LYMPHOCYTE SUBSETS SHOW MARKED DIFFERENCES IN THEIR DISTRIBUTION BETWEEN BLOOD AND THE AFFERENT AND EFFERENT LYMPH OF PERIPHERAL LYMPH NODES

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Lymphocytes continually recirculate between the bloodstream, extravascular tissues, lymph nodes, and lymph (1). This circulation of cells could provide a means whereby large numbers of lymphocytes with different antigen specificities may encounter antigen, allowing specific antigen-reactive cells to be selected out and to accumulate at sites of antigen deposition. Lymphocytes leave the blood at particular traffic sites in lymph nodes where they pass between the endothelial cells of post-capillary venules (PCV).[1] Such lymphocyte-endothelial interactions appear to be mediated by specific cell surface molecules (2, 3). From studies on afferent lymph, it is clear that there is a continuous low grade migration of lymphocytes and monocytes from blood to lymph (4). Of the lymphocytes entering a lymph node, ~10% may be derived from afferent lymph, while 85–90% are derived directly from the blood via PCV (4). Afferent lymphatics also transport antigen to the lymph node, as well as cell types other than lymphocytes, such as macrophages, dendritic cells, and under some circumstances, e.g., accute inflammation, granylocytes, and erythrocytes (4, 5). Afferent lymph has also been shown to contain fewer B lymphocytes than blood or efferent lymph (6), and afferent cells behave differently from efferent lymphocytes in graft-vs.-host reactions (6, 7).

The sheep has been used extensively for studies on lymphocyte recirculation, particularly the examination of cells migrating in afferent and efferent lymph (5, 8, 9). Here, we extend these studies using a panel of mAbs and flow cytometry to identify the phenotypes of cells in the afferent lymph and efferent lymph draining to and from a single lymph node as well as the blood of individual sheep. The striking differences in the distribution of lymphocyte subpopulations between compartments, reported in this paper, suggest that the exit of lymphocyte subpopulations (defined by our panel of mAbs) from the blood via traffic sites in lymph nodes and peripheral vascular beds is nonrandom.

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[1] *Abbreviations used in this paper:* PCV, post-capillary venule; sIg, cell surface Ig.

Materials and Methods

Animals and Surgery. Merino ewes, 8–10 mo old, were used in all experiments. They were kept in metabolism cages and given lucerne chaff and water ad libitum.

The popliteal efferent lymph duct and afferent lymph duct draining the lower part of the right hind leg were cannulated, as described by Hall and Morris (10). At the time of surgery, a cannula was placed in the jugular vein for taking blood samples.

Lymph Collection. Lymph was collected continuously in sterile polyethylene bottles that contained 1 ml of normal saline supplemented with 500 U of preservative-free heparin (Commonwealth Serum Laboratories, Melbourne, Australia), 500 IU of penicillin, and 500 μg of streptomycin (Flow Laboratories, Melbourne, Australia). The lymph collection bottles were changed at regular intervals. The cell concentration in each collection was determined using a Coulter Counter fitted with a 100-μM aperature tube (model FN; Coulter Electronics Ltd., Dunstable, Bedfordshire, UK). At least 3 d after surgery, when the changes in the lymph caused by surgery and anesthesia had subsided, lymph was collected for 8 h and the phenotype of cells in lymph and blood was determined. The popliteal lymph node was removed at the same time for preparation of cell suspensions and immunohistology.

mAbs. mAbs to sheep lymphocyte antigens were produced within the Department of Veterinary Preclinical Sciences, Melbourne University, (Melbourne, Australia) and were used as undiluted tissue culture supernatants.

mAbs were characterized by immunoprecipitation and molecular weight analysis and by immunohistology and tissue distribution. Table I summarizes the reactivities of the mAbs and gives the references describing their characterization.

Cell Suspensions. Cell suspensions of lymph nodes were prepared by gentle teasing of lymph nodes with forceps in PBS containing 1% BSA. Clumps were removed by filtering the suspension through cotton gauze, and the cells were washed three times in a solution of PBS/BSA. Peripheral blood was taken from the jugular vein in a solution of EDTA (6.2 mg/ml blood; Ajax Chemicals, Melbourne, Australia) and treated with ammonium chloride to lyse RBC. Ammonium chloride/Tris lysis buffer was prepared by mixing 9 vol of 0.83% ammonium chloride with 1 vol of 0.17 M Tris, and the pH of the Tris-NH_4Cl solution was adjusted to 7.2 before use. The lysis buffer was warmed to 37°C and 5 ml of buffer was used per 1 ml of blood. Erythrocytes usually lysed within 1–3 min. Blood lymphocytes obtained after ammonium chloride lysis were identified on the flow cytometer using 0° and 90° light scatter.

