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Light and Electron Microscopic Immunolocalization of Bovine Pregnancy-Associated Glycoprotein in the Bovine Placentome[1]

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ABSTRACT

A bovine pregnancy-associated glycoprotein (bPAG) of 67 kDa has previously been isolated from bovine fetal cotyledons. The objective of this study was to determine the cytological localization of that protein in the placentomes and possibly the cells responsible for its production.

Highly specific antisera raised against pure bPAG were used to demonstrate the cellular localization of the protein in bovine placentomes by light and electron microscopic techniques. Strong immunostaining was observed exclusively in the cytoplasm of large binucleate cells present predominantly in fetal cotyledonary tissue (villi). Some smaller weakly immunostained cells were also present in caruncular epithelium. By ultrastructural immunogold procedures, the protein was detected only within amorphous cytoplasmic granules. Granules of identical size, but weakly labeled, were found on the maternal side. All cells containing labeled granules were binucleate. These results suggest that bPAG is probably synthesized by trophoblast binucleate cells and stored in granules prior to delivery into the maternal circulation after cell migration.

INTRODUCTION

An acidic glycoprotein of 67 kDa that is associated with pregnancy (bovine pregnancy-associated glycoprotein, bPAG) was recently isolated and purified from extracts of bovine fetal cotyledons [1]. In 1982, Butler et al. [2] isolated from bovine fetal membrane extracts two proteins: pregnancy-specific proteins (PSP) A and B. PSPA was identified as α-fetoprotein and PSPB as specific to placenta. Since development of a specific RIA for PSPB by Sasser et al. [3], the presence of this protein in the maternal serum has been used as a specific serological method for pregnancy diagnosis in cattle [4, 5], sheep [6], and goats [7]. Two preliminary reports indicated that the protein is most probably a product of trophoblast binucleate cells [8, 9].

Currently it is not known if the two placental proteins (PSPB and bPAG) belong to the same family. However, such characteristics as the molecular mass (78 kDa vs. 67 kDa) [1, 10], cross-reactions with pituitary gonadotropic hormones [3], differences in peripartum mean concentrations [11], and possible accessory sources [12] seem to indicate that the two proteins may be different. Although the physiological function of bPAG in pregnancy remains unknown, its potential importance is related to the quantity of the protein produced throughout gestation and especially its dramatic serum concentration rise during the last 10 days preceding parturition [11].

Bovine PAG presents four isoforms of different pI and immunoreactivity related to the different sialic acid content of each isoform [1]. Although the protein has been well characterized and a specific RIA was developed [11], its cytological and ultrastructural origins have remained undemonstrated. In this report we describe light and electron microscope immunocytochemical investigations undertaken to establish the cellular and subcellular localizations of bPAG in bovine placentomes.

MATERIALS AND METHODS

Antiserum Production

Four rabbits weighing 3–4 kg were immunized with HPLC-purified bPAG [1] according to the procedure of Vaitukaitis et al. [13]. Briefly, rabbits received intradermal injections of 250 μg bPAG dissolved in 0.5 ml distilled water and emulsified in an equal volume of complete Freund's adjuvant (Difco, Detroit, MI). Animals received booster injections at 2-wk intervals with 250 μg bPAG in incomplete Freund's adjuvant. They were bled 1 wk after the fourth injection and then every 2 wk. Sera were tested for antibodies to bPAG in RIA. Blood was drawn at 7 and 9 wk after the first injection; these samples yielded two antisera of nearly equivalent values according to RIA. At final dilutions of 1:1 500 000, both bound 35–40% of the ^{125}I-radiolabeled bPAG [11] and were used for the present study.

Tissue Collection and Preparation

Placentomes were collected from midgestation (3–5 mo) and nearly term (7–8 mo) placentas removed from two Blanc Bleu Belge cows (one at midgestation and the other at term)

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and two Holstein Friesian cows within 15 min after slaughter at the local slaughterhouse. Gestation ages were determined by the fetal crown-rump length measurement [14].

