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# THE EFFECTS OF ANTI-CANCER AGENTS ON TRANSTRACHEAL TUMOR TRANSPLANTATION

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## ABSTRACT

An attempt was made to study the influences of anti-cancer agents on transtracheal tumor transplantation. For a preliminary investigation by the use of autoradiographic technique, the influence of Mitomycin C (MMC) was studied on DNA synthesis in epithelial cells of the rat trachea, which were regenerating following mechanical injury.

A rapid increase of DNA synthesis in regenerating epithelial cells was observed from 6 hours to 18 hours after the injury. Maximum level of DNA synthesis was maintained from 18 to 48 hours, then decreased to the same level as observed at 6 hours. After intraperitoneal administration of MMC in the dose of 1/4 or 1/2 $LD_{50}$ immediately after the injury, DNA synthesis was remarkably depressed from 6 hours to 48 hours, whereas, the value at 72 hours increased significantly as compared with the control. In case of frequent intermittent administration of MMC at 1/4 $LD_{50}$ four time every other day before and once immediately after the injury, DNA synthesis was depressed for up to 72 hours and did not recover.

Labelling index with $^3$H-Thymidine of epithelial cells of the normal trachea and bronchia was 0.5% and 0.4% respectively, and influence of MMC was not recognized except in the case of the above mentioned frequent intermittent administration.

When subcutaneously growing tissue of Yoshida sarcoma was inoculated into the bronchia of rats by inhalation transtracheally, transplantation was not established in the bronchia. By frequent intermittent administration of MMC to the animals prior to inhalation, however, transtracheal transplantation was established at a rate of 30%. By microscopic study tumor cells were found to infiltrate the bronchial wall where the mucosa of the bronchia plugged by tumor tissue had peeled off. Proliferation of the tumor cells in the bronchia was manifested by the intracellular incorporation of $^3$H-Thymidine in the peripheral area of the tumor tissue which had plugged the bronchial canal.

## INTRODUCTION

In order to understand the mechanism of transtracheal transplantation of tumor, influences of anti-cancer agents on the trachea and the bronchia were observed as fundamental study. This series of experiments was correlated with important questions described below. That is, the effects of the anti-cancer agents are unknown on the wound healing when the drugs are applied in combination with surgery for the treatment of malignant tumor. Although some reports are available on the influence of anti-cancer agents on wounds of the skin and digestive canals[1~11], nothing has been referred to the respiratory tract. In case of surgery for malignant tumor in the trachea or bronchia, the influence of drugs on wound repair of the reconstructed region or stump suture area should be taken

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into consideration. Especially when prosthesis is used for reconstruction of the trachea, its epithelization has an important meaning. Epithelization is considered to have some relation to tracheal stricture due to exuberant granulation at the anastomosis of the reconstructed region. It is also important to clarify the effects of anti-cancer agents on the complications of the respiratory tract and curing mechanism of various injuries in the trachea or bronchia. To elucidate these questions, the author observed the influence of MMC on the regenerating epithelial cells of the rat trachea by autoradiographic technique using $^{3}$H-Thymidine.

Influence of anti-cancer agent on transtracheal transplantation of experimental tumor was studied. Referring to clinical transtracheal transplantation of tumor, several reports are available stating that tumors in the oral cavity, upper respiratory tract and lungs are supposed to be transplanted into lungs via the air passages.[12~16] There have been also reports on experiments to form plumonary tumor by inhalation of tumor cells from nostrils of animals.[17,42,43] Some reports suggested that such kind of transplantation was formed clinically by not only tumor cells but also by inhalation of tumor fragments via the air passages yielding inoculative metastasis in the bronchia.[12~14] However, these have not been proved yet by experiments, and generally it is considered to be difficult to form inoculative metastasis with tumor tissue in the normal broncheal mucosa.

Clinical experience more or less suggests that tumor grows rather rapidly by the use of anti-cancer agents. In view of such adverse effect of the drug,[18~23] the author infused tumor tissue transtracheally to animals which had been pretreated with MMC. By injecting $^{3}$H-Tymidine after transplantation of the tumor into the bronchia, autoradiographic study was made on proliferation of tumor cells.

