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Subpopulations of lymphocytes in the mammary gland of sheep

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SUMMARY

The subpopulations of lymphocytes in the pregnant and non-pregnant mammary glands of the sheep were delineated by a panel of monoclonal antibodies. The most striking feature observed was that in the mammary gland of both pregnant and non-pregnant sheep the great majority of the lymphocytes in the ductal and alveolar epithelium were agranulated $CD8^+$ $CD5^-$ cells. A small subpopulation of granulated lymphocytes in the epithelium expressed the CD45R antigen but not the major histocompatibility complex (MHC) class II molecules. Other subpopulations, especially B lymphocytes, were present in much lower concentrations and were located mainly in the periductal and intralobular connective tissues. Patches of lymphocytes clustering around venules were observed and the majority of them were shown to be $CD5^+$ $CD4^+$, while some were $CD5^+$ $CD8^+$ but none were $CD45R^+$ (B cell). It is suggested that selective traffic of T cells occurs at these sites.

INTRODUCTION

Previous studies (Lee & Lascelles, 1969a, b, 1970) have reported that the mammary glands of pregnant and non-pregnant ewes were heavily infiltrated with lymphocytes and macrophages. In addition it has been shown that the concentration of these cells is sufficient to support the development of a substantial immune response following infusion of antigen (Lee & Lascelles, 1969b, 1970).

In a more recent study (Sheldrake & Husband, 1985) it was shown that intraperitoneal injection of antigen in Freund's complete adjuvant in the ewe, followed by intramammary infusion of the antigen, enhanced the number of antibody-containing cells in the mammary gland. It is now generally accepted that the IgA system in the ruminant mammary gland is poorly developed and can only be readily discerned after local antigenic stimulation during the dry period, but even then IgA in colostrum and milk secreted during the ensuing lactation represents only a small proportion of total immunoglobulin (Lascelles *et al.*, 1986).

Although it is known that the mammary gland is heavily populated with lymphoid cells, no detailed studies have been carried out to delineate their subpopulations. The present study employed a panel of monoclonal antibodies directed against six lymphocyte antigens to establish the cellular basis for the local immune response of the mammary gland of the ewe.

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MATERIALS AND METHODS

Tissues

Mammary glandular tissues were obtained from two non-pregnant primiparous ewes and three primiparous ewes about 120 days pregnant. Both mammary glands from all the ewes were free from abnormality when examined prior to tissue sampling.

For immunohistochemical studies, mammary tissue was embedded in OTC compound (Miles Scientific, Naperville, IL), snap-frozen and stored at $-70°$. Some tissues were also fixed in Bouin's fluid and processed for conventional paraffin embedding and sectioning.

For electron microscopy the glandular tissue of one 120-day pregnant ewe was fixed by perfusion of 80 ml of 0·1% glutaraldehyde in 0·1 M phosphate-buffered saline (PBS) containing 2% paraformaldehyde, through the external pudendal artery. Glandular tissues in 1-mm cubes were immersed in the same fixative or in 2% glutaraldehyde for an additional 60 min at room temperature. The tissues were then embedded in either LR-white or Araldite.

Monoclonal antibodies

All the monoclonal antibodies (mAb) used were produced in our laboratories, and their characteristics are detailed in Table 1.

Immunocytochemical staining

For most of the light microscopy studies 6 μm frozen sections were cut, fixed for 10 min in cold ethanol or acetone, air-dried and then stained using the indirect immunoperoxidase technique as previously described (Lee *et al.*, 1985). For controls

Table 1. Reactivity of monoclonal antibodies to sheep lymphocytes

Antigen	mAb clone number	Antigen distribution
CD45 (SBU-LCA)	1-28-124	All leucocytes
MHC class II	49-1	Present on B lymphocytes and activated T lymphocytes; 49-1 is monomorphic for all four subsets of sheep class II molecules
CD5 (SBU-T1)	25-91	Present on all T lymphocytes, absent from B lymphocytes
CD4 (SBU-T4)	44-38 and 44-97	Present on subset of T lymphocytes that are $CD8^-$, $SBU\text{-}T19^-$; absent from B lymphocytes
CD8 (SBU-T8)	38-65	Present on subset of T lymphocytes that are $CD4^-$, $SBU\text{-}T19^-$ absent from B lymphocytes
CD45R (SBU-p220)	20-96	Present mainly on B lymphocytes and small subset of T lymphocytes

References for the mAb are found in previous report (Lee *et al.*, 1988).

