MORPHOLOGICAL STUDIES OF UTERINE AND CERVICAL EPITHELIUM IN PSEUDOPREGNANT RABBITS

Hiroyuki SUZUKI and Yoshio TSUTSUMI

(Department of Animal Science, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan)

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Introduction

Previous studies have established that the progestational endometrium of the rabbit is characterized by fold-branching of mucosa, proliferation of the epithelium and cell fusion leading to multinucleated cells.[1,8,9,20,23,26,30] The progestational transformation of the uterine mucosa is known to be related to the hormonal events following ovulatory stimulus and to prepare consequently a favorable environment for blastocyst implantation. During the preimplantation period, intrauterine blastocysts migrate for even spacing,[6,31] whereas some unfertilized eggs in pseudopregnant rabbits begin to appear in the vagina from 4 days following ovulatory stimulus,[32] suggesting early establishment of maternal recognition of pregnancy. In a study of plasma progesterone concentrations in the rabbit, FUCHS and BELING[13] considered that the maternal ovary is sensitive to the uterine blastocysts during early preimplantation stage and that fertilized ova exert a luteotropic influence in this period.

However, the functional role of the cervix, which should be acting to hold eggs in the uterine lumen, is not fully understood. Previous studies in our laboratory showed that contractile activity of the rabbit cervix, as well as the uterus, is nearly suppressed after mating, regardless of pregnancy,[27] and that the mucosal folds of the cervix, forming a narrow lumen, are not markedly transformed during early pregnancy.[25,26] It was also demonstrated that secretory material in the cervical epithelium of pregnant rabbits is reproduced anew and released into the cervical lumen prior to implantation.[26] It is unclear how the increased amount of cervical mucus influences the maintenance of pregnancy, although considerable information is available on sperm transport through the cervical canal filled with abundant mucus.[5,14]

In the present study, the cervical mucosae of pseudopregnant rabbits were examined with light and scanning electron microscopy to investigate whether a preimplantational increase in the cervical secretory activity depends directly on the effect of ovarian steroids or on the presence of the blastocyst in the uterus. This report also deals with a study of the uterine epithelium of the same rabbits to serve as a useful indicator of the progestational effects and to examine the changes of the surface morphology during pseudopregnancy.

Materials and Methods

Thirty-two mature, female Japanese White rabbits which were reared individually in cages were given an intravenous injection of 50 IU of human chorionic gonadotropin (hCG ; Primogonyl, Schering). The day of injection was designated as Day 0. The animals were divided into eight groups which were sacrified on Days 2, 4, 5, 6, 7, 10, 14 and 18, respectively.

The reproductive tracts were immediately removed after killing, trimmed of excess fat and assigned to the following procedures.

For light microscopy (LM), one uterine horn, with the attached ipsilateral portion of the cervix, was pinned on Sealant (Dow-Corning 780) and immediately fixed in Bouin's or Carnoy's solution. After a few hours, samples were cut into smaller segments, followed by refixation for a longer period, and were embedded in paraffin. Paraffin sections of 5–7 μm thickness were stained with hematoxylin and eosin (H. E.) or azan, or by the periodic acid-Shiff (PAS) method.

Samples for scanning electron microscopy (SEM) were taken from the mid-portions of the uterine horn and portion of cervix contralateral to those subjected to the LM procedure, and the tissues were cut open longitudinally and washed gently with physiological saline solution. The tissues were then pinned on Sealant and pre-fixed with 3% glutaraldehyde in 0.1 M phosphate buffer at 4°C. After 24-hour fixation, the samples were dissected into smaller, appropriate segments, rinsed in phosphate buffer solution and post-fixed in 1% phosphate-buffered osmium tetroxide for 1–1.5 hours at 4°C. The tissues were then washed in distilled water and dehydrated in a graded series of ethanol. Following dehydration, the samples were transferred to liquid CO_2, using isoamyl acetate as the intermediate fluid for critical-point drying. Dried tissues were fastened on brass stubs and sputter-coated with gold in an Ion Sputter (JFC-1100, JEOL, LTD). All samples were subsequently observed with a JEOL JSM-200 scanning electron microscope operated at 10 kV accelerating voltage.

Results

I. Uterus

Days 2 and 4: Mucosal folds had begun to invaginate into narrow recesses on Day 2 and were subsequently branched further, forming crypts on the mucosal surface (Fig. 1). Uterine glands were increased and mostly elongated. Surface epithelium was proliferated and numerous mitoses were found in the surface and glandular epithelia, predominantly in the glands. The surface and cryptal epithelium was pseudostratified in many places, but the glandular epithelium remained simple columnar (Fig. 2), so that the differentiation between the cryptal and glandular epithelium was evident. SEM revealed that the epithelial cells around the openings of uterine glands exhibited distinct cell boundaries, similar to the estrous endometrium observed previously,[26] but at a short distance from the glandular orifices the cell boundaries had become unclear on Day 2 (Figs. 3, 4). On Day 4, non-ciliated cells were morphologically differentiated into two distinct types (Figs. 5, 6). One type exhibited a relatively flat, free surface and its boundary was delineated with crowded microvilli. The other type of cell was characterized by an apical swelling. Ciliated cells were frequently observed on Day 2, but they were decreased in number and located sporadically on Day 4. Some of the ciliated cells presented unique SEM images: those having stubby, short cilia on the overall cell surface (Fig. 6) and those with normally long cilia on the periphery of the cell surface (Fig. 4).

Days 5 and 6: The mucosal folds were remarkably branched in comparison with those in earlier stages (Fig. 7). Consequently, slender folds with narrower connective tissue cores formed relatively wide cryptal lumina. The cryptal epithelium was connected to the glandular epithelium, and the glandular orifice opened into the bottom of the cryptal lumen. The surface and cryptal epithelia were characterized by extreme crowding of the columnar cells and by cytoplasmic protrusions, resulting in an irregular border of epithelial surface (Fig. 7). In SEM, almost all of the superficial cells showed convex surfaces (Fig. 8), possibly corresponding with the apical cytoplasmic protrusions noted by LM. Microvilli on these cells were sparse or nearly absent. Apical convex surfaces were further protruded in a dome-like shape on Day 6 (Fig. 9).

