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**KEYWORDS:** BBN-induced cancer, bladder cancer, cell line, cell, surface

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# A CELL LINE DERIVED FROM BBN (N-butyl-N-[4-hydroxybutyl]-nitrosamine)-INDUCED RAT BLADDER CANCER: ESTABLISHMENT AND SCANNING ELECTRON MICROSCOPIC CELL SURFACE CHARACTERISTICS

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*Abstract.* This research was performed to establish a cell line from experimental bladder tumor and to discuss the biological characteristics of the cell line so established. Tissue cultures of epithelial cells were derived from a rat bladder cancer induced by BBN. The cells showed loss of contact inhibition and the phenomenon of piling up after several subcultures. Colonial cloning was used. The population doubling time of the wild strain and the colonial clones was about 30 h. The chromosomal mode ranged from triploid to tetraploid. Plating efficiency was well below 20%. Intraperitoneal backtransplantation into newborn Wister rats resulted in tumors in all cases. These tumors, in some parts, resembled primary transitional cell carcinoma. The major tumor cell groups, however, showed marked keratinization and the picture of squamous cell carcinoma. The nucleus/cytoplasm ratio and the numbers of nuclei, free ribosomes and intracytoplasmic microfibrils were increased. Dense microvillus arrangements characterized the electron microscopic picture. During the mitotic phase, the cells became large and globular whereas the microvilli were relatively short and were gathered profusely over the whole surface. Cells in the gap 1-synthetic phase developed lamellipodia and pseudpodia-like cytoplasmic processes and were polygonal in shape. Microvilli were present in the central part containing the nucleus, but their numbers were somewhat decreased and their height increased (scanning electron microscopy).

*Key words*: BBN-induced cancer, bladder cancer, cell line, cell surface

Tissue culture first introduced by Harrison (1) in 1907 has come to be widely used for research in specialities such as biochemistry, virology, immunology, and oncology. The attempts by Carrel (2) to culture cells from human malignant tumors and the subsequent culture of non-epithelial tumor cells by groups such as Gey (3) were of particular importance to the field of cancer research, which has gone on to make great progress since the establishment of the HeLa cell line by Gey in 1952 (4). The stage has now been reached at which various cell lines

established from both human and animal malignant tumors are available (5), and these serve as a valuable tool for basic research into anti-cancer immunochemotherapy.

The successful establishment of cell lines from human bladder tumors, the most frequent urinary tract malignancy, has been reported by Yajima (6), Rigby (7), Elliot (8), and others. Reports of experimental bladder cancer, however, have so far been limited to those of Toyoshima *et al.* (9) who established the Nara bladder cancer II from a BBN-induced tumor, of Lavin *et al.* (10) who established three cell lines (one of which was fibroblastic) from tumors induced by the combined use of N-2-fluorenylacetamide and cyclophosphamide, and of Kitagawa *et al.* (11) who established four lines from BBN-induced tumors.

The treatment of bladder cancer consists of a combination of surgery and radiotherapy, often supplemented by cryosurgery and the intravesicular instillation of chemotherapeutic agents. In order to carry out basic research at a cellular level on such methods of treatment, a cell line established from experimental bladder cancer was desirable, and a successful primary culture of BBN-induced rat bladder cancer was achieved in 1975. This paper reports the results of cloning, chromosome analysis, and backtransplanting of cultured cells together with a description of the morphology during continued subculture.

Moreover, if cell shape and alterations in surface morphology are indeed related to changes in the internal structure of cells (12), then it should be possible, to a certain extent, to gain knowledge of the intracellular changes that these reflect by study of cell morphology and surface structure. Over the past few years, the genesis of bladder carcinoma has been studied using scanning electron microscopy (13–15). This technique has also been applied to the exfoliative cytologic diagnosis of urinary tract malignant tumors. At the time of writing this paper, however, there are no reports to hand of scanning electron microscope study of the changes which occur with time in the surface structure of bladder cancer cells. In the present work, therefore, synchronized cultures of lines cloned from established bladder cancer cell lines were studied by scanning electron microscopy in regard to these points.

## MATERIALS AND METHODS

*Primary culture and subcultures.* The establishment of the primary culture has been described in part by the author's co-worker Ike (16). Briefly, the procedure was as follows: Wister strain male rats were raised with drinking water containing 0.05% BBN. The bladder cancer which developed was removed, minced to a size of approximately 2 $mm^3$, and suspended in culture medium (Eagle's MEM [Chiba Prefecture Serum Institute] + 10% fetal calf serum containing streptomycin and penicillin). This cell suspension was then placed in a TD 15 flask and cultured under stationary conditions at 37°C in an incubator. The time for

change of the culture medium was decided by visual estimation of the pH of the culture fluid. After the twentieth passage, the culture medium was changed to Eagle's MEM + 20% heat inactivated bovine serum. Whenever the cultures became confluent, they were exposed to trypsin + EDTA (later trypsin only) to disengage the cells. These were centrifuged, the supernatant discarded, the pellet resuspended in 1 cc of medium, and one-third of the cells thus obtained replated in new petri dishes or test-tubes (approximately once a week).

The morphology of cultured cells was observed with an inverse phase contrast microscope. For light microscope studies of cell cultures, sterile coverslips were placed in petri dishes until the cells formed a confluent monolayer. This was then fixed in Carnoy's solution and stained with Giemsa-Grünwald solution.

*Cloning procedure.* Single cell cloning was performed under microscopic control using microcapillaries to transfer the cells to microplates (Tokiwa's method (17). Colonial cloning was achieved by the trypsinized filter paper technique of Puck and Marcus (18).

*Measurement of growth rate.* For both the wild strain and the derived clones, the simplified replicate culture method of Katsuta *et al.* (19) was used to estimate cell proliferation. Population doubling times were calculated from the logarithmic phase of growth curves.

*Plating efficiency.* Puck's method was used to estimate plating efficiency (18). Plates were incubated in a carbon dioxide incubator for 10 days at 37°C, then stained with Giemsa-Grünwald solution. The number of colonies and the numbers of cells in each colony (*i.e.* "colony size") were counted under low power magnification.

*Chromosome preparation.* Chromosomes were obtained from subculture cells by the air drying method of Moorhead *et al.* (20). The chromosomes so obtained were stained with Giemsa-Grünwald solution, counted (100 chromosomes in metaphase), and the chromosome distribution charted.

*Tumorigenicity of cultured cells.* After trypsinization, the cultured cells were suspended in PBS at a concentration of $5 \times 10^6$ cells/ml, then backtransplanted intraperitoneally into newborn Wister strain rats (1 ml of cell suspension per rat). These rats were checked periodically for signs of tumor formation.

*Electron microscopy.* The wild strain and each of the derived clones were examined every five passages commencing with the twentieth passage. After a culture had become confluent, it was washed with PBS and new medium was added. The cells were then scraped off the bottom of the culture flask with a rubber policeman and collected by gentle centrifugation in test tubes. The supernatant was discarded and 2.6% phosphate-buffered glutaraldehyde added for sixty min. Cells were then washed three times with buffer, fixed for one hour in phosphate-buffered 1% osmium tetroxide, washed again, and stained for one hour in 0.5% uranyl acetate. Dehydration in graded-alcohol concentrations followed, then the cells were embedded in a mixture of propylene oxide and epoxy resin (21). Sections (cut on a MT-1 Porter-Blum ultramicrotome) were stained with uranyl acetate and lead citrate, then carbon coated (22). A HS-8 Hitachi electron microscope was used to examine the grids thus obtained.