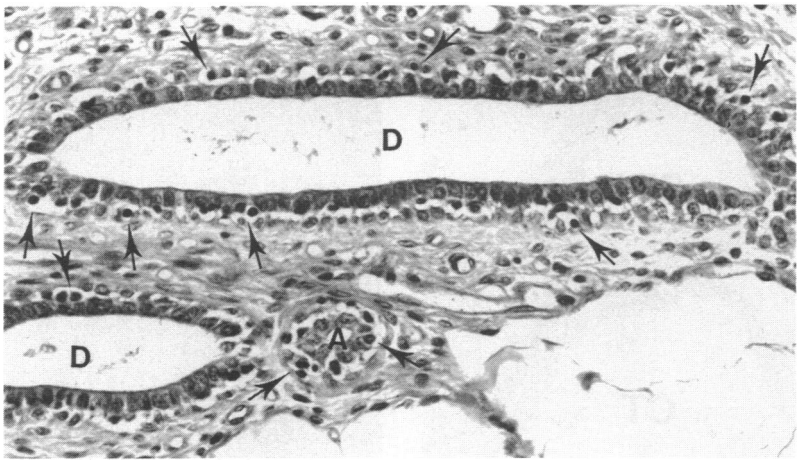

Figure 1. Haematoxylin and eosin stained section showing lymphocytes (arrows) localized within and at the basal regions of the epithelium of an alveolus (A) and two ducts (D). Non-pregnant sheep. Magnification × 300.

either PBS or 1% normal sheep serum in PBS was substituted for the mAb supernatant.

Electron microscope studies were carried out on sections mounted on 300-mesh uncoated copper grids. The grids were jet-washed with PBS and then floated with the section facing downwards on a drop of undiluted mAb supernatant for 30 min at room temperature. Following this the grid was jet-washed with PBS before being floated on a drop of 10 nm colloidal gold-labelled goat anti-mouse immunoglobulin (Janssen Pharmaceutica, Beerse, Belgium) diluted 1:20 in PBS for another 30 min at room temperature. The grid was jet-washed with PBS, distilled water and then stained with uranyl acetate and lead citrate. Control sections were stained as for light microscopy.

RESULTS

General histological observations

Examination of haematoxylin and eosin stained sections revealed that the mammary glands of non-pregnant sheep contained a series of ducts and ductules with rudimentary alveoli extending into lobules composed mainly of adipose tissues. The ducts, ductules and alveoli were lined by closely packed cuboidal epithelial cells and interspersed between them and at their basal region were lymphocytes (Fig. 1). More lymphocytes were seen in the interlobular and intralobular ducts and they were usually arranged in single file beneath the epithelium. Predominantly lymphocytes, some macrophages and the occasional mast cell were seen in the interalveolar and periductal areas.

In pregnant sheep the glands varied in lobule-alveolar maturation. In some areas ductules with rudimentary alveolar structures were observed and in others the alveoli were more mature and some secretory activity was evident. One or two lymphocytes and/or macrophages were occasionally seen in the secretion in the alveoli and ducts. However, lymphocytes were still commonly found in the alveolar and ductal epithelium though their concentration was apparently less than the dry glands.

In both pregnant and non-pregnant sheep lymphocytes were also seen in the interalveolar and interlobular areas. In addition a few large clusters around venules were observed.