Immunofluorescence and Flow Cytometry. For single-color immunofluorescent staining with mAbs, 10^6 cells were reacted with 50 μl of neat tissue culture supernatant for 30 min at 4°C, were washed three times in a solution of PBS/BSA, and were then reacted with FITC-sheep anti-mouse Ig (Silenus Laboratories, Melbourne, Australia). FITC-donkey anti-sheep Ig (Silenus Laboratories) was used to enumerate cell surface Ig^+ (sIg^+) lymphocytes. After staining, cells were fixed in a solution of 1% paraformaldehyde in PBS containing 0.1% sodium azide.

Analysis of sheep lymphocytes by flow cytometry was performed using a FACS II (Becton Dickinson FACS Systems, Sunnyvale, CA). For one-color immunofluorescent analysis or forward angle light scatter analysis, 10^4 cells were analyzed per sample and dead cells were excluded on the basis of forward light scatter.

Immunoperoxidase Staining. Tissues were submersed in OCT embedding compound (Lab-Tek, Naperville, IL), snap frozen in liquid nitrogen, and stored at −70°C. Frozen sections (3–5 μm) were placed on xylene-cleaned slides, air dried for 1 h, and fixed in absolute alcohol for 10 min at 4°C. Sections were exposed to mAbs for 1 h, washed with PBS, and then reincubated for 1 h with peroxidase-conjugated rabbit anti-mouse Ig (Dako Immunoglobulins, Copenhagen, Denmark). After washing, slides were developed in a solution of diaminobenzidene tetrahydrochloride (Sigma Chemical Co., St. Louis, MO; 0.5 mg/ml in a 0.25 M Tris, 0.14 M NaCl, pH 7.6) and H_2O_2 (0.01%). Sections were then lightly counterstained with hematoxylin.

Cytospots of efferent or afferent lymph lymphocytes were prepared using a cytocentrifuge (Shandon Southern Instruments Inc., St. Sewickley, PA), as previously described (11). Slides were stained by the immunoperoxidase method as described above for tissue sections.

Arithmetic means of the proportion of stained cells in each compartment were compared using the Mann-Whitney U test (12).

TABLE I
Reactivity of mAbs to Sheep Leukocytes

Sheep leukocyte molecule	mAb clone number	Isotype	Distribution	Reference
CD5	25-91	IgG1	Present on all T lymphocytes; absent from B lymphocytes	11
CD4	44-38	IgG2a	Present on subset of T lymphocytes	13
	44-97	IgG1	that are $CD8^-$, $SBU\text{-}T19^-$; absent from B lymphocytes	14
CD8	38-65	IgG2a	Present on subset of T lymphocytes that are $CD4^-$, $SBU\text{-}T19^-$; absent from B lymphocytes	13,14
SBU-T19	19-19	IgG1	Present on subset of T lymphocytes that are $CD4^-$, $CD8^-$; absent from B lymphocytes	14
MHC class I	41-19	IgG1	Most somatic cells	15
MHC class II	28-1	IgG1	Present on B lympohcytes and activated T lymphocytes	16
CD1	20-27	IgG1	Present on cortical thymocytes, dendritic cells and macrophages, and B cells in peripheral blood	11

Results

Phenotypic Analysis of Lymphocytes Obtained Simultaneously from Afferent Lymph, Efferent Lymph, Blood, and Popliteal Lymph Node. Lymphocytes were taken simultaneously from afferent lymph, efferent lymph, blood, and the popliteal lymph node of in-

