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# Quantitative analysis of intraepithelial large granular lymphocyte distribution and maternofetal cellular interactions in the synepitheliochorial placenta of the deer

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ABSTRACT

Intraepithelial lymphocytes are a constant feature of ruminant uterine epithelium in nonpregnant animals. Quantitation in 6 species of deer shows that the proportion of large granular lymphocytes (LGL) in this population increases markedly and continuously from the earliest implantation to the latest (midpregnant) stage examined. The size of individual granules also increases. Fetal trophectodermal binucleate cells are also present from the earliest stage in all deer examined and follow a usual ruminant life cycle: migrating and fusing with uterine epithelial cells to form trinucleate cells before releasing their granules to the maternal compartment and finally degenerating and being reabsorbed by the trophectodermal uninucleate cells. Deer LGLs were usually closely associated with the degranulating fetomaternal hybrid trinucleate cells but showed no ultrastructural changes themselves. This association indicates a dynamic interaction between deer LGL and trinucleate cells which could serve as one of the factors restricting the extent of trinucleate cell progression to a continuous syncytium as found in sheep and goats.

*Key words*: Deer; pregnancy; placenta; large granular lymphocytes.

## INTRODUCTION

Maternofetal cellular interactions are crucial in determining successful implantation and establishing the pattern of subsequent placentation (Wooding & Flint, 1994). Species with invasive placentation (man, monkeys, rodents) eliminate the uterine epithelium and establish a stable, mixed maternofetal cellular population at the maternofetal interface invariably with a major contribution from a unique subset of large granular lymphocytes (LGL) (see reviews in King & Loke, 1991; Croy, 1994). The ruminant has a noninvasive placenta with characteristic maternofetal hybrid syncytium formation but here also Lee et al. (1992) have shown in the sheep that LGLs are involved in the maternofetal accommodation. The sheep uterus is populated by a variety of lymphocytes but the LGLs form a unique population of $CD45R^+$ granulated cells which are localised exclusively in the uterine and endometrial glandular epithelium (Lee et al. 1988). In a subsequent quantitative study in the sheep (Lee et al. 1992) it was shown that from days 55 to 134 of pregnancy, the granules in these cells increased in size, and there was a significant increase also in the proportion of this cell population in the uterine epithelium but not in the endometrial glandular epithelium located in the deeper region of the stroma which is further from the maternofetal interface. This population of LGLs was uniformly distributed in the interplacentomal uterine epithelium during pregnancy, but such cells were never found in the syncytial layer of the placentomes (Gogolin-Ewens et al. 1989).

It is now well established that all ruminants so far investigated have a synepitheliochorial placenta, a mixture of maternofetal hybrid syncytium with cellular uterine epithelium apposed to fetal cellular trophectoderm (or chorion) (Wooding, 1992; Wooding & Morgan, 1993), but there are distinct differences in the degree of syncytial formation among

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the ruminant species (Wooding, 1982; Lee et al. 1986; Wooding & Flint, 1994). In the sheep, goat and the cow just after implantation, the maternofetal syncytium predominates. This situation persists in the sheep and goat placentome until parturition, but in the interplacentomal area and in the whole of the cow placentome a cellular uterine epithelium is re-established by regeneration from residual individual uterine epithelial cells. However, a constant level of binucleate cell migration persists to form transient trinucleate cells. Such differences between the ruminant species offer the opportunity to further characterise the nature of the LGL-uterine epithelial interactions by comparing the pattern of distribution of the intraepithelial granular lymphocytes in the other ruminant species with that observed in the sheep (Lee et al. 1992). Such data may promote a better understanding of the dynamics and interaction of the fetal and maternal cellular components at the maternofetal interface whose dramatic increase in area is essential for the successful growth and development of the embryo.

Some deer species are reported to have a limited region of syncytium formation (Hamilton et al. 1960; Wooding & Flint, 1994) and study was carried out to examine both the extent of binucleate cell migration and fetomaternal syncytium formation and the pattern of distribution of uterine intraepithelial granulated lymphocytes in 6 species of deer, mainly red (*Cervus elaphus*), roe (*Capreolus capreolus*) and fallow (*Dama dama*), with individual specimens of muntjac (*Muntiacus muntjak*), Chinese water (*Hydropotes inermis*) and axis (*Axis axis*) deer.

## MATERIALS AND METHODS

Gravid uteri from deer were cut out immediately after the mother was killed by captive bolt pistol (red deer), or rifle shot (other deer) (for details see Table 1). The uterine arteries were cannulated and the uterus perfused with a glutaraldehyde fixative at room temperature (4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, with 2% sucrose; or 1% glutaraldehyde plus 3% (para)formaldehyde in 0.1 M cacodylate buffer, pH 7.2, with 5% sucrose. These two fixatives were used to produce material suitable for ultrastructural examination, and immunocytochemistry respectively. Five minutes after perfusion via the maternal uterine arteries was started, fixative was also injected into the uterine lumen in early stages of pregnancy or perfused via an umbilical arterial cannula where this was possible. After perfusion with fixative for about 30 min, dyes (1% aqueous Nigrosin, Malachite Green or eosin) were perfused through the vasculature. Slices (1–2 mm) were then cut across the centre of each of 4 placentomes which showed good staining with the dye. Small (1 cm square) samples were also taken of apposed fetal and maternal layers from interplacentomal areas. The placentomal slices were fixed for a further 2–3 h and then cut into matchstick-sized pieces ($2 \times 2 \times 30$ mm) running the full depth of the placentome from the fetal to the maternal tissue. At the earlier stages of placentomal development, the entire placentome was cut off at a level just above the myometrium and subdivided as necessary. The tissue was stored in 0.1 M cacodylate buffer, pH 7.2 at 4 °C until processed.

Material from each animal was processed for structural studies through osmium tetroxide and ethanol into Araldite and for immunocytochemistry through ethanol into Araldite, glycol methacrylate, or Lowicryl K4M resin, at −20 °C (Wells, 1985).

Semithin resin sections were cut for light microscope immunocytochemistry (K4M) or LGL quantitation (osmium-Araldite or K4M or glycolmethacrylate stained with toluidine blue or periodic acid-Schiff (PAS)). Ultrathin sections were cut from Araldite blocks and stained with uranyl acetate and lead citrate. Araldite or K4M sections without osmium were stained with 1% phosphotungstic acid before electron microscope examination which selectively stains binucleate cell granules and the placental microvillar junction (Wooding, 1980).

### *Immunocytochemistry*

K4M blocks were sectioned onto coverslip fragments, dried at 60 °C for 30 min, and floated for 10 min on phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.05% thimerosal. This solution was also used for all antibody dilutions. They were then transferred to primary mouse monoclonal antibody (SBU3, see below) or rabbit polyclonal antibody (bovine placental lactogen, see below) and incubated overnight at 4 °C, followed by a PBS jetwash. After the monoclonal antibody the second antibody used was rabbit antimouse IgG at a dilution of 1:1000 for 40 min. Sections were then jetwashed with PBS before incubation for 30 min on goat antirabbit IgG coated gold colloid, 1 nm diameter (GARGI; Amersham International, Poole, UK). Sections were jetwashed in PBS and then glass distilled water and left on distilled water for 10 min before incubation for 12–20 min on the gold intensification

