Cell Tissue Res (1987) 247:667–673

Cell and Tissue Research

Trinucleate cells and the ultrastructural localisation of bovine placental lactogen

F.B.P. Wooding[1] and J.F. Beckers[2]

[1] AFRC Institute of Animal Physiology, Babraham, Cambridge, United Kingdom;
[2] Service d'obstétrique, Faculté de Médecine Vétérinaire, Brussels, Belgium

Summary. Bovine placental lactogen activity is shown by immunogold electron microscopy to be restricted to (a) the granules and the Golgi body from which they form in the bovine fetal trophectodermal binucleate cell, and (b) granules of similar size and staining reaction in trinucleate "giant" cells found in the maternal uterine epithelium throughout pregnancy. These results support the hypothesis that a fetal binucleate cell forms a maternal giant cell by migration to and fusion with a uterine epithelial cell.

Key words: Secretory granules – Cell fusion – Cell migration – Immunocytochemistry – Immunogold localisation – Placenta – Placental lactogen – Trinucleate cell – Cow

Placental lactogenic hormones (also known as chorionic somatomammotropins) have been demonstrated in a variety of mammals. They are probably of fetal origin and said to stimulate fetal growth and maternal mammary gland development (Blank et al. 1977; Porter 1980; Talamantes et al. 1980). In the few species so far investigated the lactogens appear to be produced in the placenta close to the fetomaternal interface: by syncytiotrophoblast in the human (Sciarra et al. 1963), giant cells in the mouse (Hall and Talamantes 1984), and binucleate cells in the sheep (Martal et al. 1977; Wooding 1981).

Bovine placental lactogen has recently been purified by several laboratories (Beckers et al. 1980: Murthy et al. 1982; Arima and Bremel 1983) and Verstegen et al. (1985) have shown by light-microscope immunocytochemistry that it is present in fetal binucleate cells and occasionally in the contiguous maternal uterine epithelial cell layer.

This immuno-electron-microscopical investigation was designed to establish the subcellular localisation of the hormone at the fetomaternal interface with special reference to the origin of the uterine epithelial component and the relationship of the latter with the maternal "giant" cells of previous authors (Bjorkman 1968; King et al. 1979).

Material and methods

Friesian or Friesian × Hereford cows at 22, 28, 70, 112, 150, 230 and 270 days of pregnancy and one Soay ewe at 120 days of pregnancy were killed with captive bolt pistol and the uterus removed within 10 min of death. The uterine arteries were cannulated and were perfused with 200–500 ml of glutaraldehyde fixative (see below). The uterus was then cut open and the lumen filled with fixative or the umbilical vessels were cannulated and the fetal placental circulation perfused. Total duration of perfusion was 15 min at room temperature. Individual placentomes were then cut into 1–2 mm thick slices and divided further into 1 mm square 'matchsticks' which ran from top to bottom of the placentome. These tissue pieces were placed in fixative for a further 15 or 30 min at room temperature and then stored at 4° C in 0.1 M sodium cacodylate buffer pH 7.2. The fixatives used were 1% glutaraldehyde 3% formaldehyde in 0.1 M sodium cacodylate buffer pH 7.2 with 5% sucrose; or 4% glutaraldehyde in 0.1 M Dulbecco phosphate saline, pH 7.2, with 2% sucrose. The pieces of fixed tissue were embedded in Araldite resin after dehydration in ethanol and propylene oxide (for details, see Wooding 1980) or in glycolmethacrylate resin (GMA) after dehydration using increasing concentrations of glycolmethacrylate (for details, see Spaur and Moriarty 1977). Pale gold sections were cut onto 300-mesh uncoated nickel grids.

Immunocytochemistry

Details of the isolation and characterisation of the bovine placental lactogen and the production of antibodies in rabbits have been given by Beckers et al. (1980; 1982).

Goat antirabbit-IgG coated colloidal gold particles (5 and 10 nm diameter) were obtained from Janssen Pharmaceutica, Beerse, Belgium and used at a dilution of 1:20.

The specificity of the immunolabelling was tested by incubating sections with:

(1) Adsorbed antiserum: 40 µl of a 1:100 dilution of rabbit antiserum to bovine placental lactogen was absorbed against 32 µg of pure bPL in 80 µl 0.01 M ammonium bicarbonate for use as a specificity control. Absorption was carried out by shaking the solution for 90 min at 37° C in a water bath.

(2) Preimmune rabbit serum at the same dilution as the antiserum.