*Scanning electronmicroscopy.* The cells were cultured on round cover slips for three days, then autoradiograms were prepared after pulse-labeling with $^3$H-TdR. Fraction of labeled mitosis (FLM) was calculated every 2 h through 40 h. The FLM curves were analyzed by the asymmetry method (23) and the results of these experiment were used in the following study.

Synchronous populations of cells in mitotic phase were obtained by the "shake-off" method. This method produces minimal cell damage and selects cell populations with high mitotic indices. The mitotic index in this experiment ranged from 90% to 95%. Samples of these cells were exposed to excess [$^3$H] thymidine to provide cells in the $G_1$-S range (24). These were plated on cover glasses in petri dishes and monitored periodically with phase contrast microscopy. The cover glasses were removed periodically, and the cells washed gently in physiological saline warmed to 37°C. Next, double fixation with 5% glutaraldehyde and 1% osmium tetroxide was followed by dehydration and replacement with amyl acetate. The preparations were then subjected to critical point drying before being coated with evaporated gold in an ion-coater (25). All studies were performed with a U-3 Nihon Denshi scanning electron microscope operated at 25 KV.. In addition to the above experiments, the chronological changes occurring in a single cell were studied using phase contrast microscopy.

## RESULTS

*Morphology.* In both the primary culture and later subcultures, cell migration began from one part of the tumor tissue edge. Initially the cells were a mixture of various types ranging from small ovoid to large polygonal. During the second passage, polygonal cells became predominant but there were also small numbers of large cells with copious cytoplasm and small cells with little cytoplasm. In the third passage, middle size cells were prominent with some large cells mixed in. Small cells were seen in areas where cells were thickly concentrated or piled up. Culture beyond the fifth passage after confluency resulted in dense proliferation of the small cells and marked piling up became apparent. Subcultures beyond this stage had a majority of middle size cells which piled up densely and became smaller sized cells as the duration of culture increased.

Culture beyond the twentieth passage (bovine serum used instead of fetal calf serum) did not produce any changes in the morphology or pattern of proliferation of the cells. Immediately after subculturing, the cells were globular, but proceeded to become polygonal, accompanied by large numbers of granules in the cytoplasm. Cells containing vesicles increased in number. The nuclei were large and round and the majority had 2–3 nucleoli. Various mitotic patterns were present. With proliferation and confluency of the cells, the areas where polygonal and long narrow spindle cells were mingled showed signs of the polarity characteristic of transitional epithelium. No part of the cultured cells was stained by orange-G contained in Papanicolaou staining solution, indicating a

lack of keratinized "prickle" in the cultured cells (Fig. 1-4).

Continuation of the culture resulted in marked piling up, and signs of exfoliation of the superficial cells from the major cell mass. Passage was performed at this time and the present experiments performed with one of these cell lines. The other cell line was frozen and stored.

*Single cell cloning and clonical cloning.* Single cell cloning was attempted with cells from the 40th passage (that is, which had been cultured for more than 430 days). A total of 320 individual cells (four lots of 96) were aspirated into microcapillaries, transferred to microplates, and cultured with condition medium. The results of this procedure were as follows: four produced colonies of 8 cells, 12 gave colonies of 4 cells, and 10 gave colonies of 2 cells. No other colony formation occurred. The remaining 296 cells showed a lack of cell division and died within two weeks. Because single cell cloning was so difficult, it proved necessary to use colonial cloning derived from cells of the 45th passage (that is, which had been cultured for 465 days). Ten cloning lines were achieved in this way. All of these were epithelial cells and showed slight differences in size and morphology. Clone 10 comprised polygonal cells with large nuclei and copious cytoplasm. These cells gathered together in groups (Fig. 5). Clone 2 cells had rather small nuclei and little cytoplasm (Fig. 6). Continued observation of the wild strain and the clones indicated that slight differences became evident with continued culture. With increasing subculture of each of the clones, the cells formed groups made up of small and medium sized polygonal cells. At confluency, the spindle cells showed marked similarities with the wild strain, so that the difference between the wild strain and the clones became blurred.

The growth curves for several subcultures showed that early passage was unstable but settled in line with growth. The cell count adjusted to $5 \times 10^4$ cells/ml had a population doubling time between 28 and 34 h at the logarithmic growth. This did not alter with later passages (Table 1).

The plating efficiency of the wild strain at 430 days of culture, that is, the 40th passage, was investigated. The average colony count from an inoculum of 3000 cells/3 ml was 444 (14.8%), of which two-thirds of the colonies contained only from 2 to 8 cells (Tables 2, 3). The average colony count from an inoculum of 300 cells/3 ml was 17 (5.7%). The plating efficiency was, therefore, very low and there were no colonies containing more than 32 cells. Further estimations of plating efficiency were performed but the results were all around 10%. None of the results exceeded 20%. With an inoculum of 30,000 cells/3 ml, the colonies were confluent and uncountable.

*Chromosome count.* The wild strain chromosome count at 430 culture days was between 52 and 120 with a mode of 74. At 510 culture days, the distribution was between 50 and 108 with a mode of 70. The chromosome distribution

TABLE 1. BIOLOGICAL CHARACTERS OF CULTURED CELLS. ALL LINES SHOW EPITHELIAL MORPHOLOGY WITH LOSS OF CONTACT INHIBITION

| Cells | Culture days | Doubling time (h) | Chromosome modal number | Tumorigenicity | Average survival days |
|---|---|---|---|---|---|
| Wild 1 | 115 | 76 | — | — | — |
| 2 | 430 | 34 | 74 | 4/4 | 45 |
| 3 | 510 | 32 | 70 | 4/4 | 32 |
| Clone 1 | 530 | 30 | 72 | 4/4 | 48 |
| 2 | 530 | 32 | 73 | — | — |
| 3 | 537 | 28 | 66 | 4/4 | 35 |
| 4 | 537 | 34 | 72 | — | — |
| 5 | 544 | 28 | 67 | — | — |
| 6 | 570 | 30 | 62 | 4/4 | 38 |
| 7 | 570 | 34 | 80 | — | — |
| 8 | 590 | 28 | 75 | 4/4 | 32 |
| 9 | 590 | 30 | 66 | — | — |
| 10 | 590 | 30 | 74 | 4/4 | 49 |

TABLE 2. THE PLATING EFFICIENCY OF CELLS AT DIFFERENT INOCULUM SIZE

| Number of cells inoculated per dish | Number of dishes used | Number of colonies formed (Mean±S. D.) | Plating efficiency (%) |
|---|---|---|---|
| $3\times10^4$ | 3 | Uncountable | |
| $3\times10^3$ | 3 | 444±69.4 | 14.8 |
| $3\times10^2$ | 3 | 17± 8.8 | 5.7 |

Single cell ratio; 100%.

TABLE 3. THE DISTRIBUTION OF COLONY SIZE

| Inoculum size per dish | Colony size (cells) | | | |
|---|---|---|---|---|
| | 2-8 (Mean±S. D.) | 9-32 | 33-128 | 129< |
| $3\times10^2$ | 15.6± 7.6 | 1 | 0 | 0 |
| $3\times10^3$ | 306.6±34.3 | 113.3±30.2 | 21.3±6.4 | 3.0±2.0 |

Single cell ratio; 100%.

range of the clone was fairly narrow and the mode was in the triploid to tetraploid range (Table 1).

*Backtransplanting*. Backtransplanting was attempted in the wild strain at 430 and 510 culture days and in the five clones. Tumors developed in all cases (Fig. 7). The longest rat survival was 70 days. The shortest was 30 days. The results for each of the clones are summarized in Table 1. The tumors had spread

intraperitoneally and histopathologically some parts (Fig. 8) resembled primary transitional cell carcinoma (Fig. 9). The major tumor cell groups showed marked keratinization and the picture of squamous cell carcinoma (Fig. 10).

