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ESTABLISHMENT AND MORPHOLOGIC CHARACTERIZATION OF NORMAL HUMAN ENDOMETRIUM IN VITRO

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SUMMARY

Tissue culture offers a model system with which to study the endocrine-mediated growth, differentiation, and metabolic activities of the endometrium. We have established and continue to maintain monolayer cultures of normal human endometrial epithelial cells from each phase of the menstrual cycle. At present, eight proliferative, two secretory, and two menstrual phase cultures have been established. These have been passed at least three times. One proliferative phase culture has been growing for 18 mo, and passed 10 times. Colonies of epithelioid cells as well as single cells appear in the cultures within 2 to 8 h of initial culture and maintain this appearance throughout long-term growth. The cells are periodic acid Schiff positive for carbohydrates and positive for keratin, an immunochemical marker for epithelial tissues. Studies comparing the ultrastructure of the cultures with fresh endometrial tissue revealed morphologic features common to both, including prominent nucleoli, Golgi, mitochondria-rough endoplasmic reticulum complexes, and abundant glycogen. The cells are not tumorigenic in the nude mouse and do not form colonies on soft agarose, confirming the nonneoplastic identity of the cells.

Key words: normal endometrium; tissue culture; epithelium; light microscopy; electron microscopy.

INTRODUCTION

The human endometrium is one of the most dynamic tissues in the body, undergoing morphologic and biochemical changes during the menstrual cycle. These cyclic alterations are under the control of the hypothalamus-pituitary-ovary endocrine axis. Histologically, the endometrium is a complex tissue, consisting of a surface and glandular epithelium supported by a network of fibroblasts and stromal cells unique to the endometrium. Studies of the mechanism of hormonal control of endometrial physiology are complicated by the heterogeneity of the tissue and the difficulty in separating and identifying the cell types.

Several previous reports have described the culture of human endometrium with varying degrees of success (1-9). Most reports, however, claim that the epithelial cells survived in vitro for only a few weeks and could not be subcultured (5,7-9). Trent et al. (8) isolated and subcultured epithelial cells from outgrowths of endometrial explants, and Liu and Tseng (6) also described epithelial cell lines that were propagated. Satyaswaroop et al. (7) described a method to isolate and culture intact endometrial glands free of stroma. However, these investigators had limited success with subculture, most cultures not surviving passage. More recently, Varma et al. (9) and Dorman et al. (2) reported the morphologic characterization of three cell types (epithelial, stromal, and fibroblastic) present in human endometrial monolayer cultures. These investigators have been unable to subculture the epithelial cells.

We report the establishment and characterization of cultures of human endometrial epithelial

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cells from each phase of the normal mentrual cycle. These cultures have been subcultured and maintained in vitro from 14 to 70 wk. The cells exhibit typical epithelial morphologic features and stain positively for keratin, an intracellular marker protein for epithelial cells.

Materials and Methods

Tissue acquisition. Human endometrium was acquired from patients undergoing dilatation and curettage (D & C) as a diagnostic procedure. Upon removal, portions of the tissue were prepared for tissue culture and light and electron microscopy as outlined below. The remainder of the tissue was placed in 10% buffered formalin and submitted to the Anatomic Pathology Laboratory, Naval Hospital, Bethesda, MD, for routine histologic examination. Only those tissues indicating no histologic abnormalities and which were a normal stage of the menstrual cycle were utilized for this research study.

Tissue culture. Immediately after removal, the tissue sample was placed in culture medium supplemented with penicillin-streptomycin and Garamycin (M.A. Bioproducts, Walkersville, MD) and transported to the laboratory.

The tissue was minced into 1 to 2-mm^3 pieces, placed in a 0.025% trypsin-EDTA (0.025%) solution, and digested at 37° C for 0.5 to 1 h with gentle shaking. The final volume of the digestion medium was approximately 5.0 ml. The digestion process was ended by addition of culture medium (twice the volume of the original digestion medium) supplemented with 20% fetal bovine serum. The cell suspension was washed three times with culture medium followed by centrifugation at 800 ×*g*. The cell suspension and the remaining tissue pieces were seeded into 25-cm^2 flasks in a volume of 5.0 ml and maintained as a closed system at 37° C. The initial cell numbers were not determined because many tissue pieces remained after trypsinization. The cell suspension and the tissue fragments were either combined or placed into separate vessels. The media were changed every other day or as needed. The initial culture medium consisted of Medium 199 (M.A. Bioproducts, Walkersville, MD) supplemented with glutamine (0.6 mg/ml), pyruvate (1.0 m*M*), insulin (5 μg/ml), silenium (0.005 μg/ml), transferrin (5 μg/ml), non-essential amino acids (0.1 m*M*), penicillin-streptomycin (50 U/ml), Garamycin (50 μg/ml) and 20% heat-inactivated fetal bovine serum (Sterile Systems, Inc., Logan, UT). After approximately 1 wk the medium was changed to RPMI 1640 (M.A. Bioproducts) supplemented as above.

When the monolayer cultures reached confluency, the cultures were trypsinized (0.025% trypsin-EDTA) and transferred to additional flasks. Repeated trypsinization and passage seemed to remove most fibroblast infiltration.

Microscopy. A portion of the original patient sample was prepared for light and electron microscopy and utilized as a reference for this and other protocols.

Light microscopy of cultures. The cultures were observed in situ on a twice weekly basis using inverted phase contrast optics. They were photographed using Kodak PanX black and white film.

For special staining procedures, at the initial culture or at passage, cells were seeded onto clean glass slides housed in sterile petri dishes. The dishes were maintained at 37° C, 5% CO_2. After approximately 1 to 2 wk, the slides were rinsed with warm (37° C) phosphate buffered saline and prepared as follows.

