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## Abstract

A total of 252 bladder-washing and voided specimens from normal, and inflammatory and malignant lesions were examined by a direct mapping technique, i.e., a combined use of light (LM) and scanning electron microscopy (SEM). A newly-designed mesh, which consists of a piece of gelatine-covered, osmium-impregnated and polylysine-coated glass-slip with 42 compartments/25 mm2, was used in this study. This mesh permitted us to directly correlate LM and SEM images, which resulted in a shortening of the observation time. Malignancy of exfoliated urothelial cells has been determined on the basis of the presence or absence of pleomorphic microvilli as observed by SEM. Subsequently, a new "SEM grading" system for human urinary cytology was proposed. The direct mapping technique has enhanced the accuracy of the diagnosis over conventional methods, especially in cases of noninvasive, low-grade malignant tumors of the urinary bladder.

**KEYWORDS:** urinary cytology, bladder cancer, scanning electron microscopy

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# HUMAN URINARY CYTOLOGY USING A "DIRECT MAPPING" TECHNIQUE: A COMBINED LIGHT AND SCANNING ELECTRON MICROSCOPIC INVESTIGATION

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*Abstract.* A total of 252 bladder-washing and voided specimens from normal, and inflammatory and malignant lesions were examined by a direct mapping technique, *i.e.*, a combined use of light (LM) and scanning electron microscopy (SEM). A newly-designed mesh, which consists of a piece of gelatine-covered, osmium-impregnated and polylysine-coated glass-slip with 42 compartments/25 mm², was used in this study. This mesh permitted us to directly correlate LM and SEM images, which resulted in a shortening of the observation time. Malignancy of exfoliated urothelial cells has been determined on the basis of the presence or absence of pleomorphic microvilli as observed by SEM. Subsequently, a new "SEM grading" system for human urinary cytology was proposed. The direct mapping technique has enhanced the accuracy of the diagnosis over conventional methods, especially in cases of non-invasive, low-grade malignant tumors of the urinary bladder.

*Key words:* urinary cytology, bladder cancer, scanning electron microscopy.

Scanning electron microscopy (SEM) has come to be used in various cancer studies because of its advantage in being able to demonstrate three-dimensionally the tumor cell surface (1, 2). In bladder tumors, pleomorphic microvilli (PMV) detected by SEM have been considered as a surface marker of irreversible proliferation of transitional cells in experimental animals. In our previous studies (3, 4) and others (5-7) PMV were also identified in most of the human bladder tumors examined, and were significant in differentiating tumors from other non-malignant lesions. Moreover, the degree of pleomorphism of microvilli (MV) in human bladder tumors correlated with the degree not only of histologic dedifferentiation (3, 7) but also of the loss of blood group antigens (BGA) (3, 4).

We studied the significance of PMV regarding human urinary cytology using a direct mapping technique, *i.e.*, a combined use of light microscopy (LM) and SEM. Two experiments were designed. First, urinary exfoliated cells from 180 cases with nonmalignant or malignant lesions were classified on the basis of cell surface microstructures as well as cell shapes, resulting in a SEM grading of malignancy of the exfoliated cells. Secondly, bladder washings from 72 cases with postoperated bladder tumors were evaluated with Papanicolaou's conventional classification and with the SEM grading to assess the usefulness of the direct

mapping technique.

## MATERIALS AND METHODS

*Direct Mapping Technique*

A newly-developed mesh with 42 compartments /25 mm² was prepared on osmium-impregnated and polylysine-coated glass slips (Fig. 1). Explaining in detail, a piece of a well-defatted glass-slip of 5 × 5 mm in size and 0.85 mm in thickeness was marked with a glass-knife at the left corner to assure its top corner, and the surface was covered briefly with a 10 % gelatine solution warmed at 60℃. After the gelatine set, five horizontal and six vertical lines were drawn with a razor-blade on the gelatine-coated slip, which was then fixed in osmium vapor at 37℃ for one hour, followed by fixation with 2.5 % glutaraldehyde. The slip was washed in phosphate-buffered saline (PBS) and treated with 0.1 % poly-L-lysine (molecular weight: 85,000, Sigma Co., St. Louis, Missouri). This polylysine-coated gelatine slip was used like a standard polylysine-coated slip (8) as a substrate for preparing cells in suspension for SEM.

Exfoliated cells from voiding or bladder washing were mounted on the slips prepared as above, and the following treatments were carried out on cells adhering to the substrate. After two hours of fixation with 2.5 % glutaraldehyde, slips were dipped in a 0.05 % polylysine solution to stabilize cell adhesion. The cells were then stained by a modified Giemsa method and observed by LM while wet. After photographing the cells of specific interest with LM, the specimen was prepared for SEM. Our newly-designed mesh easily permitted us to directly correlate LM and SEM images and we tentatively designated this method as the direct mapping technique.

*Preparations of Specimens for SEM*

Specimens were postfixed with 1 % osmic acid in PBS for one hour and dehydrated in a graded ethanol series. After replacing the ethanol with isoamyl acetate, the critical point drying method using liquid carbon dioxide was applied. The specimens were coated with platinum-palladium in an ion coater (IB-3, Eiko Engineering Co., Tokyo) and observed with a field emission type of SEM (HFS-2, Hitachi Co., Tokyo).

*Urinary Exfoliated Cells*

Exfoliated cells from a total of 180 patients, consisting of 71 normal, 41 bacterial cystitis including 5 cystitis cystica and 68 bladder tumors, were examined. In all the cases with bladder tumor, the diagnosis was confirmed by cystoscopy and histology. Although cells in most nonmalignant cases were collected from voided urine, bladder washings using PBS containing 2.5 % glucose (430 mOsm) were used as described below for some patients without malignancies and for almost all patients with tumors to obtain good yields of exfoliated cells. Specimens were prefixed with 0.25 % glutaraldehyde and were examined using the direct mapping technique. Exfoliated cells from 180 cases were classified based on cell surface microstructures as well as cell shapes observed by SEM. Microridges, microplicae, monomorphic MV (MMV), PMV, blebs and membrane ruffles were present on the surface of exfoliated cells, as seen on the cells from normal and tumor tissues of the bladder (3, 4). As a rule, one cell, which was the most characteristic of each specimen, was evaluated. When cell morphology was obviously different within a given specimen, two epithelial cells were evaluated. In this way, a total of 247 cells from 180 cases were classified.