*Ultrastructure.* In comparison with normal bladder mucosa cells, the nucleoli and the nucleus/cytoplasm ratio were increased (Fig. 11). Free ribosomes and intracytoplasmic microfibrils were increased, and a profuse intimate arrangement of the microvilli was characteristic. Parts of the microvilli were joined to desmosomes. Granular endoplasmic reticulum and mitochondria showed normal development but the Golgi apparatus was poorly developed. Compressed vesicles usually seen in the surface transitional epithelial layers could not be identified (Fig. 12).

Cells obtained by the shaking method were placed in petri dishes (with conditioning medium) and culture commenced. Observation with the phase contrast microscope showed that the large globular cells of the earlier period of the mitotic phase began to divide This division was completed in approximately sixty min. Two hours later, the globular shape began to change and at 8 h, the cells had spread and were putting out processes. At 16 h the cells were polygonal and at 26 h the cells reached a maximum spread over the glass surface. At 32 h loss of the cytoplasmic processes occurred and the cells became large and globular as they entered the mitotic phase. In comparison with the FLM curve which showed a cell cycle of 28.8 h ($G_1+0.3$M 15.8 h, S 9.6 h, $G_2+0.7$M 4.4 h)*, this was slightly prolonged. An analysis of the cell cycle will be discussed in a later report.

In the mitotic phase, the cells became large and globular whereas the microvilli became relatively short and were gathered profusely over the whole surface (Figs. 13, 14). After completion of the mitotic phase, early $G_1$ phase cells spread over the glass cover-slip. The microvilli remained profuse in the central area enveloping the nucleus but formed lamellipodia like the brim of a hat towards the periphery (Fig. 15). The ends formed a ruffled border (Figs. 16, 17).

Cells in the $G_1$-S phases developed lamellipodia and pseudopodia-like cytoplasmic processes and were polygonal in shape (Figs. 18, 19). Microvilli were present in the central part containing the nucleus, but their numbers were somewhat decreased and their heights increased. They extended in many directions (Fig. 20). The height and density decreased towards the periphery (Figs 18, 19). The cytoplasm spread well into the advancing parts of cell and the edge was extended as processes called fillopodia which were stuck to the surface of the glass (Fig. 21).

* M, mitotic phase; $G_1$, gap 1 phase; S, synthetic phase; $G_2$, gap 2 phase.

## DISCUSSION

The establishment of cell lines from human and animal bladder cancer is extremely difficult with only one line per research center having been developed from human tumors. Using either human or animal tumors, primary culture is performed by explantation which is easier than attempting disperse culture. This is probably because the explant cultures are not affected by agents such as trypsin, have little likelihood of developing infections, and do not undergo any decrease in their cell numbers.

Since the report of the establishment of a cell line from experimental bladder cancer by Lavin *et al.* (10) other successful lines from experimental bladder cancer have been reported by, for example, Toyoshima (9) and Kitagawa (11) and, from these, the biological character of bladder carcinoma is gradually being understood.

The population doubling time of our established cell line is approximately 30 h. Often spindle shaped cells are scattered throughout the culture and parts show the characteristic of transitional epithelium, polarity.

Normal transitional epithelial cells have fixed polarity of the Golgi apparatus. In the basement cells, the Golgi apparatus is gathered beside the nucleus; in the middle layer cells, it is above the nucleus; and on the surface side of the surface cells, it is below the nucleus, situated basally. Cytoplasmic organelles are gathered in the surface cytoplasm (26). This is thought to be the reason why bladder cancer proceeds in a fixed direction when it proliferates, and it is postulated that this character is preserved in those parts of our monolayer cell culture showing polarity.

The chromosome distribution of our established cell line, as with other experimental bladder cancer cell lines, had a mode between 70 and 80 which was less than the tetraploid range. Lavin and Koss (10) have also reported the existence of a marker chromosome due to banding. Up to the present time, the human and animal cell lines derived from malignant tumors mostly have had modes in the triploid range.

There have been no reports of successful single cell cloning from cultured tumors. In the present experiments also, plating efficiency was extremely low (5.7% with an inoculum of 300 cells/3 ml), and a long way from the 20% required to ensure successful single cell cloning. This is represented clinically by the fact that many bladder tumors are multicentric in origin and despite repeated reoccurrences, show relatively late infiltration of surrounding tissue compared to tumors of other organs. Moreover, metastasis is rare so the prognosis is comparatively good.

The clones obtained by Lavin and Koss (10) were derived from squamous

cell cancer and comprised one fibroblastic and two epithelial cell clones. Backtransplants from the two epithelial cell clones produced squamous cell carcinomas (10). Toyoshima and Ito used clones derived from transitional cell cancer but backtransplants resulted in squamous cell cancers (9). Kitagawa did not report a picture of squamous cell carcinoma (11).

Transitional epithelium is thought of as epithelium showing a structure intermediate between squamous epithelium and columnar epithelium. Both squamous epithelium and transitional epithelium are differentiated into structures suitable to contraction and extension (27), but transitional epithelium is also waterproof. However, with backtransplantation, it changes to stratified epithelium indicating that transitional epithelium itself already possesses the ability to become keratinized epithelium. It is considered, therefore, that transitional cell epithelium which became poorly differentiated stratified epithelium was involved in the Lavin and Koss (10) experiments on the course of carcinogenesis, and in the experiments on backtransplantation by Toyoshima (9) and the present author. That this was not due to culture-induced differentiation was indicated by the lack of change in morphology, growth curve or chromosome number and that transitional epithelium parts developed after backtransplants in the present work.

Differentiated transitional cell epithelium is characterized by a simplification of cell structure and a general decrease in the cytoplasmic organelles of, for example, mitochondria, Golgi apparatus and endoplasmic reticulum. The tight junctions of surface tumor cell are discernible but the compressed vesicles are said to be markedly decreased in number (26). These compressed vesicles are characteristic vesicles derived from unit membrane of parts of the intermediate and surface layer cells of transitional epithelium (28). It is known that the compressed vesicles and tight junction structures are characteristically lost in undifferentiated transitional cell cancer.

Lavin and Koss (10) studied large numbers of compressed vesicles, tonofilaments, and free ribosomes in culture cells and backtransplant cells by electron microscopy. In the present author's observations, the culture cells did not possess the functions of contraction and relaxation, so the existence of compressed vesicles was not necessary. The compressed vesicles observed by Lavin and Koss were probably the remnants of fully differentiated normal cells present amongst their culture cells.

Histological diagnosis developed from Papanicolaou staining has been applied to exfoliative cell diagnosis of urine specimens. Its effectiveness has been recognized in mass screening for occupational urinary tract tumors and follow-up of patients with urinary tract cancer, but it has only a low detection rate for the comparatively frequent, superficial papillary tumors of low malignancy (29). Moreover, in terms of urinary tract tumors, the high incidence of Class IV

(suspicious of malignant tumor) is becoming a problem. Consequently, the study of cells exfoliated in the urine by scanning electron microscopy is now being attempted. Jacobs *et al.* (30) used rats and administered N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) orally. They observed changes in the microridges of the surface of normal surface layer cells. After six weeks of administration, these were covered with small uniform microvilli, and at eight weeks, with pleomorphic microvilli. In both groups, a return to normal occurred 4–6 weeks after ceasing administration. The group that had six weeks administration was all normal 44 weeks after cessation of administration; however, the 8 week group, although normal for 42 weeks after ceasing administration, again developed hyperplastic lesions to take on the appearance of pleomorphic cells covered in pleomorphic microvilli. Jacobs' group, therefore, considered that such pleomorphic microvilli represented an irreversible alteration of bladder mucosa.