Days 7 and 10: The surface and cryptal epithelial cells had evidently changed into multinucleated cells (Figs. 10, 11). Although some non-ciliated cells remained similar in size to those of earlier stages, others had become quite large. The apical surfaces of the cells were densely covered with

short microvilli. The process of cell fusion had progressed and extended on Day 10, resulting in increased size of the cells and increased number of nuclei within a single cytoplasmic envelope (Figs. 12, 13). Signs of regressive changes, pycnosis and fragmentation of nuclei, in the multinucleated cells were only rarely observed on Day 10 (Fig. 12).

Day 14: Histological appearance of the endometrium was quite similar to that on Day 10. However, there were occasional signs of regressive changes such as nuclear disruption (Fig. 14). Moreover, cystic vacuoles containing debris of epithelial cells were frequently observed intervening between the multinucleated cells. Characteristic SEM images of ciliated cells in this stage showed sparse cilia at the central portions of the cells and microvillous surfaces at their peripheries (Fig. 15).

Day 18: The endometrial surface had become relatively smooth, displaying numerous openings of the uterine glands again. The number of nuclei within each multinucleated cell had diminished and the epithelium was thinner (Fig. 16). A degenerating appearance of the endometrium was evident in all animals examined. Mononuclear cells had appeared, and in LM their nuclei and cytoplasm were relatively translucent as compared with those of the multinucleated cells. In general, epithelial cells were covered with very small, stubby microvilli which were distributed somewhat more densely on the marginal part of the cell surface than on the central surface of the cell (Figs. 17, 18). One or more isolated, short cilia emerged on the free surface of each microvillous cell. Fully ciliated cells had increased in number and cells having normally long cilia on their peripheries were occasionally noted. On the whole, the endometrium appeared to be restored to the estrous condition.

Secretory material: Azan- and PAS-stained material was seen partially at the luminal border of the endometrium on Day 2; but no stained material existed within the epithelial cells on Days 4–7, although it was abundant in the uterine lumen. On Day 10, the secretory material in the lumen had increased further, and it had reached a maximum on Day 14. The apical cytoplasm of the endometrial cells on Day 18 was stained by both staining methods and luminal secretory material had decreased in amount.

II. Cervix

Cervical folds did not change in appearance throughout all stages examined. No consistent alterations in proportion and surface morphology of the ciliated cells were noted during pseudopregnancy, except for some minor morphological changes in the ciliated cells, such as appearance of ciliary buds, tapering and bending of tips of cilia, and limited location of

cilia on the cell surface, as reported previously.[26] There were some variations in height of the ciliated cells, but no alteration in their internal structure. Most distinct changes were noted in the non-ciliated secretory cells.

On Day 2, most non-ciliated cells contained a small amount of secretory material in their apical portion, just beneath the luminal surface (Fig. 19). Peg cells in the epithelium were rare. In SEM, microvilli on the surfaces of non-ciliated cells displayed some variations. In some cells they were elongated and/or distributed more densely as compared with their estrous appearance,[26] whereas in others they were fewer or had disappeared (Fig. 20). A few small hollows (depressions) were occasionally observed on the apical surfaces of the cells. In addition, the free surfaces of some non-ciliated cells were populated by superficial structures of various shapes and sizes (Fig. 21). These structures displayed a relatively smooth surface due to the absence of microvilli.

In three of four rabbits examined on Day 4, the secretory material within the cells had diminished in amount and peg cells had increased in number (Fig. 22). In the other animal the epithelium was more hypertrophic and the epithelial cells contained much secretory material in their supranuclear portions. In SEM, almost all non-ciliated cells displayed abundant, slender microvilli on their apical surfaces (Fig. 23).

The secretory activity of the cervical epithelium had begun to increase on Day 5 and had reached its initial peak on Day 6, when abundant secretory material (stained strongly or moderately in azan- and PAS-sections) was located in both the cells and the lumen (Fig. 24). Some of non-ciliated cells contained secretory material under the nuclei. Peg cells had decreased in number on Day 6. SEM demonstrated that the distribution of microvilli on the non-ciliated cells varied from dense to complete absence. On Day 6, the apical portions of cells having no microvilli markedly protruded into the lumen, and reached the height of the ciliary tips of adjacent ciliated cells (Fig. 25). Frequently, cytoplasmic droplets and/or buds of various shapes and sizes were observed on the free surfaces of non-ciliated cells and small globular droplets were randomly attached, also, on the cell surfaces and on the cilia (Fig. 26).

The secretory material within the cells had decreased and numerous peg cells were found on Day 7, although abundant mucus secretion still filled both the cervical and cryptal lumina. The cervical epithelium on Day 10 was similar to that of earlier stages, but one of four animals showed active production of secretory material (Fig. 27). In SEM, some non-ciliated cells had partially lost microvilli and displayed occasional cytoplasmic projections (Fig. 28).

On Day 14, there was a second peak in the secretory activity of the cervical epithelium. Increased secretory material was noted not only in the cells, but also in the cervical lumen (Fig. 29). Peg cells were frequently observed. Microvilli of non-ciliated cells had increased in density. Several normally long cilia — considerably fewer per cell than on fully ciliated cells — were observed on the apical surfaces of some microvillous cells (Fig. 30).

In both LM and SEM, the appearance of the cervical epithelium on Day 18 resembled that of the estrous state, as reported previously.[25,26] Cells containing a large or moderate amount of secretion in their supranuclear regions were predominant, and the microvilli of most cells were regularly arranged.

Cytoplasmic cell inclusions, initially noticed by LOEB and SMITH,[21] were found in the cervical epithelium throughout pseudopregnancy. From Days 2 to 6, these inclusions were observed mainly in the basal parts of the cells. On Day 7 and later, they were more frequently located in the upper cytoplasm of the nucleus. The incidence of these bodies in the supranuclear regions of the cells was maximal on Day 14, but had decreased by Day 18.

