

## MORPHOLOGICALLY STABLE EPITHELIAL VESICLES CULTURED FROM NORMAL HUMAN ENDOMETRIUM IN DEFINED MEDIA

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### SUMMARY

Epithelium from normal human endometrium was cultured as morphologically stable vesicular structures in a defined, hormone-supplemented PFMR-4 medium. The structures consisted of a single layer of polarized epithelial cells with the apical surface facing the external culture medium, and the basal surface resting on a well-defined basal lamina adjacent to the internal lumen. Vesicles were shown to (a) retain their viability for up to 3 mo. in culture, (b) to actively synthesize DNA after being cultured for over a month in a defined medium, and (c) to respond to steroid hormones. When embedded within a collagen gel, the vesicles reversed their epithelial polarity and formed branching, pseudoglandular structures. It was concluded that the three-dimensional shape of the epithelial vesicles had a critical role to play in their morphological stability, nutrient requirement, and hormone sensitivity.

*Key words:* normal human endometrium; vesicles; defined medium.

### INTRODUCTION

The complexity of the hormonal regulation of the human endometrium *in vivo* has resulted in the widespread use of monolayer cell culture as a simplified experimental approach (4,7,10,12,14,18,22,23). Unlike earlier investigators who could only maintain the epithelium as short-term primary cultures (4,10,12,14,18,23), more recent authors (3,22) claim to have serially cultivated the normal epithelial cells. In general, the monolayer epithelial cells were found to have lost their *in vivo* hormone sensitivity. However, one report (22) showed an inhibitory effect of epithelial cells by nonphysiologically high levels of progesterone. To our knowledge, no one has reported a convincing physiological response of the normal human endometrial epithelial monolayers to sex steroid hormones. It would seem, then, that some essential mediator for this response is missing in the monolayer culture system. At least part of the reason for this lack of response seems to be the use of monolayers *per se*, because studies with mammary (5,19-21) and thyroid (8,13) cell cultures have shown that maintenance of the differentiated state, which includes hormone sensitivity, required a three-dimensional cellular arrangement. Also contributing to the lack of physiological hormone sensitivity in monolayers may be the presence of the serum that is necessary for their attachment and growth. Serum is known to severely

suppress the hormonal induction of the specific functions of cultured rat ovarian cells (17).

With these considerations in mind, we have developed a simple method for culturing, in a defined nutrient medium, novel endometrial vesicles that were morphologically stable and had certain *in vivo* features absent in the monolayer cultures.

### MATERIALS AND METHODS

#### *Source of Human Endometrium*

Endometrium was obtained from hysterectomy specimens from patients ranging in age from 30 to 52 yr. Curettings were collected in sterile culture medium PFMR-4 (11) supplemented with penicillin (100 U/ml) and kanamycin (100 µg/ml) and set up in primary cultures within 2 h. Samples were obtained from proliferative, secretory, and menopausal endometria and all were histologically normal.

#### *Serum, Hormones, and Growth Factors*

Fetal bovine serum (FBS) was obtained from Irvine Scientific, Irvine, CA. Vitamin E, Vitamin A, linoleic acid, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, MO. Vitamin E and Vitamin A were prepared as concentrated stocks in acetone and dimethyl sulfoxide, respectively. Insulin (INS), triiodothyronine (T3), and transferrin (TF) were obtained from Calbiochem-Behring Corp., La Jolla, CA. Hydrocortisone (HC), estradiol-17β (E<sub>2</sub>), progesterone (P), and dexamethasone (DEX) were obtained from

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Steraloids, Inc., Wilton, NH, and prepared as concentrated stocks in ethanol that never exceeded a final concentration of 0.1% (vol/vol) in the culture medium.

**Culture medium.** The basal nutrient medium (BM) was PFMR-4 (11) containing penicillin (100 U/ml) and kanamycin sulfate (100  $\mu$ g/ml). Medium ESF-1 was PFMR-4 supplemented with INS, 5  $\mu$ g/ml; HC, 1  $\mu$ g/ml; TF, 1  $\mu$ g/ml; T3, 0.3 nM; Vitamin E, 10  $\mu$ g/ml; Vitamin A, 5  $\mu$ g/ml; and linoleic acid, 0.1  $\mu$ g/ml. Medium 199 was obtained as a dry powder from Grand Island Biological Company, Grand Island, NY.

**Primary culture.** Curettings were collected and rinsed in BM and then minced manually with scalpels into small pieces (approximately 1 to 2 mm<sup>3</sup>). Minced fragments were either added to 4 ml of the specified culture medium in 60-mm dishes and maintained as nonattached "organ-type" cultures with refeeding every 3 to 4 d or digested with collagenase (1 mg/ml BM, type IV, Worthington Biochemical Corp, Freehold, NJ) at 30° C for 2 h.

#### Percoll Fractionation of Epithelial Vesicles

A 5 to 10% discontinuous Percoll gradient (made up with BM) was used to separate the epithelial vesicular

structures from larger tissue fragments. Sedimentation under gravity for 10 min left the bulk of the vesicles in suspension with the larger tissue fragments in the pellet.

**Collagen gel culture.** Collagen was isolated from rat tails (Pel Freeze, Rogers, AR) using a standard method (16). Extracts containing about 2 mg collagen/ml were sterilized by UV irradiation and stored at 4° C before use. A mixture of 8.6 ml collagen solution, 1 ml Medium 199 (10 $\times$ ), 0.1 ml HEPES buffer [66 mg/ml distilled water (DW)], 0.5 ml NaOH (0.5 N), and 0.1 ml NaHCO<sub>3</sub> (1.2 g/10 ml DW) was added along with cultured vesicles to 35-mm petri dishes (1 ml/dish) containing an underlay of agarose gel (1 ml, 2.5%, Sea Plaque, Marine Colloids, Rockland, ME) and gelled by incubation at 37° C overnight. The layer of agarose was added as a spacer layer to preclude attachment of vesicles to the plastic surface. After gelling, cultures were refed with the appropriate liquid medium (1 ml/35-mm dish) every 2 to 3 d.

**Histology.** Vesicles were rinsed and fixed in 2% phosphate buffered paraformaldehyde, pH 7.3, at 4° C for 8 h. Fixed vesicles were then dehydrated, infiltrated, and embedded with JB4 plastic embedding medium as described in the JB4 embedding kit (Polysciences, Inc., Warrington, PA). Sections were stained with either hematoxylin and eosin (H&E) or periodic acid Schiff (PAS).