The results of their experiment are strong support for the opinion already established at the light microscope level that changes called hyperplasia consist of reversible and irreversible types.

Arai *et al.* (15) administered N-butyl-N-(4-hydroxybutyl) nitrosamine to rats and noted two types of cell, (cobblestone-like cells possessing microvilli and hemispherical cells possessing microvilli) in the areas of focal hyperplasia. The surface of the papillary carcinoma was covered in hemispherical cells of various sizes with numerous complex microvilli. This was completely different from the network of fine ridges on the surface of normal cells. Fulker *et al.* (31) have reported the presence of microvilli of various sizes in human bladder cancer also.

From findings such as the above, Jacobs *et al.* (32) considered the existence of pleomorphic microvilli an important aid to the diagnosis of irreversible superficial bladder cancer, and, in three patients with superficial bladder tumors misdiagnosed as Class II, showed, by SEM, the presence of pleomorphic microvilli. All of these were *in vivo* experiments and the interesting feature was that the pleomorphic microvilli maintained the same morphology in not only the mitotic phase, but in other phases of the cell cycle as well.

Porter and Prescott (12) used synchronized culture of Chinese hamster ovary (CHO) cells. Microvilli were prominent surface elements after mitosis and remained numerous after $G_1$ although their prominence seemed to decrease during S. Microvilli reappeared in large numbers per unit area of cell surface when the cells thickened in $G_2$.

The culture fibroblasts were globular in the mitotic phase, semiglobular in $G_1$, and proceeded to spread out flat. Microvilli were only seen in the central area and lamellipodia and fillopodia appeared towards the periphery. The cells most ressembled connective tissue cells in morphology during the S phase, at

which time the microvilli extended to the edges. In the mitotic phase, it is known that the cells again become globular (33, 34).

The changes in our cultured bladder cancer cells resembled those of L-cells cytomorphologically. The surface characteristics, however, were markedly different although changes in the surface structure were not as dramatic. Blebs were not evident either. In the mitotic phase, the whole surface had a comparatively low elevation and pleomorphic microvilli were present. The cells begin to take on a hemispherical shape (with the appearance of lamellipodia and cytoplasmic processes) and the pleomorphic microvilli concentrated in the centre extend out to the edge, decreasing in both height and profusion. Almost no microvilli are to be seen on the surface of the lamellpodia. Because, at this time, their diameters were maintained at almost a constant state, the surface membrane covering the microvilli must be supplied to the surface membrane of the spreading lamellipodia. Therefore, it would appear that the microvilli also play the role of a supply depot for cell membrane.

Our cultured bladder cancer cells already have widely spread lamellipodia by the Gl-phase period and the edge has a wavy structured membrane. With phase contrast microscopy and L-cells, Ohnishi (35) has reported that this wavy pattern is due to the lamellipodia, one part of the edge of which has advanced over the surface of the glass cover-slip. This spreads out to both sides, advances, then stands at right angles to the glass like the sails of a yacht. These sails face towards the cell centre and fall over in wide curves. They are said to be absorbed by the lamellipodia. It is thought that cell movement results from the repetition of this movement, which is thought to be due to two types of fine fibres (S type microfibrils in bundles and L-type microfibrils in a network). These two types of fibres show different reactions to Cytochalasin B (36).

With L-cells, fillopodia were seen in large numbers during the mitotic phase and took on the morphology of a heliozoan (35). Our bladder cancer cells, however, did not have any obvious fillopodia. During culture with serum-supplemented medium, cells derived from mouse spleen possessed pseudopia-like cytoplasmic processes, fillopodia, and lamellipodia, and were stuck to the surface of the walls in extension. In contrast to this, culture in medium lacking serum produced marked morphological changes. On the cell surface, only microvilli and layered folds were seen. Each of the various types of cytoplasmic processes had been lost and there was no extension or adhesion to the walls (37). Rather than migration and proliferation, therefore, these cytoplasmic processes appear to be intimately related to the ability of the cells to extend and adhere to the walls.

Microfilaments ran in large numbers in the microvilli and fillopodia. Furthermore, microfilaments have been reported in some circumstances under the surface of the pseudopodia-like cytoplasmic processes. These microfilaments,

therefore, appear to be extremely important to the maintenance of the cell in terms of its adhesiveness and extensibility.

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## Legends to Figures.

Fig. 1. Phase contrast micrograph of urinary bladder tumor cells in culture. Wild strain. 80th generation). ×150.

Fig. 2. Wild strain. (80th generation). Giemsa-Grünwald. ×100.

Fig. 3. Higher magnifiation of Fig. 2. Giemsa-Grünwald. ×400.

Fig. 4. Wild strain. (80th generation). Papanicolaou. ×250.

Fig. 5. Clone 10. (Polygonal cells with large nuclei and copious cytoplasm.) Giemsa-Grünwald. ×400.

Fig. 6. Clone 2. (Mixture of spindle cells and small polygonal cells) Giemsa-Grünwald. ×400.

Fig. 7. Multiple tumors in peritoneal cavity after intraperitoneal injection of cultured urinary bladder tumor cells. (Wild strain at 430 culture days)

Fig. 8. Histologic appearance of the tumor developed after backtransplantation. Hematoxylin and Eosin. ×400.

Fig. 9. Histologic appearance of the primary rat bladder tumor induced by BBN. Hematoxylin and Eosin. ×400.

Fig. 10. Keratinization in the tumor developed after backtransplantation. Hematoxylin and Eosin. ×250.

Fig. 11. Ultrastructure of the urinary bladder tumor cells in culture. Wild strain. (80th generation) N: nucleus. TEM.

Fig. 12. Higher magnification of Fig. 11. N: Nucleus, D: desmosome, m: microvilli, gER: granular endoplasmic reticulum. TEM.

Fig. 13. Cells in the mitotic phase. (above: being divided into two cells, below: roundly enlarged cell just before mitosis) SEM.

Fig. 14. Higher magnification of Fig. 13. Microvilli is relatively short and gathers profusely over the whole surface. SEM.

Fig. 15. Cell in early $G_1$-phase. Microvilli remained profuse in the center area enveloping the nucleus. L: lamellipodia, R: ruffles. SEM.

Fig. 16. Higher magnification of Fig. 15. SEM.