Histological procedure. Placentomes were cut into 5-mm pieces and fixed in Bouin's solution for 48 h, immersed in 70% ethanol for 48 h, dehydrated in graded ethanol, and embedded in paraffin. Sections 6 μm thick were cut and serially collected on glass slides coated with glue for immunostaining.

Electron microscopic procedure. Placentomes were cut into 1–2-mm-thick slices and divided further into 1-mm^2 matchstick-like pieces that ran from top to bottom of the placentome. The tissue pieces were dropped immediately into 4% glutaraldehyde (in 0.1 M PBS, pH 7.2) for 30 min at room temperature (RT) and then postfixed in 1% osmium tetroxide in 0.1 M veronal acetate buffer, pH 7.2, for 2 h. Tissues were then rinsed with PBS, dehydrated in graded isopropylic alcohol and propylene dioxide, and embedded in epon. Ultrathin sections of 90 nm were picked up on nickel grids.

Immunohistochemical Staining

Bovine PAG was detected by the antiserum R498 raised in a rabbit against the pool of the 4 isoforms [1] and by the peroxidase-antiperoxidase procedure. All incubations were carried out at RT with rocking in a moist chamber, and all dilutions and washes were done using Tris-buffer saline, pH 7.6, with 10% normal swine serum. After dewaxing, rehydration, and washing off of the paraffin, undiluted normal swine serum (Dako Ltd., High Wycombe, Bucks, UK) was applied for 15 min to block nonspecific binding. Sections were then incubated for 1 h in the primary antibody, which was the antiserum anti-bPAG (1:50). For negative control, the primary antibody was the antiserum anti-bPAG (1:50) previously absorbed with pure bPAG (250 μg/ml). Thereafter, sections were incubated in 1:20 diluted swine-anti-rabbit IgG antiserum (Dako Ltd.) for 30 min and then in 1:20 diluted soluble complex of horseradish peroxidase and rabbit-anti-horseradish peroxidase for 30 min. A solution of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) was added for 5 min. Sections were counterstained with the Carazzi hematoxylin for 8 min and rinsed in distilled water and then in running tap water for 10 min. Thereafter, sections were dipped in ethyl alcohol (95%), in absolute methanol (twice), and washed with xylol (twice). Coverslips were mounted with Canada balsam. Slides were examined and photographed with a Leitz photomicroscope. Specificity controls consisted of preabsorption of the antibody to bPAG with pure bPAG (250 μg/ml) for 17 h.

Immunocytochemical Technique

The rabbit antiserum to bPAG was diluted 1:50 in PBS containing 10% normal goat serum (NGS). The marker was 15 nm colloidal gold coated with goat-anti-rabbit IgG (Amersham Corp., Arlington Heights, IL). Goat-anti-rabbit IgG was diluted 1:20 in PBS containing 2% NGS.

The grid-mounted sections were incubated in PBS containing 2% NGS for 10 min at RT and then with the first primary antiserum for 2 h at RT in a moist chamber. Thereafter, sections were washed twice (5 min each) with PBS. Finally, sections were incubated in the gold-labeled goat-anti-rabbit IgG for 1 h, washed with PBS, rinsed twice with distilled water, and counterstained with uranyl acetate. For control, the immunostaining was tested by absorbing the anti-bPAG antiserum with purified bPAG.

Rabbit preimmune sera were not tested for either light or electron microscopic investigations.