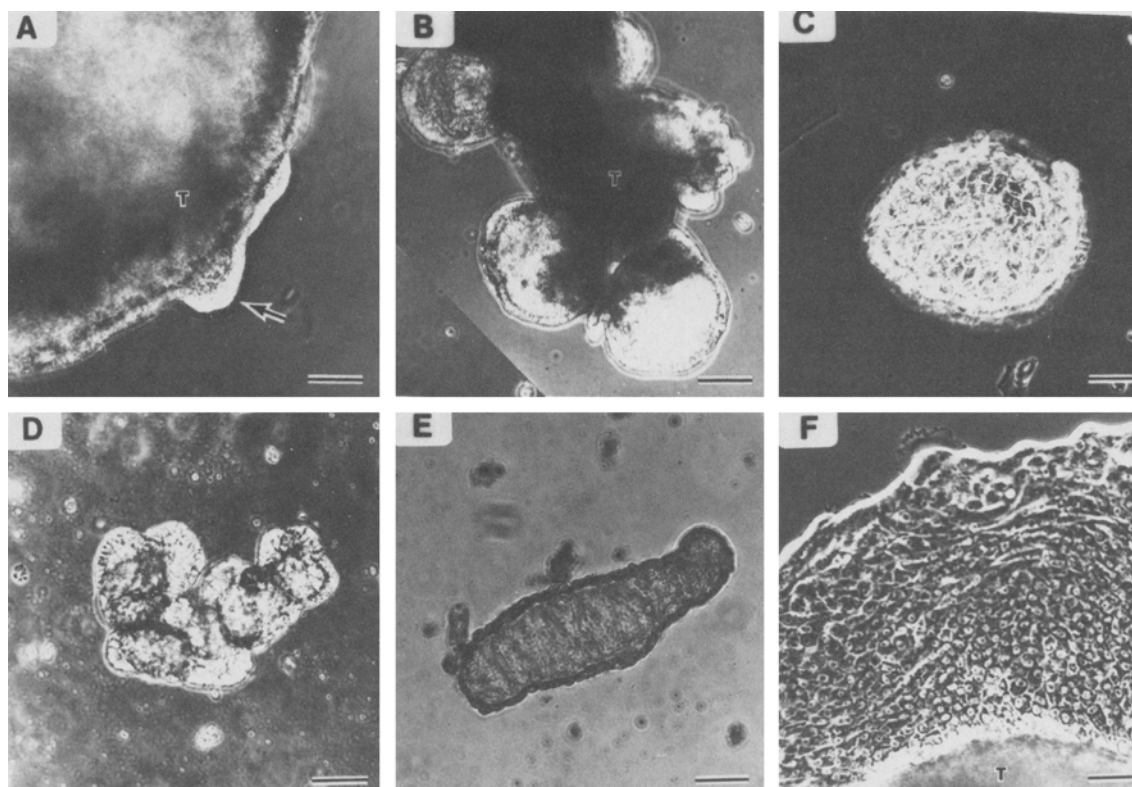


FIG. 1. Formation and variety of endometrial vesicular structures cultured from a normal proliferative endometrium in defined media. Phase contrast. Bar = 100  $\mu$ m.  $\times$ 108. A, epithelial protrusions (arrow) from periphery of tissue (T) after 8 d culture in BM. B, dilated vesicles attached to tissue after 8 d culture in BM. C, free floating vesicle after 8 d culture in ESF-1 medium. D, complex vesicular structure after 8 d culture in ESF-1 medium. E, simple cylindrical vesicle after 2 d culture in ESF-1 medium. F, floating epithelial monolayer on air-liquid interface emerging from the tissue (T) after 8 d culture in BM.

**Transmission electron microscopy (TEM).** Vesicles were fixed and processed as described previously (11).

**Autoradiography.** Cultures were grown in thymidine-free BM containing 1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]TdR (21.8 Ci/mmol, New England Nuclear, Boston, MA). Labeled vesicles were fixed and processed for JB4 histology as described above. Mounted sections were rinsed with cold (4° C) trichloroacetic acid (5%, vol/vol), rinsed with distilled water, and covered with Kodak AR10 striping film and stored at 4° C. Autoradiographs were developed after 57 d with Kodak D19 developer. The labeling index was determined as the percentage of cells with labeled nuclei.

## RESULTS

We shall relate here how novel endometrial epithelial vesicle cultures were derived from nonenzymatically treated floating tissue fragments and how they behaved in different culture media.

### *Production of Epithelial Vesicles*

The initial observation of endometrial vesicles resulted from a comparison of the effect of collagenase digestion versus nonenzymatic treatment on the subsequent culture of the epithelial cells. Minced endometrial fragments were either added directly to BM with or without 10% serum (vol/vol) or digested with collagenase before culture. As found before (12), collagenase digested the stromal tissue and released epithelial glandular structures which adhered to the petri dish and formed epithelial monolayer outgrowths with a high plating efficiency. These cultures degenerated after about 2 wk as observed previously (12).

However, the unique behavior of the nonenzyme-treated fragments was of potentially greater interest. Unlike the collagenase digested tissue, the untreated fragments generally remained floating in the culture medium. In the defined BM the fragments rarely attached, and when they did they showed little or no cellular outgrowth. In contrast, the addition of serum (10%, vol/vol) to the BM resulted in a greater attachment of fragments which gave rise mostly to stromal cellular outgrowths. Continued observation of the floating fragments derived from a proliferative endometrium revealed an interesting behavior of the epithelial component. After about a week in culture in either of the two defined media, BM or ESF, the epithelial activity at the periphery (Fig. 1 A) of the floating tissue pieces resulted in the formation of hollow epithelial vesicles (Fig. 1 B). These spherical vesicles eventually detached from the parent tissue and became free floating (Fig. 1 C). These vesicles, which could reach 500 to 1000  $\mu\text{m}$  in size, exhibited great structural diversity, from simple spherical (Fig. 1 C) to more complex forms (Fig. 1 D). Cylindrical forms (Fig. 1 E) were very rare and appeared to result from "spill" glands (produced by the mincing), which subsequently "re-annealed" to form a hollow gland. Also of interest was the ability of the epithelium to occasionally form a cellular monolayer on the liquid-air in-

terphase (Fig. 1 F), an observation that was associated only with cultures in defined medium.

Generally, however, the overall majority of epithelial structures were of the three-dimensional, spherical variety. Monitoring the vesicle development with phase contrast microscopy indicated that the majority of vesicles were derived largely from glandular elements and to a lesser extent, from the surface epithelium. Comparison of cultures derived from eight different endometria showed that the rate of vesicle formation was very similar for both secretory (four samples) and proliferative endometria (two samples), but was noticeably slower for menopausal tissue (two samples). Vesicles were observed to be morphologically stable for up to at least 3 mo.