To confirm the glandular nature of the cultured cells, the cells were stained for periodic acid Schiff (PAS). The epithelial, glandular, and stromal cells will stain with PAS. For PAS staining, the slides were fixed with 10% neutral buffered formalin (pH 7.5), stained with PAS, and counterstained with hematoxylin using routine techniques (10).

Keratin proteins are now recognized as ubiquitous in human, rabbit, and rodent epithelial tissues and epithelial cultured cells (11,12). To determine that the endometrial cultures were composed of the surface and glandular epithelial cells rather than stromal elements (stromal and fibroblasts), slide preparations of the cultures were stained for keratin. Only the epithelial glandular and surface epithelial cells will stain for keratin proteins. The cells were cultured on glass slides, as mentioned above, and fixed after 1 to 2 wk in absolute cold methanol. The slides were stained using the peroxidase-antiperoxidase (PAP) technique available as the Immunoperoxidase Staining Kit (Immulok Histoset, Scientific Products, State College, PA). After staining and cover slip mounting, the slides were viewed in a Leitz Laborlux microscope and photographed. Reddish brown staining is considered a positive reaction for keratin. For control, a slide was stained without the antikeratin antibody. This slide did not exhibit a red-brown stain. In

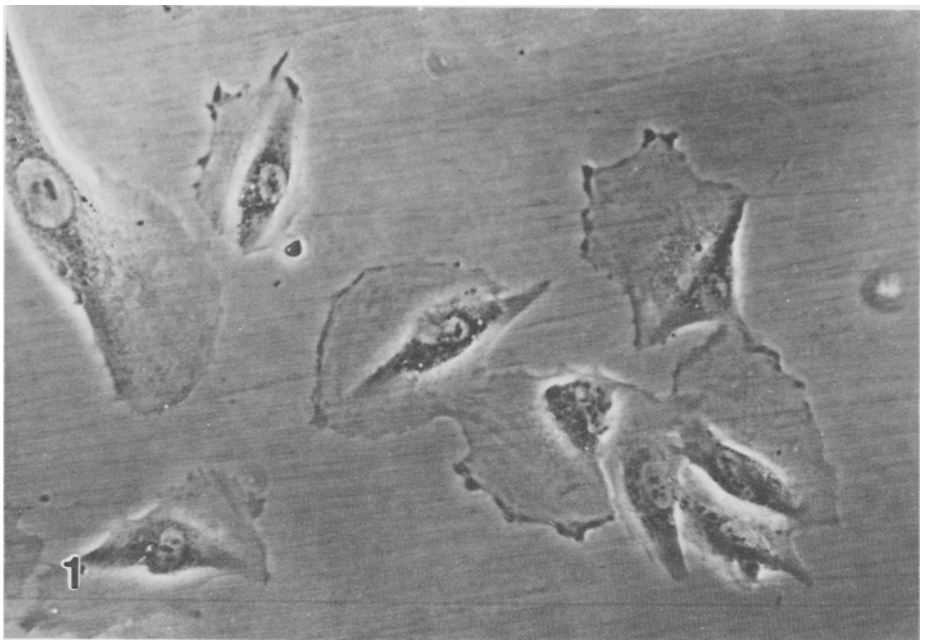


FIG. 1. Inverted phase contrast photograph of normal human endometrium in vitro. The cultures were composed of flat, pancake-shaped epithelioid cells, with a prominent nucleus and nucleolus. A few binucleated cells were present. The nucleus was surrounded by dense granules. ×200.

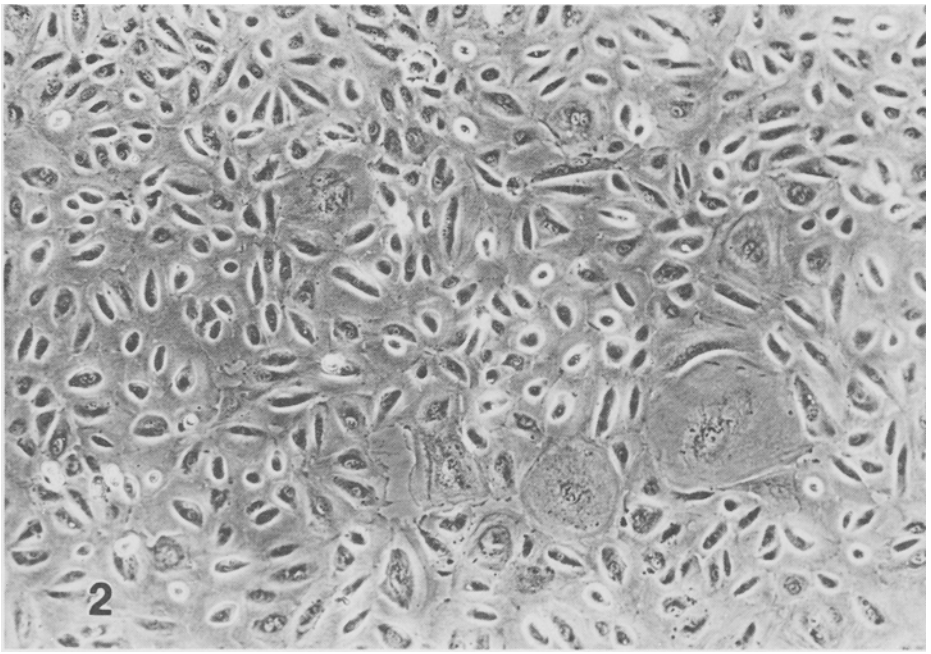


FIG. 2. Confluent monolayer culture of normal human endometrium in culture. The cells are pancake-shaped epithelioid cells. ×150.

addition, a positive control tissue (epidermis) was stained for keratin using this technique.