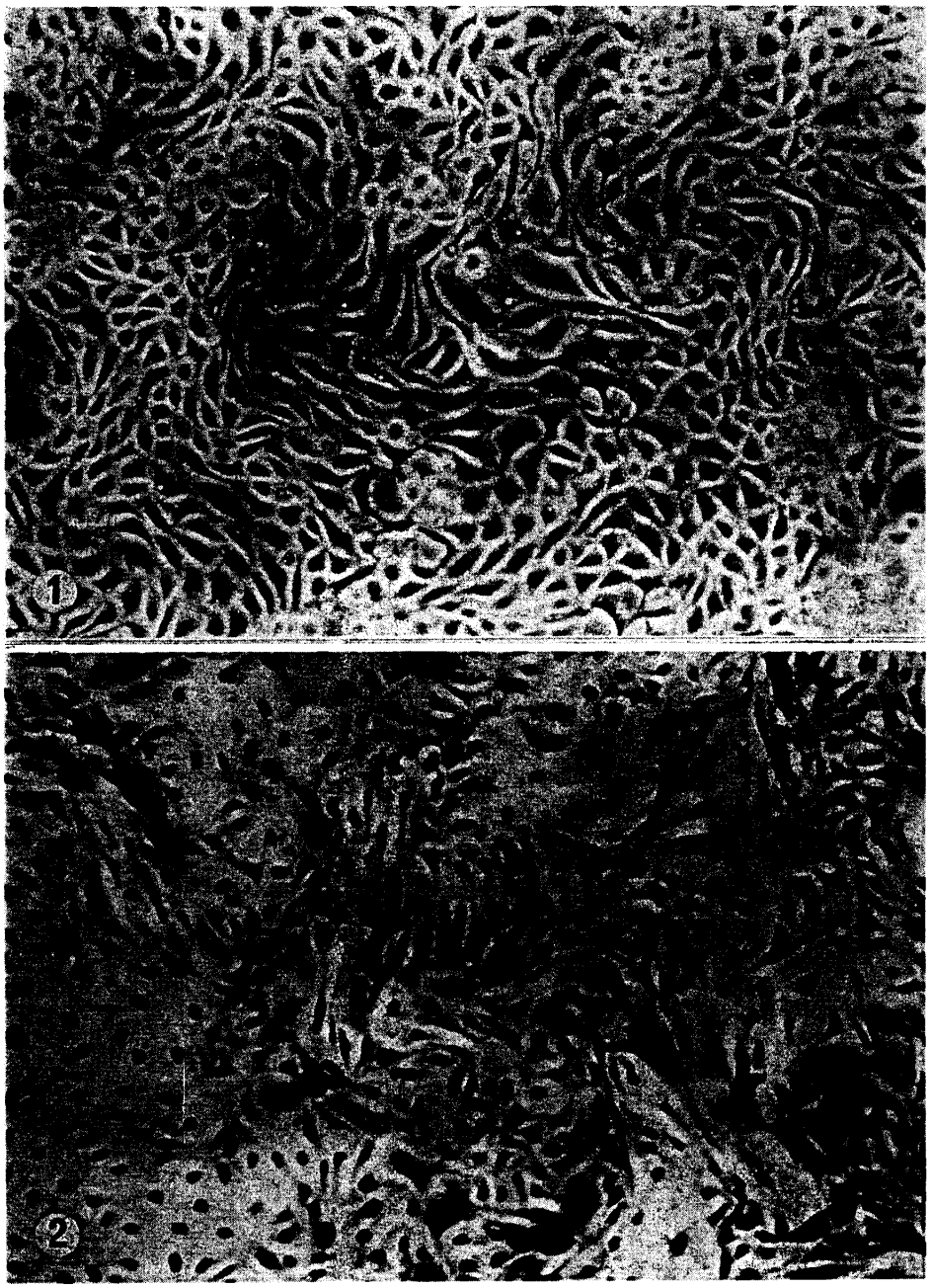
Fig. 17. Ruffles in Fig. 15. F: fillopodia. SEM.

Fig. 18. Cell in $G_1$-S phase. SEM.

Fig. 19. Cell in S phase. Microvilli present in the central part containing the nucleus. SEM.

Fig. 20. Higher magnification of Fig. 19. The numbers of microvilli are somewhat decreased and their heights increased. SEM.

