

An immunological cryo-ultrastructural study of a sequential appearance of proteins in placental binucleate cells in early pregnancy in the cow*

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Summary. Using the most sensitive immunocytochemical method available, on ultrathin frozen sections, the results in this paper demonstrate that bovine placental lactogen (bPL) is present in the earliest fetal binucleate cells found at 21 days *post coitum* in the trophoctoderm. A second protein, the SBU-3 antigen, which is absent in the early stages of pregnancy appears abruptly in the binucleate cell granules at 30 days *post coitum* coincident with the start of villus development. Subsequently, the granules contain both bPL and the SBU-3 antigen. This sequential production of unlike proteins indicates that the binucleate cell has different functions depending on the stage of pregnancy and has important roles to play both at implantation and in villus development.

Keywords: immunology; ultrastructure; proteins; early pregnancy; cow

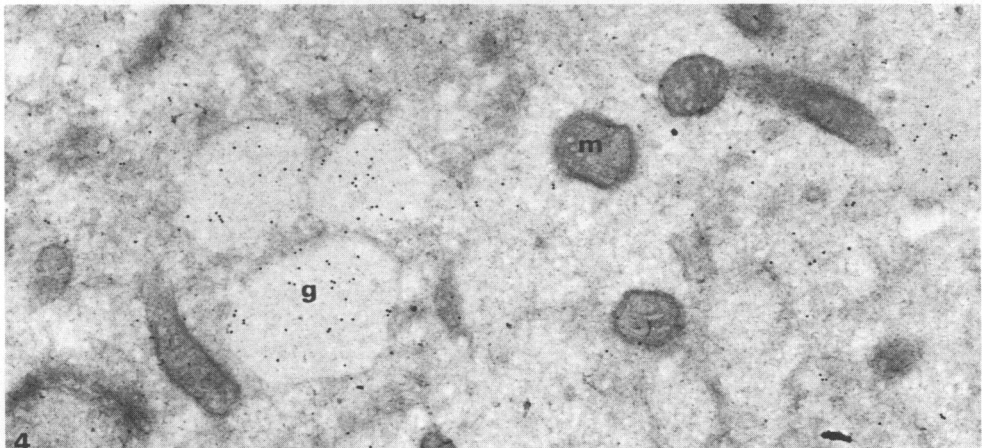
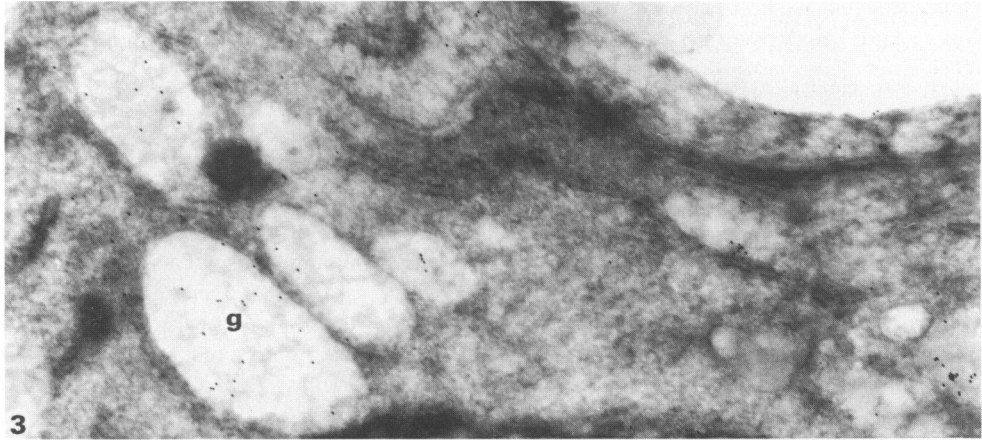
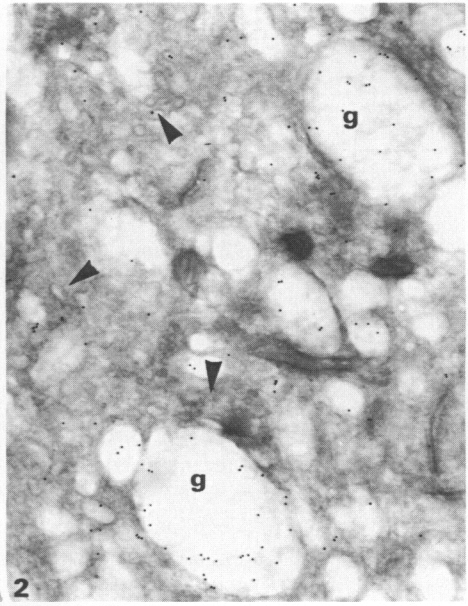
Introduction

