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Autoradiography of the Tissues of Mice Infected with Ectromelia Virus Using ^{3}H-Thymidine

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SUMMARY

"A" and "B" type inclusions in mouse liver cells infected with ectromelia virus were demonstrated in tissue sections as well as in smear preparations. "B" type inclusions could also be seen in the epithelial cells of the kidney, pancreas, lung and intestine, in the mesenchymal cells of the lymph nodes, thymus and bone marrow, and often in the cytoplasm of muscle cells in the intestinal wall.

Intensive incorporation of ^{3}H-thymidine into "B" type inclusions was demonstrated in these infected tissues. Relatively intensive accumulation of silver grains mainly on the edge of "B" type inclusions was seen by thin sectioning at a level of 1μ. This suggests that synthesis of viral DNA is most active at the surface of the "B" type inclusions. The wide range of susceptible cells including cells which do not show any active nuclear DNA synthesis, reveals that cytoplasmic viral DNA synthesis occurs independently of host nuclear DNA synthesis.

INTRODUCTION

Since Marchal (1930) isolated ectromelia virus as "a hitherto undescribed virus", it has been shown that the virus elicits eosinophilic cytoplasmic inclusions in cells of several tissues of mice. Fenner (1949) gave "a review about mousepox (infectious ectromelia of mice)" and reported histological findings in the liver. Mims (1959a, 1959b) using the fluorescent antibody technique, showed that when ectromelia virus was injected intravenously into mice it was taken up by the littoral cells lining the liver sinusoids. Then, viruses reached hepatic cells by "growing through" littoral cells, and caused infected foci in the hepatic cells. However, the appearance of the inclusions in the liver cells has not been described.

Two kinds of inclusions were found in Ehrlich ascites tumor cells infected with ectromelia virus (Kato, 1955; Kato *et al.*, 1955). The one which appears earlier and stains reddish purple with Giemsa solution was named a "B" type inclusion ("B"), and the other which appears later, stains pale blue and contains acidophilic particles was named an "A" type inclusion ("A"). These workers found that the "A" corresponded to the so-called Marchal body. Later, "A" and

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"B" were demonstrated in smear preparations of infected mouse liver by Oketani (1959). Further studies have revealed that "B" type inclusion formation is common to all kinds of poxvirus infection and has been proved to be the site of viral antigen (Takahashi *et al.*, 1959; Kameyama *et al.*, 1959; Kato *et al.*, 1959). Through the autoradiographic studies on the tissue culture cells infected with several poxviruses including ectromelia virus, "B" has been turned out to be an intrinsic site of viral DNA synthesis (Kato *et al.*, 1960a, 1960b, 1962).

In the experiments reported in this paper, ectromelia virus infected mice were exposed to ^{3}H-thymidine. Incorporation of ^{3}H-thymidine into DNA in the infected tissues was studied autoradiographically.

MATERIALS AND METHODS

1. *Virus*

The virus suspensions used throughout the present work were fluorocarbon (Daifron S3) purified supernatants after centrifugation of a 30 per cent liver emulsion from moribund mice infected with the Hampstead strain of ectromelia virus. The supernatant was diluted with Hanks' solution. The infectivity of the final diluent was about 10^6 $TCID_{50}$ per ml.

2. *Mice*

ddO mice, weighing 20 gr were used.

3. *Isotope*

Tritiated thymidine (nominally-6T), purchased from the Radiochemical Centre, Amersham, England (specific activity 2.5 C/mM) was used at a level of 200 μC / ml.

4. *Route of infection*

Aliquots of 0.2 ml of virus suspensions (2×10^5 $TCID_{50}$) were inoculated into mice intraperitoneally. Mice were sacrified at 8, 15, 20, 24, 48 and 72 hours after infection. One hour before death, each mouse was injected intraperitoneally, with 0.5 ml of ^{3}H-thymidine solution (100 μC per mouse).

