Immunology 1989 **66** 312–317

Site-directed differences in the immune response to the fetus

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Accepted for publication 3 October 1988

SUMMARY

Major differences in the maternal immune response to the fetus were observed in the placentomes and in the interplacentomal regions of the pregnant sheep uterus. Firstly, fewer lymphocytes were detected in the placentomes compared to the interplacentomal regions and to non-pregnant uterine tissue (Lee, Gogolin-Ewens & Brandon, 1988). Secondly, a large population of CD45R$^+$ granulated lymphocytes was uniformly distributed in the interplacentomal uterine epithelium throughout pregnancy but never in the syncytial layer of the placentomes. Thirdly, monoclonal antibodies specific for the CD5 antigen consistently stained the endothelium of blood vessels within the placentomes but never blood vessels in the interplacentomal areas. Finally, OLA class I antigens were present on the interplacentomal uterine epithelial cells and on the maternal stromal cells, but no staining of the trophoblast or syncytium was observed. These observations suggest that different mechanisms to prevent immune rejection of the fetus may operate in the placentomes where trophoblast invasion of the maternal tissue occurs compared to the interplacentomal regions.

INTRODUCTION

It is well established that paternal antigens are expressed on some fetal membranes in direct contact with maternal tissue. The mechanism by which the fetus escapes immune rejection is still unresolved but it is obvious that the response of the pregnant female to paternal alloantigens is quite different from that seen to conventional allografts (Head & Billingham, 1986) particularly as large numbers of lymphocytes expressing T-cell markers do not appear to be present in murine and human decidua. Kabawat *et al.* (1985) have shown that very few T cells are present at the site of implantation in the human placenta. Several studies (Bulmer & Johnson, 1984; Bulmer & Sunderland, 1984; Ritson & Bulmer, 1987) have reported that most leucocyte common antigen (LCA$^+$) cells present in placental tissues in the first trimester are either macrophages or decidual lymphocytes characterized by a unique combination of cell surface markers: CD3$^-$, CD5$^-$ and CD8$^-$ but CD2$^+$, CD7$^+$, OKT10$^+$ and NKH1$^+$. Furthermore, it has been suggested that different populations of suppressor cells present in murine decidua may be involved in the process of preventing immune rejection (reviewed in Clark *et al.*, 1986, 1987), and Athanassakis *et al.* (1987) and Wegmann (1987, 1988) have provided evidence supporting an 'immunotrophic' role for maternal T cells which may act via the lymphokines IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) to promote placental growth.

Ruminants provide an excellent opportunity to study the direct relationship between immune cells present in the placenta and the invading fetal tissue. In the sheep, trophoblast invasion of maternal tissue occurs only in the placentomes (pregnant caruncles) beginning about Day 16–18 of gestation (Boshier, 1969; King, Atkinson & Robertson, 1982) and is characterized by fusion of fetal trophoblast cells with the maternal epithelium to form a syncytial layer (Wooding, 1980; Lee *et al.*, 1985). The number of immune cells present in the placentomes and the distribution of lymphocyte subsets can be directly contrasted to the adjacent interplacentomal (intercaruncular) areas where the trophoblast does not invade the maternal tissue and where the uterine epithelium remains intact throughout most of pregnancy. In cattle, Vander Wielen & King (1984) have reported a significant reduction in the number of lymphocytes present in the uterine epithelium between 19 and 27 days after fertilization; however, no further characterization of these cells was carried out. In this paper, we present an analysis of immune cells present in the pregnant sheep uterus using a panel of monoclonal antibodies (mAb) reactive against a number of ovine lymphocyte surface markers and ovine major histocompatibility complex (OLA) class I and class II antigens. We show that there is an apparent reduction in the number of lymphocytes in the placentomes of the pregnant sheep compared to the interplacentomal regions and to the non-pregnant caruncles (Lee *et al.*, 1988). In addition, major differences in cell populations expressing these antigens were detected in the placentomes and interplacentomal regions.