Placental lactogenic hormones have been shown to be produced by fetal binucleate cells (BNC) in sheep (Martal *et al.*, 1977; Wooding, 1981; Morgan *et al.*, 1987) and cows (Verstegen *et al.*, 1985; Wooding & Beckers, 1987), throughout pregnancy. They are said to act as fetal growth hormones and change the mother's metabolism to benefit the fetus (Thordarson *et al.*, 1987; Freemark *et al.*, 1987). Morgan *et al.* (1987) using ultrathin frozen sections for electron microscopy (EM) localized ovine placental lactogen (oPL) to BNC granules in sheep at the earliest stage of placenta formation, coincident with implantation at 17 days *post coitum* (*p.c.*). However, the SBU-3 antigen (Gogolin-Ewens *et al.*, 1986) could not be demonstrated in BNC granules before the development of the fetal cotyledonary villi at a later stage (29 days *p.c.*). SBU-3 is a monoclonal antibody which recognizes an as yet uncharacterized glycoprotein found only in BNC granules and the Golgi bodies which produce them. Wooding & Beckers (1987), using resin sections, found that bPL localization in cows was very weak before 28 days *p.c.* The present more sensitive cryosection study was undertaken to establish whether bPL was synthesized by the earliest BNC produced by the chorion (between 17 and 21 days *p.c.*) and whether the later appearance of SBU-3 in the BNC coincided with villus formation in the placenta of the cow, as in the sheep.

Materials and Methods

Friesian cows at 18, 20, 21, 23, 30, 37, 135 and 260 days *post coitum* (*p.c.*) were killed by captive bolt pistol. The intact uteri were immediately removed and fixed by perfusion through both uterine arteries with 1% glutaraldehyde plus 3% (para)formaldehyde in 0.1 M cacodylate buffer containing 5% sucrose. In addition 5–10 ml fixative were injected into

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the uterine lumen 10 min after perfusion started. Total perfusion time was 20 min at room temperature. The uteri at Days 135 and 260 *p.c.* were also perfused via the fetal umbilical arteries. The uteri were then cut up into 5-cm lengths and left in fixative for a further 40 min, after which time they were rinsed thoroughly and stored in 0.1 M-cacodylate buffer.

