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Ultrastructural anomalies in the fetal small intestine indicate that fetal swallowing is important for normal development: an experimental study

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Summary. Fetal swallowing is established early in development and if fetal ingestion is prevented, the gastrointestinal (GI) tract fails to grow normally. In this article we describe the ultrastructural features of GI tissues developing in the absence of swallowing, in the fetal sheep. We have noted a number of defects in enterocyte morphology. These include abnormal or absence of microvilli, inappropriate cell extrusion, glycogen accumulation and altered lysosomal morphology. Many of these changes resemble those seen in malnourished infants. It is possible that fetal ingestion provides a significant source of nutrients, ensuring adequate GI tract growth in utero, in addition to specific growth factors which may be present in ingested fluid.

Key words: Fetus – Small intestine – Ultrastructure – Malnutrition

Introduction

It has been suggested for some time that fetal ingestion in utero might play a part in the normal developmental processes. This suggestion arises from consideration of at least two sets of circumstances. Firstly, the fetus might derive nutritional input from swallowed fluid (in addition to that supplied by the placenta). In support of this proposal is the reported association between reduced body growth and congenital oesophageal atresia (Pierro et al. 1987). Secondly, the development and growth of the gastro-intestinal (GI) tract itself might be dependent on ingested sources of nutrition, and the presence of putative (and well-characterized) growth factors in the ingested fluid.

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There are now a considerable number of studies, including our own, which have demonstrated that the experimental abolition of fetal ingestion (by fetal oesophageal ligation or fistulation) results in a marked reduction in the development and absorptive capacity of the GI tract in fetal rabbits (Wesson et al. 1984; Mulvihill et al. 1985, 1986, 1989; Jacobs et al. 1989), fetal rats (Morikawa et al. 1988), and fetal sheep (Trahair et al. 1986a; Avila and Harding 1991). Experiments in rabbits and rats have been conducted over only relatively short periods (during the last third of gestation), and so may not mimic congenital oesophageal atresia. Since fetal swallowing begins very early in development (in man, as early as 11 weeks; Herbst 1981), it is preferable to start the period of experimental intervention as early as possible, thereby more effectively excluding swallowed factors from the GI tract.

Our previous long-term experiments in fetal sheep have examined fetal growth in the absence of swallowed fluid over the period from 90 to 136 days' gestation (term = 147 days) (Trahair et al. 1986a; Avila et al. 1991). The present study extends the period of study further, with surgical blockade of the oesophagus being performed considerably earlier, at 60 days. While fetal body growth is not affected by these procedures, we have consistently noted that GI development is retarded. More particularly, it is the development of the small intestinal mucosa which is most sensitive to fetal ingestion.

In the present study we report for the first time the ultrastructural features of the intestinal epithelial cells from ovine fetuses aged 136 days' gestation, where the oesophagus was ligated at 60 days.

Materials and methods

At 60–65 days of gestation, date-mated ewes underwent surgery. Using sterile techniques and halothane anaesthesia, the fetus was exposed and the mid-cervical oesophagus ligated. The skin incision was closed, and the fetus returned to the uterus. After recovery

(2–5 days), the ewe was returned to the farm. At approximately 120 days of pregnancy, the ewe was again anaesthetized and additional surgery performed. In four fetuses, the continuity of the oesophagus was re-established by inserting a small length of Silastic tube to replace the ligated section. In one fetus, stainless steel electrodes were implanted in the laryngeal adductor muscles to determine whether normal episodes of swallowing movements were present after the surgical reconstruction. Recordings from these electrodes confirmed that electrical activity typical of fetal swallowing was present. In three fetuses a sham procedure was performed (ligation, followed by sham reconstruction). In one of the sham fetuses, catheters were inserted into the jugular vein and amniotic sac to examine the effects of oesophageal ligation on fluid and blood chemistry. All values for fetal haematocrit, PO_2, PCO_2, pH, electrolytes, and osmolality were within the normal range for age-matched fetal sheep.