RESULTS

Light Microscopic Investigations

Sections of cow placentomes removed from either mid-gestation or term placenta were strongly immunohistochemically stained when antiserum anti-bPAG raised in rabbits was used. Immunostaining was especially strong in the cytoplasm of a population of large cells located predominantly within the chorionic villi that interdigitated with caruncular tissue (Fig. 1, a and b). These large cells were binucleate (Fig. 1c). Although the other immunostained cells appeared mononucleate (open arrows), they were similar in shape and size to binucleate cells. All large (binucleate) cells present in the fetal villi were specifically stained. Large (binucleate) cells were seen throughout the fetal villi, more often near the microvillus boundary that separates the chorionic villi from the caruncular septum (Fig. 1b, arrows). In the caruncular epithelium, some smaller cells, not obviously binucleate, were discretely stained (Fig. 1b. arrowheads). An isolated binucleate cell can be seen between the caruncular and the trophoblastic epithelia (large solid arrow).

The specificity of the immunolocalization was demonstrated by the abolition of the staining when antibodies to bPAG had been previously absorbed with pure bPAG (Fig. 2a).

Electron Microscopic Investigations

Gold labeling revealed the presence of bPAG on the fetal side (trophoblast) exclusively over binucleate cell granules (Fig. 3a; arrows). The intensity of gold labeling of each granule was fairly uniform in all binucleate cells. Gold labeling was not observed over the nuclei (Fig. 3a) nor mitochondria (Fig. 3b).

Labeled granules of equivalent size to those found in fetal binucleate cells were also found in large binucleate cells in the maternal uterine epithelium. However, in the caruncular epithelium, the granule labeling was more discrete than in the trophectoderm (Fig. 3c, arrows).

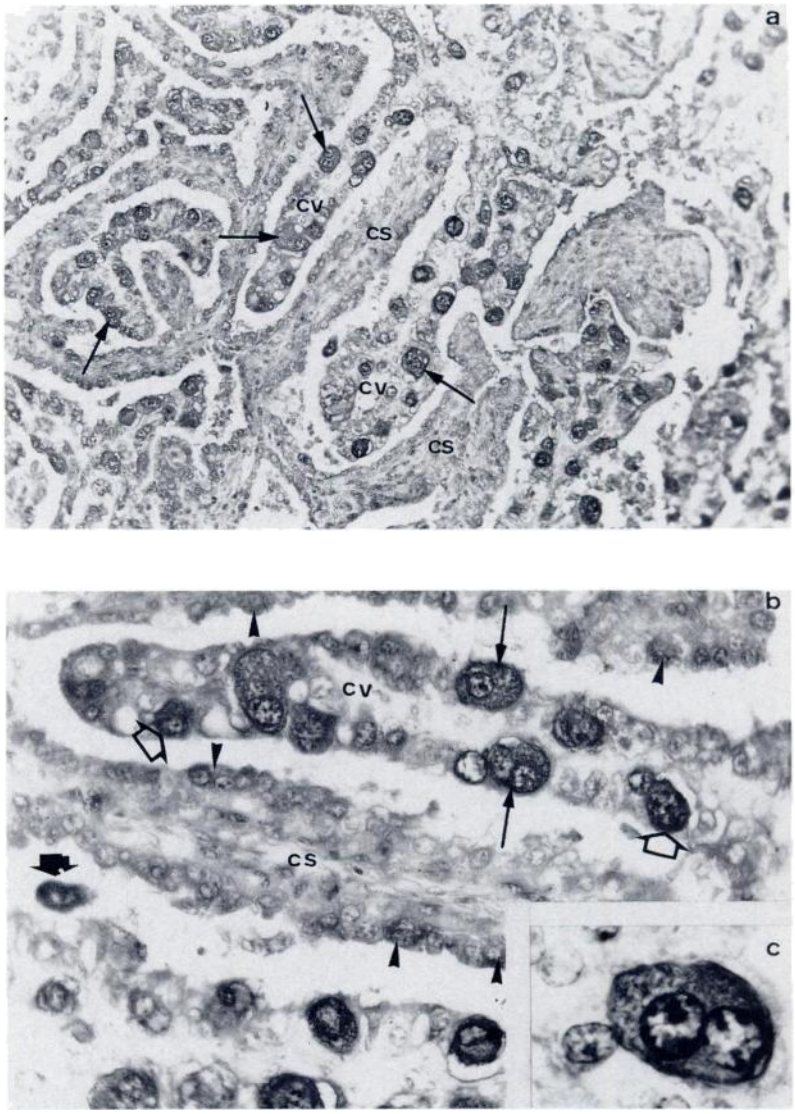

FIG. 1. Light microscopic investigations of cow placentome (midgestation to term).