Immunochemical staining of lymphocyte subpopulations

The pattern of distribution of lymphocytes positive for the markers CD45, MHC class II, CD5, CD4, CD8, and CD45R (Figs 2a–f) was generally similar both in the glands from pregnant and non-pregnant animals. The mammary glandular tissue was heavily populated with leucocytes as numerous

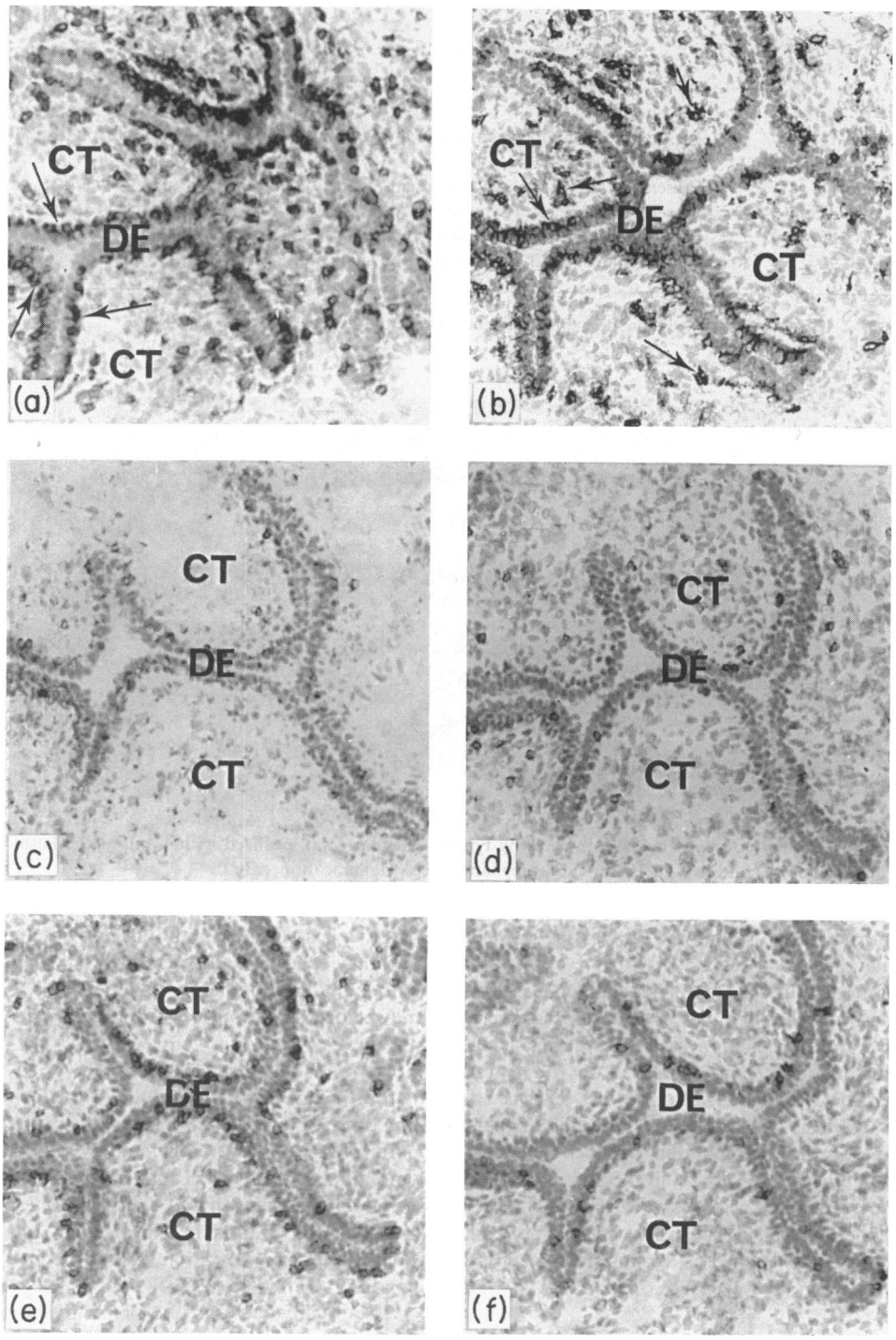

Figure 2. Immunoperoxidase staining of sections of an interlobular duct from a non-pregnant sheep with mAb against (a) CD45, (b) MHC class II, (c) CD5, (d) CD4, (e) CD8 and (f) CD45R antigens. (a) Numerous $CD45^+$ cells are present in the connective tissues (CT) and the ductal epithelium (DE). Note the single file arrangement of positive cells (arrows) at the basal regions of the ductal epithelium. (b) Most of the MHC class II^+ cells in the connective tissues and at the basal regions of the ductal epithelium are either stellate or spindle shaped (arrows). (c) Few $CD5^+$ cells are present in either the surrounding connective tissues or the ductal epithelium. (d) More $CD4^+$ cells are present in the connective tissues than in the ductal epithelium. (e) Many $CD8^+$ cells are localized within and at the basal regions of the ductal epithelium, but some are present in the surrounding connective tissues. (f) The few $CD45R^+$ cells are localized exclusively in the ductal epithelium. Magnification (a–f) $\times 160$.