Samples of perfused cotyledons or caruncular areas where the blastocyst was adherent to the uterine epithelium were dissected out under a binocular microscope and orientated on the heads of metal pins (Reichert cryomicrotome supports) so that sections would be cut at right angles to the caruncular surface. The samples were then frozen by immersion in liquid nitrogen and sectioned at -100°C with a Reichert OM FC4D cryo-ultramicrotome. The sections were transferred from the dry frozen glass knife, on the bottom of droplets of 2.3 M-sucrose in 0.1 M-phosphate buffer (pH 7.2), to touch down on and adhere to celloidin-covered 150-mesh nickel grids. The grids were floated section side down on a sequence of three drops of buffer for 10 min each to remove the sucrose. The sections, firmly adherent to the celloidin on their grids, were then incubated on a series of droplets of solutions on Nescio film in a Petri dish for immunogold localization of bPL or the SBU-3 antigen. Characterization of the bPL F, bPL B, and monoclonal SBU-3 antibodies are described by Murthy *et al.* (1982), Beckers *et al.* (1980, 1982) and Gogolin-Ewens *et al.* (1986), respectively. The first incubation was on heated (60°C , 10 min) whole sheep serum diluted 1:10 with 0.1 M-Dulbecco phosphate-buffered saline (pH 7.2) containing 1% bovine serum albumin and 0.01% Thimerosal (a bacteriostatic) for 10 min. (The above buffer was used for all antibody and gold dilutions.) The sections were then blotted free of excess heated whole sheep serum and incubated for 16 h at 4°C on anti-bPL at dilutions of 1:40 and 1:80 respectively, or for 1 h on undiluted hybridoma supernatant for SBU-3. After washing the grids with buffer from a wash bottle they were floated for 30 min on a drop of a 1/25 dilution of Bioclin 10 nm colloidal gold particles coated with goat anti-rabbit (or goat anti-mouse for SBU-3) IgG (BioClin, Agar Scientific, Stansted, UK). They were then jet washed with buffer and floated on drops of 2% aqueous osmium tetroxide for 5 min to enhance the contrast and washed by floating on 4 drops of distilled water (jet washing with distilled water washed off the celloidin films). The sections were then stained on drops of 0.25% aqueous methyl cellulose (Sigma, Poole, Dorset, UK: Cat No. MO262: viscosity of a 2% aqueous solution at 25°C approximately 400 centipoises). After 5 min the methyl cellulose has permeated sufficiently to act when dry as a reinforcing matrix for the sections, the excess is blotted off and the sections allowed to dry.

As specificity controls the initial antibody was replaced by heated whole sheep serum or by anti-bPL absorbed with pure bPL (the SBU-3 antigen is not yet available). Counts of the gold particles were made on the electron microscope. A wire loop attached to a pointer can be swung into the field of view of the JEOL 100C electron microscope and superimposed on the screen. This provides a constant size and the magnification of the image is selected to give between 5 and 25 gold particles within the area of the loop when positioned over the relevant cellular area. This magnification can be established quickly by an initial rapid qualitative scan of the section. Examples of the organelle or cell area under study (granule, cytoplasm, RER, Golgi body etc) are then selected at random, that is at a magnification at which the gold particles are not visible. The gold particles within the loop are then counted using the $\times 10$ eyepiece and recorded together with the magnification. From these two figures, given the area of the loop, the frequency of gold particles/ μm^2 of each cellular inclusion can readily be calculated. This procedure is far quicker and as accurate (to within 5–7%) as counting gold particles on standard magnification prints.

Results

Localization of bPL

At 18 and 20 days *p.c.* no mature BNC with granules were found but the occasional young BNC was identified, which showed no label in their rare granules. At 21 days *p.c.* a few more BNC were found but most of these were immature, containing few granules with a low level of gold labelling (Figs 1, 2, 3; Table 1). By 23–25 days the granule label has almost doubled (Figs 4, 5a, 5b; Table 1).

Fig. 1. Ultrathin frozen section of BNC (b) apposed to uterine epithelium (u). Note mitochondria (arrows) occasional granules (small arrowheads) and Golgi area between the white arrowheads. 21 days *p.c.*; $\times 2800$.

Fig. 2. Ultrathin frozen section of BNC Golgi body showing gold labelling using anti bPL B. Golgi cisternae and small vesicles (arrowheads), granules (g). 21 days *p.c.*; $\times 20\ 000$.

Fig. 3. Ultrathin frozen section of BNC granules (g) showing gold labelling using anti bPL F. 21 days *p.c.*; $\times 20\ 000$.

Fig. 4. Ultrathin frozen section of BNC granules (g) showing gold labelling using anti bPL F. Mitochondria (m) are unlabelled. 23 days *p.c.*; $\times 23\ 000$.