a) Chorionic villi (cv) interdigitating with the caruncular septum (cs). Note that the bPAG-positive cells (arrows) are distributed quite evenly in the trophoblast of chorionic villi. Indirect immunoperoxidase method, counterstained with hematoxylin. ×387.

b) Bovine PAG-positive binucleate cells (small arrows) with cytoplasmic portion protruding towards the caruncular epithelium. An isolated (probably a migrating) binucleate cell can be seen between the caruncular and trophoblast epithelia (solid large arrow). Shrunken binucleate cells with a scanty cytoplasm are seen in the caruncular epithelium (arrowheads). Open arrows indicate apparently mononucleate large cells. Indirect immunoperoxidase method, counterstained with hematoxylin. ×992.

c) The inset shows a bPAG-positive binucleate cell. Note the strongly stained cytoplasm and the two nuclei of equivalent size. × 2 650.

Absorption of antibodies to bPAG with purified bPAG abolished labeling in all such treated control sections (Fig. 2b).

DISCUSSION

Since Aschheim and Zondeck [15] in human and Cole and Hart [16] in animal species revealed the endocrine role of the placenta, many proteins and hormones produced by this organ have been isolated and purified. Most have the same physiological effects as those present in normal non-pregnant adults [17]. Some of these proteins, such as bPAG, are pregnancy-associated; others, such as SP1 [18] and PSPB [2], are pregnancy-specific.

Bovine PAG has been purified to homogeneity from bovine fetal cotyledons as described elsewhere [1]. Antisera raised in rabbits against highly purified bPAG were specific in that cross-reactions were not observed with other placental proteins or hormones (SP1, bovine placental lacto-