## MATERIALS AND METHODS

Animals used were male rats of Wistar strain weighing from 150 to 200g. The wound on the tracheal epithelium was made as follows; Animals were anesthetized with ether inhalation. Skin incision was made in the neck and the trachea was incised for 2 mm in length below the thyroid cartilage on the midline. Tracheal mucosa was then curetted lightly up to the tracheal bifurcation with a cupper curette 2 mm in width. The wound in the skin was closed with No. 3 silk thread. Proper sterile technic and environmental control was practiced for these operations.

$^{3}$H-Thymidine (Radiochemical Center, Amersham; specific activity 5 c/mM) as a solution of 1 $\mu$c/g in 0.4 ml of physiological saline solution was administered intraperitoneally 45 minutes prior to sacrifice. The animals curetted on the trachea were sacrificed after intraperitoneal administration of 20 mg thiopentobarbital at 6, 12, 18, 24, 48 and 72 hours. The removed trachea was fixed with 10% formalin, and embedded in paraffin. Sections were made on the sagittal plane 4 u in thickness. Autoradiography was performed in accordance with the Dipping method (Sakura $NR_2$ emulsion). After 4 weeks exposure and development with Konidol developer, the specimens were stained with hematoxylin and eosin and studied by light microscope.

Labelling index of regenerated epithelial cells was obtained as percentage of the labelled nuclei by counting 1,000 regenerated epithelial cells.

MMC was administered intraperitoneally to the animals. (Fig. 1) The $LD_{50}$ of MMC was 2.5 mg/kg, i.p.[24] To group 1, 1/12 $LD_{50}$ of MMC was given immediately after the curettage. To group 2, 1/4 $LD_{50}$, and to group 3, 1/2 $LD_{50}$ respectively of MMC were given immediately after the curettage. To group 4, 1/4 $LD_{50}$ of MMC was given 4 times

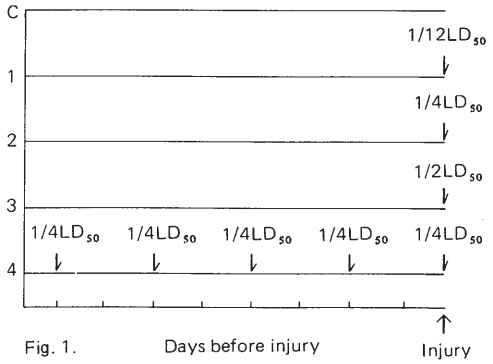


Fig. 1. Schedules of MMC administration. Control group (C); treated groups (1 through 4).

Fig. 2. Schematic presentation of tumor inoculation into bronchus.

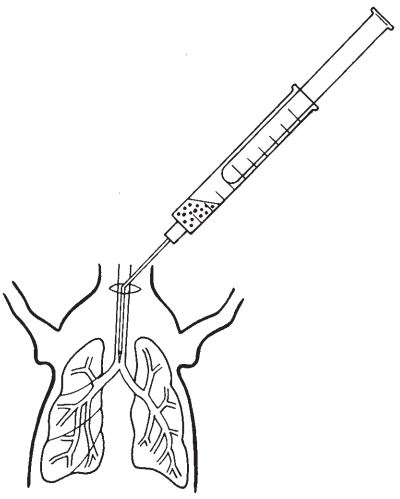

Fig. 2.

every other day before, and once immediately after the curettage. Every group consisted of 18 animals.

Labelling index was studied on the epithelial cells of the normal trachea and bronchia of rats which were sacrificed immediately after the completion of MMC given 5 times every other day intraperitoneally in a dose of 1/4 $LD_{50}$.

Animals used for transtracheal transplantation of the tumor were male rats of Wistar strain weighing from 150 to 200 g. Solid tumor of Yoshida sarcoma was prepared by transplanting ascites tumor cells subcutaneously in the back. The tumor tissue infused into the bronchia was obtained from a solid tumor 1 week after inoculation. Tumor tissues were minced into pieces of 0.8 $mm^3$ size with a pair of scissors, excluding necrotic lesion, washed with phosphate buffered saline solution (PBS) and filtered with a gauze to eliminate free cellular components.