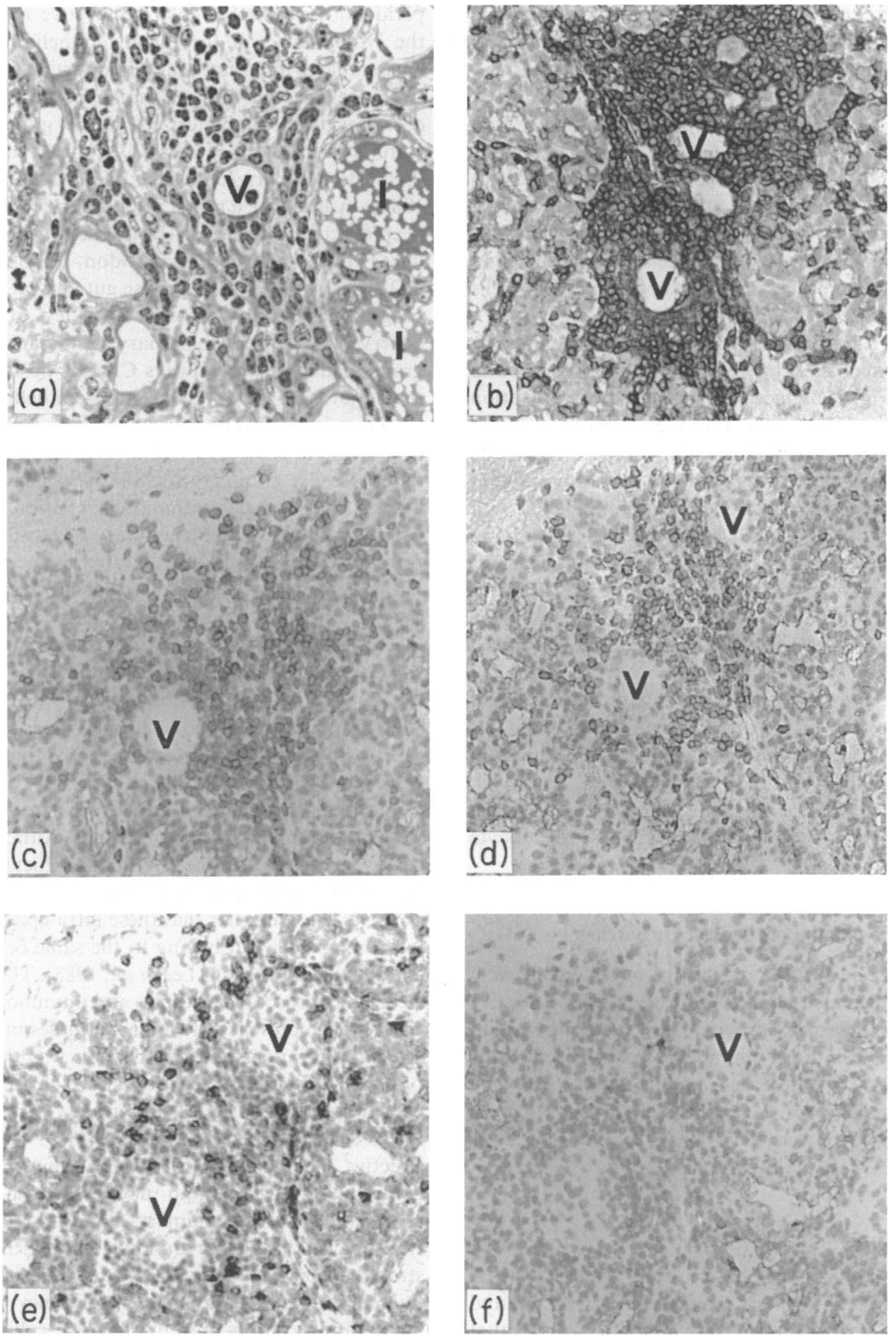

Figure 3. (a) Semithin plastic section showing a venule (v) surrounded by a cluster of lymphocytes. The alveolar lumina (L) are filled with secretion. Day 120 pregnant. 1% toluidine blue in 1% borax. Magnification × 400. (b–f) Immunoperoxidase staining of sections from a patch of lymphocytes in the interlobular area with mAbs against (b) CD45, (c) CD5, (d) CD4, (e) CD8 and (f) CD45R antigens. Note that virtually all the cells around the venules (V) are $CD45^+$ (b), a large proportion of them are $CD5^+$ (c) and $CD4^+$ (d), some are $CD8^+$ (e) and hardly any cell is $CD45R^+$ (f). Magnification (b–f) × 160.

$CD45^+$ cells were seen in the alveolar and ductal epithelium, the interalveolar and interlobular areas. The larger ducts in the interlobular areas had apparently more positive cells, which sometimes formed a single file along the subepithelial region (Fig. 2a). The majority of these resembled lymphocytes while some appeared to be macrophages.

Only the occasional $CD5^+$ cell was observed in the alveolar and ductal epithelium while relatively more $CD5^+$ cells were observed in the connective tissues around the alveoli and ducts (Fig. 2c) and they were weakly stained. $CD4^+$ cells were also

seen usually located in the connective tissues around alveoli and ducts and occasionally at the basal region of the alveolar and ductal epithelium (Fig. 2d), suggesting that most of the CD5$^+$ cells were probably CD4$^+$ cells.

The most striking feature was the large population of CD8$^+$ cells seen in both the alveolar and ductal epithelium. More cells were usually seen in the ductal region where they sometimes formed a single file beneath the epithelium (Fig. 2e). Some CD8$^+$ cells were also seen in the periductal and interalveolar connective tissue. Since only a few CD5$^+$ cells were observed in the alveolar and ductal epithelium, the majority of CD8$^+$ cells were probably CD5$^-$.