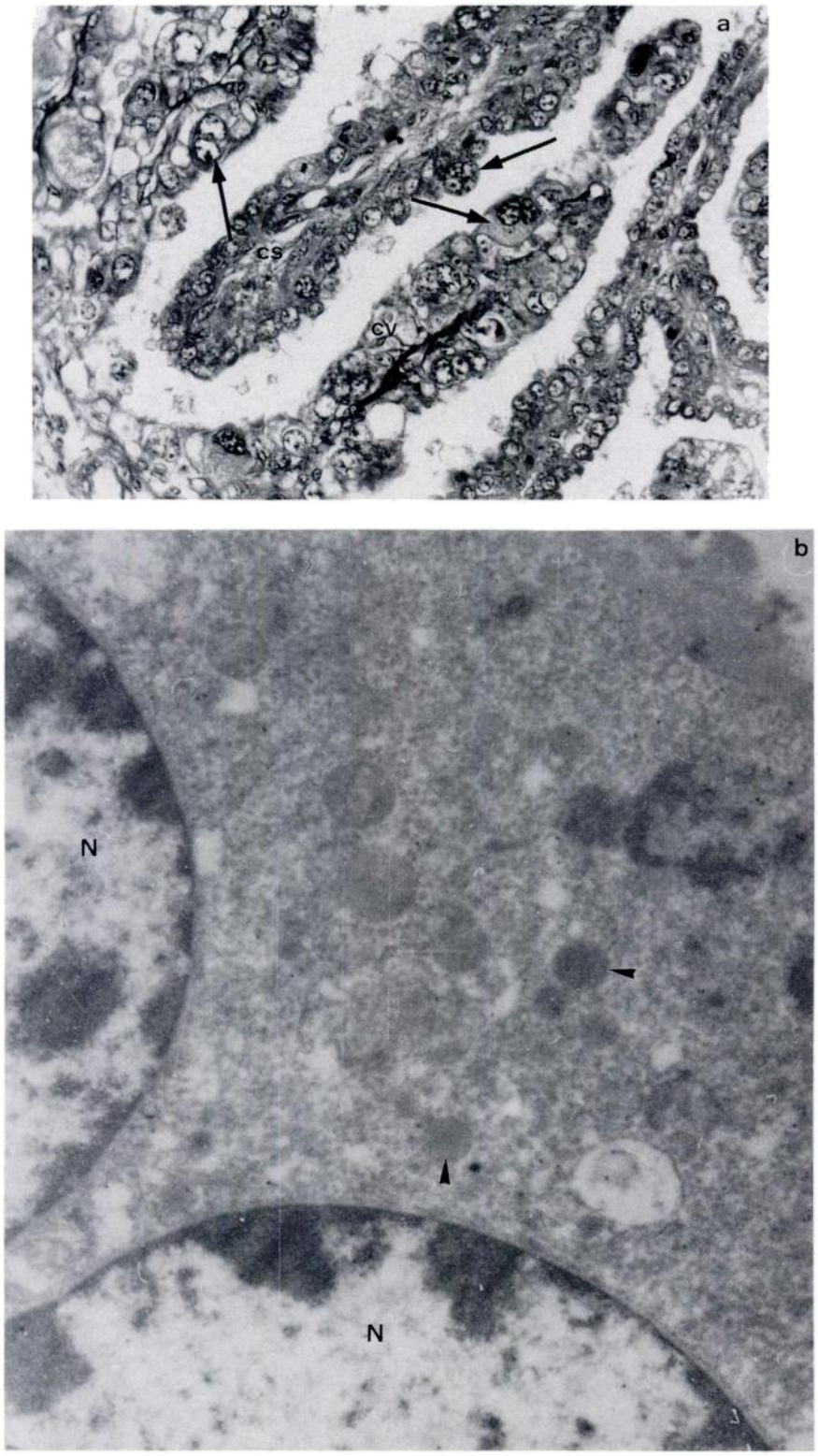

FIG. 2. Negative controls: tissue sections were treated with preabsorbed anti-bPAG antiserum. a) Absence of immunostaining in binucleate cells (arrows). ×992. b) Lack of labeling of granules (arrowheads). Preabsorption of antibody with purified bPAG resulted in an abolition of immunostaining in binucleate cells and labeling of granules. ×26 280.