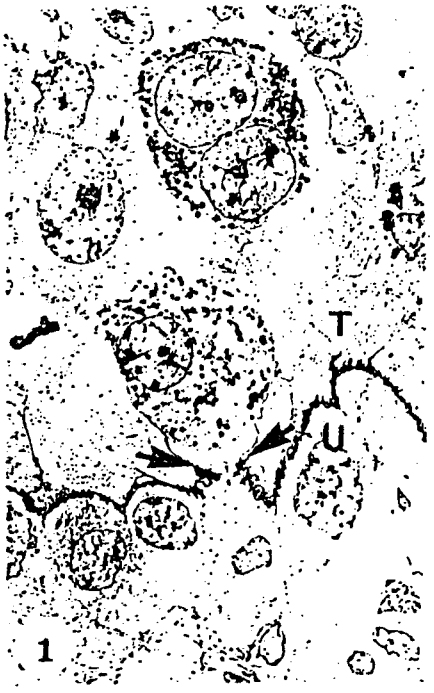
(3) Antiguinea-pig IgG gold instead of antirabbit IgG gold.

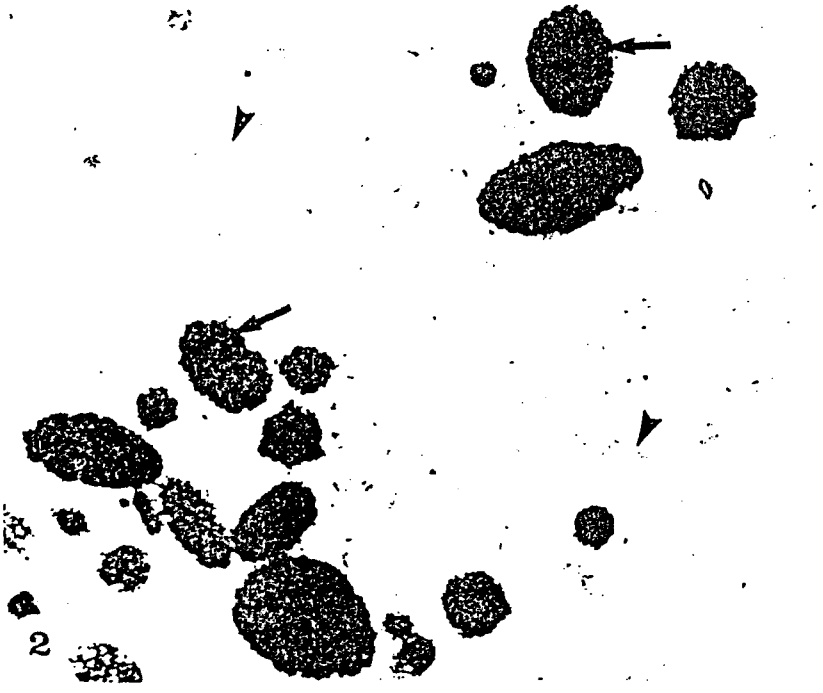
(4) Antirabbit IgG gold alone.

No specific labelling was seen on any of these sections.

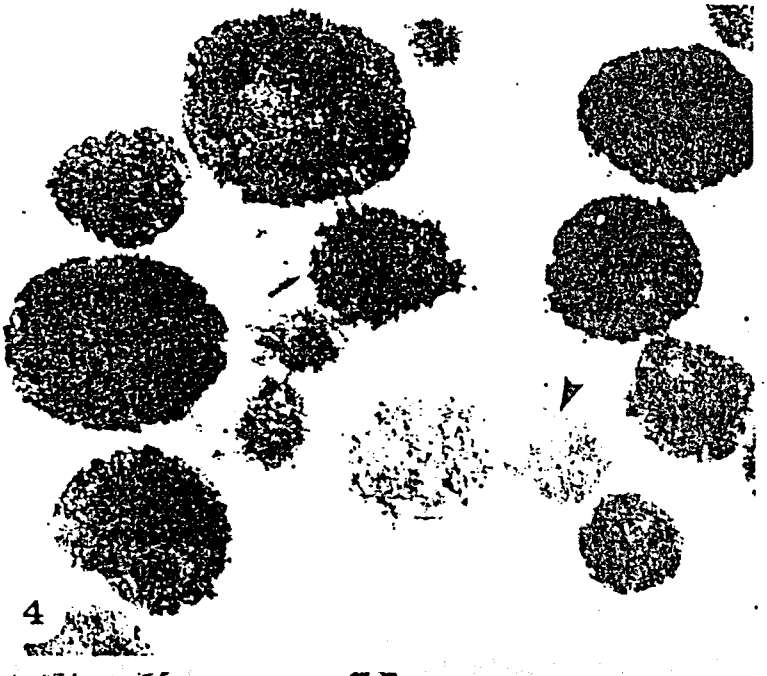
The immunogold staining procedure of the sections on the grids was the same as that detailed in Theodosis et al. (1986). Briefly, the grids were floated on 10% whole sheep serum (60 min), antibody at 1:200 dilution (18 h at 4° C),

Send offprint requests to: Dr. F.B.P. Wooding, AFRC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, United Kingdom