5. *Sectioning, autoradiography and staining*

The organs were fixed in absolute ethanol containing 5 per cent glacial acetic acid at 4° C for 24 hours. During fixation, the organs were sliced in half every one or two hours, until finally they were about 0.2 mm thick. The fixed tissues were dehydrated with cooled ethanol, embedded in paraffin and sectioned at a thickness of 1 or 3 μ. Then the sections were placed on clean cover slips 11×45 mm square, without using any adhesive such as egg-albumin. The procedure of fixation brought about results satisfactory to the staining of section preparation with Giemsa solution.

Autoradiograms were made, according to the techniques of Messier and Leblond (1957) and Joftes (1959) with a little modification. After treatment with 0.2 N perchloric acid at 4°C for 60 min to remove acid soluble molecules, the cover slips were rinsed thoroughly with distilled water. They were dipped into the Eastman Kodak NTB2 emulsion in the sol phase at 45°C. Then the cover slips were kept in test tubes with small holes in the bottoms, which were disigned by us.

These tubes were stored vertically for 10 days in a dark chamber at low temperature to avoid relaxation of the emulsion. The moisture in the chamber was maintained at about 40 per cent by introducing saturated $CaCl_2$ solution into the chamber. Silica gel was also used tentatively to keep humidity low. The low humidity increased the number of grains intensively. The thickness of the emulsion sheets after dipping was about 5μ. Developing was carried out according to the Kodak formula 19b at 18°C for 5 min.

Tissue smears were also fixed in methanol, treated with perchloric acid and dipped as described above.

After the cover slips had been developed, fixed and washed gently, they were dried in an air-cooler, and stained with Giemsa solution (pH 6.4) or hematoxylin-eosin at under 18°C. Some were stained with Feulgen's reagent before dipping. Finally the cover slips were mounted with Canadian balsam, and the emulsion sheets on the opposite side (without cells) were stripped off completely.

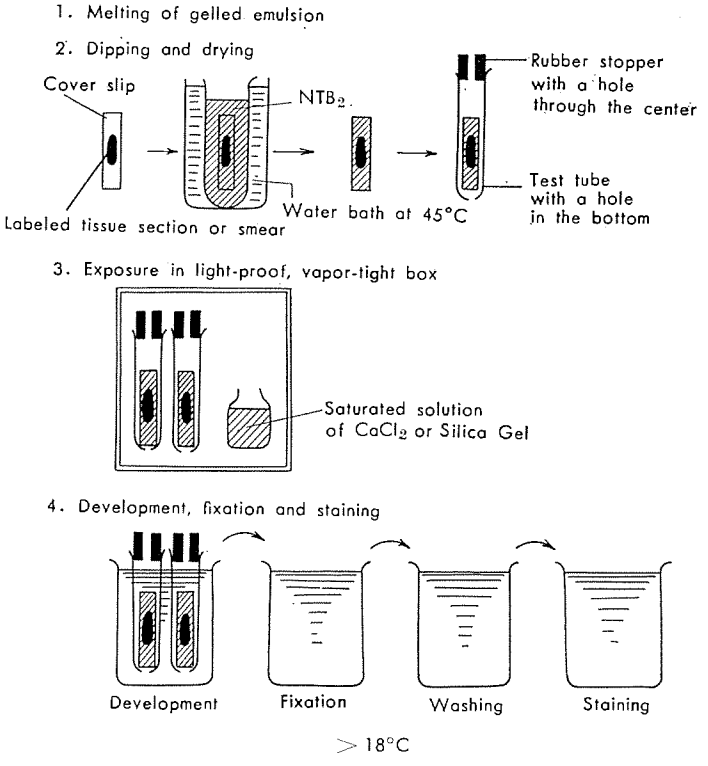

Fig. 1 Process of Dipping Autoradiography

Test tubes with small holes in the bottom were very convenient for developing, washing and staining the cover slips after dipping. They permitted the carrying out of these successive procedures without touching the cover slip.

The procedure for dipping autoradiography is shown in Fig. 1.

RESULTS

Liver :

General pathology

Liver damage began as a multiple focal necrosis which was distributed throughout the organ regardless of the lobular architecture. At this stage the sinusoids frequently filled with hyaline thrombi, and the cytoplasm of the liver cell was swollen and hyaline. On Giemsa staining, the reddish purple "B" s in the

cytoplasm of degenerated liver cells became apparent. There was very little evidence for the appearance of wandering cells. These findings were seen within 24 hours after infection (Fig. 2).