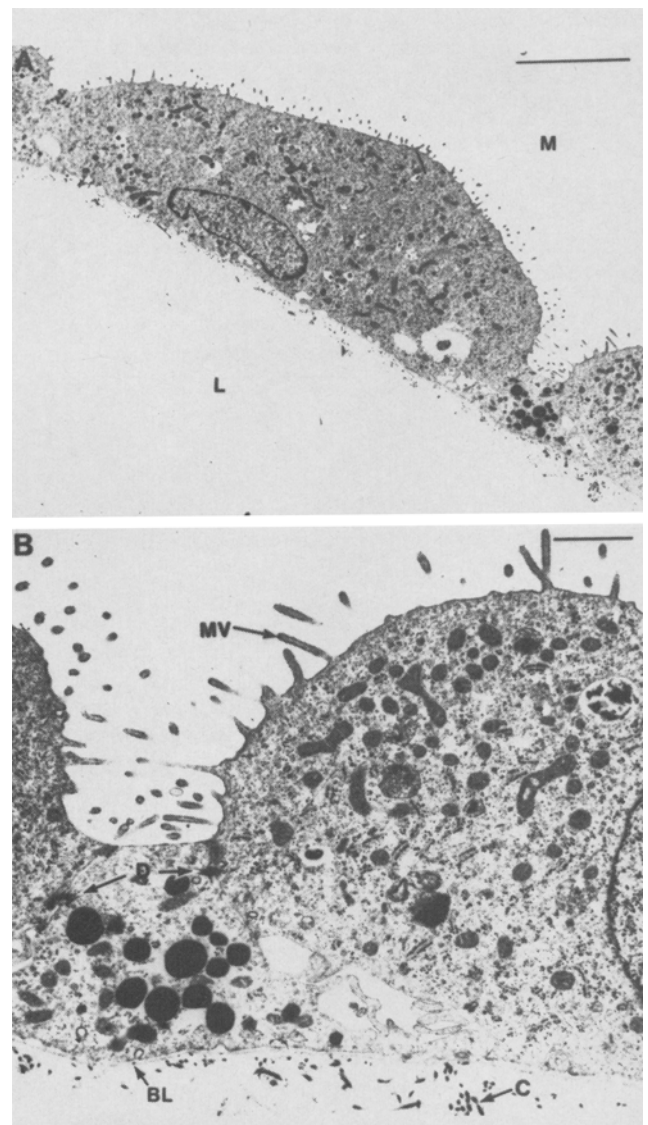


FIG. 2. Ultrastructure of a vesicle after 15 d culture in BM supplemented with 5% FBS. A, low power transmission electron micrograph of the vesicle (L=Lumen; M=culture medium). Bar = 5  $\mu\text{m}$ .  $\times 1840$ . B, high power detail of A showing microvilli (MV), desmosomelike junctions (D), the basal lamina (BL), and collagen fibrils (C). Bar = 1  $\mu\text{m}$ .  $\times 6400$ .



FIG. 3. DNA synthesis in vesicles cultured from a normal, proliferative endometrium in BM supplemented with INS (5  $\mu\text{g}/\text{ml}$ ) and HC ( $10^{-7}$  M). Autoradiograph showing active DNA synthesis in both vesicle (V) and monolayer (M) cultures after 36 d H&E. Bar = 50  $\mu\text{m}$ .  $\times 463$ .

in serum-containing BM and up to at least 1.5 mo. in defined BM.

As regards the optimal medium for promoting vesicle production, it was observed that the simple BM was as good as, if not superior to, the more sophisticated, hormonally supplemented ESF-1 medium. However, additional studies with BM have shown that the production, morphological appearance, and viability of vesicles were noticeably improved after the addition of HC ( $10^{-7}$  M) and INS (5  $\mu\text{g}/\text{ml}$ ). Therefore, HC (or DEX) and INS were routinely added to the BM. The added advantage of using defined media in this culture system was to promote the rapid degeneration of the stromal cellular components while at the same time leaving the epithelial vesicles intact. In contrast, the presence of serum partially helped to retain stromal cell viability and resulted in mixed stromal epithelial vesicular structures.

#### Characterization of Vesicles

Ultrastructural studies using the transmission electron microscope confirmed that the vesicles, derived from a secretory endometrium cultured in BM containing 10% serum, consisted of a single layer of epithelial cells (Fig. 2 A) possessing tight junctions, desmosomelike structures and microvilli on their apical surfaces (Fig. 2 B). The cells showed a cuboidal morphology with a similar polarity to that observed in vivo. The "external" (or

apical) surface facing the medium bore the microvilli, whereas the "internal" or basal cell surface rested on a well-defined basal lamina, which in turn was associated with an underlying layer of loose collagen fibrils. In addition, the nuclei were located on the basal cell surface.

TABLE 1

EFFECT OF HORMONES ON DNA SYNTHESIS IN VESICULAR AND FLOATING MONOLAYER CELLS IN A DEFINED MEDIUM (BM) SUPPLEMENTED WITH INS (5  $\mu\text{g}/\text{ml}$ ) AND HC ( $10^{-7}$  M)

	Labeling Index (%) <sup>a</sup>	
	Vesicles	Monolayers
Control	55.8 $\pm$ 3.8	60.6 $\pm$ 2.1
Progesterone ( $10^{-7}$ M)	31.6 $\pm$ 3.2 <sup>b</sup>	61.3 $\pm$ 2.3 <sup>c</sup>
Progesterone ( $10^{-7}$ M) and Estradiol ( $10^{-8}$ M)	27.3 $\pm$ 3.8 <sup>d</sup>	N.D.

<sup>a</sup>Derived from counts on at least 500 cells from at least eight different sections  $\pm$  SE.

<sup>b</sup>Student's *t*-test comparison with control showed significance ( $P < 0.02$ ).

<sup>c</sup>Student's *t*-test comparison with vesicles-progesterone value showed significance ( $P < 0.01$ ).

<sup>d</sup>Student's *t*-test comparison with control showed significance ( $P < 0.01$ ).

N.D. = Not Done.