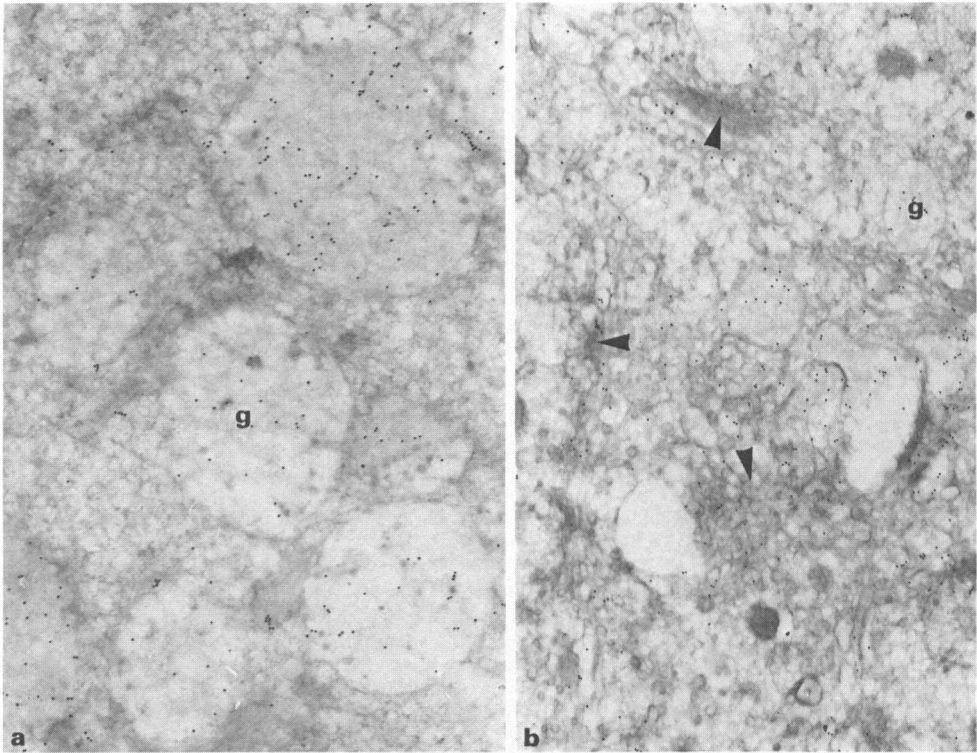


Fig. 5. Ultrathin frozen sections of BNC granules (g) (5a) and Golgi body cisternae (arrowheads) (5b) showing the strictly localized gold labelling using anti bPL F. (a) 25 days *p.c.*; $\times 23\ 000$; (b) 25 days *p.c.*; $\times 25\ 000$.

Similar results were obtained with both bPL B and F at 21 and 23 days *p.c.* but the bPL F was effective at lower dilution with a lower background level so was used for the remainder of the study. There was a further rise in gold labelling between 25 and 30 days *p.c.* with a slight increase to a fairly constant peak value between 135 and 260 days. BNC Golgi body label was approximately half that of their granules (Figs 2 and 5b; Table 1). Uterine 'giant' cell granule label was first detected at 23 days *p.c.* and was consistently less than BNC granule label (Table 1). Particle counts on sections treated with absorbed purified antibody (bPL) or heated whole sheep serum showed no area with gold particle frequency of $>0.7 \pm 0.2$ per μm^2 ($n = 10$ for each).

Localization of SBU-3

No localization of SBU-3 was found before 30 days *p.c.* At 30 days, three groups of BNC in the trophectoderm could be distinguished by the degree of their granule labelling. No labelling was found in 5 BNCs, a low level of labelling was found in 11 and a high level of granule labelling was found in 55 of the BNCs counted (Figs 6a, 6b; Table 2). From 37 to 260 days, all BNC granules showed a similar high level of label which increased slightly at the later stages. The pattern of SBU-3 labelling in BNC and uterine 'giant' cells was similar to that of bPL, i.e. the BNC Golgi body label was approximately half that seen on their granules, and uterine 'giant' cell granule label was consistently less than that found in BNC granules (Table 2). Uninucleate cells never showed any labelling significantly above background level over any organelle with either SBU-3 or bPL (Tables 1 and 2). Particle counts on sections treated with heated whole sheep serum showed no area with gold particle frequency of $>0.4 \pm 0.2$ per μm^2 ($n = 10$).