Later, focal necrosis developed and areas fused with each other forming sublobular lesions (Fig. 3). There was much monocytic infiltration all over the liver parenchyma. Often damaged blood vessels were seen (Fig. 5). In later stages the "B"s became diffuse in the cytoplasm and stained less than the compact "B"s seen at an early stage. "A"s staining pale blue were rarely seen. The appearance of the bile ducts and branches of the venous and arterial system within the liver still remained normal.

From 48 to 72 hours after infection, liver damages spread rapidly and finally subtotal liver necrosis developed (Fig. 4). The sinusoids filled with monocytes, degenerated liver cells and extruded "B"s. No lobular architecture was discernible, and islets of liver cells free from infection and sometimes showing slight regeneration were seen in the necrotic areas. The number of "B"s in a single cell increased to ten or more. These were compact and small in size. These facts showed that massive infection had occurred at this stage following the sublobular damage. Animals all died between 70 to 80 hours after infection.

It seems that the liver damage of mice caused by ectromelia infection resembles human acute yellow liver atrophy.

Smear preparations of the infected liver cells were made and stained with Giemsa solution. In the smear taken 48 hours after infection, there were many "B" bearing liver cells and sometimes "A"s were encounterred (Fig. 6, 7).

Autoradiography

One hour after the intraperitoneal inoculation of ^{3}H-thymidine, mice were sacrificed and the organs were immediately fixed in cool alcohol to stop enzymatic activity of DNA polymerase and kinase.

The autoradiogram of control liver showed that most of the liver cells contained no silver grains. The percentages of labelled parenchymal liver cells were less than 0.01 per cent. In other words, there was very little evidence of active DNA synthesis in control liver.

On the other hand, in infected liver, there was intensive accumulation of silver grains in the cytoplasmic area corresponding to the location of the "B". The most intensive accumulation of silver grains was seen on "B"s of 5μ to 6μ in diameter, in sections of 3μ thickness, after 10 days exposure. There were few or no grains on diffuse "B"s. No grains were found in the area corresponding to "A"s, no matter what their size (Fig. 10 and 11).

In 1μ serial sectioning after 10 days exposure, it was found that 60 to 70 per cent of the grains were present in the area around the edge of the "B"s. They gave the appearance of a crown (Fig. 12, 13 and 14). The incorporation of ^{3}H-thymidine into liver parenchyma near the peritoneum seemed relatively more

intensive than into that of the inner part.

Autoradiogram of the "B" type inclusions found in the liver cells in the smear preparations also showed the defenite incorporation of ^{3}H-thymidine into them (Fig. 8, 9).

Spleen and other organs:

The damage of the spleen seemed to progress more rapidly than that of the liver. Infected cells, most of which were cells of the reticulo-endothelial system, became loosened but remained in the networks of the sinuses. In these cells there were many typical "A"s as well as "B"s. Often the "A"s were larger than the nuclei (Fig. 15).

At a late stage of infection many compact "B"s could be seen in the epithelial cells of the kidney, pancreas, lung and intestine, in the mesenchymal cells of the lymph nodes, thymus and bone marrow, and often in the cytoplasm of muscle cells in the intestinal wall (Fig. 18).

Autoradiograms of the spleen and other organs showed an intensive accumulation of silver grains on the "B"s, as seen in the liver. No grains were found on the "A"s, which could be easily seen in the cells of the reticulo-endothelial system in the spleen and mesenterium (Fig. 15, 16, 17, 18).

DISCUSSION

Many authors who stained liver section with hematoxylin-eosin or Mann's solution, have reported that it is very difficult to find inclusions in the liver of mice infected with ectromelia virus. It was probably because of the prevailed idea that the so-called Marchal body was an only kind of inclusion of ectromelia virus. And these double staining methods are suitable for demonstrating "A" type inclusion (Marchal body), but do not demonstrate well the "B" type inclusions (Kato *et al.*, 1962). In fact, "A" type inclusions (Marchal bodies) were very few, but by no means absent.

