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Failure of endometrial cup development in the donkey-in-horse model of equine abortion

A. C. ENDERS[1], SALLY MEADOWS[2], FRANCESCA STEWART[2] AND W. R. ALLEN[2]

[1] *Department of Cell Biology and Human Anatomy, School of Medicine, University of California, Davis, California, USA and* [2] *T.B.A. Equine Fertility Unit, Newmarket, Suffolk, UK*

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ABSTRACT

The mature preinvasive chorionic girdles of horse, mule, donkey and extraspecies donkey-in-horse conceptuses, and the very young endometrial cups on d 37 of gestation in mares carrying horse, mule and transferred donkey-in-horse conceptuses, were compared histologically and ultrastructurally to determine possible mechanisms underlying failure of endometrial cup development in the donkey-in-horse model of equine abortion. The progenitor chorionic girdle from the failing donkey-in-horse pregnancy was similar in size to the normal donkey chorionic girdle but the trophoblast cells within the former were smaller, less organised and showed definite signs of degeneration and pyknosis. In the 37 d endometrial cups both in the horse and mule pregnancies, the recently invaded, differentiated and enlarging endometrial cup cells had penetrated deeply into the endometrial stroma and were becoming tightly packed between the persisting endometrial glands. In the donkey-in-horse pregnancy, on the other hand, relatively few donkey chorionic girdle cells had begun the invasion process and the majority of these, having penetrated and dislodged the horse luminal epithelium, did not penetrate the basement membrane beneath. Very few cells had reached the endometrial stroma and these had already attracted considerable numbers of lymphocytes to the area. It is concluded that unknown factors in the horse uterus affect adversely all phases of the development, attachment and invasion of donkey chorionic girdle cells, thereby leading to very little or no endometrial cup development and equine chorionic gonadotrophin secretion in the extraspecific donkey-in-horse pregnancy created by embryo transfer.

Key words: Equids; pregnancy; chorionic girdles.

INTRODUCTION

The equine endometrial cup reaction comprises the development of a series of small, saucer-shaped endometrial protuberances in a circle around the conceptus at the base of the gravid horn between days (d) 40 and 120 of the ±340 d gestation period in the mare and other equine species (Cole & Goss, 1943). Each cup consists of a densely packed mass of large binucleate epithelioid-type cells interspersed between dilated endometrial glands and occasional blood vessels. These large endometrial cup cells secrete the gonadotrophic hormone, equine chorionic gonadotrophin (eCG; Clegg et al. 1954) which expresses both FSH-like and LH-like biological activities (Cole, 1936; Stewart et al. 1977) and reaches the maternal circulation via a network of large lymph sinuses that develops beneath each cup (Amoroso, 1952; Allen, 1982*a*). The secretory function and lifespan of the endometrial cups is cut short by a vigorous cytotoxic cell-mediated maternal immune response against foreign antigens expressed by the cup cells. This hastens their death and the dehiscence of the necrotic cups off the surface of the endometrium around d 100–120 (Amoroso, 1955; Allen, 1975).

The large eCG-secreting endometrial cup cells are actually fetal trophoblast cells which originate from a discrete annulate band of hyperplastic cells, the

Correspondence to Dr Allen C. Enders, Department of Cell Biology and Human Anatomy, University of California, Davis, CA 95616-8643, USA.

chorionic girdle. This develops between 25 and 34 d after ovulation around the circumference of the spherical conceptus in the region of abutment of the regressing yolk sac and enlarging allantoic membranes (Allen & Moor, 1972). Between d 35 and 38, the chorionic girdle adheres to the overlying endometrial epithelium and the cells at the surface of the girdle begin to invade the maternal tissue vigorously by dislodging and phagocytosing the epithelial cells on the lumenal surface and in the apical portions of the endometrial glands, before passing through the basement membranes to enter the stroma (Allen et al. 1973; Enders & Liu, 1991*a*, *b*). Here they complete their differentiation into endometrial cup cells by becoming sessile, rounding up and enlarging greatly so that they become tightly packed together (Hamilton et al. 1973).

From a series of interspecies matings and embryo transfer experiments carried out over many years it has been possible to demonstrate that fetal genotype and uterine environment exert marked interacting influences on endometrial cup development and eCG secretion rates by governing the width, general development and invasiveness of the progenitor chorionic girdle (Allen, 1969, 1975, 1982*a*; Allen et al. 1987, 1993). For example, mares (*Equus caballus*, 2n = 64) and Jenny donkeys (*E. asinus*, 2n = 62) can both conceive and carry to term viable interspecific hybrids (mule and hinny respectively, both 2n = 63) and Jenny donkeys will successfully carry horse embryos transferred to them (Allen, 1975, 1982*a*). However, only occasionally will mares carry transferred donkey embryos to term and surgical removal of such extraspecific donkey-in-horse conceptuses between 53 and 87 d after ovulation has revealed a complete absence of endometrial cups in the uteri of the surrogate horse mothers concurrent with an absence of eCG in their blood (Allen et al. 1987). More recently, genetically identical mule demi-embryos, made by bisecting an interspecies mule morula on the 6th day after ovulation (Skidmore et al. 1989) were transferred to separate surrogate mothers, one a horse and the other a donkey. This resulted in the development of broad, and well developed endometrial cups which secreted high concentrations of eCG into the blood of the Jenny donkey recipient, compared with low serum eCG concentrations and small, narrow prematurely necrotic cups in the horse recipient. This demonstrated convincingly that uterine factors can exert an overriding influence on chorionic girdle and endometrial cup development (Allen et al. 1993).

The transferred donkey-in-horse pregnancy constitutes a model of gestation failure which is not only predictable but also includes failure of endometrial cup formation and the resulting lack of eCG secretion (Allen et al. 1987). Many questions arise from this placental malfunction early in pregnancy. Is it caused by (1) a lack of development of the donkey chorionic girdle in the horse uterus; (2) a lack of differentiation of cells within the chorionic girdle; (3) an inability of donkey chorionic girdle cells to invade the horse endometrium, or (4) a lack of differentiation of girdle cells into endometrial cup cells once they have entered the endometrium? To examine these questions, chorionic girdle formation and invasion were examined histologically and ultrastructurally in 2 donkey-in-horse pregnancies and the results compared with these events in normal intraspecies horse and donkey pregnancies and in interspecies mule pregnancies.

MATERIALS AND METHODS

Equine pregnancies

Two intraspecies horse, 2 interspecies mule and 1 intraspecies donkey pregnancies were established by inseminating mares or a Jenny donkey on alternate days during oestrus with fresh, extended semen from a pony stallion or a Jack donkey. Two donkey-in-horse pregnancies were created by surgically transferring donkey blastocysts recovered nonsurgically on d 7 after ovulation to the uteri of 2 unmated synchronised recipient mares, as described previously by Allen (1982*b*). Ovulation in the inseminated animals, and in the donor donkeys and recipient mares, was diagnosed by measuring a rise in progesterone concentrations in peripheral plasma samples recovered daily during and after oestrus (Allen & Sanderson, 1987). Pregnancy was diagnosed initially at d 14–17, and monitored fortnightly thereafter, by ultrasound scanning of the uterus (Simpson et al. 1982).

Horse, donkey, mule and donkey-in-horse conceptuses were recovered on d 33 or 34 of gestation for examination of chorionic girdle development and a horse, mule and donkey-in-horse conceptuses were perfuse-fixed in utero to examine chorionic girdle invasion into the endometrium.

Collection and preparation of tissues

The horse, mule and donkey-in-horse conceptuses were recovered nonsurgically on d 33 or 34 after ovulation from their respective mothers by large volume uterine lavage via a 14 mm diameter endo-

tracheal tube passed through the cervix. The donkey conceptus was recovered on d 33 by ventral midline surgical hysterotomy performed under general anaesthesia. Representative portions of chorion between the allantochorion and the trilaminar choriovitelline membrane, including the chorionic girdle, were dissected free and fixed in glutaraldehyde-formaldehyde fixative. They were then processed for light microscopy and for scanning and transmission electron microscopy as described below.

After killing on d 37, the uteri were removed intact from the 3 horses carrying, respectively, a horse, an interspecies mule and a transferred donkey conceptus. In each case, initial aldehyde fixation was induced by a catheter introduced into the caudal end of the artery supplying the uterine horn containing the conceptus. The conceptuses were then exposed by dissecting a square of tissue out the antimesometrial surface of the gravid uterine horn and the regions containing the embryo within its amnion, the regressing yolk sac and the girdle remnant identified. The endometrial surface was examined carefully for the presence of developing endometrial cups and portions of these, together with pieces of the remnants of the chorionic girdle, were dissected free and prepared for histological and cytological examination. The initial perfuse-fixation with 4% formaldehyde: 0.5% glutaraldehyde in 0.1 M phosphate buffer was followed, either by immersion in this fixative, or by transfer to a 2% formaldehyde: 2% glutaraldehyde mixture.