Fig. 21. Higher magnification of Fig. 19. Fillopodia are stuck to the surface of the glass. SEM.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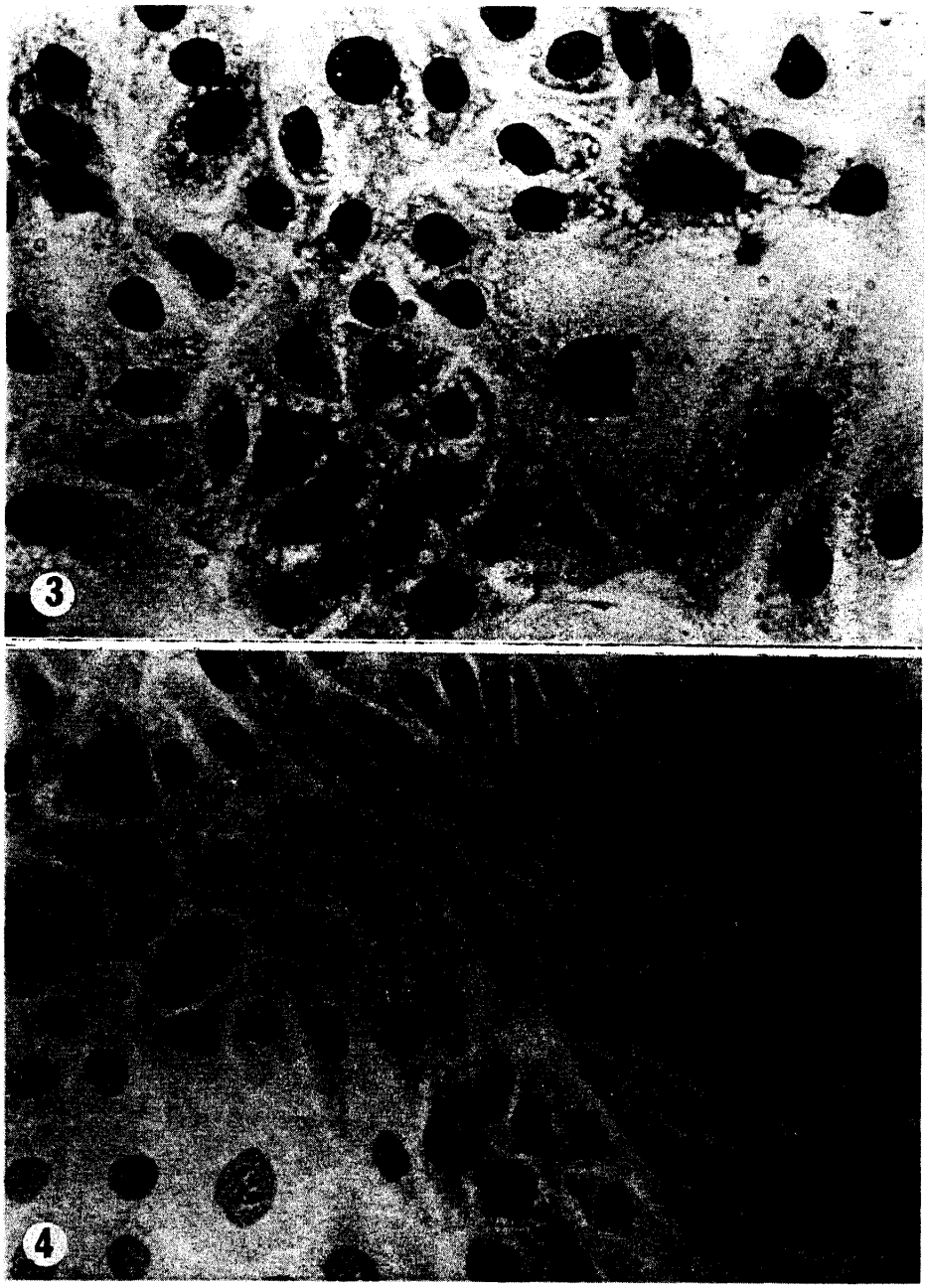
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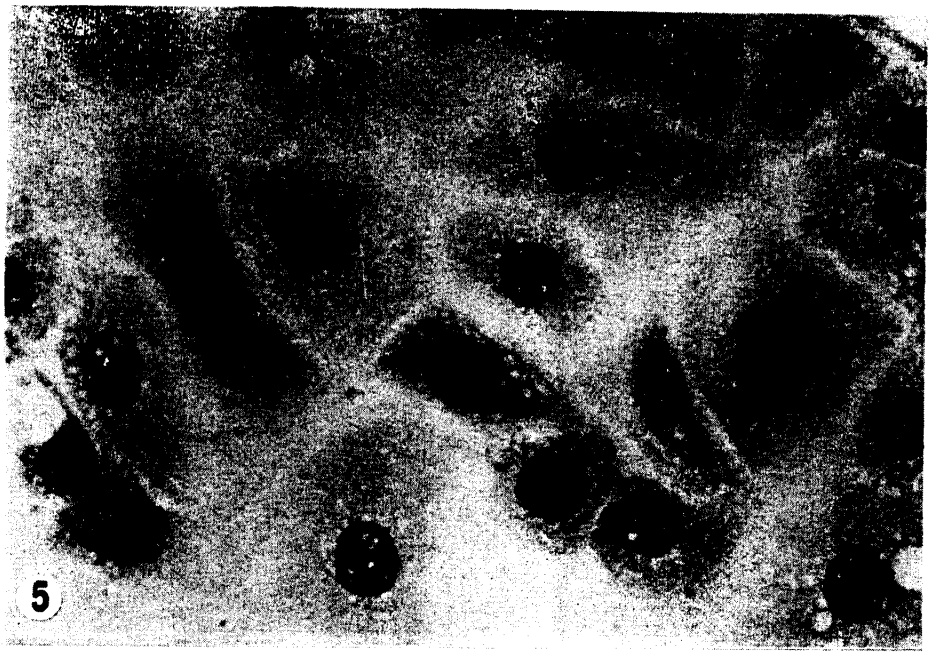
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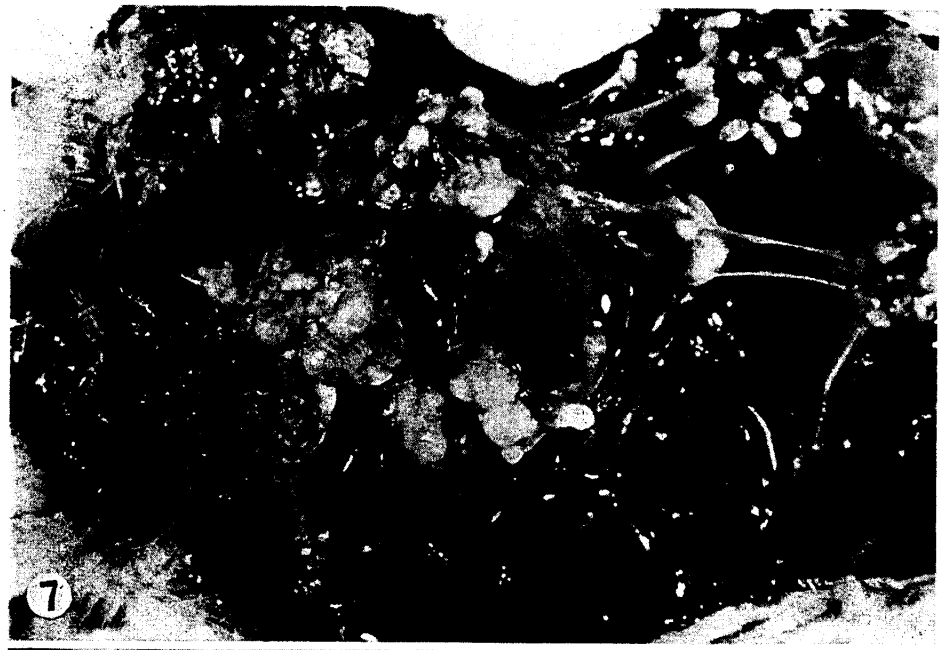

