# Leucocyte phenotypes in involuting and fully involuted mammary glandular tissues and secretions of sheep

**L. TATARCZUCH, C. PHILIP, R. BISCHOF AND C. S. LEE**

*Department of Veterinary Science, The University of Melbourne, Victoria, Australia*

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ABSTRACT

Mammary glandular tissues and mammary secretions were obtained from sheep at 2–60 d after weaning to study the leucocyte phenotypes associated with mammary involution. From 2–4 d after weaning, neutrophils were the predominant leucocytes in the alveolar and ductal lumina. Lymphocytes were present in the alveolar and ductal epithelium, interalveolar and periductal areas. Most of the lymphocytes in the alveolar and ductal epithelium (IEL) were $CD8^+$, some were $CD45R^+$ and few were $CD4^+$. In the periductal clusters and in the interalveolar areas most of the lymphocytes were $CD4^+$. There was a significant increase ($P < 0.05$) in the percentages of $CD45R^+$ granulated IEL from 2 to 7 d after weaning, and this paralleled the increase in the percentages of apoptotic cells in the glandular epithelium. By 7–60 d after weaning, most cells within the alveolar and ductal lumina were macrophages followed by predominantly $CD8^+$ lymphocytes. $CD8^+$ lymphocytes were still predominant in the alveolar and ductal epithelium while $CD4^+$ cells were predominant in the interalveolar areas. Very few $\gamma\delta^+$ T cells were observed at all the stages examined. The cells in the mammary secretions correlated with those observed in the alveolar and ductal lumina. At the early stages of involution, the neutrophils and macrophages were heavily laden with lipid droplets, casein and cellular debris. The most interesting feature was the presence of cells either with extensive cytoplasmic processes ($LCA^+$MHC class $II^+$) or cytoplasmic veils ($LCA^+$MHC class $II^+CD1^+$), probably dendritic cells. It is concluded that the cellular constituents of the mammary gland at the latter part of involution may afford the mammary gland more resistance to infection than the lactating gland and the gland at early stages of involution. The $CD45R^+$IEL may trigger apoptotic cell death in the mammary glandular epithelium during mammary involution.

*Key words*: Lactation; granulated lymphocytes; mammary regression; dendritic cells.

## INTRODUCTION

It has been reported that the early period of mammary gland involution coincides with a period of acutely increased susceptibility to intramammary infection (Nickerson, 1989; Oliver & Sordillo, 1989). One of the reasons given was that this could be due to the loss of the physical barrier by sloughing of the alveolar epithelium. Our recent studies (Lee et al. 1996; Tatarczuch et al. 1997) excluded this possibility as it was clearly shown that the process of apoptosis enabled the deletion of the alveolar epithelial cells without the loss of the integrity of the epithelial lining of the gland.

Our previous studies (Lee et al. 1989) showed that in the mammary gland of both nonpregnant and pregnant sheep, the great majority of the lymphocytes in the ductal and alveolar epithelium were agranulated and that they belong to the $CD8^+CD5^-$ phenotype followed by $CD45R^+$ cells and $CD5^+CD4^+$ cells. In our subsequent studies (Lee et al. 1992*a*) in which killed *Staphylococcus aureus* were infused into the mammary gland of nonpregnant and pregnant sheep, numerous $CD45R^+$MHC class $II^+$ B cells and antibody producing plasma cells were found, indicating that the lymphocyte phenotypes in nonpregnant and pregnant sheep mammary gland are capable of mounting an immune response. However, the dramatic changes occurring during involution are likely to have a profound effect on the resident immune cell

Correspondence to Dr C. S. Lee, Department of Veterinary Science, The University of Melbourne, 3010, Victoria, Australia. Fax: +61 3 9344 7374; e-mail: c.lee@vet.unimelb.edu.au

population and the proportion of the different phenotypes of leucocytes which have different roles in the immune mechanism. It is possible that the leucocyte types, especially the lymphocytes infiltrating into the gland at this stage are not sufficient to afford the gland protection. The phagocytic leucocytes residing in the alveolar and ductal lumina are probably preoccupied in removing the accumulated lipid droplets and milk protein and they are replete with engulfed material and may therefore be incapacitated.

The present study was designed to define the leucocyte phenotypes both in the mammary glandular tissues and mammary secretions during mammary involution in the sheep.

## MATERIALS AND METHODS

### *Animals*

To delineate the leucocyte type, especially the lymphocyte phenotypes, in the mammary glandular tissues at various stages of mammary involution 18 pregnant and 3 lactating sheep were used. In addition, another 18 pregnant sheep were used to study the leucocyte types and the lymphocyte phenotypes in the mammary secretions collected at various stages of mammary involution. The reason for not collecting mammary secretion from the same sheep for mammary glandular tissue studies was to avoid imposing artifactual changes to the glandular tissues through hand milking and infusion of normal saline for harvesting cells in involuting glands.

### *Tissues*

The 18 pregnant sheep to be used to delineate the lymphocyte phenotypes in the mammary glandular tissues at various stages of mammary involution were randomly allocated to 6 groups of 3. Mammary glandular tissues were obtained from the sheep at 2 (n = 3), 4 (3), 7 (3), 15 (3), 30 (3) and 60 (3) d after lambs were weaned 5 d after birth for light microscopy, immunohistochemistry and electron microscopy. Tissues were also obtained from the 3 lactating sheep. All animals were killed by an overdose of pentobarbitone sodium (Nembutal, Ceva Chemicals, Victoria, Australia) prior to tissue collection.

### *Antibodies*

A panel of monoclonal antibodies (mAb) (kindly provided by Dr M. R. Brandon, Centre for Animal Biotechnology, The University of Melbourne, Victoria, Australia), raised against ovine leucocyte surface antigens, was used for immunoperoxidase staining. These antibodies included anti-CD45R (Mackay et al. 1987); anti-CD8 (Maddox et al. 1985*b*); anti-CD5 (Mackay et al. 1985); anti-CD4 (Maddox et al. 1985*b*); anti-CD45 (Maddox et al. 1985*a*); anti-SW1 (Mackay et al. 1986); anti-MHC class II (Puri et al. 1987) and anti-γδTCR (Mackay et al. 1989).

### *Light microscopy*

Specimens for routine light microscopy were obtained from the lower, middle and upper regions of the left mammary gland. At each level, 3 samples were taken, fixed in Bouin's fixative for 24 h, processed and embedded by conventional methods. Paraffin sections (4–5 μm) were prepared and stained routinely with haematoxylin and eosin.

### *Immunohistochemistry*

For immunohistochemical studies, mammary tissues were embedded in OCT compound (Miles Scientific, Naperville, IL), snap-frozen using liquid nitrogen and stored at −70 °C. Frozen sections (6 μm) were fixed for 10 min in cold ethanol, air-dried and then stained, using the indirect immunoperoxidase technique as previously described (Lee et al. 1985). Briefly, tissue sections were incubated with the first antibody for 45 min at room temperature. Slides were washed with phosphate-buffered saline (PBS) and then incubated with peroxidase-conjugated rabbit antimouse Ig (DAKO-Immunoglobulins, Glostrup, Denmark) diluted 1:50 in PBS containing 5% normal sheep serum for 45 min at room temperature. After thorough washing in PBS, sections were incubated in 0.06% (w/v) diaminobenzidine tetrahydrochloride (Sigma Chemicals, St Louis, MO) in PBS containing 0.05% (v/v) hydrogen peroxide for 10 min at room temperature. For controls either PBS or 1% normal sheep serum in PBS was substituted for the first antibody.

### *Electron microscopy*

The right mammary glands were fixed by perfusion via the external pudendal artery with a fixative containing 2.8% paraformaldehyde, 5% glutaraldehyde and 0.6% picric acid in 0.15 M cacodylate buffer, pH 7.2. After initial fixation of the whole gland, small samples of mammary tissue were taken

from the lower, middle and upper regions of the gland. These samples of tissue were then cut into smaller pieces of $\sim 1\,mm^3$ while immersed in the fixative. The tissues were placed in fresh fixative for a minimum of 12 h at 4 °C before being processed. After washing 4 times in cacodylate buffer, the samples were postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature on a vertical rotator. The samples were then washed in distilled water twice and dehydrated in a series of graded acetone solutions before being embedded in Araldite (Biorad, USA). The blocks were polymerised at 60 °C for 48 h. Some specimens were stained en bloc during dehydration using a solution containing 2% uranyl acetate in 70% acetone.