Table 1. *Incidence of intraepithelial lymphocytes as a percentage of the total uterine epithelial cells in the uterus of nonpregnant and pregnant deer*

| Group | Species of deer | Reproductive state of uterus | Percentages of lymphocytes | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | LGL | | NGL | | TL | |
| | | | COT | ICOT | COT | ICOT | COT | ICOT |
| 1 | Red | Nonpregnant | 0.7 | 0.7 | 10.2 | 11.5 | 10.9 | 12.2 |
| | Red | Nonpregnant | 0.7 | 0.7 | 10.3 | 11.6 | 11.0 | 12.3 |
| | Roe | Nonpregnant | 0.1 | 0.2 | 2.5 | 4.9 | 2.6 | 5.1 |
| 2 | Roe | Pregnant (4 mm CRL) | 6.5 | 2.7 | 5.7 | 5.3 | 12.2 | 8.0 |
| | Red | Pregnant (5 mm CRL) | 2.1 | 2.7 | 11.4 | 8.1 | 13.5 | 10.8 |
| | Red | Pregnant (10 mm CRL) | 1.1 | — | 2.6 | — | 3.7 | — |
| | Red | Pregnant (13 mm CRL) | 1.4 | — | 1.6 | — | 3.0 | — |
| | Red | Pregnant (14 mm CRL) | 2.4 | 3.2 | 2.0 | 3.7 | 4.4 | 6.9 |
| | Fallow | Pregnant (15 mm CRL) | 2.5 | 1.5 | 5.1 | 2.0 | 7.6 | 3.5 |
| | Red | Pregnant (15 mm CRL) | 1.5 | — | 0.8 | — | 2.3 | — |
| | Red | Pregnant (18 mm CRL) | — | 4.8 | — | 3.9 | — | 8.7 |
| | Red | Pregnant (20 mm CRL) | 1.6 | — | 0.7 | — | 2.3 | — |
| | Fallow | Pregnant (20 mm CRL) | 2.6 | 3.7 | 1.8 | 3.1 | 4.4 | 6.8 |
| | Fallow | Pregnant (25 mm CRL) | 3.1 | 4.1 | 2.7 | 3.4 | 5.8 | 7.5 |
| 3 | Chinese water | Pregnant (30 mm CRL) | 4.3 | 6.8 | 1.6 | 2.6 | 5.9 | 9.4 |
| | Roe | Pregnant (30 mm CRL) | 7.8 | 5.7 | 2 | 1.6 | 9.8 | 7.3 |
| | Red | Pregnant (38 mm CRL) | 3.7 | 11.5 | 1.4 | 3.4 | 5.1 | 14.9 |
| | Red | Pregnant (80 mm CRL) | 4.0 | — | 0.9 | — | 4.9 | — |
| | Roe | Pregnant (80 mm CRL) | 8.4 | 4.9 | 3.2 | 2.0 | 11.6 | 6.9 |
| | Muntjac | Pregnant midterm | 5.1 | — | 0.8 | — | 5.9 | — |
| | Axis | Pregnant midterm | 8.8 | — | 2.0 | — | 10.8 | — |

CRL, crown–rump length; LGL, large granulated lymphocytes; NGL, nongranulated lymphocytes; TL, total lymphocytes; COT, caruncular or placentomal area; ICOT, intercaruncular or interplacentomal area.

reagent (Amersham). The label visibility was monitored on the light microscope. The coverslip pieces were finally jetwashed in distilled water, dried and mounted permanently for photography. The same sequence was used for the polyclonal antibody, but the secondary antibody step was omitted.

*Antibody origin*

The antibodies have been characterised previously, and were all raised against ovine or bovine placental preparations: SBU3 (Gogolin-Ewens et al. 1986) a mouse monoclonal reacting with placentomal binucleate cell granules; bPL (Murthy et al. 1982), a rabbit polyclonal raised against purified bovine placental lactogen and reacting with all binucleate cell granules in cow and deer. Presumably the antibovine placental lactogen is reacting with deer placental lactogen but this molecule has not yet been isolated.

A panel of monoclonal antibodies (mAb) (kindly provided by Dr M. R. Brandon, Centre for Animal Biotechnology, 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*a*); anti-CD5 (Mackay et al. 1985); anti-CD4 (Mackay et al. 1985*a*); anti-CD45 (Maddox et al. 1985*b*); anti-T19 (Mackay et al. 1986); anti-MHC class II (Puri et al. 1987) and anti-$\gamma\delta$TcR (Mackay et al. 1989).

*Quantitation of cells*

Araldite K4M or glycolmethacrylate sections (1 μm) stained with 1% toluidine blue in 1% borax or PAS were used for quantitation of granulated lymphocytes in uterine and endometrial glandular epithelium. Only cells with a prominent nucleus were included in the count. Cells counted were epithelial cells, granulated lymphocytes, nongranulated lymphocytes and trinucleate cells. These last cells were considered to originate from the migration and fusion of one fetal binucleate cell with one uterine epithelial cell, and are therefore found only in the uterine epithelium. The cells have 3 nuclei, 2 large fetal nuclei and 1 smaller paler maternal nucleus, but not all will necessarily be

found on any one section through such a large cell. Other characteristics of trinucleate cells—PAS-positive granule content, the density and size of the nucleus—could sometimes be used to identify such cells unequivocally if only 1 or 2 nuclei were present, but counting criteria were rigidly applied and the figures probably indicate minimum values only. A total of 40 microscopic fields were counted for each sample with a × 100 oil immersion objective.

Statistical analysis was carried out with Student's t test to assess if there was any significant difference ($P < 0.01$) within and between the means of the sample groups. All values are reported as mean ± standard deviation.

## RESULTS

Twenty-one deer uteri (for details, see Table 1), 3 nonpregnant (group 1; Table 2) and 18 at different stages of pregnancy (groups 2 and 3; Table 1) were obtained. Histological examination indicated that the cell structure and interactions at the maternofetal interface in the different species were very similar and the extent of placentomal development correlated consistently with the crown–rump length (CRL) of the fetus. For analytical purposes it was therefore considered legitimate to group different species into the two arbitrary categories 'early' (4–25 mm CRL) and 'mid' (30–80 mm CRL) pregnancy but quantitative results for individual animals are given in Table 1.

### *Nonpregnant uterus*

Histological examination of 1 μm sections revealed that the cellular organisation of both the caruncular and intercaruncular uterine epithelium was generally either cuboidal or columnar, although areas with pseudostratified epithelium were not uncommon. Intraepithelial lymphocytes were frequently observed and were usually interspersed between the basal regions of the uterine epithelial cells. Closer examination showed that most of these lymphocytes lacked granules although the occasional cell was seen with 1–3 metachromatic granules usually located at the indented region of the nucleus. The concentration of intraepithelial lymphocytes both in the caruncular and intercaruncular areas was similar, and both these regions contained more nongranulated than granulated intraepithelial lymphocytes. The few lymphocytes scattered in the underlying stroma showed no granules in their cytoplasm. Mast cells characterised by an oval nucleus and numerous metachromatic granules were sometimes seen in the underlying stroma.

### *Early pregnant uterus*

The earliest pregnant uteri available were from a roe deer (uterus contained a fetus with 4 mm CRL) and a red deer (fetus with 5 mm CRL). Examination of sections of both showed an equivalent range of cell structures and organisation. The trophectodermal (or chorionic) epithelium was flat and closely apposed to the uterine epithelium with interdigitated microvilli in both caruncular and intercaruncular areas (Fig. 1). The trophectoderm was formed by 2 cell types: a continuous sheet of uninucleate cells with intraepithelial binucleate cells. The binucleate cells located at the basal region of the trophectoderm were usually small and immature as they contained few granules in their cytoplasm (Fig. 1, arrowheads). The mature fully granulated binucleate cells were larger and were usually located at the apical region of the trophectoderm. Some were seen with extensive cytoplasmic

Table 2. *Mean of the percentages (± S.D.) of intraepithelial lymphocytes in the uterine epithelium of nonpregnant and pregnant deer*

| Group | Reproductive state of uterus | Percentages of lymphocytes | | | | | |
|---|---|---|---|---|---|---|---|
| | | LGL | | NGL | | TL | |
| | | COT | ICOT | COT | ICOT | COT | ICOT |
| 1 | Nonpregnant | 0.5 (0.3) | 0.5 (0.3) | 7.5 (4.5) | 9.3 (3.8) | 8.1 (4.8) | 9.8 (4.1) |
| 2 | 4–25 mm CRL | 2.5 (1.6) | 3.2 (1.1) | 3.4 (3.2) | 4.2 (2.0) | 5.9 (4.0) | 7.4 (2.2) |
| 3 | 30 mm CRL midterm | 6.0 (2.2) | 7.2 (3.0) | 1.7 (0.8) | 2.4 (0.8) | 7.7 (2.9) | 9.6 (3.7) |

Figures in brackets are standard deviations. For abbreviations see Table 1.