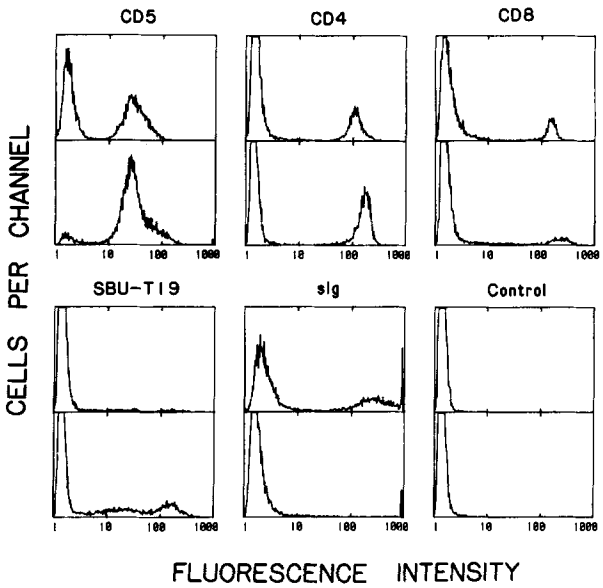

FIGURE 1. FACS analysis of lymphocytes from efferent lymph (*top profile in each panel*) and afferent lymph (*bottom profile in each panel*) stained with mAbs. Macrophages and monocytes were excluded from analysis by gating on 0° and 90° light scatter. Fluorescence intensity is log scale.

TABLE II

Distribution of Lymphocyte Subpopulations between Popliteal Lymph Node, Blood, and the Afferent and Efferent Lymph of a Popliteal Lymph Node

Cell source	Mean percentage cells stained (SE)*						
	CD5	CD4	CD8	SBU-T19	MHC II	sIg	MHC I
Efferent lymph	60.3 (6.5)	38.8 (7.7)	14.0 (1.8)§	10.0 (3.1)	39.5 (7.4)	32.0 (7.0)	100.00
Afferent lymph	89.8 (1.3)‡	38.5 (3.7)	12.8 (1.1)‖	27.8 (2.2)‡	35.3 (3.8)	7.3 (0.3)‡	100.00
Peripheral blood	57.5 (5.6)	14.3 (2.7)‡	8.5 (0.5)§‖	11.8 (2.1)	29.8 (3.8)§	17.5 (5.3)§	100.00
Lymph node	55.5 (6.2)	33.8 (3.9)	13.3 (3.1)	6.5 (1.9)	45.3 (4.9)§	32.3 (3.1)§	100.00

* Arithmetic means of four sheep. Comparisons of the percentages of each subpopulation between the compartments were made using the Mann-Whitney U test. Unless specified, there were no significant differences between compartments as $p \leq 0.05$.

‡ Significantly different at $p \leq 0.05$ from other values in the same column.

§ Significantly different at $p \leq 0.05$ from the other values in the column with the same footnote.

‖ Significantly different at $p \leq 0.05$ from the other values in the column with the same footnote.

dividual sheep, and the cells were reacted with the panel of mAbs (Table I) and were analyzed using flow cytometry.

The distribution of lymphocyte subsets analyzed using flow cytometry is shown in Fig. 1 and Table II. $CD5^+$ lymphocytes were the major lymphocyte subpopulation in all compartments examined. The higher percentage of $CD5^+$ lymphocytes in afferent lymph was statistically different from the percentage of $CD5^+$ lymphocytes in blood, efferent lymph, or in the lymph node, and was associated with a much lower percentage of sIg^+ lymphocytes. Another difference in the distribution of $CD5^+$ lymphocytes was that nearly 25% of T cells in afferent lymph were SBU-$T19^+$. This was in marked contrast to the much lower percentage of SBU-$T19^+$ cells in the lymph node, the efferent lymph draining the node, or the blood supplying the node.

The SBU-$T19^+$ lymphocyte subset in sheep is unique in that these cells are $CD5^+$, but $CD4^-$, $CD8^-$, and sIg^-. The cellular lineage of these cells was uncertain, al-

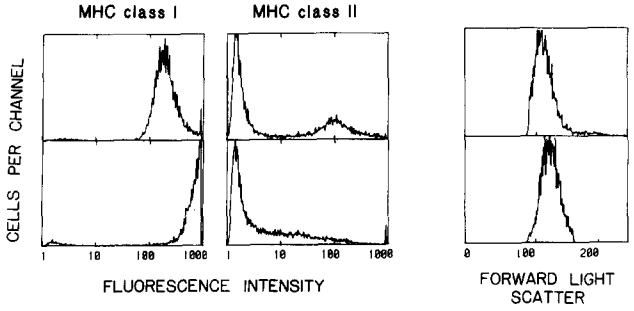

FIGURE 2. MHC antigen expression and cell size of afferent and efferent lymphocytes. Top profiles show efferent lymphocytes, and bottom profiles show afferent lymphocytes.