Discussion

Surface features of the rabbit endometrium during pseudopregnancy, as revealed by SEM, agree with previous observations made by LM and transmission electron microscopy.[8,9,20,30] The present LM observations confirm the developmental phases of the progestational endometrium in the rabbit reported by DAVIES and HOFFMAN,[8] who indicated the following subdivision: (1) priming phase (Days 0-1), (2) proliferative phase (Days 1-4), (3) phase of epithelial relayering and folding (Days 4-6), (4) phase of cell fusion (Days 6-8), (5) maximal progestational phase (Days 8-13) and (6) declining phase (after Day 14). Furthermore, morphological changes occurring during the first week of pseudopregnancy were found to be almost identical with those described for the same period in pregnancy.[1,3,16,20,23,26] In the pregnant rabbits, subsequent symplasmic changes have been observed at the areas in contact with the blastocysts, but such changes were not seen at all during pseudopregnancy. LARSEN[20] considered that the symplasma is formed by a local effect of the blastocyst, while multinucleated cells are formed under progestational influence.

The degenerative changes in the endometrium in late pseudopregnancy observed here confirm the previous LM findings of TSUTSUMI and HAFEZ,[30] who reported that sloughing off of the syncytial epithelium begins at 13 to 15 days after sterile mating; but that the syncytium in the epithelium remaines until 20 days, and that at 15-16 days the uterine glands are reduced

in number and length and the surface of the folds becomes smooth. From the surface features of cells lining the endometrium, regressive changes were evident on Day 18.

SEM observations on the endometrium, as well as the cervical epithelium, revealed that the ciliated cells have stubby, short cilia or cilia only at their peripheries. The former types are considered to be immature, developing cilia as demonstrated by other SEM investigators;[2,10,12,24] and the latter type may result from the process of ciliation observed in mouse oviductal epithelium by DIRKSEN,[10] who mentioned that cilia grow first at the periphery of the cell and then fill the central area. By contrast, cells having cilia only in their central portions were observed in the endometrium on Day 14, although it remains unclear whether this type of cells represents ciliation or deciliation. The present results, together with previous findings,[26] suggest that the rabbit uterus and cervix possess at least two individual types of ciliary generation.

Relatively little change occurred in the cervical mucosa, in spite of considerable alteration in the endometrium. Changes occurring in the cervix appeared to be limited to the non-ciliated secretory cells. During pseudopregnancy, the secretory activity of the cells displayed two peaks, on Days 6 and 14, respectively. Since the first peak in secretory activity in the cervix has been observed also in normally mated rabbits,[26] apparently the secretory activity in the cervical epithelium depends on ovarian influence, and not on the local effect of the embryo (at least during the preimplantation stage).

In most animals, it has been believed that estrogens cause an increase in the height and secretory activity of the cervical epithelium, while progestins decrease them (reviewed by EL-BANNA and HAFEZ[11]). Similarly, estrogens function in the maintenance of the cervical epithelium of the rabbit. Ovariectomy results in the disappearance of secretory granules and patchy deciliation, but estrogen treatment causes a rapid return to the normal state in intact animals.[22,24]

Plasma estrogen and progesterone concentrations start to increase before implantation in the pregnant rabbit.[7,18] In pregnant rabbits plasma progesterone continues to rise until mid-pregnancy,[7] whereas in the pseudopregnant rabbit it begins to decrease after a peak reached on Days 7 to 9, followed by a further decline towards the control levels by 15–18 days of pseudopregnancy.[19,23] On the other hand, plasma estradiol concentrations change relatively little during pseudopregnancy[4] and pregnancy[7]. CHALLIS *et al.*[7] have demonstrated that the level of estradiol-17β fluctuates somewhat, with peaks on Days 6, 15 and 30 of pregnancy. It is worth noting that

the two peaks in cervical secretory activity in the present study correspond with the first two peaks in plasma estrogen concentrations in the pregnant rabbit reported by CHALLIS *et al.*[7]

The present results, together with that of a previous study,[26] clearly suggest that the secretory activity of cervical epithelium is regulated mainly by the ovarian steroids — estrogens rather than progestins. Therefore, it is assumed that the morphological constancy of the cervix, even under a progestational effect, may be due to relatively constant levels of plasma estrogen, and that postovulatory changes occurring in the non-ciliated cells of the cervix may be a result of their higher sensitivity to this steroid than that of the ciliated cells.

Cytoplasmic cell inclusions are known to occur usually in the rabbit cervical epithelium at estrus, during pregnancy and in late pseudopregnancy.[15, 17,21,26,29] The present study showed that inclusions occur in the cervical epithelium at any time during pseudopregnancy. Cytoplasmic cell inclusions were confined to the basal parts of the cells during early pseudopregnancy, but many were located in the supranuclear region thereafter, suggesting that estrogen induces their production and progesterone provokes their release. Our results support a previous view of LOEB and SMITH[21] and HAFEZ *et al.*[15], who mentioned that cytoplasmic cell inclusions move upwards toward the cervical lumen and are dischanged into the lumen.

Summary

The uterine and cervical epithelia of hCG-induced pseudopregnant rabbits were studied with light and scanning electrone microscopy. The endometrial epithelium of Days 2 and 4 was characterized by proliferation and pseudostratification, followed by rearrangement of the cells to a single layer for two additional days (Days 5 and 6), when the epithelium was characterized by crowded nuclei and apical cytoplasmic protrusions. By Days 7 to 10, all superficial epithelial cells were transformed into large, multinucleated cells. Degenerative signs in the uterine epithelium were evident on Day 14 and subsequently the endometrium appeared to be restored to its estrous state.

The cervix showed relatively little change in morphology of the mucosal folds and in the proportion of ciliated cells. More distinct changes occurred in the non-ciliated cells, in which secretory activity showed two peaks, on Days 6 and 14, respectively.

It is concluded that cervical activity in the rabbit is regulated mainly by the ovarian steroids, espcially estrogen, and that is organ is more tolerant to progestins than is the uterus.