Semithin sections (1 μm) were mounted on glass microscope slides and stained with a solution of 1% methylene blue in 1% borax. Ultrathin sections were collected on acetone-cleaned uncoated 200-mesh copper grids. These sections were contrasted with a 5% aqueous solution of uranyl acetate for 10 min and then with Reynold's lead citrate for 10 min.

*Cells in mammary secretions*

The 18 pregnant sheep used to study the leucocyte phenotypes in mammary involution secretions were randomly allocated to 6 groups of 3. The secretions were collected at 2 (n = 3), 4 (3), 7 (3), 15 (3), 30 (3) and 60 (3) d after lambs were weaned 5 d after birth. For glands at more advanced stages (15–60 d) of involution, 15 ml of sterile physiological saline were infused into the glands to harvest the cells. Cells from the secretion collected were isolated by centrifugation (400 g/10 min) at 4 °C. The cell pellets were resuspended in a small amount of saline (containing 2% BSA). Some cells were fixed for electron microscope immunohistochemical studies and some were used for preparing cytospots for immunocytochemical staining.

*Preparation of cytospots for immunocytochemistry*

After cells in mammary secretions were isolated by centrifugation, the cells were resuspended and diluted to the appropriate concentration ($4 \times 10^6$/ml) and 200 μl placed into a plastic well mounted onto a polylysine coated glass slide. The cell suspension was allowed to settle by gravity for 30 min at room temperature. Cytospots prepared in this manner were either stained with Giemsa stain or the immunoperoxidase method using a panel of monoclonal antibodies.

*Differential mammary cell count*

For differential cell count, cytospots stained with 10% Giemsa stain were used. A total of 200 cells were counted for each sample with a ×100 oil immersion objective. Based on morphological features and staining affinity, the cells were classified as neutrophils, monocytes/macrophages, lymphocytes or epithelial cells.

*Immunocytochemical staining of cytospots and leucocyte phenotype cell count*

The cytospots prepared from the mammary involution secretions were stained by the indirect immunoperoxidase technique in the same manner as described above to differentiate the leucocyte phenotypes. A total of 20 microscopic fields for each sample were counted with a ×100 oil immersion objective.

*Immunocytochemical staining of cells in mammary secretions for electron microscopy*

The immunogold-silver staining of cells in mammary secretions with a panel of monoclonal antibodies raised against sheep leucocyte surface antigens was performed according to a modified method described by Otsuki et al. (1990). Briefly, 200 μl aliquots of cell suspensions were fixed with 0.05% glutaraldehyde in PBS for 20 min at 4 °C. After 2 washings with PBS (containing 1% BSA), the cells were incubated overnight at 4 °C in 200 μl of primary monoclonal antibodies. The cells were then washed twice with PBS before incubation for 4 h at 4 °C with goat antimouse IgG conjugated with 5 nm gold particles (Amersham International, Poole, UK) at a dilution of 1:10 or 1:20. After further washings with PBS, the cells were fixed with 2% glutaraldehyde in PBS for 1 h, postfixed in 0.5% osmium tetroxide for 1 h at room temperature and incubated in 200 μl of silver enhancement developer (Amersham) for 7 min. The cells were then washed twice with distilled water and routinely dehydrated in acetone and embedded in Procure-Araldite resin (ProSCiTech). During dehydration, the cells were stained en bloc with 2% uranyl acetate in 70% acetone.

*Quantitation of apoptotic cells, granulated lymphocytes and nongranulated lymphocytes*

Araldite sections (1 μm) stained with 1% methylene blue in 1% borax were used for quantitation of

apoptotic cells, granulated and nongranulated lymphocytes. Only cells with a prominent nucleus were counted. Cells counted were large mononucleated cells (epithelial cells or macrophages), apoptotic cells (epithelial cells or macrophages containing apoptotic bodies also counted as apoptotic cells), granulated lymphocytes and nongranulated lymphocytes. A total of 60 microscopic fields were counted for the 3 samples collected from each gland with a $\times 100$ oil immersion objective.

### *Statistical analyses*

Statistical analysis was carried out with Student's $t$ test to assess if there was any significant difference ($P < 0.05$) between means of the sample groups. All values are reported as mean $\pm$ standard deviation of the mean (S.D.)

## RESULTS

### *General light and electron microscopic observations*

Light microscopic examination of haematoxylin and eosin stained sections revealed that from 2 to 4 d after weaning neutrophils were the predominant leucocytes in the ductal and alveolar lumina (Fig. 1*a*) and some were also seen in the interalveolar areas (Fig. 1*a*). Lymphocytes were also present but they were usually localised in or at the basal region of the alveolar and ductal epithelium (Fig. 1*b*) and the interalveolar areas. In 1 μm Araldite sections and ultrathin sections, some of the intraepithelial lymphocytes were seen with several cytoplasmic granules (Fig. 1*c*, *d*). Clusters of lymphocytes were seen in some interalveolar and periductal areas (Fig. 2*a*, *b*), and large clusters were occasionally found within lymphatic vessels located in the interlobular areas (Fig. 2*c*). Macrophages were located in the interalveolar areas and glandular epithelium. Similar to our previous report (Tatarczuch et al. 1997), apoptotic cells were also observed in the alveolar epithelium where they appeared to peak at 4 d after weaning.

At 7 d the architecture of most alveoli was still well defined though most of them were lined with highly vacuolated epithelial cells. The most striking feature was that most of the lumina of alveoli and ducts displayed predominantly foamy cells (Fig. 2*d*) and some neutrophils. Lymphocytes were still the predominant leucocyte type in the parenchyma and they were usually seen in the ductal and alveolar epithelium and a high proportion of them were granulated. Sometimes clusters of lymphocytes were seen in periductal and interalveolar areas.

By 15 d after weaning the alveoli had reduced markedly in size and lymphocytes were commonly found interspersed between the highly vacuolated alveolar epithelial cells. Large aggregates of lymphocytes were sometimes found in the periductal connective tissues and the interalveolar areas. Numerous foamy cells were prominent in the lumen of most alveoli and ducts (as seen at 7 d after weaning), while intraepithelial lymphocytes were commonly found in the ductal epithelium.

The glands at 30 and 60 d appeared to be completely involuted. Due to an increase in interalveolar connective tissues the lobular pattern became very conspicuous. Lymphocytes were now far more numerous than other types of leucocytes and they were located usually in close association with the alveolar and ductal epithelium. Some intraepithelial granulated lymphocytes were present, but their granules were fewer and much smaller than those observed at 2–7 d after weaning. The predominant cell type in the lumina of alveoli and ducts were the foamy cells, though some lymphocytes were found.

### *Immunocytochemical staining of leucocytes*

A panel of monoclonal antibodies was used to define the leucocyte types, especially the lymphocyte phenotypes and the differentiation of foamy macrophages from alveolar and ductal epithelial cells. It was revealed that the mammary glandular tissue was heavily populated with leucocytes as numerous $CD45^+$ cells were observed in the alveolar and ductal epithelium, the interalveolar areas and the periductal connective tissues. At all stages of involution, most of the intraepithelial lymphocytes, especially those in the ductal epithelium were of the CD8 phenotype (Fig. 3*a*). However, $CD4^+$ and $CD45R^+$ lymphocytes were also found in the alveolar and ductal epithelium albeit in a lower concentration, and very few $\gamma\delta^+$ cells were seen. The clusters of cells seen occasionally in the interalveolar areas and periductal connective tissues consisted predominantly of $CD5^+$ (Fig. 3*b*) and $CD4^+$ cells, and some $CD8^+$ cells.