At 136 days' gestation, the ewe was killed by barbiturate overdose. The uterus was quickly excised and allantoic and amniotic fluid collected for volume and composition estimation. The fetus was removed and tissue samples from both the small and large intestine were quickly taken from routinely used sampling points: proximal (within 5 cm distal to ligament of Treitz), mid, and distal (5 cm proximal to ileo-cecal junction) small intestine, and proximal colon (5 cm distal to ileo-cecal junction). Samples of tissue were fixed for electron microscopy in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. In addition, a small number of archival samples were examined to provide an overview of normal developmental features which are relevant for the changes noted in the present study. After routine processing and sectioning, sections were examined in a Jeol or Hitachi transmission electron microscope. Samples for light microscopy were fixed in buffered formal saline and routinely processed and sectioned.

Results

We have published a comprehensive description of normal fetal sheep GI development elsewhere (Trahair and Robinson 1986). To summarize: enterocyte differentiation begins at about 35–40 days' gestation. The immature enterocytes are irregularly columnar and bulge into the lumen. They possess an extensive intracytoplasmic store of glycogen. Development is polarized along the length of the intestine, with changes first appearing in proximal regions. Soon after fetal swallowing begins (at least as early as 60 days, unpublished observations) enterocytes develop a specialized system for uptake and intracellular trafficking (Fig. 1A). This system, called the apical endocytic complex (AEC), is made up of an extensive network of vesicles and tubules in the apical region of the cell which are responsible for the sorting of material taken up from the lumen into pathways destined either for lysosomal degradation of transcellular transfer. The lysosomal components associated with the AEC expand and the enterocytes develop a large supranuclear vacuole. By 125 days vacuolated enterocytes are still well developed in mid to distal regions but have almost totally disappeared from proximal regions (Figs. 1B and 2). The AEC is still present in distal regions for at least the first few days after birth.

The fetal intestine near term is hypomature after developing for 71–76 days in the absence of ingested fluid,