Electron microscopy of cultures. Selected flasks were fixed in Karnovsky's fixative (2.5% glutaraldehyde 2% paraformaldehyde) for 1 to 2 h and postfixed in 2% osmium tetroxide for 1 h. The flasks were further processed, and the cultures were embedded in situ in Epon using the technique of Douglas et al. (13). The specimens were sectioned on a Sorvall MT-5000 ultramicrotome; thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate. The specimens were viewed in a Zeiss 10A transmission electron microscope and photographed using Kodak plate film. Representative cultures were examined in the transmission electron microscope approximately every 2 mo during the entire culture period to determine if long-term culture modified the cell ultrastructure.

RESULTS

Establishment of cultures. There are currently 12 normal proliferative phase cultures, ranging from 15 to 70 wk (mean = 24.8 ± 4.3 wk) in culture. Additionally, five secretory phase cultures ranging from 14 to 26 wk (mean = 19.2 ± 2.8 wk) in vitro and two menstrual phase cultures 22 to 24 wk (mean = 23 ± 1.0 wk) in vitro are currently being maintained by this laboratory.

Efforts to develop a suitable whole cell steroid (estrogen and progesterone) receptor-binding assay for cultures of normal and malignant endometrium have been unsuccessful in this laboratory and thus could not be used as a criterion for characterization of these cells.

Morphology. Within 24 to 48 h the cells attached to the flask and assumed a polygonal, epithelioid appearance. With the first medium change, contaminating red and white blood cells were removed. There seemed to be no qualitative difference between the cells during each stage of the cycle. The epithelial cells appeared as both single, pancake-shaped epithelioid cells (Fig. 1), small colonies of epithelioid cells, and dense colonies of cells (Figs. 2 and 3). The cells composing the dense colonies appeared as both large polygonal-shaped cells (Fig. 2) and elongated cells that grew as whorls (Fig. 3). Both the single cells and colonies continued to grow and all cultures have been subcultured. The cultures reached confluency some 4 to 6 wk after plating in control medium (no added hormones).

At least 95% of the cells composing all cultures (proliferative, secretory, and menstrual phases)

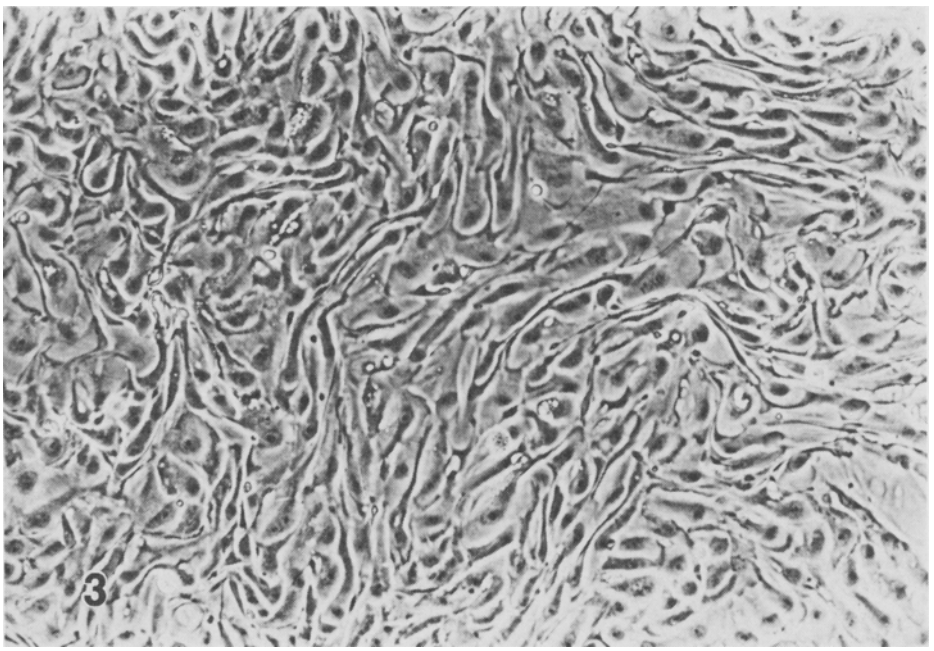
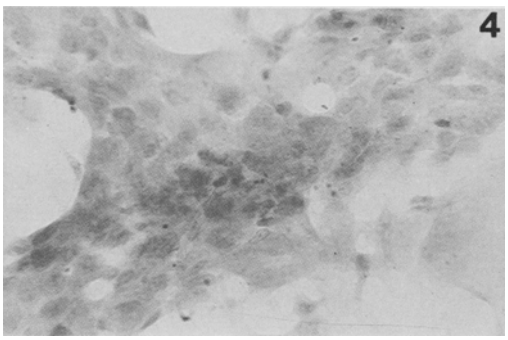
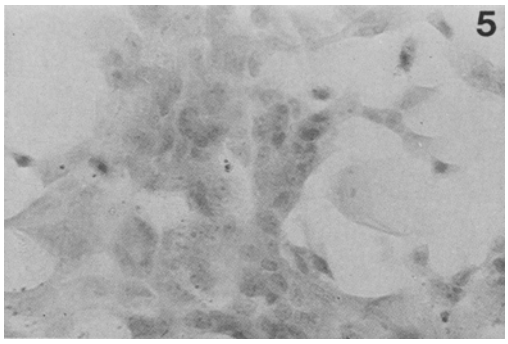