Most of the tissues were processed immediately, but some were retained in phosphate buffer for 2 wk. After postfixation in osmium tetroxide, selected portions of the tissues were dehydrated and embedded in Araldite epoxy resin. These blocks were sectioned at 1 μm and stained with azure B for light microscope examination. Selected regions were thin-sectioned for examination in a Phillips 400 transmission electron microscope. Tissues from both fixatives were prepared for light microscope examination by embedding them in paraffin wax, whereas others were embedded in LR White resin. Tissues to be examined by scanning electron microscopy (SEM) were dehydrated in acetone, critical-point dried, coated with gold in a sputter-coater, and examined in a Phillips 501 scanning electron microscope.

RESULTS

Preinvasion chorionic girdles

The chorionic girdles of the d 34 donkey-in-horse conceptus and the d 33 horse, mule and donkey conceptuses were compared grossly and histologically (Figs 1, 2). Only the horse and donkey-in-horse specimens were examined ultrastructurally (Figs 3–6, Fig. 8).

The horse girdle at d 33 was broad, deep and compact (Figs 1*a*, 3*a*) and its lumenal surface was flattened in appearance due to the compressive effect of uterine tone; it was covered by a continuous layer of darkly stained hyperplastic epithelial cells showing mitotic figures. In section, most of the girdle showed the typical pattern of stratified columnar cells arranged in clefts and furrows (Figs 2*a*, 4*a*). A few areas towards the yolk sac side of the girdle still displayed the simple ridged structure typical of the earlier stages of girdle formation (Fig. 3*a*). Nevertheless, most of the trophoblast cells in the girdle were already binucleate (Fig. 4*a*) but with some uninucleate cells appearing in both basal and superficial regions. Mitotic figures were seen at all levels through the depth of the girdle. Although the distance between the point of separation of the allantois from the chorion to the start of the girdle varied slightly around the circumference of the conceptus, it averaged only about 0.5 mm. Fine blood vessels diverging from the allantois passed through this area and penetrated into the first 0.5–1.0 mm width of the 4 mm wide girdle.

The mule girdle was appreciably narrower than the horse girdle at the same stage of gestation (cf. Fig. 1*a*, *b*) but was otherwise histologically similar with a flattened apical surface covered by an apparently continuous layer of hyperplastic trophoblast cells (Fig. 2*b*). The donkey girdle was much narrower still and it was generally less compacted (Fig. 1*c*). It was seen clearly to be composed of finger-like projections of trophoblast cells that had not become flattened on the surface like the horse and mule girdles, perhaps due to lower myometrial tone in the uterus of the donkey (Fig. 2*c*). The widths of these girdles clearly correlated with the previously described differences in the amounts of endometrial cup tissue and maternal eCG concentrations in these 3 types of pregnancy (Allen, 1975, 1982*a*; Allen et al. 1993).

The donkey-in-horse girdle was even narrower than its donkey-in-donkey counterpart, being less than 1 mm wide in most places, although there were a few regions where it became slightly broader (Figs 1*d*, 3*b*). Although quite deep, the girdle had a generally disorganised appearance, without the development of an organised layer of cells on the apical surface. Indeed, small clumps of mostly mononucleate girdle cells appeared close to being dislodged from the surface of the girdle while deeper in the tissue cells were condensed with dense nuclei, and abnormal

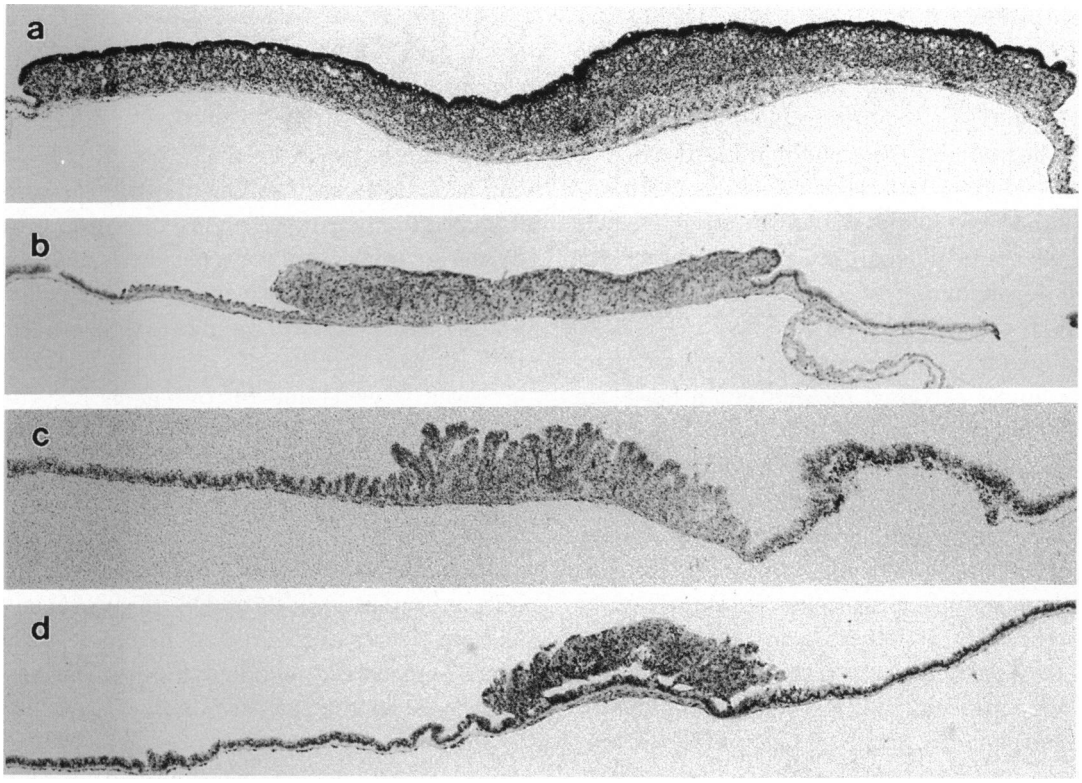

Fig. 1. Low power photomicrographs of sections through the entire chorionic girdle and the adjacent allantochorionic and choriovitelline membranes of a 33 d intraspecies horse (*a*), interspecies mule (*b*) and interspecies donkey (*c*), and a 34 d extraspecies donkey-in-horse conceptuses (*d*). Note the declining widths of the girdles. × 25.

extracellular spaces were present within the girdle (Figs 2*d*, 3*c*). In sections of the donkey-in-horse girdle, a continuous basal layer of uninucleate trophoblast could be identified on which sat clusters of binucleate cells arranged in ridges, and sometimes in patches (Fig. 4*b*); many of the more superficial binucleate cells on these ridges appeared 'leached' (Fig. 4*c*). Overall, the degenerate appearance of some of the trophoblast cells, their poor attachment to one another and the patchy nature of the whole girdle gave the impression that cell death and sloughing were occurring. Nevertheless, mitotic figures were abundant, both basally and in the more superficial regions. Thus, the narrowness of the girdle, and especially the patchy nature of the broader areas, suggested that irregular development and rapid demise of the girdle cells was taking place, rather than a lack of cell division yielding insufficient cells.

Transmission electron microscopy revealed considerable diversity in the trophoblast cells making up the donkey-in-horse girdle. Some contained the apical vesicles typical of noninvasive trophoblast cells of the allantochorion, others resembled the basal noninvasive cells of the chorionic girdle, and others were binucleate with typical surface microvilli similar to the invasive girdle cells of the horse and mule (Fig. 5). However, there were also many cells with highly dilated granular endoplasmic reticulum and some apparently leached cells that were only loosely associated with adjacent normal cells (Fig. 6). The most common form of these degenerating cells had deeply indented nuclei, dilated endoplasmic reticulum, small rod-like mitochondria and lacked microvilli. Only occasionally were there additional features of apoptosis such as blebbing of cytoplasm, and these cells remained closely associated with one another. More rarely individual cells with expanded nuclei and mitochondria were found. These cells may indicate either secondary necrosis or primary necrosis of cells isolated from their normal relationships by surrounding apoptic cells. A few cells with the cells showed the ectoplasmic processes associated with migration into the endometrium (Enders & Liu, 1991*b*) but these tended to be smaller than those seen in the normal horse and donkey girdles.