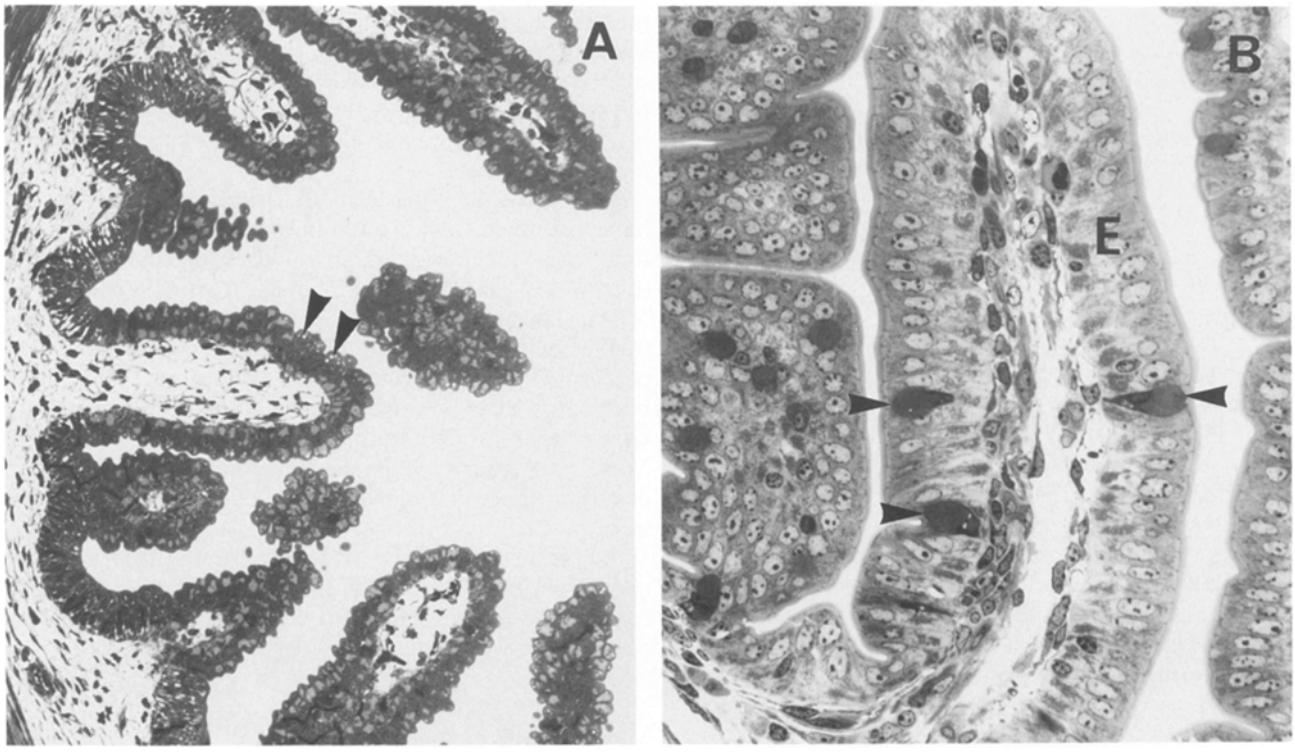

Fig. 1. A Light microscopy (LM) of proximal small intestine at 70 days' gestation. Short villi are lined with immature glycogen-filled enterocytes which bulge into the lumen. The villus tip enterocytes have begun to develop clear apical vesicles (*arrows*) which are part of the apical enterocytic complex (AEC). Crypts are not present. ×125. **B** LM of proximal small intestine at 125 days' gestation. The epithelium (*E*) is regularly columnar and well differentiated. Well-developed goblet cells are present (*arrows*). No elements of the AEC are visible. ×500

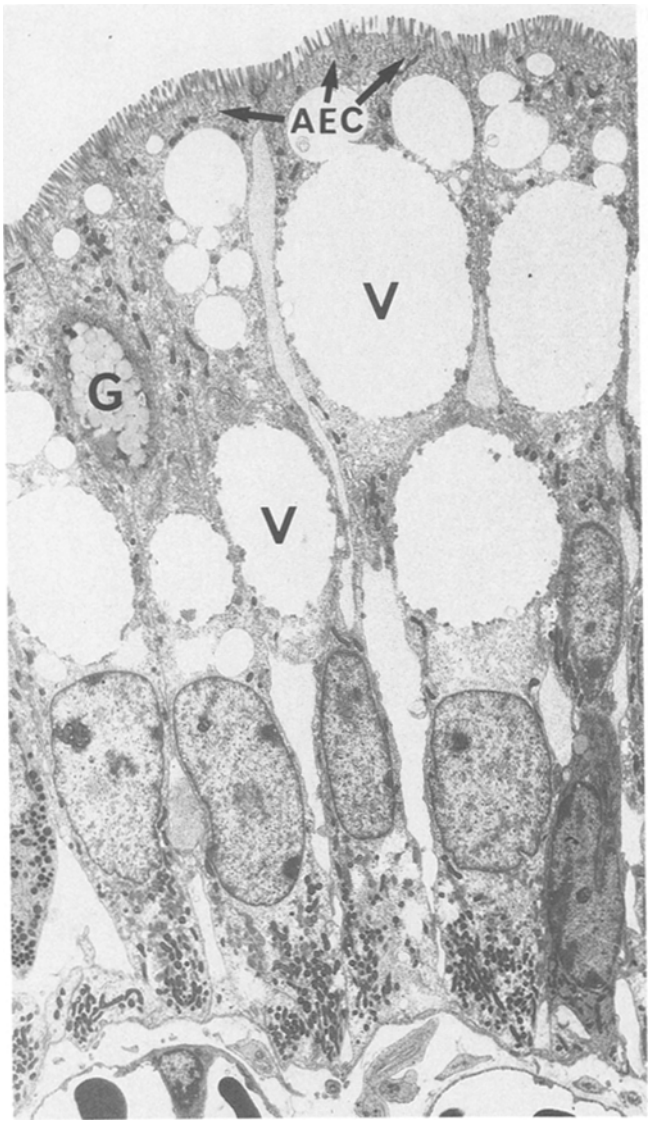

Fig. 2. Electron microscopy (EM) of distal small intestine at 142 days' gestation. Tall enterocytes have a well-developed AEC and prominent supranuclear vacuoles (*V*). Well-developed goblet cells are present (*G*). ×2300

as a result of experimental obstruction of the oesophagus. Instead of being regularly columnar, the mucosal cells bulge into the lumen, deeply accentuating the fissures on villus profiles (Fig. 3A). Foci of cells (groups of 4–5) which are extensively vesiculated are found throughout the entire intestine (Fig. 3B). These may be cells in the process of being extruded. Apart from the numerous vesicles, there are no other ultrastructural anomalies; in particular, mitochondria appear normal and do not indicate cell death.