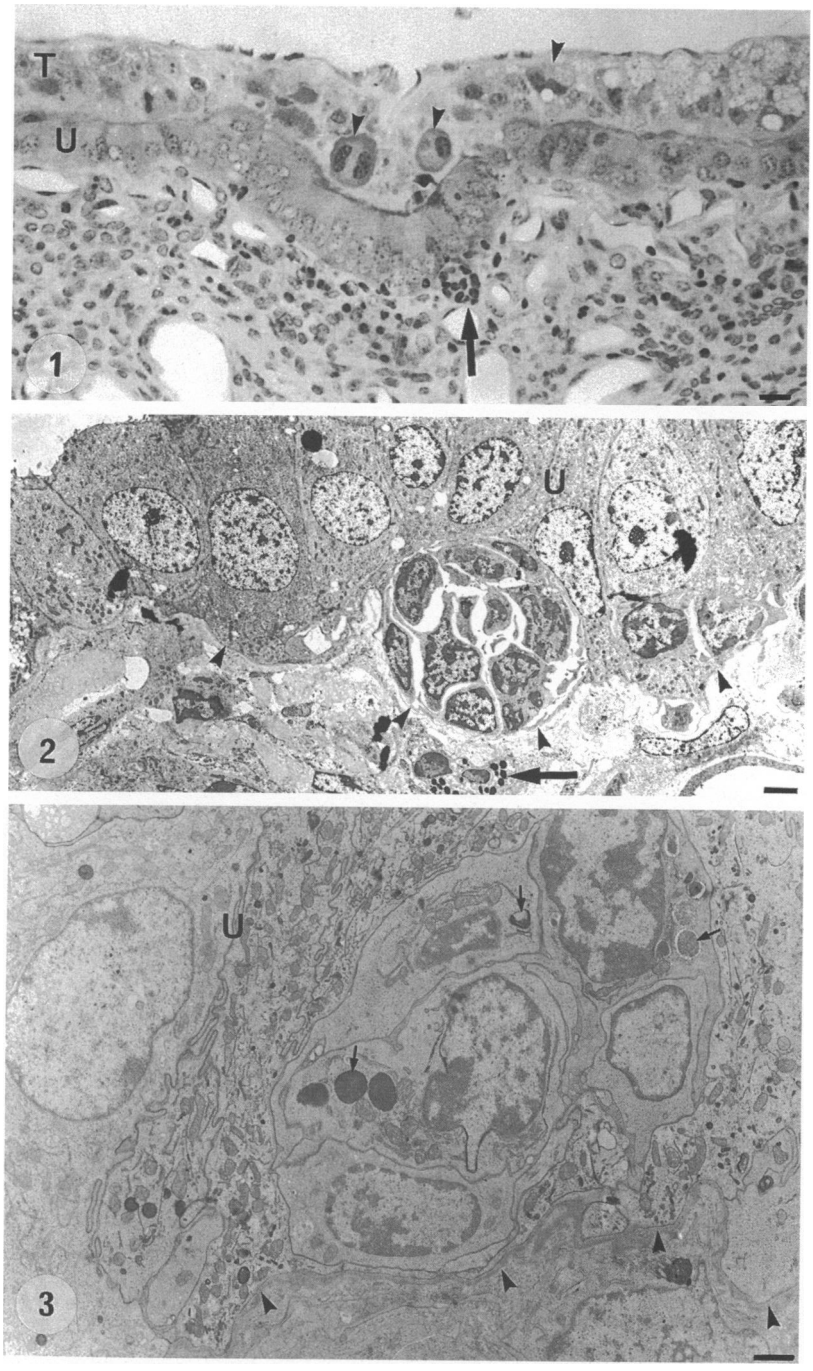



Fig. 1. Light micrograph of a section from a pregnant uterus at the earliest stage of implantation available. Note the presence of a spherical focal aggregate of lymphocytes (arrow) located at the basal region of the uterine epithelium (U). Trophectoderm (T) with intraepithelial binucleate cells (arrowheads). Red deer, 5 mm CRL. Bar, 2 μm.

Fig. 2. Electron micrograph of another focal aggregate of lymphocytes from the same red deer specimen as in Figure 4 confirming that the aggregate of lymphocytes is located above the basement membrane (arrowheads) of the uterine epithelium (U). The granulated stromal cell (arrow) is a pigment cell. Roe deer, 4 mm CRL. Bar, 2 μm.

Fig. 3. Higher magnification of a similar aggregate from the earliest roe deer pregnancy showing that most of the lymphocytes are granulated (arrows). The basement membrane of the uterine epithelium (U) is indicated by arrowheads. Roe deer, 4 mm CRL. Bar, 1 μm.

processes extending towards the maternal epithelium, thus flattening the microvillar junction with granules concentrated in the opposite, fetal trophectoderm directed, pole of the cell (Fig. 4). On the maternal side the continuous flat sheet of uninucleate uterine epithelial cells contained a significant increase in the number of intraepithelial large granulated lymphocytes compared with the nonpregnant state both in

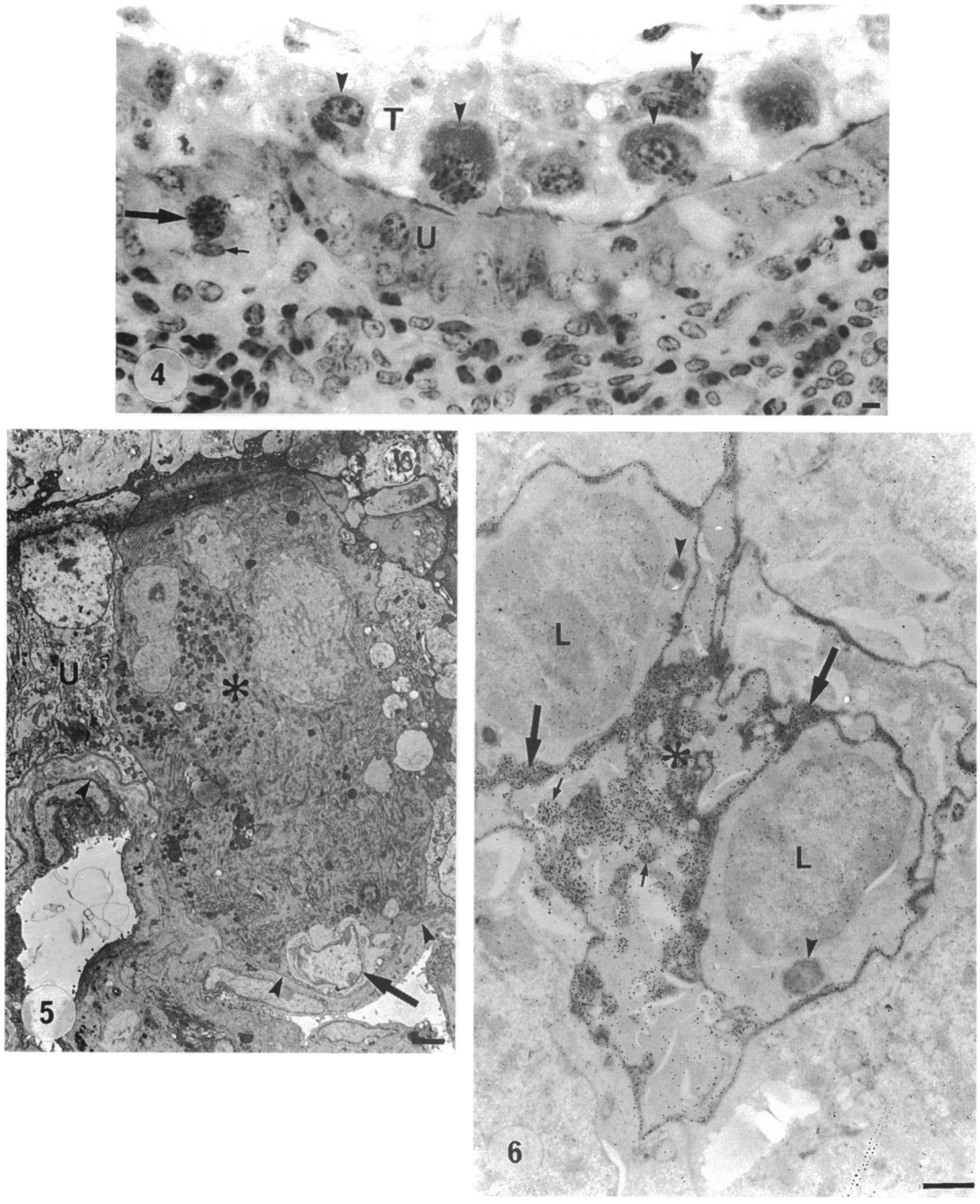



Fig. 4. Light micrograph of a section from an early pregnant uterus showing a trinucleate cell characterised by having 2 large nuclei (large arrow) resembling those of the binucleate cells in the trophoblast and a small paler nucleus (small arrow) resembling that of a uterine epithelial cell. Several mature binucleate cells are present (arrowheads) in the trophectoderm (T) apposing the uterine epithelium (U). Red deer, 5 mm CRL. Bar, 2 μm.