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LEGEND OF FIGURES

PLATE 1
EXPLANATION OF FIGURES

Fig. 1. Endometrium on Day 2 in LM showing proliferation and partial pseudostratification in epithelium. Surface epithelium invaginates into narrow recesses. Glandular epithelium is still lined with simple columnar cells. H. E. ×80

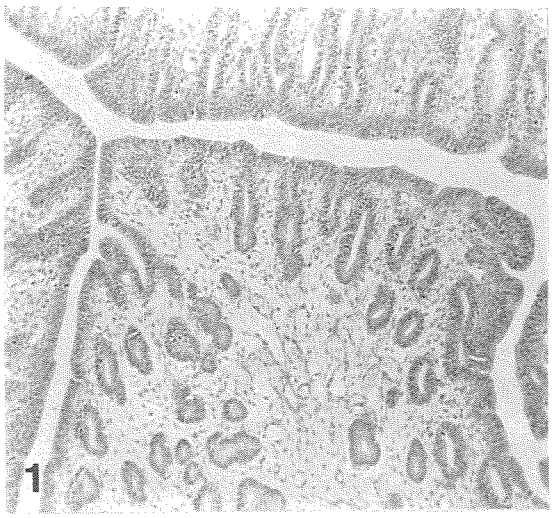
Fig. 2. Endometrium on Day 4 in LM. Invagination of the mucosal folds is advanced, forming crypts. Surface and cryptal epithelium is pseudostratified, but the glandular epithelium remains a simple layer. H. E. ×160

Fig. 3. Endometrial surface on Day 2 in SEM. Cell boundaries around glandular openings are clearly outlined, but at a distance from the openings they become unclear.

Fig. 4. Higher magnification of the endometrial surface on Day 2 in SEM. Normally long cilia have emerged to a limited degree at the periphery of the cell, and the microvillous surface is apparent in the center portion. A uterine glandular opening is noted.

Fig. 5. Endometrial surface on Day 4 in SEM, illustrating two types of non-ciliated cells. Cells of one type display relatively smooth surfaces and cells of the other type form convex apical surfaces.

Fig. 6. Higher magnification of the endometrial surface on Day 4. Short cilia emerge in clusters among microvillous cells, easily distinguished from microvilli by their greater diameter.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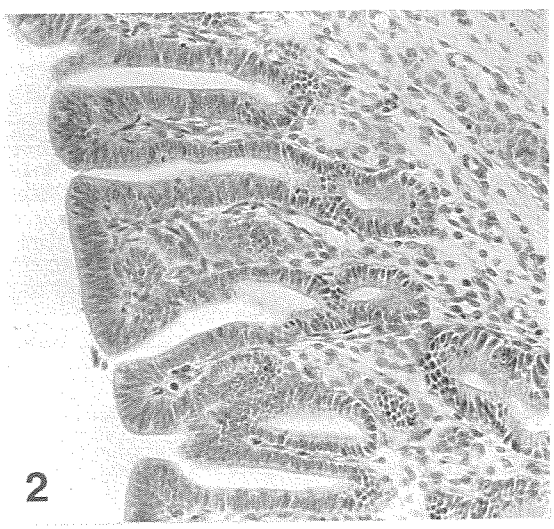
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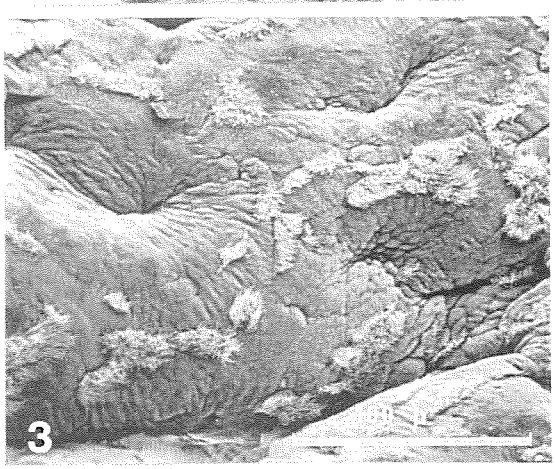
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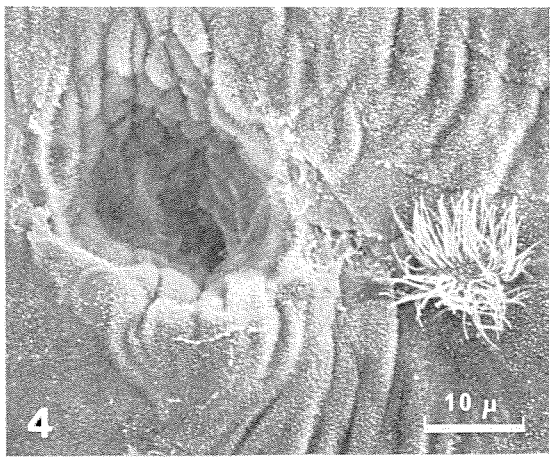
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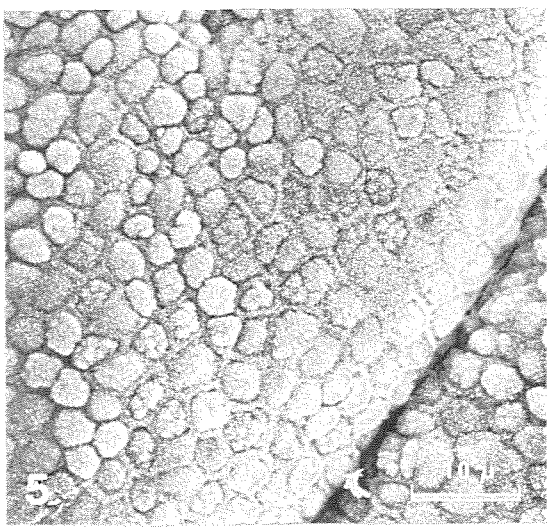
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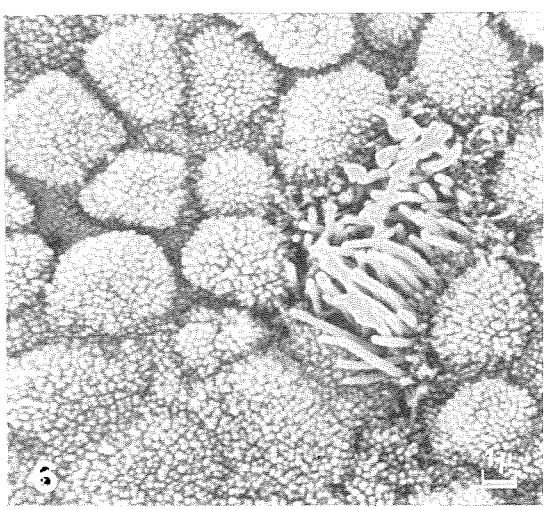
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PLATE 2
EXPLANATION OF FIGURES