The animals were anesthetized with ether. After incising the neck skin lesion, the trachea was cut 2 mm in length below the thyroid cartilage. From this incised opening of the trachea a teflon tube connected to a subcutaneous injector was inserted into the trachea near the bifurcation. By this injector approximately 15 pieces of minced tumor tissue in 0.2 ml PBS were momentally infused with 0.2 ml of air. (Fig. 2)

To the groups pretreated with MMC, 1/4 $LD_{50}$ of MMC was given intraperitoneally five times every other day before tumor infusion and immediately after the last administration of MMC the tumor tissues were introduced. $^3$H-Thymidine (1 $\mu$c/g) was injected intraperitoneally 72 hours after tumor inoculation, and 45 minutes later all the animals were sacrificed by intraperitoneal administration of 20 mg thiopentobarbital. The lungs, with the trachea and bronchia were removed, fixed with 10% formalin and embedded in parafin. Histological sections of the organs were cut serially at a thickness of 4$\mu$ in the frontal plane to observe lodgment and proliferation of the tumor.

Autoradiography was performed using 20 animals in the same way as stated above by the Dipping method.

Untreated group was observed as to lodgment and proliferation of the tumor everyday from 24 hours to the 7th day after the tumor inoculation. Three rats each were sacrificed

daily to prepare serial sections in the same manner as that for the treated group.

The sections of the MMC treated and untreated groups were then stained with hematoxylin and eosin and studied by the light microscope.

## RESULTS

### I. Effects of MMC on epithelia of the trachea and bronchia

Labelling index of normal epithelial cells of the trachea and bronchia were 0.5% and 0.4% respectively. Labelling index immediately after completion of MMC administration at 1/4 $LD_{50}$ five times every other day was 0.2% in the trachea and bronchia.

The histological findings of the trachea immediately after curetting the mucosa was similar in both control and MMC groups. Epithelial cells of the trachea peeled off nearly completely, and the basal cells were scattered on the basement membrane. The basement membrane was also partly injured.

Table 1. Effects of MMC on the incoporation of $H^3$-Thymidine into DNA of the regenerative cells of trachea.

| Group | Labelling index ± SE* | | | | | |
|---|---|---|---|---|---|---|
| | 6h | 12h | 18h | 24h | 48h | 72h |
| Control | 2.3 ± 0.71 | 9.8 ± 1.01 | 28.0 ± 1.64 | 25.5 ± 1.51 | 27.3 ± 1.38 | 2.7 ± 0.75 |
| 1 | 4.5 ± 0.83 | 10.1 ± 1.52 | 27.5 ± 1.69 | 26.3 ± 1.23 | 24.1 ± 2.26 | 3.8 ± 0.72 |
| 2 | 0.9 ± 0.28 | 4.8 ± 0.67 | 5.1 ± 0.47 | 14.5 ± 2.10 | 13.9 ± 0.95 | 9.4 ± 1.50 |
| 3 | 0.8 ± 0.16 | 3.1 ± 0.58 | 4.5 ± 0.41 | 14.9 ± 0.86 | 11.7 ± 1.02 | 8.9 ± 1.08 |
| 4 | 0.4 ± 0.09 | 4.3 ± 0.50 | 4.8 ± 0.83 | 15.3 ± 1.12 | 18.3 ± 1.28 | 0.5 ± 0.12 |

* SE indicates standard error.

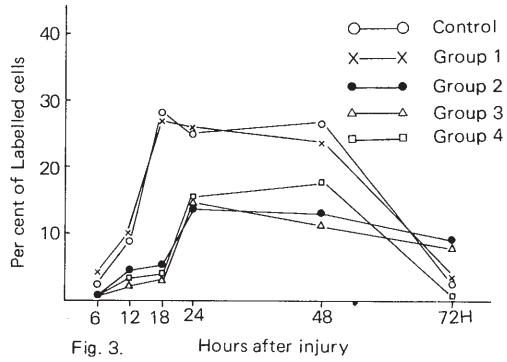


Fig. 3.