A few lymphocytes expressing the surface marker CD45R were found located almost exclusively in both the alveolar and ductal epithelium (Fig. 2f). Since this antigen survives fixation (Lee, Gogolin-Ewens & Brandon, 1988), electron microscope immunocytochemical studies with the immunoglold technique was used to examine the morphology of these cells. It was found that few of the intra-epithelial lymphocytes were labelled with gold particles. Some of the labelled cells were seen containing one to four granules while others contained no granules. Though only a limited number of tissue specimens was examined, granulated lymphocytes were always uniformly surface labelled with gold particles while many agranulated lymphocytes were unlabelled.

Cells expressing the MHC class II antigens were found mainly in the connective tissues around the alveoli and ducts and some in the subepithelial region. Only the occasional cell appeared lymphocyte-like, whereas most of them were oval, stellate or spindle shaped (Fig. 2b), suggesting that they belonged to cells of the macrophage/dendritic/histiocyte lineage.

Immunocytochemical staining of lymphocyte patches

In paraffin and plastic sections, patches of lymphocytes were sometimes seen in the intralobular and periductal connective tissues and some of these patches were seen clustering around venules (Fig. 3a). The indirect immunoperoxidase technique revealed that virtually all these cells were CD45$^+$ (Fig. 3b), whereas the great majority of these cells were CD5$^+$ (Fig. 3c) and CD4$^+$ (Fig. 3d), some were CD8$^+$ (Fig. 3e), few were class II$^+$ and none was CD45R$^+$ (Fig. 3f).

DISCUSSION

The present studies confirm previous reports (Lee & Lascelles, 1969a, b, 1970) that the mammary glands of non-pregnant and pregnant ewes are heavily populated by lymphocytes and that they are usually located adjacent to the epithelium of ducts and alveoli. Relatively fewer lymphocytes were observed in lobules in which the alveoli were more advanced in development. This apparent decline in lymphocyte concentration could be due to diluting effect, a consequence of the rapid and extensive proliferation of the alveoli in the glands of pregnant animals.