TABLE III

Hourly Output of Lymphocyte Subpopulations from Afferent and Efferent Popliteal Lymph from an Individual Sheep

Small lymphocyte population	Output of cells/hour			
	Afferent lymph		Efferent lymph	
	$\times 10^{-6}$	%	$\times 10^{-6}$	%
Total	0.96		54.01	
CD5	0.85	87	27.70	54
CD4	0.24	32	15.92	31
CD8	0.07	9	5.64	11
MHC class II	0.24	25	18.55	36
sIg	0.06	8	17.42	34
SBU-T19	0.24	25	3.60	7

Percentage staining was assessed by indirect immunofluorescence and flow cytometry. Cell outputs and fluorescent staining were determined from an 8-h collection of lymph collected at least 3 d after surgery, at a time when the lymph output was regarded as physiologically normal and free from RBC and granulocyte contamination.

though they were originally described as T cells (14) based on the fact they are $CD5^+$ and appear in the thymus during early ontogeny (17). Recently, however, using a rabbit anti-human CD3 antisera, immunoprecipitation of CD3 molecules prepared from a lysate made from purified SBU-$T19^+$ cells showed the presence of three CD3 bands migrating at 19 kD, 20 kD, and 24 kD. CD4 or CD8 antigens could not be precipated from the lysate. (Hein, W., M.-F. Beya, R. Kudo, C. Mackay, and S. McClure, manuscript in preparation).

The percentage of $CD4^+$ lymphocytes was much higher in the lymph node and in afferent and efferent lymph compared with blood although there was no significant difference in the distribution of $CD4^+$ cells between the two lymph compartments and the lymph node. The percentage of $CD8^+$ lymphocytes was higher in afferent and efferent lymph than in peripheral blood but there were no significant differences between the lymph node and the two lymph compartments.

A further lymphocyte subset that was $CD5^+$ but negative for all other markers was present in peripheral blood (22%) and afferent lymph (11%) but could not be demonstrated in the lymph node or efferent lymph. The percentage of null cells (cells that did not express either CD5 of sIg) also varied between the compartments examined. For example, the percentage of null cells in afferent and efferent lymph was <8% compared with up to 25% null cells in blood.

Expression of MHC Antigens on Cells in Different Lymphoid Compartments. Lymphocytes from afferent lymph, efferent lymph, the lymph node and blood were analyzed for expression of MHC class I and class II antigens by immunofluorescent staining and flow cytometry. mAbs used for analysis were reacted with cells under saturating antibody-binding conditions. The percentage of MHC class II^+ lymphocytes was the same in all compartments except the lymph node where the percentage of MHC class II^+ lymphocytes was significantly higher than blood. A striking difference in the distribution of MHC class II^+ lymphocytes was found in afferent lymph compared with other compartments where nearly 30% of T cells were MHC class II^+.

All lymphocytes in blood, efferent and afferent lymph, and lymph nodes were MHC

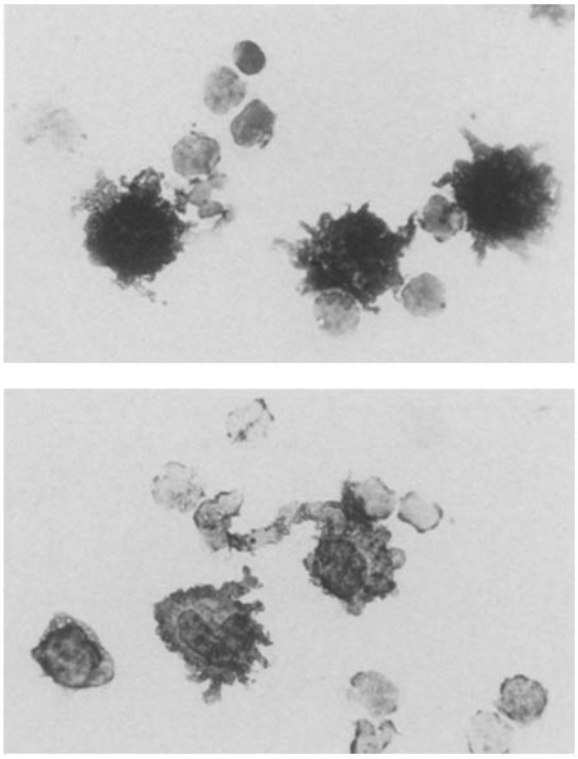

FIGURE 3. Immunoperoxidase staining of afferent lymphatic cells by mAbs. CD1 (20-27), showing reactivity with large macrophage/dendritic cells, but not with lymphocytes (*top*; × 160). MHC class II (28-1), showing intense staining of macrophages and dendritic cells and staining of some lymphocytes (*bottom*; × 160).