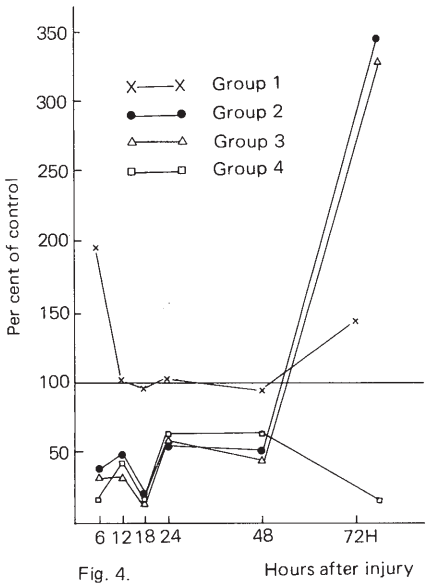


Fig. 4.

Fig. 3. Labelling index curves of control group and MMC treated groups. $^3$H-Thymidine was injected 45 minutes before sacrifice.

Fig. 4. Labelling index curve presented as the per cent of controls.

Table 1 shows labelling index of regenerated epithelial cells from six hours to 72 hours after the curettage. Fig. 3 illustrates labelling index of every group, while Fig. 4 indicates that of MMC groups in percentage against the control. Epithelial cells of the normal tracheal mucosa regenerated rapidly after the curettage and its DNA synthesis increased considerably from six hours after the injury, reaching a peak by 18 hours, which was maintained for 48 hours. It decreased nearly to the value of 6 hours by 72 hours. In the group treated with 1/12 $LD_{50}$ of MMC, DNA synthesis of regenerated epithelial cells was not inhibited and the values at 6 hours and 72 hours after the injury were rather greater than the control. In the groups given 1/4 $LD_{50}$ and 1/2 $LD_{50}$ the inhibition was remarkable from six hours to 48 hours, but an increase at 72 hours following the depression was significant as compared with the control. Between both groups, however, no significant difference was observed. This means that frequent administration of MMC in rather small dose causes more interference than the others given the same total dose. In group 4 to which MMC was given every other day before the injury during the lifespan period of tracheal epithelial cells and once after the injury, synthesis of DNA was inhibited and a recovery was not observed.

The labelling index of epithelial cells in the uninjured portion indicated a value exceeding 0.5% at every hour, except group 4, and renewal of the epithelial cells was free from any noticeable influence. Although regenerated epithelial cells were easily affected by MMC, the epithelial cells under normal conditions were not affected by MMC unless intermittent massive adminstration was applied.

II. Effects of MMC on tumor treansplantation

In the groups pretreated with MMC, inoculative transplantation in the bronchia was established at a rate of 30%. Number of the tumor tissue plugged in the bronchia was less than 2, and the emboliform portion was limited to the branching point of the bronchia. Although the center of the tumor tissue plugged in the bronchia was degenerating, tumor cells in the peripheral area showed infiltrative proliferation as revealed by the autoradiography which indicated incorporation of $^{3}$H-Thymidine in the tumor cells. Proliferation of fibroblasts was remarkable in some tumoral lesions, which was supposed to be a host reaction against organized foreign matters.

Epithelial cells of the bronchia were swollen and edematous, and a part of the epithelium peeled off. The tumor infiltrated the bronchial wall via submucosa to muscular layer, growing from the portion which showed injured epithelium, as described above.

Labelling index of tumor cells was 6.0% in the bronchia 31% in the bronchial submucous coat and 32% in the muscular layer. It indicated that tumor cells which infiltrate the bronchial wall have strong proliferability. (Fig. 5)

In the untreated group, lymphatic tissue in the bronchial wall proliferated remarkably from 24 hours after introduction of the tumor tissues, while no lodgment and proliferation of the tumor was observed in the bronchial wall and in the lungs. The infused tumor pieces were apparently all expectorated, and no tumor was recognized on the incised neck lesion, either.