Endometrial cups

The 3 d 37 conceptus chambers within the 3 perfuse-fixed horse uteri were similar in size, as were the

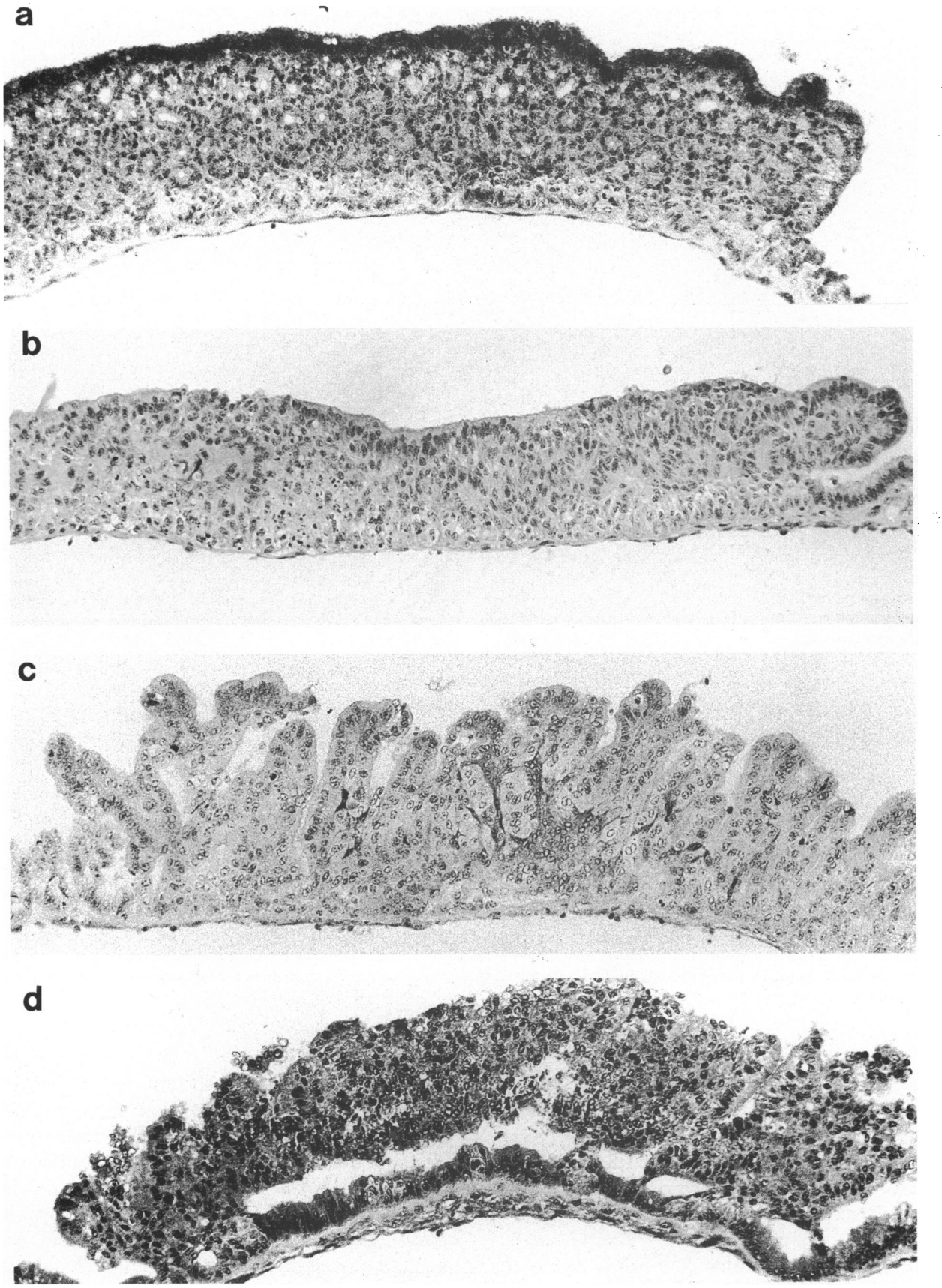

Fig. 2. Higher power photomicrographs of the same sections in Figure 1. Note the compact and flattened appearance of (*a*) the horse and (*b*) the mule girdles compared with the still fingerlike processes of cells in (*c*) the donkey girdle. The extraspecies donkey-in-horse girdle (*d*) is beginning to separate from the underlying layer of trophoblast and it shows clear signs of cell necrosis and general degeneration. × 100.

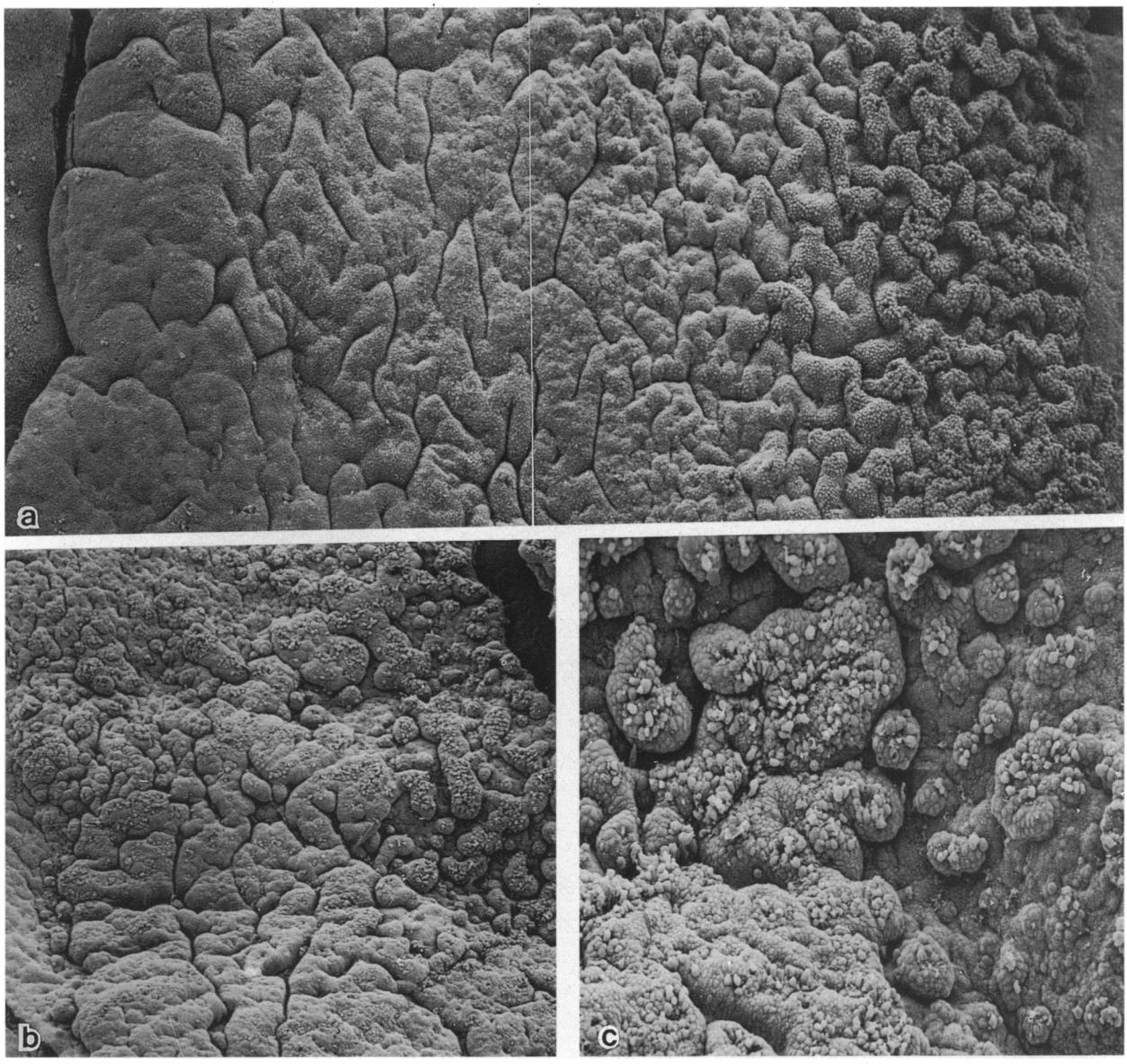

Fig. 3. Scanning electron micrographs of the apical surface of the 33 d horse chorionic girdle (*a*) and the 34 d donkey-in-horse chorionic girdle (*b*, *c*). (*a*) On the left (allantoic side) of the photograph the girdle has assumed its mature form as a solid mass of cells with clefts and pits, whereas on the right (yolk sac) side the girdle is more irregular in shape and the girdle cells occur in patches. These are composed of clusters of cells, the more superficial of which are only loosely attached to the other cells. (*b*) The donkey-in-horse girdle is narrower and more irregular in shape. (*c*) The girdle cells appear in patches, each of which can be seen to be composed of a cluster of cells. × 80 (*a*, *b*) and × 160 (*c*).

crown-rump lengths of the 3 fetuses (19–20 mm). However, while the uteri containing the horse and mule conceptuses showed development of abundant endometrial cups, examination of the endometrial surface of the uterus containing the 37 d donkey-in-horse conceptus revealed only 2 small areas of commencing cup development.