*Comparison of the Efficacy of Papanicolaou Staining with that of the Direct Mapping Technique*

To evaluate the efficacy of the direct mapping technique, bladder washings of another 72 patients with postoperated bladder tumors were examined using both Papanicolaou staining

and the direct mapping technique. After cystoscopy using sterilized PBS containing 2.5 % glucose (GPBS, pH 7.2, 430 mOsm), 40-50 ml GPBS was reinstillated to the bladder and cells were collected by washing with 20-30 times of pumping through a cystoscope. The resulting bladder washings were cooled in an ice bath, and the cells were collected immediately by low-speed centrifugation and fixed in 0.25 % glutaraldehyde in PBS. Two of the above-described meshes were prepared for each specimen and examined by the direct mapping technique. The remaining cells were examined by the Papanicolaou staining method. Cytologic evaluation by the Papanicolaou was followed according to the criteria proposed by Papanicolaou in 1954, and that by the direct mapping technique was based mainly on the cell surface microstructures. For histologic grading reference was made to the "General rule for clinical and pathological studies on bladder cancer", published by the Japanese Urological Association and Japanese Pathological Society in October 1980 (Kanahara Shuppan, Tokyo).

## RESULTS

*Direct Mapping Technique*

Each line and compartment of the newly-developed mesh was clearly identifiable by LM and SEM, and staining quality by Giemsa in a wet preparation was also satisfactory enough for identification of the exfoliated cells. The cells photographed by LM were easily detected by SEM (Fig. 2). Cells which adhered to the substrate did not become detached during subsequent treatments. Moreover, the osmification of gelatine for making the substrate conductive enhanced the quality of SEM images by eliminating charging artefacts (9).

*Cell Surface Microstructures of Exfoliated Cells*

As shown in Table 1, 247 cells from 180 patients with normal, inflammatory and tumorous bladders were classified according to cell shapes and cell surface microstructures. Most benign urothelial cells identified by LM (137/163) were flat and polygonal, and had microridges, microplicae and MMV, *i.e.*, regular or short MV, on the cell surface (Fig. 3). The surface of approximately 50 % (84/163) of all benign cells were covered by microridges (Fig. 3 a, b). Most of the microridges were flat (Fig. 3b) as compared to those seen in luminal cells of the bladder, probably due to an artefact arrising at the time of cell detachment from the bladder surface. On the other hand, microplicae were well preserved, and approximately one third (54/163) of the benign cell surfaces were covered with microplicae. In particular, almost all large, flat squamous cells were covered predominantly by microplicae (Fig. 3c). MV on the benign cell surfaces were found in approximately 20 % (38/191) of the cases; most of them (28/38) had also microridges and microplicae as seen in Fig. 3c, d. In spite of the presence of MV, cells predominantly having microridges and microplicae are classified under the groups "microridges" and "microplicae" in Table 1, and the number of cells so classified is expressed in brackets in the group "monomorphic MV". In general, MV on benign cells were short, and stubby (Fig. 3c, d), with only 3 % (5/163) showing pleomorphism. Cells in two of these five cases with PMV were obviously flat, resembled cells from the urethral epithelia, and considered normal by SEM,

TABLE 1. CELL SURFACE MICROSTRUCTURES ON 247 URINARY EXFOLIATED CELLS FROM 180 PATIENTS[a]

| Cell shapes / Cell surface microstructures | Normal and inflammatory condition | | | Bladder tumors | | |
|---|---|---|---|---|---|---|
| | Flat & Polygonal | Spherical | Subtotal | Thick & Polygonal | Spherical | Subtotal |
| Microridges | 74 | 10 | 84 | 0 | (6[c]) | (6[c]) |
| Microplicae | 49 | 5 | 54 | 0 | 0 | 0 |
| Monomorphic MV[b] | 5 (27) | 0 | 5 (27[d]) | 0 | 8 | 8[f] |
| Pleomorphic MV[b] | 2 | 3 (1) | 5 (1[e]) | 19 | 51 | 70[g] |
| Without SMS[h] | 7 | 8 | 15 | 0 | 6 | 6 |
| Total | 137 | 26 | 163 | 19 | 65 | 84 |

*a*) The number in brackets indicates predominance of other cell surface structures. *b*) Microvilli. *c*) With predominant monomorphic MV (2/6) or pleomorphic MV (4/6). *d*) With predominant microridges or microplicae. *e*) With predominant microplicae. *f*) With blebs (2/8). *g*) With blebs (20/70) or membrane ruffles (9/70). *h*) Cell surface microstructures.

although they had a mild pleomorphism of MV (Fig. 4).

The cells from inflammatory lesions showed flatter microridges than those described above and often lost their characteristic cell surface microstructures, although microplicae were relatively well preserved.

Cancer cells identified by LM were thicker than benign cells and were mostly (65/84) round. MV, identified in 94 % (78/84) of the cells, had variable diameters and lengths with pleomorphic configurations (Figs. 5, 7), being obviously different in morphology from those on benign cells. In most cases MV were distributed densely as shown in Fig. 5, whereas in some cases the MV were rather sparsely distributed ,being localized on a few areas of the cell surface (Figs. 6, 7). Approximately 10 % (8/78) of the MV were found on the surface of large spherical cells, lacked villous pleomorphism and were regarded as MMV (Fig. 6b). The overall cellular pattern of these spherical cells, however, differed from that in benign cells, although MV were morphologically similar. Blebs or bleb-like structures (Fig. 6c) were present in 22/84 (26 %) cells. Membrane ruffles, resembling those on leukocytes and leukemic cells, were also found in 9/84 (11 %) cells. About 7 % (6/84) of the malignant cells lost their characteristic surface structures; they were round and had only a few blebs and membranous holes or pits, probably due to cell degeneration.

*SEM Grading of Exfoliated Cells*

Based on observations of 247 cells, we propose a new SEM grading system (Table 2). S-1 is characterized by the presence of normal cell surface microstructures (NSS), such as microridges, microplicae or MMV, on the surface of flat,

TABLE 2. SEM GRADING OF URINARY EXFOLIATED CELLS

S-1: Presence of NSS[a] on flat and polygonal cells.
S-2: Absence of NSS on flat and polygonal cells.
S-3: Absence of NSS and TSSS[b] on spherical cells.
S-4: Presence of TSSS on pleomorphic cells.
S-5: Presence of TSSS on grouped S-4 cells.

*a*) Normal cell surface microstructures, *i.e.*, microridges, microplicae and monomorphic microvilli. *b*) Tumor specific cell surface microstructures, *i.e.*, pleomorphic microvilli with or witout blebs and/or membrane ruffles.

TABLE 3. CORRELATION OF SEM GRADING WITH PAPANICOLAOU'S CLASSIFICATION

| SEM grading / Papanicolaou | S-1 | S-2 | S-3 | S-4 | S-5 |
|---|---|---|---|---|---|
| I | | | □ | ◉ | |
| II | ■□□□□□ □□□□□□ □□□□□□ □□□□□ | □□□□ | □□ | □□■■■■ | ■■■ |
| III | □□□□□ | □ | ■□□□ | ■■■■■■ | ■ |
| IV | | | ■■ | ■■■ | |
| V | | | ■■ | ■■■■■ | ■■■ |

■: Tumor recurrence (+) by cystoscopy. □: Tumor recurrence (−) by cystoscopy. ◉: Tumor recurrence (+) one month after cystoscopy.

polygonal cells. S-2 is similar to S-1 but lacking NSS. S-4 is represented by the presence of tumor specific cell surface structures (TSSS) including PMV on the suface of polygonal or round cells with more increased thickness of each cell as compared to the cells of S-1 or -2. S-5 consists of the S-4 cells in clusters. S-3 is characterized by round cells, on which NSS and TSSS are absent. As exceptions to the above classification, round cells with NSS are regarded as S-1, flat cells with a slight degree of PMV as S-2 (Fig. 4), and large spherical cells showing obvious malignant features because of a distributing pattern of MV, which lacks pleomorphism, are as S-4 (Fig. 6b).

