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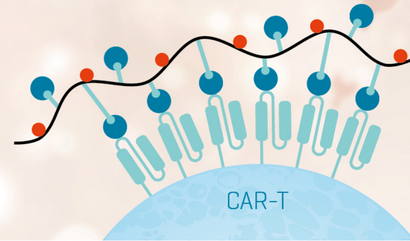

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CELLULAR DETERMINANTS OF MAMMARY CELL-MEDIATED IMMUNITY IN THE RAT

I. The Migration of Radioisotopically Labeled T Lymphocytes[1]

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Current theories about the cellular basis of mammary gland immunity are based primarily on the migratory behavior of B lymphocytes bearing intracytoplasmic IgA. These B cells presumably constitute an intestinal pool that circulates independently of the peripheral B cell pool and provides a source of plasma cell precursors for secretory tissues. The hypothesis of a common, yet independent, mucosal immune system has not been applied to mammary gland cell-mediated immunity (CMI). The present study was undertaken, therefore, to compare the migration of T lymphoblasts from gut-associated mesenteric lymph nodes (MLN) with that of their counterparts recovered from cervical lymph nodes (CLN). When labeled with ^{3}H-thymidine and adoptively transferred to lactating recipients, MLN and CLN T lymphoblasts demonstrated equal affinities for the mammary gland. This result suggests that the mammary gland can draw from both circulating pools of T cells (intestinal and peripheral). T cell migration to the mammary gland was found to increase 7- to 10-fold with the onset of lactation and remained high during the first 2 wk postpartum. Activation of MLN and CLN T cells by preculture with Con A greatly increased the proportion of large cells but did not alter cell accumulation in mammary tissues. These results, discussed in the context of recent observations regarding T cell locomotion and circulating lymphocyte subsets, suggest that CMI in the mammary gland may not depend solely on oral immunization for its immunologic specificity.

Studies of the cellular basis of colostral secretory immunity have recently provided a model system for local immunity at extra-intestinal sites. With regard to the mammary gland B lymphocyte, a clear picture is now emerging as to its migratory characteristics and the properties of glandular development controlling its accumulation in peri-alveolar connective tissues (1-10). Based on histologic evidence (1-4) we now know that IgA-secreting plasma cells begin to accumulate in the mammary parenchyma of the rat and mouse shortly before parturition and constitute the predominant immunoglobulin-secreting cell type. This process continues throughout lactation (1, 2) and is probably triggered by hormone-induced changes in the exocrine gland (5, 6). During lactation, mammary tissue is also receptive to adoptively transferred B lymphocytes, particularly those capable of IgA synthesis and secretion (2-4, 6-8). Mammary-seeking B cells are thought to be large, cycling cells (11-14) belonging to a pool of lymphocytes with affinity for secretory tissues and associated lymphoid structures, especially those of the small intestine (2, 4, 8). This premise is derived from the observation that intestine-seeking B cells prepared from mesenteric lymph nodes can be labeled with ^{3}H-thymidine (^{3}H-TdR)[2] and will migrate to the mammary gland when adoptively transferred to syngeneic lactating recipients (2-4). On the basis of these observations, a number of investigators have persuasively argued that colostral antibody content reflects immunizing events within the intestinal lymphoid tissues and depends, at least in part, on the migration of gut-derived antibody-forming cell precursors to distant secretory organs (2-5, 7, 8). This hypothesis in turn is further supported by the specificity of naturally occurring colostral antibody (15-17) and antibody responses arising after intentional oral immunization (8, 18-22).

The cellular basis of colostral cell-mediated immunity (CMI) is not as clearly defined. Indeed, although some support for an intestinal derivation of colostral CMI has been forthcoming (23-25), certain notable inconsistencies have also emerged. For instance, human colostrum contains antigen-reactive T lymphocytes specific for at least 1 species of enterobacteria (K1^{+} *Escherichia coli*), but it apparently lacks CMI to *Candida albicans* (23), which is normal flora for the human gastrointestinal tract (26). For this reason, we embarked on a study designed to elucidate the origins of T lymphocytes in the lactating rat mammary gland using standard radioisotopic and adoptive transfer techniques. Reported here are data suggesting that, in the rat, T lymphocyte migration to the mammary gland is not restricted by the same processes governing B lymphocyte migration. More specifically, mammary gland T cells appear to be equally derived from peripheral and intestinal lymphoid organs, suggesting that colostral CMI may not depend solely on oral immunization for its immunologic specificity.