Histological examination of the early endometrial cup tissue from the uterus containing the horse conceptus showed active invasion of the endometrium by binucleate chorionic girdle cells and appreciable hypertrophy of these cells to form typical cup cells (Fig. 7*a*). In general, the cups were broad and deep and, judging from the depth of cell invasion, it was estimated that the girdle cells had begun to invade the endometrium about 2 d previously. In many of the sections, enlarged binucleate endometrial cup cells could be seen in the lumina of lymph vessels. One small cup was developing in the trough between adjacent endometrial folds, rather than at the apex of a fold. The invasion process in this cup was less extensive than in the others, the individual cup cells

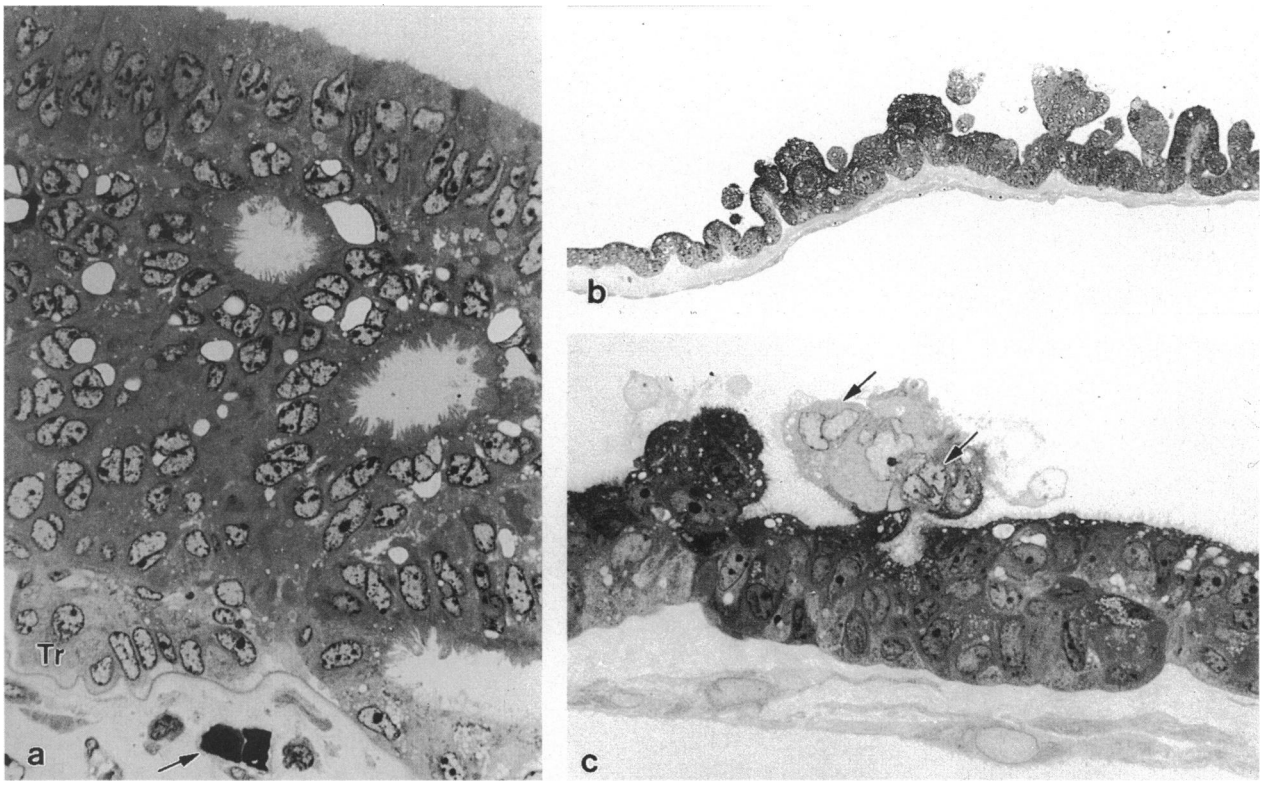

Fig. 4. Photomicrographs of the 33 d (*a*) and the 34 d donkey-in-horse chorionic girdles (*b*, *c*). (*a*) In the allantoic margin of the horse girdle, the majority of cells are seen to be binucleate. Note also the capillary and a few mononucleate basal trophoblast cells (Tr) underlying this portion of the girdle. (*b*, *c*) Donkey-in-horse girdle in a region similar to that seen in Figure 3*c*. Note the irregular clusters of the superficial girdle cells. There are a few binucleate cells (arrows) in the projection and several leached cells are associated with the surface of the trophoblast. ×600 (*a*, *c*) and ×100 (*b*).

were smaller and more lymphocytes appeared to have accumulated around the cup.

In the uterus containing the mule conceptus, the cups were somewhat narrower and invasion had not occurred to the same depth as in the cups in the uterus containing the horse fetus. Nonetheless, the invading chorionic girdle cells were hypertrophied and they occupied most of the available stroma between endometrial glands (Fig. 7*b*). Overall, the cups were similar to those of the intraspecies horse conceptus except that the cup cells at the periphery of each cup were less tightly clumped together. Focal accumulations of lymphocytes were present at the periphery of the cup in some sections as had been observed in the intraspecies horse cup. Cup cells were found within one lymph vessel at the margin of a mule cup.

In the smaller of the 2 areas of girdle attachment in the donkey-in-horse pregnancy, almost all the chorionic girdle cells were still on the surface of the endometrium (Fig. 8*a*, *b*), that is, they had penetrated to the basal lamina of the lumenal epithelium but few, if any, had entered the underlying stroma (Fig. 8*a*). In the larger area, on the other hand, some binucleate girdle cells had managed to invade into the endometrial stroma (Figs 7*c*, 8*c*). However, this invasion was much more superficial than in the horse and mule cups, many fewer cells were present in the endometrial stroma and those that were formed a less compact mass. In addition, abundant lymphocytes were clustered around these few invaded donkey fetal cup cells which had begun to hypertrophy but were not as large as their counterparts in the horse and mule cups. Nevertheless, the differentiated cup cells exhibited the same large nucleoli and development of extensive endoplasmic reticulum seen in horse and mule cup cells (Fig. 9).

Residual chorionic girdle

The region of chorion between the underlying attachments of the allantois and yolk sac which persisted after separation of the chorionic girdle was examined in the 3 d 37 conceptuses. In the horse, small blood vessels extended beneath the trophoblast of the allantochorion as far as a distinct ridge in the chorion (Fig. 10*a*). This ridge was composed of folds of trophoblast sitting on a thick basement membrane, beneath which was a thickened fibroblast-rich stroma covered by a thin mesothelium on the exocoelomic