T
U
1


2


T
U
*
3


4


T
U
5

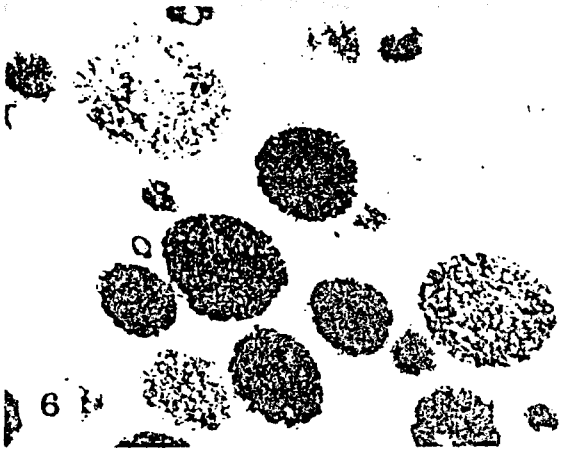

6

Table 1. Gold particle counts over granules and adjacent cytoplasm of binucleate cells (BNC) from placentomes of pregnant cows (Means±S.E.M.)

Days pregnant[a]	Number of BNC sampled[b]	Gold particles per BNC granule[b]	Granule areas (μm^2)	Gold particles per 500 μm^2	
				Granules[b]	Cytoplasm[b]
22	5	2±1	3.7±0.2	145±50	40±20
28	5	5±1	4.3±0.5	540±95	36± 9
50	10	8±5	4.6±0.4	870±195	30±15
150	10	15±7	5.8±0.5	1100±215	37±30
150[c]	10	1±1	5.6±0.8	34±20	10± 3
230	10	16±5	5.3±0.4	1500±295	50±20

[a] One animal sampled at each time
[b] At each time areas from 5–10 BNC including 150–300 granules and adjacent areas (totalling 2000–5000 μm^2) without granules were counted
[c] Antibody adsorbed with antigen before use (see Methods)

washed with Tris buffer, floated on antirabbit IgG gold at 1:20 dilution (60 min) washed with tris buffer and glass distilled water, fixed with 2.5% glutaraldeyhde in distilled water and finally washed in distilled water. Sections were stained with 1% phosphotungstic acid (PTA) in 3.5% HCl for 15 min at 60° C (Wooding 1980). Gold particle counts were carried out by projecting negatives at constant magnification onto a digitiser pad connected to an Apple IIE. Granule or cytoplasmic areas were defined with a stylus and the number of gold particles in each area counted and recorded.

Results

The micrographs presented are all of material processed without osmium fixation and stained, after immunolabelling, with phosphotungstic acid. This selective staining highlights the microvillar junction, nuclei and binucleate cell granules leaving the rest of the cytoplasm low in contrast, thus simplifying the search for gold localisation. The gold labelling of the granules was heavier and more consistent after 2% glutaraldehyde fixation and glycol methacrylate embedding, but Araldite gave a better preservation of ultrastructural detail (e.g. Fig. 2). 4% Glutaraldehyde or osmium treatment reduced the labelling to an erratic low level.

Gold labelling indicating the presence of bovine placental lactogen was found in the fetal trophectoderm of the placentomes exclusively over binucleate cell granules (Figs. 2, 4, 8, 11) and the binucleate cell Golgi region (Figs. 10, 11). All the granules in fetal binucleate cells were labelled, including those in mature migrating binucleate cells at, or partly across the microvillar junction (Figs. 1–6). The level of gold labelling of individual granules was fairly uniform in any one binucleate cell and appeared unrelated to the wide variations in phosphotungstic acid staining characteristically found (Table 1, Fig. 4). No Golgi region labelling was seen in any other trophectodermal or uterine epithelial cell.

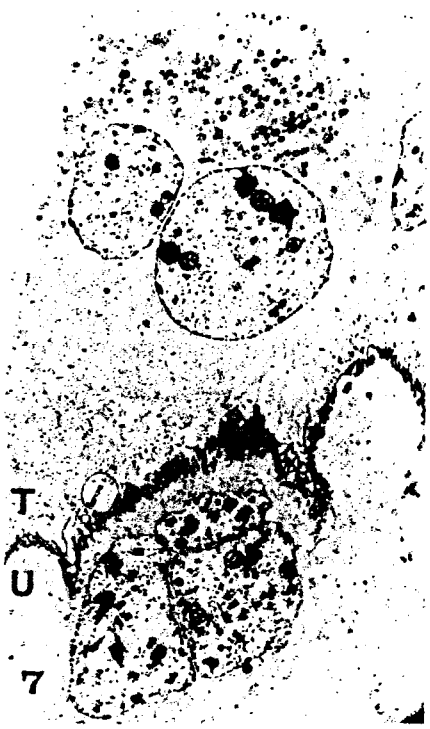
Gold labelled granules of equivalent size and PTA staining characteristics to those in the fetal binucleate cells were also found in a few cells in the maternal uterine epithelium (Figs. 5, 6). Such cells frequently had up to a maximum of three nuclei, with two frequently showing a very different density to the third (Figs. 12, 13). These nuclei were packed closely together and such cells are probably equivalent to the (maternal) "giant cells" of previous authors. A maximum of two out of the three nuclei had small 60–80 nm diameter dense masses scattered in the chromatin (Figs. 14, 15) the only other placental nuclei showing such structures were the mature fetal binucleate cells (Fig. 14).

