IL-2–like and IFN antiviral activity by isolated blood, lymph node, and mammary parenchyma T lymphocytes 48 hr after incubation with Con A is shown in Table II. Significantly lower levels of IL-2–like activity were observed in culture supernatants from peripheral blood ($P < 0.001$), lymph node ($P < 0.05$), and mammary parenchyma ($P = 0.056$) mononuclear cells isolated from postpartum animals when compared with cells from mid-lactating animals. Each mononuclear cell source isolated from postpartum animals also produced less IFN than mid-lactating animals. However, only cells isolated from the peripheral blood had significantly lower ($P < 0.01$) IFN activity than cells from lactating cows.

**Cytotoxic Activity.** Figure 3 demonstrates spontaneous (IL-2–unstimulated) and activated (IL-2–stimulated) cytotoxic activity of peripheral blood, lymph node, and mammary lymphoid cells against K562 target cells at an effector:target of 100:1. Similar levels of spontaneous lymphocyte cytotoxicity were observed among the different tissue locations and lactational stages. Interleukin-2 stimulation significantly increased cytotoxic activity of isolated lymphocytes from both peripheral blood and lymph node of mid-lactating cows ($P < 0.01$ and $P < 0.05$, respectively). However, addition of IL-2 did not enhance the cytotoxicity of those lymphocytes collected from postpartum animals. Moreover, the cytotoxic activity of mammary parenchyma lymphocytes did not rise above

**Table I.** Proliferation of Isolated Mononuclear Cells from the Blood, Lymph Node, and Mammary Gland Parenchyma Tissue of Mid-Lactating and Postpartum Dairy Cows to Various Mitogens

| Tissue | Con A | PHA | PWM | IL-2 |
|---|---|---|---|---|
| | | (increased cpm$^a$ × $10^3$) | | |
| Blood | | | | |
| Mid-lactating | 130 ± 23 | 119 ± 19$^b$ | 113 ± 26 | 100 ± 21$^b$ |
| Postpartum | 113 ± 23 | 44 ± 19 | 59 ± 26 | 22 ± 25 |
| Lymph node | | | | |
| Mid-lactating | 177 ± 22 | 122 ± 19$^b$ | 102 ± 26 | 86 ± 21 |
| Postpartum | 130 ± 22 | 39 ± 22 | 64 ± 26 | 45 ± 21 |
| Parenchyma | | | | |
| Mid-lactating | 88 ± 22$^c$ | 132 ± 19$^c$ | 124 ± 26 | 74 ± 21$^b$ |
| Postpartum | 56 ± 22 | 22 ± 19 | 65 ± 26 | 9 ± 21 |

[a] Proliferation expressed as increased cpm over control cultures. Values are least square mean ± SEM, $n = 4$.
[b] Significantly different with respect to stage of lactation ($P < 0.05$).
[c] Significantly different with respect to stage of lactation ($P < 0.001$).

**Table II.** Cytokine Production[a] by Isolated Mononuclear Cells from the Blood, Lymph Node, and Mammary Parenchyma Tissue of Mid-Lactating and Postpartum Dairy Cows

| Cytokine | Lactation stage | Blood | Lymph node | Parenchyma |
|---|---|---|---|---|
| IL-2[b] | Mid-lactating | 60.2 ± 7.9[c] | 47.9 ± 11.2[d] | 46.7 ± 11.2 |
| (SI) | Postpartum | 3.0 ± 6.9 | 13.0 ± 6.9 | 14.8 ± 8.4 |
| IFN[e] | Mid-lactating | 146.6 ± 19.5[f] | 378.4 ± 166.4 | 269.5 ± 81.6 |
| (U/ml) | Postpartum | 33.0 ± 16.9 | 291.4 ± 144.1 | 90.9 ± 66.6 |

[a] Mononuclear cells stimulated with 0.5 μg/well Con A for 48 hr at 37°C, 5% $CO_2$.
[b] Activity expressed as a stimulation index (stimulated cpm/unstimulated cpm) Values are least square mean ± SEM, $n = 4$.
[c] Significantly different with respect to stage of lactation ($P < 0.001$).
[d] Significantly different with respect to stage of lactation ($P < 0.05$).
[e] Activity expressed as units/ml. Values are least square mean ± SEM, $n = 4$.
[f] Significantly different with respect to stage of lactation ($P < 0.01$).

spontaneous levels following IL-2 stimulation, regardless of lactational stage.