A striking feature was that at 4 d, there was a fair number of MHC class $II^+$ cells (Fig. 3*c*) interspersed between the alveolar and ductal epithelial cells which were $CD45^-$ and MHC class $II^-$ (Fig. 3*c*). At 7 and 15 d after weaning, numerous highly vacuolated cells were observed within the lumen of alveoli and ducts and immunohistochemical staining revealed that these cells were $CD45^+$, MHC class $II^+$ (Fig. 3*d*, *e*) but

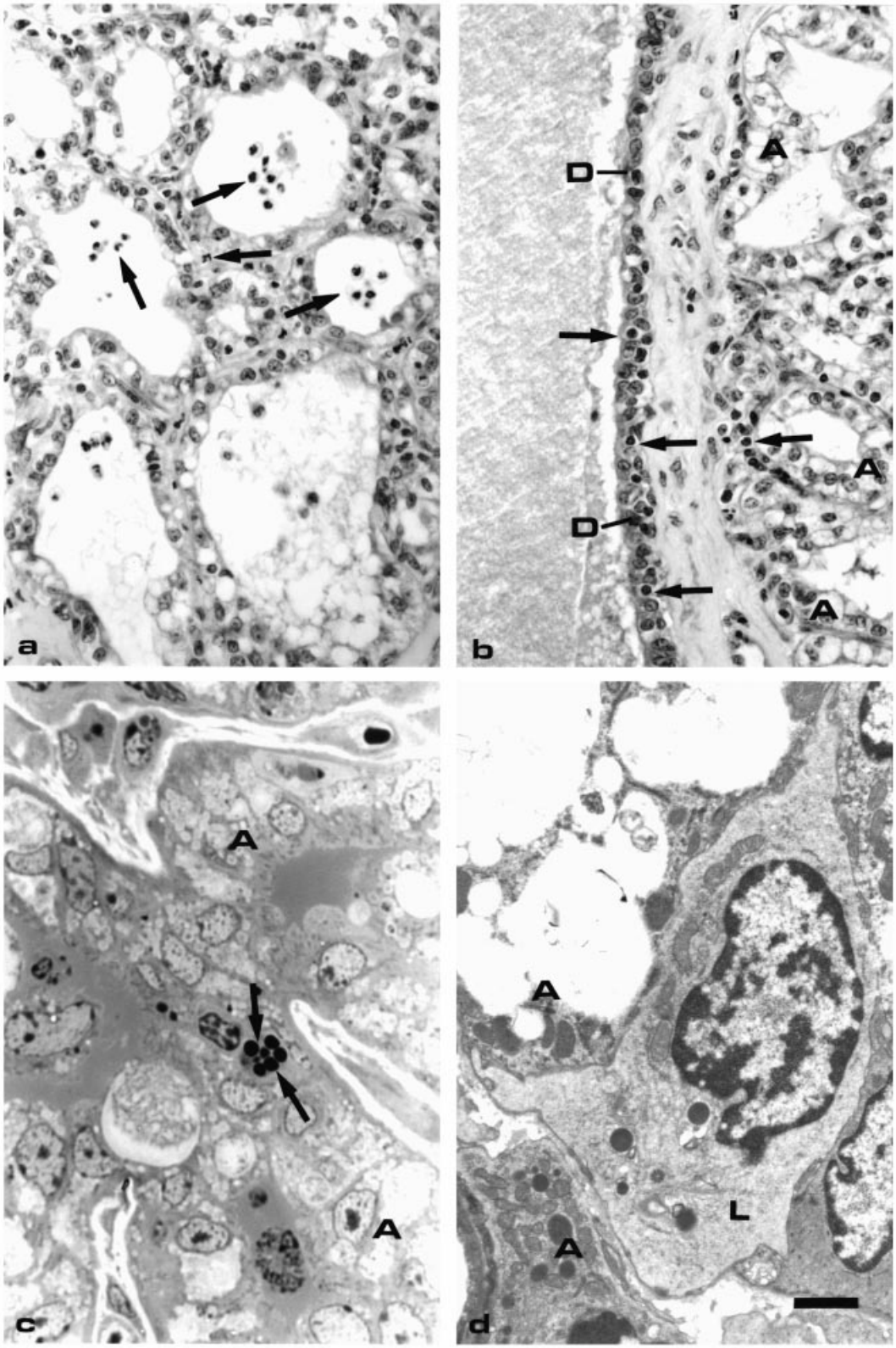


Fig. 1. Leucocytes in mammary gland tissue during early stages of involution (2–4 d after weaning). (*a*) Neutrophils (arrows) in alveolar lumina and interalveolar areas; H & E, × 337. (*b*) Lymphocytes (arrows) within and at the basal regions of the ductal (D) and alveolar (A) epithelium; H & E, × 337. (*c*) Intraepithelial lymphocyte seen with numerous granules (arrows) in its cytoplasm. A, alveolar epithelium; 1 % methylene blue in 1 % borax, × 756. (*d*) Electron micrograph showing an intraepithelial granulated lymphocyte (L) intervening between alveolar epithelial cells (A), Bar, 1 μm.

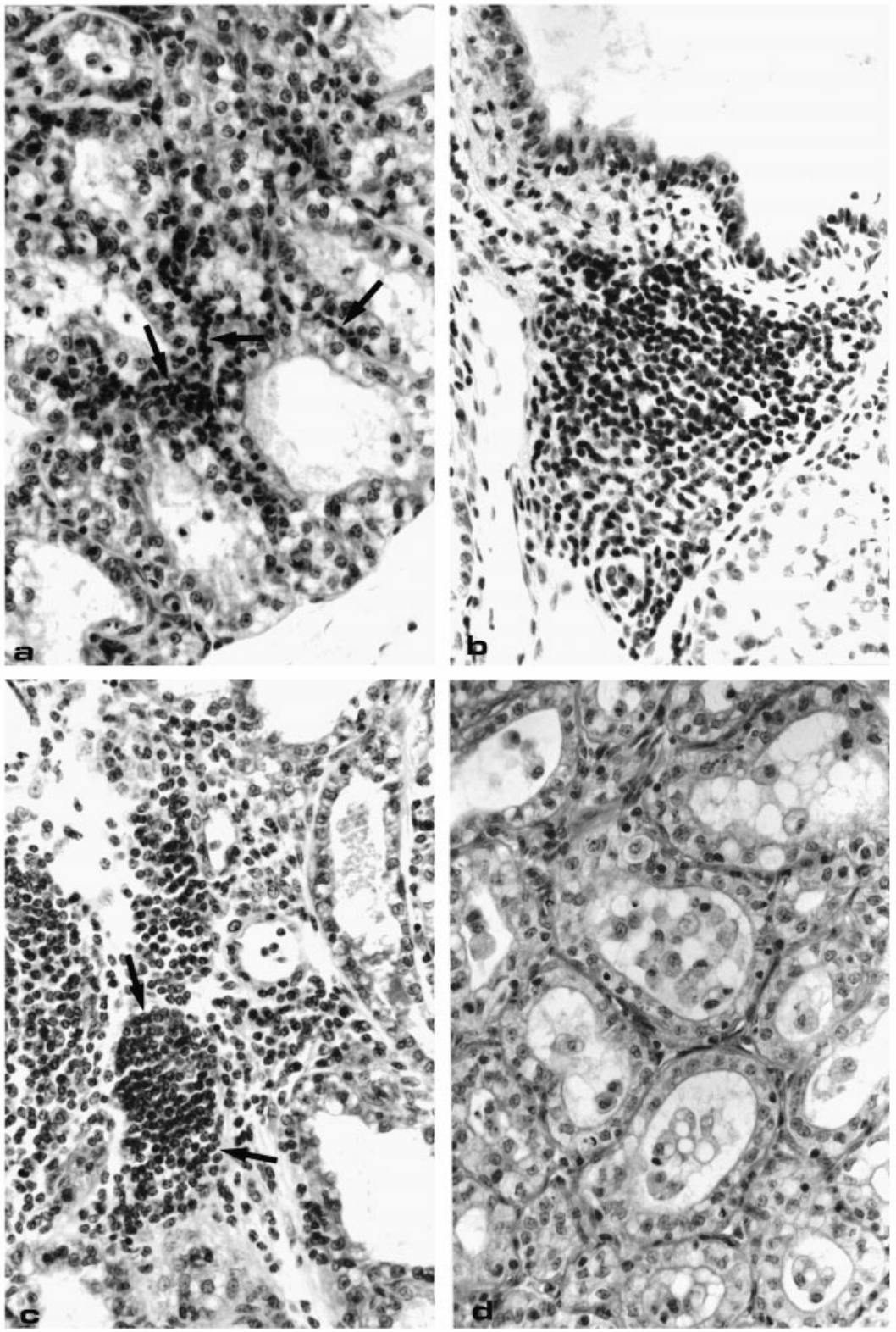


Fig. 2. (*a*) Numerous lymphocytes (arrows) in interalveolar areas, 4 d after weaning; H & E, ×337. (*b*) Cluster of lymphocytes in the periductal area, 4 d after weaning; H & E, ×337. (*c*) Numerous lymphocytes within a lymphatic vessel (arrows) located in the interlobular connective tissue, 4 d after weaning; H & E, ×450. (*d*) Most of the cells within the alveolar and ductal lumina are vacuolated macrophages, 7 d after weaning; H & E, ×337.