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MATERIALS AND METHODS

Tissues

Uterine tissues from pregnant sheep of various gestational ages were obtained at an abattoir within 15 min of slaughter and

processed immediately as described below. The stages of pregnancy were estimated by measuring the fetal crown–rump length (Barcroft, 1952). In addition, five uteri were obtained from timed matings of ewes with rams fitted with a sire sine harness. Day 0 was taken as the day the ewe was marked by the ram. One uterus (Day 9) was obtained from a ewe that had been superovulated by administering six injections of ovine follicle-stimulating hormone over a 3-day period beginning 10 days after insertion of a 60 mg progesterone sponge (Repromap, Upjonn). A total of 24 uteri from pregnant sheep between 9 and 125 days of gestation were included in this investigation. Days 9 ($n=2$), 19, 30 and 40 ($n=1$ each) were obtained from timed matings and Days 30–35 ($n=3$), 36–40 ($n=4$), 50–55 ($n=2$), 60–70 ($n=4$), 71–80 ($n=2$), 81–90 ($n=2$), 100 ($n=1$) and 125 ($n=1$) were abattoir material.

Pregnant uterine tissues were taken from the placentomes and interplacentomal regions, embedded in O.C.T. Compound (Miles Scientific, Naperville IL), snap-frozen and stored at −70° or fixed in 95% cold ethanol and processed according to Sainte-Marie (1962). Similar tissues were also prepared for electron microscopy as described previously (Lee *et al.*, 1988).

Immunohistological studies

All mAbs used in this study were produced in our laboratory. The properties of the mAbs directed against CD45 (SBU-LCA; mAb 1-28), CD5 (SBU-T1; mAb 25-91), CD4 (SBU-T4; mAb 44-38 and 44-97), CD8 (SBU-T8; mAb 38-65), SBU-T19 (mAb 19-19), CD45R (p220; mAb 20-96) and OLA class II (mAb 49-1) have been summarized previously (Lee *et al.*, 1988). MAb 41-19, which reacts with OLA class I antigens (Gogolin-Ewens *et al.*, 1985), was also used in this study.

For immunohistological studies, frozen sections (6 μm) fixed for 10 min in cold ethanol or acetone containing 0·6% (v/v) H_2O_2 to inhibit endogenous peroxidase were labelled using the indirect-immunoperoxidase technique and counterstained with haematoxylin (Lee *et al.*, 1985). Tissue sections for electron microscopic examination were labelled using the immunogold procedure (Lee *et al.*, 1988). Control slides for light and electron microscopy were stained using tissue culture medium in place of hybridoma supernatant. Tissue sections were also routinely stained with haematoxylin and eosin.

RESULTS

Distribution of lymphocytes in the placentome and interplacentomal area

Prior to the establishment of chorionic villi, lymphocytes were commonly found in and at the base of the caruncular epithelium. Some lymphocytes were also scattered in the caruncular stroma and in the connective tissues adjacent to the basal regions of the caruncular epithelium. By the time the chorionic villi and caruncular septa were formed and had established intimate contact (about 30–35 days), lymphocytes were never observed in the syncytial layer covering the caruncular septa (Fig. 1a). Semithin sections of the placentomes failed to reveal the presence of any granulated lymphocytes in the syncytium. Lymphocytes were always present in the connective tissues in the caruncular septa (Fig. 1a) but were fewer in number than in the interplacentomal stroma. This pattern persisted throughout the period studied.