Microvillus morphology is also affected focally. In some areas microvilli are abnormally blunted or branched or are entirely absent (Fig. 4A). In other areas microvillus-like structures are present, but these do not possess filamentous cores, nor are there any elements of the terminal web below the brush border. Examination at higher power confirms that some of these structures are in fact sections through the AEC, where the tubules are arranged perpendicularly to the luminal membrane, giving the illusion of a normal brush border (Fig. 4B). Careful examination of their inner membrane leaflet confirms the presence of the characteristic particulate coating which is a feature of the AEC components. In adult tissues and tissues of normal fetuses, microvilli are tallest in the mid-villus region, while the microvilli of crypt cells are rudimentary and sparse. In contrast to this distribution, in tissues from the fetuses from the present study, the microvilli of some crypt cells are long, up to two to three times the length of (more mature) villus enterocytes (Fig. 4D).

The disappearance of the AEC is delayed after fetal oesophageal ligation. It is still present (and well developed) in proximal small intestinal cells (in normal fetuses, the AEC is virtually absent by 125 days or more Fig. 4C). In distal regions the AEC is well developed, as is typical for fetuses at this age. In addition to the large lysosomal vacuoles, lysosomes and multivesiculate bodies are abundant in cells from all regions, ranging in size from small to large and of varying electron density, both light and dark (Fig. 5A). Lamellar and crystalline inclusions are found in lysosomes throughout the intestine, more particularly in the colon (Fig. 5B). In crypt enterocytes, many dense bodies can be found in apical regions, below the developing brush border. The presence in the crypt lumen of similar dense bodies and flocculent material suggests that they are released from the apical region of crypt cells (Fig. 5A). In other crypt enterocytes, clear vesicles are found in the apical region of the cells.

Light microscopic periodic acid-Schiff (PAS) staining (with and without diastase treatment) demonstrates that there are extensive glycogen deposits within the cytoplasm and the vacuolar structures. Electron microscopic examination confirms the presence of these intracellular pools of the glycogen (Fig. 5C, D). The vacuolar profiles are often distorted by the extent of glycogen accumulation. In some vacuoles glycogen is present as a discrete cluster, or otherwise the glycogen is dispersed throughout the vacuole contents. All the above features are present in both groups of fetuses, that is to say, fetuses with or without reconstructive surgery.

Discussion

For some time now, experiments performed in our laboratories (Trahair et al. 1986a; Avila et al. 1991) and in the laboratories of other groups (Wesson et al. 1984; Mulvihill et al. 1985, 1986, 1989; Morikawa et al. 1988; Jacobs et al. 1989) have shown consistently that when surgical intervention prevents ingestion in utero, the growth of GI tissues is retarded. In addition, a variable degree of body growth retardation, depending on the timing of the disruption to fetal ingestion, may also occur. The reversibility of these effects after the re-introduction of amniotic fluid (Mulvihill et al. 1985) taken together with results of studies demonstrating the benefi-