MATERIALS AND METHODS

Animals. Female Fisher strain rats weighting 150 to 200 g were obtained from Charles River (Wilmington, MA) or Microbiological Associates (Bethesda, MD) and maintained at the

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[2] Abbreviations used in this paper: CLN, cervical lymph node; CMI, cell-mediated immunity; FITC, fluorescein isothiocyanate; ^{3}H-TdR, tritiated thymidine; ^{125}I-UdR, ^{125}I-deoxyuridine; MLN, mesenteric lymph node; TdR, thymidine.

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University of Kansas Medical Center Animal Laboratories. For studies requiring lactating animals, Fisher females were mated with Dark Agouti (DA) males and were used as cell recipients from 1 to 5 days or 9 to 13 days postpartum. Lactating recipients were left with their litters throughout the experiments.

Preparation of cell suspensions. Intestinal (i.e., mesenteric) or peripheral (i.e., superficial cervical) lymph nodes (MLN and CLN, respectively) were obtained from normal virgin Fisher females. Monodispersed cells were prepared by gently teasing the nodes in Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY) containing 10% calf serum and pressing the resultant cell suspension through a stainless steel screen (40 mesh). Viabilities were determined by trypan blue exclusion and ranged from 85 to 95%. Cells were labeled with ^{3}H-TdR (spec. act. = 57 Ci/mmol; Schwarz/Mann, Orangeburg, NY) by incubating them at a concentration of 5×10^7 cells/ml for 90 min at 37°C in HBSS containing 10% calf serum and 25 μCi ^{3}H-TdR/ml. After labeling, the cells were washed 4 times in HBSS–10% calf serum, and 8 to 10×10^7 viable cells in 0.5 to 1.0 ml were injected via the lateral tail vein into ether anesthetized virgin or lactating recipients. Duplicate 20-μl aliquots of the injection inocula were retained to determine the amount of injected radioactivity.

Preparation of T cell suspensions. Mesenteric and cervical T lymphocytes were prepared with nylon wool columns according to the method of Julius *et al.* (27). The cells were subjected to 2 successive nylon wool fractionations to achieve maximum enrichment, and viabilities were always >95%. The resultant nonadherent T cells were labeled with ^{3}H-TdR as described above, and 8 to 10×10^7 were injected i.v. The degree of T cell enrichment was determined by indirect membrane immunofluorescence with antisera that are described below. Mesenteric T cell preparations were found to be >91% T^+, <3% IgM^+, and cervical T cell preparations were >95% T^+, <1% IgM^+.

In vitro concanavalin A (Con A) stimulation. Populations of MLN and CLN cells were cultured *in vitro* at a concentration of 1×10^6 cells/ml in RPMI 1640 medium containing 5% fresh rat serum, 4 mM L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml. (Lewis × Brown Norway)F_1 animals were used as serum donors for most cultures. Ten-milliliter volumes of cells were cultured in 50 ml plastic tissue culture flasks (Falcon No. 3013) in the upright position. Con A (Sigma, St. Louis, MO) at a final concentration of 5 μg/ml was added to each flask, which was then incubated for 3 days at 37°C in a humid atmosphere of 5% CO_2. On the 3rd day, the cultures were labeled with 1 μCi ^{3}H-TdR/ml for 7 to 8 hr. The cells were then washed 4 times, and 1 to 2×10^7 cells were injected i.v. into lactating recipients. Samples of each culture were subjected to cytocentrifugation and Giemsa staining and always consisted of greater than 75% large lymphoblasts (>12 μ diameter). Analysis of cultured cells for ^{3}H-TdR uptake by liquid scintillation counting revealed that Con A-treated MLN cells had a median stimulation index[3] of 100.4 and Con A treated CLN cells demonstrated a median stimulation index of 49.3.

Control cell populations were exposed to Con A for 90 min, labeled with ^{3}H-TdR, and washed 4 times. Eighty to 100 million labeled cells were then injected i.v. into lactating recipients. Examination of these cell populations revealed <5% large lymphocytes (>12 μ diameter), even if the cells were maintained under tissue culture conditions for an additional 24 hr.

In vivo distribution of labeled cells. Twenty to 24 hr after cell transfer, recipients were killed by ether anesthesia and cervical dislocation. Tissues were removed and weighed, samples were solubilized in Soluene-350 (Packard Instruments, Downers Grove, IL), and radioactivity was determined by liquid scintillation counting (Beckman LS 7000, St. Louis, MO). Care was taken to dissect mammary tissue free of lymph nodes. Intestinal contents and Peyer's patches were removed before processing. All results were corrected for background counts and quenching by the external standard method using individual tissue quench curves. Results were expressed as either percent injected radioactivity per gram recipient tissue or percent injected radioactivity per organ.