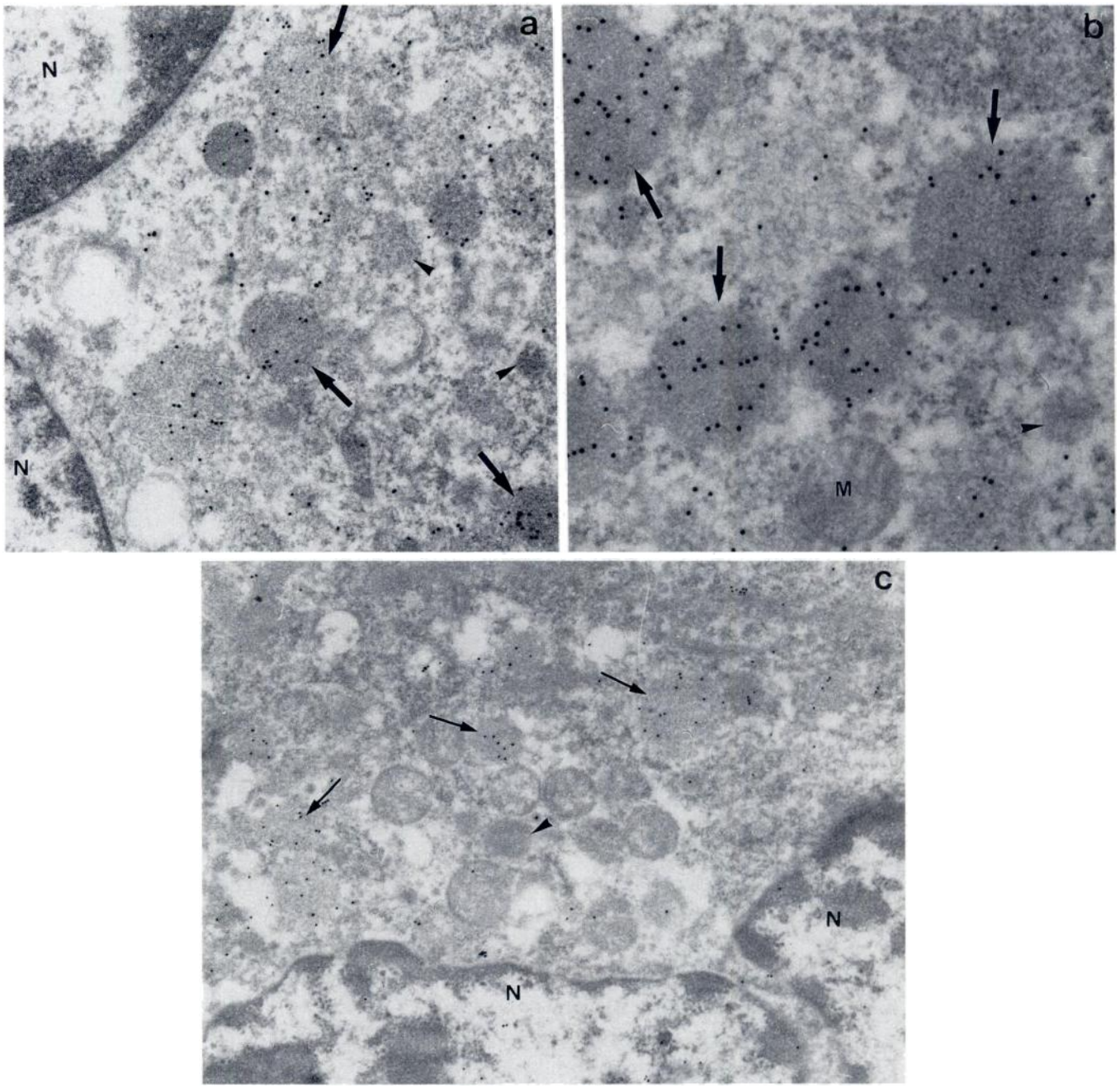

FIG. 3. Electron microscopic investigations of cow placentome (midgestation to term).

a) Binucleate cell located in the trophoblast epithelium. Note the two nuclei (N) and the gold particle labeling of the granules (arrows) and some unlabeled granules (arrowheads). × 36 450.

b) Trophoblastic binucleate cell cytoplasm. Note the heavy and uniform gold particle labeling of the granules (arrows) and the lack of label over the mitochondria (M) and some granules (arrowhead). × 54 600.

c) Binucleate cell located in the caruncular epithelium. Note the two nuclei (N) and the presence of some weakly labeled (arrows) and unlabeled (arrowheads) granules. ×19 200.

gens [bPL], eCG) or with gonadotropic hormones (bovine LH and porcine FSH) and serum proteins (AFP and BSA) [11].

The most characteristic element of the ruminant placenta is the presence of binucleate cells in the trophectoderm. They represent approximately 20% of the total number of trophectodermal cells and are present in constant proportions from implantation to parturition [19]. Because they are stained with periodic acid-Schiff and phosphotungstic acid, they are supposed to be rich in glycoproteins [20], as are bPL, PSPB, etc. In addition, steroids such as progesterone are also synthesized in binucleate cells [21]. In

the present report, the specific immunostaining of large-cell cytoplasm indicated that the bPAG is produced by, or at least stored, in these cells. The production site of bPAG could be confirmed by in vitro study. The majority of binucleate cells were found within the trophoblast epithelium and in close proximity to the feto-maternal boundary. Some stained giant cells exhibited cytoplasmic protrusions towards the caruncular epithelium (Fig. 1b, large arrowheads). They might be the migrating binucleate cells described by other workers [22–24]. Smaller weakly stained cells present in the caruncular epithelium were probably binucleate cells that had migrated across and become shrunken with a scanty cytoplasm [25] containing less bPAG-positive material. These findings are well correlated with the hypothesis that binucleate cells migrate into the caruncular epithelium to deliver their secretory product into the maternal circulation.