*Comparison of the Efficacy of Papanicolaou Staining with the Direct Mapping Technique*

Bladder washings from another 72 patients with postoperated bladder tumors were examined both with Papanicolaou staining and the direct mapping technique. Out of these 72 cases, there were 31 cases with tumor recurrence observed by cystoscopy, one with recurrence one month after the first cystoscopic examination, and 40 without recurrence. The above one patient showed class I of urinary cytology by Papanicolaou while S-4 by SEM grading, and one month later proved to have a low-grade tumor by cystoscopy together with a biopsy. Histologically, all 32 recurrent tumors were transitional cell carcinoma except for one case of adenocarcinoma. As to tumor differentiation, there were seven cases with grade 1, 18 with grade 2, and seven with grade 3.

Table 3 shows the correlation between SEM grading and Papanicolaou's classification. Positive SEM grading (S-4 and S-5) was found in eight of nine cases of classes I to II by Papanicolaou, and in seven of eight cases of class III; all of these 17 cases were proved to have tumors cystoscopically and histologically. On the other hand, 15 cases with positive Papanicolaou had recurrent tumors. Although a positive SEM grading was found in 11 of these 15 cases, the remaining four were classified as S-3 by SEM because of the loss of surface microstructures.

Table 4 shows the correlation of Papanicolaou's classification and SEM grading according to the grade of tumor differentiation. Seven cases with grade 1 tumors were all below class III by Papanicolaou whereas six out of the seven were S-4 to -5 by SEM grading. Of eighteen cases with grade 2 tumors three were in classes I to II, five in class III and 10 in classes IV to V, while there were three in S-3 and 15 in S-4 to -5. There were seven grade 3 cases, of which two cases in class III were positive by SEM grading and two S-3 cases were positive by Papanicolaou. Accordingly, SEM grading was far superior to Papanicolaou's classification for low-grade tumors, while the two methods had almost equal efficacy for high-grade tumors.

Fig. 7 shows an example demonstrating the usefulness of SEM in a recurrent tumor case of grade 2 in which cell clusters could have been classified at most class III by LM while the cells had PMV of the S-5 by SEM. Bladder tumors are often complicated by infection which may result in misinterpretation of the observations when using Papanicolaou's classification. Fig. 8 shows cells obtained from a high-grade tumor with infection. PMV were well preserved in spite of the infection, and differed in surface morphology from leukocyte clusters.

PMV on exfoliated tumor cells were almost always identified regardless of the tumor grade, but the degree of pleomorphism of the microvilli and cells tended to increase with higher tumor grades, that is, low-grade tumors often had less-pleomorphic MV on the surface of large spherical cells (Fig. 6c), apart from con-

TABLE 4. CORRELATION OF PAPANICOLAOU'S CLASSIFICATION AND SEM GRADING ACCORDING TO TUMOR GRADE

| Tumor grade* | No. of cases | Papanicolaou | | | SEM grading | | |
|---|---|---|---|---|---|---|---|
| | | I-II | III | IV-V | 1-2 | 3 | 4-5 |
| Grade 1 | 7 | 6 | 1 | 0 | 1 | 0 | 6 |
| Grade 2 | 18 | 3 | 5 | 10 | 0 | 3 | 15 |
| Grade 3 | 7 | 0 | 2 | 5 | 0 | 2 | 5 |
| Total | 32 | 9 | 8 | 15 | 1 | 5 | 26 |

*Based on the "General rule for clinical and pathological studies on bladder cancer", published by the Japanese Urological Association and Japanese Pathological Society in October 1980 (Kanahara Shuppan, Tokyo).

TABLE 5. COMPARISON OF PAPANICOLAOU'S CLASSIFICATION AND SEM GRADING, WITH SEM GRADING PLUS LM FINDINGS BY GIEMSA STAINING

| | Papanicolaou | SEM grading | SEM grading plus LM findings[a] |
|---|---|---|---|
| Positive rate (%) | 20.8 (15/72)[b] | 38.9 (28/72) | 44.4 (32/72) |
| Detectability (%) | 20.8 (15/72) | 36.1 (26/72) | 41.7 (30/72) |
| Sensitivity (%) | 46.9 (15/32) | 81.2 (26/32)[c] | 93.8 (30/32)[c] |
| Specificity (%) | 100 (40/40) | 95.0 (38/40) | 95.0 (38/40) |
| False-positive (%) | 0 (0/40) | 5.0 (2/40) | 5.0 (2/40) |
| False-negative (%) | 53.1 (17/32) | 18.8 (6/32)[c] | 6.2 (2/32)[d] |
| Validity (%) | 146.9 | 176.2 | 188.8 |

*a*) LM findings by Giemsa staining were applied particularly to S-3 of SEM grading. *b*) Brackets indicate the number of positive cases out of the cases examined. *c*) and *d*) Statistical difference between Papanicolaou's classification: *c*) $p < 0.05$, and *d*) $p < 0.01$.

ventional PMV (Fig. 5). Some exfoliated cells from high-grade tumors were smooth and spherical without characteristic surface microstructures and had numerous membrane holes or pits. These cells often had the classical nuclear abnormalities of cancer cells under LM using Papanicolaou or Giemsa staining. There were five cases of S-3 despite the presence of tumor recurrence. Four of these five were observed to have nuclear abnormalities with Giemsa staining performed during the direct mapping technique, and can be reclassified to be positive.

Therefore, in S-3, the nuclear abnormality revealed by Giemsa staining, as a part of the direct mapping technique, was regarded to be an important finding. Table 5 shows the comparison of (i) Papanicolaou's classification, (ii) SEM grading and (iii) SEM grading plus LM findings by Giemsa staining in which (iii) was particularly applied to S-3 cases. The sensitivity proved to be 46.9 % (15/32) by (i), 81.2 % (26/32) by (ii), and 93.8 % (30/32) by (iii).

