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IMMUNOELECTRON MICROSCOPY OF CELL POPULATIONS IN REGIONAL AND CENTRAL LYMPH OF SHEEP

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ABSTRACT

The immunoreactivity and the ultrastructural localization of monoclonal anti-sheep lymphocyte antibodies conjugated with colloidal gold particles were examined in free-floating cells of sheep central lymph from the thoracic duct, postnodal lymph draining either the popliteal nodes or the mesenteric nodes, and prenodal lymph draining the pregnant uterus. The monoclonal antibodies used in this study were SBU-T_1 (CD5), SBU-T_4 (CD4), SBU-T_8 (CD8), SBU-II (anti DR antibody), and E_{53} which are reported to be sheep homologues of human T_1, T_4, T_8, HLA-DR, and pan B cell antibodies, respectively. Colloidal gold particles were evenly distributed or segmentally aggregated on the surfaces of lymphocytes and macrophages incubated with monoclonal antibodies and in vesicles in the cytoplasm of anti DR antibody labeled macrophages. Not only did CD5 labeled cells show a high percentage in each regional lymph examined, but the percentage of CD4 labeled cells was consistently higher than that of CD8 labeled cells. Moreover, the immunoreactivity of CD8 labeled cells was specific among lymph from the different regions. The sum of the percentages of CD4 and CD8 labeled cells was less than the percentage of CD5 labeled cells, indicating the presence of a minor T cell subpopulation which was $CD5^+$, $CD4^-$, and $CD8^-$. A characteristic finding was a high percentage of CD8 labeled cells and many abnormal eosinophils in uterine prenodal lymph in pregnant sheep. Taken together the results showed that variously labeled immunoreactive cells are distributed somewhat differently in lymph derived from different organ sites.

A number of studies have been carried out on lymph cell phenotypes and frequencies in sheep popliteal (1), precapsular (2) postnodal lymph and in human skin prenodal lymph (3). They have shown some differences in cell concentration and cell type between post- and prenodal lymph with prenodal lymph containing fewer cells but greater percentage of monocytes, macrophages, and granulocytes than postnodal lymph (4,5). It has also been claimed that phenotypes and frequencies of migrating cells in high endothelial venules and lymphatic capillaries differ among various lymphoid tissues (6,7). To help clarify these issues, we examined in a single species (the sheep) the circulating cell phenotypes and ultrastructural features of postnodal lymph draining the popliteal lymph nodes, the mesenteric nodes, central thoracic duct, and prenodal lymph cells of the pregnant uterus.

MATERIALS AND METHODS

Animals

Merino sheep provided the lymph

required for this study. Sheep were housed indoors in pens or cages and fed lucerne chaff. The various lymphatic vessels were cannulated by methods originally described by Lascelles & Morris (8), Hall & Morris (9). Central lymph was collected from the thoracic duct and postnodal lymph from the popliteal nodes and the mesenteric nodes in different sheep. Prenodal lymph from the uterus was obtained from three pregnant sheep (50-60 days gestational age) mated following estrous synchronization with intravaginal sponges containing synthetic progestins.

Monoclonal antibodies (mAbs)

SBU-T_1 (CD5), -T_4 (CD4), -T_8 (CD8), and - II (anti DR antibody) used in this study were prepared from supernatant fluid and purchased from Dr. M.R. Brandon, Embryology Research Unit, School of Veterinary Science, University of Melbourne, Australia. These mAbs were prepared against cell surface antigens of sheep lymphocytes and are reported to be the sheep homologues of human T_1, T_4, T_8, and HLA-DR, respectively (10-12). Monoclonal antibody E_{53} which is claimed to recognize B cells in Peyer's patch follicles, splenic follicles and germinal centers of lymph nodes was supplied by the Basel Institute for Immunology, Basel, Switzerland (13).