Giemsa staining is the most preferable staining for "B" type inclusions. In the present paper, the section preparations were stained with Giemsa solution. The difficulties of staining tissue sections with Giemsa solution were overcome by improving the procedure as follows. The organ is sliced as thin as possible, fixed in cool ethanol and the dehydrated as quickly as possible. In the infected liver, "B" type inclusions were present in abundance. The number of cells bearing "B" type inclusions increased in parallel with the progress of liver damage. The sudden appearance of numerous compact "B" type inclusions at a late stage of infection showed that massive virus production occurred at a moribund stage of the mice.

Intensive incorporation of ^{3}H-thymidine into "B" type inclusions *in vivo* seemed similar to the phenomenon seen in vitro (Kato *et al.*, 1960a). The fact that we

could not find any silver grains on the "A" type inclusions also agreed with the results of in vitro experiments. It can be, therefore, safely said that the "B" type inclusions *in vivo* are also the site of viral DNA synthesis as well as virus antigen shown by fluorescent antibody technique (Kameyama *et al.*, 1959).

In our present work, silver grains were seen arranged around the "B" type inclusions like a crown in sections of 1μ. In our previous tissue culture works, however, grains seemed to cover over the whole surface of the "B" type inclusion which was not sectioned. The results suggest that the synthesis of viral DNA in the "B" type inclusion is more active in the area adjacent to the boundary between the inclusion and cytoplasm.

From the present work it is clear that a wide range of tissue cells are susceptible to poxvirus infection as indicated by the formation of "B" type inclusions in their cytoplasm. Extensive studies have been carried out on the incorporation of ^{3}H-thymidine and ^{3}H-uridine into several tissues of mice (Messier and Leblond, 1960; Amano and Leblond, unpublished). From the point of view of nucleic acid synthesis, the intensive incorporation of ^{3}H-thymidine into the "B" type inclusions was seen in the cytoplasm of cells where there was normally little or no active nuclear DNA synthesis. These cells included liver cells, muscle cells and epithelial cells of the kidney. A similar intensive incorporation of ^{3}H-thymidine was seen in cells showing more active DNA synthesis under normal physiological conditions, such as cells of the reticulo-endothelial system in the spleen, the lymph gland and bone marrow. Therefore it is considered that the poxviruses are able to multiply regardless of the normal level of nuclear DNA synthesis of the host cells. The liver cell-ectromelia virus system *in vivo* has been found to provide a typical example of dissociation of the synthesis of host nuclear and viral cytoplasmic DNA.

It may be of interest to notice that all these cells susceptible to ectromelia virus are the cells showing RNA metabolism. The similar results have been obtained using cowpox virus and several tissues of rabbits and human bone marrow cells (unpublished). Two compounds, *i. e.*, 5, 6-Dichloro-1-β-*D*- ribofuranosyl-benzimidazole and RNase, are considered to have an inhibitary action upon RNA metabolism. The pretreatment of L cells susceptible to ectromelia virus, by these two compounds caused apparent suppression of the "B" type inclusion formation (Ikegami *et al.*, 1960). These results probably indicate that the condition of *living host-cells* may not be a minimal requirement for virus multiplication and that *living cells showing RNA metabolism* might be a further requirement, while it is not necessary that the host cells show nuclear DNA metabolism for the virus to multiply.

The results also suggest that the mechanism controlling nuclear DNA synthesis of liver cells in the physiological state is probably not a direct action upon DNA synthesis itself, but an action upon the division mechanism of the cell.

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EXPLANATION OF PLATES

All preparations were stained with Giemsa solution. Figs. 2-4. show the progress of liver lesion. Focal necrosis (Fig. 2) advances to sublobular damage (Fig. 3) and finally to subtotal necrosis (Fig. 4). In Fig. 4 no lobular architecture is disernible. In Fig. 5 note the damage of a blood vessel (portal vein).

Fig. 6 and 7. Smear preparation of liver 48 hours after infection. In Fig. 6, there are many scattered compact "B" type inclusions in the cytoplasm. In Fig. 7, note several "A" type inclusions (indicated by arrows) and diffuse "B" type incusion.

Fig. 8 and 9 are autoradiograms of smear preparations of the liver. Intensive accumulation of silver grains corresponding to the "B" type inclusions are seen.