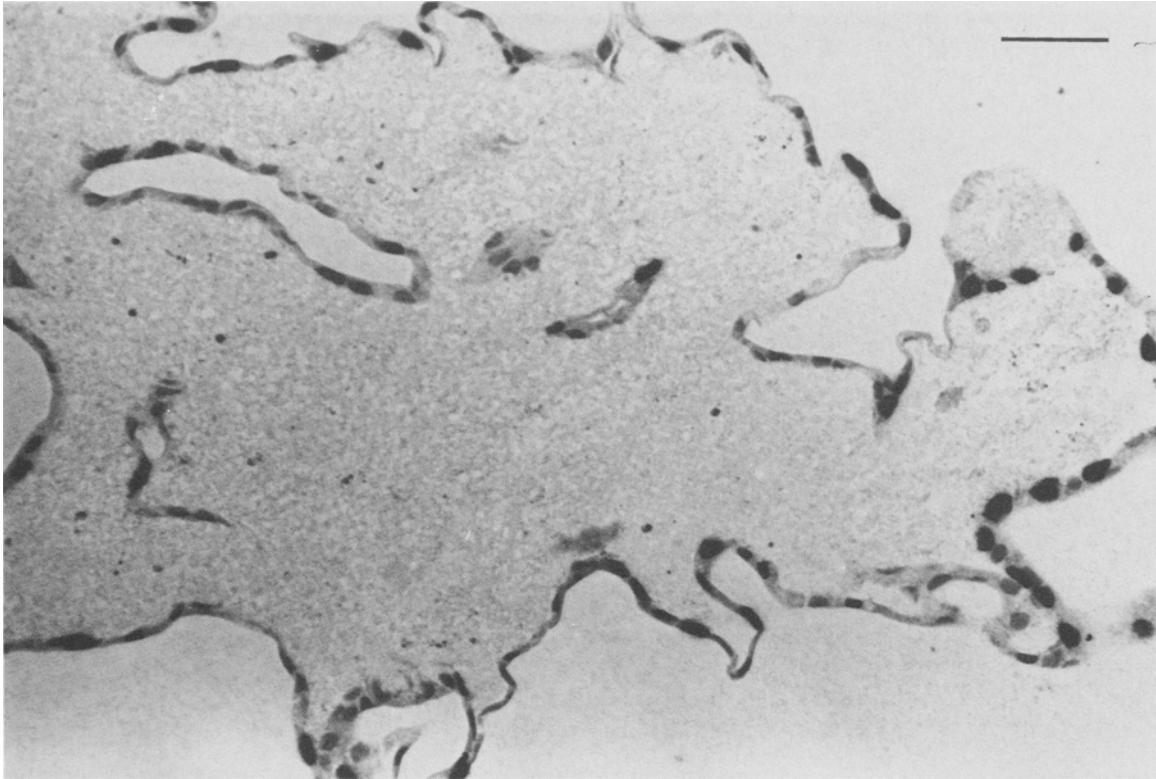


FIG. 4. Effect of progesterone on DNA synthesis in vesicles. Autoradiograph of vesicle previously cultured as described in Fig. 3, except progesterone ( $10^{-7}$  M) was added to the BM for the last 3 d H&E. Bar = 50  $\mu$ m.  $\times 289$ .

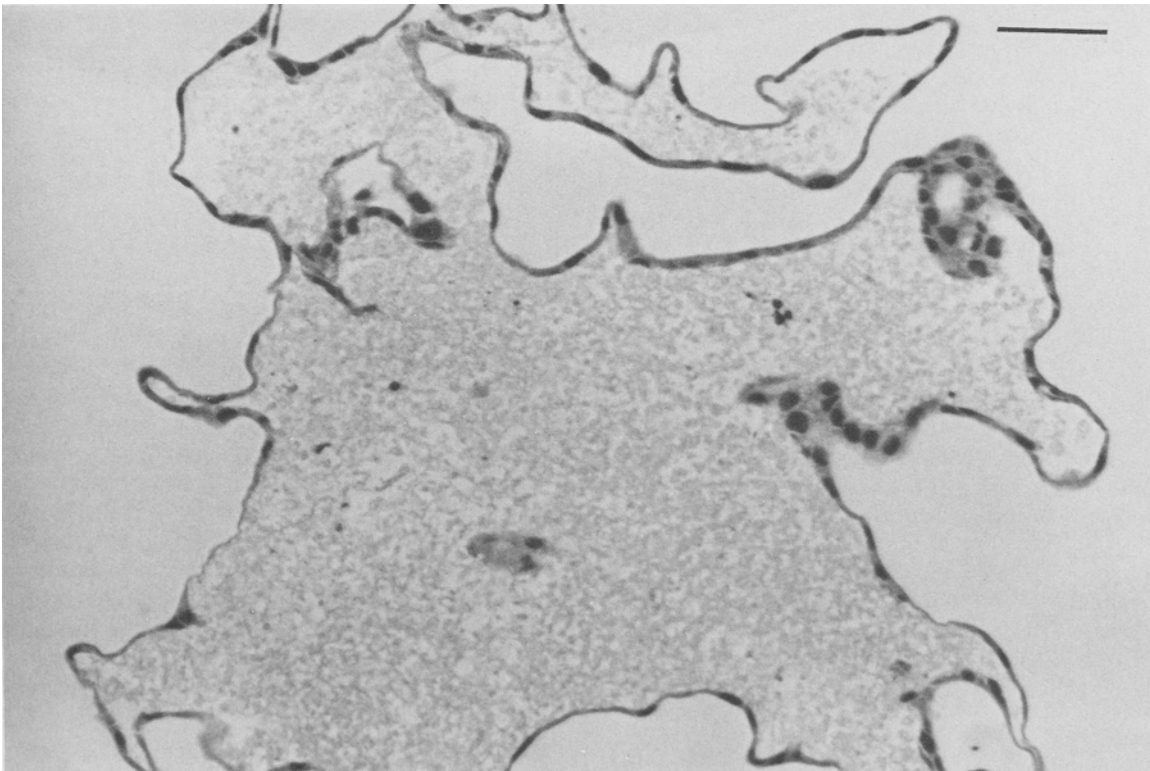


FIG. 5. Unusual looplike structures of vesicles treated with progesterone as described for Fig. 4. Periodic acid Schiff (PAS) stain. Bar = 50  $\mu$ m.  $\times 292$ .

Abundant mitochondria and rough endoplasmic reticulum were consistent with secretory activity. Occasionally, cells with dense staining bodies (Fig. 2 A, B) and with very little rough endoplasmic reticulum (Fig. 2 B) were observed. Some vesicles contained an amorphous material, presumably dead stromal cell debris, which however, did not seem to affect the epithelial morphology or viability. Cilia were not observed in these cultures.

#### *DNA Synthesis and Effects of Hormones*

To determine if the vesicles were produced *de novo* or were merely rearrangements of existing epithelium, vesicles were examined for active DNA synthesis by means of [<sup>3</sup>H]Tdr autoradiography. Vesicles derived from a proliferative endometrium and cultured for 33 d in BM were separated from large tissue fragments by Percoll fractionation on a 5 to 10% discontinuous gradient. After fractionation, some vesicles formed floating monolayers at the air-liquid boundary. Both the vesicles and the monolayers were then resuspended in thymidine-free BM containing HC ( $10^{-7}$  M) and INS (5  $\mu$ g/ml). Cultures were then pulsed for 3 d with [<sup>3</sup>H]Tdr in the above media with no addition (control), with progesterone ( $10^{-7}$  M), or with estradiol and progesterone ( $10^{-8}$ ,  $10^{-7}$  M, respectively). All cultures, including the control, contained a final ethanol concentration of 0.1% (vol/vol).

The autoradiograph (Fig. 3) of the control culture showed labeled nuclei in both the vesicle and in the

floating monolayer, and confirmed that DNA synthesis was actively occurring in these normal epithelial cells in this defined nutrient medium. It should also be noted (Fig. 3) that although the labeling index for controls was similar for both vesicles and floating monolayers (approximately 60%, Table 1), the epithelial morphology differed markedly. The monolayer contained large, flat, vacuolated cells with a pale cytoplasm, whereas the vesicle contained small, cuboidal cells with a dark cytoplasm (Fig. 3).

Progesterone had two notable effects. First, it significantly reduced the labeled index of epithelial cells in the vesicles but not in the monolayers (Table 1, Fig. 4), implying a sensitivity of vesicular cells to progesterone that had been lost by the monolayer cells. Second, progesterone seemed to induce highly irregular vesicular shapes (Figs. 4-6). The surface epithelium formed numerous cellular protrusions or looplike structures. It was also clear that the epithelial population was heterogeneous. Two morphologically distinct cell types were identified. The flat, elongated, surface epithelium contrasted with clusters of darkly staining, cuboidal cells that seemed to be forming glandularlike structures with lumens (Figs. 5,6).