Fig. 5. Electron micrograph of a section from an early pregnant uterus. Note that the trinucleate cell (asterisk) has virtually surrounded an LGL (arrow) located on the basement membrane (arrowheads) of the uterine epithelium (U). Roe deer, 5 mm CRL. Bar, 2 μm.

Fig. 6. Electron micrograph of a section from an early pregnant uterus stained by the immunogold technique for the localisation of placental lactogen (immunoreactivity using antibovine placental lactogen). Note that the trinucleate cell granules (small arrows) and their exocytosed content (large arrows) located in the intercellular space around the cytoplasm of the trinucleate cells (asterisk) and associated LGLs (L) are heavily labelled with gold particles. The LGL granules (arrowheads) are not labelled. Roe deer, 4 mm CRL. K4M resin-immunogold. Bar, 1 μm.

placentomal and interplacentomal regions (Table 2). Some of these lymphocytes were found in spherical focal aggregates which were only seen at this earliest stage of implantation (Figs 2, 3) and examination with the electron microscope showed that most were granulated (Fig. 3). The uterine epithelium also

contained trinucleate and polynucleate cells, formed by migration of the fetal binucleate cells from the trophectodermal epithelium and their fusion with individual uterine epithelial cells or their derivatives. In favourable sections these fetomaternal hybrid trinucleate cells showed 2 or more large nuclei resembling those of the fetal binucleate and a smaller paler nucleus resembling that of a uterine epithelial cell (Fig. 4) but it was not usually possible to recognise differences in nuclear appearance at the electron microscope level (Fig. 5). LGLs were usually seen in close contact with these tri or polynucleated cells (Figs 5, 6). Close examination showed that both the trinucleate cells and polynucleate cells contained PAS and bovine placental lactogen positive granules similar to those in the binucleate cells in the trophoblast but all concentrated at the base of the uterine epithelium, i.e. maternally directed. Use of the immunogold technique at the ultrastructural level clearly demonstrated that bovine placental lactogen was present in the granules (Fig. 6) and that they exocytosed their content into the intercellular space around the trinucleate cell associated LGL in the uterine epithelium. The situation in the interplacentomal areas was basically similar to that observed in the caruncular areas.

As pregnancy advanced (uteri with fetuses of 10–15 mm CRL) sporadic sites of villus formation were evident. In these regions the trophoblast began to indent the caruncular epithelium (Figs 7, 8). The caruncular epithelium apposing the tip of these very early villi were usually seen with several layers of cells (Figs 8, 10) and mitotic figures were commonly observed. Placental lactogen immunoreactivity served as a valuable marker for binucleate cells and their derivatives in the uterine epithelium (Figs 7–11).

In pregnant uteri with fetuses of 20–25 mm CRL (red and fallow deer), chorionic villus formation in the caruncular area became more distinct. These villi were in the form of finger-like projections extending into the caruncle (Figs 10, 11). Similar to the earlier stage, there was also thickening of the caruncular epithelium apposed to the tip of the chorinoic villi. One other very noticeable feature was the presence of higher concentrations of bovine placental lactogen-positive binucleate cells at the tips of the chorionic villi (Fig. 10). Migration of binucleate cells to form trinucleate cells and polynucleate cells with the maternal uterine epithelium was evident from the distribution of placental lactogen immunoreactivity (Figs 7–11) but there was no evidence of any increase in frequency of migration (Table 3). The distribution of binucleate cells in the interplacentomal trophectoderm and trinucleate cells in the interplacentomal uterine epithelium (Table 1) was basically similar to that observed in the roe deer, as was the fragmentation and degeneration of the trinucleate cells with chorionic phagocytosis of the residues (see under 'midpregnancy', Figs 12, 13, 15).

The increased frequency of intraepithelial LGLs in both the placentomal and interplacentomal uterine epithelium seen in the 4 or 5 mm conceptuses was maintained (Tables 1, 2). None of the focal collections of intraepithelial lymphocytes seen at the basal region of the uterine epithelium in the 4 or 5 mm conceptuses (Figs 1, 2) were seen at this or any later stage. In contrast to the nonpregnant uteri, far fewer nongranulated intraepithelial lymphocytes were found in both the placentomal and interplacentomal uterine epithelium.

### *Midpregnant uteri*

At midpregnancy, the placentomes were well established. The chorionic villi had increased considerably in length and secondary villi had developed. The trophectoderm around the tips of the chorionic villi had a high concentration of binucleate cells and the uterine epithelium at the basal region of the caruncular crypts were formed by several layers of cells. Fewer binucleate cells were seen from the midregion to the base of the chorionic villi. The percentage of trinucleate cells observed in the placentomal uterine epithelium was similar to that observed at the earlier stages of pregnancy (Table 1) but the proliferation of the fetomaternal interface layers makes good examples of the trinucleate cell formation and subsequent degeneration sequence easier to find. The following description is valid from the earliest implantation to the latest stage examined. Binucleate cells (deriving from any uninucleate cell) differentiate out of contact with either trophectodermal basement membrane or tight junction (e.g. Figs 1, 4, 9, 10, 16, 19). No evidence for any exocytosis into the fetal epithelium was found in the mature, i.e. fully granulated binucleate cells, which then migrate up to and through the fetal trophectodermal tight junction, while maintaining that seal. Few examples of membrane fusion of a binucleate cell to a uterine epithelial cell were found but the consequence was commonplace, with granules streaming down to the basolateral surfaces of the trinculeate cell before their release by exocytosis (Figs 17, 18). Evidence of granule release came from immunogold identification of placental lactogen or SBU3 antigens in the trinucleate cell granules and in the basolateral intercellular space