The success of gold labeling of binucleate cell granules with a very low background demonstrated the validity of the antisera used. These findings suggest that bPAG, probably produced by trophoblast binucleate cells, is stored in granules formed from the Golgi cisternae [19]. The granules are thought to be delivered in the maternal circulation after cell migration.

The presence of labeled granules of identical size in the cytoplasm of giant (binucleate) cells in the caruncular epithelium (Fig. 3c) and the bPAG concentrations, higher in maternal than in fetal circulation [11], was the consequence of binucleate cell migration, which ended in the fusion of each binucleate cell with a uterine epithelial cell to form a (trinucleate) maternal giant cell that is well known to be transient [19]. As observed with the light microscope, the granules on the maternal side were weakly labeled. This might have been the consequence of the delivery of the binucleate cell granule content to the maternal circulation. The process continued from implantation to term, corresponding to the period when bPAG is detected in the maternal circulation [11]. As with bPL and ovine PL (oPL) [26, 27], it can be concluded that bPAG is closely related to the life history of binucleate cells. The only difference from these placental hormones is that bPAG is essentially secreted in the maternal circulation where the concentration, especially at the end of gestation, is almost 100-fold higher than in fetal blood [11]. Throughout gestation in cows [28] and in sheep [29], placental lactogen concentrations in fetal blood are consistently higher than in maternal blood. As suggested by Wooding [26] and Verstegen et al. [30] for bPL, the presence of relatively small amounts of bPAG in fetal blood may be due to the fact that binucleate cells release their content before, or as an alternative to, migration. However, neither for bPL [27] nor for bPAG could evidence be found for binucleate cell degranulation or exocytosis on the fetal side. Since no immunoreactivity was observed in the extracellular space around binucleate cells, the process of bPAG delivery remains obscure.

All large (binucleate) cells, both fetal and maternal, were stained; however, it was not obvious in the light microscopic investigations that all stained cells were binucleate. Although some immunostained cells appeared mononucleate, they were similar in shape and size to binucleate cells. Thus it was more probable that the cells that seemed to have a single nucleus were in fact binucleate cells that appeared mononucleate due either to the section or to the microscopic focusing. This was confirmed by the examination of serial sections and by slight modification of microscopic focusing. The electron microscopic investigations showed that all cells containing labeled granules were binucleate. This might suggest that, in ruminant placenta, bPAG is exclusively synthesized by, or at least stored in, binucleate cells, which are also responsible for the synthesis of many other placental proteins and hormones. Consequently, it is probable that this intensive activity of binucleate cells accounts for their transient life.

The mechanism of the increase in serum concentration of bPAG, and especially of the dramatic rise of this concentration within the last 10 days of the gestation remains unclear. This dramatic increase of serum bPAG concentration may be related to its biological function(s). Xie et al. [31] demonstrated that bPAG and its ovine homolog belong to the aspartic proteinase family but do not appear to be enzymatically active as proteinases, although the substrate-binding clefts on the molecules are probably conserved. Clearly such molecules may still be capable of binding polypeptide ligands. So bPAG could well be important in the remodeling of the placental attachment or in the placental release mechanism that allows the villi to be pulled out of the maternal crypts after parturition. This would mean that cows with retained placentas should have lower serum bPAG concentrations at the end of gestation. However, further study is necessary to determine the role(s) that bPAG may have during gestation and especially at parturition and postpartum.

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