$CD4^-$, $CD8^-$ and $CD45R^-$, thus indicating that they were macrophages and not desquamated epithelial cells.

In fully involuted glands (30 and 60 d after weaning), the lymphocyte was the predominant cell type. They were predominantly $CD8^+$ and were

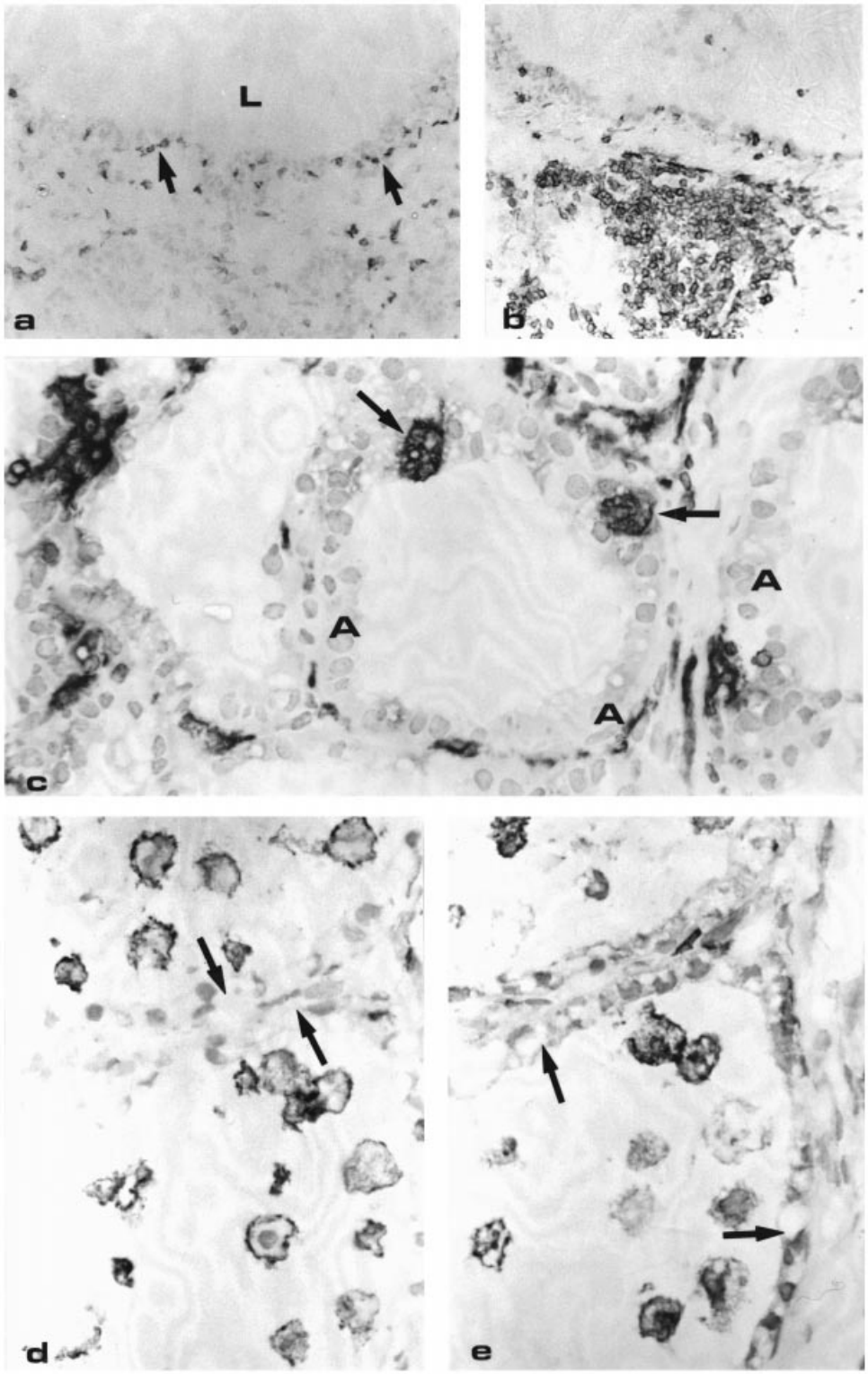


Fig. 3. (*a*) Most of the intraepithelial lymphocytes are $CD8^+$ (arrows). L, lumen of duct. 4 d after weaning; indirect immunoperoxidase method, ×160. (*b*) Most of the cells within the periductal cluster are $CD5^+$ (and $CD4^+$) T lymphocytes; indirect immunoperoxidase method, ×160. (*c*) MHC class $II^+$ macrophages (arrows) interspersed between alveolar epithelial cells (A) which are MHC class $II^-$, 4 d after weaning; indirect immunoperoxidase method, ×640. (*d*, *e*) The vacuolated cells within the alveolar lumina are $LCA^+$ and MHC class $II^+$, whereas the alveolar epithelial cells (arrows) are $LCA^-$ and MHC class $II^-$, 15 d after weaning; indirect immunoperoxidase method, ×640.

usually located within and beneath the remnants of alveolar and ductal epithelium. Sometimes $CD45^+$ MHC class $II^+$ vacuolated cells were seen in the lumina of some alveoli and ducts. The basal region of

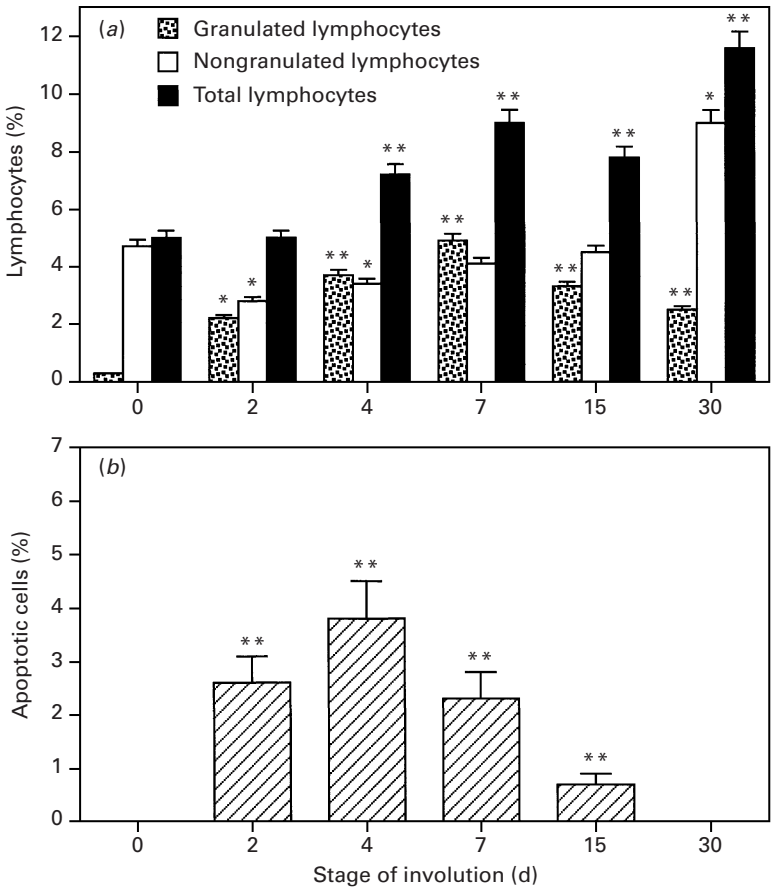


Fig. 4. Proportions of (*a*) lymphocytes and (*b*) apoptotic cells in the mammary glandular epithelium during mammary involution (values represent mean % ± S.D.; * $P < 0.05$, ** $P < 0.01$ significant difference compared to d 0 timepoint; n = 3 sheep/group).

the alveolar and ductal epithelium was frequently seen with cells containing MHC class $II^+$ cytoplasmic processes. These were presumably dendritic cells.