Cell suspensions

The cell concentration in lymph was measured with a model Fn Coulter Counter (Coulter Electronics, Dunstable, U.K.). The cells in the lymph were freshly collected and were held at 4°C until treated with fixative. One ml of cell suspensions containing a minimum of 1.50×10^7 cells/ml and 4ml of 0.1M cacodylate buffer supplemented with 1% bovine serum albumin (BSA), pH 7.4 (cacodylate/BSA) was pipetted into each of seven conical bottomed test tubes. One tube was used for each of the control, CD5, CD4, CD8, anti DR antibody, and E_{53}, and double immunogold staining for both CD4 and anti DR antibody labeled cells. The suspended cells in each tube were mixed well and were centrifuged at 200xg for five min. The cells were washed and centrifuged three times with 5ml of cacodylate/BSA taking care not to disturb the cell pellet when pipetting the wash buffer. One ml of cells resuspended to 1.0×10^7 cells/ml per test tube was transferred to EM embedding capsules and centrifuged at 200xg for five min. The supernatant wash buffer was removed by pipette.

Single immunogold staining (IGS) and double IGS

The specificity of IGS was checked in control lymph incubated in colloidal gold particles without primary mAb. MAbs were diluted 1:10 or 1:20 with a buffer composed of 20mM Tris buffered saline (pH 8.2) supplemented with 1% BSA (Tris/BSA). 100μl of antibody was added to the cell pellet. The cells were mixed well and incubated with the primary mAb for 30 min. After incubation, the cells were washed twice with Tris/BSA and the cell pellet then incubated for a further 30 min with 100μl of colloidal gold particles (5 or 15nm diameter) conjugated to goat anti-mouse Ig or protein A (Sigma, St. Louis, Mo., USA) diluted either 1:20 or 1:40 with Tris/BSA. After two washes, the cells were fixed with 2ml of 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 30 min, then post-fixed with 2% osmium tetroxide in 0.1M cacodylate buffer for 20 min at room temperature. After two washes with 0.1M cacodylate buffer, the cells were stained "en bloc" with 1% uranyl acetate in 0.1M cacodylate buffer for 10 min and then dehydrated and embedded in Spurrs resin according to routine procedures. Ultrathin sections (60-80nm) were prepared and examined in a Hitachi H 7000 electron microscope. The numbers of CD5, CD4, CD8, anti DR antibody and E_{53} labeled cells and the ultrastructural localization of these mAbs recognized by the presence of colloidal gold particles on their cell surfaces were examined and counted in montage preparations of elec-

Table 1
Percentage of Cells (mean ± SD) Labeled with Various Anti-Sheep Lymphocyte mAbs in Lymph from Different Regions

mAb	Thoracic duct lymph*(5)	Popliteal lymph(7)	Mesenteric lymph(5)	Uterine lymph†(3)
CD5	63.9±6.8	73.4±8.1	60.4±10.5	69.1±5.5
CD4	42.4±7.3	51.4±6.0	52.4±4.7	35.6±3.8
CD8	13.4±4.6	9.5±2.1	4.5±0.4	21.5±2.4
anti DR	38.6±4.6	24.4±7.3	51.3±3.8	39.8±7.6
E_{53}	11.8±4.3	12.2±4.6	11.0±3.9	<1

*Numbers in parentheses indicate the number of sheep studied.
†Uterine lymph was collected from three pregnant sheep (50-60 days gestation).

tron micrographs photographed at 8,000 magnification. Dead cells were excluded from counting procedures. For double IGS, two reactions were carried out using the method described above; the first reaction used mAb anti DR antibody combined with 5nm colloidal gold particles conjugated to goat anti-mouse Ig, and the second a combination of mAb CD4 and 15nm colloidal gold particles conjugated to goat anti-mouse Ig.

RESULTS

Lymph and cell concentration

Postnodal lymph collected from the popliteal lymph nodes and prenodal pregnant uterine lymph were pale clear yellow whereas postnodal lymph from the mesenteric lymph nodes was "milky." Central lymph from the thoracic duct was a clear brownish or sometimes turbid reddish color, indicating the presence of red blood cells.

The cell concentrations in postnodal lymph from the popliteal nodes, mesenteric nodes, thoracic duct and prenodal uterine lymph were 0.84±0.79 (10^7/ml), 1.47±0.67 (10^7/ml), 1.59±0.86 (10^7/ml), and 1.07±0.32 (10^5/ml), respectively. Uterine prenodal lymph, therefore, contained fewer cells and a large volume of lymph was needed to form a cell pellet.