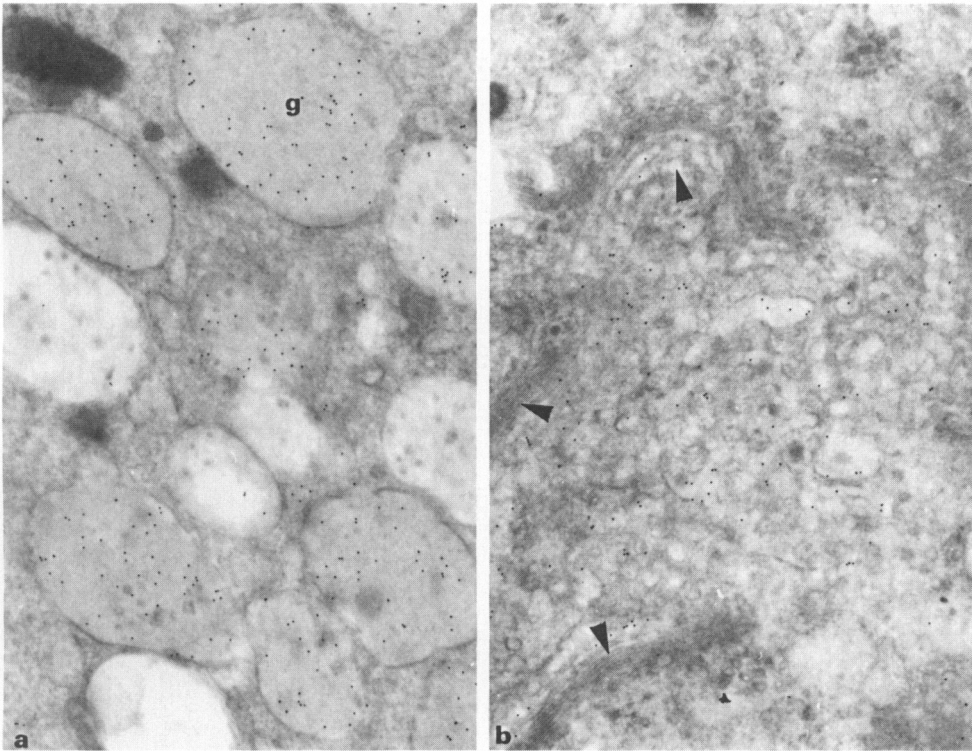


Fig. 6. Ultrathin frozen sections of BNC granules (g) (6a) and Golgi body cisternae (arrowheads), (6b) showing the localized gold labelling of the anti SBU-3 antigen. 30 days *p.c.*; $\times 23\ 000$.

Discussion

The results clearly show that bovine trophodermal binucleate cells (BNC) synthesize and store two defined proteins in sequence at specific times. The youngest conceptuses with mature granulated BNC were found at 21 days *p.c.* and immunolocalization on ultrathin cryo-sections showed that granules and Golgi bodies both contained bovine placental lactogen (bPL). When the fetal BNC had migrated to form fetomaternal tri- and multi-nucleate cells by fusion with uterine epithelial cells then these minisyncytia frequently showed granules, but never Golgi bodies, containing bPL. This confirms the hypothesis that bPL is synthesized only by mature fetal BNC. BNC have been reported in the trophoderm as early as 17–18 days *p.c.* (Greenstein *et al.*, 1958) with no significant population before 20 days *p.c.* (Wooding, 1983). If the bovine BNC needs about 48 h to differentiate, as shown for the very similar ovine BNC (Wooding *et al.*, 1981), then few mature BNC would be expected in cows before 19–20 days *p.c.* The absence of bPL-containing BNC in the material at 20 days could be due to the necessarily small samples used for cryo-ultramicrotomy allied to the wide variation in development between these early caruncular implantation sites and the inherent plus or minus 1 days inaccuracy of mating dates. BNCs containing bPL were first found at 21 days *p.c.* in this study, but they may be present 1 or 2 days earlier in small numbers. The amount of bPL per unit volume of granule increases far more in the first 30 days *p.c.* than in the subsequent 260 days. This is quite different from the high concentrations of oPL found in the earliest ewe BNC granules (17 days *p.c.*; Morgan *et al.*, 1987) but the reason for this considerable species disparity is at present unknown. BNC ultrastructure shows no change during implantation in cows or sheep.