Fig. 7. Endometrium on Day 5 in LM, showing delicately branched mucosal folds with narrow connective-tissue cores. The epithelium is clearly packed with cells which are characterized by extreme crowding of nuclei and by apical cytoplasmic protrusions. H. E. ×320

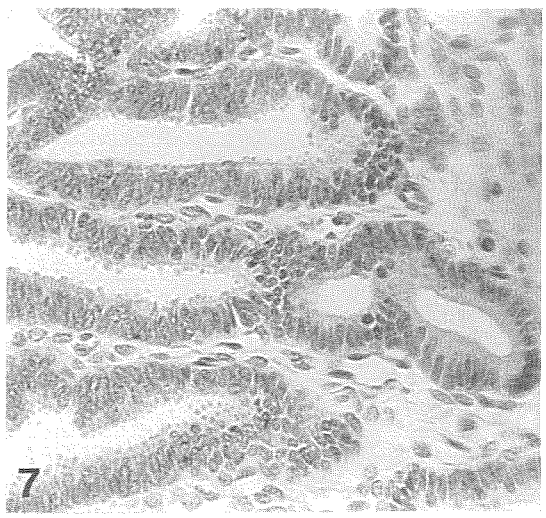
Fig. 8. Endometrial surface on Day 5 in SEM, showing dome-like protrusions of the apical parts of cells. Microvilli on these protrusions are sparsely distributed.

Fig. 9. Endometrial surface on Day 6 in SEM. The apical protrusions of cells show fewer microvilli.

Fig. 10. Endometrium on Day 7 in LM. The process of cell fusion is evident. Several nuclei are enveloped with a single cell membrane. H. E. ×320

Fig. 11. Endometrial surface on Day 7 in SEM, showing large cells of various sizes. Microvilli densely distributed on the cell surfaces.

Fig. 12. Endometrium on Day 10 in LM, showing increased size of cells and nuclear masses. Note pycnosis and fragmentation of nuclei. H. E. ×320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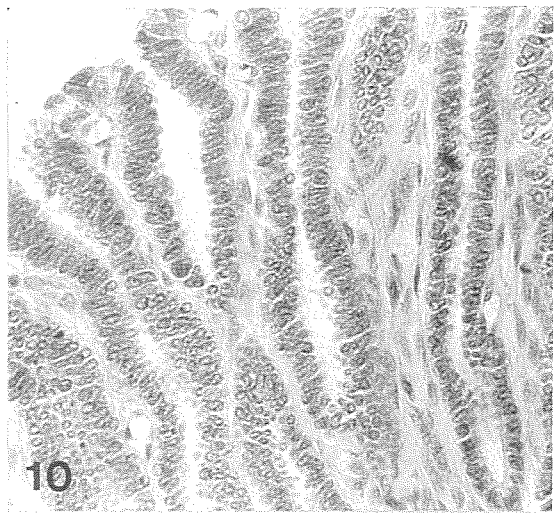
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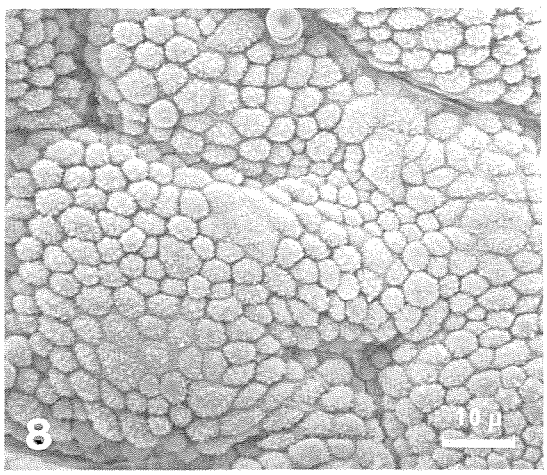
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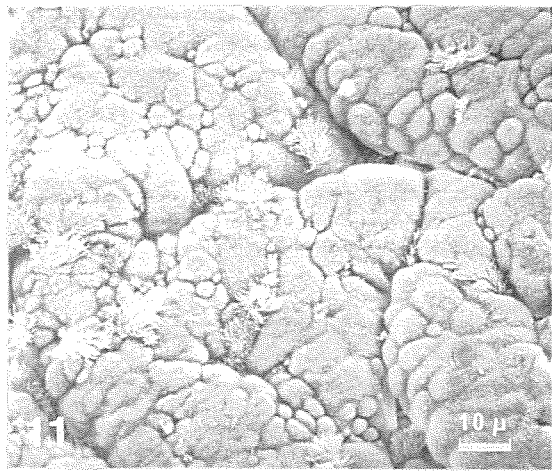
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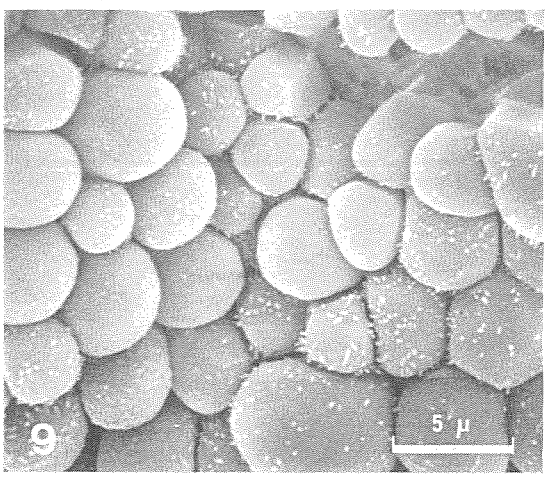
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5 μ

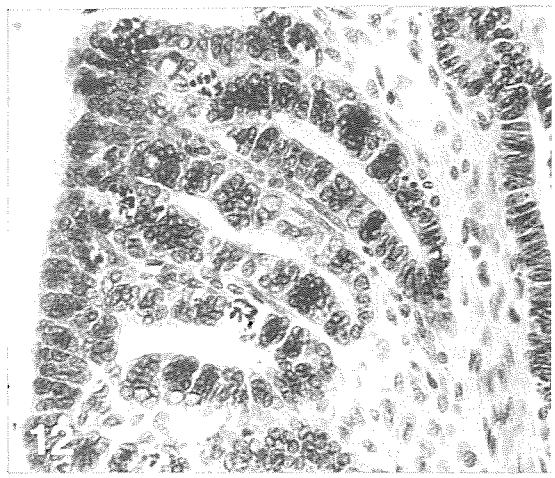

12

PLATE 3
EXPLANATION OF FIGURES

Fig. 13. Endometrial surface on Day 10 in SEM, showing advanced cell fusion.