A combination of estradiol and progesterone also promoted irregular vesicular shapes (Fig. 7 A), but in addition it caused a morphological degenerative appearance possibly associated with hypersecretory activity. This hormonal combination also had the unique effect of

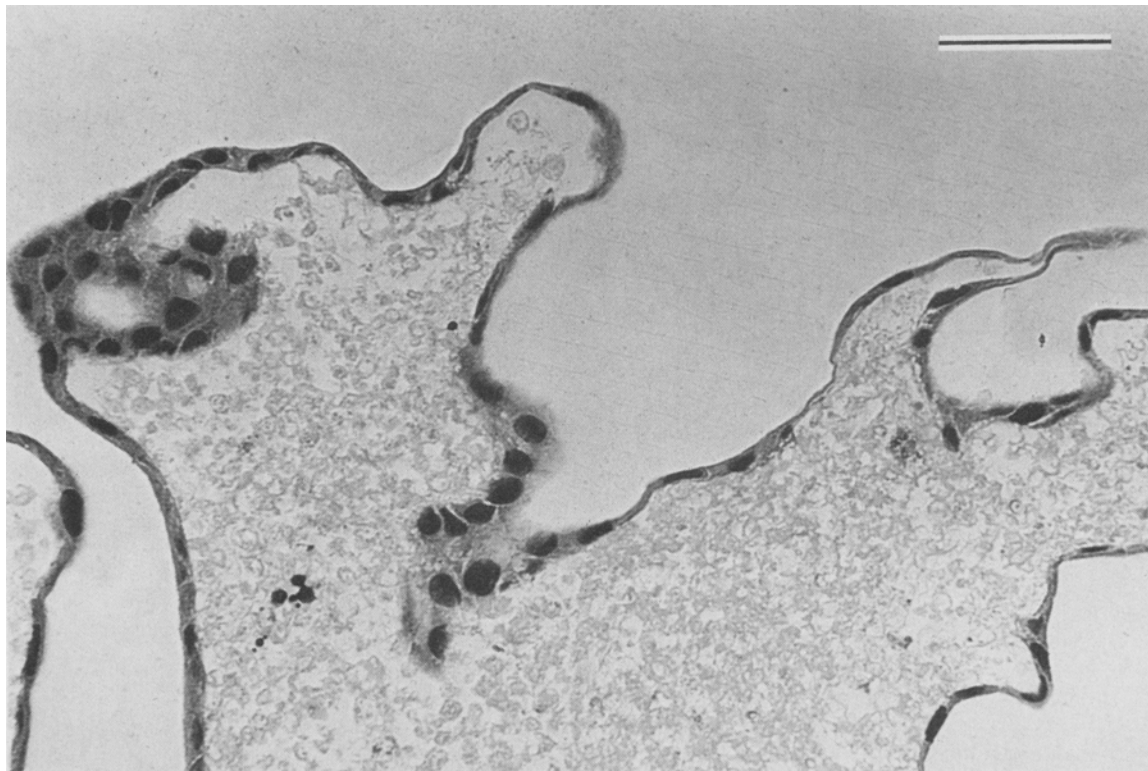


FIG. 6. High power detail of Fig. 5 showing a thin, squamous cell type and also a more cuboidal, darkly staining cell which associated in clusters and formed lumens. PAS stain. Bar = 50  $\mu$ m.  $\times 459$ .

being associated with the appearance of both subnuclear vacuoles and cilia (Fig. 7 B).

#### Vesicle Behavior in Collagen Gels

Because collagen maintains epithelial function *in vitro* (see ref. 24 for review), we embedded the endometrial vesicles within a hydrated collagen gel in an attempt to induce a morphogenesis similar to that observed for the *in vivo* endometrium. Vesicles, derived from a late proliferative endometrium, were cultured for 13 d in BM supplemented with DEX (1  $\mu\text{g}/\text{ml}$ ), HC ( $10^{-7}$  M), TF (5  $\mu\text{g}/\text{ml}$ ), and BSA (1 mg/ml) before embedding within a collagen gel. The vesicles initially embedded were typically spherical, but after 6 d culture in the collagen they oriented themselves into what seemed to be pseudoglandular structures (Fig. 8). Whether the development of these structures was due to either localized cellular growth at the point of origin of the collagen stress fibers or to selective cellular attachment to the collagen with a mere physical rearrangement is not known. Nonetheless, there was a striking similarity between the narrow, epithelial vesicles embedded in the collagen (Fig. 8 A-D) and the narrow glands observed in the intact endometrium (Fig. 9). Cuboidal-columnar epithelium lined both the *in vitro* vesicles (Fig. 8 A-D) and the *in vivo* glands (Fig. 9). Also of interest was the appearance of growing branch points in the cultured vesicles (Fig. 8 D), although these could also be simple folds in the epithelial sheet.

A direct ultrastructural comparison of vesicles cultured (a) in BM, (b) in BM with 10% FBS, or (c) embedded in collagen with the original late proliferative endometrial tissue (Fig. 10 A) was also instructive. The height of the cuboidal epithelium of all types of cultured vesicles (Fig. 10 B-D) was approximately half the height (10 to 15  $\mu\text{m}$ ) of the pseudostratified columnar epithelium (approximately 30  $\mu\text{m}$ ) of the intact tissue (Fig. 10 A). In general, all the vesicles were more representative of a secretory endometrium (rather than late proliferative) as evidenced by the presence of numerous mitochondria and large vacuoles which characterize the secretory phase cell. The vesicles cultured in liquid medium supplemented with 10% serum (Fig. 10 B) more closely resembled the parent tissue epithelium in that they possessed a normal cellular polarity with minimal intercellular spaces. On the other hand, the vesicles cultured in the defined BM medium, although maintaining a normal polarity, exhibited extensive intercellular spaces (Fig. 10 C).

However, the vesicles cultured within the collagen gel showed the most remarkable *in vitro* structural modification. It was observed that the vesicles embedded in collagen had undergone a reversal of cellular polarity (Fig. 10 D). The vesicles had in effect turned inside out. The external, apical surface, with the microvilli and tight junctions, was now facing the internal lumen and had replaced the previous basal cell surface along with its basal lamina. However, the new basal cell surface, now facing the external collagen matrix, did not have a reformed basal lamina. Also particularly noticeable in the

collagen embedded vesicles compared to the liquid cultures was (a) the increased levels of well-defined desmosomes (not shown), (b) the presence of increased amounts of dilated rough endoplasmic reticulum (not shown); and (c) the presence of increased amounts of both subnuclear and supranuclear vacuoles. However, the vesicles in collagen showed a morphological degeneration after about only 10 d, whereas the vesicles in liquid media maintained their morphological stability for a substantially longer period.