## Discussion

Although lymphocytes play a central role in the immune response, little information is available in either human or bovine models concerning the function of resident lymphocyte populations or those that traffic into the mammary gland during disease. Our laboratory has shown previously that during lactation, mammary gland lymphocytes may play a role in early nonspecific as well as specific defenses against mastitis (16). Therefore, this study investigated in greater depth the phenotypic and functional role of mammary lymphoid populations in order to elucidate the significance of these effector cells during periods of increased susceptibility to mastitis.

A significant finding of the current study was the observed shift in the relative proportions of leukocytes obtained from mammary parenchyma when compared with those obtained from either blood or lymph node tissues. For the first time, this study demonstrated that $CD8^+$ lymphocytes are the predominant T lymphocyte phenotype in the tissues of healthy mammary glands, with a CD4:CD8 ratio of less than 0.5 during both lactational periods. This directly contrasts with the CD4:CD8 ratio of cells obtained from blood and lymph node tissues, which was consistently >1. This is similar to results observed in human milk where a higher proportion of $CD8^+$ than $CD4^+$ T lymphocytes are present compared with that of peripheral blood (8, 25). However, the functional significance for the elevated frequency of $CD8^+$ over $CD4^+$ lymphocytes in both milk and mammary tissue of humans and bovines has not been thoroughly defined. This conserved difference suggests that there is preferential trafficking of

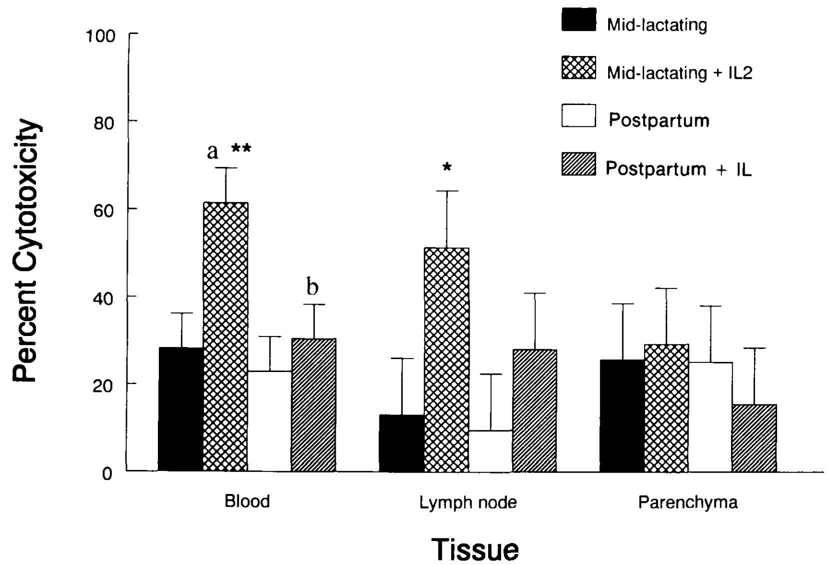

**Figure 3.** Cytotoxicity of isolated bovine blood, lymph node, and mammary parenchyma lymphocytes cultured for 48 hr in the presence or absence (control) of recombinant human interleukin-2. Data is expressed as mean percentage cytotoxic activity against K562 target cells at effector:target ratio of 100:1 ± SEM. Solid bars represent spontaneous (control) cultures from mid-lactating cows. Cross-hatched bars represent interleukin-2–stimulated cultures from mid-lactating cows. Unfilled bars represent spontaneous cultures from postpartum cows. Hatched bars represent interleukin-2–stimulated cultures from postpartum cows. *Significantly different from control cultures; $P < 0.05$. **Significantly different from control cultures; $P < 0.01$. [a,b]Significantly different with respect to stage of lactation within IL-2–stimulated cultures; $P < 0.05$.

$CD8^+$ phenotypes into the mammary gland under normal conditions in both species.