After adsorption of the antibody with purified bovine placental lactogen (Fig. 9) or omission of any one of the immunolabelling reagents, no labelling above background levels was seen on any of the sections. Granules in placentome binucleate cells from 120 days pregnant sheep showed no labelling after antibovine placental lactogen incubation. The labelling of the bovine granules (Table 1) was weakest in 22-days pregnancy placentomes, stronger at 28 days

Figs. 1, 2. Cow placentome, 150 days post coitum (dpc), Araldite embedding. **Fig. 1** shows two granulated binucleate cells in the fetal trophectodermal epithelium (*T*). One has migrated up to the microvillar junction (at *arrows*). No granules are visible in the maternal uterine epithelial cells (*U*) ×1300. **Fig. 2** shows a detail from the migrating binucleate cell, note the heavy and uniform gold particle labelling of the granules (*arrows*) and the lack of label over the numerous small mitochondria (*arrowheads*). This labelling pattern was found in all placentomal binucleate cells at this stage of pregnancy. ×20000

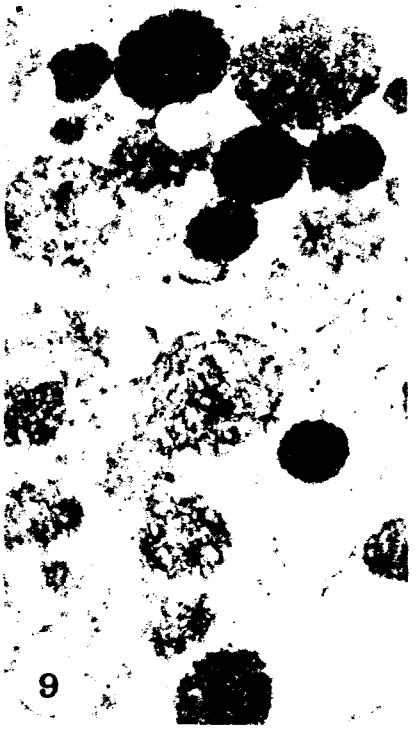
Figs. 3, 4. Cow placentome, 150 dpc, Araldite embedding. **Fig. 3.** shows a binucleate cell just after fusion with a maternal uterine epithelial cell (*asterisk*). This fusion interrupts (between *arrows*) the microvillar junction between trophectoderm (*T*) and uterine epithelium (*U*) ×3000. **Fig. 4** shows the variation in phosphotungstic acid staining but uniformity of gold labelling of the binucleate cell granules. The unlabelled granule (*arrowhead*) may be a lysosome rather than a secretory granule. ×40000

Figs. 5, 6. Cow placentome, 150 dpc, Araldite embedding. **Fig. 5** shows a trinucleate (or "maternal giant") cell in the maternal uterine epithelium (*U*) with numerous granules equivalent in phosphotungstic acid staining reaction to those of the fetal binucleate cells in Figs. 1 and 3. Note that the microvillar junction staining is interrupted above the trinucleate cell (between *arrows*). It is suggested that this micrograph represents a stage in binucleate cell migration subsequent to that shown in Fig. 3. The granules and the nuclei are now completely within the uterine epithelial cell, but the microvillar junction and its staining has not yet been fully restored. ×1800. **Fig. 6** shows that the granules in such cells have an equivalent level of gold labelling to those in fetal binucleate cells. ×21000