## DISCUSSION

Since MMC affects normal tissue, especially cells in the DNA synthetic period, it injures bone marrow and intestinal epithelium, where cellular renewal is rapid.[25~29] According to the report of Shorter *et al.*[30,31] the lifespan of epithelial cells in the trachea and bronchia of normal rats is 6–7 days, and that of intestinal epithelium about 2 days.[32] Therefore, the side effect of MMC against normal epithelium of the trachea or bronchia is considered to be much less than against intestinal epithelium. It is also considered that proliferating host cells in the wound during the healing process after the injury is inhibited considerably by MMC. However, the effect of MMC on the repair of the tracheal epithelium is apparently different from that on the repair of the intestinal epithelium. Histology of the tracheal epithelium is different from that of the intestinal epithelium in which generative cells are only localized in the crypts. Generating time of the cells is also different between both epithelia. It is also considered that the effect is somewhat related to drug transition in the tissue, activation and inactivation of the respective organ with such drug.

Wiznitzer *et al.*[33] reported that in the cure of wound on the intestinal anastomosis of rats, intraperitoneal administration of MMC at 0.28 mg/kg ($\fallingdotseq$ 1/8 $LD_{50}$ in rat) caused no significant difference in tensile strength at the union and assay of hydroxyproline. Since the cytocidal action of MMC is known to depend on its concentration,[34,35] it is necessary to study such action for every concentration. It is considered appropriate to measure such injury with the effect on DNA synthesizability of proliferating cells at the wound.

From data obtained by the author it is assumed that administration of MMC at 1/12 $LD_{50}$ did not inhibit the renewal rate of epithelial cells in the trachea at all but increased the rate at 6 and 72 hours more than that in the control. The reason for such increase is not clear, but it is assumed that renewal mechanism of tissue casuses acceleration of DNA synthesis. Similar phenomenon has been reported on crypt cells of the intestine of mice, when a comparatively smaller amount of MMC was given.[36] By local application of Uracil to skin trauma, epithelization of the skin was accelerated[37] and by administration of various anti-cancer agents to tumor cells similar phenomena were observed.[21,22,38] Such adverse effect of anti-cancer agent is an extremely interesting phenomenon.

In the 1/4 $LD_{50}$ and 1/2 $LD_{50}$ group an increase in value at 72 hours is considered to be due to DNA synthesis being controlled easier by the administration of MMC which has retarded renewal of epithelial cells.

In group 4, the reason for no increase in value observed at 72 hours was assumed to be due to the damage of generative cells of the tracheal epithelium before the injury.

Recently, reconstructive operation of the trachea and bronchus has been increasingly performed in the surgery of lung cancer, in order to preserve the intact lobe or to repair the respiratory tract after resection of the primary or secondary tumor of the trachea.[39] However, no report states the use of anti-cancer agent in such operation. The reason is probably because of the side effects of anti-cancer agents as adjuvant therapy concerned to this operation has not been completely clarified yet.

The author studied first the influence of MMC on the regenerating epithelial cells of the trachea. When converted by the formula of Freireich *et al.*[40], MMC in a dose of 1/4 $LD_{50}$ to a rat corresponds with 4 mg/50 kg of clinical dose. From the author's data the effect of MMC on tracheal trauma is readily observed at the clinical dose. Therefore, it is considered necessary to pay sufficient consideration to pre- and post-operative adjuvant chemotherapy.

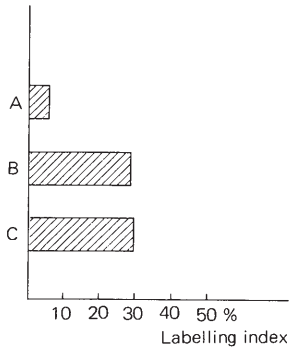


Fig. 5.

Fig. 5. Comparisons of labelling index of Yoshida sarcoma in bronchus and bronchial wall. intrabronchus (A), submucosal (B), intramucular (C). $H^3$-Thymidine was injected 45 minutes before sacrifice.