class I^+. However, Fig. 2 demonstrates that there was a much higher density of MHC class I antigens expressed on lymphocytes in afferent lymph than on lymphocytes in blood or efferent lymph. Forward angle light scatter (cell size) analysis revealed that afferent lymph lymphocytes were only slightly larger than lymphocytes from efferent lymph (Fig. 2).

Output of Lymphocyte Subpopulations from Afferent and Efferent Popliteal Lymph. Table III shows the hourly output of lymphocytes from popliteal efferent and afferent lymph of an individual sheep. The hourly output of small lymphocytes from efferent lymph was 50 times greater than from afferent lymph. Only one afferent lymphatic was cannulated, however, the total input of small lymphocytes from afferent lymph does not exceed 5-10% of the output from efferent lymph (5).

Clearly, the output of lymphocyte subpopulations from afferent lymph, no matter how enriched for a given lymphocyte subpopulation, cannot account for differences in the outputs of lymphocyte subsets observed in efferent lymph. This is apparent even for subpopulations in efferent lymph such as SBU-$T19^+$ cells where the hourly output was only $\sim 3\text{-}6 \times 10^6$ yet the total afferent hourly output was an estimated 10^6. This was despite the fact that SBU-$T19^+$ cells were present in four times the concentration in afferent lymph than efferent lymph.

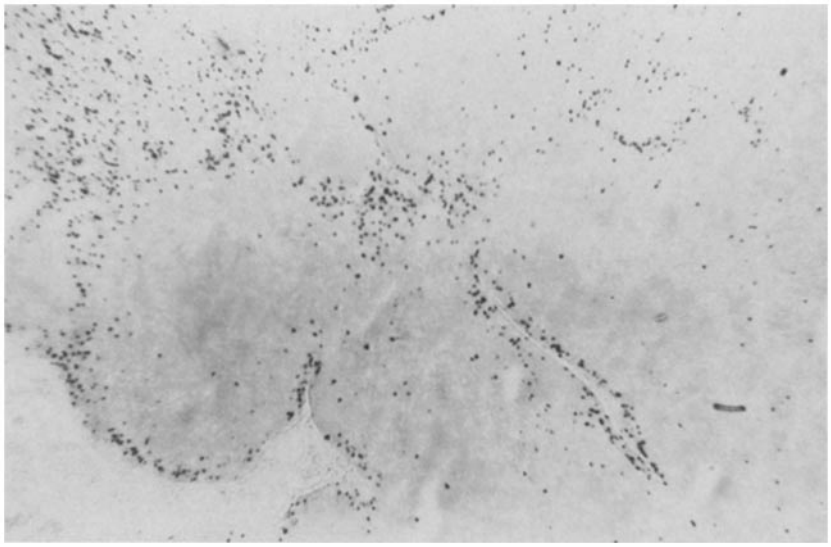

FIGURE 4. Section of sheep popliteal lymph node showing the distribution of SBU-T19$^+$ (× 100). Stained cells were localized to the subcapsular sinus, trabeculae, and to the medulla, but were largely absent from B cell follicles and T cell domains.

Immunoperoxidase Staining of Cytocentrifuged Afferent Lymphatic Cells. The phenotype of macrophages in afferent lymph was examined using immunoperoxidase staining on cytospots. All macrophages showed strong expression of MHC class II antigens (Fig. 3). CD1 was expressed on virtually all macrophages, but not by lymphocytes (Fig. 3). Many of the macrophages possessed long intensely stained "veils" and were taken to represent dendritic cells. CD4 was also expressed on ~50% of macrophages.

Distribution of SBU-T19$^+$ Cells in Lymph Nodes of Sheep. Fig. 4 shows a peripheral lymph node stained for SBU-T19. SBU-T19$^+$ cells were prominent in the outer cortex close to the subcapsular sinus and were also associated with trabeculae and medullary sinuses. SBU-T19$^+$ cells were usually scattered sparsely throughout the deep cortex and medulla and were absent from B cells follicles.