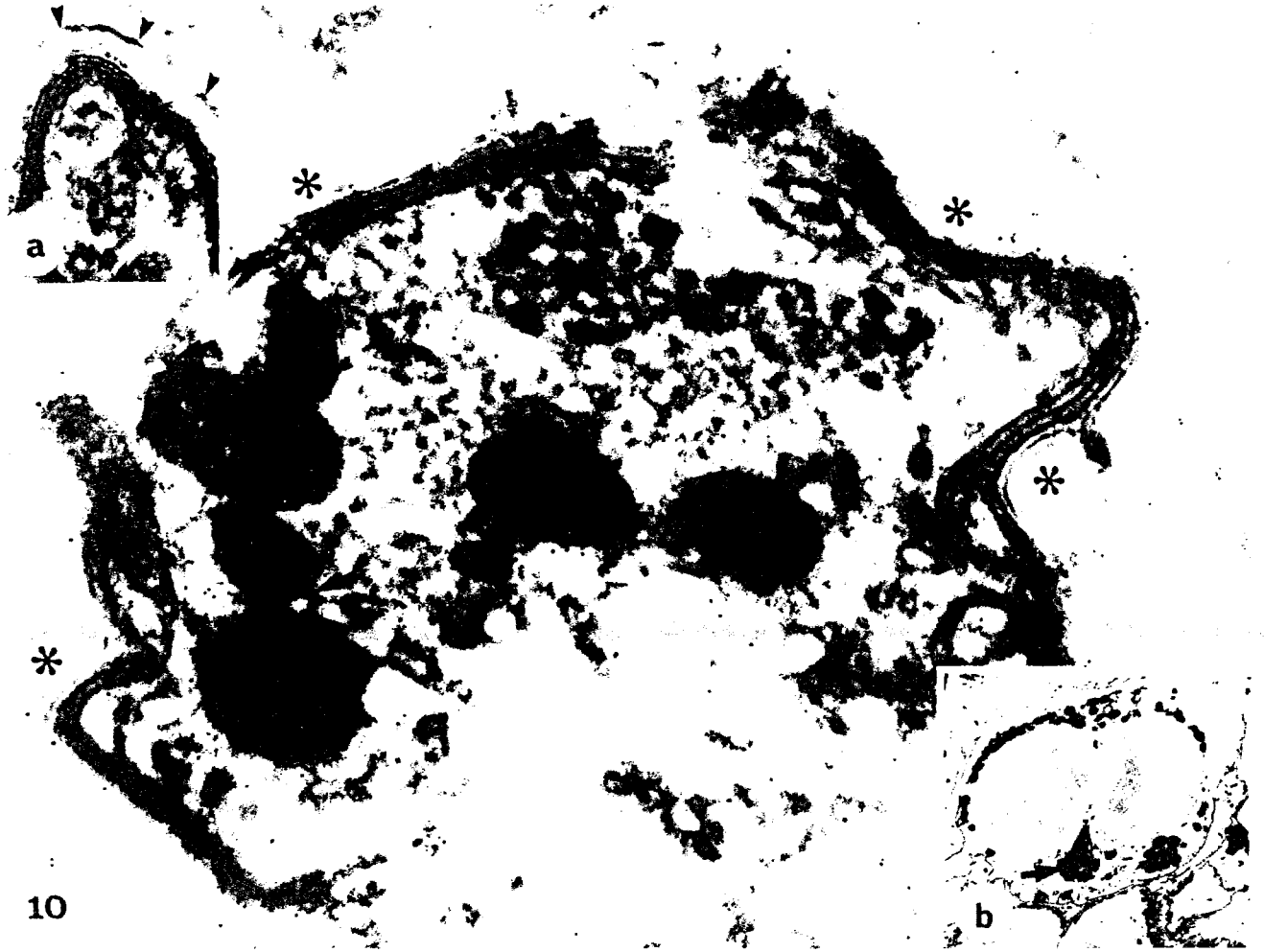


7

8



9



10

(Fig. 11) and reached a plateau of intensity in the 150- to 230-days pregnancy specimens (Figs. 2, 4, 6).

Gold labelling was found only over fetal binucleate cells or maternal giant cells. No evidence for exocytosis such as granule cores or labeling in the intercellular space around these two cell types was observed. No other cells, connective tissue or intercellular spaces showed any labelling above background levels.

Discussion

This report provides the first evidence that bovine placental lactogen activity is found solely in fetal binucleate cell granules and the Golgi cisternae from which those granules form. The presence of labeled granules of equivalent size and staining characteristics in cells half way across the microvillar junction and in trinucleate cells (referred to as maternal giant cells by previous authors Bjorkman 1968; King et al. 1979) in the maternal uterine epithelium supports the concept of binucleate cell migration (Wooding and Wathes 1980). It is unlikely that these granules are synthesised on the maternal side since none of the Golgi bodies in the maternal uninucleate or trinucleate cells show any labelling indicative of placental lactogen synthesis.

These results confirm the light-microscope immunolocalisation of bovine placental lactogen in fetal binucleate cells by Verstegen et al. (1985) and fully support their suggestion that the bPL positive material present between their artifactually separated fetal and maternal layers and in an occasional maternal uterine epithelial cell results from binucleate cell migration.