During the postpartum period, decreased proliferation to T lymphocyte mitogens was observed in isolated lymphocytes from all three tissues. Similarly, reduced lymphocyte proliferation was reported for blood and lacteal mononuclear cells isolated from early lactation women (18, 25). The relatively higher proportion of monocyte to lymphocyte populations in both humans (14) and bovines may partially explain the reduced proliferative responses to T lymphocyte mitogens during the postpartum period. The fact that the proportion of B lymphocytes remained constant during both periods may explain why proliferative responses to PWM stimulation (T and B lymphocyte mitogen) were not decreased significantly in this study. Although postpartum mammary parenchyma tissue and lymph node cells demonstrated shifts in the percentages of lymphocytes and monocytes similar to those of the blood, these changes were not significant when compared with cells isolated during lactation. These findings suggest that other factors also are involved in suppressing proliferative responses at this time within these localized tissue compartments.

Cells isolated from all three tissue sources postpartum exhibited a reduced ability to produce IL-2 and IFN, which may be one underlying reason for immunosuppression during this period. IL-2 and IFN are among the many T lymphocyte–derived cytokines of major importance in the regulation of a variety of immune responses including lymphocyte proliferation (26). Decreased IL-2 and IFN production postpartum is most likely due to the lower relative percentage of $CD4^+$ lymphocytes present in tissues during this time. Fewer cells capable of producing these growth promoting cytokines may partially explain the lower proliferative responses of mammary parenchyma lymphocytes during the postpartum period in the absence of significant reductions in $CD2^+$ lymphocytes. Lower cytokine production also may be related to the $CD4{:}CD8^+$ ratio which favors a higher percentage of $CD8^+$ lymphocytes that are capable of exerting a suppressive effect. In fact, several other immunosuppressive disease models have been correlated with reduced CD4:CD8 ratios (27, 28). Although monocyte/macrophage and B-cell percentages in postpartum animals increased or did not change, decreased IFN product may result from altered functional capabilities of these cell types as well.

The relative importance of cytotoxic lymphocytes in the surveillance of tumor progression in the breast has been recognized. However, the functional role of these lymphocytes in mammary gland immunity against bacterial challenge are not well understood. Cytotoxic lymphocytes may play a scavenging role, removing old or damaged secretory cells whose presence could increase the susceptibility of the mammary gland to infections (9). Therefore, the cytotoxic activity of bovine lymphocytes isolated during both the postpartum and mid-lactation periods was examined in this study. Regardless of lactational period, lymphocytes isolated from all three tissues demonstrated low spontaneous cytotoxic ability. Stimulation with IL-2 elevated cytotoxic ability in blood and lymph node lymphocytes isolated from mid-lactating but not from postpartum animals. However, IL-2 did not induce a higher cytotoxic ability in mammary parenchyma lymphocytes isolated from either lactational period. The inability to stimulate cytotoxic activity by lymphocytes from postpartum cows or those obtained from mammary parenchyma suggests that either a lower proportion of cells capable of mediating cytotoxic function are present in the cultures, or that these cells have a decreased level of activation compared with cells obtained from mid-lactating animals.

Relative to peripheral blood, both humans and ruminants express greater levels of $\gamma\delta$ T lymphocytes in mammary secretions and mammary parenchyma respectively (8). The finding that $\gamma\delta$ T lymphocyte percentages decrease significantly in the mammary parenchyma during the postpartum period raises interesting questions about the potential effector functions of these newly discovered cells that preferentially home to epithelial surfaces (29). Although the function of $\gamma\delta$ T lymphocytes is still speculative, there are indications that some $\gamma\delta$ T lymphocytes can mediate cytotoxicity with variable involvement of MHC (30). The role of $\gamma\delta$ T lymphocytes in mammary gland defense remains to be elucidated. However, a significant reduction in the proportion of these cells during times of increased susceptibility to disease suggests that they may constitute an essential line of defense against mastitis-causing bacteria.

In conclusion, early prevention of mastitis is effective in reducing the chances of progression and recurrence of infection (3). Preventing mastitis in lactating women is important for it is a potentially serious infection that can compromise milk quality and secretion in current and subsequent lactations. Data from this study indicate that alterations in leukocyte trafficking and diminished mammary parenchyma lymphocyte functions in bovines play a central role in increased susceptibility to infection. Therefore, targeting lymphocytes for immunoregulation may be strategic in controlling both human and bovine mastitis.

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