Reactivity of anti-sheep lymphocyte mAb

At least 400 cells were counted to formulate an estimate of the proportions of the respective mAb labeled cells. Cells reacting with the mAbs had numerous immunogold particles around the surface membrane. Most negative cells had no particles. The percentages of CD5, CD4, CD8, anti DR antibody, and E_{53} labeled cells were calculated from the ratio of cells labeled with gold particles to the total number of lymphocytes, and that of eosinophils and macrophages to total cells, respectively. The reactivity of mAb CD5, CD4, CD8, anti DR antibody, and E_{53} against sheep lymphoid cells is shown in *Table 1*. The combined percentage of cells staining for CD4 and CD8 were consistently less than CD5. Also, the percentage of CD4 cells was uniformly greater than that of CD8 labeled cells. The percentage of CD8 labeled cells varied among lymph from the different regions, although lymph showed similar percentages of CD4 labeled cells. Specifically, uterine prenodal lymph of the pregnant sheep contained the most CD8 labeled cells: lymphocytes in uterine prenodal lymph consisted of 21.5% $CD8^+$ cells whereas lymphocytes in other postnodal lymph samples were only 4.5-13.2% $CD8^+$. The percentage staining of E_{53} in the prenodal uterine lymph was less than 1%, in contrast to 11.0-12.2% in lymph from the other regions.

The samples of uterine prenodal lymph also contained large numbers of eosinophils (63.7±5.3%) and macrophages (9.8±3.0%) compared with postnodal lymph from other regions where the percentage of the eosinophils and macrophages was less than 1% to 4.5±0.9% of macrophages in mesenteric postnodal lymph.

The ultrastructure of cell populations

Lymphocytes in all specimens possessed rounded nuclei with peripheral chromatin, free ribosomes and mitochondria. Centrioles were prominent. There were few morphological differences between CD5, CD4, CD8, and E_{53} labeled cells. However, large granular lymphocytes were seen occasionally in all lymph samples which were distinguished from CD5, CD4, CD8, and E_{53} labeled cells by their intracytoplasmic electron-dense granules. They were $CD5^+$, $CD4^-$, and $CD8^-$. Plasma cells were consistently absent from all lymph samples.

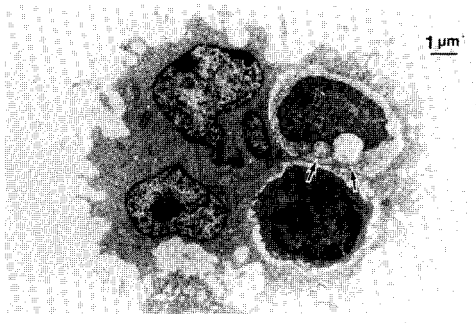

Fig. 1. A macrophage in prenodal lymph from the pregnant uterus engulfing two lymphocytes of which one shows an ill-defined nuclear membrane and ruptured mitochondria (arrows) consistent with degeneration (x6900).

Two types of macrophages were detected by electron microscopy in uterine prenodal lymph: one type possessed an irregularly shaped nucleus with a prominent nucleolus, multiple vesicles, many free ribosomes, lysosomes, and pseudopodis, and often contained one or two degenerated lymphocytes within the cytoplasm *(Fig. 1)*. The engulfed lymphocytes showed poorly defined nuclear membranes and ruptured mitochondria. The other macrophage type characteristically displayed abundant rough endoplasmic reticulum within the electron-lucent cytoplasm *(Fig. 2)*.

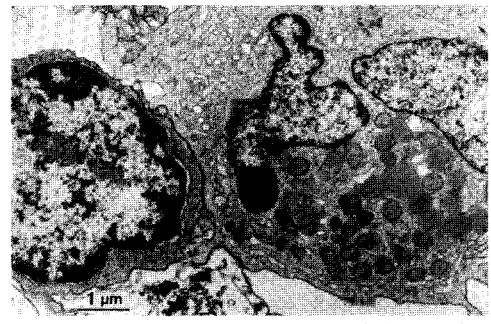

Fig. 2. An interdigitating-like cell in uterine prenodal lymph labeled with 5nm colloidal gold particles against DR antibody. The electron-lucent cytoplasm and the presence of abundant rough endoplasmic reticulum are characteristic. Note the dense aggregation of colloidal gold particles at the interface between a DR^+ labeled lymphocyte and a macrophage (x13,800).