*Lymphocyte and apoptotic cell counts*

The above studies showed that there was an apparent increase in the number of intraepithelial granulated lymphocytes from 2 to 30 d after weaning, and there were more and larger granules in these cells especially from 2 to 7 d after weaning. Consequently, a quantitative study was carried out, based on 60 microscopic fields on 1 μm Araldite sections with a × 100 oil immersion objective. As shown in Figure 4*a*, there was a significant increase ($P < 0.05$) in the percentage of granulated lymphocytes (2.2 %) from 2 days after weaning. This increase coincided with the significant increase ($P < 0.01$) in the percentage of apoptotic cells which reached a peak (3.8 %) 4 d after weaning (Fig. 4*b*). However, the percentage of granulated lymphocytes reached a peak on d 7 (4.9 %) and it declined to 2.5 % by 30 d, by which time no apoptotic bodies were detected (Fig. 4*a*, *b*). The increase in granulated lymphocytes was accompanied by a significant decline ($P < 0.05$) in the nongranulated lymphocytes at 2 d after weaning. From 4 to 15 d, the nongranulated lymphocytes returned to their original levels (3.4–4.5 %), followed by a significant increase ($P < 0.05$) by 30 d after weaning. There was a significant increase in the total percentages of lymphocytes from 4 to 30 d after weaning ($P < 0.01$).

*Cells in mammary secretions*

From 2 to 4 d after weaning, neutrophils (Fig. 5*a*) were the predominant leucocytes followed by macrophages and lymphocytes. Following this, there was a decline in neutrophils ($P < 0.01$), and macrophages (Fig. 5*b*) became the predominant cell type from 7 d ($P < 0.01$), with lymphocytes also seen to gradually increase from this point (Fig. 6). Electron microscopy

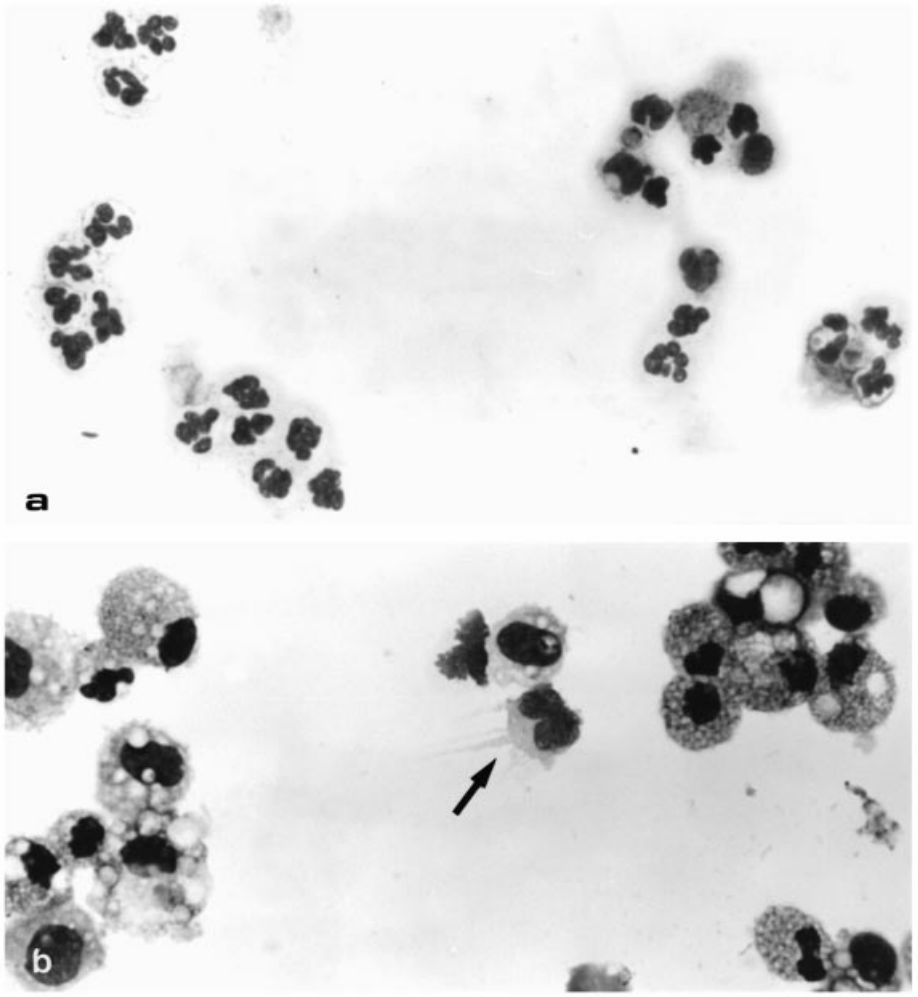


Fig. 5. Cytospot preparation of cells derived from a sample of mammary secretion illustrating (*a*) the predominance of neutrophils at 2 d after weaning; Giemsa stain, ×300, and (*b*) the predominance of vacuolated macrophages present at 15 d after weaning. The cell marked with an arrow with a lobulated nucleus and long cytoplasmic processes is probably a dendritic cell; Giemsa stain, ×560.

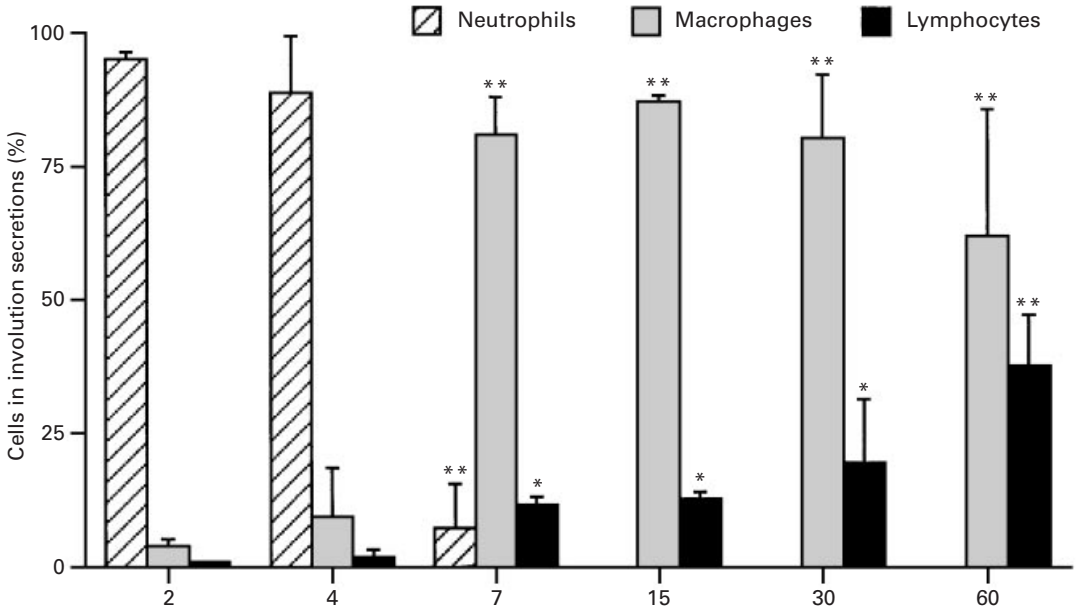


Fig. 6. Changes observed in % of neutrophils, macrophages and lymphocytes in involution secretions following weaning (values represent mean % ± S.D.; * $P < 0.05$, ** $P < 0.01$ significant difference compared with 2 timepoint; n = 3 sheep/group).