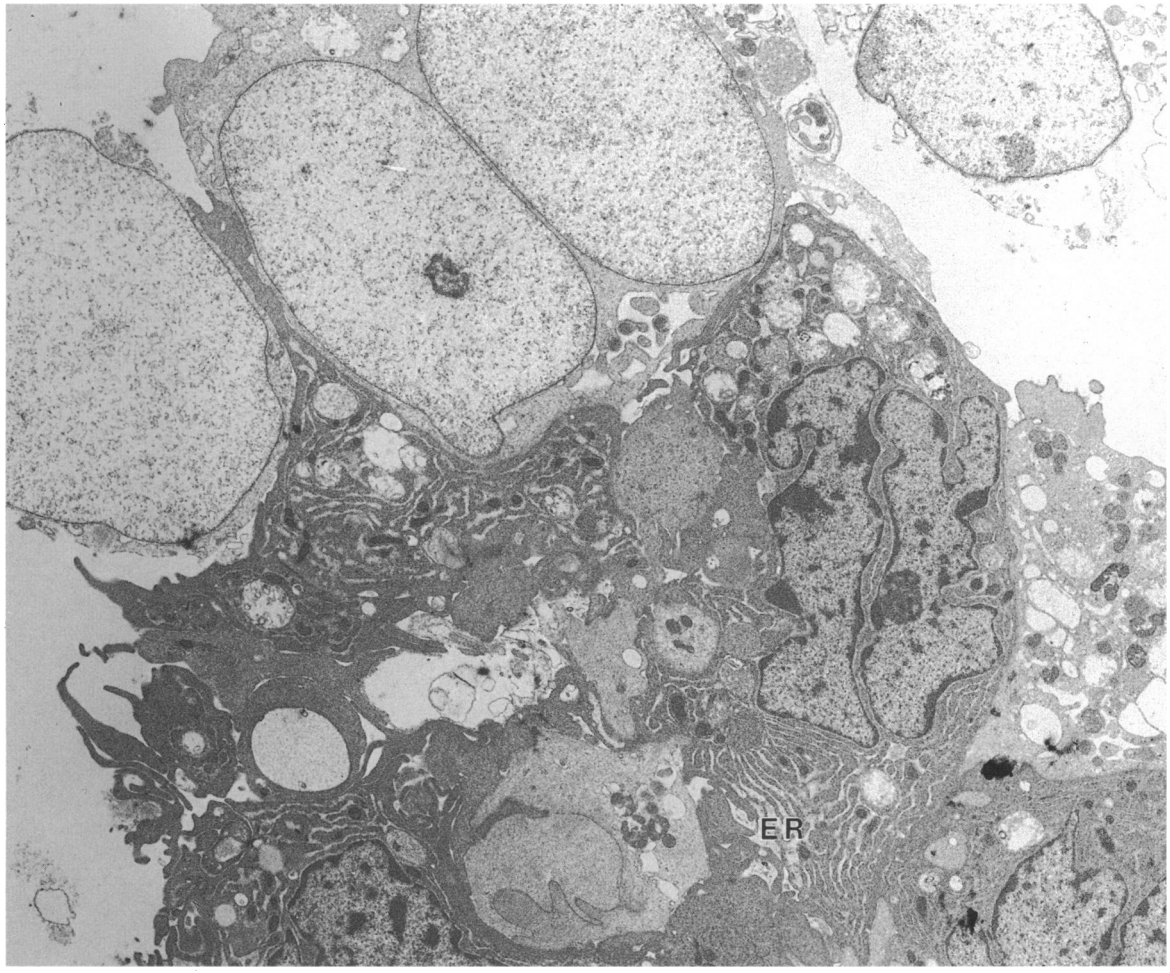

Fig. 5. Electron micrograph of the 34 d donkey-in-horse girdle showing a mixture of binucleate cells with extensive endoplasmic reticulum (ER), pale binucleate cells and degenerating cells. × 7000.

side. Beyond this came a smooth area of chorion covered by low columnar or cuboidal trophoblast cells which was clearly the portion of membranes that had underlain the chorionic girdle before its invasion of the endometrium. This, in turn, was followed by a series of smaller circumferential ridges, each composed of a layer of tall trophoblast cells on a thick basement membrane which marked the transition back to the vascularised trilaminar yolk sac placenta. The heights and number of these minor yolk sac associated folds varied around the circumference of the chorionic sac. The surface of the major fold of chorion at the point of separation of the allantois from the trophoblast was composed principally of columnar trophoblast cells, but occasional patches of binucleate cells were also present on the apical surface (Fig. 10*a*). These were presumed to be remnants of the chorionic girdle that had not managed to invade the endometrium.

The mule conceptus also exhibited a rather broad elevated ridge of chorion on the allantoic side of the smooth area of flattened trophoblast cells from which the chorionic girdle had dehisced. However, fewer patches of binucleate cell chorionic girdle remnants persisted on this ridge compared with the horse. In the donkey-in-horse conceptus, on the other hand, the persisting ridge of chorion on the allantoic side of the site of the chorionic girdle was much narrower. Furthermore, it was situated further away from the point of separation of the allantois than in the horse and mule conceptuses, which meant that the fine blood vessels from the allantochorion did not reach as far as the ridge. With the exception of 2 clusters of binucleate cells showing clear evidence of degeneration, very few binucleate chorionic girdle cells persisted on the ridge (Fig. 10*b*). As in the horse and mule, smaller trophoblast ridges encircling the chorionic sac were present near the yolk sac end of the girdle remnant. These were covered by mononucleated columnar trophoblast cells containing lipid droplets

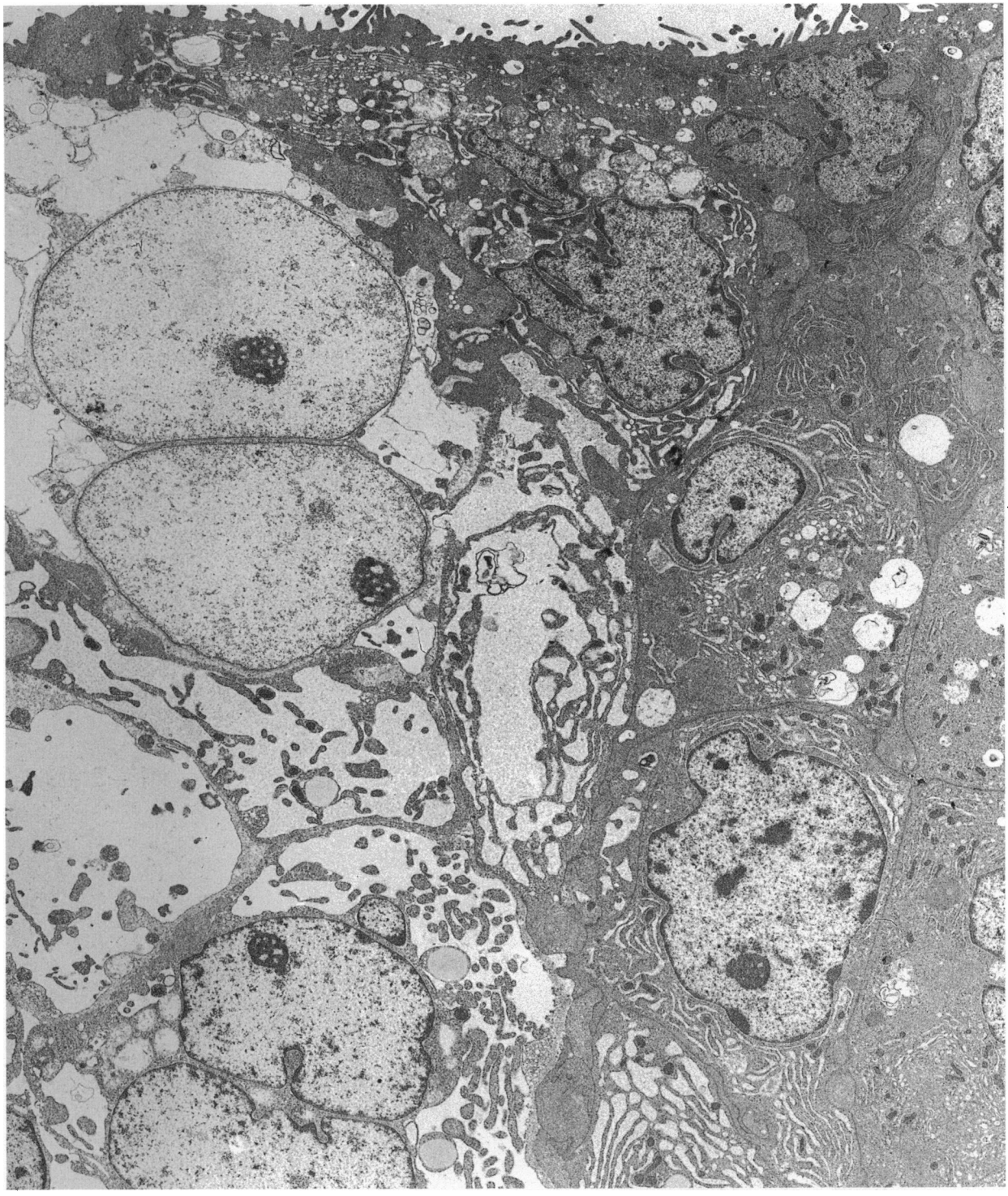

Fig. 6. Electron micrograph of the 34 d donkey-in-horse girdle showing the juxtaposition of normal girdle cells (right) and girdle cells with abnormally dilated endoplasmic reticulum (left). × 6400.

and apical vesicles, which differed substantially in appearance from the binucleate girdle cells. Clearly then, the very small amount of developing cup tissue encountered in the endometrium of the mare carrying the donkey was not due simply to the failure of the binucleate trophoblast cells to leave the chorionic girdle when it became attached to the endometrium, since fewer, rather than more, girdle cells remained behind on the chorion ridges compared with the horse and mule conceptuses.