Discussion

The experiments described in this report are the first to examine simultaneously the distribution of lymphocyte subsets circulating in blood and in the afferent lymph and efferent lymph of a peripheral lymph node. Within these compartments, marked differences in the percentages of certain lymphocyte subsets were apparent, which suggests that certain lymphocyte subsets may leave the blood with differing efficiencies. Lymphocyte subpopulations also appear to be extracted from the blood at different rates by the lymph node as opposed to subcutaneous vascular endothelium.

The distribution of lymphocytes within different anatomical sites throughout the body depends upon both the delivery of cells to a tissue by the blood and the capacity of lymphocytes to cross specialized endothelium at those sites. Analysis of lymphocyte recirculation in sheep has demonstrated that there are at least three subpopulations of lymphocytes that recirculate by different pathways. Lymphocytes from

intestinal lymph recirculate preferentially back into intestinal lymph, whereas lymphocytes from peripheral lymph nodes recirculate preferentially back into peripheral lymph node efferent lymph (6, 18, 19). A third population of lymphocytes has been shown to recirculate preferentially to lymph draining sites of chronic inflammation (20). The exit of circulating lymphocytes from blood may be controlled by specific molecular interactions between lymphocytes and endothelial cells (21). Thus different lymphocyte-endothelial cell interactions may occur in various tissues enabling lymphocytes to migrate preferentially to peripheral lymph nodes, to gut, or to sites of inflammation (3, 21, 22).

The differences in the distribution of lymphocyte subpopulations between the various lymphoid compartments reported in this paper may result from lymphocyte subsets bearing different migration-related cell surface molecules. Accordingly, endothelial cells would express complementary cell surface molecules that would differ from vascular bed to vascular bed either in the nature of the molecules expressed or in their number.

In this study, $CD5^+$ lymphocytes were present in a much higher concentration in afferent lymph than in blood or efferent lymph. However, traffic endothelium in lymph nodes transmitted many more $CD5^+$ cells than in subcutaneous vascular beds (see Table III) although the delivery of lymphocytes to both vascular beds was estimated to be similar. It follows that the lymph node vascular bed as a whole is far more efficient at transmitting $CD5^+$ lymphocytes into efferent lymph than the subcutaneous vascular bed as a whole is in transmitting $CD5^+$ lymphocytes into afferent lymph. The enrichment of $CD5^+$ cells compared with B cells in afferent lymph as opposed to efferent lymph could be explained if subcutaneous vascular endothelium transmits $CD5^+$ lymphocytes in preference to B lymphocytes, either by positively selecting for the former or negatively selecting for the latter. Differences in the migration of T lymphocyte subsets at lymph nodes and the skin and subcutaneous tissues could be explained by the expression of different cell migration-related cell surface molecules on each lymphocyte subset associated with quantitative differences in the expression of complementary molecules on traffic endothelium. For example, if the complementary molecule to a migration-related cell surface molecule specific for $SBU\text{-}T19^+$ lymphocytes was expressed in greater numbers on subcutaneous vascular endothelium than on endothelium in lymph nodes, $SBU\text{-}T19^+$ lymphocytes may bind to endothelium more efficiently in the subcutaneous tissues resulting in a higher extraction rate for $SBU\text{-}T19^+$ cells from blood into afferent lymph than for blood into efferent lymph. If this notion is correct, the endothelium, not the lymphocyte, is the key factor in regulating the nonrandom exit of lymphocyte subsets from different vascular beds.

The higher concentration of T lymphocytes and their subpopulations in afferent lymph compared with blood could also be explained if T lymphocytes were dividing in the tissue spaces after they left the blood and before they appeared in afferent lymph. The modal transit time of T lymphocytes from blood to afferent lymph is $\sim$18–20 h and is the same as the transit time of T lymphocytes from blood through the lymph node into efferent lymph (23). Such an explanation would require a high turnover of T lymphocytes in the tissue spaces for which there is no evidence. However, there is evidence that in a nonstimulated lymph node such as those examined in our study, < 2–3 of lymphocytes in efferent lymph are newly formed (4), so the

higher concentration of $CD4^+$ lymphocytes in efferent lymph compared with blood could not be due to the proliferation of $CD4^+$ lymphocytes in the lymph node.