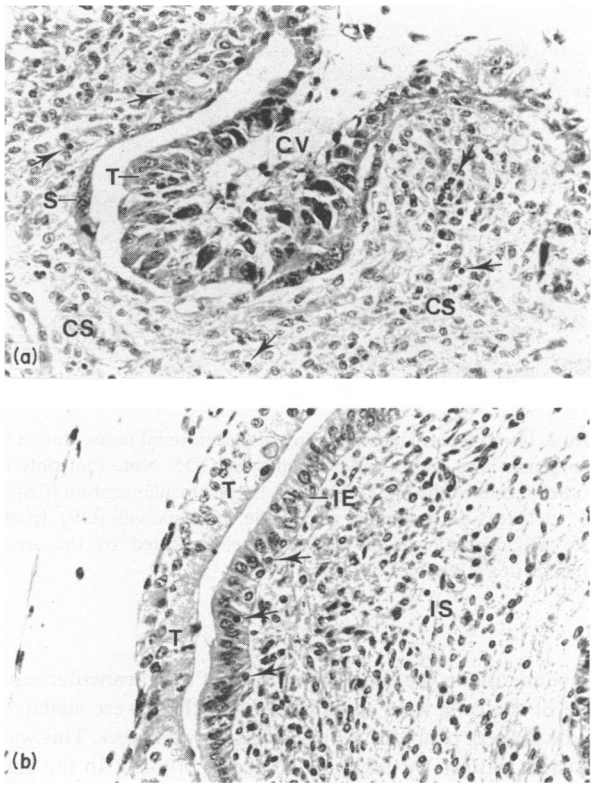

Figure 1. (a, b) Haematoxylin and eosin-stained section showing distribution of lymphocytes (arrows) in a placentome (a) and interplacentomal region (b). (a) In the placentome, note that there is no infiltration of lymphocytes into the syncytia (S) covering the caruncular septum (CS), whereas a few lymphocytes (arrows) are present in the caruncular septum. (b) In the interplacentomal region, several lymphocytes are present in the interplacentomal uterine epithelium (IE). T = trophoblast; IS = interplacentomal stroma; 30–35 days pregnant (×220).

The distribution of lymphocytes in the interplacentomal areas was similar to that reported for non-pregnant ewes (Lee *et al.*, 1988). Numerous lymphocytes were consistently found in the uterine epithelium, the basal region of the epithelium (Fig. 1b), the endometrial glandular epithelium and the connective tissues in the interplacentomal stroma closely associated with the epithelium and the endometrial glands. At about 30–40 days of gestation when some patches of the uterine epithelium had degenerated, it was difficult to distinguish pyknotic nuclei from lymphocytes; however, in regions where the epithelium remained normal, lymphocytes were frequently observed in both the epithelium and subepithelial sites. As pregnancy progressed the uterine epithelium re-established its uniformity and a similar pattern of lymphocyte distribution was evident through to Day 125 of pregnancy. Closer examination of semithin plastic sections showed that many of the intra-epithelial lymphocytes contained several large granules. Electron microscopy revealed that these granules were electron dense and each was bounded by a membrane.

Distribution of lymphocyte subsets in the placentome and interplacentomal area

CD45 (SBU-LCA). Numerous LCA^+ cells were observed in the early stages of pregnancy at the base of the caruncular

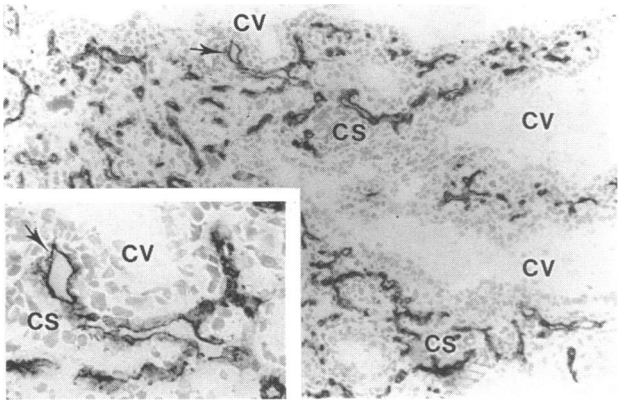

Figure 2. Immunoperoxidase staining of placentomal tissue from a 66-day pregnant ewe with a mAb specific for CD5. Note that only the endothelial cells of the blood vessels in the caruncular septum (CS) are CD5$^+$, whereas no staining is seen in the chorionic villi (CV). Inset is higher magnification of the blood vessel indicated by the arrow. (Magnification × 99; inset × 248).

epithelium and in the connective tissue of the caruncular septa. Some of the cells were rounded, while others were stellate or spindle shaped, probably representing macrophages. This was a consistent pattern throughout the period studied. In the interplacentomal region, numerous CD45$^+$ cells were present in all areas, especially within the uterine and endometrial glandular epithelium and in the connective tissue surrounding the glands. In addition, cells weakly expressing LCA were scattered in the mesenchyme of the fetal chorion.