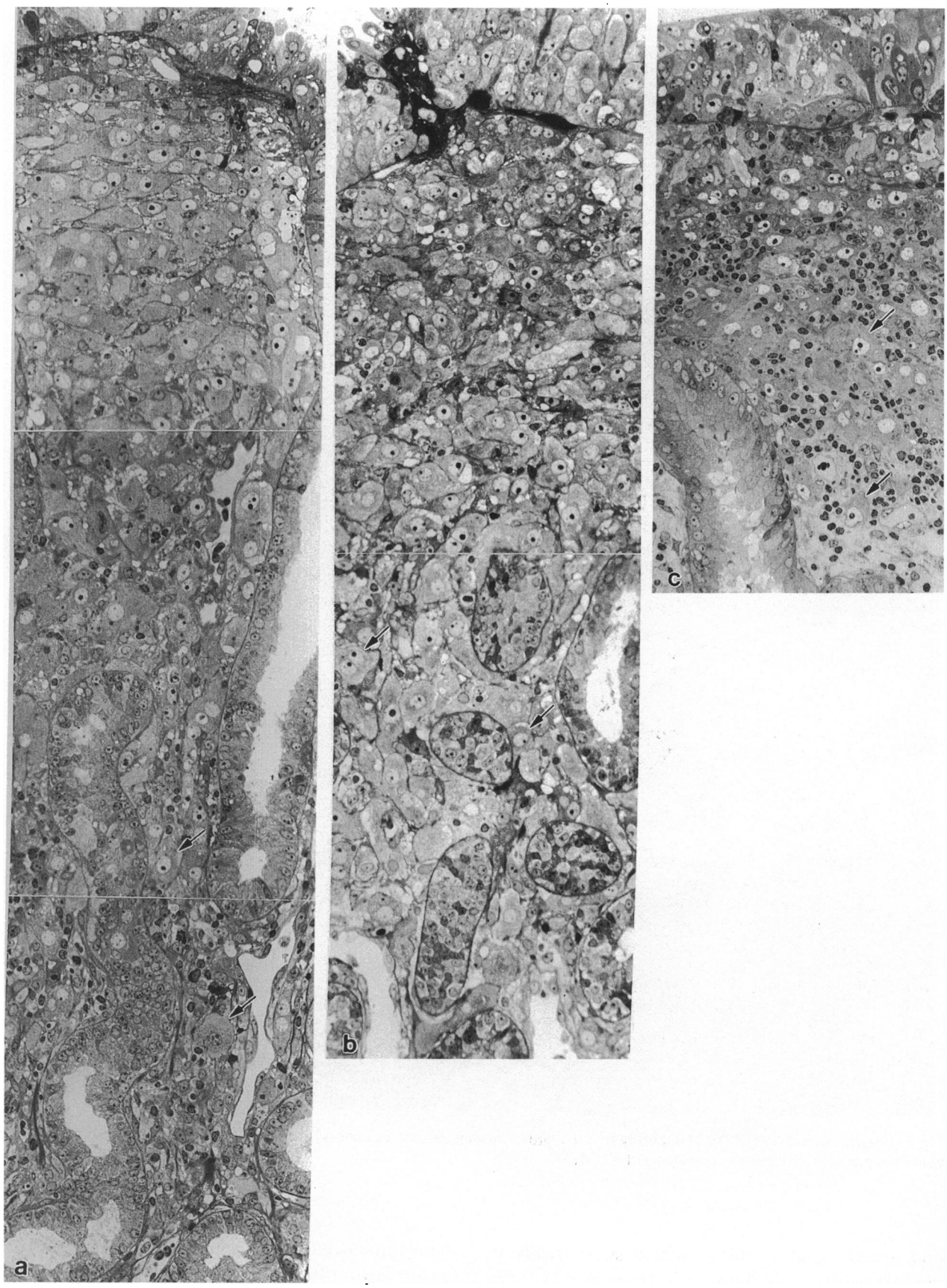

Fig. 7. Developing endometrial cups from the intraspecies horse (*a*), the interspecies mule (*b*), and the extraspecies donkey-in-horse (*c*) pregnancies on d 37 after ovulation. Note that the enlarging cup cells (arrows) with their prominent nucleoli are penetrating deeply into the endometrium in the horse cup but less deeply in the mule cup; this reduced depth of invasion reflects the smaller number of invading cells

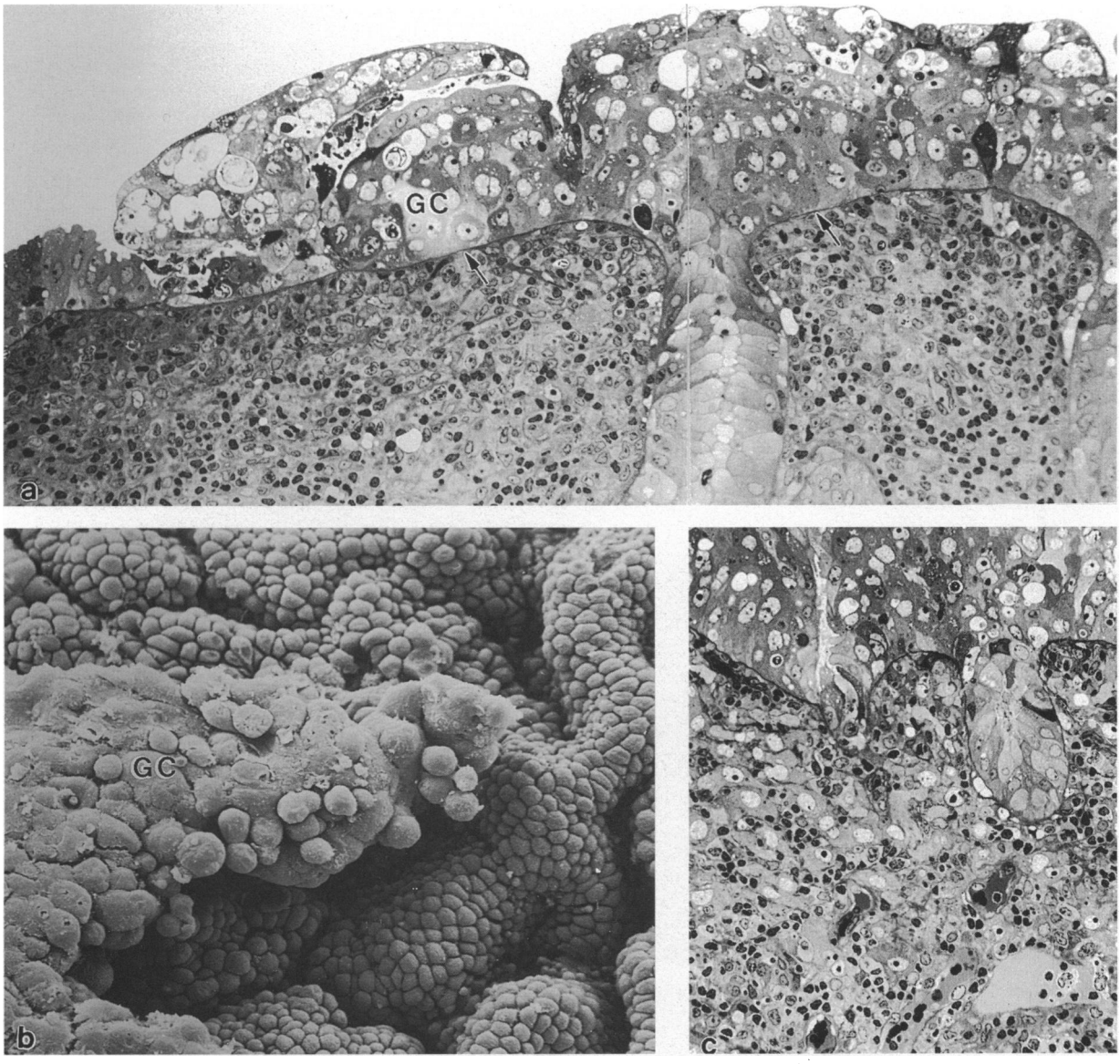

Fig. 8. The extraspecies donkey-in-horse endometrial cup on d 37 after ovulation. (*a*) Light micrograph and (*b*) scanning electron micrograph of the donkey chorionic girdle sitting on top of the horse endometrium. In both micrographs the large girdle cells (GC) are superficial and are either (*a*) above the basal lamina (arrows) after the uterine luminal epithelium has been displaced, or (*b*) above the epithelial surface. (*c*) Section through the deepest region of the only area where invading girdle cells had extended into the stroma of the endometrium. The small dark nuclei of numerous lymphocytes are seen accumulated in the stroma beneath the invading donkey girdle cells. × 250 (*a*, *c*) and × 320 (*b*).