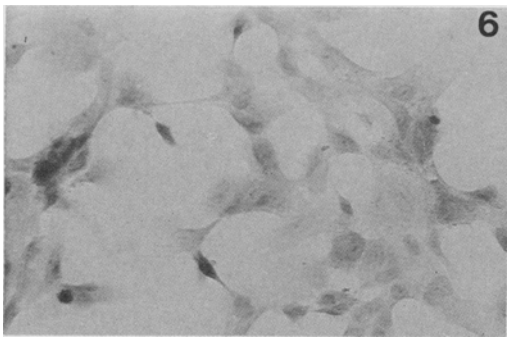
FIG. 3. Confluent colony of normal human endometrium in culture. Dense colonies often assumed the appearance of whorls of epithelioid cells with elongated cell processes. ×150.

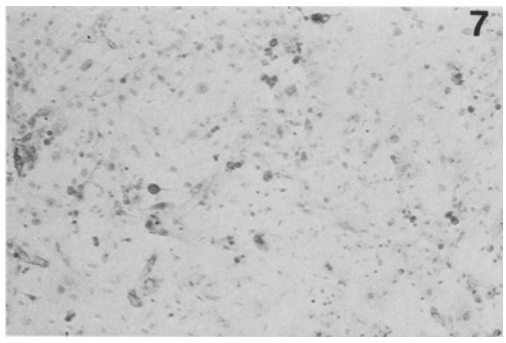
are PAS positive for carbohydrates (Fig. 4–6) and stain positively for keratin, a marker for epithelial tissues/cells (Figs. 7–9). With long-term culture, the numbers of nonkeratin positive cells present in the cultures seemed to be reduced as a result of repeated trypsinization, i.e. passage.

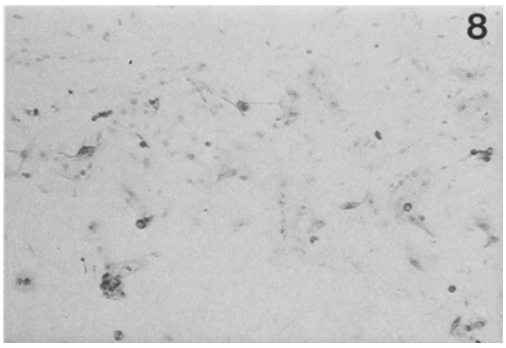
At least 1×10^6 cells were implanted in pellet form into each nude (athymic) mouse. Within a 3.5 mo observation period, the normal cell cultures did not form tumors when injected into two out of two nude mice. (Malignant cells readily form tumors when injected into nude mice; the











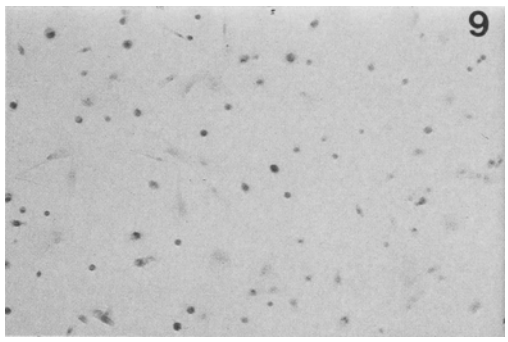


FIG. 4. Periodic acid Schiff-positive proliferative phase culture. PAS stain. ×140.

FIG. 5. Periodic acid Schiff-positive secretory phase (normal) endometrium in vitro. ×140.

FIG. 6. Periodic acid Schiff-positive culture of normal menstrual phase endometrium. ×140.

FIG. 7. Normal proliferative phase culture stained using immunoperoxidase method for keratin. A red-brown stain is positive for keratin. ×56.

FIG. 8. Normal secretory phase culture stained using the immunoperoxidase method for keratin. A red-brown stain is positive for keratin. ×56.

FIG. 9. Normal menstrual phase culture of human endometrium stained with the immunoperoxidase method for keratin. A red-brown stain is positive for keratin. ×56.

normal lag period for development of the tumors has been 6 to 8 wk in our mice colony.) Additionally, when 10^3 - 3×10^3 cells were seeded into agarose plates, the cells (at all concentra-