## DISCUSSION

Urinary cytology by LM has proved to be valuable for the diagnosis of bladder cancers in man when the lesions are relatively high in histologic grade. It has been, however, of little value for the detection of relatively common, superficial, low-grade tumors (10). Recently, several investigators (11-13) indicated the potential value of SEM for the diagnosis of low-grade and noninvasive bladder tumors, although no systematic studies have been reported. SEM is very helpful for observation of minute structures on the cell surface, but lacks information as to intracellular structures including nuclear configurations. As indicated by Albrecht and Wetzel (14), cell surface microstructures observed by SEM often cannot be reliably interpreted for the diagnosis of a malignancy. A few SEM studies (13, 15) of urinary exfoliated cells positively identified by LM have been reported, but they were based on observations of few patients and, furthermore, emphasized the lack of diagnostic advantages of SEM. We have developed a more reliable and useful

technique, which we refer to as the direct mapping technique. This technique has the following advantages over previous methods: 1) Lines of the mesh do not disturb clear cell images, since far sharper lines can be drawn on the gelatine-coated slips than by conventional techniques, such as the use of a diamond marker on coverslips. 2) Clear identification of all the given cells is possible by LM. 3) Cells photographed by LM can easily be detected with SEM. 4) Osmification of the gelatine-coated substrate results in enhanced quality of the SEM image by eliminating charging artefacts. 5) The technique itself is inexpensive and reproducible because one can avoid the redundancy for preparing the meshes. With this technique, we examined a total of 252 nonmalignant and malignant lesions to assess the potential value of SEM for the diagnosis of bladder tumors.

The SEM study on bladder tumors was initiated by Fulker, Cooper and Tanaka, (16) and since then a large body of data has accumulated on human as well as experimental animal materials. One of the most significant findings was the identification of pleomorphic microvilli (PMV) on the bladder tumor cell surface. Using Fischer rats fed by N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide, Jacobs *et al.* (17) found the PMV to be a reliable marker of irreversible, proliferative changes in the urothelium. They then observed PMV on cells from all 17 of the patients studied with transitional cell carcinoma of the urinary bladder, regardless of the tumor grade (7); similar results were obtained by Merk *et al.* (5) and Nelson *et al.* (6).

Blood group antigens (BGA) are present on the normal urothelial cell surface, and their progressive disappearance from the neoplastic epithelial surface was related to an increased malignant potentiality of tumors. Using an immuno-SEM technique (3, 4, 9), we also examined biopsied materials from 51 cases with transitional cell carcinoma of the bladder in an association with the loss of BGA from neoplastic cell surfaces. Cell surface patterns were classified into five main types and variability in appearance increased not only with an increase in tumor grade but also with a greater degree of BGA loss. PMV were observed on all tumor cells except for type 1 cells, which was covered by evenly-distributed monomorphic microvilli (MMV) and flattened, waving microridges. Type 1 cells resembled the exfoliated cells from low-grade tumors shown in Fig. 6c and also were found only in biopsied materials from low-grade tumors. All the type 1 cells had well-preserved BGA, and type 1 is regarded as a cell type indicating a very early stage of malignancy.

As stated before, the purpose of cytologic approach by SEM is the diagnosis of low-grade and noninvasive lesions, which cannot be detected by conventional LM alone. In other words, the early lesion including the type 1 cell can or cannot be identified by the SEM cytology. Therefore, the cells as shown in Fig. 6b were exceptionally regarded as S-4 by SEM grading, *i.e.*, malignant cells; these cells are lacking in villous pleomorphism, but are certainly different from normal cells in the distribution of villi, loss of normal cell surface microstructures (NSS) and cellular morphology of spherical shape and enlarged size. These were seven cases

of grade 1 tumors out of the 32 recurrent cases. Cells from two of the seven cases belonged to the cell type described above. The number of false-positive case was, however, not influenced even though we grouped this type of cell as positive.

Regarding the efficacy of SEM cytology, Domagala *et al.* (13) examined 200 urothelial cancer cells, which were identified by LM and studied by SEM; these cells were derived from one patient with papillary carcinoma of grade Ⅱ-Ⅲ. They stated: "While about 30 % of malignant urothelial cells were endowed with numerous, densely packed, long, irregular, pleomorphic MV and about 10 % of the cancer cells had surfaces covered by numerous but uniform MV, the majority of cancer cells had smooth surfaces with no MV, with few MV remnants or with membrane holes or pits. On the other hand, some benign urothelial cells were covered by irregular, although usually short and fairly monotonous, MV or MV-like processes. Even some squamous epithelial cells had numerous, irregular MV-like processes next to the usual microridges. Thus, the use of SEM for the diagnosis of human bladder cancer is fraught with considerable danger at this time". From SEM cytologic view points, however, one would expect this particular case to be malignant, when about 30 % of the 200 urothelial cells from the one patient had PMV on the surface of round or oval cells. Our previous observations on biopsied specimens (3, 4) indicated that bladder tumors have a wide range of cell surface structures, that is, there are some tumors made of relatively monomorphous cells whereas others are composed of smooth-surfaced cells and of cells with varied degrees of pleomorphism. We (3, 4) have grouped the latter type of tumors to type 3, which was mostly found in tumors of grades 2 and 3. Therefore, it is reasonable to find some cells lacking pleomorphism in a given tumor, the majority of which is made of pleomorphic cells with PMV. Smooth-surfaced cells in the case examined by Domagala *et al.* may have outnumbered other types of cells. Criteria of malignancy should depend on not only the presence of PMV but also the presence or absence of NSS, such as microridges or microplicae, and the change of cell morphology, *i.e.*, from flat, polygonal to round. For instance, the mere presence of "irregular MV-like processes" on the surface of flat squamous cells dose not indicate a malignancy, if one can identify "the usual microridges", or microplicae (20) strictly speaking, on the surface.

Another point regarding urinary exfoliated cell cytology by SEM is the way of collecting and preparing the urine. Since urine *per se* is nonphysiologic in the sense of pH and osmolarity, exfoliated cells, in particular, their surface structures are artificially influenced. The degree of cell degeneration entirely depends on the time lapse of cell exposure to the air prior to the preparation; most of markedly degenerated cells become spherical and show numerous holes or pits on the cell membrane. In order to minimize the above problem, we have used bladder washings instead of voided urine, and fixed exfoliated cells immediately after collection. Our preliminary experiments indicated better preservation of cell surface

microstructures of exfoliated cells with a solution of about 400 mOsm than with a physiologic saline solution. In the present experiments, therefore, PBS containing 2.5 % glucose (430 mOsm) was tried for both cystoscopy and bladder washing. Cystoscopy or catheterization decreases the contamination of degenerated cells with numerous holes or pits, which are floating in the bladder or remaining in residual urine, and the following bladder washings enhance the yield of exfoliated cells.