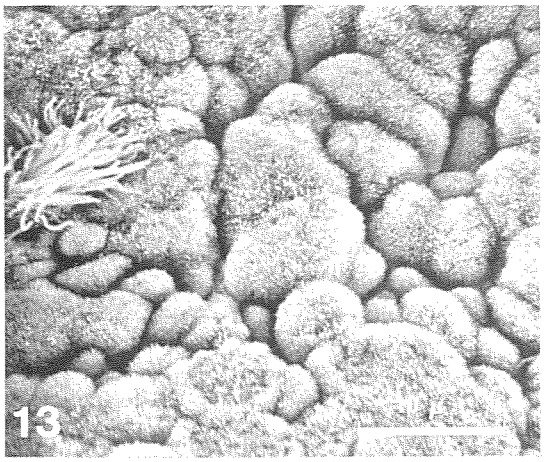
Fig. 14. Endometrium on Day 14 in LM, showing frequent signs of epithelial regression. Note pycnosis of nuclei and cystic vacuoles containing debris of epithelial cells. H. E. ×320

Fig. 15. Endometrial surface on Day 14 in SEM, illustrating a characteristic ciliated cell. Cilia emerge only at the central portion of the cell. Note swollen cilia among normal cilia.

Fig. 16. Endometrium on Day 18 in LM. Surface epithelium has become thin and the number of nuclei within each cell is decreased. H. E. ×320

Fig. 17. Endometrial surface on Day 18 in SEM, showing relatively smooth surface and increasing number of ciliated cells. Location and size of glandular openings are similar to those of the estrous endometrium.

Fig. 18. Higher magnification of an area in Fig. 17. Note very small, stubby microvilli and one or more single cilia.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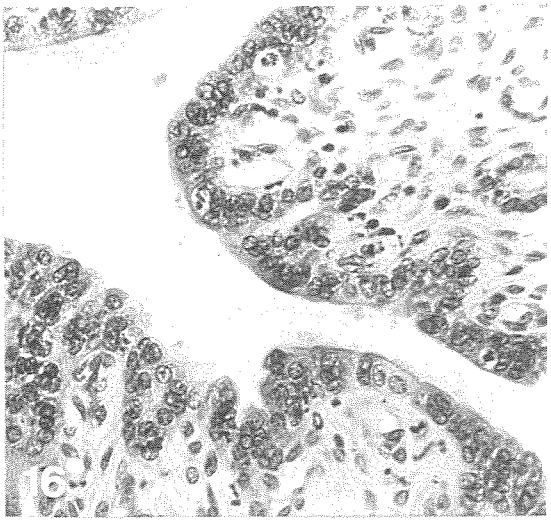
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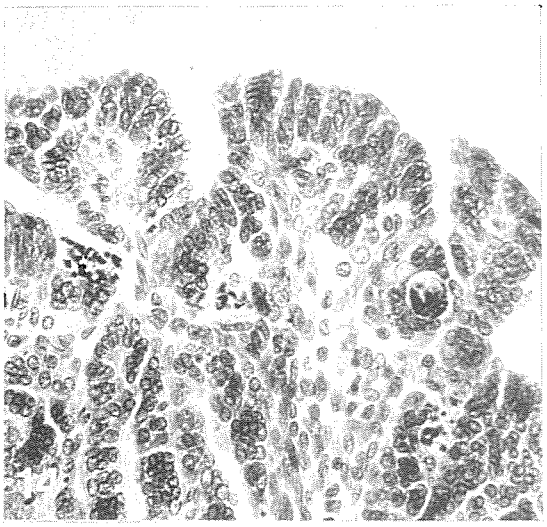

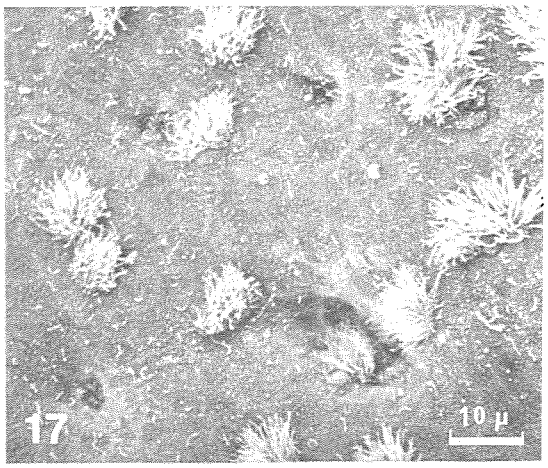
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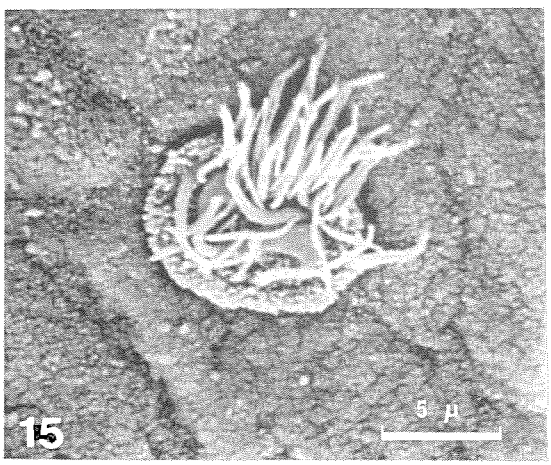
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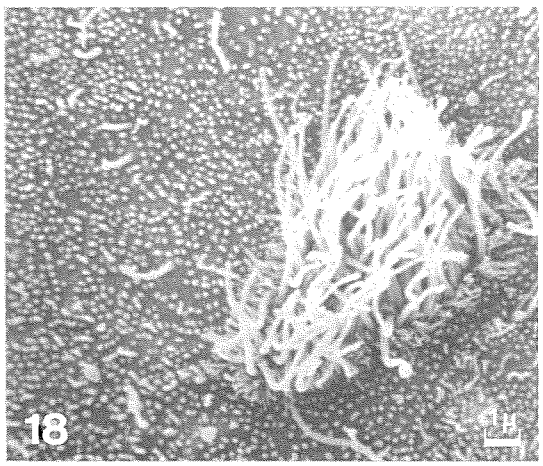