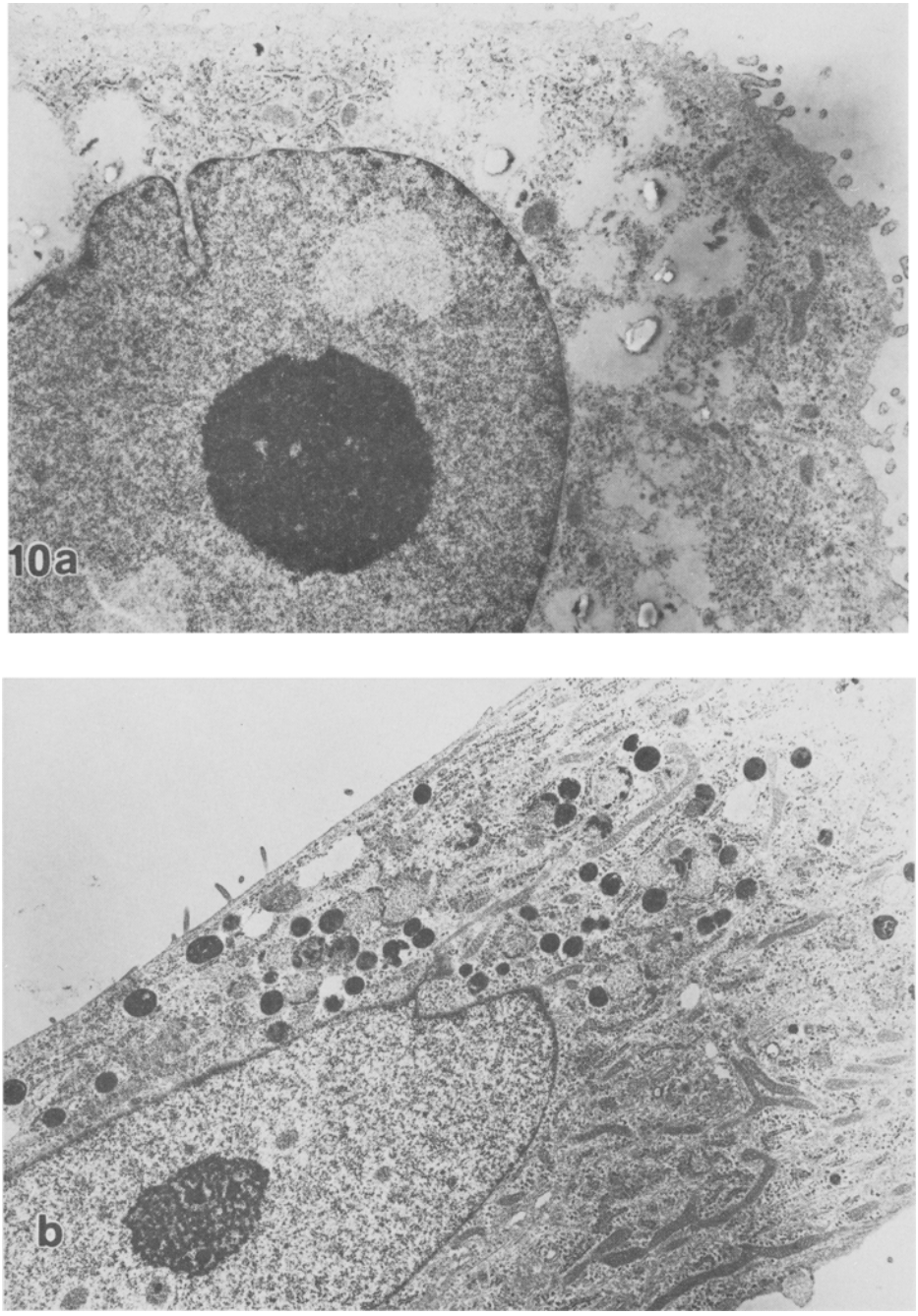


FIG. 10 *a* and *b*. Electron micrographs of normal human proliferative phase endometrial cells in culture. This cell exhibited a large nucleus with a prominent nucleolus. The nucleus was surrounded by large droplets of lipid, which appeared as the dense granules seen in the light micrograph. The cells contained numerous profiles of RER in association with pleomorphic mitochondria. Note particularly the surface microvilli, which characterizes these cells as epithelial cells. ×10 000; ×4725.

tions) did not form colonies on the semisolid medium (14). This confirms the nonneoplastic identity of the cells, the cells maintaining a normal character after short- and long-term culture.

Electron microscopy. Figures 10–14 are representative electron micrographs of normal proliferative, secretory, and menstrual phase endometrium that have been maintained in vitro for approximately 20, 16, and 16 wk, respectively.