Antisera. Rabbit anti-rat IgM, goat anti-rabbit IgG, and rabbit anti-mouse IgG were obtained from Miles Laboratories (Elkhart, IN). The goat anti-rabbit IgG and the rabbit anti-mouse IgG were conjugated with fluorescein isothiocyanate (FITC) according to the method of Wood and Beutner (28) and adsorbed twice with rat spleen cells. All antisera underwent heat inactivation (56°C for 10 min) and ultracentrifugation for 60 min at 135,000 × G before used. Aliquots were stored at −70°C.

For preparation of rabbit anti-rat T cell antiserum, 6 to 10×10^7 pooled MLN and CLN nylon-nonadherent T cells were emulsified in complete Freund's adjuvant and injected subcutaneously into New Zealand White rabbits. This injection procedure was repeated 3 times at weekly intervals. Nine days after the last injection, the animals were bled. The serum was adsorbed twice with rat liver and bone marrow cells and then subjected to heat inactivation and ultracentrifugation. T cells were also detected using a mouse monoclonal IgG antibody against rat thymocyte membranes (29) (W3/13 HLK, Accurate Chemical and Scientific Corp., Hicksville, NY) with virtually identical results.

Indirect Immunofluorescence. For the detection of IgM^+ cells, aliquots containing 1 million cells were incubated with an optimum concentration (1:80) of the IgG fraction of rabbit anti-rat IgM for 30 min, washed, and then exposed to an optimum concentration (1:50) of FITC-goat anti-rabbit IgG. Labeled cells were examined for surface fluorescence using a Zeiss fluorescence microscope equipped with an HBO 200 light source, Schott BG12 excitation filter, and Schott 65 and 50 barrier filters. At 400× magnification, 200 cells were examined, and only cells showing discrete surface patches were scored as positive. Under these conditions the rabbit anti-rat IgM reacted with 30 to 38% of MLN or CLN cells, 35 to 45% of spleen cells, and 0.5% of thymocytes. Each of these cell types were also incubated with either normal rabbit serum and FITC-goat anti-rabbit IgG or FITC-goat anti-rabbit IgG alone to test for possible cytophilic antibodies in these reagents and always showed <2% labeling.

To detect T cells, aliquots containing 1 million cells were incubated with an optimum concentration (1:80) of rabbit anti-rat T cell antiserum for 30 min, washed, and incubated with a 1:50 dilution of FITC-goat anti-rabbit IgG. The cells were again washed, and surface fluorescence was evaluated as above. This antiserum reacted with 55 to 65% of MLN or CLN cells, 45 to 55% of spleen cells, 95% of thymocytes, and <1% of bone marrow cells. Controls similar to those described above were employed with each assay. Alternatively, T cells were enumerated with an optimum concentration (1:200) of mouse monoclonal IgG antibody against rat thymocyte membranes. A 1:50 dilution of FITC-rabbit anti-mouse IgG was then applied, and surface fluorescence was evaluated as above. This antiserum reacted with 65 to 75% of MLN or CLN cells, 45 to 55% of spleen cells,

[3] Stimulation index $= \dfrac{\text{cpm, Con A-treated cells}}{\text{cpm, untreated cells}}$.

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and 98% of thymocytes. Each of these cell types, when incubated with either a 1:80 dilution of normal mouse serum and FITC-rabbit anti-mouse IgG or FITC-rabbit anti-mouse IgG alone, always exhibited <1% labeling.

Autoradiography. ^{3}H-TdR-labeled cell preparations were deposited on glass microscope slides by cytocentrifugation, dipped in photographic emulsion (NTB-3; Eastman-Kodak, Rochester, NY), and developed after 48 hr. The autoradiographs were then stained with Wright-Giemsa and examined for labeled cells by light microscopy.

Statistical analysis. Calculation of the means and standard deviations were performed, and p values were determined by nonpaired, 2-tailed Student's *t*-test. Significance levels of $p<0.05$ and $p<0.01$ were used.

RESULTS

To compare the ability of peripheral and intestinal lymphoid cells to accumulate in rat mammary tissues, CLN and MLN cells (respectively) were labeled with ^{3}H-TdR and injected into lactating syngeneic recipients. Similar to the findings reported by others (2, 30), we observed that MLN lymphoblasts migrated to the small intestine and associated lymph nodes of lactating adoptive recipients in far greater numbers than did cervical lymphoblasts (Table I).The rat mammary gland, however, was unable to discriminate between the 2 cell types and accumulated equal quantities of CLN and MLN cells. This is in contrast to reports concerning the homing of mouse lymph node cells (mesenteric *vs* "peripheral") in which mesenteric cells generally migrated more efficiently to the lactating mammary gland than did peripheral lymph node cells (2–4).