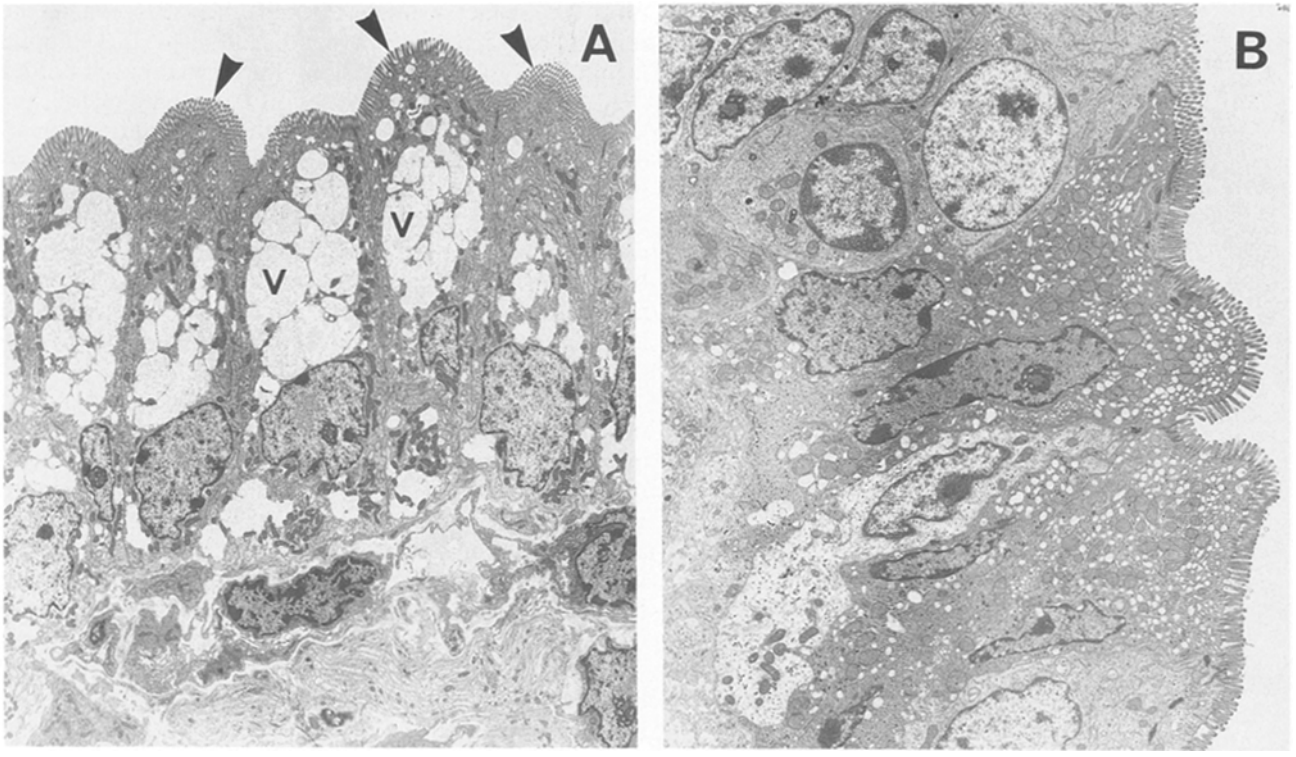

Fig. 3. A EM of distal small intestine at 136 days' gestation after oesophageal ligation. Enterocytes bulge into the lumen (*arrows*) and vacuoles (*V*) are well developed. ×3200. **B** EM of proximal small intestine at 136 days' gestation after oesophageal ligation (with reconstruction). A group of cells are conspicuously vesiculated, possibly in the process of being extruded from the side of the villus. ×4100

cial effects of amniotic fluid on GI organ culture (Calvert 1981; Calvert et al. 1983), cell culture (Mulvihill et al. 1989) and pancreatic explant culture (Dunger et al. 1990), suggests that amniotic fluid, which is a major (though not the only) component of swallowed fluid, participates in the control of normal growth of GI tissues.

The ultrastructural observations made in the present study suggest that in the absence of fetal ingestion, in addition to retardation of GI development, a more acute and localized tissue substrate deprivation takes place, resulting in a series of pathologies which are morphologically similar to those which have been reported in cases of malnutrition in infancy. In a number of publications describing intestinal ultrastructure in malnutrition or associated diseases, all features we have noted in the present study have been reported (Brunser et al. 1976; Campos et al. 1979; Shiner et al. 1990). These included shorter villi, microvillus anomalies, (including branching and denudement), lysosomal expansion, epithelial cell extrusion and cell lysis. Some authors suggest that there might be a common immunological basis for many of these changes (Shiner et al. 1990), especially considering that bacterial infection often compounds the effects of the low nutrition state. Without discounting this possibility, our studies suggest that the GI tissues themselves are especially sensitive to local nutritional levels to the extent that they waste, or fail to develop normally, in the absence of local nutrition. Indeed the very high rate of protein turnover in both the mature and fetal (Kishnamurti et al. 1987) GI tract might, at least in part, explain the basis of this heightened susceptibility.