Referring to transtracheal metastasis of tumor, generally, questions of the implantation metastasis of tumor on epithelium are discussed. In the bronchia digestive enzymes do not exist, which is considered to be less improbable than in the alimentary tract for establishment of transplantation in this system.[41] However, in the bronchia expectoration of foreign matter and cleansing by muco-ciliary system are considered to be barrier of inoculative metastasis of tumor to broncheal mucosa, and problems of lowered viability of tumor cells freed in the respiratory tract must be also considered.

The experimental study to make plumonary tumor by transplantation of tumor cells transtracheally was initiated by Furth.[42] He made plumonary tumor and Leukemia by giving carcinoma and Leukemia cells to mice through the nostrils to inhale. Takahashi *et al.*[43] made plumonary tumor in a high percentage by transplantation of cells of various strains transtracheally to mice and rats, and reported on it in detail. They reported that the plumonary tumor formation ratio, location and form of proliferation were different by various tumoral series and that the tumor formation ratio of Yoshida sarcoma was 100%. Although its proliferation is apparent in the alveoli and the interstitium, it is not clear that via what channel tumor cells appear in the plumonary interstitium.

On the other hand, with tumor tissue pieces, invasion of tumor into the lung is considered to be due to proliferative infiltration of tumor cells. However, it seems difficult for tumor cells to proliferate in the healthy bronchia without any direct contact with interstitia of the host.[44] In the author's experiments it was not observed that tumor tissue was plugged to form inoculative transplantation on the normal bronchial mucosa. Almost all of the tumor inhaled into the bronchea seem to be expectorated.

It is not apparent how much MMC influences expectorability in the respiratory tract and muco-ciliary system of rats, but emaciation by systemic massive administration may cause weakness of expectorability.

As to plumonary disturbance with MMC, Shimosato *et al.*[45] have reported that plumonary angiopathy and its secondary change was characteristic, whereas plumonary angiopathy caused by Bleomycin was known to be dose independent.[46,47] There has been no report available on disturbance caused by systemic administration of MMC to the bronchia, but it is apparent from the author's experiments that one time administration of MMC causes no disturbance at all in normal epithelial cells of the bronchia. However, if a clinical dose is administered frequently at a short intervals, renewal may be inhibited even in the normal epithelium. When edematous change and desquamation of the epithelium are readily induced by the drug, tumor tissue apparently plugs the bronchia to cause decubitic situation

of the bronchial epithelium, which is reciprocal. Renewal of the injured epithelium may be retarded by MMC.

Mizota *et al.*[48] produced gastric tumor of rats in a high percentage by injecting Yoshida sarcoma into the gastric lumen, where a gastric ulcer had been made experimentally. In the bronchia associated with insufficient expectoration, bronchial epithelium is disturbed to cause plugging of tumor tissue. Such tumor cells in direct contact with the interstitia of the host may be given an environment to proliferate infiltratively from the bronchial wall into the lungs. Such mechanism can also be established by administration of MMC. Identification of $^{3}$H-Thymidine incorporation in tumor cells plugging the bronchia presents a reasonable basis for establishment of tumor proliferation in such a system. For such metastatic formation it is also considered that administration of MMC give influences both general and local immunity of the host. The above indicates that in chemotherapy for malignant tumor the method of drug administration becomes very difficult.

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## LEGENDS TO PHOTOS

Photo. 1. Tracheal epithelium immediately after curettage.

Photo. 2. Tracheal epithelium immediately after curettage, following 5 times administration of 1/4 $LD_{50}$ of MMC.

Photo. 3~8. Tracheal epithelium from 6 to 72 hours after curettage, control and 5 times administration of 1/4 $LD_{50}$ of MMC before curettage, Autoradiograph. H. and E. stain, x 450. Photo. 3. Tracheal epithelium, at 6 hours, control. Photo. 4. Tracheal epithelium at 6 hours, MMC treated. Photo. 5. at 18 hours, control. Photo. 6. at 18 hours, MMC treated. Photo. 7. at 72 hours, control. Photo. 8. at 72 hours, MMC treated.

Photo. 9. Macroscopic appearance of the lung innoculated tumor fragments into bronchus, showing partial atelectasis of the left lobe.