To investigate the determinants of mammary CMI, we prepared T cell-enriched populations of MLN and CLN cells by nylon wool adherence, labeled the cells with ^{3}H-TdR, and injected them into lactating recipients. T lymphocytes (>91% T^+, <3% IgM^+) evidenced virtually the same propensity for mammary tissue as did the unfractionated cells, with cervical T cells homing in numbers equivalent to those seen with the gut-derived population. Thus, the mammary gland seemed to be equally receptive to radiolabeled cervical and mesenteric T lymphoblasts. Mesenteric T cells were detected in significantly greater numbers in the small intestine than were T lymphoblasts prepared from CLN, suggesting that these 2 secretory tissues accumulated different numbers and/or different subsets of T cells from the circulating lymphocyte pool.

As the tendency for B cell accumulation in mammary tissues of adoptive recipients has been shown to depend on the stage of lactation of the recipient (1, 2), we next examined the effect of glandular development on rat T lymphocyte migration. Syngeneic recipients were used that were either virgin, postpartum and early in the lactation period (1 to 5 days), or lactating and 9 to 13 days postpartum. For the lactating recipients, the newborns were left with their mothers and allowed to suckle until the time of sacrifice and tissue recovery. Since comparisons between these groups were greatly affected by overall pregnancy-associated changes in organ weights (1), the data in Table II are reported as the percentage of injected radioactivity recovered per organ rather than per gram tissue weight. Viewed in this way it is apparent that the most significant change in the pattern of T lymphocyte migration accompanying the onset of lactation is a 7- to 10-fold increase in T cell migration to the mammary gland. On a per weight basis (% injected radioactivity/g tissue), lactating mammary tissue demonstrated a 3- to 4-fold greater accumulation of labeled T lymphocytes than did virgin mammary tissue (data not shown). The tendency of the rat mammary gland to trap circulating T lymphoblasts continued well into lactation and was seen with cells derived from CLN as well as MLN.

Parrott *et al.* (30, 31) have cautioned that accurate comparisons of homing tendencies between various ^{3}H-TdR-labeled lymphoblast populations may be affected by the relative proportions of lymphoblasts and radiolabeled cells in the 2 populations. We examined our preparations by autoradiography and found that labeled cells were distributed exclusively among the large cell fractions (>12 μ diameter) in all cell populations (Table III). Similar percentages of unfractionated MLN and CLN cells incorporated ^{3}H-TdR, and the proportions were not altered significantly by nylon wool-adherence columns. Overall, these data are comparable to those reported by Guy-Grand *et al.* (14) for the mouse and argue that comparisons between our 2 T cell populations, which are actually based on lymphoblast activity, are justifiable.

Realizing that ^{3}H-TdR labeled exclusively large T lymphoblasts, we were interested in determining whether techniques that enrich lymphocyte populations with lymphoblasts might increase nonspecific T cell entrapment in mammary tissues. Such an effect has been reported for lymphocyte migration to the lung shortly after adoptive transfer (12). Culturing lymph node cells with Con A for 3 days dramatically increased the proportion of large cycling cells as well as the proportion of labeled lymphoblasts detected by autoradiography (Table III). However, surface Con A is also known to alter lymphocyte migration nonspecifically (32–36), presumably by membrane

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TABLE I

Migration of ^{3}H-thymidine-labeled mesenteric and cervical lymph node cells or T lymphocytes in lactating adoptive recipients[a]

Recipient Tissues	Lymph Node Cells			Lymph Node T Lymphocytes		
	Mesenteric (n = 5)	Cervical (n = 5)	MLN/CLN	Mesenteric (n = 5)	Cervical (n = 5)	MLN/CLN
Mammary gland	0.30 ± 0.15	0.28 ± 0.09	1.1	0.32 ± 0.05	0.29 ± 0.07	1.1
Small intestine	6.69 ± 1.75	2.08 ± 0.44	3.2**[b]	3.46 ± 1.12	2.16 ± 0.89	1.6*
MLN	6.00 ± 0.98	1.84 ± 0.53	3.3**	4.27 ± 0.77	5.71 ± 3.48	0.8
CLN	2.11 ± 0.54	2.70 ± 0.19	0.8*	2.74 ± 0.48	5.47 ± 3.07	0.5*
Spleen	3.02 ± 0.93	3.61 ± 0.44	0.8	5.99 ± 0.73	4.71 ± 1.95	1.3
Lung	1.07 ± 0.23	3.92 ± 1.31	0.3**	0.71 ± 0.37	1.54 ± 0.56	0.5*

[a] Either unfractionated lymph node cells or nylon wool purified T lymphocytes were prepared, labeled with ^{3}H-TdR and 8 to 10 × 10^7 cells were injected into syngeneic lactating recipients (1 to 5 days postpartum). Twenty to 24 hr later, the recipients were sacrificed, tissues were collected and radioactivity was determined. The results of several such experiments are expressed as % injected radioactivity/gram of recipient tissue or as a ratio of % injected MLN to % injected CLN cells.