CD5 (SBU-T1). The immunoperoxidase staining pattern observed in the placentomes with mAb 25-91 was very striking. Figure 2 shows the endothelium of the arterioles and capillaries in the placentomal septum stain very intensely for the CD5 antigen. These structures were identified as blood vessels by histological criteria. This was confirmed by reacting serial sections with mAb 41-19, an OLA class I mAb that characteristically reacts strongly with blood vessel endothelium (Gogolin-Ewens *et al.*, 1985). A few CD5$^+$ lymphocytes were also seen scattered in the connective tissue of the caruncular septa. These CD5$^+$ lymphocytes were detected as early as Day 9 of pregnancy in similar numbers to that seen in the non-pregnant uterus; however, the CD5 staining of blood vessel endothelium was not observed until about Day 30 of pregnancy. No staining of cells or blood vessels in the chorionic villi which interdigitated with the caruncular septum was observed. In the interplacentomal region, the CD5 antigen was not detected on blood vessels or capillaries which were positively stained by the mAb 41-19 (Fig. 3a,b), although some CD5$^+$ lymphocytes were present along the base of the interplacentomal uterine epithelium and in the underlying connective tissue (Fig. 3c).

CD4 (SBU-T4). In early stages of pregnancy (less than Day 40) some CD4$^+$ cells were present in the stroma of the caruncular septa; however, after this time the number of cells expressing CD4 decreased considerably. In the interplacentomal region some CD4$^+$ cells were scattered in the denser connective tissue layer near the epithelium; however, no CD4$^+$ cells were present within the epithelium (Fig. 3d). This pattern did not change as pregnancy progressed.

CD8 (SBU-T8). A few CD8$^+$ lymphocytes were observed among the stromal cells of the caruncular septa throughout pregnancy. CD8$^+$ lymphocytes were also observed throughout the period studied in the interplacentomal stroma and in the connective tissue near the endometrial glands. After Day 50 of pregnancy, CD8$^+$ lymphocytes had also infiltrated into the interplacentomal uterine epithelium (Fig. 3e). Since no CD5$^+$ cells were observed in the epithelium, these CD8$^+$, CD5$^-$ cells are probably similar to those found in the ovine gut (Gorrell *et al.*, 1988) and mammary gland (Lee, Meeusen & Brandon, 1989).

SBU-T19. SBU-T19 represents a third subset of T cells in sheep which are CD4$^-$ and CD8$^-$ and which comprise 10–15% of peripheral blood lymphocytes (Mackay, Maddox & Brandon, 1986). A few SBU-T19$^+$ cells were present in the placentomes throughout the period studied, but there was an apparent decrease in number after about Day 40 of gestation. Very few SBU-T19$^+$ cells were present in the stroma of the interplacentomal region and none were observed in the interplacentomal uterine and endometrial glandular epithelium.

CD45R (SBU-p220). MAb 20-96 recognizes a restricted epitope of LCA, designated CD45R, present on B cells and on a small unique subset (5–6%) of peripheral blood lymphocytes (Mackay, Maddox & Brandon, 1987). We have also shown that this antibody reacts with numerous granulated lymphocytes present in non-pregnant caruncular and intercaruncular epithelium (Lee *et al.*, 1988). In pregnant uterine tissue, no CD45R$^+$ granulated cells were detectable in any region of the placentome; however, a large population of CD45R$^+$ lymphocytes was present in the interplacentomal uterine epithelium (Fig. 3g). Comparison of consecutive sections stained with monoclonal antibodies against CD5 (Fig. 3c) and OLA class II (Fig. 3f) revealed that these intra-epithelial CD45R$^+$ cells were CD5$^-$, OLA class II$^-$. Analysis using both light (Fig. 4a) and electron microscopy (Fig. 4b) showed that the CD45R$^+$ cells in pregnant uterine epithelium were granulated but that the granules were larger and more numerous than observed in comparable cells in non-pregnant tissue.

Numerous CD45R$^+$ cells were present in the pregnant caruncular stroma in the early stages of pregnancy, but after about Days 30–40 of pregnancy their number declined dramatically. Presumably these were B cells since there was also OLA class II staining of cells in this region.