Concerning the diagnosis of low-grade and noninvasive lesions described above (Table 4), six out of seven Patients with recurrent tumors were positive by SEM grading while all were negative by Papanicolaou's classification, which indicates the superiority of the former. Clinical data indicate that superficial low-grade tumors of the bladder recur at a frequency of approximately 70 %, and 10 to 20 % of them progress to invasive or metastatic tumors (21), thus, SEM cytology can be very useful in the future for close clinical follow-up of high-risk patients. Based on our clinical data (22) and others (18, 19) ,the loss of BGA reflects the degree of functional dedifferentiation, *i.e.*, of malignant potential, of tumor cells. Therefore, a combined use of immuno-SEM using T4-bacteriophages as a marker (23) for the loss of BGA with direct mapping technique for surface morphology can be promising for the early detection of lesions which may progress to high-grade tumors. The presence or absence of BGA on the surface of exfoliated cells and also of biopsied or surgically resected tumor tissues has been studied in our laboratory. Our preliminary results indicate that, as seen in Fig. 6c, the cells with less pleomorphic MV are more likely to have BGA than those with more pleomorphic MV (Fig. 5).

Fukushima *et al.* (24) in 1981 reported that when the hyperplasia of the rat urinary bladder, induced by surgical incision, freeze-ulceration or formalin instillation, was marked to form nodular and papillary pattern, occasional epithelial cells had PMV on their surface. These lesions were reversible and did not progress to carcinoma over a 2-year observation period. In the areas of hyperplasia, the cells with PMV were often flat and polygonal, varying little in shape and size. The PMV themselves were few, generally very long and frequently present on the cells in cluster.[24] The relationship of reversible hyperplasia in man with that of artificial induction in experimental animals remains to be elucidated. The postoperative urinary cytology in the patients with suprapubic prostatectomy or partial cystectomy has been followed under a rigid schedule in our clinic.

High-grade tumors, in particular, often lose their surface microstructures due probably to cell degeneration. Most of these tumors, however, still possess nuclear abnormalities as seen by LM. Thus, a combination of SEM with LM will decrease the probability of misinterpretation of cytologic findings. Furthermore, through shortening the time for observing specimens under SEM, the simplified direct mapping technique by LM and SEM may also be useful as a routine screening procedure for high-risk individuals, such as patients with postoperated bladder

tumors and also workers exposed to known or suspected bladder carcinogens.

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## LEGENDS FOR FIGURES

Fig. 1. Direct mapping technique.

Fig. 2. LM and SEM of urinary exfoliated cells on the newly-developed mesh. (a) Low-power view by SEM showing clear each line and compartment (×30). (b) LM view of one compartment showing clear crossed lines (×150). (c) SEM view of the compartment in (b) (×150).

Fig. 3. Normal urothelial cells by SEM. (a) Prominent microridges on a flat, polygonal cell (×2,800). Inset: The identical cell by Giemsa staining. (b) Flattened microridges on a flat, polygonal cell (×1,800). (c) Microplicae on two flat squamous cells (×600). Inset: Two identical cells straddling a vertical line by Giemsa staining. (d) Coexistence of microplicae and microvilli on a cell with several facets (×1,500).

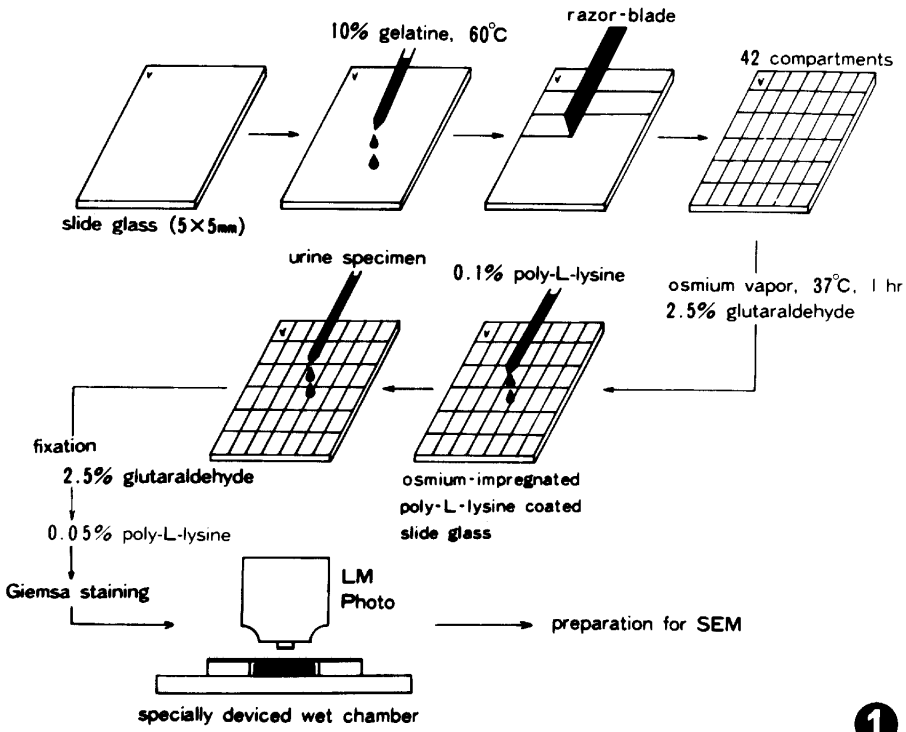
Fig. 4. Mildly pleomorphic microvilli on a flat cell (×21,000).

Fig. 5. Typical pleomorphic microvilli on two cancer cells (×11,000). Inset: A cell cluster consisting of identical, thickened, polygonal cells.

Fig. 6. A cell cluster derived from a patient with a low-grade bladder cancer. (a) Low-power view (×1,700). Inset: The identical cell cluster by Giemsa staining. (b) Monomorphic microvilli on a few conglomerated, large spherical cells (×5,200). (c) Bleb or bleb-like structures on a polygonal, thickened cell (×6,300).

Fig. 7. Pleomorphic microvilli of S-5 on a cell cluster derived from a patient with a grade 2 bladder tumor (×4,200). Inset: The identical cell cluster by Giemsa staining.

Fig. 8. The cells obtained from a high-grade tumor with infection. (a) Pleomorphic microvilli on a cancer cell found in the cell cluster at the top of the inset (×11,000). (b) Pleomorphic microvilli-like surface structures and membrane ruffles on two leukocytes found in the cell cluster at the bottom of the inset (×11,000).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fixation
2.5% glutaraldehyde
osmium-impregnated
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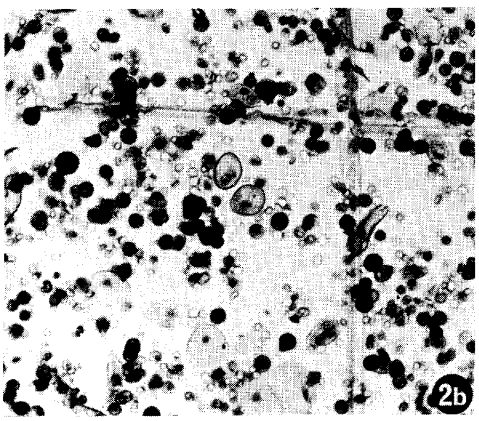
LM
Photo
preparation for SEM
specially deviced wet chamber
1