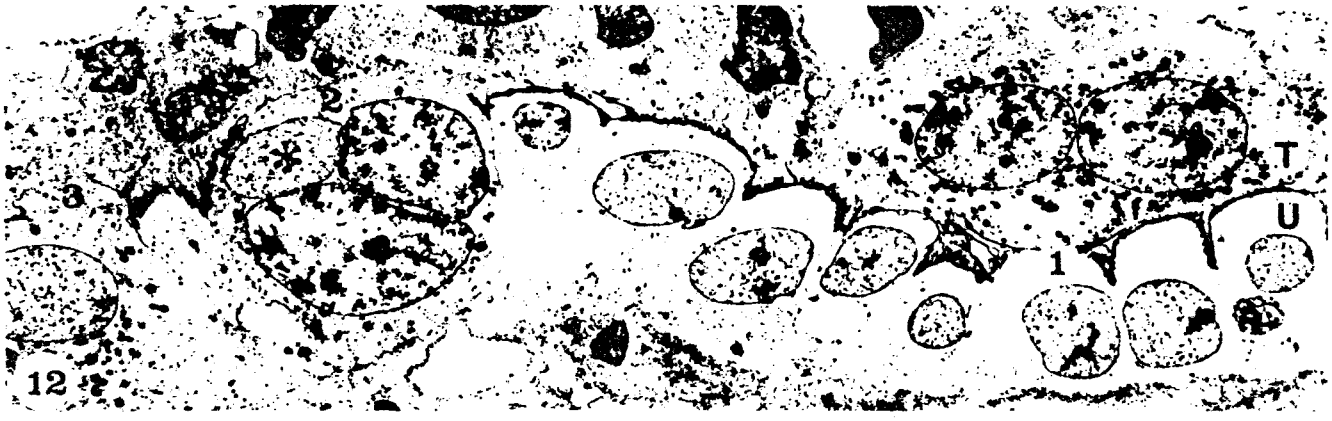
Wooding and Wathes (1980) have presented morphological evidence for bovine binucleate cell migration and Wooding (1982) suggested that each migration ends in fusion with a uterine epithelial cell to form a (trinucleate) maternal giant cell. The results in this paper support this, for no maternal giant cells with more than three nuclei were observed and the nuclei frequently comprised two like and one unlike on grounds of electron density or characteristic nucleoplasmic structures. Such structures were only observed elsewhere in the placenta in mature fetal binucleate cell nuclei so the morphological and lactogen localisation results provide further evidence for the idea that migration and fusion are the normal fate of bovine fetal binucleate cells. The ovine placental binucleate cell has been shown to have a similar life history (Wooding 1980, 1982, 1984; Lee et al. 1985) delivering its characteristic granules and forming a persistent fetomaternal syncytium at the fetomaternal interface. In the cow the trinucleate (maternal giant) cells are transient, die after releasing their granules and are resorbed by the fetal trophectoderm (Wooding and Wathes 1980; Wooding 1982).

Bovine binucleate cell migration thus makes no permanent structural contribution but serves only to deliver the granules, the only source of bPL identified so far, close to the maternal circulation. The labelling of the granules indicates a gradual increase in their bPL concentration during early pregnancy to a fairly constant level by mid pregnancy. This correlates better with the slow rise in bPL in the maternal serum throughout pregnancy than with the continuous fall reported for the fetal serum from 75 days of pregnancy (the 75-day sample was the earliest fetal sample assayed Beckers et al. 1982). Although the maternal serum levels of bPL are consistently 10–15 times lower than the fetal during gestation (Beckers et al. 1982) the far greater volume of maternal blood means that it probably contains most of the bPL synthesised in the first half of pregnancy. Bovine and ovine placental lactogens are molecules of similar size and since the latter cannot cross from maternal to fetal blood down a concentration gradient (Grandis and Handwerger 1983) it seems unlikely that bPL could pass the same way against a gradient. Throughout gestation in the cow (Beckers et al. 1982) and for more than two thirds (100/145) of pregnancy in the sheep (Chan et al. 1978) placental lactogen concentrations in the fetal blood are consistently higher than in maternal blood. This is in marked contrast to monkeys and humans where maternal levels are reported always higher (see Chan et al. 1978). The origin of the fetal serum bPL is obscure unless, as Wooding (1981) and Verstegen et al. (1985) have suggested, binucleate cells can release granules prior to, or as an alternative to, migration. In this and in earlier studies no morphological evidence was found for binucleate cell degranulation or exocytosis on the fetal side and in this study no trace of bPL immunoreactivity was found in the extracellular space around fetal binucleate cell or maternal giant cell. However, since exocytosis is so transient an event and diffusion of granule content liable to be rapid it will require more careful study with material processed for optimum membrane preservation to uncover the details of the process. This paper provides further evidence for the uniformity of structure and function in the ruminant binucleate cell. Placental lactogen granule production, migration, and fetomaternal cell fusion coupled with granule delivery to the maternal tissues are all common features. However the origin of the placental lactogen in the fetal circulation remains a puzzle and supports recent evidence for subpopulations of binucleate cells which may have different functions despite very similar structure (Lee et al. 1985).