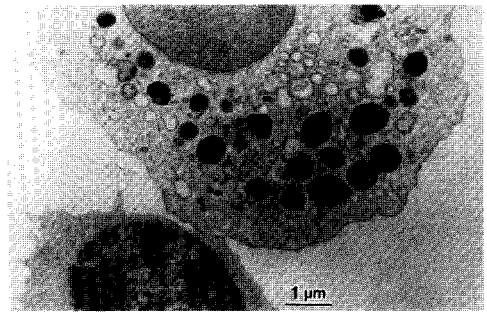

Fig. 3. Part of an eosinophil in pregnant uterine prenodal lymph showing characteristic granules with crystalloid cores in the cytoplasm and an attachment to a CD4 labeled cell (x12,800).

The nuclei of the eosinophils in uterine prenodal lymph were varied in form and possessed evenly distributed chromatin. Their cytoplasm contained moderate numbers of mitochondria, vesicles and one or more pseudopod-like processes. The most characteristic feature was the presence of granules with crystalloid cores *(Fig. 3)*. Eosinophils were sometimes attached to CD4 or CD8 labeled lymphocytes or macrophages as seen in *Fig. 3*. Luteal cells were seen

occasionally in the uterine prenodal lymph and were the largest in the entire cell populations. They possessed one round or oval nucleus with evenly distributed chromatin, abundant rough endoplasmic reticulum, free ribosomes, moderate numbers of mitochondria and several pseudopod-like processes.

Ultrastructural localization of mAbs conjugated with colloidal gold particles

Colloidal gold particles were evenly distributed or periodically aggregated on the surfaces of the cells incubated with each mAb, except vesicles in the cytoplasm of anti DR antibody labeled macrophages. E_{53} conjugated with colloidal gold particles was observed on the cell surface but not in the cytoplasm of lymphocytes. Eosinophils and luteal cells showed no colloidal gold particles in their cell surfaces. Control cells failed to exhibit specific staining with colloidal gold particles *(Fig. 4)*.

Fig. 4. Electron photomicrograph showing "control" lymph cells incubated in colloidal gold particles without primary mAb in popliteal postnodal lymph. There is minimal background staining with colloidal gold particles (arrow) (x16,800).

The number of 5nm colloidal gold particles attached to cell surfaces was uniformly greater than that of 15nm colloidal gold particles in each specimen. Double IGS showed abundant 5nm colloidal gold particles at the interface between anti DR antibody labeled cells, while 15nm colloidal gold particles were scattered on the surface of CD4 labeled cells *(Fig. 5)*.

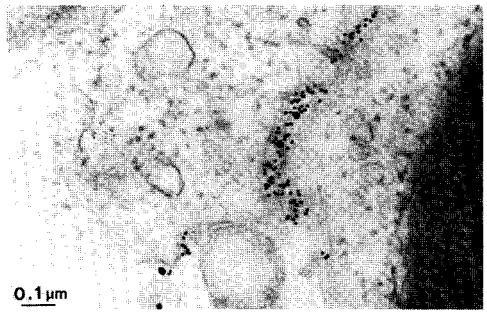

Fig. 5. Part of two lymphocytes in mesenteric postnodal lymph showing clearly the immunolocation of DR^+ conjugated to 5nm colloidal gold particles and CD4 conjugated to 15nm colloidal gold particles on the cell surfaces at high magnification (x83,700).

DISCUSSION

It is generally accepted that lymphocytes leave the blood circulation via high endothelial venules or blood capillaries in lymphoid or non-lymphoid tissues and enter lymphatic capillaries (5-7,9,14). Moreover, it has been confirmed recently that lymphocytes leaving the bloodstream are highly selected by the wall of high endothelial venules or blood capillaries in tissues (15-17). Therefore, it may be inferred that lymph cell phenotypes and frequencies in the postnodal and prenodal lymph reflect the local immunoresponsiveness and its physiological significance in lymphoid and non-lymphoid tissues. Lymph cells examined in this study were obtained in sheep from central lymph of the thoracic duct, postnodal lymph of popliteal nodes and mesenteric nodes, and prenodal lymph of the pregnant uterus. Uterine prenodal lymph contained fewer cells but more macrophages and eosinophils than the other postnodal lymphs and these data were consistent with the contents of postnodal and prenodal lymph as reported earlier (4,5).