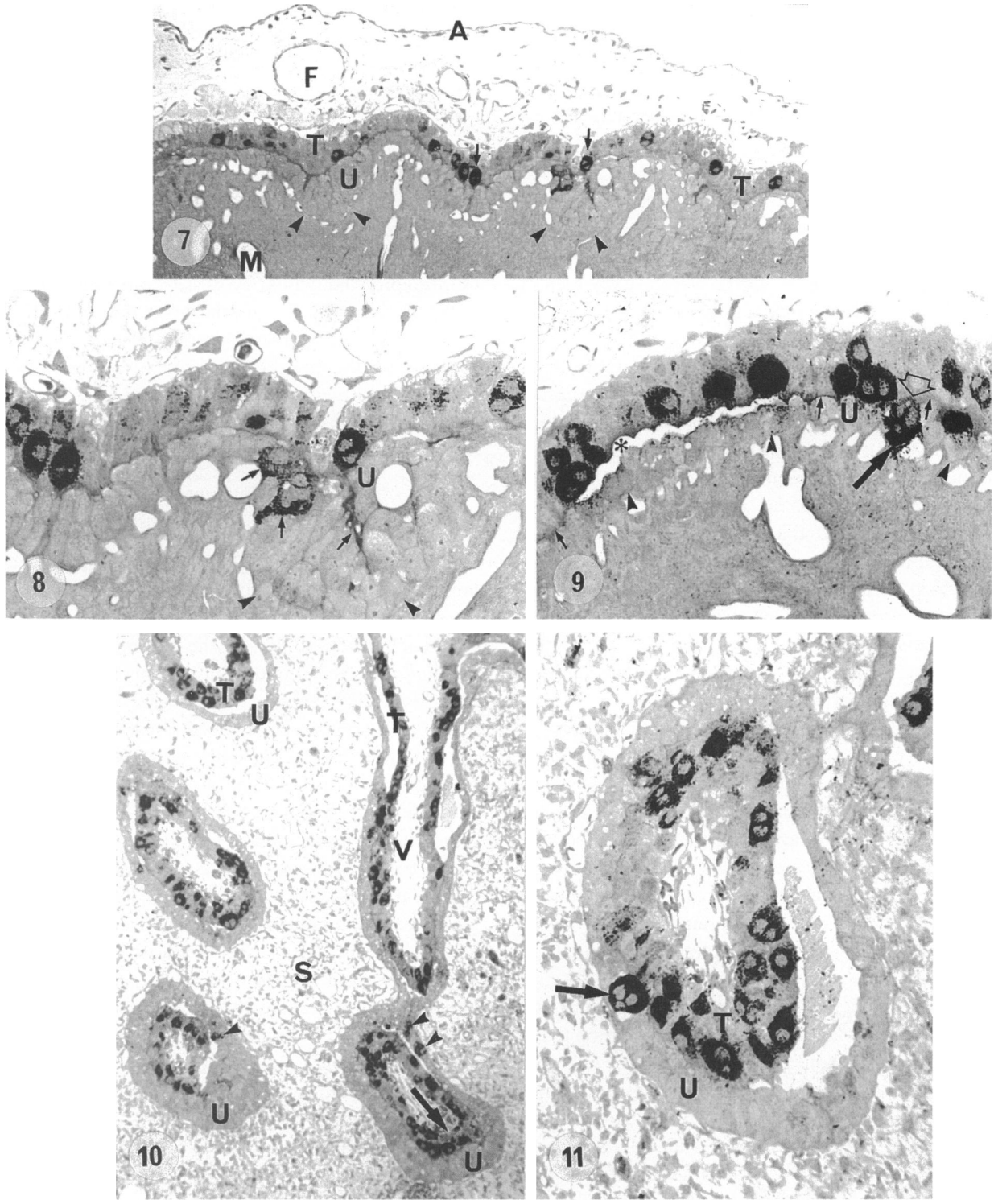



Fig. 7. Light micrograph of a section from an early pregnant uterus stained by the immunogold-silver enhancement technique for the localisation of placental lactogen immunoreactivity. There are localised thickenings (arrowheads) of the uterine epithelium (U) each indented by the trophoblast (T) which we interpret as initiation sites of chorionic villi. Some of these show binucleate cells containing placental lactogen at their putative tips (arrows). A, allantoic epithelium; F, fetal blood vessel; M, maternal blood vessel. Fallow deer 15 mm CRL. K4M resin-immunogold × 120.

Fig. 8. Higher magnification of an area shown in Figure 10 confirming that the caruncular epithelium apposing the putative rudiments of chorionic villi is formed by several layers of cells (arrowheads). Note that the trinucleate cells (arrows) interspersed between the caruncular epithelium cells (U) also contain placental lactogen immunoreactivity indicating that they are derived from the labelled trophoblast binucleate cells. Fallow deer, 15 mm CRL. K4M resin-immunogold, × 270.

Fig. 9. Light micrograph of an area from an early pregnant uterus showing a trinucleate cell (open arrow) labelled heavily for placental lactogen localised in the caruncular epithelium (U). All the PL-containing granules are at the maternal pole of the trinucleate cell (large

Table 3. *Trinucleated (TNC) plus polynucleate (PNC) cells as a percentage (±S.D.) of total cells in the placentomal (COT) and interplacentomal (ICOT) uterine epithelium of the 2 groups of pregnant deer*

| Group | Pregnant uterus | Percentages of TNC plus PNC | |
|---|---|---|---|
| | | COT | ICOT |
| 2 | 4–25 mm CRL | 1.2 (0.5) n = 11 | 1.0 (0.6) n = 11 |
| 3 | 30 mm CRL, midterm | 0.8 (0.7) n = 7 | 0.2 (0.1) n = 7 |

Figures in brackets are standard deviations. For abbreviations see Table 1.

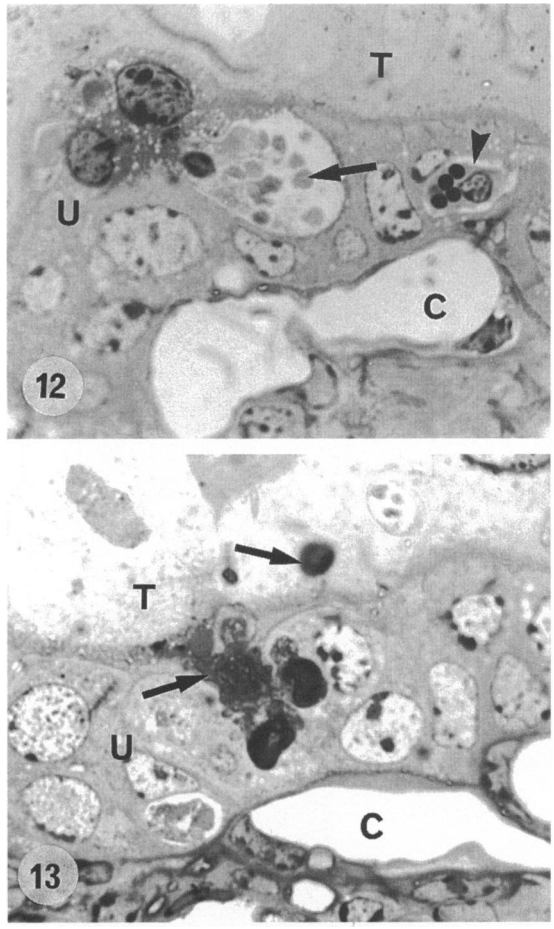



Fig. 12, 13. Light micrographs of sections from a uterus containing a 30 mm CRL fetus showing firstly blebbing (arrow, Fig. 12) of the trinucleate cells cytoplasm, and finally phagocytosis of the trinucleate cell residue (Fig. 13, dark pyknotic mass indicated by arrow) by uninucleate trophoblast cells (T). U, uterine epithelium; C, maternal capillary. There is usually an LGL (large arrowhead) near a trinucleate cell (Fig. 13). Chinese water deer, 30 mm CRL, ×1100.

around the trinucleate cells (Fig. 14). At this stage the trinucleate cells often displayed fragmentation or blebbing of the cytoplasm (Figs 12, 13). LGL were usually associated with this and all other stages of the trinucleate cell life cycle but showed no ultrastructural changes at any time (Figs 12, 15, 17, 18). After degranulation the trinucleate cell nuclei shrank into dense pyknotic masses (Figs 13, 15), the remaining cytoplasm darkening (Fig. 13) or becoming full of small vesicles (Fig. 15). Equivalent residues were often seen at the microvillar junction (Fig. 15) apparently being phagocytosed by the uninucleate trophectodermal cells and similar inclusions were seen in the adjacent trophectoderm (Figs 13, 15) but not in the uterine epithelium. The frequency of such trophectodermal inclusions did not appear to increase during pregnancy. This describes the normal history of trinucleate cells in the deer uterine epithelium where pentanucleate and syncytial plaques are rarely seen. However, in the uterine epithelium apposed to the bases of the chorionic villi frequent trinucleate cells and small areas of syncytium interspersed with uterine epithelial cells and LGLs were consistently found (Fig. 19). The derivation of the trinucleate cells and syncytial areas from binucleate cell migration was confirmed by placental lactogen immunoreactivity (Fig. 20). LGL were present at a higher frequency among these trinucleate cells and syncytial patches (Fig. 19) than in the more uniform uterine epithelium (Figs 16, 17) seen elsewhere on the villus.

The trophectodermal epithelium in the interplacentomal areas was in close contact with the interplacentomal uterine epithelium. Numerous PAS-

arrow), ready for exocytosis. The microvillar junction, partly artifactually separated (asterisk), is indicated by small arrows, the uterine epithelium basement membrane by arrowheads. Fallow deer, 15 mm CRL. Glutaraldehyde-K4M resin-immunogold, ×250.

Fig. 10. Light micrograph of an immunogold-treated section from a uterus containing a fetus of 20 mm CRL. At this stage the villi (V) are in the form of finger-like projections extending into the caruncle (S). Note the presence of numerous binucleate cells containing placental lactogen in the trophoblast (T), especially at the tips of the villi (arrow). The uterine epithelium (U) apposing the tips of the villi is formed by several layers of cells and contains several placental lactogen containing cells, i.e. trinucleate cells (arrowheads). Fallow deer, 20 mm CRL. Glutaraldehyde-K4M resin-immunogold, ×100.

Fig. 11. Higher magnification of a comparable area to that of Figure 13 showing a villus tip with numerous labelled binucleate cells in the trophoblast (T) and a fortunate section of a trinucleate cell (arrow) in the uterine epithelium (U) clearly demonstrating all 3 nuclei, the result of a recent binucleate cell migration and fusion with a uterine epithelial cell. The granules containing bovine placental lactogen will subsequently stream down to the maternal side as they have already done in the trinucleate cells in Figures 11 and 12. Fallow deer, 20 mm CRL. Glutaraldehyde-K4M resin-immunogold, ×260.