#### DISCUSSION

We have described here an unusual, organ-type culture method for the development and culture of epithelial

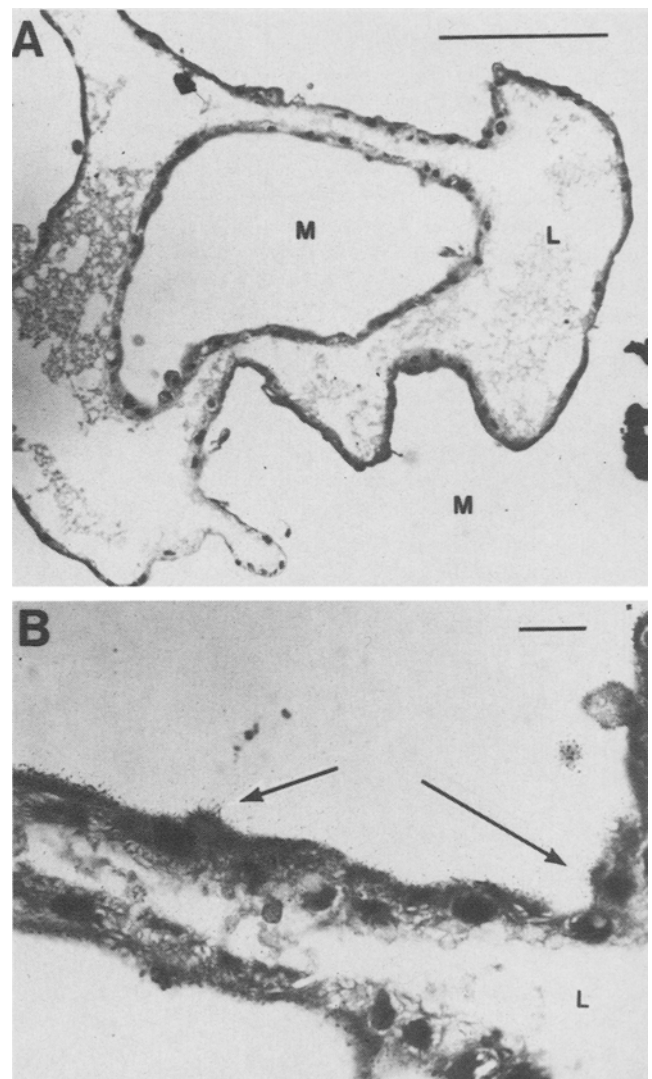


FIG. 7. Effect of the combination of estradiol ( $10^{-8}$  M) and progesterone ( $10^{-7}$  M) on the morphology of vesicles cultured as described in Fig. 3. A, after 33 d in BM followed by a 3-d treatment with the above hormones, the vesicles showed irregular cellular shapes, surface blebbing, and morphological degeneration (M=medium; L=lumen). PAS stain. Bar = 100  $\mu\text{m}$ .  $\times 217$ . B, high power detail of A showing abundant subnuclear vacuoles, surface blebs, and cilia (arrows). PAS stain. Bar = 10  $\mu\text{m}$ .  $\times 862$ .

vesicles from normal human endometrium. These vesicles were morphologically stable and were character-

ized by an apical, microvilli-bearing surface toward the external nutrient medium, whereas the basal surface contained a well-defined basal lamina facing the internal lumen. Two important requirements for vesicle formation were (a) the avoidance of collagenase digestion of the basal lamina after tissue mincing and (b) the use of serum-free, defined media. Although the nonenzymatic requirement was the more crucial of the two, the removal of serum had the important effect of causing an accelerated degeneration of the stromal cells, presumably due to their specific lipid requirement (2), which is normally obtained in conventional monolayer culture from the serum supplement. The use of defined media therefore actually favored the selective survival of the epithelium as well as providing the added benefits of having no undefined components present. Moreover, the omission of the serum actually seemed more important than the type of defined medium used. For instance, the ESF-1 medium, which was a complex mixture of hormonal and vitamin supplements, showed no material improvement over the use of the simpler PFMR-4 medium supplemented only with HC and INS although it should be noted that vesicles cultured in PFMR-4 alone were generally less viable. The cultured vesicles were more tedious to handle than the monolayer cultures and not as easily quantifiable; nonetheless this nonconventional culture system has several distinctive advantages over its conventional counterpart.

First, is the prolonged stability of the vesicles compared to the short-term viability of epithelial monolayers (4,10,12,14,18,23). Morphological viability ranged up to about 100 d for vesicles cultured in BM supplemented with HC ( $10^{-7}$  M) and INS (5  $\mu\text{g}/\text{ml}$ ) but only up to about 15 d for epithelial monolayer explants (12). In addition the epithelial cells within the vesicles more closely resembled the *in vivo* epithelial cells than did the epithelial cells derived from monolayer explants. Particularly prominent in this respect was a well-defined basal lamina present on the vesicle epithelial cells but absent on monolayer epithelial cells. However, the presence of this structure cannot entirely account for the epithelial stability. On occasions where the vesicles formed floating monolayers on the air-liquid interface, even though there was a basal lamina present, the cells nevertheless still underwent vacuolation and degeneration. It seems, then, that there is some inherent property of the three dimensional configuration that is important for prolonged morphological stability.

One interesting aspect of epithelial morphology within the vesicles was the occasional presence of cells with