Major histocompatibility complex antigens in the placentome and interplacentomal area

OLA class I. Most stromal cells in the placentomes expressed OLA class I antigens. The epithelium was also positive with mAb 41-19 prior to Day 19. In later stages, however, the syncytial layer of the placentomes was consistently negative. In the interplacentomal regions, the uterine and endometrial grandular epithelium and the connective tissues were all positive throughout the period studied. OLA class I antigens were not detected on the trophoblast with mAb 41-19, although positive staining was detected on cells just beneath the trophoblast which probably represented vascular endothelium.

OLA class II. Cells expressing OLA class II were present in the placentomes throughout pregnancy, although more positive cells were detectable in earlier pregnancy (less than Days 50–60) than in later stages. Similarly, in the interplacentomal stroma

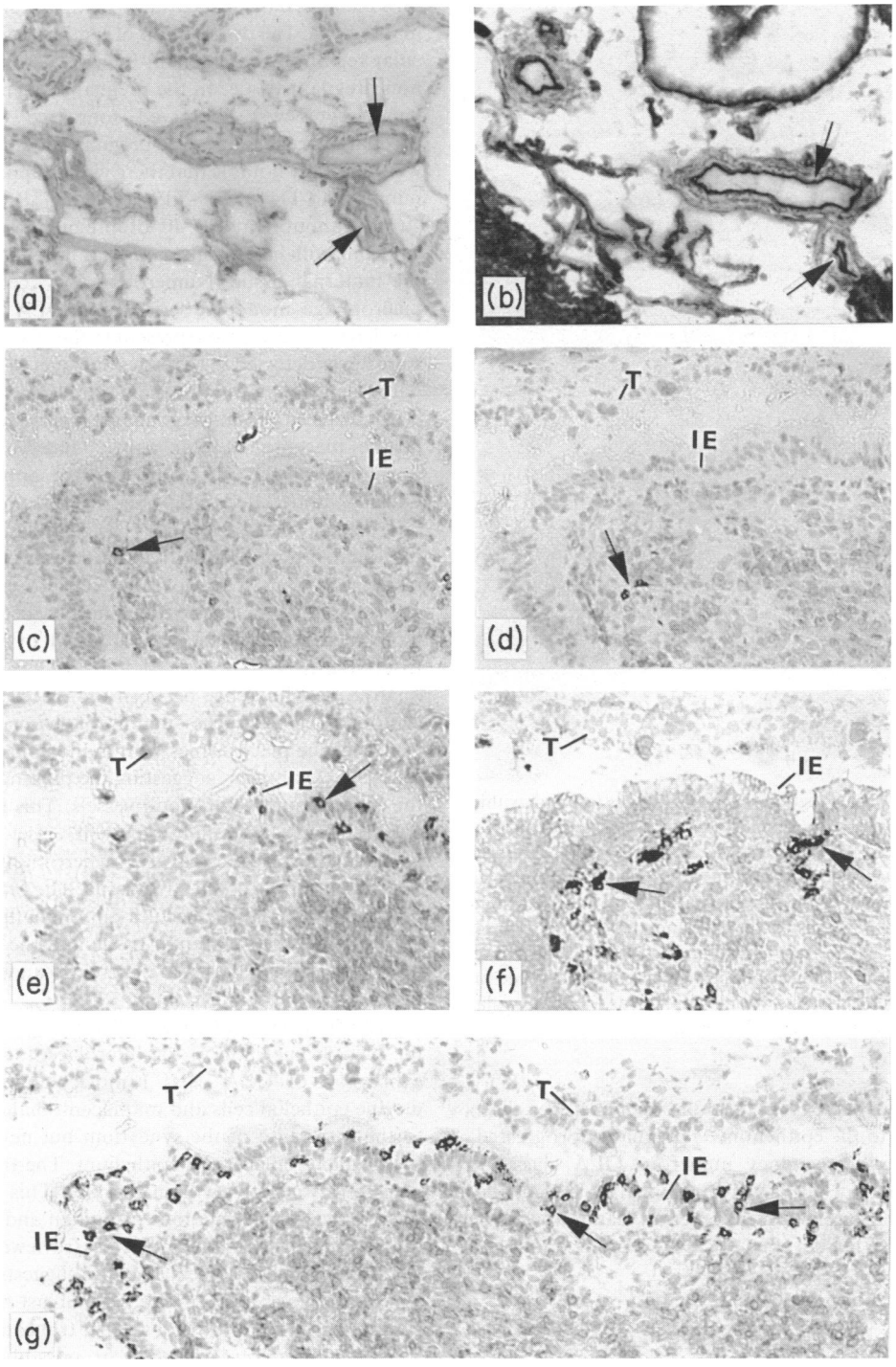

Figure 3. Immunoperoxidase staining of uterine tissue from the interplacentomal area of a Day 66 (a,b) and a Day 86 (c-g) pregnant ewe with mAbs against CD5 (a,c), OLA class I (b), CD4 (d), CD8 (e), OLA class II (f) and CD45R (g). (a, b) Sections of the same arteries (arrows) in the interplacentomal area showing that the endothelium of the blood vessels identified by intense staining with OLA class I (b) are clearly $CD5^-$ (a). (c–g) Sections from the same interplacentomal area with arrows indicating the presence of only a few $CD5^+$ (c) and $CD4^+$ (d) cells in the connective tissues beneath the uterine epithelium (IE), some $CD8^+$ cells in the uterine epithelium (e), spindle- and stellate-shaped OLA class II^+ cells in the connective tissue immediately beneath the interplacentomal uterine epithelium (f) and numerous $CD45R^+$ cells located exclusively in the uterine epithelium (g). T = trophoblast; magnification (a,b) × 162, (c–g) × 144.

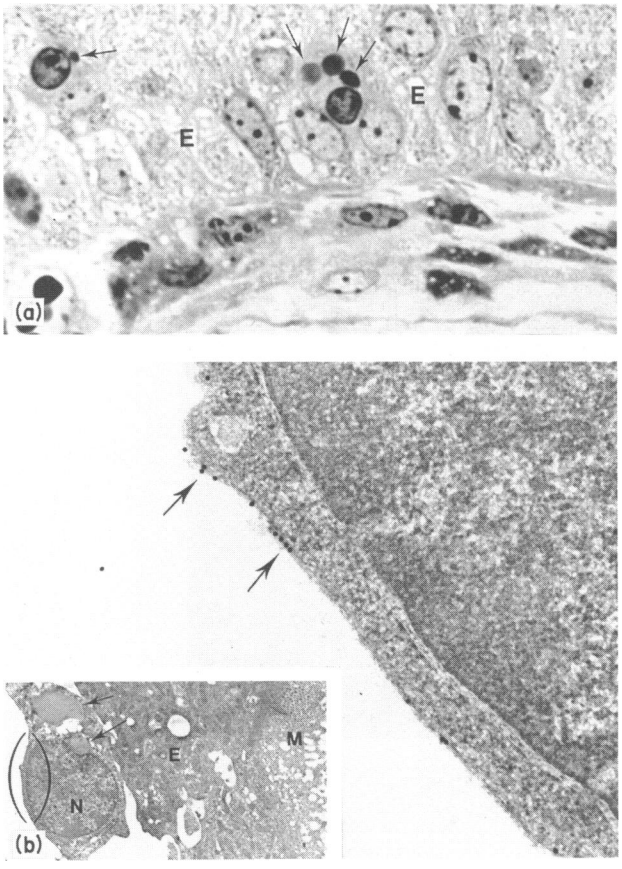

Figure 4. (a) Semithin plastic section showing granules (arrows) within the cytoplasm of two granular lymphocytes in the interplacentomal uterine epithelium (E); 1% toluene blue in 1% borax; magnification × 1200. (b) Electron micrograph of a granulated lymphocyte located at the basal region of the interplacentomal uterine epithelium (E) stained with mAb 20–96 using the immunogold technique (inset magnification × 3202). Higher magnification (× 45,936) of the bracketed area in the inset clearly shows gold particles (arrows) localized on the surface of the cell. G = granules within lymphocyte; M = microvillar junction; N = nucleus of lymphocyte. Uranyl acetate and lead citrate.

there was an apparent decrease in the number of positive cells in the region adjacent to the epithelium as pregnancy progressed. However, throughout pregnancy numerous OLA class II$^+$ stellate and spindle-shaped cells were prominent in the connective tissue around the base of the endometrial glands and at the base of the uterine epithelium (Fig. 3f). OLA class II staining of cells in the chorion was not detected.