[b] * $p < 0.05$ (Student's *t* test) comparing MLN cell and CLN cell recipients; ** $p < 0.01$ (Student's *t* test) comparing MLN cell and CLN cell recipients.

TABLE II

Effect of lactation status on the migration of ^{3}H-thymidine T lymphocytes to the rat mammary gland[a]

Recipient Tissues	Cells Injected: / Recipient Status:	MLN T Lymphocytes: Virgin	MLN T Lymphocytes: Lactating (1–5 days)[b]	Lac/Vir	MLN T Lymphocytes: Lactating (9–13 days)	Lac/Vir	CLN T Lymphocytes: Virgin	CLN T Lymphocytes: Lactating (1–5 days)	Lac/Vir	CLN T Lymphocytes: Lactating (9–13 days)	Lac/Vir
Mammary gland		0.27 ± 0.10	2.72 ± 0.24	10.1**[c]	2.35 ± 0.15	8.7**	0.33 ± 0.10	2.23 ± 0.77	6.8**	2.17 ± 0.41	6.8**
Small intestine		19.32 ± 5.34	15.19 ± 3.38	0.8	21.08 ± 2.60	1.1	6.71 ± 2.28	8.44 ± 2.33	1.3	5.80 ± 1.18	0.9
MLN		0.69 ± 0.13	0.94 ± 0.25	1.4	0.37 ± 0.13	0.5**	0.17 ± 0.03	0.69 ± 0.30	4.1**	0.20 ± 0.06	1.2
CLN		0.72 ± 0.29	0.54 ± 0.08	0.8	0.18 ± 0.06	0.3**	0.50 ± 0.15	0.56 ± 0.25	1.1	0.23 ± 0.07	0.5*
Spleen		2.94 ± 1.05	3.49 ± 0.82	1.2	2.12 ± 0.43	0.7	2.33 ± 0.50	2.63 ± 1.09	1.1	1.94 ± 0.63	0.8
Lung		1.78 ± 0.38	1.19 ± 0.45	0.7*	0.44 ± 0.10	0.2**	3.29 ± 0.78	1.56 ± 0.57	0.5**	0.68 ± 0.20	0.2**

[a] Virgin or lactating recipients were injected with 8 to 10 × 10^7 ^{3}H-TdR labeled T lymphocytes and treated as described in Table I. Results are expressed as % injected radioactivity/organ to adjust for large changes in organ weights observed in lactating animals (e.g., 2.0- to 2.9-fold increase in mammary gland weight; 1.5- to 2.7-fold increase in small intestine weight).

[b] Lactating recipients were either 1 to 5 days or 9 to 13 days postpartum (n = 5 to 6 animals/group).

[c] * $p < 0.05$ (Student's t test) comparing virgin and lactating recipients; ** $p < 0.01$ (Student's t test) comparing virgin and lactating recipients.

TABLE III

Distribution of size and radiolabel (^{3}H-thymidine) among lymph node lymphocytes

Cell Type	% Large Cells (>12 μ)	% Labeled Cells[a]	Size Distribution (Diameter) among Labeled Cells (%): <12 μ	12–15 μ	>15 μ
MLN cells	8.5 ± 1.7	3.1 ± 1.1	0	63.8 ± 7.8	36.2 ± 7.8
CLN cells	7.6 ± 2.0	2.3 ± 0.6	0	64.9 ± 8.5	35.1 ± 8.5
MLN T cells	5.4 ± 1.0	2.0 ± 0.5	0	64.5 ± 6.5	35.5 ± 6.5
CLN T cells	5.5 ± 0.8	1.9 ± 0.6	0	60.3 ± 10.3	39.7 ± 10.3
MLN blasts[b]	88.2 ± 4.2	71.4 ± 1.6	0	24.3 ± 5.5	75.4 ± 5.9
CLN blasts	89.8 ± 3.4	62.0 ± 3.9	0	24.2 ± 7.7	75.8 ± 7.7

[a] The percentage of labeled cells was determined by examining autoradiographs of cytocentrifuge preparations of ^{3}H-TdR labeled cell inocula.