2a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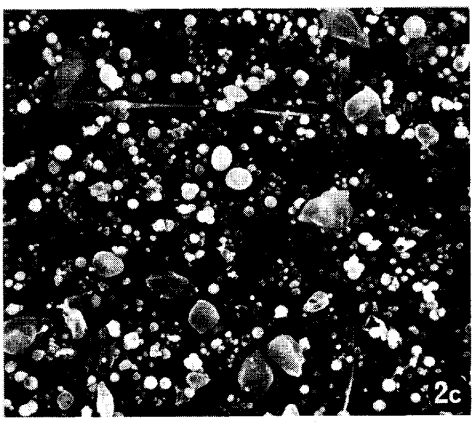
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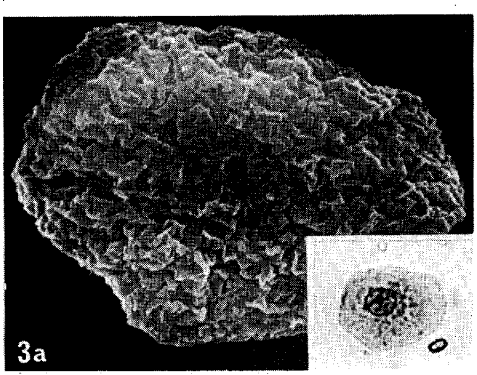
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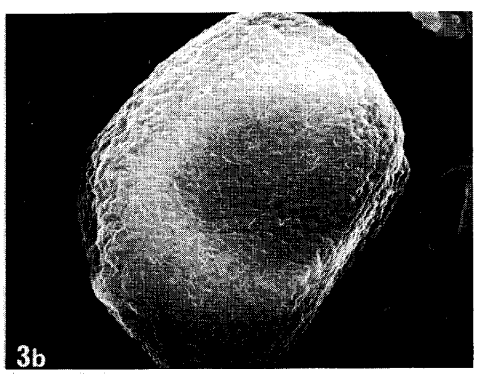
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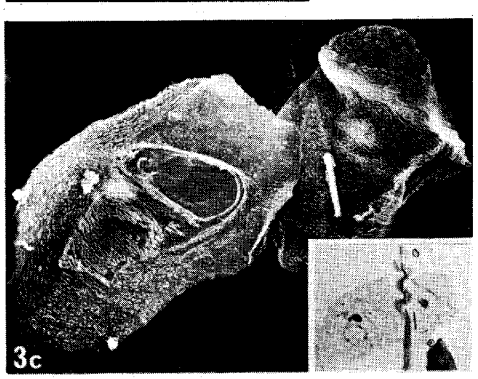