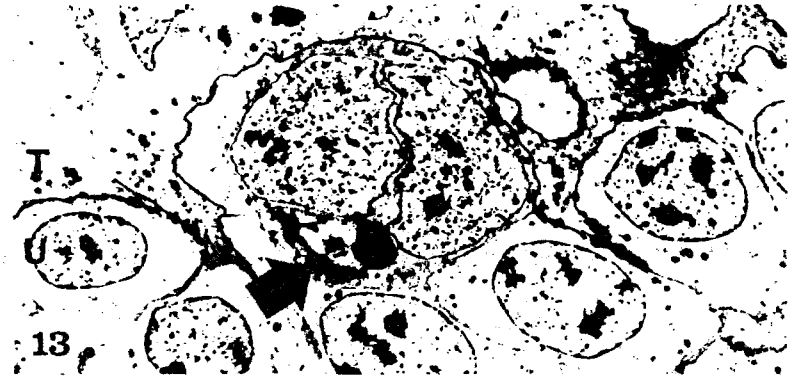
Figs. 7, 8, 9. Cow placentome, 150 dpc, Araldite embedding. **Fig. 7** shows a fetal binucleate cell and maternal trinucleate cell. The latter represents the final stage of migration and, compared with Fig. 5, the granules have been released and the microvillar junction between trophectoderm (*T*) and uterine epithelium (*U*) reformed. ×1700. **Figs. 8, 9** show sections through the binucleate cell of **Fig. 7** at different levels; **Fig. 8** treated with 1:300 antibovine placental lactogen (abPL) serum, **Fig. 9** with 1:300 abPL serum absorbed against purified bPL. No gold labelling above background levels is seen on **Fig. 9**. **Figs. 8** and **9** both ×27000

Fig. 10. Cow placentome, 150 dpc, Glycolmethacrylate embedding. *Inset* **b** shows that with acrylate embedding the binucleate cell Golgi body stains much more densely than with araldite and can be clearly seen (*arrow*) even at low power. ×2000. On the main micrograph at a higher magnification (at a different level) it appears that secretory granules are forming from the reticulated innermost (trans) cisternum (*arrows*) of the Golgi. There seems to be more gold label on the "trans" side than on the more weakly stained or unstained cisternae on the "cis" side (*asterisks*) ×38000. *Inset* **a** shows that the extent of the mostly unstained "cis" Golgi cisternae is defined by a strand of endoplasmic reticulum (*arrowheads*) which sometimes stains after acrylate embedding. ×18000

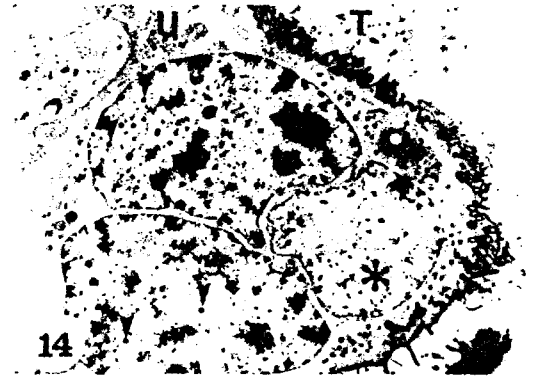
11



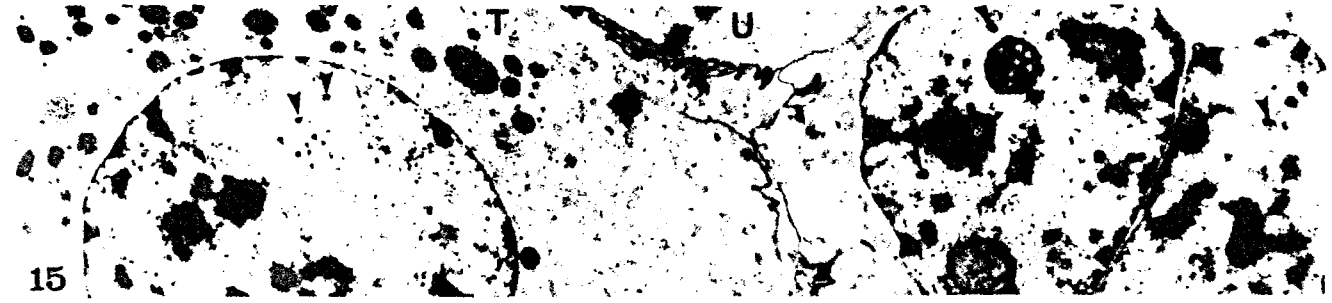
12



13



14



15

Fig. 11. Cow placentome, 28 dpc, Glycolmethacrylate embedding. Gold labelling on binucleate cell granules and Golgi body is somewhat less uniform but still clear. The reticulated "trans" Golgi cisternum (*solid arrow*) is forming granules which are labelled; the "cis" face of the Golgi is unstained and unlabelled (*asterisks*). × 31 000

Fig. 12. Cow placentome, 50 dpc, Araldite embedding. The fetal trophectoderm (*T*) is separated from the maternal uterine epithelium (*U*) by the microvillar junction which is interrupted in three places. At *1* by a fetal binucleate cell at the start of migration, and at *2* and *3*, by maternal 'giant' cells. The 'giant' cell at *2* has all three nuclei on this plane of section. Two of these have a density of chromatin very similar to that in the binucleate cell nuclei and very different from the third (*asterisk*) which closely resembles the maternal uterine epithelial cell nuclei. × 2100