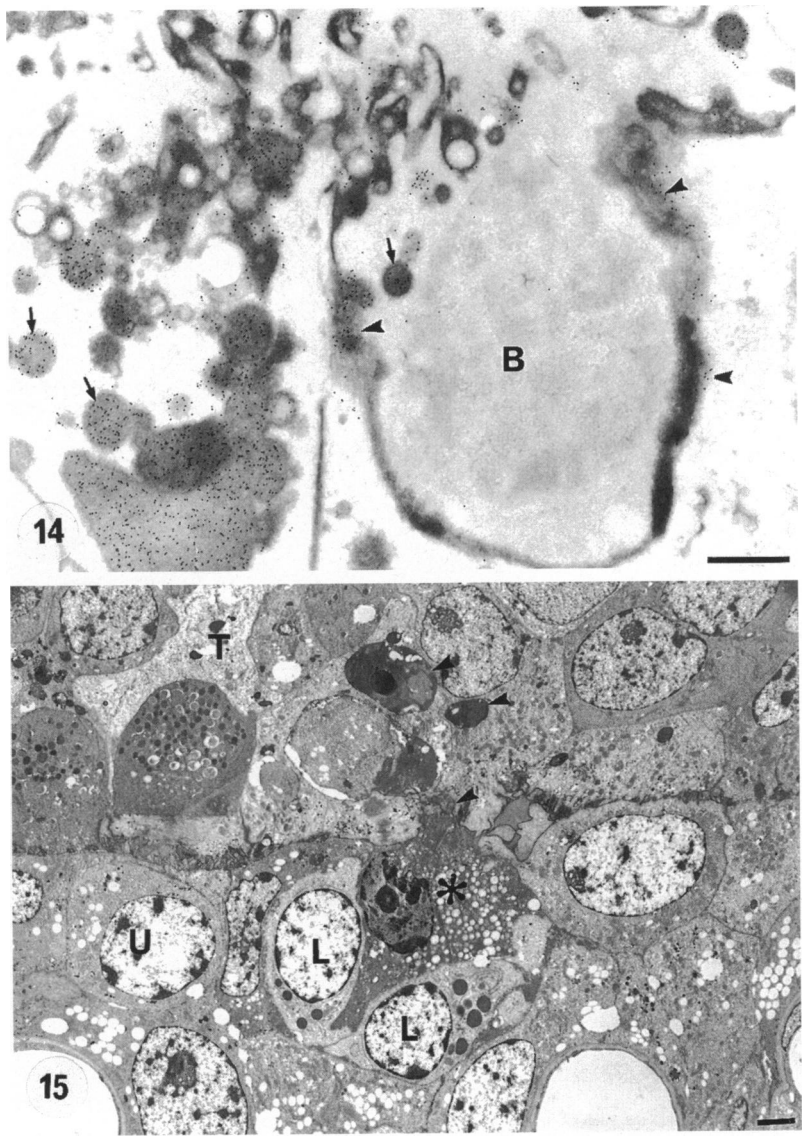



Fig. 14. Electron micrograph of a section from a midpregnant uterus stained by the immunogold technique for the localisation of placental lactogen. Note that the trinucleate cell granules (arrows) and the exocytosed material from the trinucleate cell granules localised in the intercellular space (arrowheads) around the cytoplasmic bleb (B) are heavily labelled with gold particles. Red deer, 80 mm CRL. K4M resin-immunogold. Bar, 1 μm.

Fig. 15. Electron micrograph of a section from a midpregnant uterus. Note that located at the microvillar junction is a trinucleate cell residue (asterisk) with fragments (arrowheads) being phagocytosed by uninucleate trophoblast cells (T). The portion of the pyknotic trinucleate cell which is still within the caruncular epithelium (U) is in close contact with 2 LGLs (L). Roe deer, 80 mm CRL. Phosphotungstic acid-uranyl acetate. Bar, 2 μm.

positive binucleate cells were observed in the trophectoderm. The percentage of trinucleate cells in the interplacentomal uterine epithelium was significantly lower ($P < 0.05$) than at the earlier stages of pregnancy (Table 3).

At this stage of pregnancy there was a further dramatic increase in the proportion of LGLs in both the placentomal and interplacentomal uterine epithelium, which were found at all levels in the epithelium (compare Figs 15 and 17). Mitotic figures were seen in LGLs at all stages of pregnancy examined (Fig. 21). Most of these cells contained several large electron dense granules each of which was bounded by a membrane. The Golgi apparatus was distinct and gave rise to vesicles of various sizes, each of which contained electron dense material (Fig. 22). These vesicles were usually close to the electron dense granules, most of which were significantly larger than those found at earlier stages. The remaining cytoplasm typically showed a few rough enodplasmic reticulum cisternae, mitochondria, free ribosomes and centrioles, features similar to those of lymphocytes.

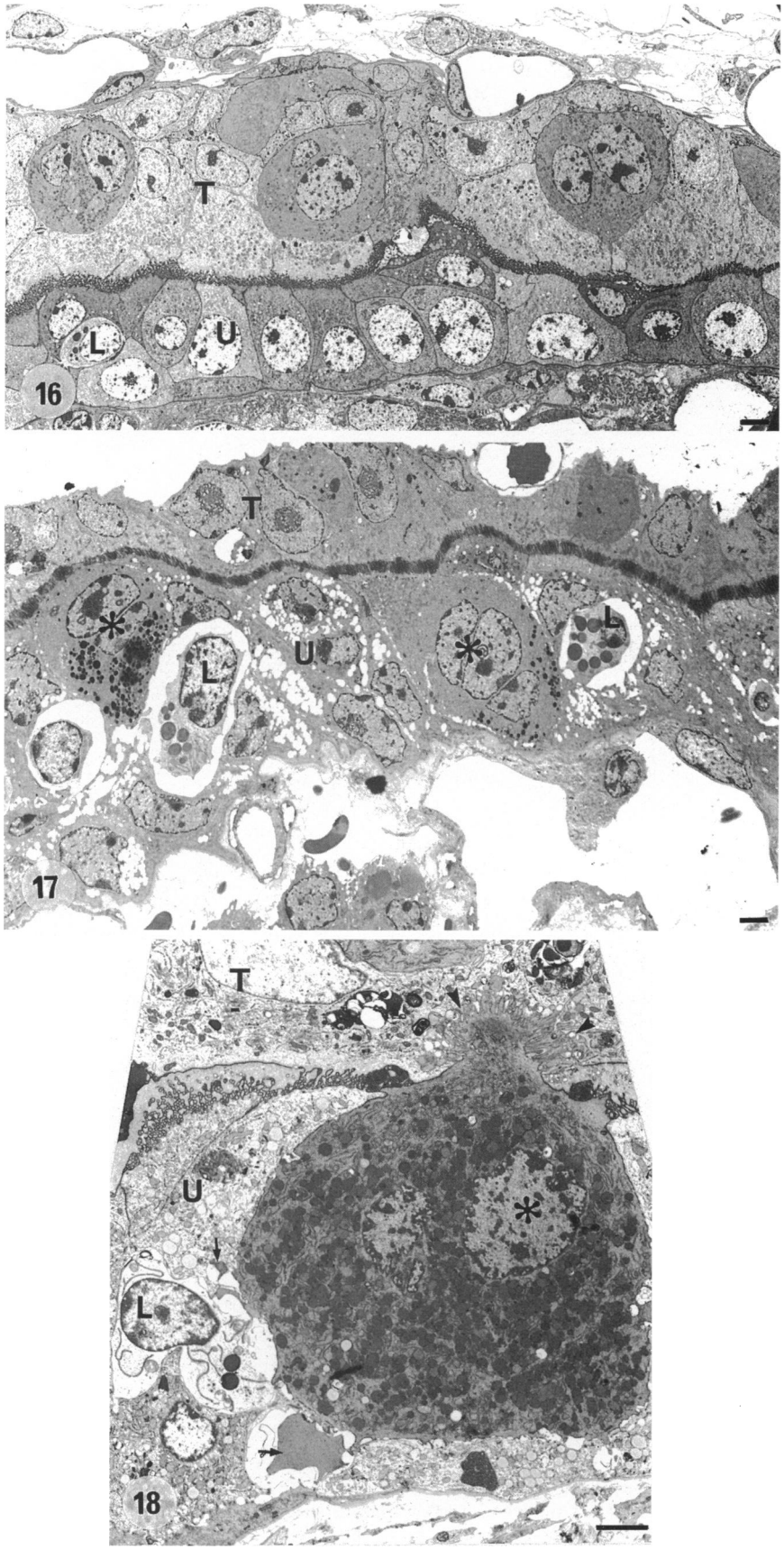
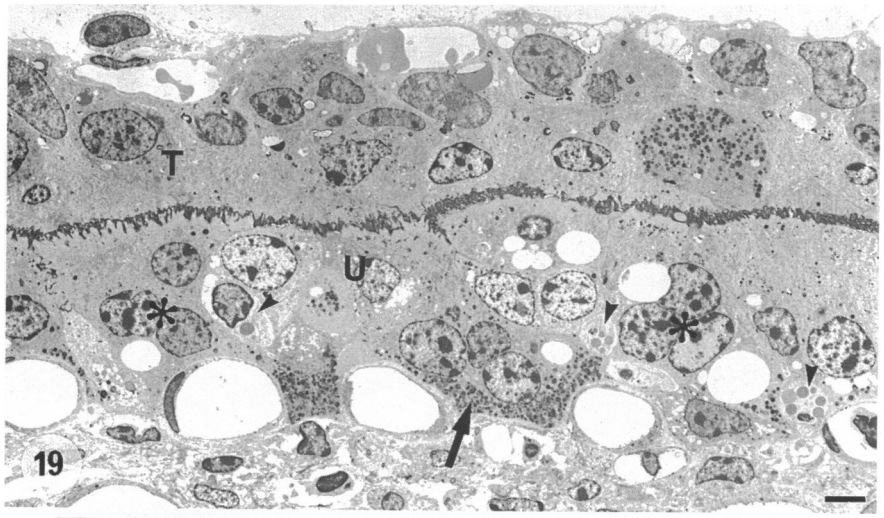