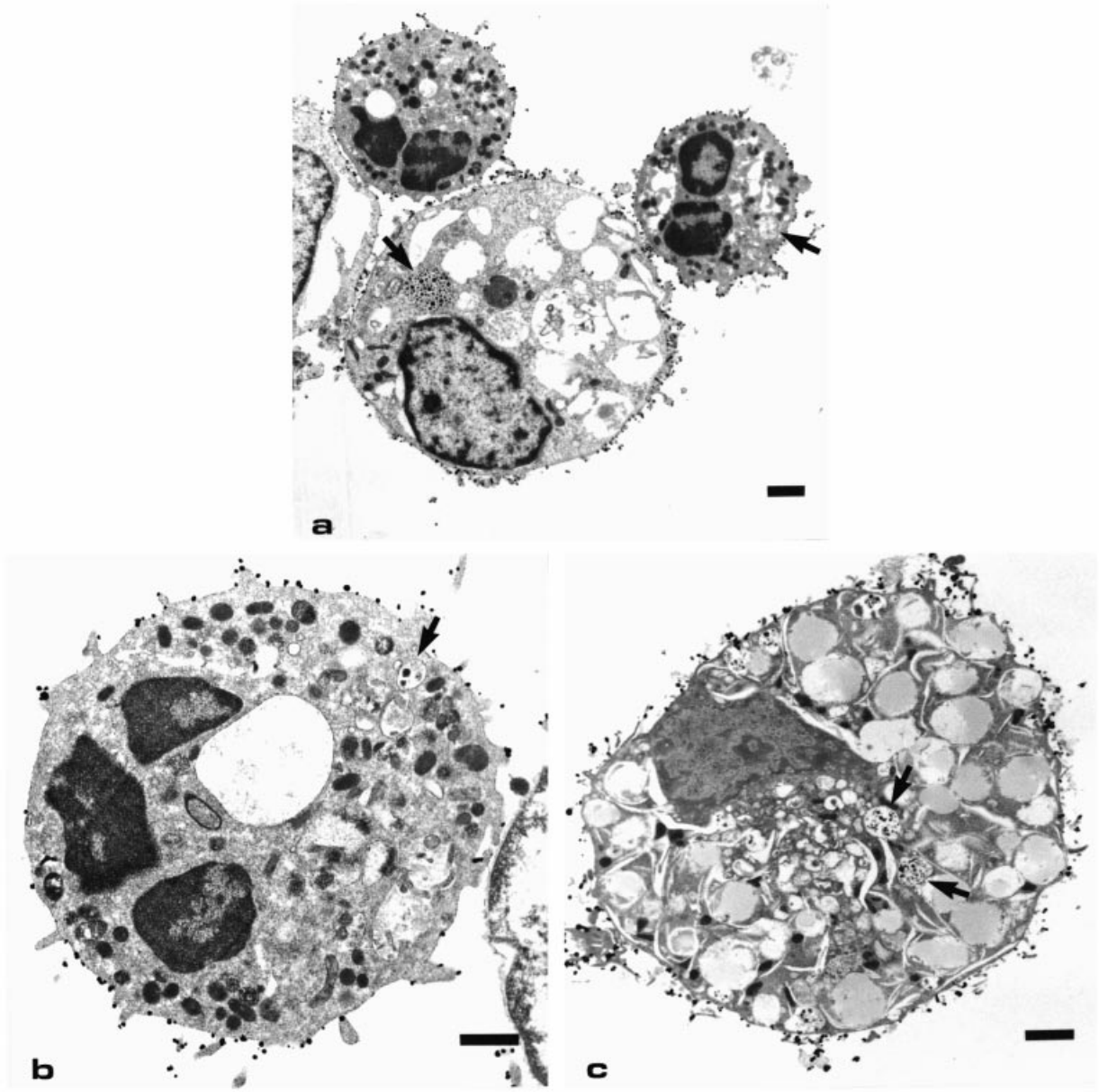


Fig. 7. Electron micrographs showing that both neutrophils (*a*, *b*) and macrophages (*a*, *c*) are laden with lipids, and casein (arrows). Note that both these cells are labelled positively with monoclonal antibodies raised against LCA. Immunogold–silver staining method. Bars, 1 μm (*a*, *c*); 0.5 μm (*b*).

confirmed that the vacuoles seen in foamy cells (Fig. 2*d*) were lipids and in addition casein and cellular debris were also taken up by these cells (Fig. 7*a–c*).

Immunocytochemical staining revealed that from d 7 there was an increase in the proportion of cells staining positive for MHC class II (Fig. 8*a*) in mammary secretions, an observation which closely paralleled the dramatic increase seen in macrophages at this time (Fig. 6). Most of the lymphocytes were T cells based on staining with the T cell specific markers CD5, CD8 and CD4. $CD8^+$ lymphocytes (Fig. 8*b*) were the predominant subpopulation at all stages, followed by $CD4^+$ cells. In contrast, there were very few $\gamma\delta^+$ T cells or B cells ($CD45R^+$) seen in mammary secretions. Quantitative studies revealed that the increase in lymphocytes in mammary secretions from d 7 involved an increase in both CD8 and CD4 T cells (data not shown).

A striking feature of the mammary involution secretions was the presence of a small subpopulation of cells ($< 1\%$ of all leucocytes in mammary secretions) with extensive cytoplasmic processes (Figs 5*b*, 9*a*, *b*). These cells were $LCA^+$ and MHC class $II^+$, suggesting that they were most likely dendritic cells. Other cells possibly related to these, which were few in number and displayed a lobulated nucleus and cytoplasmic veil, were $LCA^+$, MHC class $II^{high}$ and expressed CD1 (present on dendritic cells) (Fig. 9*c*, *d*).

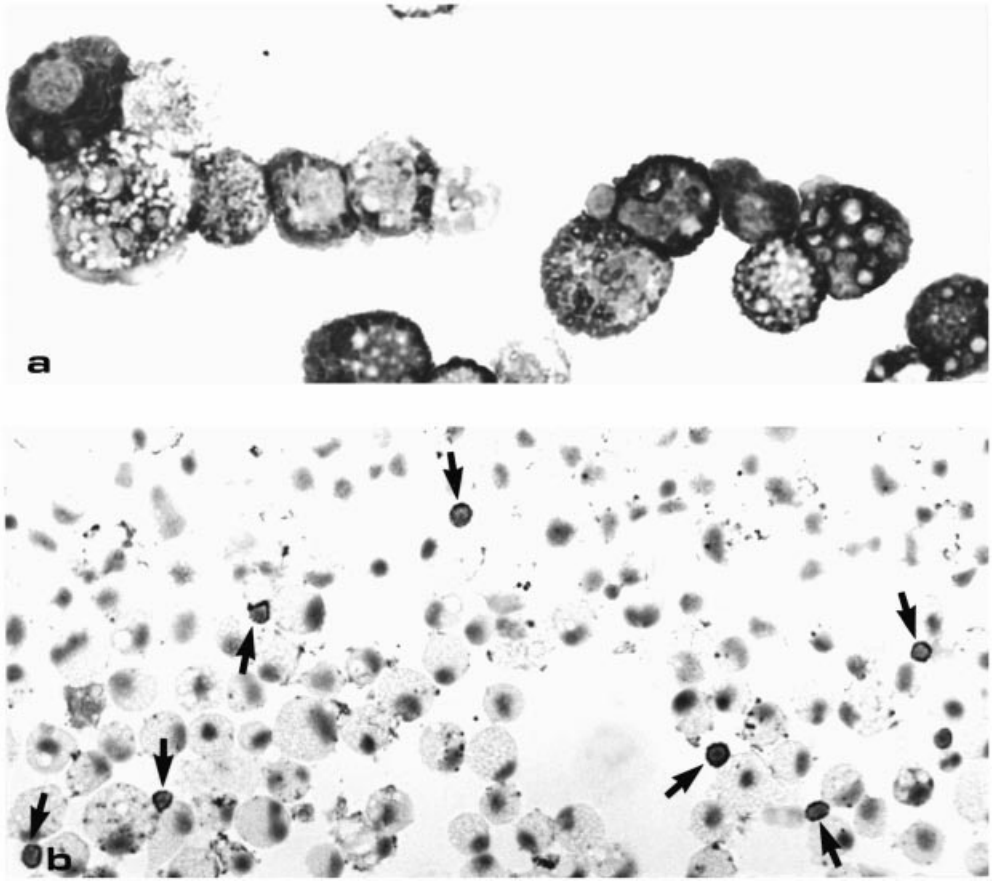


Fig. 8. Cytospot preparation of cells derived from a sample of mammary secretion at 15 d after weaning illustrating (*a*) the predominance of MHC class $II^+$ cells and (*b*) that most of the lymphocytes (arrows) are $CD8^+$; indirect immunoperoxidase method, $\times 400$.