The percentages of $CD4^+$ and $CD8^+$ lymphocytes in sheep blood were much lower than those reported for human blood. Typically, the $CD4^+$ and $CD8^+$ subsets represent 20–25% and 10–12% of sheep PBL, respectively (13), although in this study the values for these subsets were even lower. Sheep blood also has a higher percentage of B cells than human blood, as well as 10–15% $SBU\text{-}T19^+$ cells. An analogous population in human blood to SBU-T19 could be represented by the $CD3^+$, $CD5^+$, $CD4^-$, and $CD8^-$ subset that expresses the γ chain of the T cell receptor (24). Human and mouse lymphokine-activated killer effector cells, but not precursor cells, can show a similar phenotype to SBU-T19 cells, although $CD3^+$, $CD4^-$, and $CD8^-$ T cells in human peripheral blood constitute only 3% of lymphocytes (25).

The SBU-T19 subset exhibited an interesting distribution in lymph nodes where they were located mainly in the subcapsular cortex and adjacent to trabeculae. Few $SBU\text{-}T19^+$ cells were observed within conventional T cell or B cell domains. The high concentration of $SBU\text{-}T19^+$ lymphocytes in afferent lymph could partly account for their unusual distribution in the lymph node although it must be conceded that the majority of $SBU\text{-}T19^+$ lymphocytes in lymph nodes at any one time will have entered the lymph node via lymph node vascular endothelium. Any consideration of the contribution to the output of $SBU\text{-}T19^+$ lymphocytes in efferent lymph by cells from afferent lymph should take into account that, as far as we are aware, afferent lymph lymphocytes have never actually been shown to migrate into efferent lymph.

The majority of lymphcytes in afferent lymph express greater amounts of MHC class I antigen than lymphocytes in efferent lymph or blood. We have no explanation for how this occurs. We also have no explanation for the large numbers of MHC class II^+ T lymphocytes in afferent lymph although in both cases it seems likely that altered expression of MHC antigens occurs after lymphocytes have left the blood and before they have entered afferent lymph. There is no direct evidence to support this proposition nor is there any evidence to support the notion that such lymphocytes have migrated preferentially into afferent lymph because they bear MHC class II antigens. The observed expression of MHC antigens on lymphocytes in afferent lymph is all the more confusing in view of their anergy in graft-vs.-host reactions as reported by Scollay & Lafferty (7). Studies on human afferent lymph have also shown a high percentage of T cells that express MHC class II antigens (26). Other studies on afferent lymph cells in sheep have not demonstrated large numbers of MHC class II^+ cells, although increased levels of MHC class II expression were observed after antigenic stimulation. The same study reported complete *de novo* synthesis by efferent T cells from undetectable levels to nearly double the number of class II molecules found on B cells after immunological stimulation (27).

10–20% of cells in afferent lymph are large macrophage-like cells. Their presence in afferent lymph raises the question of their origin and subsequent fate after entering the node since they are not seen in efferent lymph. The phenotypic analysis of afferent lymph macrophages reported here supports the idea that at least a proportion of these cells are Langerhans' cells that have migrated from the skin. All afferent lymph dendritic cells were intensely MHC class II^+ and $CD1^+$ while ~50% were $CD4^+$, similar to the phenotype of Langerhans' cells in human epidermis (28). The $CD1^+$ veiled dendritic cells in afferent lymph may be Langerhans' cells that have

left the epidermis, but the relationship of these cells to Langerhans' cells is difficult to define in sheep because they do not contain Birbeck granules although they do phagocytose immune complexes in vivo (29).

Summary

The surface phenotypes (CD1, CD4, CD5, CD8, SBU-T19, MHC class I, MHC class II, and sIg) of cells in blood, lymph nodes, and lymph were determined to examine simultaneously the distribution of lymphocyte subsets circulating in blood, afferent lymph, and efferent lymph of a peripheral lymph node. Marked differences in the percentage of certain lymphocyte subsets were apparent within the compartments examined, suggesting that lymphocyte subsets leave the blood with differing efficiencies. Lymphocyte subsets also appeared to be extracted from the blood at different rates by lymph node as opposed to subcutaneous vascular endothelium. Endothelial cells in different vascular beds may express different numbers of molecules complementary to a set of migration-related cell surface molecules specific for each lymphocyte subset. Accordingly, the vascular endothelium would be the key factor in regulating nonrandom cell migration.

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