Table 1. Counts of gold particles representing bovine placental lactogen localization on sections of trophoctoderm apposed to uterine epithelium

		Days post coitum									
		21	21	23	23	25	30	37	135	260	
Antibody Dilution		abPL B 1:40	abPL F 1:80	abPL B 1:40	abPL F 1:80	abPL F 1:80	abPL F 1:80	abPL F 1:80	abPL F 1:80	abPL F 1:80	abPL F 1:80
Chorion binucleate cell (BNC)											
Granules		12.0 ± 1.0 (24)	12.0 ± 1.0 (46)	24.0 ± 1.5 (15)	21.0 ± 1.5 (40)	21.0 ± 2.0 (34)	61.0 ± 4.0 (24)	51.0 ± 7.0 (13)	76.0 ± 5.5 (11)	72.0 ± 6.0 (12)	
Golgi body		7.0 ± 1.0 (17)	6.0 ± 1.0 (15)	9.0 ± 0.5 (20)	8.0 ± 0.5 (15)	13.0 ± 1.0 (14)	27.0 ± 1.5 (12)	26.0 ± 1.5 (15)	23.0 ± 4.0 (14)	27.0 ± 1.5 (16)	
Cytoplasm		1.0 ± 0.5 (14)	1.5 ± 0.5 (52)	2.0 ± 0.5 (21)	4.0 ± 0.5 (40)	1.5 ± 0.5 (44)	4.5 ± 0.5 (27)	6.5 ± 0.5 (18)	8.0 ± 0.5 (15)	7.0 ± 1.0 (15)	
Uterine epithelium											
Giant cell granules				22.0 ± 1.5 (14)	14.5 ± 1.0 (31)		40.0 ± 3.0 (24)		60.0 ± 2.5 (15)	60.0 ± 3.0 (13)	
Giant cell cytoplasm			7.5 ± 0.5 (38)	2.5 ± 0.5 (18)			8.0 ± 1.0 (30)		7.0 ± 0.5 (15)	9.0 ± 1.0 (15)	
Chorion uninucleate cell cytoplasm		2.0 ± 0.5 (11)	3.0 ± 0.5 (46)	2.5 ± 0.5 (21)	4.0 ± 0.5 (40)	2.0 ± 0.5 (44)	5.0 ± 0.5 (29)	7.0 ± 0.5 (13)	9.0 ± 0.5 (12)	8.0 ± 1.0 (12)	

Values are mean ± s.e. of 1 μm^2 areas, chosen at random, from the number of samples indicated in parentheses. All sections were incubated with antibody at 4°C for 16 h.

Table 2. Counts of gold particles representing SBU-3 antigen localization on sections of trophoctoderm apposed to uterine epithelium