In addition to the specific abnormal appearances noted, overall, the intestinal epithelium was less mature, when compared to age-matched normal fetuses. These observations are consistent with the view that amniotic fluid possesses many specific GI growth modulating properties. We have previously noted that during normal development, proximal regions are the first to develop specific morphological features. Many of these (e.g. the development of the AEC) first appear coincident with the onset of fetal swallowing (Trahair and Robinson 1986), perhaps because of the enhanced interaction with ingested material afforded by their more proximal location. This being the case, it is also possible that the passage of ingested material might be the basis for programming and maintenance of the proximo-distal gradient of maturation which persists throughout development (Trahair et al. 1985; Trahair and Robinson 1986), even after region-specific experimental perturbation (Trahair et al. 1987a, b). The surgical techniques used in studies of adaptation (e.g. resection, transplantation and transposition) will be an important future development of our fetal experimental models.

We did not attempt to quantify the extent of the altered morphology in the present study because of the focal nature of the "lesions". The presence of the features in both groups of fetuses, irrespective of whether the oesophagus was reconstructed or not, suggests that any growth promoting effects of swallowed factors after

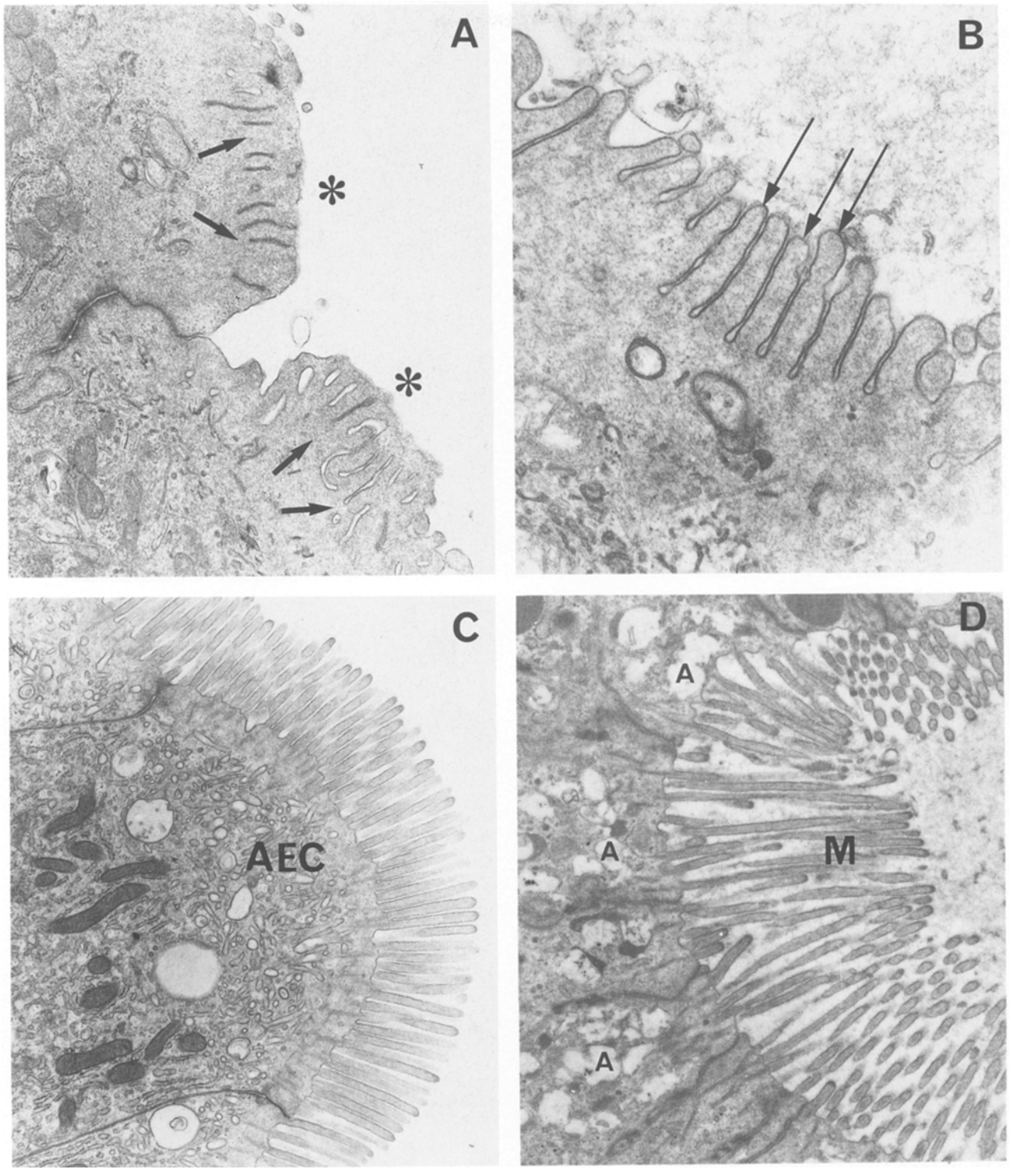

Fig. 4. **A** EM of affected enterocytes showing total loss of brush border (*asterisks*) and re-arrangement of AEC components (*arrows*). ×18500. **B** Higher power detail of area of loss of brush border. Microvillus-like structures are present in regions where the AEC tubules line up perpendicularly to the apical membrane (*arrows*). There is no terminal web present. ×32000. **C** Bulging enterocyte from the proximal region demonstrating well-developed AEC which is normally absent (or very sparse) by this age. ×15000. **D** Crypt enterocytes with abnormally long microvilli (*M*) and apical vesicles (*A*). ×13000

surgical reconstruction of the oesophagus at 120 days of gestation were not sufficient to induce compensatory changes within the time course of the experiments. This is quite likely to be the case since our previous experiments have shown that renewal of the epithelium of fetal intestine requires between 8 and 20 days [for 136- and 115-day-old normal fetuses, respectively (Trahair et al. 1986b)]. In addition, a recent study has shown that in