DISCUSSION

There was no general retardation of development of the donkey conceptus in the uterus of the mare since both the fetus and its membranes were approximately the same size in all 3 combinations examined. Furthermore, it was apparent that girdle cells from the donkey conceptus were capable of invading the luminal epithelium, passing through the basement membrane and differentiating into cup cells in the endometrial stroma. However, the very small number of differentiated cup cells present in the endometrium of the surrogate mare and the lack of residual binucleate cells in the remaining girdle tissue both suggest too few girdle cells that were sufficiently mature and in the appropriate condition to invade the

from the less well developed mule chorionic girdle. The donkey-in-horse cup (*c*) is also sectioned through its deepest region, but the invading girdle cells extend only a short way into the endometrium and are much smaller. × 250.

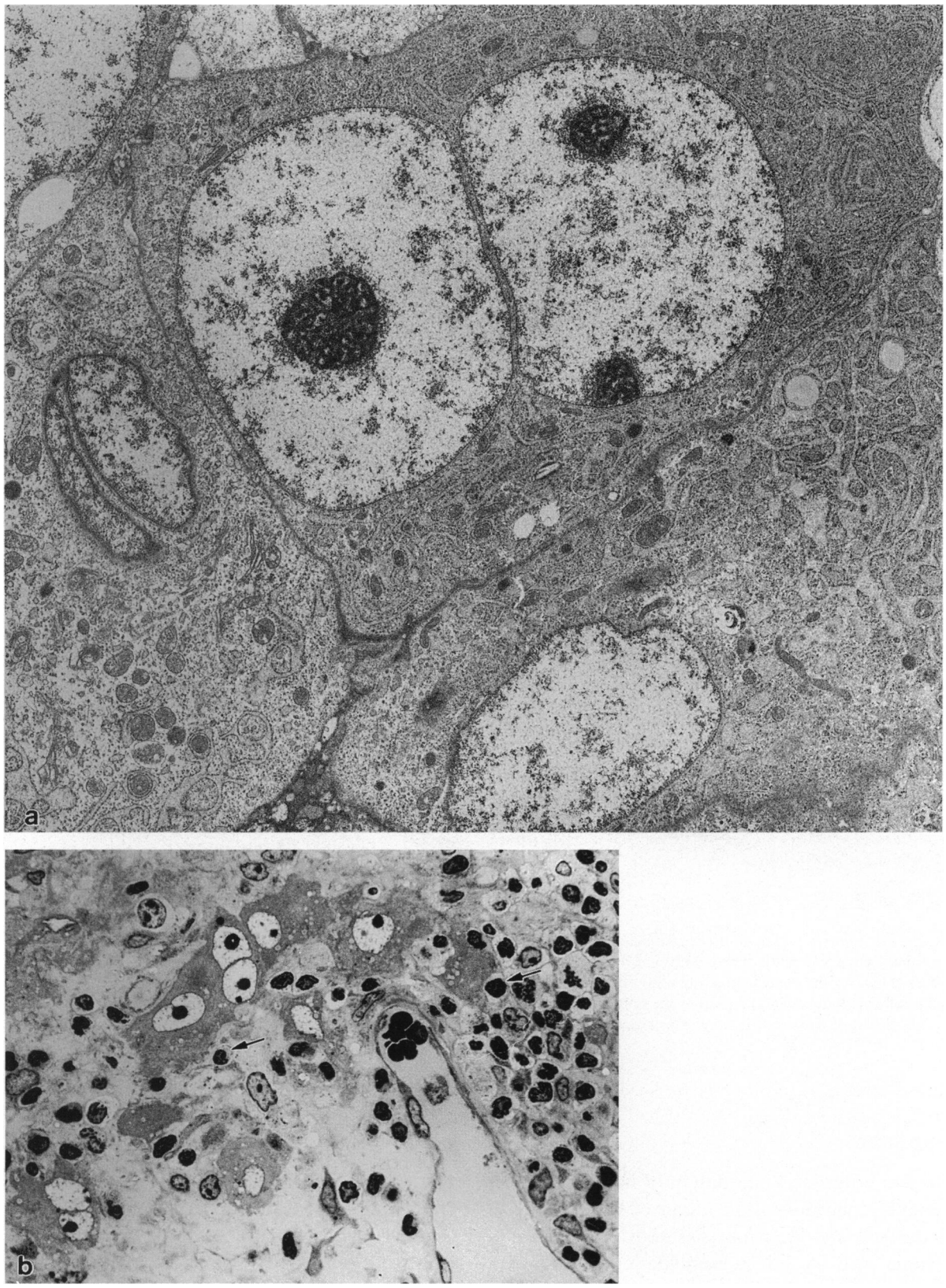

Fig. 9. Newly differentiated endometrial cup cells at the periphery of the cup in the extraspecies donkey-in-horse pregnancy. (*a*) Extensive differentiation of the granular endoplasmic reticulum is apparent in these binucleate cup cells. (*b*) Note the dense cluster of maternal lymphocytes in the area, some of which are already beginning to indent the cytoplasm of the cup cells (arrow). (*a*) × 7200, (*b*) × 650.

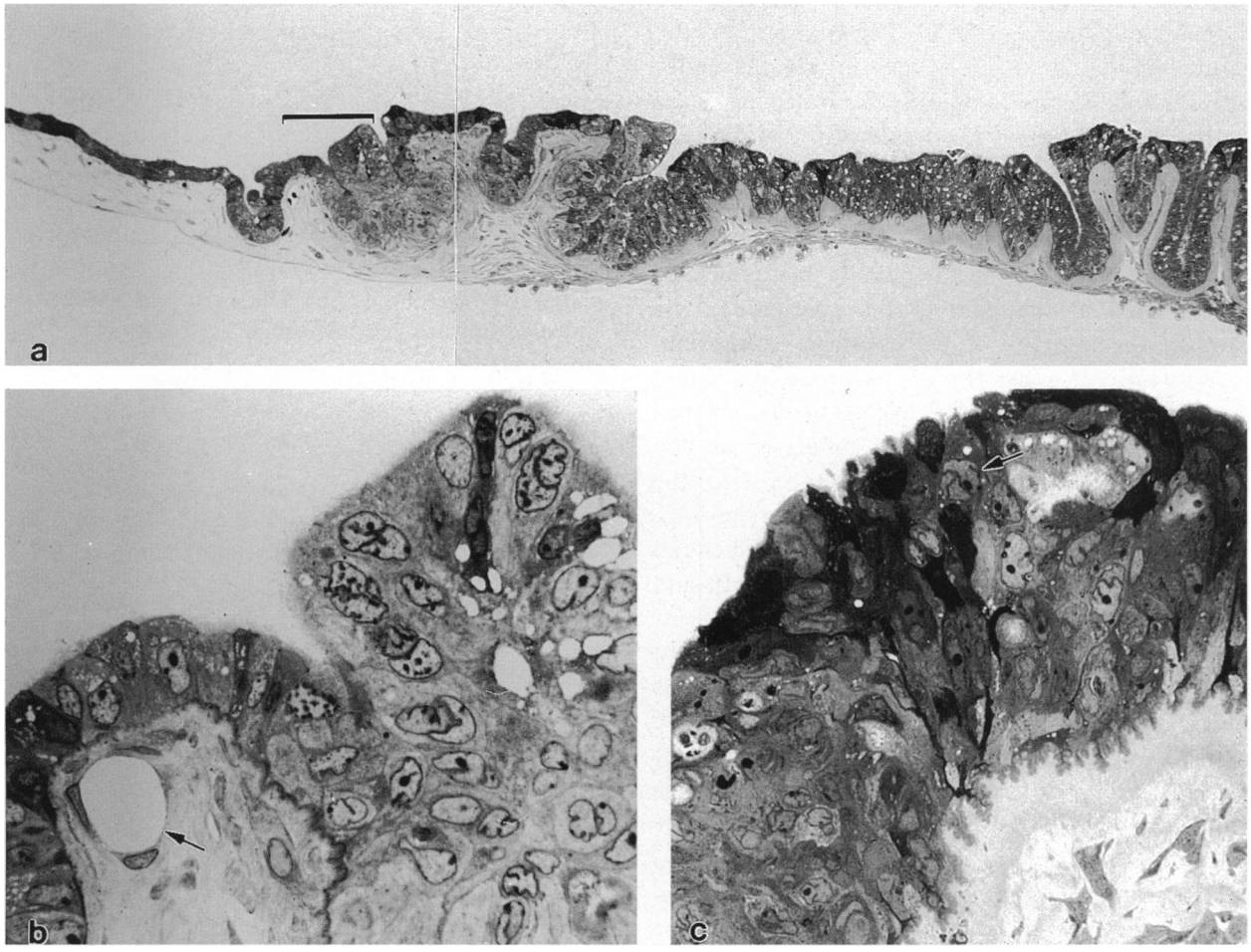

Fig. 10. Region of the chorion remaining beneath the chorionic girdle following the latter's separation to invade the maternal endometrium to form the endometrial cups in the horse (*a*, *b*) and the donkey-in-horse conceptus (*c*). (*a*) The allantoic side is to the left. The folded region in the centre is underlain by a thick pad of connective tissue. (*b*) Area in (*a*) indicated by a bracket. Note the persistence of binucleate girdle cells and the fetal capillary (arrow) towards the allantoic side. (*c*) A few binucleate cells (arrow) can be seen in the disorganised ridge of the donkey-in-horse conceptus. (*a*) × 100 and (*b*, *c*) × 600.

endometrium at the appropriate time. Nevertheless, our finding that at least some donkey chorionic girdle cells can invade the endometrium of the mare in vivo corresponds to the observation of Meadows et al. (1995) in vitro that donkey chorionic girdle cells cocultured with a number of tissues, including homologous and heterologous horse endometrium, can invade the host tissue.