DISCUSSION

Our previous study (Lee *et al.*, 1988) showed that in the non-pregnant sheep uterus, lymphocytes are uniformly present in both the caruncle and intercaruncular uterine epithelium and stroma. Immunoperoxidase staining of these cells using monoclonal antibodies to ovine lymphocyte surface markers showed that while some CD5$^+$ lymphocytes were detectable, few cells expressed the CD4, CD8 or SBU-T19 antigens. The predominant subpopulation of lymphocytes in the non-pregnant caruncle and intercaruncular epithelium was strongly CD45R$^+$, weakly CD5$^+$ (if detectable) granulated non-B cells. The present study shows that pregnancy is accompanied by changes in the immune cells present in the placentomes compared to the interplacentomal regions. The distribution of lymphocytes in the intercaruncular region of the pregnant uterus was similar to that seen in non-pregnant tissue. In the pregnant caruncle, however, fewer lymphocytes were observed compared to either the non-pregnant caruncle or to the pregnant intercaruncular region. It was especially notable that there was an apparent decrease in the number of CD4$^+$, SBU-T19$^+$ and CD45R$^+$ lymphocytes in the stroma about Day 30–40 of pregnancy by which time the chorionic villi had formed and established intimate contact with the maternal tissue. Numerous LCA$^+$ cells, probably of the macrophage–monocyte series, some OLA class II$^+$ cells and a few T lymphocytes were present in the stroma, but lymphocytes were never detected in the syncytial layer of the placentomes. In particular, none of the unusual population of CD45R$^+$ granulated cells seen in non-pregnant uterine epithelium were detected in the placentome after approximately Day 19 when the formation of the syncytium became apparent, whereas the CD45R$^+$ granulated cells persisted throughout pregnancy in the interplacentomal epithelium. The granules within the interplacentomal intra-epithelial lymphocytes were larger and more numerous than in comparable cells found in non-pregnant uterine epithelium, suggesting that these cells may have become more metabolically active as a result of the presence of the trophoblast.

Another difference between the placentomes and interplacentomal regions is the staining of the endothelium of blood vessels in the placentomes with monoclonal antibodies specific for the CD5 antigen, suggesting the presence of this antigen on the luminal surface of blood vessels. This is contrasted by the complete lack of staining of blood vessel endothelium in the adjacent interplacentomal areas where many more lymphocytes are present in the connective tissue. The biochemical similarity between the CD5 molecules immunoaffinity purified from thymus and from placental tissue and the functional implications of this observation are currently under further investigation.

The final major difference in lymphocyte antigens detected in the placentomes and in the interplacentomal regions was the expression of OLA class I molecules on interplacentomal uterine epithelial cells and on placentomal epithelial cells prior to the formation of the syncytium but never on the syncytial layer which replaces the epithelium. The trophoblast was also consistently negative with mAb 41-19. This is contrary to recent reports that subpopulations of human and mouse trophoblast cells express MHC class I molecules (reviewed in Head, Drake & Zuckerman, 1987). Recent evidence suggests that the HLA class I molecules expressed on the trophoblast may not be classical MHC antigens, but rather a smaller truncated molecule (Ellis *et al.*, 1986; Stern *et al.*, 1987) or possibly a novel molecule analogous to mouse Qa (Head *et al.*, 1987). It remains a possibility that a variant OLA class I molecule is expressed on sheep trophoblast in a form not detected by the mAb 41-19.

These results suggest that the maternal response to the presence of the fetus is different in the interplacentomal regions compared to the placentomes where intimate contact between fetal membranes and maternal tissue occurs. It is possible, for example, that a fetally derived antigen such as SBU-3, which is produced by binucleate cells present at the tips of the invading chorionic villi but not in the interplacentomal region (Lee *et al.*,

1985), may function to suppress the maternal response. On the other hand, differential lymphocyte migration may be occurring in the placentomes. Experiments assessing these possibilities are in progress.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Health & Medical Research Council of Australia. Dr K. Gogolin-Ewens is an Australian Meat and Livestock Research Development Corporation Research Fellow. Dr W. R. Mercer is the recipient of an NH&MRC postgraduate research fellowship. The authors wish to thank G. Barcham, P. Smith and K. Snibson for their technical assistance.

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