18
1μ

PLATE 4
EXPLANATION OF FIGURES

Fig. 19. Cervical mucosa on Day 2 in LM. Cells containing a little secretory material are common. Azan. ×320

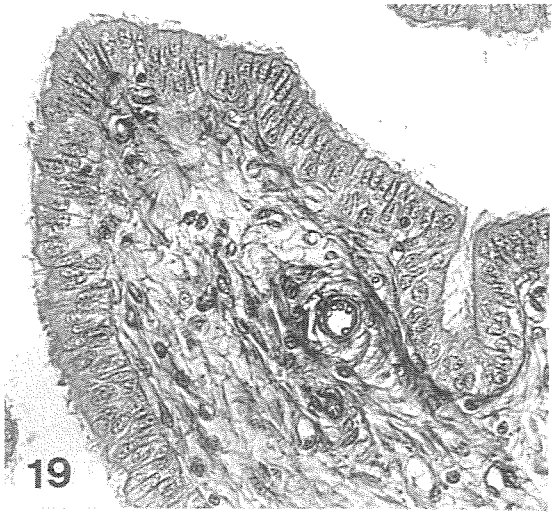
Fig. 20. Surface of cervical epithelium on Day 2 in SEM. Microvilli on the non-ciliated cells have partly disappeared. Note a small hollow on the apical surface (arrow).

Fig. 21. Cytoplasmic projections and buds on the cervical epithelium on Day 2. One projection is still connected to the apical surface of cell, suggesting an apocrine secretion.

Fig. 22. Cervical mucosa on Day 4 in LM. Secretory material within the cells is diminished. Peg cells are commonly noted. Azan. ×320

Fig. 23. Surface of cervical epithelium on Day 4 in SEM, showing regular arrangement of microvilli. Note a few small hollows on the surface of a cell.

Fig. 24. Cervical mucosa on Day 6 in LM, showing abundant secretory material in both cells and lumen. Azan. ×320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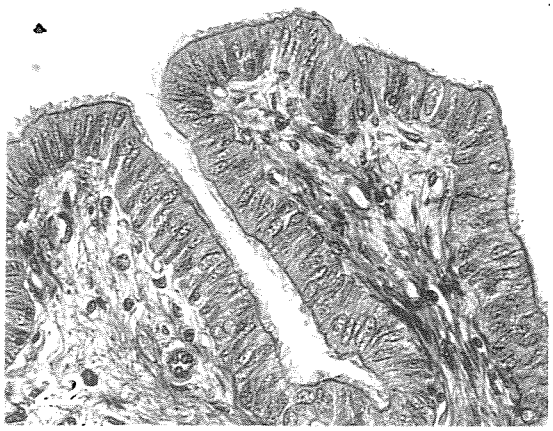
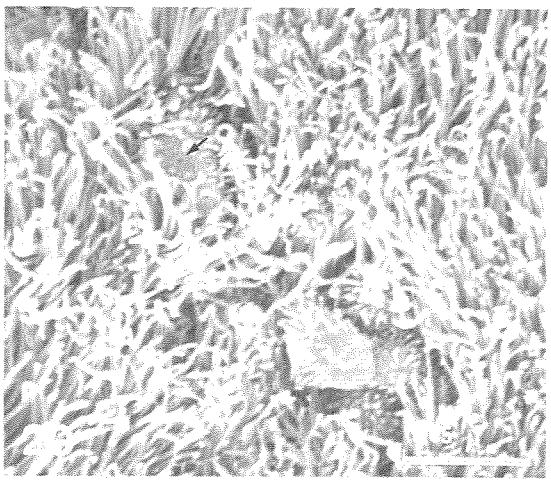
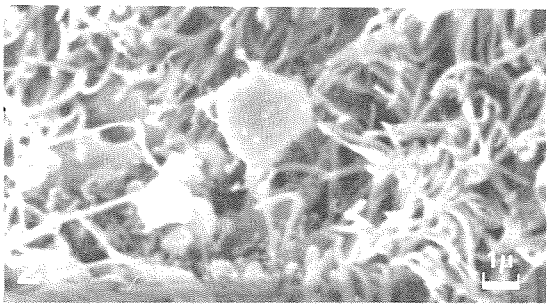






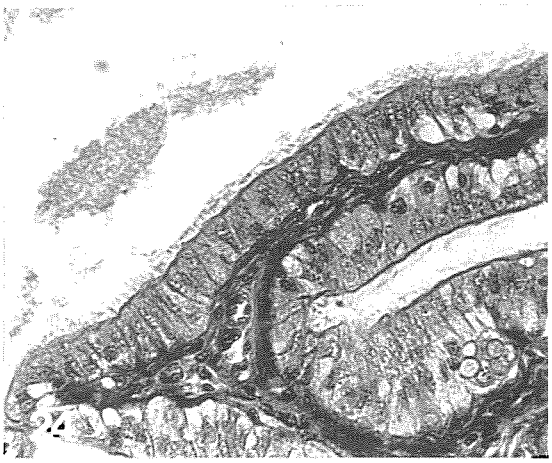


PLATE 5
EXPLANATION OF FIGURES

Fig. 25. Surface of cervical epithelium on Day 6 in SEM, showing swollen non-ciliated cells. Their apical surfaces have very few or no microvilli.