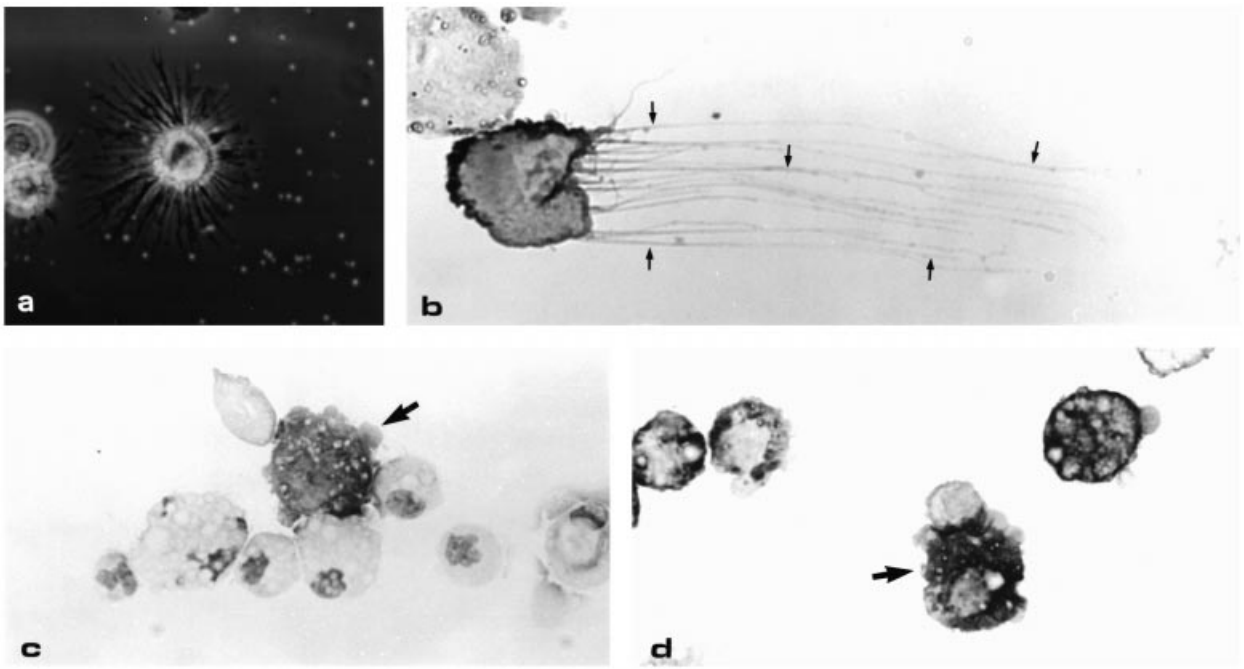


Fig. 9. (*a*) Cell with extensive cytoplasmic processes; phase contrast microscopy of a cell suspension derived from a sample of mammary secretion at 15 d after weaning, $\times 640$. (*b–d*) Cytospot preparation of cells derived from samples of mammary secretion at 15 d after weaning illustrating (*b*) MHC class $II^+$ cells with extensive cytoplasmic processes (arrows); indirect immunoperoxidase method, $\times 1008$. (*c*) a $CD1^+$ cell with many cytoplasmic veils (arrow). Note that all the other cells are not stained; indirect immunoperoxidase method, $\times 640$. (*d*) Cell (arrow) presenting similar morphology to the $CD1^+$ cell in Fig. 9*c*, is stained intensely with MHC class II; indirect immunoperoxidase method, $\times 640$.

## DISCUSSION

The results of this study confirmed our previous observation (Tatarczuch et al. 1997) that the mammary gland of the ewe involutes by apoptosis, a process which allows deletion of cells without the loss of the basic architecture and the integrity of the epithelial lining of the gland. The mammary glands were completely involuted by 30 d after weaning.

The present study also revealed that during the early stages of involution, 2–7 d, numerous leucocytes infiltrated into the mammary glands. It appeared that there was selective compartmentalisation of leucocyte phenotypes within the involuting gland. During the early stages, the phagocytic cells, predominantly neutrophils followed by macrophages were found in the alveolar and ductal lumina. Influx of these 2 cell types into these regions was for the removal of casein, lipid droplets and cellular debris, an activity vital in the involution process. Electron microscopic examination indeed confirmed earlier observations (Lee & Outteridge, 1981) that these cells were heavily laden with casein, lipid droplets and cellular debris. It is likely that this may partly account for the observation that the mammary gland is more susceptible to infection at the early stages of involution as these cells are replete with engulfed materials and are incapacitated. However, susceptibility of the gland to infection at this time could also be due to other factors. Recent studies by Ayoub & Yang (1997) on mammary involution of the cow revealed that there were high levels of transforming growth factor beta (TGF-β) in the mammary secretions of involuting glands. They indicated that the high levels of TGF-β and the absence of interleukin-2 (IL-2) in secretions of involuting mammary glands in contrast to the dry glands, reflected the immunosuppressive nature of dry gland secretions. This may predispose the gland to higher incidence of infection at the beginning of involution. In addition, it is well documented that casein also has an immunosuppressive effect (Cooray, 1996).

One striking observation in this study was the presence of a small subpopulation of MHC class $II^+$, $CD1^+$ cells showing similar morphological characteristics as dendritic cells in the involution secretions collected at 15–30 d after weaning. There was also a concomitant increase of lymphocytes with a greater number of $CD8^+$ cells than $CD4^+$ cells, an observation consistent with that of Persson-Waller & Colditz (1998). In other parts of the body, for example the respiratory tract, the primary role of dendritic cells is in the surveillance of antigen, which they sequester and store in immunogenic form for subsequent presentation to T cells (Holt et al. 1992, 1993). The local cytokine mielieu may play a significant role in the changes seen in the presence of leucocytes in involution secretions. Relevant perhaps to the appearance of dendritic cells in mammary involution secretions, recent in vitro studies (Geissmann et al. 1998) reported that TGF-β, in the presence of granulocyte/macrophage colony-stimulating factor and IL-4, induced differentiation of human peripheral blood monocytes into dendritic Langerhans cells. It is possible that the TGF-β-rich microenvironment provided by the involution secretion at this time (Ayoub & Yang, 1997) is sufficient to stimulate cells of the monocyte/macrophage series in the involution secretion to differentiate into dendritic cells. Such a sequence of events could occur in the involuted mammary gland and may partly account for the observation that the dry gland is more resistant to infection than the lactating gland and gland at the early stages of involution (Sordillo et al. 1987). The role of dendritic cells in the mammary gland is currently under investigation in our laboratory.

It appeared that most of the lymphocytes entering the mammary gland had a tendency to remain in the interalveolar areas, periductal areas and in the epithelium of alveoli and ducts. Relatively few lymphocytes entered the lumina of alveoli and ducts. This observation is consistent with our previous studies on pregnant and nonpregnant mammary glands of ewes (Lee et al. 1989).

The frequent appearance of lymphocytes within lymphatic vessels suggests that quite a large proportion of the lymphocytes infiltrating into the mammary gland are returned to the regional lymph node for recirculation. The recirculation of lymphocytes is a highly complex and dynamic process and there is selective entry or homing of lymphocytes to certain tissues which is controlled principally by interaction between adhesion molecules on lymphocytes and their complementing ligands on vascular endothelium. Our previous (Lee et al. 1989) and present studies indicated that there exists a separate and organised distribution of different leucocyte populations in the mammary tissue. To date, little is known about which adhesion molecule(s) are important in regulating the selective traffic of various lymphocyte subpopulations. Indeed there are still gaps in our understanding of the molecular mechanism regulating lymphocyte homing and the physiological consequences of these mechanisms in the mammary gland.