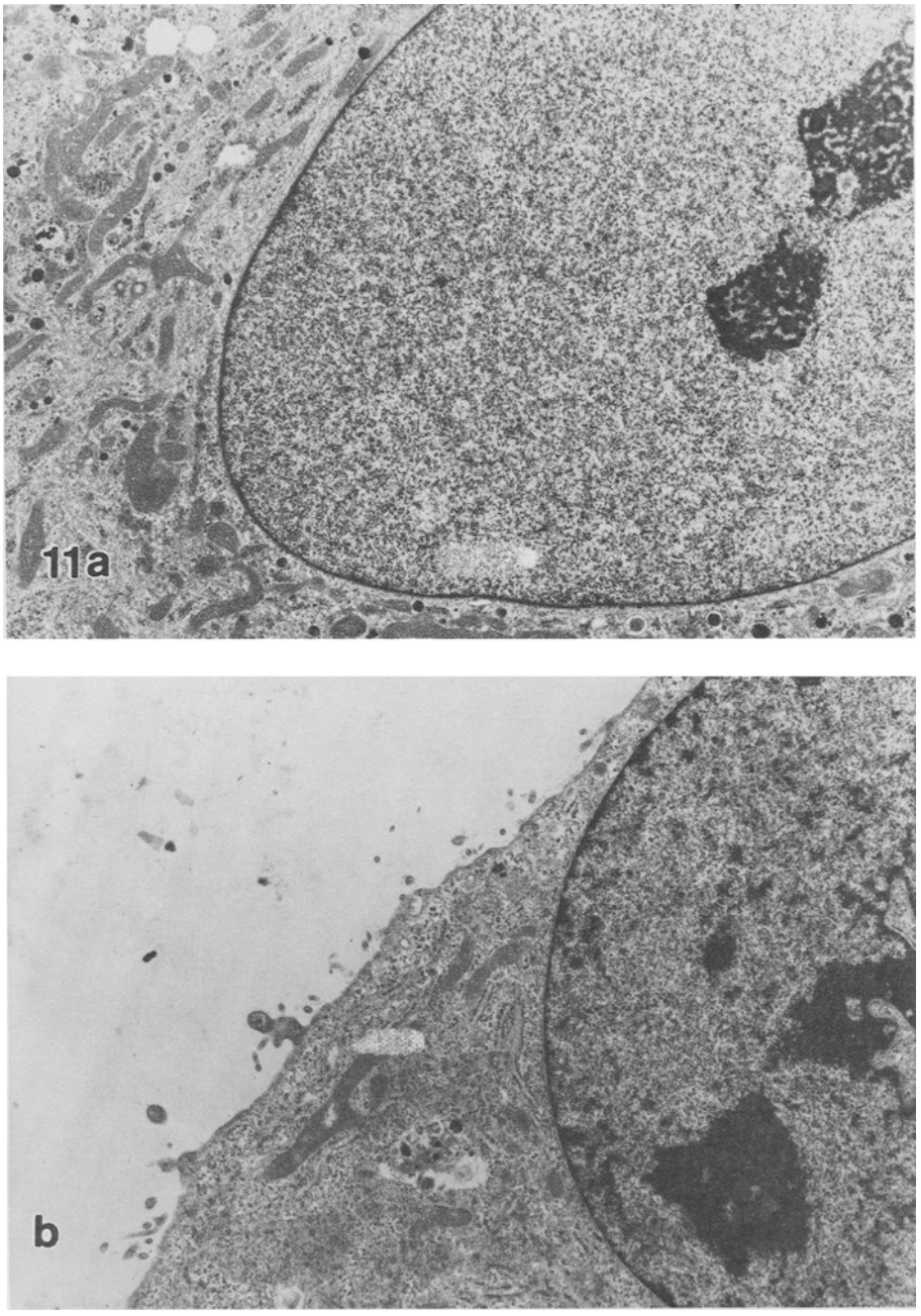


FIG. 11 *a* and *b*. Electron micrographs of normal secretory phase endometrial cells in vitro. Note the numerous pleomorphic mitochondria and the surface filopodia and microvilli. ×11 000; ×10 000.

Representative cultures from each group, examined approximately every 2 mo, seemed to maintain the characteristic ultrastructural features throughout the long-term culture period. The proliferative phase cells were characterized by a large nucleus with a prominent nucleolus

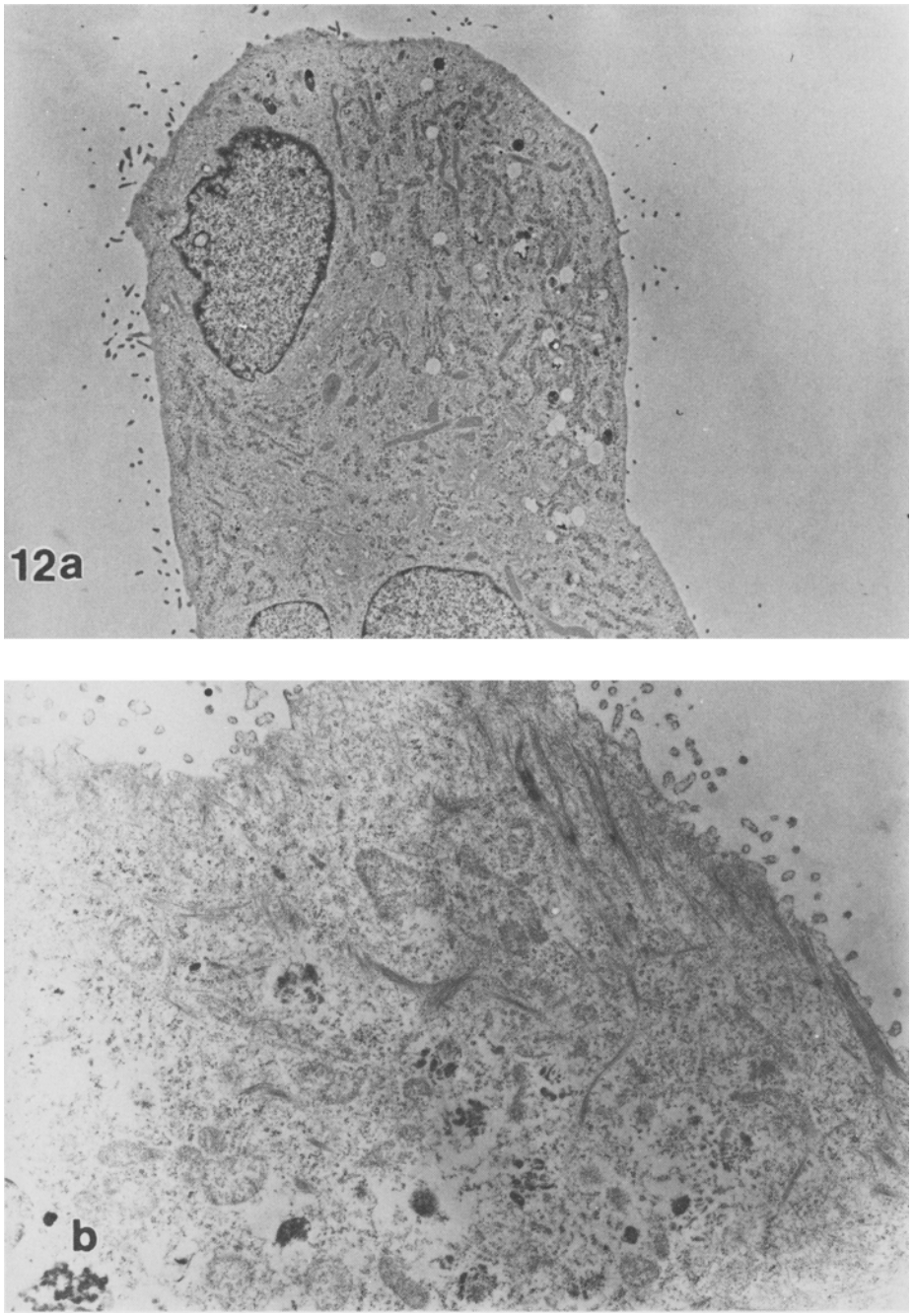


FIG. 12 *a* and *b*. Electron micrographs of normal menstrual phase endometrial cells in vitro. The cells exhibit numerous pleomorphic mitochondria and several lysosomes in the perinuclear region. Note the numerous surface microvilli. ×3000; ×5000.