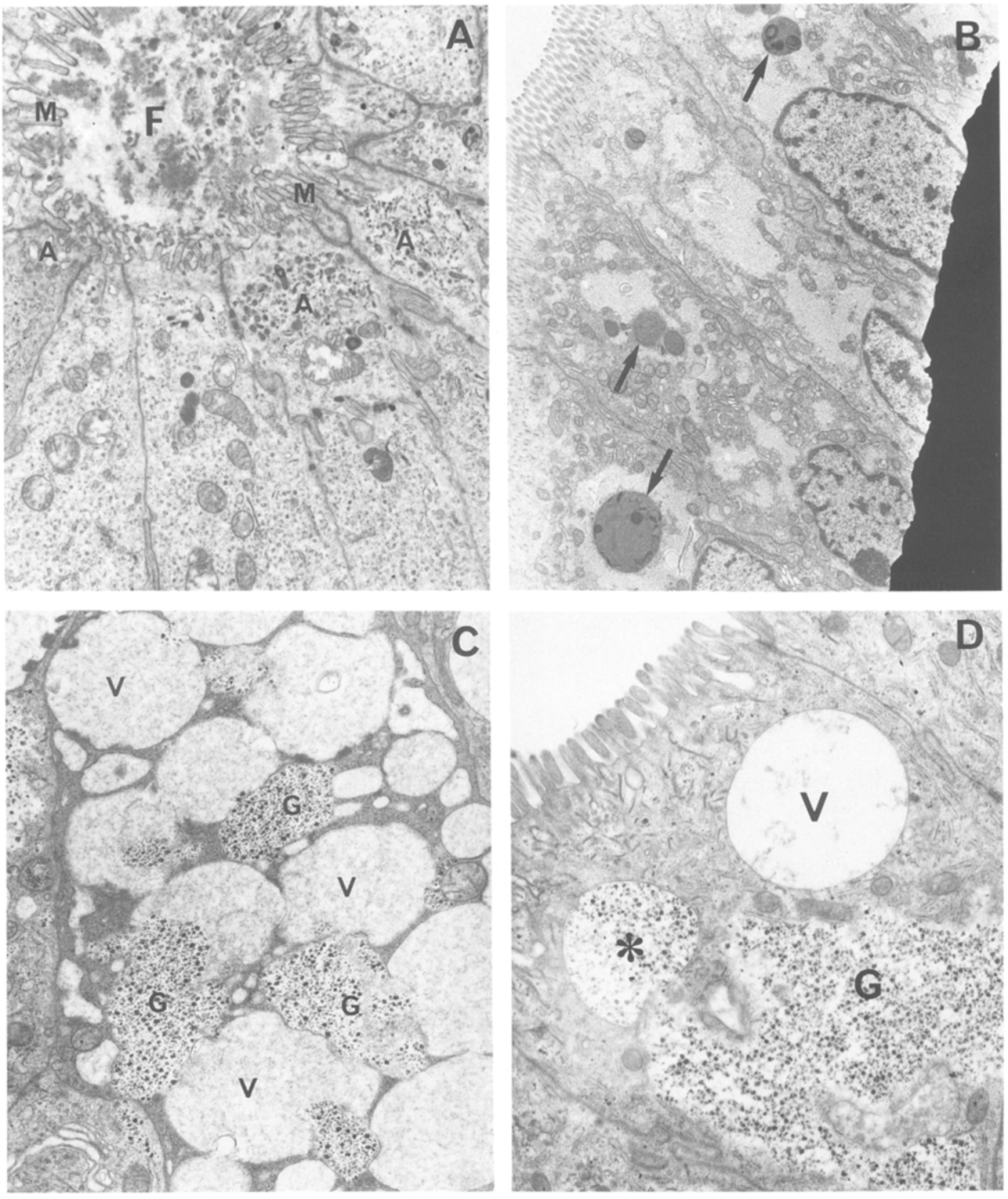

Fig. 5. **A** Crypt enterocytes with irregular brush border (*M*) and a wide variety of apical vesicles and tubules (*A*). The lumen is filled with flocculent material and membrane vesicle fragments (*F*). ×10000. **B** Colonic absorptive enterocytes with a well-developed store of cytoplasmic glycogen (*G*) and numerous dense body inclusions (*arrows*). ×6000. **C**, **D** Glycogen accumulation (*G*) in vacuolated enterocytes. Glycogen stores protrude into the vacuolar structures. In some cells, the glycogen is membrane bound (*asterisk*), presumably within the cytoplasmic vacuoles. ×12000

fetuses whose oesophagus has been ligated (90–136 days), cellular renewal times are about 12 days (Avila et al. 1991). With only approximately 14 days on average, between oesophageal reconstruction and tissue collection at 136 days of gestation, complete epithelial cell renewal might not have occurred in all fetuses, and cells affected prior to reconstruction would still most likely be present.

Although our experiments do not throw any further light on the general issue of a possible nutritive role of swallowed fluid for whole body or organ growth, it should be noted that calculations based on the composition of amniotic fluid and the volumes swallowed suggest that the human fetus could derive up to 15% of its daily requirement for total body protein deposition from ingestion (Gitlin et al. 1972). This input could become critical for maintenance of normal body growth rates as gestation proceeds, especially in the face of declining placental delivery of nutrients (and reduced placental growth rates), as occurs with the approach of normal term (Jacobs et al. 1989), or in the case of an underlying placental dysfunction, or even maternal under-nutrition (Owens et al. 1989). The lack of effect of oesophageal blockade on fetal body weight in our three independent groups of experiments might