[b] MLN or CLN cells were stimulated with Con A for 72 hr (see Table V).

TABLE IV

Effect of brief concanavalin A treatment[a] *on the localization of ^{3}H-thymidine-labeled lymph node lymphocytes in lactating adoptive recipients*

Recipient Tissues	MLN Cells: Untreated (n = 5)	MLN Cells: Con A-treated (n = 4)	CLN Cells: Untreated (n = 5)	CLN Cells: Con A-treated (n = 4)
Mammary gland	0.30 ± 0.15	0.41 ± 0.11	0.28 ± 0.09	0.38 ± 0.06
Small intestine	6.69 ± 1.75	3.37 ± 0.93**[b]	2.08 ± 0.44	0.74 ± 0.14**
MLN	6.00 ± 0.98	3.26 ± 0.55**	1.84 ± 0.53	2.62 ± 0.84
CLN	2.11 ± 0.54	2.49 ± 0.55	2.70 ± 0.19	3.43 ± 1.22
Spleen	3.02 ± 0.93	3.49 ± 0.60	3.61 ± 0.44	4.15 ± 1.44
Lung	1.07 ± 0.23	1.04 ± 0.30	3.92 ± 0.13	0.88 ± 0.31**

[a] MLN or CLN cells were either labeled with ^{3}H-TdR or labeled and simultaneously treated with concanavalin A (5 μg/ml) for 90 min, washed extensively and injected into lactating recipients (1 to 5 days postpartum). After 20 to 24 hr tissues were recovered and radioactivity determined. Data is expressed as % injected radioactivity/gram of recipient tissue.

[b] ** $p < 0.01$ (Student's t test) comparing untreated to Con A-treated cell recipients

alterations induced by surface-bound lectin (32, 33, 36). Among its effects is a reduction in lymphocyte homing to the small intestine of adoptive recipients (32). To assure ourselves that similar nonspecific effects did not occur that might affect migration to the mammary gland, we treated MLN and CLN cells with Con A for 90 min while the cells were also being labeled with ^{3}H-TdR. Each preparation was then washed extensively and injected into lactating recipients. Control cells maintained in culture for an additional 24 hr showed no increase in the proportion of large (>12 μ diameter) cells. As reported by others (32), cells treated briefly with Con A evidenced reduced migration to the small intestine as well as to the MLN (Table IV). No such altered cell homing to the mammary gland was observed with either lymph node cell type, suggesting that homing to these 2 secretory organs was not an equivalent process. These results also indicated that lymph node cells stimulated by Con A could be utilized in adoptive transfer studies, since residual Con A on their surfaces had no effect on their localization to the mammary glands of lactating recipients.

MLN and CLN lymphocytes were then prepared, treated with Con A for 3 days, labeled with ^{3}H-TdR, and washed. Autoradiography revealed that these preparations consisted primarily of lymphoblasts (88 to 90%; Table III) and that 62 to 71% were labeled. Immunofluorescence demonstrated 3% IgM^+, 76% T^+ cells among Con A lymphoblasts. Twenty to 24 hr after transfer to lactating recipients, tissues were examined for radioactivity. The mammary glands showed essentially the same degree of cell accumulation with these large lymphoblast preparations (Table V) as with preparations consisting predominantly of small lymphocytes (Table IV). By contrast, the lung, small intestine, and spleen trapped significantly more Con A lymphoblasts than control Con A-treated cells. These results with mammary gland homing were obtained whether MLN or CLN T lymphoblasts were injected and suggest that T cell

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TABLE V

Migration of ^{3}H-thymidine labeled Con A lymphoblasts in lactating adoptive recipients[a]

Recipient Tissues	MLN Cells (n = 4)		CLN Cells (n = 5)	
	Con A lymphoblasts	Blasts/control[b]	Con A lymphoblasts	Blasts/control
Mammary gland	0.55 ± 0.21	1.3	0.38 ± 0.10	1.0
Small intestine	2.49 ± 0.62	0.7	2.02 ± 0.20	2.7**[c]
MLN	4.26 ± 0.92	1.3	4.32 ± 1.08	1.6*
CLN	1.75 ± 0.32	0.7*	1.64 ± 0.34	0.5*
Spleen	6.47 ± 1.75	1.9*	4.32 ± 1.08	1.1
Lung	2.23 ± 1.48	2.1	2.25 ± 0.28	2.6**

[a] Mesenteric or cervical lymph node cells were cultured with 5 μg concanavalin A/ml for 72 hr and then labeled with ^{3}H-TdR. After extensive washing 7 to 12 × 10^6 viable cells were injected i.v. into lactating (1 to 5 days postpartum) recipients and 20 to 24 hr later radioactivity of recipient tissues was assessed. Data is expressed as % injected radioactivity/gram.