Our other finding in the present experiment, that the donkey-in-horse girdle on d 34 was exceptionally narrow and contained irregular clusters of cells rather than a dense, stratified band of columnar cells, also points to a general deficiency of girdle cells prior to invasion of the endometrium. Why then do so few mature, invasive cells develop in this type of girdle? The surprising observation of an abundance of mitotic figures in the donkey-in-horse girdle perhaps indicates that the low cell number does not result from any lack of stimulation of cell division. On the contrary, the leached and apparently sloughing binucleate cells seen in the d 34 donkey-in-horse girdle suggest that cell death may be an important factor in the phenomenon.

It should be noted that the sequence of events surrounding differentiation of the girdle cells around the time of their invasion of the endometrium is normally precise: large pseudopodia develop on individual binucleate girdle cells (Enders & Liu, 1991*a*) and these indent and force their way between and through endometrial epithelial cells on the day of invasion (Allen et al. 1973). Presumably, the turgor of the enlarging conceptus and the considerable tonicity of the myometrium work together to keep the girdle juxtaposed to the lumenal surface of the epithelial cells on top of the endometrial folds, thereby allowing initial penetration of this layer (Enders & Liu, 1991*b*). Starting on the day of endometrial invasion, and continuing during the next day, spaces appear within the girdle tissue that separate most of the superficial

binucleate cells from the deeper binucleate cells and the basal mononucleate cells. Thus, within 2 d the mature differentiated girdle cells invade the endometrium and become physically separated from the chorion (Allen et al. 1973). Some of the girdle cells remaining on the surface of the endometrium degenerate, as do most of the residual deeper binucleate girdle cells that have not managed to become attached to the endometrium. The remaining mononucleate cells situated at the base of the chorionic girdle maintain the integrity of the chorionic epithelium. Consequently, if either the timing or the sequential progression of the 3 major events in endometrial invasion (formation of invasion processes on the leading chorionic girdle cells, migration into the endometrial epithelium, separation from the chorion), or the timing of cell death in the girdle cells which fail to invade were to be disrupted, random death and sloughing of cells could be expected to occur.

Based on their previous studies of intra, inter and transferred extraspecies equine pregnancies, and the effects of transferring whole and bisected mule embryos to recipients of differing genotype, Allen et al. (1993) proposed a theoretical model of chorionic girdle development and invasion in which paternally derived growth factors and maternally derived trophoblast growth factor receptors were assigned a species-related numerical value and the host uterine environment was assigned an inhibitory value. The sum of these values represented the expected breadth of the chorionic girdle and the size of the resulting endometrial cups. Although the model correctly predicted the development of a very small chorionic girdle on the donkey-in-horse model of pregnancy, the findings in the present study seem to suggest that the inhibitory influence of uterine environment goes beyond merely limiting the size and general development of the chorionic girdle. It disrupts all 3 phases of the invasion process as well.

Finally, our interesting incidental observation that, in the d 33 horse chorionic girdle the general differentiation of the binucleate cells had progressed to the stage of forming a stratified columnar epithelium with pits and crevasses, whereas on the yolk sac side of the girdle the trophoblast cells were still arranged in simple folds or ridges, fits well with the hypothesis of Stewart et al. (1995) that differentiation of the discrete chorionic girdle is induced by one or more growth factors secreted by the vascularised mesoderm of the allantois. Whether this stimulus reaches the girdle region of the chorion by virtue of the latter's juxtaposition to the underlying point of separation of the allantois from the chorion, or via the small blood vessels which penetrate beneath the allantoic side of the chorionic girdle, remains to be determined.

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REFERENCES

ALLEN WR (1969) Factors influencing pregnant mare serum gonadotrophin production. *Nature (Lond)* **223**, 64–66.

ALLEN WR (1975) The influence of fetal genotype upon endometrial cup development and PMSG and progestagen production in equids. *Journal of Reproduction and Fertility*, Suppl. 23, 405–414.

ALLEN WR (1982*a*) Immunological aspects of the equine endometrial cup reaction and the effect of xenogeneic pregnancy in horses and donkeys. *Journal of Reproduction and Fertility*, Suppl. 31, 57–94.

ALLEN WR (1982*b*) Embryo transfer in the horse. In *Mammalian Egg Transfer* (ed. C. E. Adams), pp. 135–154. Florida: CRC Press.

ALLEN WR, MOOR RM (1972) The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. *Journal of Reproduction and Fertility* **29**, 313–316.

ALLEN WR, HAMILTON DW, MOOR RM (1973) The origin of the equine endometrial cups. II. Invasion of the endometrium by trophoblast. *Anatomical Record* **177**, 485–502.

ALLEN WR, KYDD JH, BOYLE MS, ANTCZAK DF (1987) Extraspecific donkey-in-horse pregnancy as a model of early fetal death. *Journal of Reproduction and Fertility*, Suppl. 35, 197–209.

ALLEN WR, SANDERSON MW (1987) The value of a rapid progesterone assay (AELIA) in equine stud veterinary medicine and management. *Proceedings of the 9th Bain-Fallon Memorial Lectures*, pp. 75–82. Sydney: AEVA.

ALLEN WR, SKIDMORE JA, STEWART F, ANTCZAK DF (1993) Effects of fetal genotype and uterine environment on placental development in equids. *Journal of Reproduction and Fertility* **97**, 55–60.

AMOROSO EC (1952) Placentation. In *Marshall's Physiology of Reproduction*, 3rd edn, (ed. A. S. Parkes), vol. 2, pp. 127–311. London: Longmans Green.

AMOROSO EC (1955) Endocrinology of pregnancy. *British Medical Bulletin* **11**, 117–125.

CLEGG MT, BODA JM, COLE HH (1954) The endometrial cups and allantochorionic pouches in the mare with emphasis on the source of equine gonadotropin. *Endocrinology* **54**, 448–463.

COLE HH (1936) On the biological properties of mare gonadotropic-hormone. *American Journal of Anatomy* **59**, 299–332.

COLE HH, GOSS H (1943) The source of equine gonadotropin. In *Essays in Biology in Honor of H. M. Evans*, (ed. T. Coles), pp. 107–119. Berkeley: University of California Press.

ENDERS AC, LIU IKM (1991*a*) Trophoblast-uterine interactions during equine chorionic girdle cell maturation, migration and transformation. *American Journal of Anatomy* **192**, 366–381.

ENDERS AC, LIU IKM (1991*b*) Lodgement of the equine blastocyst in the uterus from fixation through endometrial cup formation. *Journal of Reproduction and Fertility*, Suppl. 44, 427–438.

HAMILTON DW, ALLEN WR, MOOR RM (1973) The origin of the equine endometrial cups. III. Light and electron microscopic

study of fully developed equine endometrial cups. *Anatomical Record* **177**, 503–518.

MEADOWS SJ, ALLEN WR, ANTCZAK DF (1995) Factors affecting the maturation and invasiveness *in vitro* of chorionic girdle cells of different genotypes. *Biology of Reproduction*, Monograph Series I, 171–180.

SIMPSON DJ, GREENWOOD RES, RICKETS SW, ROSSDALE PD, SANDERSON M, ALLEN WR (1982) Use of ultrasound echography for early diagnosis of single and twin pregnancy in the mare. *Journal of Reproduction and Fertility*, Suppl. 32, 431–439.

SKIDMORE JA, BOYLE MS, ALLEN WR (1989) Micromanipulation of equine embryos to produce monozygotic twins. *Equine Veterinary Journal*, Suppl. 8, 126–128.

STEWART F, ALLEN WR, MOOR RM (1977) Influence of foetal genotype on the follicle-stimulating hormone: luteinizing hormone ratio of pregnant mare serum gonadotrophin. *Journal of Endocrinology* **73**, 419–425.

STEWART F, LENNARD SN, ALLEN WR (1995) Mechanisms controlling formation of the equine chorionic girdle. *Biology of Reproduction*, Monograph Series I, 151–159.