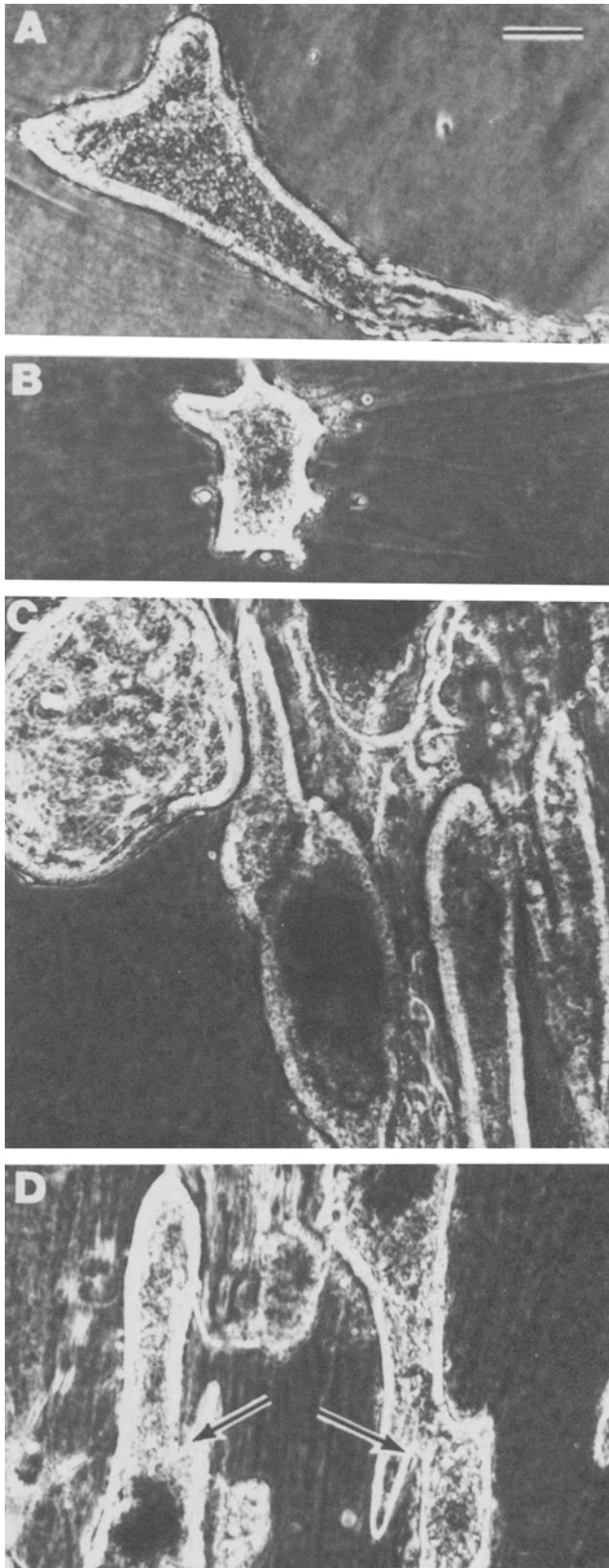


FIG. 8. Effect of collagen gel on the morphology of vesicles derived from a normal, proliferative endometrium. Vesicles, initially cultured in BM supplemented with DEX, INS, TF, and BSA as given in the text, were embedded and cultured within a collagen gel for 6 d. *A, B*, nonspherical vesicles with cuboidal epithelium. Bar = 100  $\mu\text{m}$ .  $\times 148$ . *C*, long, narrow, "glandular-like" vesicles lined with cuboidal epithelium. Bar = 100  $\mu\text{m}$ .  $\times 148$ . *D*, Branching points (arrows) on the long, narrow vesicles. Bar = 100  $\mu\text{m}$ .  $\times 148$ .



dark-staining bodies. Similar cells have previously been observed in mouse choroid plexus tissue *in vivo* (6) but their specific function has not been determined. Their presence in the endometrial vesicles is not therefore thought to represent an artifact of the culture process. In fact, further use of this culture system may help resolve the function of this conspicuous cell type.

Second, endometrial epithelial cells have demonstrated a relatively simpler nutrient requirement when cultured as vesicles as compared to when they were cultured as cell monolayers. Active DNA synthesis in vesicle epithelial cells required only a simple basal medium (PFMR-4) supplemented with HC and INS, a nutrient medium that would not support the attachment or outgrowth of conventional monolayer epithelial cells. In view of the acknowledged difficulty of culturing normal human epithelium as monolayers in conventional media, let alone in a defined medium, it is amazing that the vesicles were actively synthesizing DNA after having been cultured for about a month in the simple, defined PFMR-4 nutrient medium. It seems clear, then, that the nutrient requirement of the endometrial epithelial cell is a function of intercellular, spacial relationships or cell shape or both. Here again it seems logical to attribute the simplified nutrient requirement of the vesicles to their three-dimensional form.

Third, the epithelial cells displayed a sensitivity to sex steroid hormones only when arranged as vesicles and not

as cell monolayers. Progesterone, at physiological levels ( $10^{-7}$  M), selectively inhibited DNA synthesis in the vesicle epithelial cells but not in the floating monolayer cells. However, attached epithelial monolayers have previously (22) been shown to be inhibited by progesterone, but only at a much higher concentration ( $10^{-5}$  M). The vesicles also responded to a progesterone-estrogen combination with hypersecretory activity and by the development of cilia and subnuclear vacuoles, both features that are known to be estrogen-inducible *in vivo*. These observations are not surprising because it is now known that three-dimensional cellular shapes are important in the differentiation and physiological hormone sensitivities of both mammary (5,19,20,21) and thyroid (8,13) epithelium. It is important to note also that in the progesterone-treated vesicles the epithelial population was morphologically heterogeneous, consisting of two morphologically distinct cell types. One cell type was squamous and the other was cuboidal and glandularlike. This aspect of cellular heterogeneity within a single epithelium may be of particular importance in understanding the cellular mechanisms of hormonal stimulation of this classic target epithelium. It is not known if either of the two cell types are specific cell targets for progesterone or estradiol in this particular experiment. However, this culture system does provide the potential for answering the important question of whether the steroid hormones, or combinations thereof, act either focally on specific epithelial cells or whether they act more generally on the epithelium in a nonspecific "blanket" fashion.

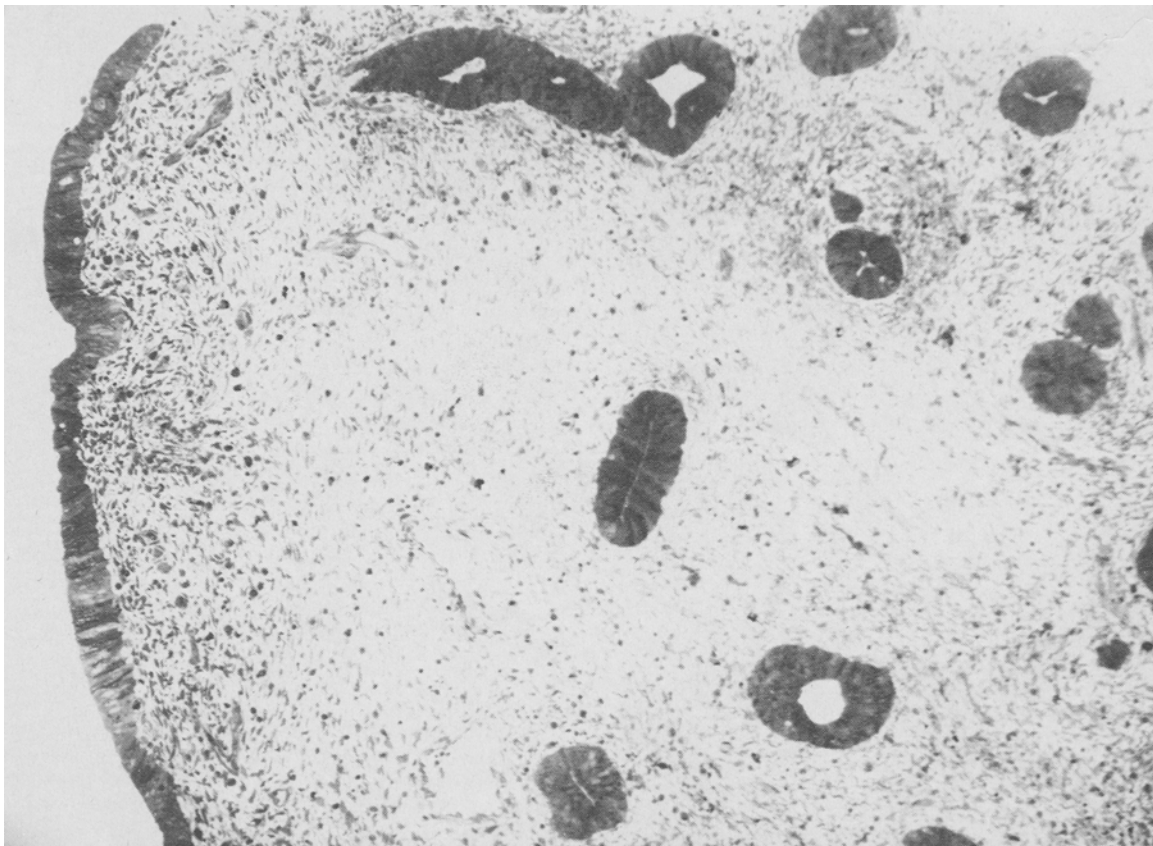


FIG. 9. Intact endometrial biopsy of the normal, proliferative endometrium described in Fig. 8. H&E.  $\times 119$ .