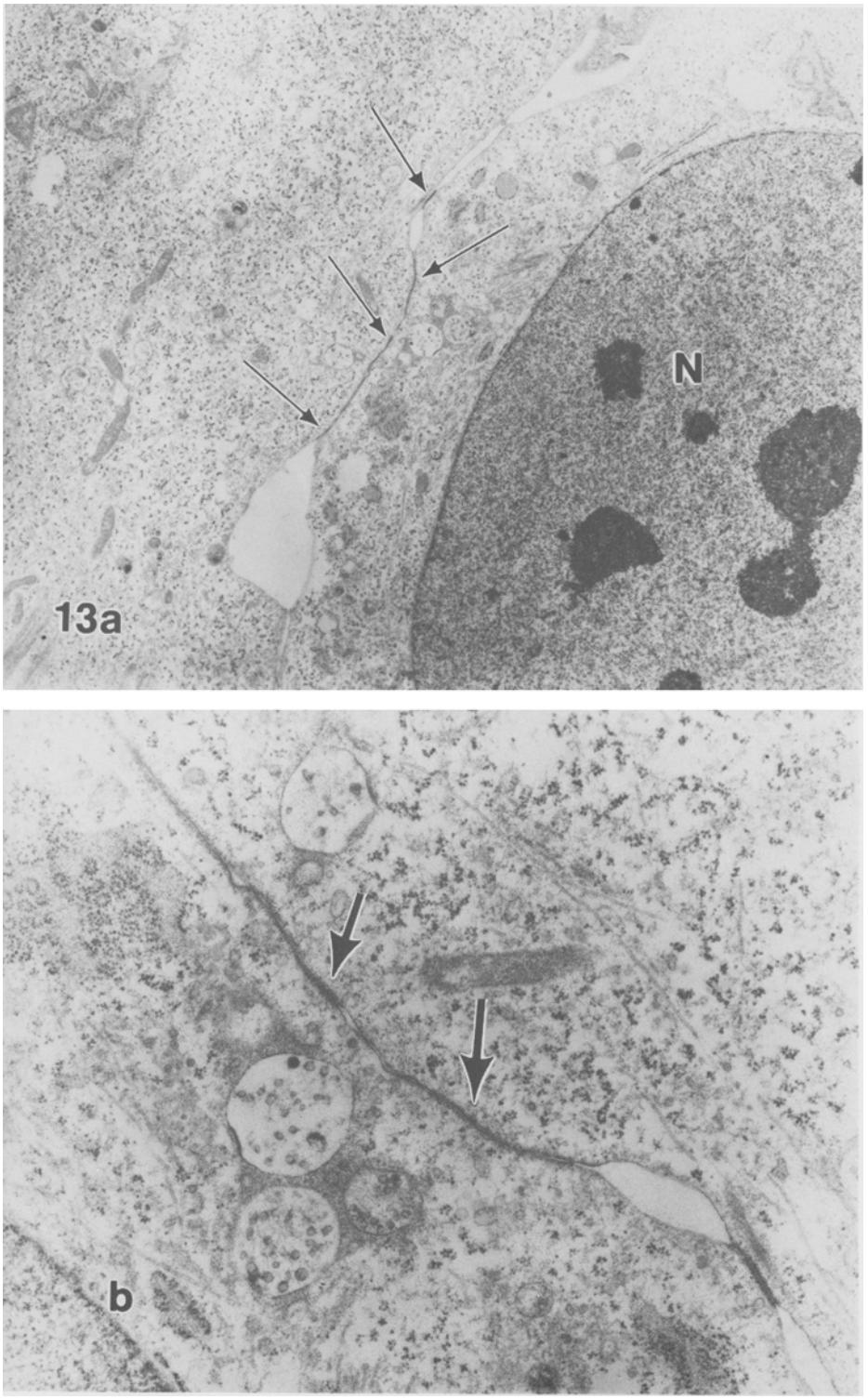


FIG. 13. *a*, Low power electron micrograph of two endometrial epithelial cells in culture. Note the junctional complexes at the arrows. These are shown to better advantage at higher power in *b*. *N* = nucleus. ×4000. *b*, Higher power of the junctional complex shown in the previous micrograph. These junctions are typical of epithelial cells. ×15 000.

(Fig. 10 *a*, *b*). The cells exhibited numerous mitochondria with associated rough endoplasmic reticulum (RER). The exposed surface of the cells exhibited numerous microvilli, a characteristic of epithelial cells and the endometrial epithelial glandular component in situ (Fig. 10 *a*, *b*).

Figure 11 *a*, *b* are electron micrographs of normal secretory phase endometrium in vitro. The cells were characterized by a large nucleus, a prominent nucleolus, and numerous pleomorphic mitochondria (Fig. 11 *a*, *b*). There was no evidence of a nucleolar channel system characteristic of the secretory phase, presumably due to the lack of progesterone in the culture medium. These cells also exhibited surface microvilli and filopodia characteristic of epithelial cells (Fig. 11 *a*).