Photo. 10~11. Microscopic appearance of tumor in bronchus 3 days after innoculation. Autoradiograph. H. and E. stain, x 450.

Photo. 12~13. Yoshida-sarcoma infiltrated into bronchial wall. Autoradiograph. H. and E. stain, x 450.

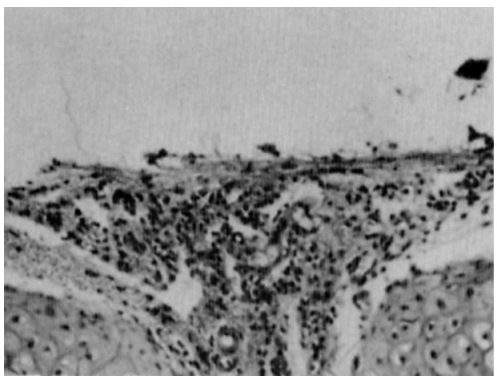
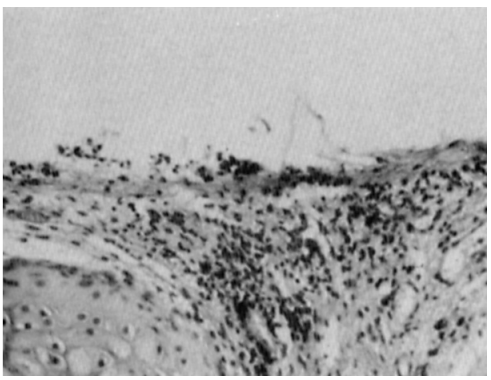


Photo. 1



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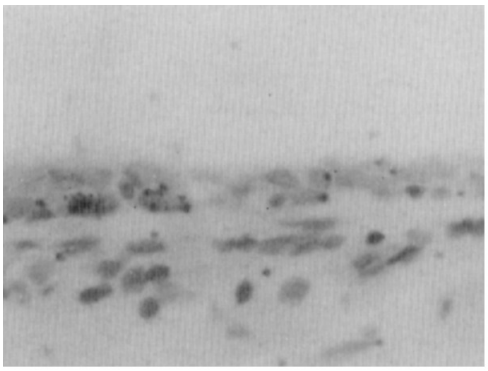

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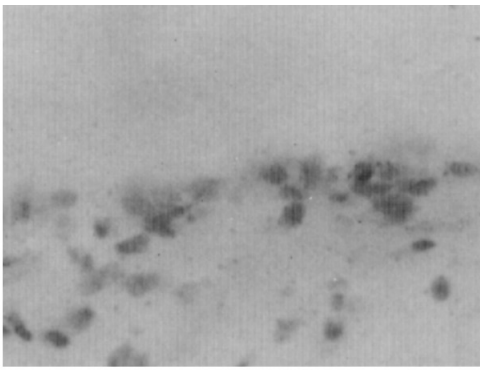

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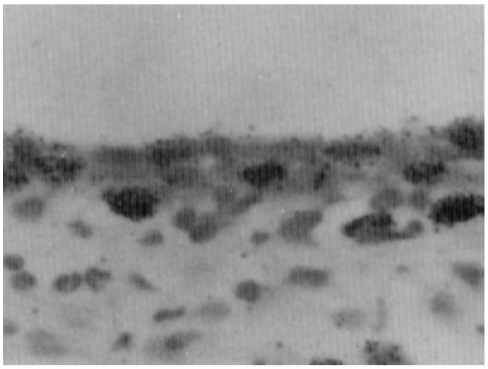

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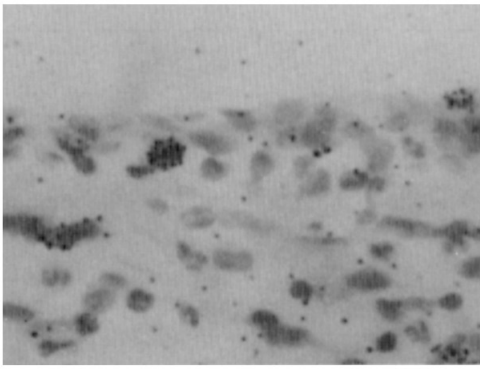