[b] Control cells were treated with Con A for only 90 min, labeled and injected (see Table IV).

[c] * $p < 0.05$ (Student's t test) comparing Con A lymphoblasts with control cell recipients (Table IV); ** $p < 0.01$ (Student's t test) comparing Con A lymphoblasts with control cell recipients (Table IV).

affinity for the mammary gland requires more than simply the increase in cell size that accompanies lymphocyte activation.

DISCUSSION

The principal impetus for the experiments reported here was the suspicion, based on functional analyses of human colostral T cells, that mammary CMI may not be derived exclusively from gut-associated lymphoid tissues. In studying lymphocyte migration in the rat, we observed that the lactating mammary gland was indeed equally receptive to peripheral and intestine-associated lymphocytes. This was in contrast to the small intestine, which accumulated greater numbers of radiolabeled MLN than CLN. These results are somewhat inconsistent with the reports of Roux *et al.* (2) and McDermott and Bienenstock (4), who demonstrated preferential homing of ^{125}I-deoxyuridine- (^{125}I-UdR) or ^{3}H-TdR-labeled MLN cells to the mouse small intestine and mammary gland when compared with the migration of peripheral lymph node cells. We feel this most likely represents a species difference (rat *vs* mouse) rather than essential differences in the way the experiments were performed.

One essential difference between our experimental design and those of others (2, 7, 13) was the choice of CLN cells to represent "peripheral" lymphoid tissue. This choice was based on the following considerations: i) Prior adoptive transfer studies of secretory immunity that demonstrated preferential homing of MLN cells to the small intestine used a mixture of peripheral node cells, including the superficial and deep cervical, for control populations (2, 7, 13). ii) The axillary, brachial, and inguinal nodes drain the skin and its appendages (including the mammary gland) in the thoracic and abdominal areas. Since cells from these nodes might be expected to demonstrate an enhanced affinity for the mammary gland, we decided not to include them. The most appropriate peripheral lymph node might have been the popliteal, since it drains neither secretory nor mammary tissue, but the number of cells from these nodes was inadequate to perform the studies described. iii) It was apparent from preliminary studies that rat CLN cells showed the same pattern of migration in adoptive recipients as had been published for other peripheral lymph node cells in the mouse and rat (13, 14). Griscelli *et al.* (13), for example, compared cervical and pooled peripheral lymph node cells and found no differences in their homing characteristics in adoptive recipients.

When purified T lymphoblasts from these 2 lymph nodes were compared (Table II), MLN T cells showed a greater tendency to return to the small intestine of both virgin and lactating recipients than did their CLN counterparts, whereas MLN and CLN T lymphoblasts evidenced an equal affinity for the mammary gland. Thus, these 2 secretory sites (small intestine and mammary gland) were decidedly different as targets of migrating T cells, and it is apparent that the rat mammary gland is not as restricted in its sampling of circulating T lymphoblasts as is the small intestine.

These findings are generally in agreement with those of Rose *et al.* (37–39) and Parrott (30) who have reported that ^{125}I-UdR-labeled mouse MLN T lymphoblasts homed more readily to the small intestine than did oxazolone-stimulated peripheral T lymphoblasts. Cell migration to the mouse mammary gland, however, was exhibited by both unfractionated mesenteric lymphoblasts and peripheral T lymphoblasts (30, 39). Since peripheral T blasts could also enter the peritoneal cavity and sites of inflammation, whereas MLN lymphoblasts could not (39), the authors suggested that large T lymphoblasts can be assigned to 2 distinct pools of circulating cells. Our current results suggest that mammary T cells, unlike intestinal T cells, are derived in part from both pools.

A distinction can also be made between T and B lymphoblast migration to the mammary gland. Although both involve large cycling cells that accumulate in larger numbers with the onset of lactation (1, 2, 30; Table II), mammary gland plasma cell precursors are significantly more frequent in MLN than in CLN (2–4, 8). Thus, Roux *et al.* (2) reported that the numbers of IgA$^+$ ^{125}I-UdR-labeled MLN cells that homed to the mouse mammary gland increased with lactation, remained high throughout the first 10 days postpartum, and at all times far outnumbered IgA$^+$ B cells derived from peripheral lymph nodes. Using autoradiographic techniques, McDermott and Bienenstock (4) have provided similar data for a single animal at 19 days of gestation. Likewise, T lymphoblasts in the present study exhibited an increased access to the postpartum mammary gland that continued into the second week of lactation; however, the homing efficiencies of peripheral and mesenteric T cells were nearly identical.