In summary, it seems that the three-dimensional shape of the epithelial vesicles has a critical role to play in their morphological stability, nutrient requirement, and hormone sensitivity.

Finally, it is pertinent to ask if these endometrial vesicle structures have any *in vivo* relevance. These vesicles do have an *in vivo* similarity and show at least an interesting preliminary hormonal sensitivity. However, all the endometrial samples studied, irrespective of their origin within the menstrual cycle, yielded vesicles with a secretory phenotype. This is probably due to the absence of some humoral or mesenchymal factor necessary for

maintaining the original differentiated state. Using collagen gel as a first step toward reconstructing a more *in vivo* tissue structure yielded interesting results. The vesicles embedded within the gel responded morphogenetically by forming long, narrow, branching structures characteristic of glands from a proliferative endometrium. Most surprising, however, the collagen induced a reversal of epithelial cell polarity, the collagen inducing the apical surface to change into a basal surface. This reversal of polarity by collagen has been shown before for cultured thyroid follicles (9) and possibly represents a common response of glandular cells to the collagen. The

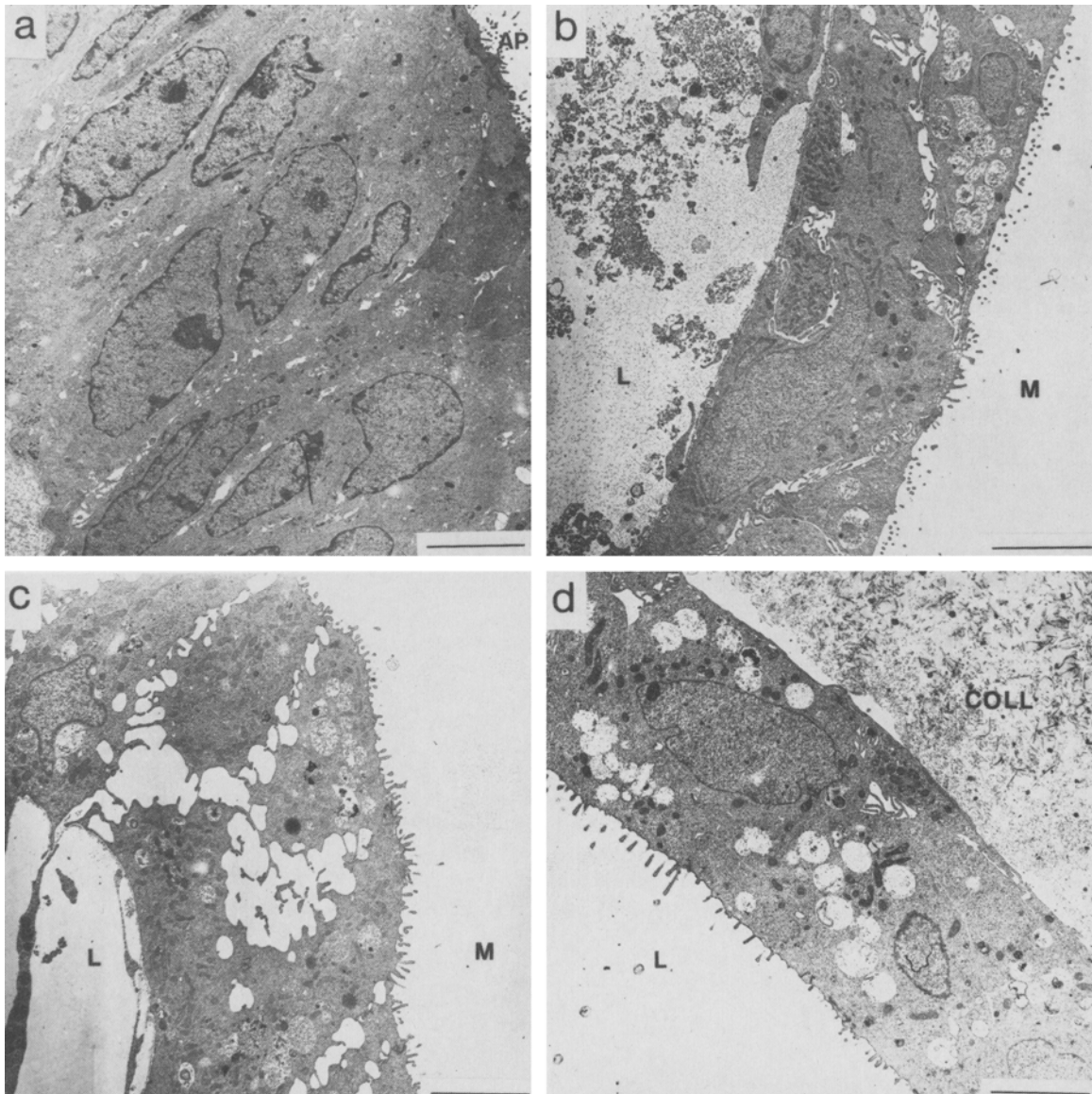


FIG. 10. Ultrastructural comparison of vesicles cultured in different media with their original, noncultured, endometrial epithelium. *Bar* = 5  $\mu$ m.  $\times 1917$ . *a*, original tissue from a normal, proliferative endometrium (as described for Fig. 8) before culture. *Note*: Tall, pseudostratified, columnar epithelium typical of the proliferative phase (*AP*=apical surface). *b*, vesicles cultured in BM with 10% FBS for 10 d (*M*=medium; *L*=lumen). *c*, vesicles cultured in BM for 10 d (*M*=medium; *L*=lumen). *d*, vesicles cultured within collagen gel for 6 d exactly as described for Fig. 8. *Note*: The vesicles have reversed their polarity (*L*=lumen; *COLL*=collagen).

collagen gel system provides a useful model for future studies on the effects of stromal cells or hormones or both on vesicle growth and differentiation.

More generally, epithelial cyst formation has been shown before in cultures of choroid plexus (1), thyroid (9), and even in insect cultures (15). This three-dimensional tissue arrangement seems to be one of the most profound requirements for normal epithelial cell function. Continued studies on the endometrial vesicles could therefore be expected to unravel the complex hormonal mechanisms that regulate the growth and differentiation of this cell type.

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