Fig. 16. Electron micrograph of a section from a midpregnant uterus showing the midregion of a chorionic villus, the trophoblast (T) of which contains 3 intraepithelial binucleate cells and is in close contact with a uniform layer of caruncular epithelium (U). Note that only a single LGL (L) is present. Chinese water deer, 30 mm CRL. Bar, 4 μm.



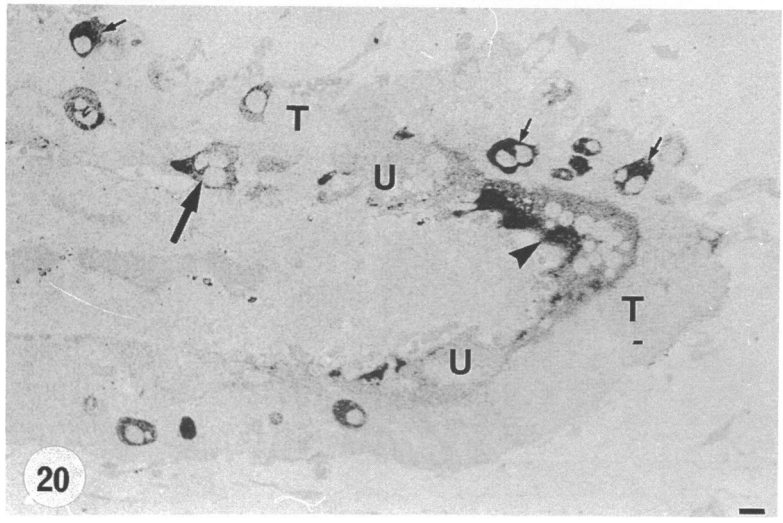




Fig. 19. Electron micrograph of a section from the base of a midpregnant chorionic villus. Note that the uterine epithelium (U) in this region is seen with patches of syncytium (arrow) and trinucleate cells (asterisks). Once again the frequency of LGLs (arrowheads) in this region is higher than that in the midregion of the chorionic villus (shown in Fig. 16) and they are located in close proximity to the trinucleate cells and syncytium. Roe deer, 80 mm CRL. Phosphotungstic acid-uranyl acetate. Bar, 4 μm.

Fig. 20. Light micrograph of a section from a midpregnant placentome stained by the immunogold-silver enhancement technique for the localisation of bovine placental lactogen. Note that the binucleate cell (small arrows) in the trophoblast (T) and the trinucleate cell (large arrow) and syncytium (arrowhead) in the caruncular epithelium (U) are all positively labelled, confirming that both the trinucleate cell and syncytium are derived from binucleate cell migration. Red deer, 80 mm CRL. K4M resin-immunogold. Bar, 10 μm.

There was a marked decline in the proportions of nongranulated lymphocytes both in the placentomal and interplacentomal uterine epithelium.

*Quantitation of intraepithelial lymphocytes*

The above studies indicated a dramatic increase in the proportions of intraepithelial granulated lymphocytes both in the placentomal and interplacentomal uterine epithelium as pregnancy advanced. This trend was accompanied by a concomitant decline in the proportions of intraepithelial nongranulated lymphocytes in both these regions. Consequently, a quantitative study based on 40 microscopic fields on Araldite or acrylic sections with a × 100 oil immersion objective was carried out to substantiate this observation. As

Fig. 17. Electron micrograph of a section from a midpregnant uterus towards the base region of a chorionic villus. Note that the uterine epithelium (U) apposed to the trophoblast (T) in this region often contains trinucleate cells (asterisks) closely associated with LGLs (L). Chinese water deer, 30 mm CRL. Phosphotungstic acid-uranyl acetate. Bar, 2 μm.

Fig. 18. Electron micrograph of a section from a midpregnant uterus. Note that a trinucleate cell (asterisk) displays blebbing of its cytoplasm (arrows) and is located in the uterine epithelium (U) in close association with an LGL (L). The basal pole of the trinucleate cell still maintains contact with the uninucleate cell of the trophoblast (T) which is phagocytosing the vesiculating remnants of the binucleate cell plasmalemma (arrowheads). Roe deer, 80 mm CRL. Bar, 2 μm.

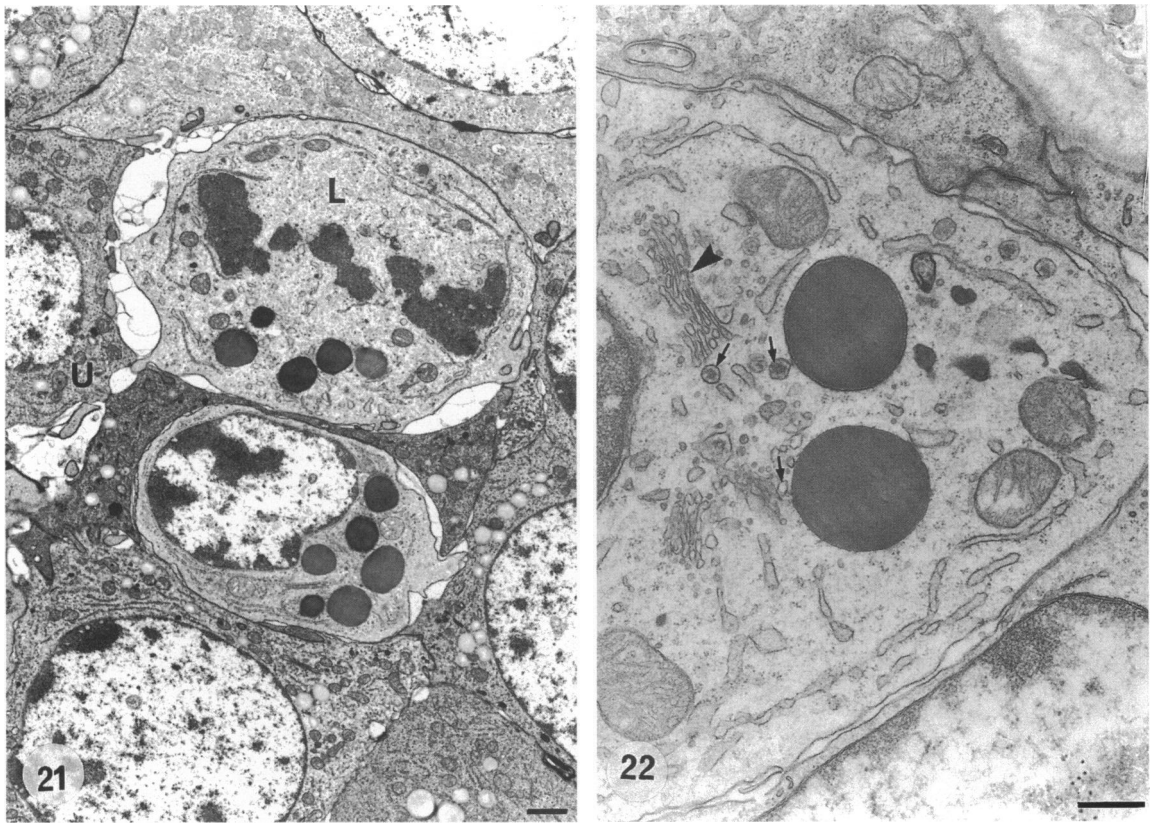



Fig. 21. Electron micrograph from a midpregnant placentome showing 2 LGLs in the uterine epithelium (U). Note that one of the LGLs (L) is in mitosis. Chinese water deer, 30 mm CRL. Bar, 1 μm.