Fig. 10-14. Autoradiograms of liver sections. Figs. 10 and 11, 72 hours after infection (section 3μ thick). Figs. 12-14 show the crown-like arrangement of silver grains at the edge of the "B" type inclusion (section 1μ thick).

Fig. 15. Autoradiogram of a spleen section, 48 hours after infection. Note several large "A" type inclusions which are free from silver grains. "A" type inclusions were indicated by arrows.

Fig. 16 and 17. Autoradiograms of mesenterium sections. The cells lining the right side of the figure are endothelial cells of the peritoneum. Many sites of silver grain accumulation which corresponds to "B" type inclusions, are seen. "A" type inclusions are free from silver grains.

Fig. 18. Autoradiogram of the intestinal wall. Note the grain accumulation in the cytoplasmic area of the muscle cell.

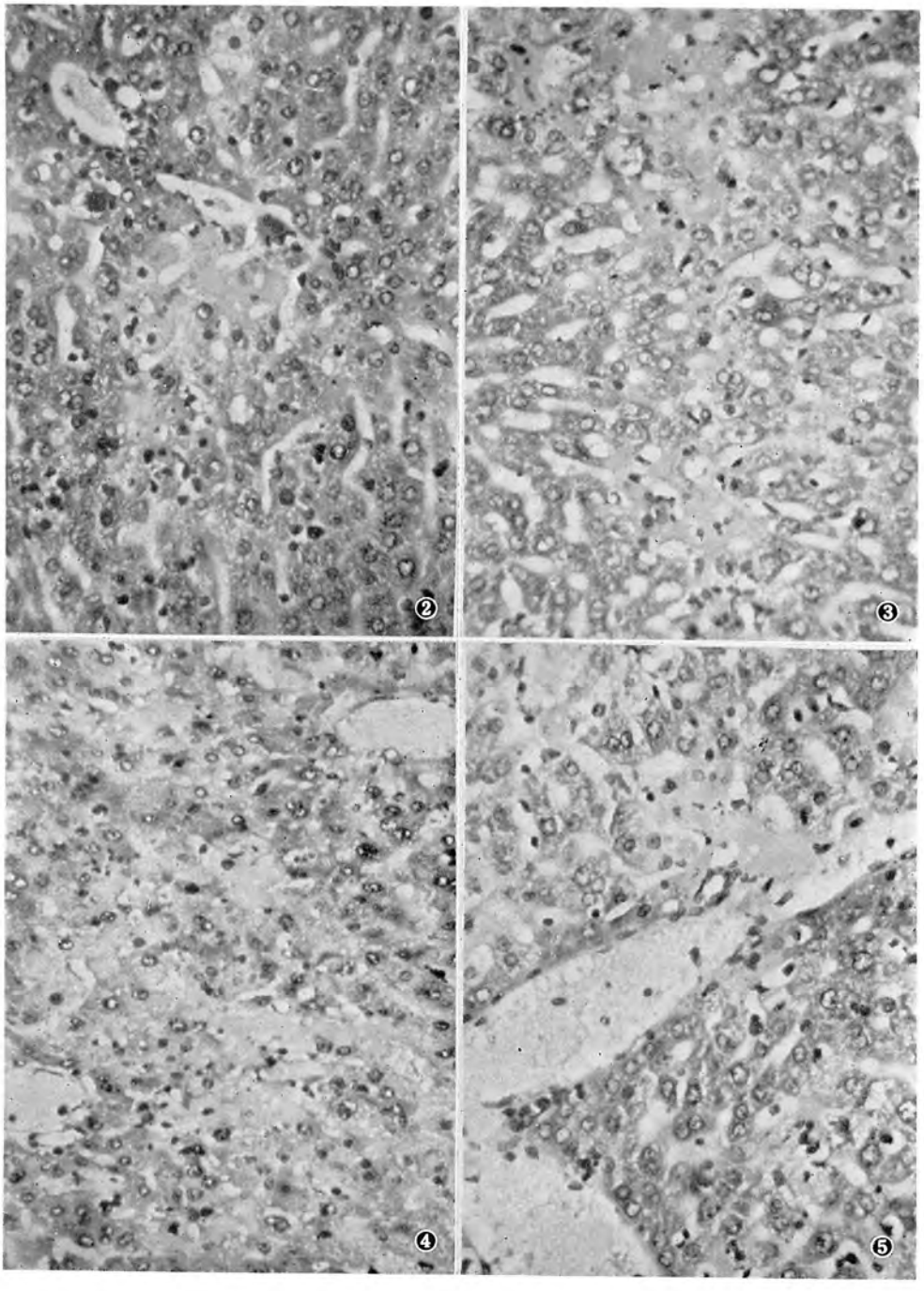

Fig. 2～5

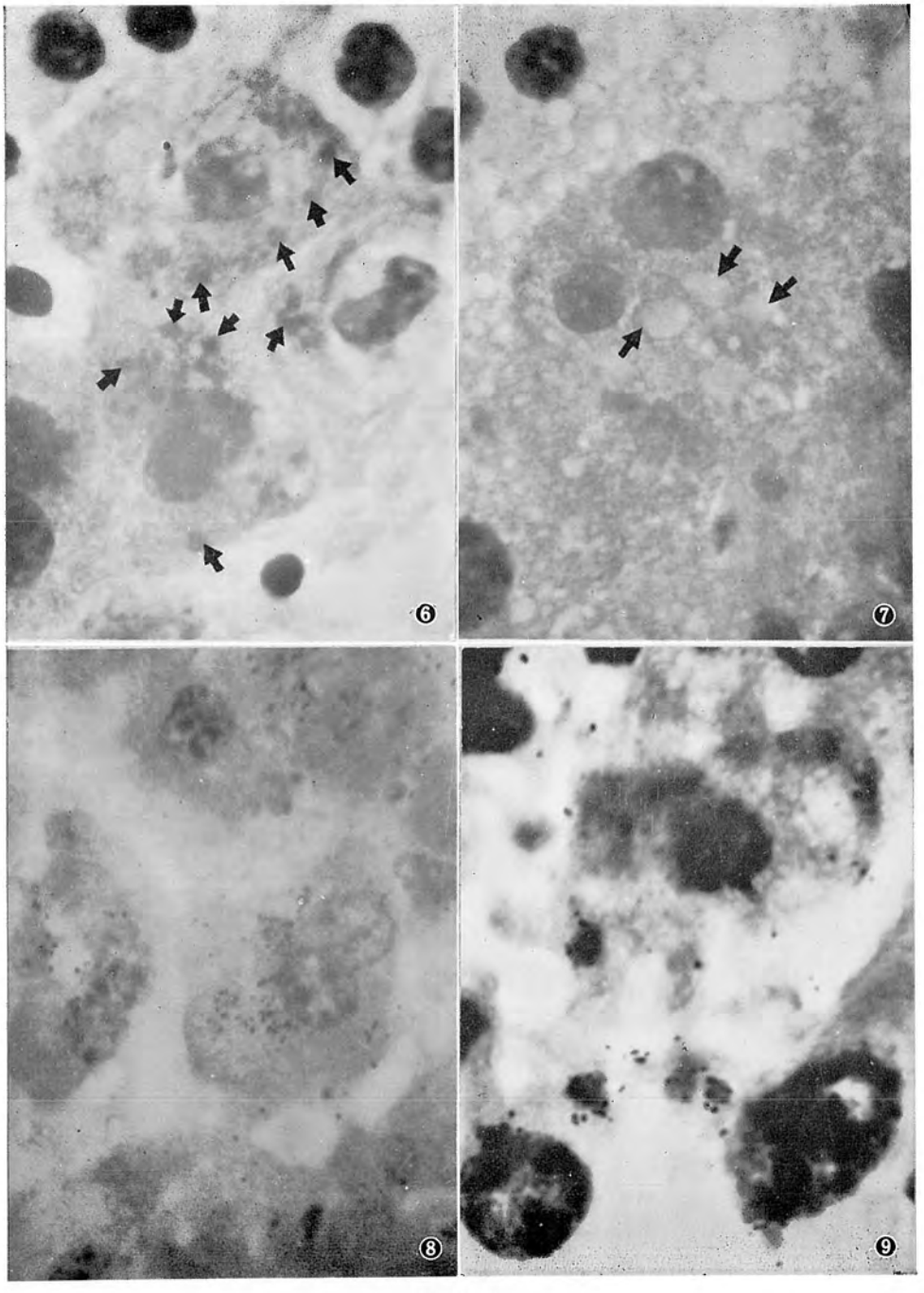

Fig. 6~9

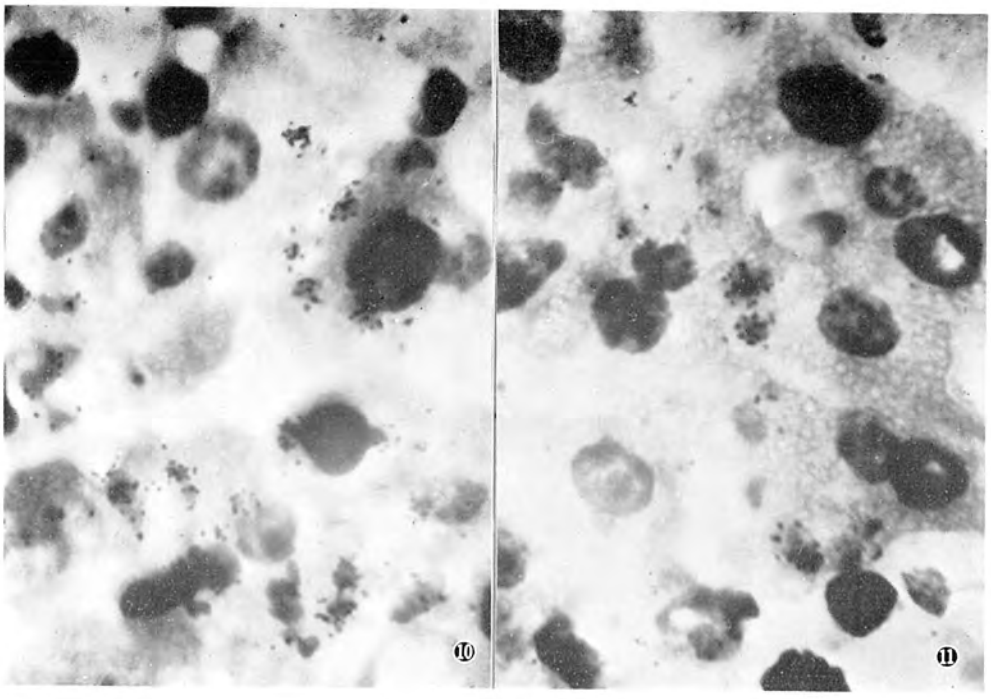

Fig. 10～11

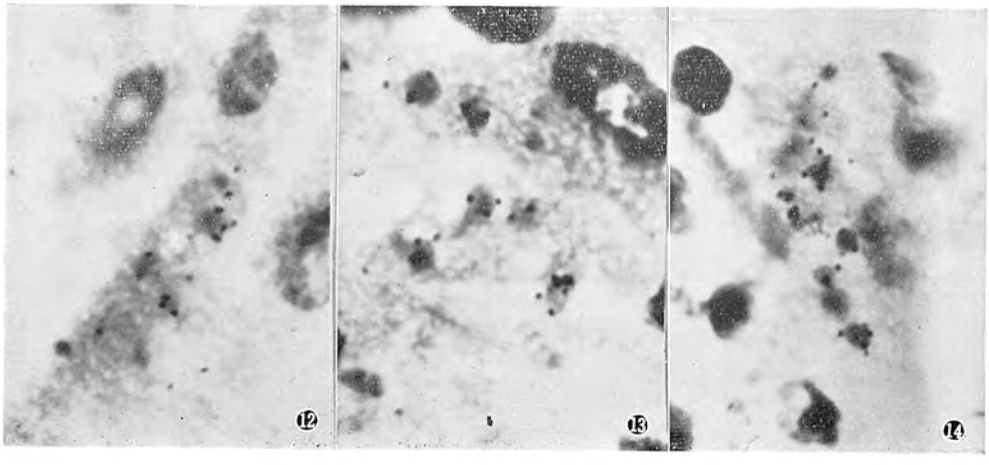

Fig. 12～14

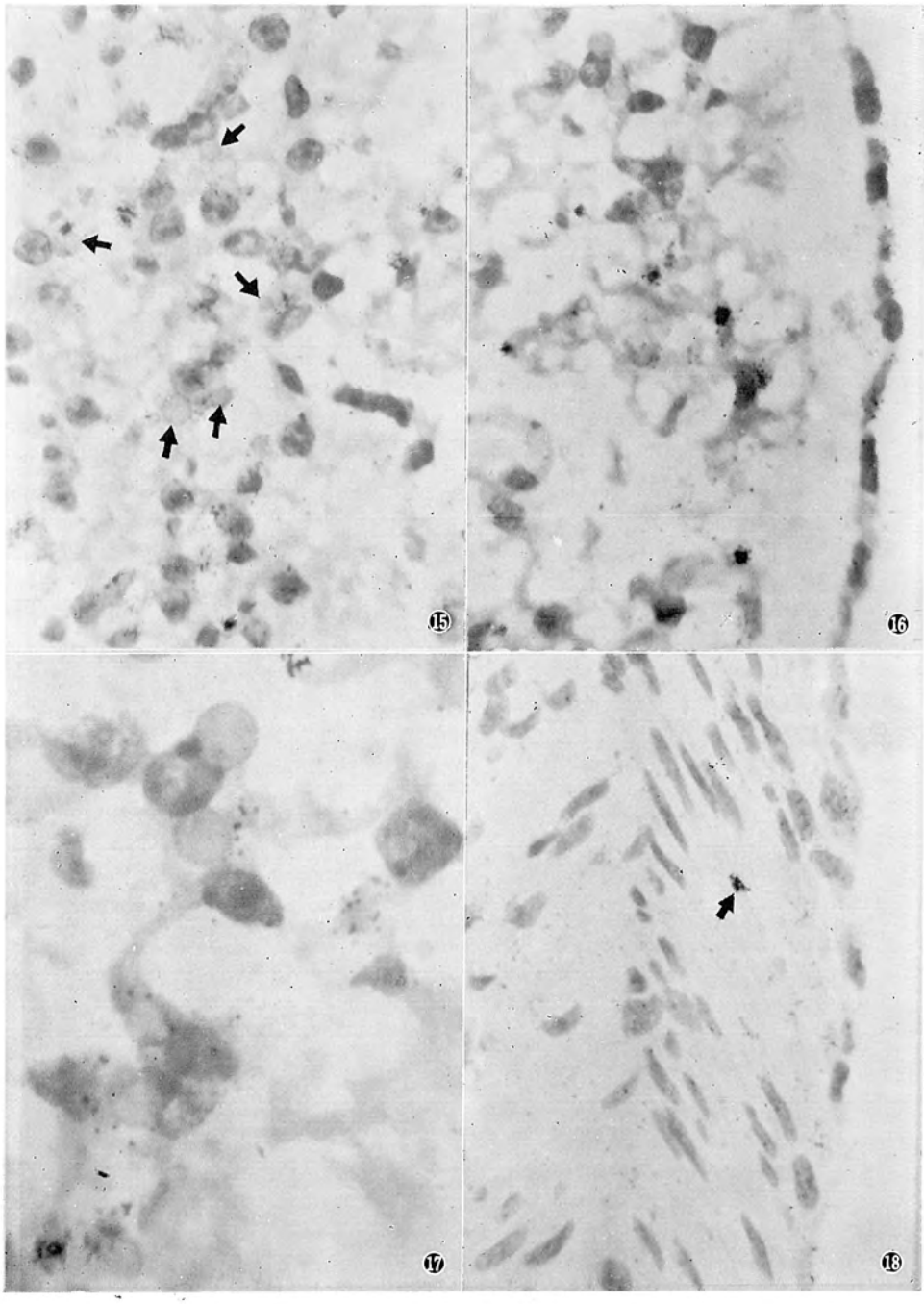

Fig. 15～18