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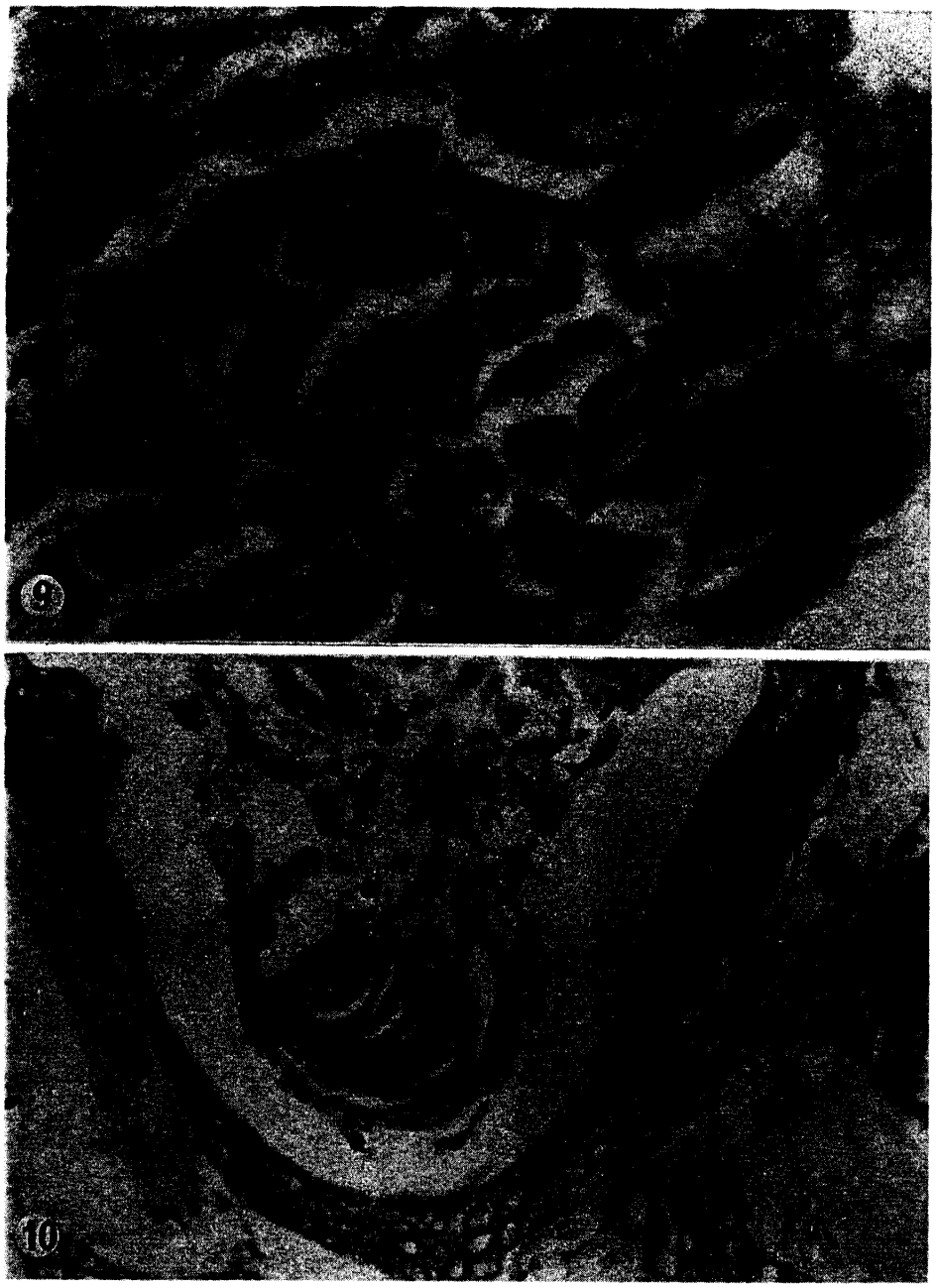
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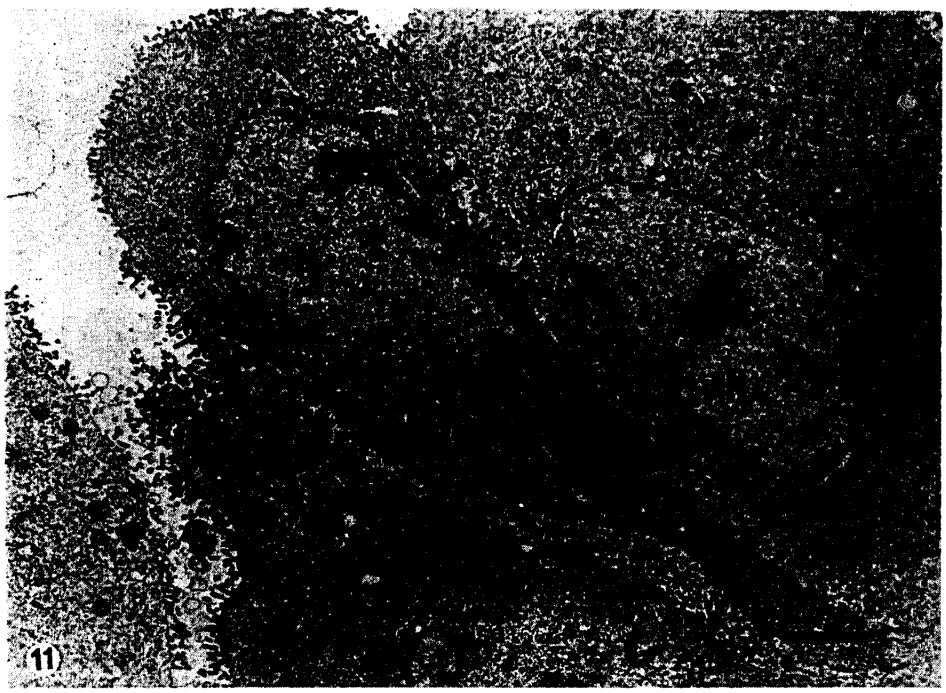
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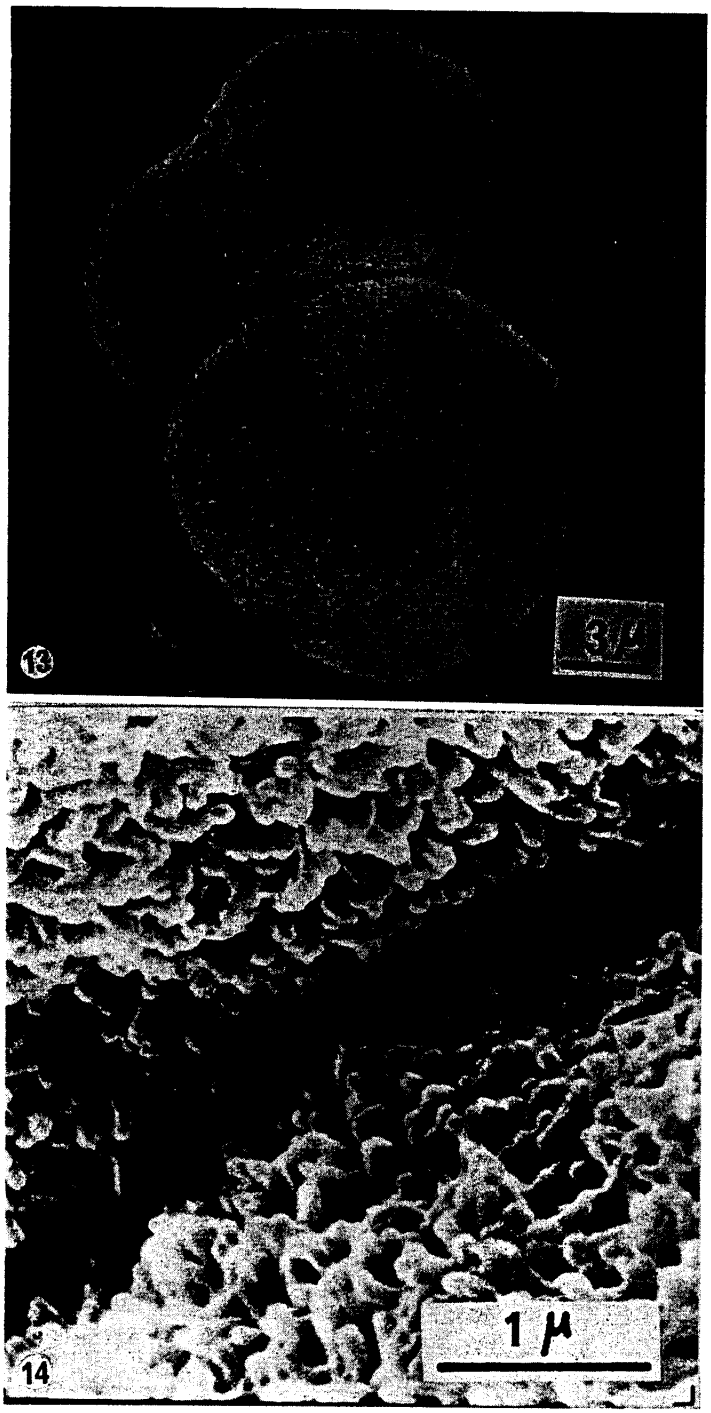