Fig. 22. Higher magnification of an LGL from a midpregnant uterus. Note the 2 large membrane bounded granules. Numerous small vesicular structures (arrows), are located in close association with the large granules and with the Golgi body (arrowhead). Roe deer, 80 mm CRL. Bar, 0.5 μm.

shown in Table 2, the percentage ($0.5 \pm 0.3$) of LGLs in the nonpregnant caruncular epithelium was significantly lower ($P < 0.05$) than the percentage ($7.5 \pm 4.5$) of the nongranulated lymphocytes. However, in early pregnancy, the percentages of LGLs both in the placentomal ($2.5 \pm 1.6$) and interplacentomal ($3.2 \pm 1.1$) uterine epithelium increased, but only the increase in the interplacentomal area was significantly greater ($P < 0.01$) than the percentage of LGLs in the intercaruncular uterine epithelium of the nonpregnant uteri. By midpregnancy the percentage of LGLs in the placentomal uterine epithelium was significantly higher than those in the placentomal uterine epithelium of early pregnant uteri ($P < 0.01$). Similarly, the percentage of LGLs in the interplacentomal uterine epithelium was significantly higher than those in the interplacentomal uterine epithelium of early pregnant uteri ($P < 0.01$).

In contrast to the LGLs, there was a continuous decline in the percentages of nongranulated lymphocytes as pregnancy progressed, but the decreases from nonpregnant to early pregnant and early to midpregnant were not significant. Only the overall reductions in nongranulated lymphocyte percentages between nonpregnant and midpregnant were significant ($P < 0.01$) both for placentomal and interplacentomal regions.

The percentage of nongranulated lymphocytes was significantly lower than that of LGL ($P < 0.01$) in the placentomal uterine epithelium of midpregnant uteri. This was also observed to be the case ($P < 0.05$) in the interplacentomal uterine epithelium.

The slight decline in the total lymphocyte population both in the placentomal and interplacentomal uterine epithelium at early pregnancy was followed by a slight elevation at midpregnancy, but these changes were not significant.

Although individual LGLs with large granules were seen at early stages there was a definite increase in average granule size as pregnancy progressed. Mitosis of LGLs with either large or small granules was observed at all stages (Fig. 21).

The frequency of intraepithelial lymphocytes in gland epithelium was at a similar low level to that in

the nonpregnant uterine epithelium and did not vary significantly during pregnancy (data not shown).

*Immunocytochemical staining of leucocytes with monoclonal antibodies to sheep leucocytes*

None of the monoclonal antibodies raised against sheep leucocytes cross-reacted with the leucocytes on unfixed frozen or resin sections of the nonpregnant and pregnant deer uteri.

## DISCUSSION

The results of this study clearly show that in deer placenta, although there is no appreciable change in the frequency of intraepithelial lymphocytes in the uterine epithelium during pregnancy, there is a significant alteration in the ratio of types of lymphocyte. The LGL increases continuously as a percentage of the total with a concomitant decline of the nongranular type. As yet it has not been possible to immunologically characterise the deer cells unequivocally as large granular lymphocytes. However their similarities in structure, location and development to the ovine variety (Lee et al. 1992) make such an identification very likely.

Light and electron microscope observations also confirm that as in all other ruminants (Wooding & Flint, 1994) so far examined, 15–20% of the fetal trophectodermal cells in deer become binucleate, develop granules and eventually migrate to fuse with uterine epithelial cells. This forms usually trinucleate but occasionally multinucleate maternofetal hybrid uterine epithelial cells (depending on species) from which the fetal binucleate cell derived granule contents are released to the maternal circulation establishing a major route of fetomaternal communication from implantation to term.

The uterine epithelial trinucleate cells are usually in close contact with one or more LGL, although this correlation needs to be conclusively established morphometrically. So far no structural changes have been seen in LGL corresponding to the degranulation of the trinucleate cells. The LGL in deer are present in increasing frequency throughout the entire placentomal and interplacentomal uterine epithelium during pregnancy and so constitute a vastly larger total population than in sheep, where they are only found in the interplacentomal area which is less than 10% of the total from midpregnancy. They also increase at the earliest implantation stage examined in deer compared with midpregnancy in sheep (Lee et al. 1992). This early increase is coincident with the appearance of focal aggregates of the intraepithelial lymphocytes suggesting a possible clonal expansion of the population. Mitosis of individual LGLs within the epithelium has been observed here and previously in sheep (Lee et al. 1992) but no division stages have yet been seen in these aggregates.

No similar groupings have been seen in previous investigations of LGL populations in sheep where the uterine epithelium is modified to a fetomaternal syncytium at implantation. No LGLs have been found in such uniquely ruminant syncytium, and it is only when the interplacentomal syncytium is replaced by cellular uterine epithelium regenerating from gland mouths that significant numbers of intraepithelial LGLs appear and accumulate.

In the sheep and goat (Wooding & Flint, 1994) the placentomal area after implantation remains syncytial in nature and the enormous increase in area during placental growth throughout pregnancy is sustained entirely by binucleate cell migration from the trophectoderm. However in the deer there is no evidence from this or earlier studies (Lee et al. 1986) of any significant syncytium formation at implantation; binucleate cell migration and fusion occur at similar frequency to that found in sheep and goat but only produce transient trinucleate cells in the uterine epithelium except at the apex of the maternal villi where small patches of syncytium can usually be found (Hamilton et al. 1960).

The observed LGL association with trinucleate cells indicates an interaction which could serve to inhibit trinucleate cell expansion into syncytium. The early proliferation of LGLs would ensure the rapid demise of trinucleate cells after they have degranulated and favour maintenance and growth of a cellular uterine epithelium. The LGLs could have a direct natural killer function or, since no morphological evidence of this has been observed, act as a back up system if the normal programmed cell death of the trinucleate cell after degranulation fails to occur.

Fetal syncytium is very invasive in other placental systems (Wooding & Flint, 1994). In ruminants it could be that its invasiveness is limited initially by the maternal contribution to its hybrid fetomaternal constitution and subsequently by an LGL monitoring contribution. The LGL and the fetomaternal hybrid trinucleate cell would form a dynamic system restricting the fetal invasiveness but maintaining the fetomaternal dialogue.

In most species of deer examined at midpregnancy there is a restricted region at the fetal end of the maternal villi where small patches of syncytium are consistently found (Hamilton et al. 1960). The frequency of LGLs in this epithelial layer of uni-

nucleate cells mixed in with syncytial patches is significantly increased, again suggesting a dynamic balance between the two.

This is a similar role to that postulated for LGLs in the human (King & Loke, 1991) and rodent (Croy, 1994) placental bed or decidua and, as in these systems, the deer LGL population increases by division in situ rather than continuous recruitment from a stem cell population. Again, no evidence for any direct interactions between invading human extravillous trophoblast and LGLs has been found so far but the temporal and developmental correlation between the two cell populations strongly supports a functional connection.

This initial accommodation would rely on paracrine and autocrine interactions based on specific cytokine production and could include the production of signals acting locally on placental growth as well as on maternal and fetal systems outside the placenta. This could play a central role in producing the multi-functional adaptations necessary for a successful pregnancy. The exocytosis of granules from the deer (and other ruminants) trinucleate cells is the first direct evidence for fetal signals delivered directly to the LGL surface but no evidence for prior or coincident LGL modification or degranulation has yet been found. However, there is evidence for a progressive increase in the size of the granules and the metabolic activity of the LGLs in deer (this study) and sheep (Lee et al. 1992) as pregnancy progresses, supporting suggestions of an active monitoring (and secretory?) role in the uterine epithelium initiated and reinforced by interaction with trinucleate cells. This would be a further development of the LGLs normal role of maintenance and repair of an epithelium by growth factor secretion which has recently been demonstrated for resident $\gamma\delta$T lymphyocytes in skin and gut epithelium (Boismenu & Havran, 1994). Meeusen et al. (1993) have shown that the sheep interplacentomal LGLs are $CD8^+CD45R^+\gamma\delta TcR^+$; unfortunately the deer LGLs do not cross-react with any of the human or ovine antibody markers available but on morphological and developmental grounds seem likely to be of similar phenotype.

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