Lymphocytes within the glandular epithelia, es-

pecially the ductal epithelia, were mainly $CD8^+$. This observation is consistent with our previous study on the mammary glands of nonpregnant and pregnant ewes (Lee et al. 1989). Electron microscope studies revealed that some of the intraepithelial lymphocytes were $CD45R^+$ and they were seen with several large granules within their cytoplasm. The morphological features of these cells were similar to the granulated lymphocytes (LGLs) observed in the interplacentomal areas in the placenta of the sheep (Lee et al. 1988, 1992*b*), cow (Lee et al. 1997) and deer (Lee et al. 1995). It was implicated that in the deer these cells may play a role in inhibiting the trinucleate cells to expand into a syncytium or in destroying the trinucleate cells. Although the role of these cells during mammary involution is unknown, their significant increase from 2 d after weaning and reaching a peak at 7 d which coincided with apoptosis of alveolar epithelial cells (Fig. 4), suggests that they may play a role in inducing apoptosis of the glandular epithelium. However, the increase in the proportions of LGLs and apoptotic cells from 2 to 7 d might have been a consequence of changes in the size of the epithelial cells due to the accumulation of lipid droplets and casein. Notwithstanding, the number and size of the granules within the LGLs increased over this period. Recent studies (Gamen et al. 1998) indicated that granulysin which is released by cytotoxic T lymphocytes and NK cells could induce apoptosis. Further studies are necessary to identify the content of the granules in the LGLs.

From the foregoing, it is evident that involution of the mammary gland is associated with extensive infiltration of leucocytes into the mammary gland and that there is compartmentalisation of the leucocyte phenotypes. Although there was an increase in lymphocyte concentrations in the involution secretion with advancement of involution, most of the lymphocytes resided in the glandular tissues and most of the intraepithelial lymphocytes were $CD8^+$. Since there were very few $\gamma\delta TCR^+$ cells in the glandular epithelium, it is possible that most of the $CD8^+$ intraepithelial lymphocytes belong to the $CD8^+$ $CD45R^+$ $\gamma\delta TCR^-$ subpopulation reported by Meeusen et al. (1993). The phagocytic leucocytes, both neutrophils and macrophages, were destined for the alveolar and ductal lumina. The presence of macrophages, dendritic cells, $CD8^+$ and $CD4^+$ lymphocytes in involution secretion of involuted glands may afford the involuted mammary gland more resistance to infection than lactating glands and glands at early stages of involution. In addition, the high concentration of humoral defence factors such as lactoferrin (Smith & Schanbacher, 1977) in the secretion of involuted glands may also provide the dry gland better protection. It is evident that the fully involuted mammary gland provides a suitable microenvironment for mounting an immune response.

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## REFERENCES

AYOUB IA, YANG TJ (1997) The regulatory role of transforming growth factor-beta in activation of milk mononuclear cells. *American Journal of Reproductive Immunology* **38**, 121–128.

COORAY R (1996) Casein effects on the myeloperoxidase-mediated oxygen-dependent bactericidal activity of bovine neutrophils. *Veterinary Immunology and Immunopathology* **51**, 55–65.

GAMEN S, HANSON DA, KASPAR A, NAVAL J, KRENSKY AM, ANEL A (1998) Granulysin-iduced apoptosis. I. Involvement of at least two distinct pathways. *Journal of Immunology* **161**, 1758–1764.

GEISSMANN F, PROST C, MONNET J-P, DY M, BROUSSE N, HERMINE O (1998) Transforming growth factor β, in the presence of granulocyte/macrophage colony stimulating factor and Interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *Journal of Experimental Medicine* **187**, 961–966.

HOLT PG, OLIVER J, McMENAMIN C, SCHON-HEGRAD MA (1992) Studies on the surface phenotype and functions of dendritic cells in parenchymal lung tissue of the rat. *Immunology* **75**, 582–587.

HOLT PG, OLIVER J, BILYK N, McMENAMIN C, McMENAMIN PG, KRAAL G et al. (1993) Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *Journal of Experimental Medicine* **177**, 397–407.

LEE CS, OUTTERIDGE PM (1981) Leucocytes of sheep colostrum, milk and involution secretion, with particular reference to ultrastructure and lymphocyte sub-populations. *Journal of Dairy Research* **48**, 225–237.

LEE CS, GOGOLIN-EWENS K, WHITE TR, BRANDON MR (1985) Studies on the distribution of binucleate cells in the placenta of the sheep with a monoclonal antibody SBU-3. *Journal of Anatomy* **140**, 565–576.

LEE CS, GOGOLIN-EWENS K, BRANDON MR (1988) Identification of a unique lymphocyte subpopulation in the sheep uterus. *Immunology* **63**, 157–164.

LEE CS, MEEUSEN E, BRANDON MR (1989) Subpopulations of lymphocytes in the mammary gland of sheep. *Immunology* **63**, 157–164.

LEE CS, MEEUSEN E, BRANDON MR (1992*a*) Local immunity in the mammary gland. *Veterinary Immunology and Immunopathology* **32**, 1–11.

LEE CS, MEEUSEN E, GOGOLIN-EWENS KJ, BRANDON MR (1992*b*) Quantitative and qualitative changes in the intraepithelial lymphocyte population in the uterus of the non-pregnant and pregnant sheep. *American Journal of Reproductive Immunology* **28**, 90–96.

LEE CS, WOODING FBP, MORGAN G (1995) Quantitative analysis of intraepithelial large granular lymphocyte distribution

and maternofetal cellular interactions in synepitheliochorial placenta of the deer. *Journal of Anatomy* **187**, 445–460.

LEE CS, TATARCZUCH L, PHILIP C (1996) Mechanism of mammary gland involution in the sheep. *Proceedings of the Anatomical Society of Australia and New Zealand* **33**, 20

LEE CS, WOODING FBP, MORGAN G (1997) Quantitative analysis throughout pregnancy of intraepithelial large granular and non-granular lymphocyte distributions in the synepitheliochorial placenta of the cow. *Placenta* **18**, 675–681.

MACKAY CR, MADDOX JF, GOGOLIN-EWENS KJ, BRANDON MR (1985) Characterization of two sheep lymphocyte differentiation antigens SBU-T1 and SBU-T6. *Immunology* **55**, 729–738.

MACKAY CR, MADDOX JF, BRANDON MR (1986) Three distinct subpopulations of sheep T lymphocytes. *European Journal of Immunology* **16**, 19–25.

MACKAY CR, MADDOX JF, BRANDON MR (1987) A monoclonal antibody to the p220 component of sheep LCA identifies B cells and a unique lymphocyte subset. *Cellular Immunology* **110**, 46–53.

MACKAY CR, BEYA MF, MATZINGEN P (1989) γδ cells express a unique surface molecule during thymic development. *European Journal of Immunology* **19**, 1477–1485.

MADDOX JF, MACKAY CR, BRANDON MR (1985*a*) The sheep analogue of leucocyte common antigen (LCA). *Immunology* **55**, 347–353.

MADDOX JF, MACKAY CR, BRANDON MR (1985*b*) Surface antigens, SBU-T4 and SBU-T8 of sheep T lymphocyte subsets defined by monoclonal antibodies. *Immunology* **55**, 739–748.

MEEUSEN E, FOX A, BRANDON M, LEE C-S (1993) Activation of uterine intraepithelial γδ T cell receptor-positive lymphocytes during pregnancy. *European Journal of Immunology* **23**, 1112–1117.

NICKERSON SC (1989) Immunological aspects of mammary involution. *Journal of Dairy Science* **72**, 1665–1678.

OLIVER SP, SORDILLO LM (1989) Approaches to the manipulation of mammary involution. *Journal of Dairy Science* **72**, 1647–1664.

OTSUKI Y, MAXWELL LE, MAGARI S, KUBO H (1990) Immunogold–silver staining method for light and electron microscopic detection of lymphocyte cell surface antigens with monoclonal antibodies. *Journal of Histochemistry and Cytochemistry* **38**, 1215–1221.

PERSSON-WALLER K, COLDITZ IG (1998) Expression of surface antigens on blood and mammary leukocytes in lactating and dry ewes. *Veterinary Immunology and Immunopathology* **62**, 273–278.

PURI NK, GORRELL MD, BRANDON MR (1987) Sheep class II molecules. I. Immunochemical characterization. *Immunology* **62**, 567–573.

SMITH KL, SCHANBACHER FL (1977) Lactoferrin as a factor of resistance to infection of the bovine mammary gland. *Journal of the American Veterinary Medical Association* **170**, 1124–1227.

SORDILLO LM, NICKERSON SC, AKERS RM, OLIVER SP (1987) Secretion composition during bovine mammary involution and the relationship with mastitis. *International Journal of Biochemistry* **19**, 1165–1172.

TATARCZUCH L, PHILIP C, LEE CS (1997) Involution of the sheep mammary gland. *Journal of Anatomy* **190**, 405–416.