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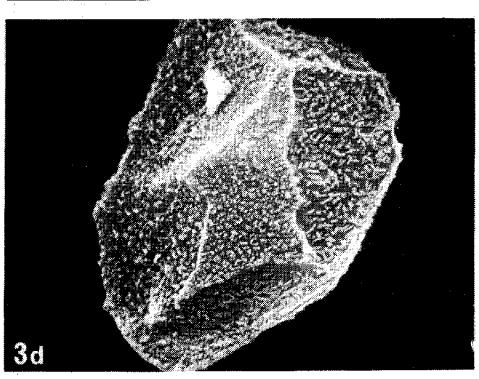
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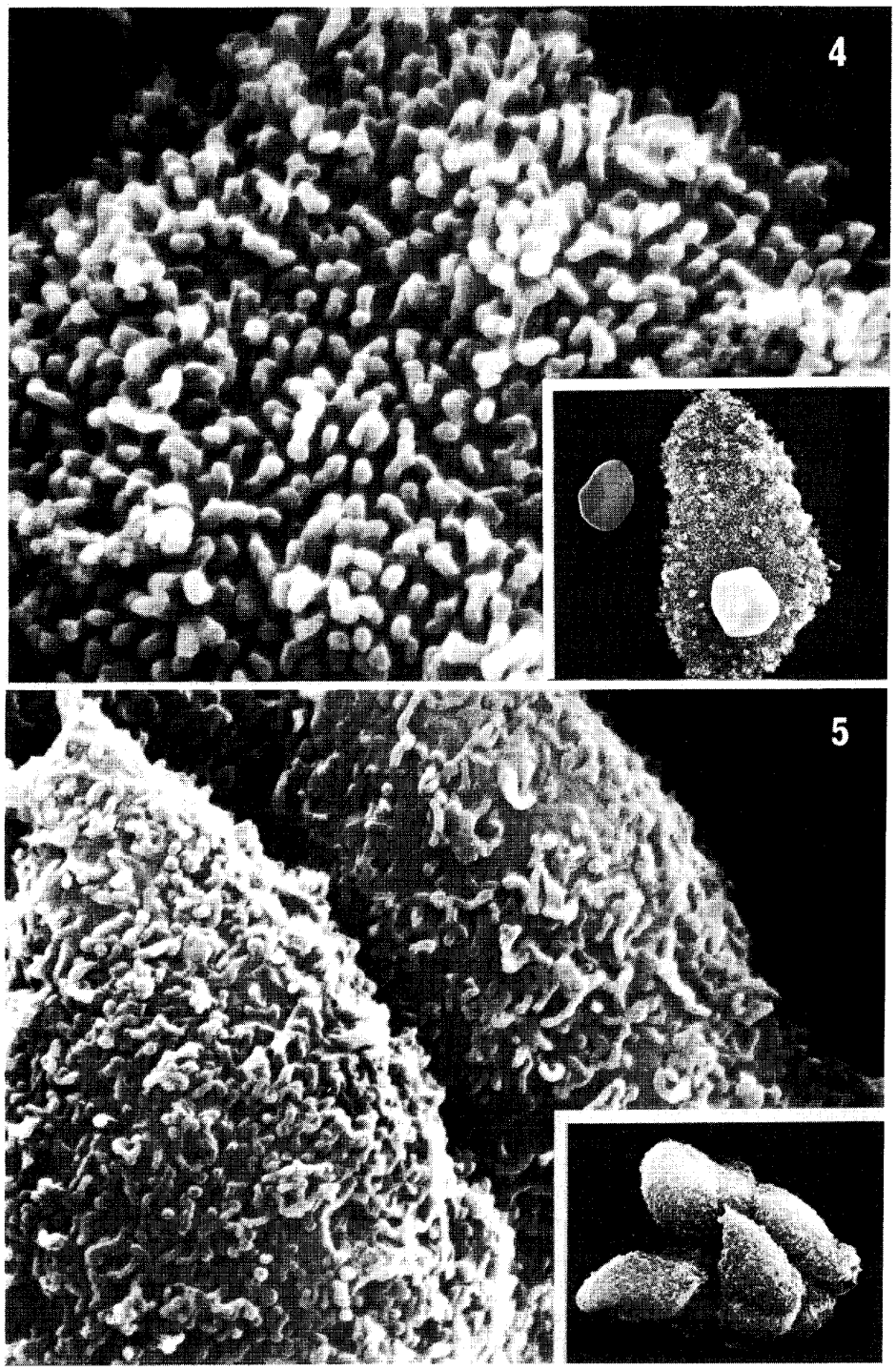
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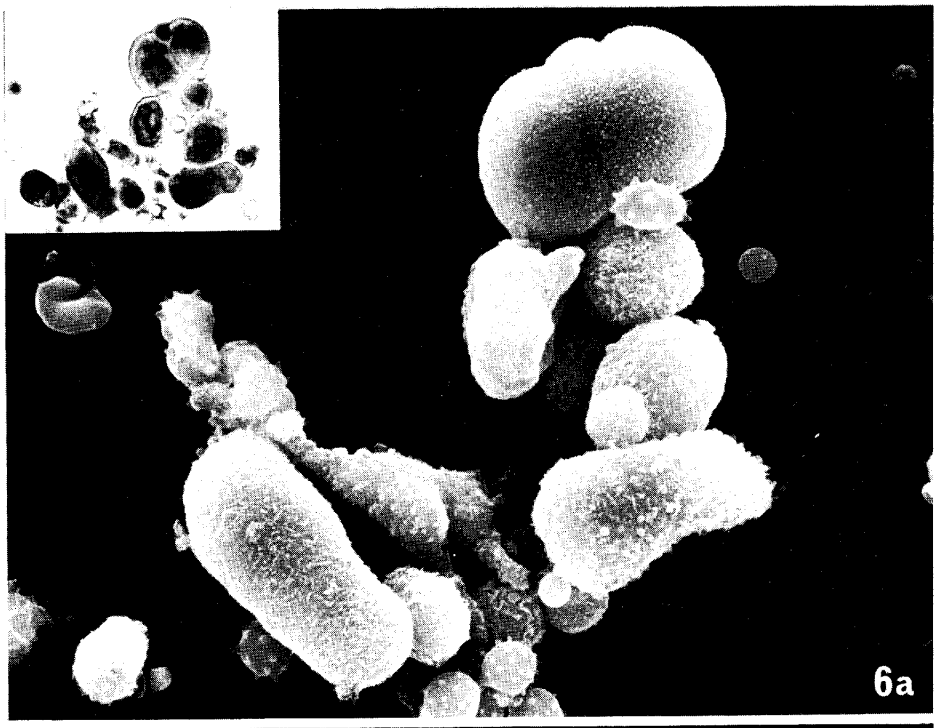
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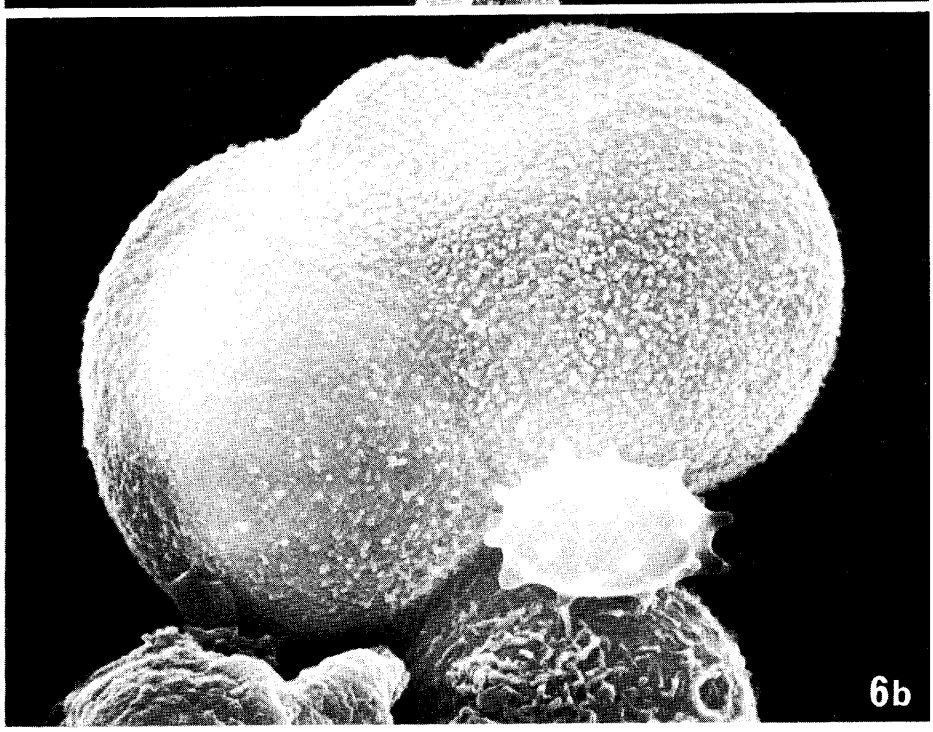