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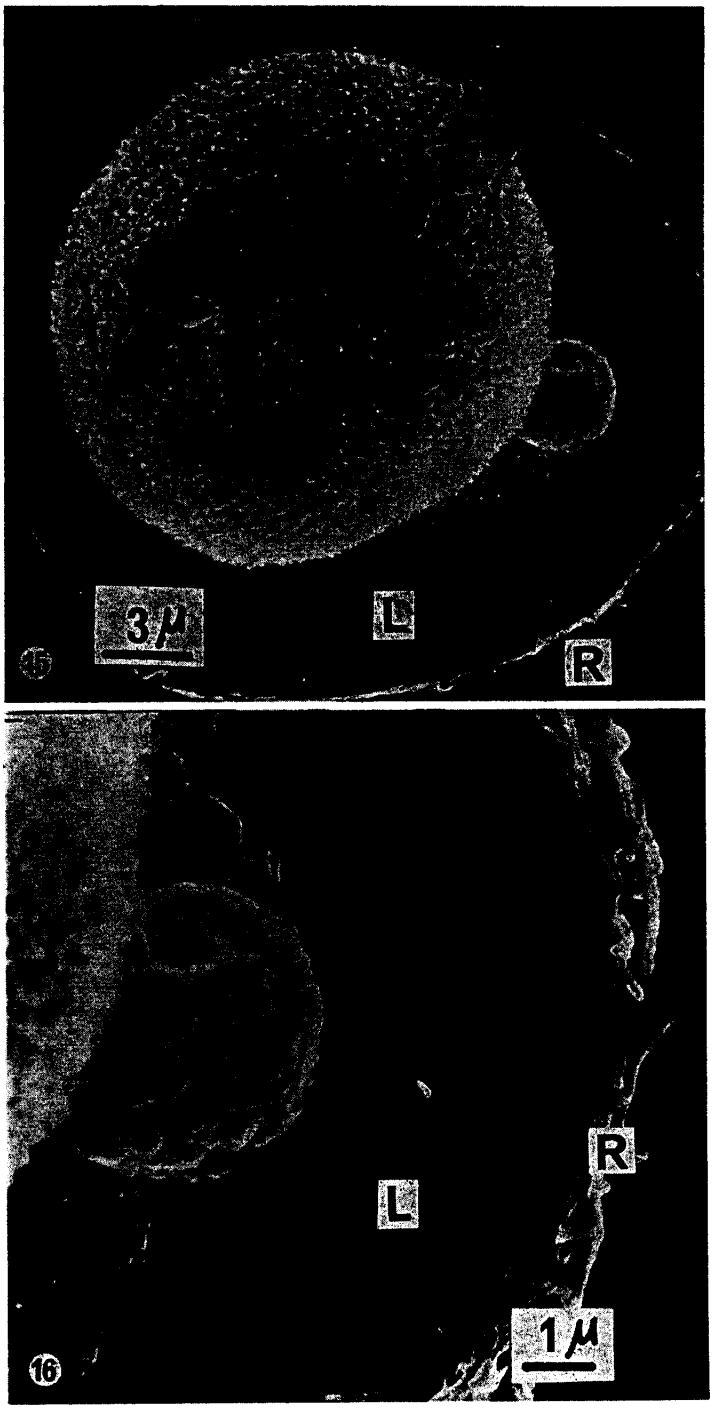
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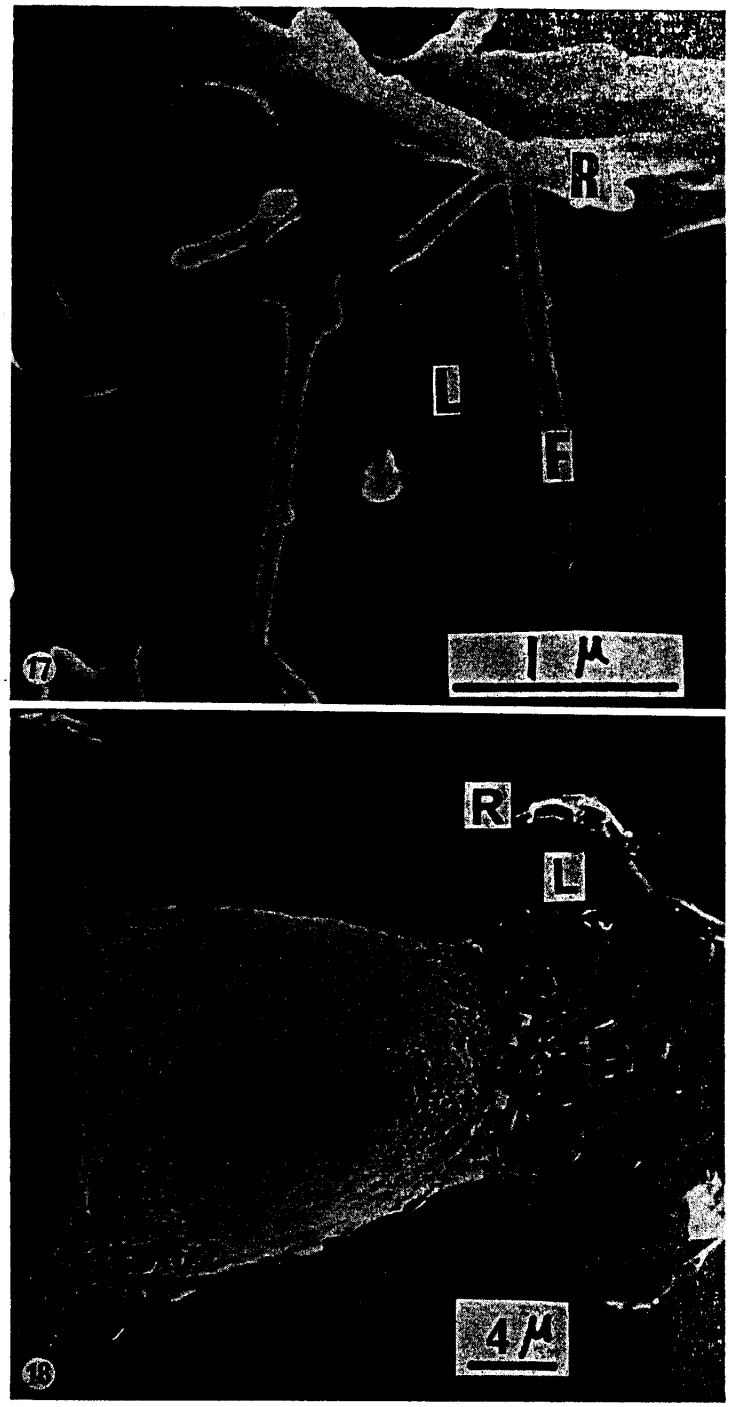
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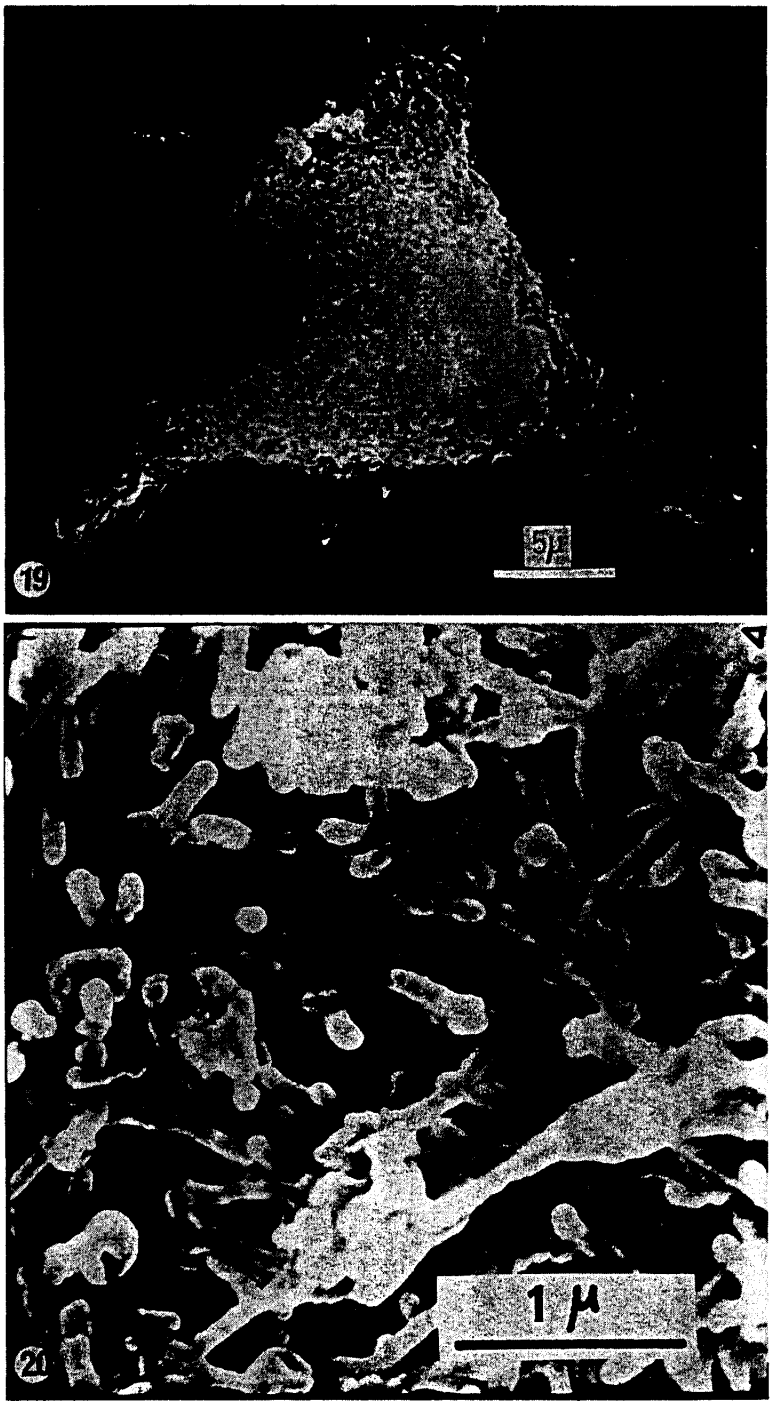
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