	Days post coitum*				
	25	30	37	135	260
Antibody, undiluted hybridoma supernatant	aSBU-3	aSBU-3	aSBU-3	aSBU-3	aSBU-3
Chorion binucleate cell (BNC)					
Granules	0.5 ± 0.5 (10)	0.5 ± 0.5 (5) 3.0 ± 0.5 (11) 20.0 ± 1.5 (55)	26.0 ± 1.5 (16)	32.0 ± 2.0 (50)	33.0 ± 5.5 (31)
Golgi body		5.0 ± 1.0 (16)	6.0 ± 1.0 (15)	12.0 ± 1.0 (11)	12.0 ± 1.0 (13)
Cytoplasm	0.5 ± 0.5 (10)	0.5 ± 0.5 (23)	0.5 ± 0.5 (10)	0.5 ± 0.5 (13)	0.5 ± 0.5 (15)
Uterine epithelium					
Giant cell granules		1.0 ± 0.5 (20)	12.0 ± 1.0 (12)	23.0 ± 1.0 (34)	24.0 ± 2.0 (25)
Giant cell cytoplasm		0.5 ± 0.5 (10)	0.5 ± 0.5 (10)	0.5 ± 0.5 (10)	0.5 ± 0.5 (14)
Chorion uninucleate cell cytoplasm	1.0 ± 0.5 (10)	0.5 ± 0.5 (24)	0.5 ± 0.5 (10)	0.5 ± 0.5 (13)	0.5 ± 0.5 (19)

*At 18, 20, 21 and 23 days, no areas in any cells counted higher than the background level (0.5 ± 0.5). Values are mean ± s.e. of 1 μm² areas, chosen at random from the number of samples indicated in parenthesis. All sections were incubated with antibody at 4°C for 16 h.

A second protein apparently unrelated to bPL but defined so far only by its monoclonal antibody (SBU-3: Gogolin-Ewens *et al.*, 1986) and found in a wide variety of ruminant BNC granules from mid- to late pregnancy (Lee *et al.*, 1985, 1986) was shown in this study to appear first in bovine BNC granules and Golgi bodies at 30 days *p.c.* Subsequently, it is a consistent granule constituent together with bPL. This increase in the number of proteins synthesized by the BNC is essentially similar to that found in the sheep with ovine placental lactogen and SBU-3 (Morgan *et al.*, 1987). In the sheep the multinucleate syncytial plaques produced by the BNC migration into the uterine epithelium persist throughout pregnancy. However, the fetomaternal tri- and multi-nucleated cells produced at implantation in the bovine uterine epithelium are ephemeral and are rapidly displaced by regrowth of residual uterine epithelial cells. Bovine BNC migrate throughout pregnancy but only produce transient trinucleate cells (Wooding, 1982). The only consistent result of BNC migration in the cow is delivery of the BNC granule content to the maternal compartment from implantation to term (Wooding, 1987). The process starts several days too late to be associated with maintenance of the maternal corpus luteum but it does correlate with the need for immunomodulation by the mother to prevent rejection of the conceptus. Clark (1985) and Chaouat (1987) have provided evidence for rodents which suggests that trophoctodermal secretions can recruit specific elements of the mother's immune system to ensure a local suppression of the immune response. In the cow the tri- and multinucleated plaques may form a more significant physical barrier against immune attack than the cellular uterine epithelium they replace. They may provide sufficient immune camouflage until a more permanent fetomaternal accommodation is reached possibly partly as a result of exocytosis of BNC granules into the maternal compartment. From their cytochemical reactivity there are undoubtedly constituents in the BNC granules at this stage

other than the bPL demonstrated here, but as yet there is no evidence for defined immunological functions for any of the granule contents.

The BNC do not synthesize the protein to which SBU-3 is the monoclonal antibody until 30 days *p.c.* coincident with the start of villus formation. The development of the cotyledonary villi provides the increase in fetomaternal exchange area necessary to supply nutrients for growth of the fetus. BNC are concentrated at the tips of the forming villi, and it is possible that release of their granule content, which now includes the SBU-3 antigen, facilitates the tissue remodelling which accompanies villus development. The process does not proceed by erosion of and penetration into maternal tissue, but rather a mutual elaboration of fetal and maternal surfaces. Unlike bPL the SBU-3 antigen never reaches the maternal blood (Gogolin-Ewens *et al.*, 1986) and so a local function is indicated. The SBU-3 antigen and other granule constituents could be involved in producing the considerable plastic deformation and massive cellular proliferation necessary for this process of cotyledonary villus development.

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