therefore appear surprising. While there may be significant species differences in this regard (humans and rabbits, compared to sheep), it should be noted that the end point of our experiments (at 136 days' gestation) was 10–14 days prior to normal term; hence placental delivery of nutrients might not have reached restrictive levels in a well-fed ewe. With these observations in mind, it would be interesting to speculate what the levels of luminal nutrition might be in cases of fetal growth retardation, since at least one model for intra-uterine growth retardation has shown perhaps the most dramatic GI growth retardation and altered growth which has been observed to date (Avila et al. 1990).

Analyses of amniotic fluid have demonstrated the presence of a very wide range of growth factors and hormones (Schindler 1982), many of which have known GI growth-promoting activities. The lung liquid (and buccal secretions) which also contributes to the fluid ingested (Harding et al. 1984) also contains known growth factors, e.g. epidermal growth factor (Stahlman et al. 1989) and insulin-like growth factor (Spencer et al. 1983). In the fetal and suckling rat it has been shown that the AEC is responsible for the intact transepithelial transfer of milk-borne growth factors and hormones (Siminoski et al. 1986; Gonnella et al. 1987, 1989; Weaver et al. 1990). It is significant therefore that we have noted that the AEC first appears in proximal small intestine coincident with the onset of swallowing (Trahair and Robinson 1986). In addition to any nutritive benefit from swallowed fluid, growth factors could cross the epithelium via the AEC and influence growth in the GI tract or elsewhere in the fetus. In support of this idea, a recent study in the fetal sheep by our colleagues demonstrated reduced liver and pancreatic growth after oesophageal fistulation (Avila et al. 1991).

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