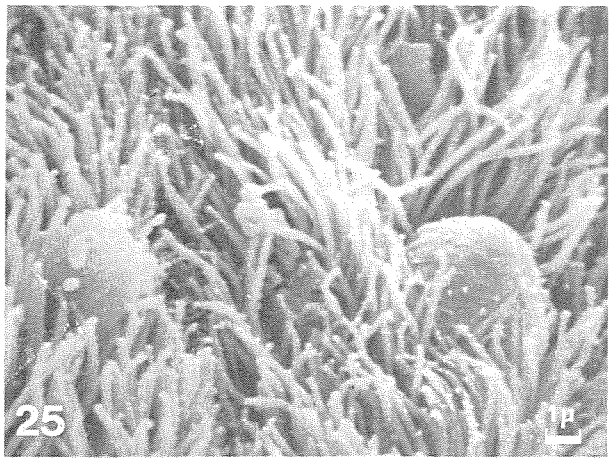
Fig. 26. Surface of cervical epithelium on Day 6 in SEM. Cytoplasmic droplets or buds of various shapes and sizes are attached on the cell surfaces and the cilia.

Fig. 27. Cervical mucosa on Day 10 in LM, showing the resumption of secretory activity in the cells. Azan. ×320

Fig. 28. Surface of cervical epithelium on Day 10 in SEM. Microvilli of non-ciliated cells are frequently lost, showing occasional cytoplasmic swelling.

Fig. 29. Cervical mucosa on Day 14 in LM. The secretory cells show intense production of mucin. Azan. ×320

Fig. 30. Surface of cervical epithelium on Day 14 in SEM. Some microvillous cells display several ciliary shafts of various length on their surfaces.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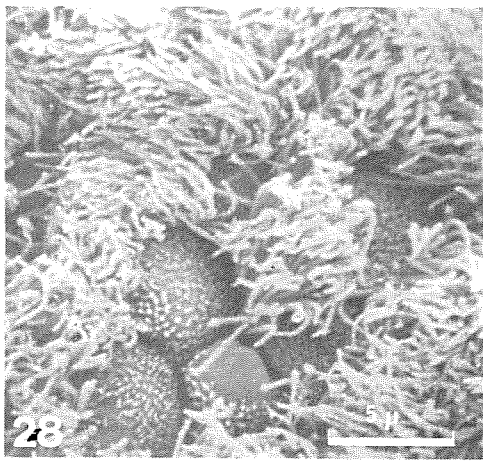
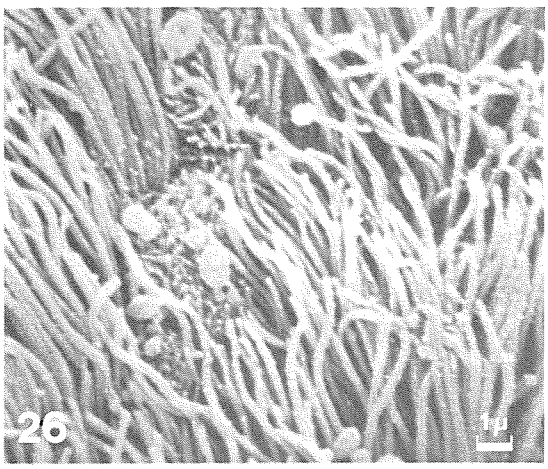
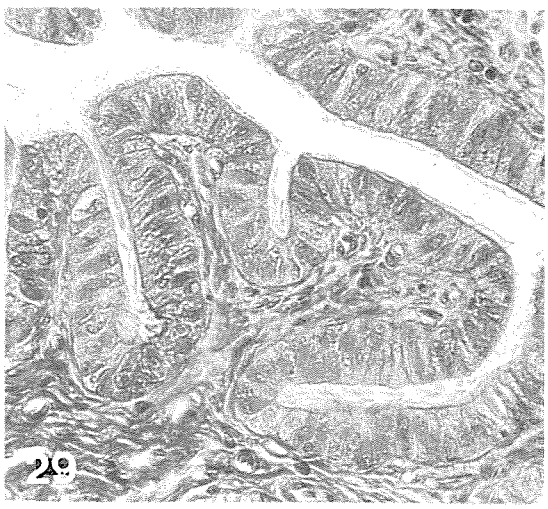
1μ


28
5μ


26
1μ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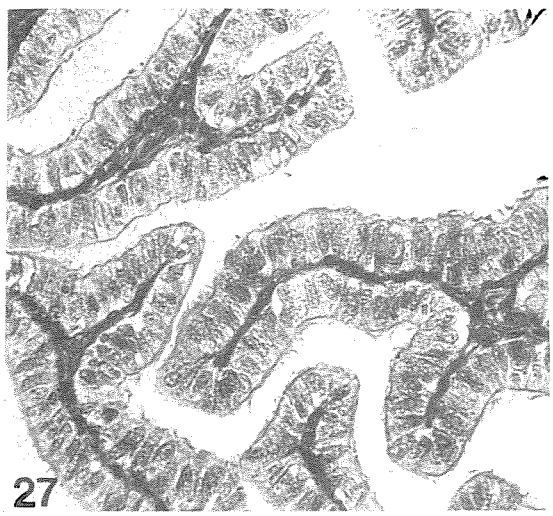
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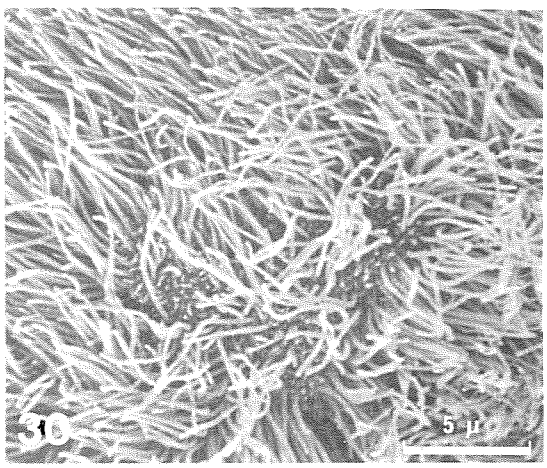
30
5μ