In vitro studies of locomotion by T lymphocytes have demonstrated that stimulation of these cells by polyclonal activators (e.g., PHA or Con A) can enhance their chemokinetic and chemotactic activities (40–42). These results initially suggested that the induction of lymphoblasts favored enhanced locomotion (40), but subsequent reports have demonstrated that both small and large T cells can be so affected by preculture and activation (42, 43). For this reason, one might predict that activation of T cells with Con A *in vitro* might alter their migration *in vivo*. Since casein, a major protein constituent in colostrum and milk, is an effective chemotactic agent for Con A-activated T cells (41, 42), we were interested in examining T lymphoblast migration to the mammary gland of lactating adoptive recipients. We found that Con A pretreatment of rat lymph node cells (>75% T$^+$) did not alter their attraction to the

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mammary gland, although alterations in cell migration to other organs were observed. We would not interpret this observation as inconsistent with *in vitro* studies of T cell locomotion, but it does indicate that generation of lymphoblasts *per se* is not adequate for enhanced mammary gland homing.

Large scale reutilization of free or released radiolabel would alter the interpretation of our data as well as that of others, but we do not consider this a particularly serious problem for the following reasons: i) In the present study, at least 75% of the radioactivity detected in recipient tissues was still associated with donor lymphoid cells 24 hr after transfer. This conclusion was derived from 2 experiments. Injections of a million-fold excess of nonradioactive thymidine (TdR) (44, 45) (125 μg TdR/g body weight 3 times within 24 hr) together with ^{3}H-TdR-labeled MLN cells reduced recoverable radioactivity in individual recipient tissues by 25 to 30%. When approximately 1 μCi (9×10^5 cpm) of free ^{3}H-TdR was injected i.v. into lactating recipients, recovery of radioactivity was never greater than 25% of that seen with an equivalent injection dose of cells (i.e., $\sim 10^6$ cpm/10^8 cells). This level compares favorably with that reported by Rose *et al.* (37), who assessed the problem of ^{125}I-UdR reutilization in a similar system. Therefore, even if all of the ^{3}H-TdR were released from labeled cells (which is unlikely), one would not obtain the data reported in this manuscript. ii) We have recently begun using autoradiography to assess the migration of ^{3}H-TdR-labeled T cells in lactating animals. Recipient tissues assayed in this fashion have demonstrated labeled cells in frequencies directly proportional to the distribution of radioactivity assessed by liquid scintillation counting. Previous reports (2, 4, 13, 14) have demonstrated that both techniques produce equivalent results when utilized to monitor lymphoblast migration to the small intestine or mammary glands, and our data concerning T lymphoblast accumulation within the small intestine are in complete agreement with these reports.

Several authors have commented on the possible competition between the small intestine and the mammary gland as target tissues for lymphoblast migration (2, 30, 39, 46, 47). The discussion has arisen from observations made by Love and Ogilvie (46) that radiolabeled thoracic duct lymphocytes (TDL), which normally migrated to the parasitized small intestine, were diverted to the mammary gland during lactation. Although the same proportion of injected TDL homed to the small intestine in both groups of recipients, the small intestines of lactating animals had increased 2.5-fold in weight. Thus, on a radioactivity per gram basis, small intestine localization was less in lactating recipients. We observed a similar phenomenon with purified T lymphoblasts (Table II). This is an important distinction and can be lost when comparisons are made on a radioactivity per organ basis. In either case, we would argue that by itself this data does not distinguish between the 2 current hypotheses explaining lymphocyte migration to the mammary gland. The first proposes that the mammary gland and the small intestine share a single common migrating cell population, and total cell accumulation is increased during lactation by the availability of more target tissue (2–4, 8). The second suggests that there are indeed 2 subsets of circulating lymphoblasts, a "gut-associated" subset capable of migrating to either organ and a "peripheral" subset accumulated by the lactating mammary gland but not by the intestine (30, 39). Although the first hypothesis may be adequate to explain MLN-derived B cell migration into the gland, it clearly does not deal well with the activity of T lymphoblasts, since migration to the mammary gland, but not to the intestine, is increased with the onset of lactation (Table II). Final resolution of this issue depends on direct evidence that 2 antigenically distinct T cell subsets exist with characteristic migratory behavior.

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