Figures 12 *a*, *b* are representative micrographs of menstrual phase endometrium in culture. These cells also exhibited large nuclei and numerous pleomorphic mitochondria. Lipid droplets and lysosomes seemed more numerous. Surface microvilli were also typical of the cultured cells.

Figures 13 *a*, *b* demonstrate the junctional complexes that are typical of epithelial cells and that aided the characterization of these cultured endometrial cells as epithelial components of the endometrium. The cells were joined by typical tight junctional complexes, as well as the gap or nexus-type of junction. Desmosomes were also present.

Figure 14 is an electron micrograph of a proliferative phase cell after approximately 12 mo in culture. The cell exhibited abundant dilated rough endoplasmic reticulum, characteristic of endometrial epithelium, as well as abundant lipid and ribosomal whorls. The cells retained ultrastructural characteristics of normal endometrium and seemed metabolically active after long-term culture.

Discussion

We have established and continue to maintain cultures of the epithelial component of normal human endometrium from each stage of the menstrual cycle. These cells continue to grow in culture and have been subcultured.

The epithelial identity of these cells has been confirmed using morphologic criteria. The cells are PAS positive for carbohydrates, a characteristic of the glandular component of human

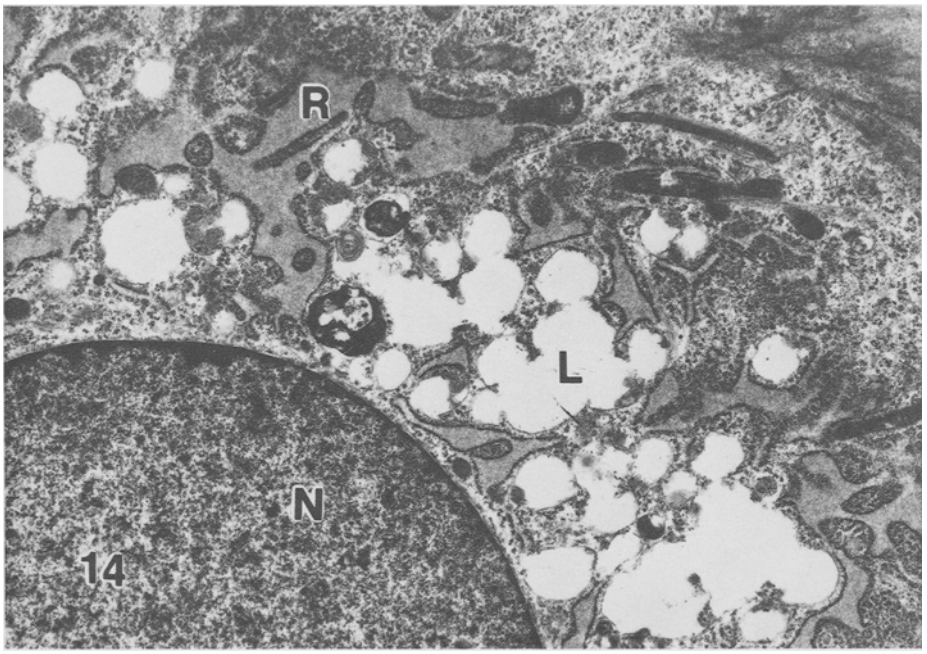


FIG. 14. Electron micrograph of proliferative phase cell after approximately 12 mo in vitro. Note the dilated RER (*R*) and abundant lipid (*L*). *N* = nucleus. ×10 000.

endometrium. Most importantly, the cells stain positively for keratin, a marker for epithelial tissues and cells (11,12). One would not expect elements of the stroma to stain for keratin, inasmuch as these cells are of mesenchymal origin and keratin is found only in epithelial cells of ectodermal origin. The majority of cells present in the cultures stained positive for keratin. In early cultures, several cells were present that did not stain for keratin. These cells were thought to be contaminating stromal cells or fibroblasts. With long-term culture, the percentage of these "contaminating" cells was reduced to less than 5%.

Ultrastructurally, the cells exhibit features characteristic of epithelial cells and the endometrium. These features include surface microvilli and junctional complexes typical of epithelia, as well as a large nucleus and a prominent nucleolus, perinuclear filaments, abundant glycogen, and large mitochondria with associated rough endoplasmic reticulum (4,5).

After long-term culture (longer than 4 wk) the epithelial cells were implanted into the nude (athymic) mouse and were seeded onto semisolid medium. These procedures are used routinely to assay for neoplasmic cells or tissues (14) and were used in this study to confirm that the cells had not transformed after long-term culture. The cells, including those grown in vitro for 70 wk, did not form tumors in the nude mouse and did not form colonies on semisolid medium.

Varma et al. (9) and Dorman et al. (2) have reported the culture and identification of three cell types present in endometrial cultures. We could not distinguish three cell types, but only one cell type. Furthermore, the epithelial identity of the cells described in this study has been unequivocally confirmed by special staining for keratin. Additionally, the cells described in this protocol exhibit numerous surface microvilli, dilated RER and mitochondria—RER complexes characteristic of endometrial surface and glandular epithelial cells (5).

We continue to examine these normal endometrial cultures throughout the culture period. The growth rate of the cells is relatively slow, confluency being reached in 4 to 6 wk. Growth studies and steroid-receptor analyses are currently in progress or planned. Additionally, we are investigating the use of steroid supplements to the medium for increasing growth rate.

The in vitro system provides an easily manipulated and controlled experimental model for the study of endometrial epithelial physiology. With the establishment and characterization of normal human endometrial cell lines the study of the physiology of the normal endometrial cell and its malignant transformation will become the forefront of basic research.

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