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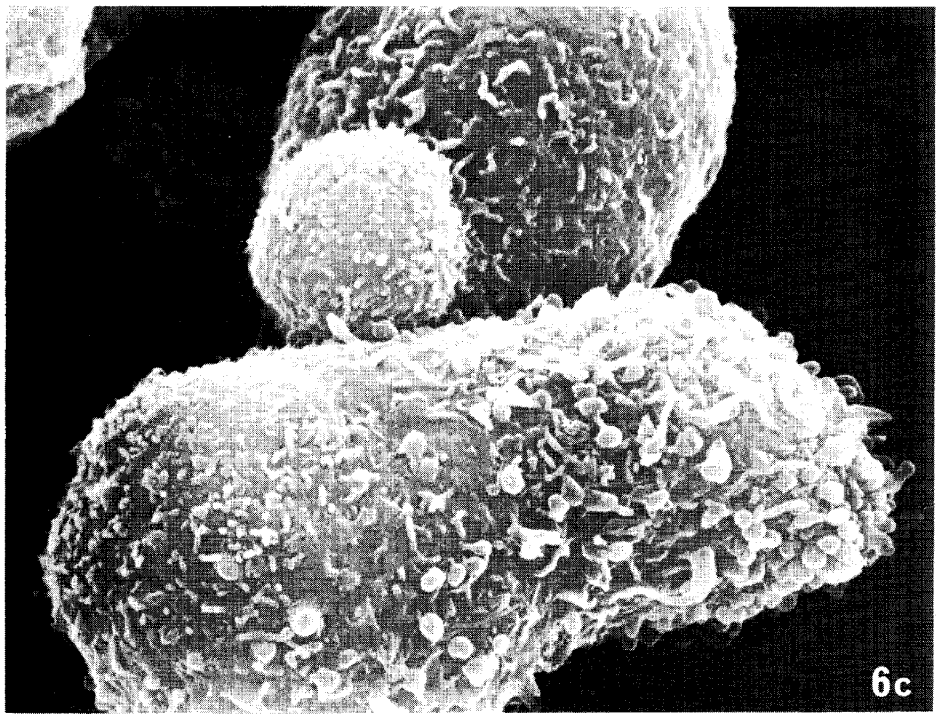
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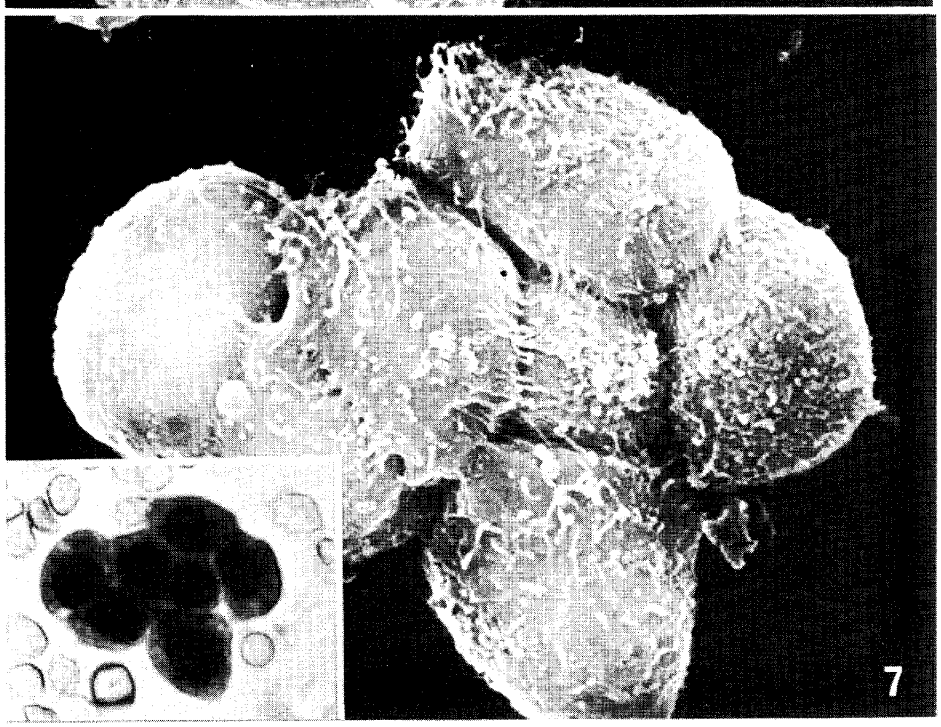
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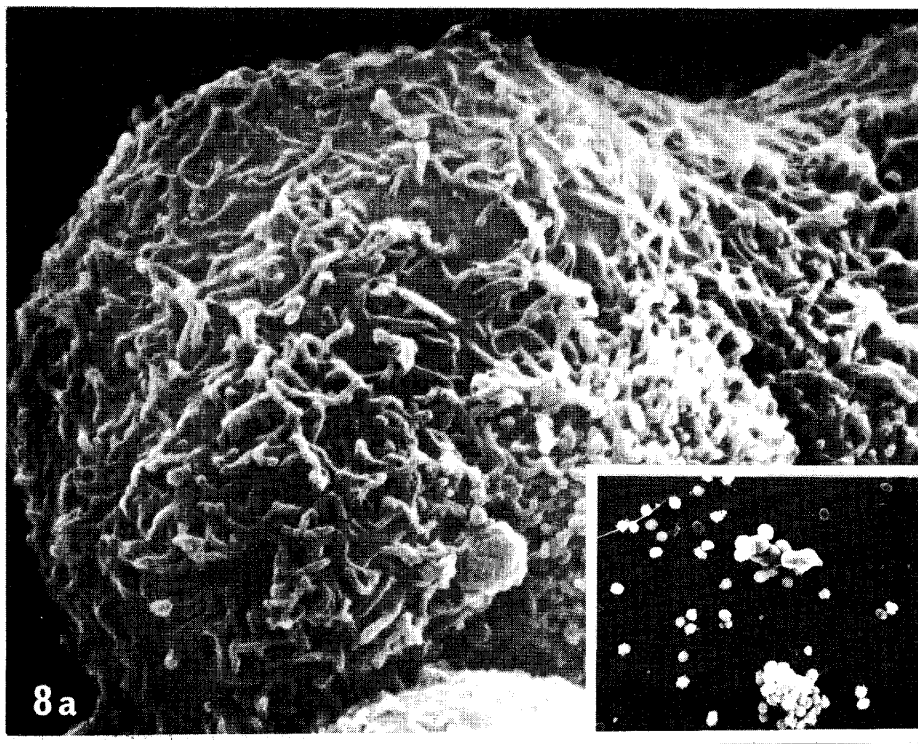
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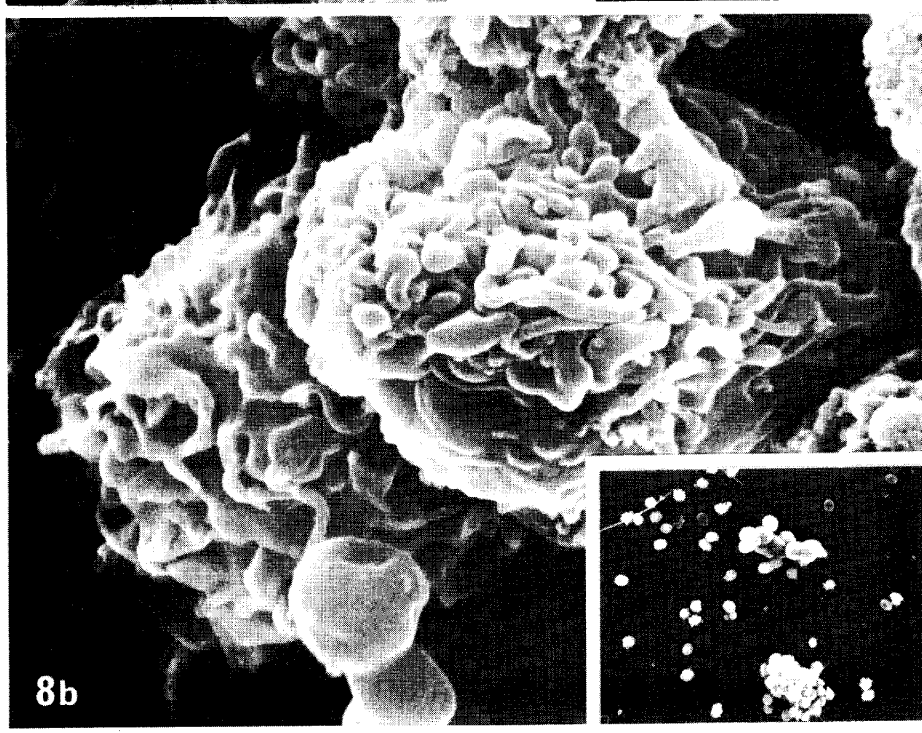
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