This report describes the reactivity of five anti-sheep lymphocyte mAbs with cells in lymph from various sources. The

reactivity of mAbs (CD5, CD4, and CD8) in the popliteal postnodal lymph revealed by immunoelectron microscopy in this study was very similar to that in the precapsular postnodal lymph as determined by indirect immunofluorescence and flow microfluorometry (2). Lymph from different regions consistently showed a high percentage of CD5 labeled cells in contrast to the low percentage of E_{53} labeled cell, although the sum of the percentage of CD4 and CD8 labeled cells was less than that of CD5 labeled cells. These data suggest the presence of a small population of T-cells which are $CD5^+$, $CD4^-$, and $CD8^-$, a finding consistent with an earlier report by Mackay et al (2) using mAb 19-19. Moreover, lymphocytes with large electron-dense bodies within the cytoplasm noted in our study were labeled with $CD5^+$ but not with $CD4^-$ or $CD8^-$. These 19-19 labeled cells and large granular lymphocytes of natural killer cells may be small populations of T cells. Similar detailed information on lymph cell phenotypes in human skin prenodal lymph has already been described by Lukomska et al (16). Our percentage of lymph cell phenotypes and visceral lymph agree in general with their findings except for the high percentage of anti DR^+ cell observed. This difference may relate to use of a different specificity of anti DR antibody, different species, or different site sampled for lymph.

A noteworthy finding in our study was the high percentage of $CD8^+$ cells in the uterine prenodal lymph of pregnant sheep. Daya et al (18) and Morris et al (19) previously reported that T cells in the human uterine endometrium and the epithelium of the human oviduct were predominantly T cytotoxic or suppressor lymphocytes, respectively. This finding supports the notion that T cytotoxic/suppressor lymphocytes play an important role in local immunoreaction in the uterus (18) and the oviduct (19).

Another notable finding was large numbers of eosinophils in pregnant uterine prenodal lymph. It is long recognized that circulating eosinophils are affected by specific hormones. For example, the administration of glucocorticoids prevents uterine eosinophilia and edema (20). Moreover, uterine eosinophils are bone marrow-derived (21), possess a cell-surface estrogen receptor (22), and are sharply increased by the administration of estrogen (23,24). Nonetheless, the exact role of eosinophils in reproductive physiology has not been clarified. Although no hormonal analysis in lymph and blood was done in this study, we previously observed (unpublished) that the estrogen level between 50 and 60 days of gestation is higher than that of nonpregnant sheep. Because eosinophils were sometimes attached to CD4 or CD8 labeled lymphocytes in uterine prenodal lymph, it is possible that regulation of T cells takes place by eosinophils underlying varying estrogenic stimulation.

Two kinds of macrophages were distinguished by electron microscopy. Both types were anti DR $antibody^+$. Macrophages with typical fine structure act mainly as phagocytes, engulfing degenerated cells and waste products, as observed in prenodal lymph from the liver (5). The fine structure of the other macrophage type was similar to that of the "interdigitating cells" described in the bronchus-associated lymphoid tissue of the rat (7). These macrophages often attached to lymphocytes, but did not ingest lymphocytes within their cytoplasm. This finding supports the concept of mutual cooperation *in vivo* between these two cell types as suggested by Olszewski (3).

The ultrastructural localization of E_{53} has not been fully elucidated. Some suggest localization either in the cytoplasm or on the cell surface although this mAb recognizes all B cells in tissue sections (13). Immunoelectron microscopy of sheep lymph showed that the ultralocalization of E_{53} conjugated with colloidal gold particles was only on the surface of E_{53} labeled cells. Moreover, most of the E_{53} labeled cells in lymph were lymphocytes and none plasma cells.

In short, these experiments provide in regional lymph of sheep the relative numbers of subsets of lymphocytes identi-

fied by anti-sheep lymphocyte mAbs and also the ultralocalization of mAbs conjugated with colloidal gold particles as well as the fine structures of lymph lymphocytes and macrophages.

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