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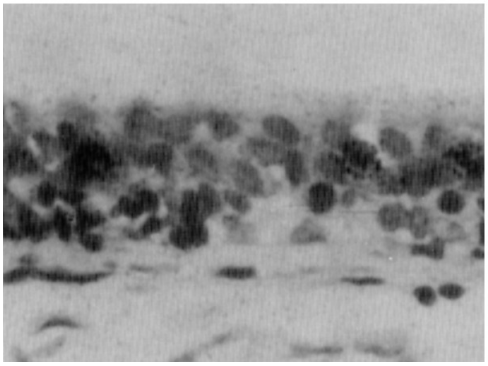

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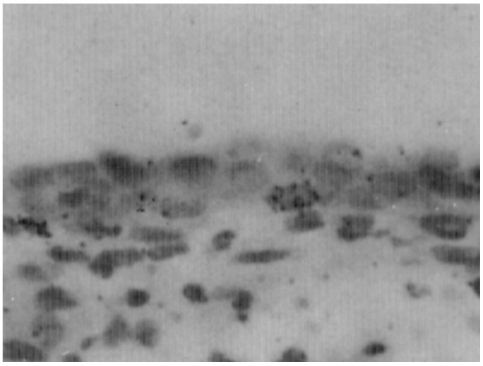


Photo. 8

Photo. 9

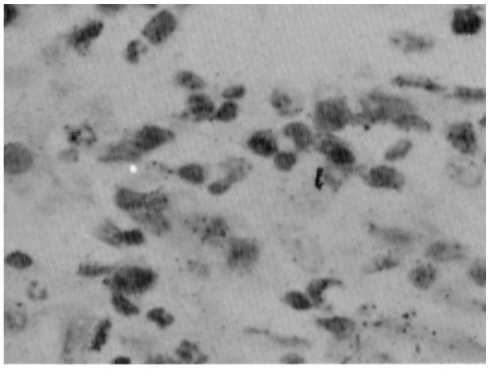
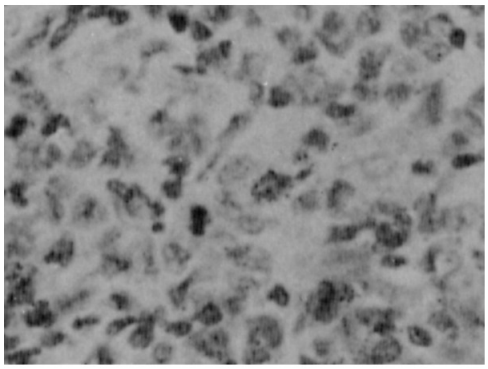


Photo. 10



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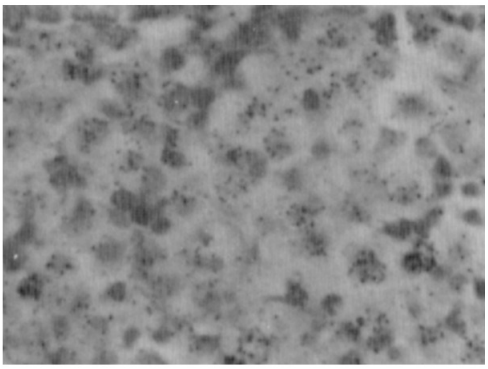

Photo. 12

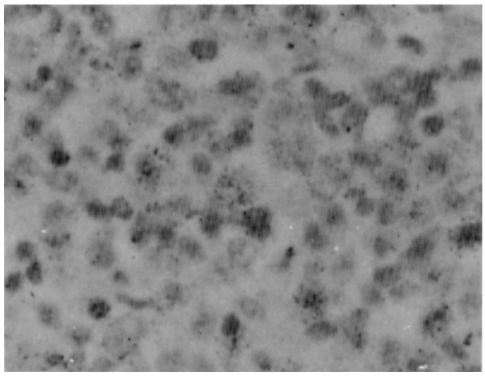

Photo. 13