Immunocytochemical studies of frozen sections revealed that the most predominant cell type in the mammary glands of both pregnant and non-pregnant ewes was CD8$^+$. These CD8$^+$ cells were mainly located in the intra-epithelial region and often arranged in single file beneath the ductal epithelium. There were fewer CD4$^+$ cells and most of these were localized in the periductal and interalveolar connective tissues. Since most of the lymphocytes in the ductal epithelium were CD5$^-$, it is reasonable to infer that a large proportion of the intra-epithelial CD8$^+$ cells were CD5$^-$. A similar population of CD8$^+$ CD5$^-$ cells has been characterized in the gut epithelium of man (Janossy *et al.*, 1982), rats (Lyscom & Brueton, 1982), mice (Schrader, Scollay & Battye, 1983) and sheep (Gorrell *et al.*, 1988). In addition, a major population of CD8$^+$ CD5$^-$ cells has been described in the rat (Bouwens & Wisse, 1987) and sheep liver (Meeusen, Gorrell & Brandon, 1988). The majority of the CD8$^+$ CD5$^-$ cells in the mouse gut and rat liver were shown to be granulated. In contrast, only a few granulated cells were observed in the sheep mammary glandular epithelium and these granulated cells were always CD45R positive, suggesting that the majority of CD8$^+$ CD5$^-$ intra-epithelial lymphocytes were not granulated. It is possible that CD8$^+$ CD5$^-$ cells in the sheep differ from other species in that they lack cytoplasmic granules. Alternatively, granulation could be dependent on the state of activation of the cells with more activation occurring in the gut compartment while the mammary glands of healthy virgin and primiparous sheep have probably not encountered extraneous antigens. In this respect, a drastic change in the size of cytoplasmic granules has been observed in the intra-epithelial CD45R$^+$ lymphocytes of the uterus in pregnant sheep (Gogolin-Ewens *et al.*, 1989). However, the role of these CD8$^+$ CD5$^-$ intra-epithelial lymphocytes is unknown.

B lymphocytes have both MHC class II and the CD45R (p220) antigen on their surface. The finding that only the occasional MHC class II$^+$ and CD45R$^+$ lymphocyte-like cells were seen in the connective tissue indicates that very few B cells were present in the mammary glands. Although more CD45R$^+$ cells were observed in the alveolar and ductal epithelium, hardly any of the intra-epithelial lymphocytes were MHC class II positive. This suggests that these intra-epithelial CD45R$^+$ cells were not B cells but belong to the same cell type found in the endometrium of sheep (Lee *et al.*, 1988). This was confirmed by electron microscopical studies and immunogold staining which revealed the presence of similar granules in their cytoplasm.

Another interesting observation was the presence of lymphocytes clustering around venules. The great majority of these cells were CD5$^+$ CD4$^+$, some were CD5$^+$ CD8$^+$, only a few MHC class II$^+$ and none was CD45R$^+$. These may be sites for selective T-cell migration, an equivalent of the thymus-dependent zone in the lymph node. B cells were very poorly represented in the mammary gland and their absence in the lymphocyte clusters surrounding the vessels was striking. This strongly supports the first of the three mechanisms proposed by Lascelles *et al.* (1985), i.e. a deficiency of endothelial cell membrane receptors preventing B-cell traffic through the blood vessels, to explain the poor IgA response of the ovine mammary gland.

From the foregoing, it is evident that the mammary gland is populated by numerous subpopulations of lymphocytes, the most predominant of which bear the CD8$^+$ CD5$^-$ phenotype followed by CD4$^+$ CD5$^+$ and CD45R$^+$ MHC class II$^-$ cells. B cells are also present, though in small numbers. Since all the tissues were from healthy primiparous ewes it is assumed that we had examined a pattern of lymphocyte distribution in a normal physiological state. Further studies are currently being conducted to examine if there are any alterations in the lymphocyte pattern following local immunization.

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