Fig. 13. Cow placentome, 112 dpc, Araldite embedding. Maternal trinucleate cell with two nuclei clearly different from the third (*arrow*). Fetal trophectoderm (*T*); maternal uterine epithelium (*U*). × 2500

Fig. 14. Cow placentome, 270 dpc, Araldite embedding. This maternal trinucleate cell has only two nuclei showing uniformly sized round, dense particles (*arrowheads*). The third nucleus (*asterisk*) had none (on several planes of section) and had much less dense chromatin, reinforcing the 'two plus one' appearance. *T* fetal trophectoderm; *U* maternal uterine epithelium. × 3000

Fig. 15. Cow placentome, 150 dpc, Araldite embedding. Both the binucleate cell nucleus in the trophectoderm (*T*) and the maternal giant cell nuclei in the uterine epithelium (*U*) contain discrete dense nucleoplasmic dots (*arrowheads*). Such structures have not been observed in any other placental nuclei. × 6000

Acknowledgements. This work was partly supported by the "Institut pour l'encouragement de la recherche scientifique dans l'industrie et l'agriculture" (IRSIA, rue de Crayer, 6, 1050 Brussels, Belgium).

The excellent technical assistance of Sue Johnson and Geoffrey Morgan is gratefully acknowledged.

References

Arima Y, Bremel RD (1983) Purification and characterisation of bovine placental lactogen. Endocrinology 113:2186–2184

Beckers JF, Fromont-Lienard CH; Van der Zwalmen P, Wouters-Ballman P, Ectors F (1980) Isolement d'une hormone placentaire bovine présentant un réactivité analogue à la prolactine et à l'hormone de croissance. Ann Vet Med 124:585–601

Beckers JF, Decoster R, Wouters-Ballman P, Fromont-Lienard CH, Van der Zwalmen P, Ectors F (1982) Dosage radioimmunologique de l'hormone placentaire somatotrope et mammotrope bovine. Ann Vet Med 126.9–21

Bjorkman N (1968) Fine structure of cryptal and trophectodermal giant cells in the bovine placentome. J Ultrastruct Res 24:249–258

Blank MS, Chan JSD, Friesen HG (1977) Placental lactogens, new developments. J Steroid Biochem 8:403–414

Chan JSD, Robertson HA, Friesen HG (1978) Maternal and fetal concentrations of ovine placental lactogen measured by radioimmunoassay. Endocrinology 102:1606–1613

Grandis AS, Handwerger S (1983) Differential effects of ornithine on placental lactogen and growth hormone secretion in the pregnant ewe and fetus. J Endocrinol 97:175–178

Hall J, Talamantes F (1984) Immunocytochemical localisation of mouse placental lactogen in the mouse placenta. J Histochem Cytochem 32:379-381

King GJ, Atkinson BA; Robertson HA (1979) Development of the bovine placentome during the second month of gestation. J Reprod Fertil 55:173–180

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. J Anat 140:565–576

Martal J, Djiane J, Dubois MP (1977) Immunofluorescent localisation of ovine placental lactogen. Cell Tissue Res 184:427–433

Murthy GS, Schellenberg C, Friesen HG (1982) Purification and characterisation of bovine placental lactogen. Endocrinology 111:2117–2124

Porter DG (1980) Fetomaternal relationships: the actions and the control of certain hormones. Placenta 1:259–274

Sciarra JJ, Kaplan SL, Grumbach MM (1963) Localisation of anti-human growth hormone serum within the human placenta: evidence for a human chorionic 'growth hormone prolactin'. Nature 199:1005–1006

Spaur RC, Moriarty GC (1977) Improvements of glycolmethacrylate: its use as on embedding medium for electron microscopic studies. J Histochem Cytochem 25:163–174

Talamantes F, Ogren L, Markoff E, Woodard S, Madrid J (1980) Phylogenetic distribution, regulation of secretion and prolactin like effects of placental lactogens. Fed Proc 39:2582–2587

Theodosis DT, Wooding FBP, Sheldrick EL, Flint APF (1986) Ultrastructural localisation of oxytocin and neurophysin in the ovine corpus luteum. Cell Tissue Res 243:129–135

Verstegen J, Fellman D, Beckers JF (1985) Immunodetection of bovine chorionic somatomammotropin. Acta Endocrinol 109:403–410

Wooding FBP (1980) Electron microscopic localisation of binucleate cells in the sheep placenta using phosphotungstic acid. Biol Reprod 22:357–365

Wooding FBP (1981) Localisation of ovine placental lactogen in sheep placentomes by electron microscope immunocytochemistry. J Reprod Fertil 62:15–19

Wooding FBP (1982) The role of the binucleate cell in ruminant placental structure. J Reprod Fertil [Suppl] 31:31–39

Wooding FBP (1984) Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Am J Anat 170:233–250

Wooding FBP, Wathes DC (1980) Binucleate cell migration in the bovine placentome. J Reprod Fertil 59:425–430

Accepted August 28, 1986