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Electron Microscopical and Histochemical Studies of the Spontaneous Tumors of *Xenopus laevis*

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ABSTRACT—Spontaneous tumors were found in eight *Xenopus laevis* (African clawed toads), and their various properties were studied. In five of the eight cases, the primary tumor appeared on the back and infiltrated the muscles of the abdomen and thighs. Destruction of muscle fibers by the tumor cells was observed. Virus particles were found in the middle and outer layers of the original tumor mass. They had apparently first appeared in the nucleus and then aggregated near the nuclear membrane, finally spilling out into the cytoplasm. Based on their movement from nucleus to cytoplasm and morphological form and size, the virus particles were presumed to be of the herpes type. DOPA tests revealed premelanophore cells, with tyrosinase activity but no melanin granules, in the tumors. The tumors appeared to consist of melanophoma cells and granules in different stages of differentiation. It was possible to transplant the tumors to normal toads, four out of fifteen becoming progressive.

INTRODUCTION

There are markedly fewer reports on spontaneous tumors in amphibians than in other vertebrates [1–3]. Lucké renal adenocarcinoma in *Rana pipiens* [4, 5] and Japanese newt papilloma in *Cynops pyrrhogaster* [6–8] have been investigated as amphibian tumors, but reports on other amphibian tumors have been sporadic.

Although African clawed toads (*Xenopus laevis*) are used as experimental animals world-wide, there have been few reports on tumors in this species [9–16].

At the rate of 10,000 per annum, we surveyed about 40,000 adult *X. laevis* breeding in an artificial pond between 1983 and 1986, and found eight large tumor-bearing toads. Examinations previous to this study had shown such tumors to be of melanophoma form and to contain virus particles [16]. Cell cultures derived from those tumors were established, and four cell lines containing both melanoma and neuroma cells had been obtained [15].

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The eight tumor-bearing toads were used in the present study to further examine certain characteristics and properties of the tumors.

MATERIALS AND METHODS

Animal

Tumors were found in a total eight of 40,000 African clawed toads (*Xenopus laevis*) examined between 1983 and 1986. They were maintained at 20°C in a plastic aquarium until the time of autopsy [16]. Each toad, having a large tumor on its back, as shown in Figure 1, was anesthetized with MS-222 (Sankyo Co.) and subjected to gross biopsy.

Preparation for transmission electron microscopy

As shown in Figure 1, the original spontaneous tumor appeared under the skin of the back and infiltrated muscles of the abdomen and the right thigh. Tissue samples were taken from both the primary tumor and the infiltrated regions, and small blocks were prepared. For electron microscope examination, specimens were fixed in a buffered (0.05 M phosphate buffer, pH 7.2) 2.5% glutaraldehyde solution containing 2% paraform-

aldehyde and 0.1% picric acid, and embedded in Epon. Sections were stained with 2% uranyl acetate and lead citrate and examined with a JEM-100C electron microscope.

DOPA Test

Part of the tumor tissue was removed and sliced into sections approximately 3×3×1 mm which were then fixed for 30 min in 5% formalin embedded in ice. They were rinsed in a 0.1 M phosphate buffer. Tyrosinase activity against 3,4-dihydroxyphenylalanine (L-DOPA) and L-tyrosine was examined histochemically in accordance with Lerner's method [17]. Incubation of the specimens in the reaction mixture was performed at 25°C. In the control experiments, either a substrate-free reaction mixture, heat-inactivated specimens (10 min in boiling water), or a reaction mixture to which 10 mM sodium diethyldithiocarbamate had been added as an inhibitor was used. After incubation, specimens were again fixed in Bouin's solution, embedded in paraffin, sectioned and counterstained with Körnechrot.

Transplantation Study

Fifteen unaffected normal control toads, 7 males and 8 females, were selected from the laboratory breeding colony as tumor transplant recipients. Their body surface was wiped with 70% ethanol.

Part of tumor tissue obtained from the original 8 affected toads was cut into 2 mm^3 cubes. Each cube was inserted into a small pocket created under the skin of a control toad's back with a sharp knife. After the operation, each recipient toad was covered with cotton gauze soaked in an antibiotic (Kanamycin-sulfate; Banyu Co., Tokyo) solution for a few days to protect the injured region from infection. They were then observed for 8 months.

RESULTS

Gross Observations

A spontaneous original tumor was found under the skin of the back, as shown in Figure 1, of five of the eight original toads, on the legs of two, and on the abdomen of one. In this study, only the tumors on the backs of the five toads were used as starting material. Vertical section specimens obtained from these five primary tumors demonstrated a thin cartilage plate connecting to the scapula. There were two layers of tumor tissue, one above and one below the cartilage. The five tumors did not remain within the primary region but infiltrated into muscles of such other regions as the abdominal wall (*m. obliquus externus*) and right thigh (*m. cruralis* and *m. glutaeus magnus*).

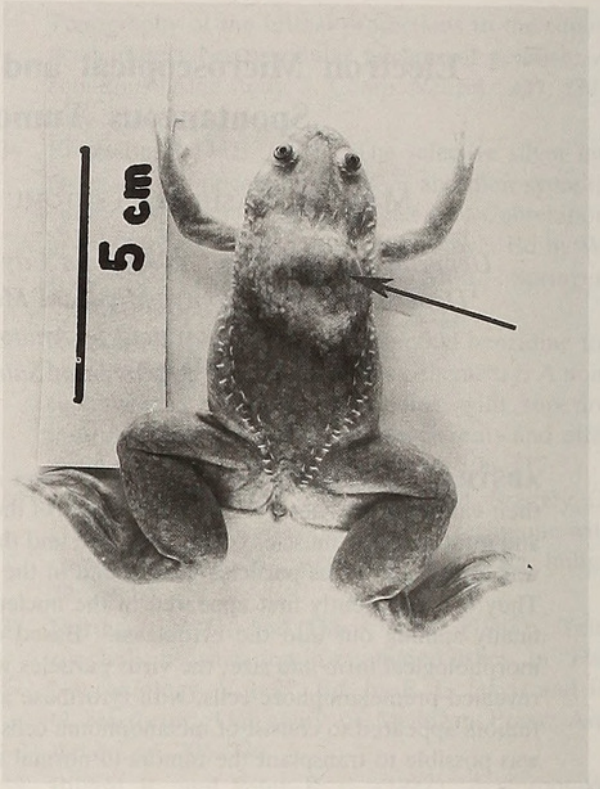


FIG. 1. Tumor-bearing *Xenopus*. The tumor (arrow) is seen on its back.

Electron microscope observations

Presence of Virus Tumor tissue could be separated into three layers under low power magnification (Fig. 2A) The inner layer was necrotic but no virus particles could be seen in the observed cells (162 cells, 0%). Active tumor cells were found in the middle layer, and many (35 of 241 cells, 14.5%) contained virus particles. In the outer layer, tumor cells were narrower and more concentrated, but fewer (4 of 196 cells, 2.0%) contained virus particles than in the middle layer.

The virus particles in the middle layer were then examined at higher magnifications. They had first

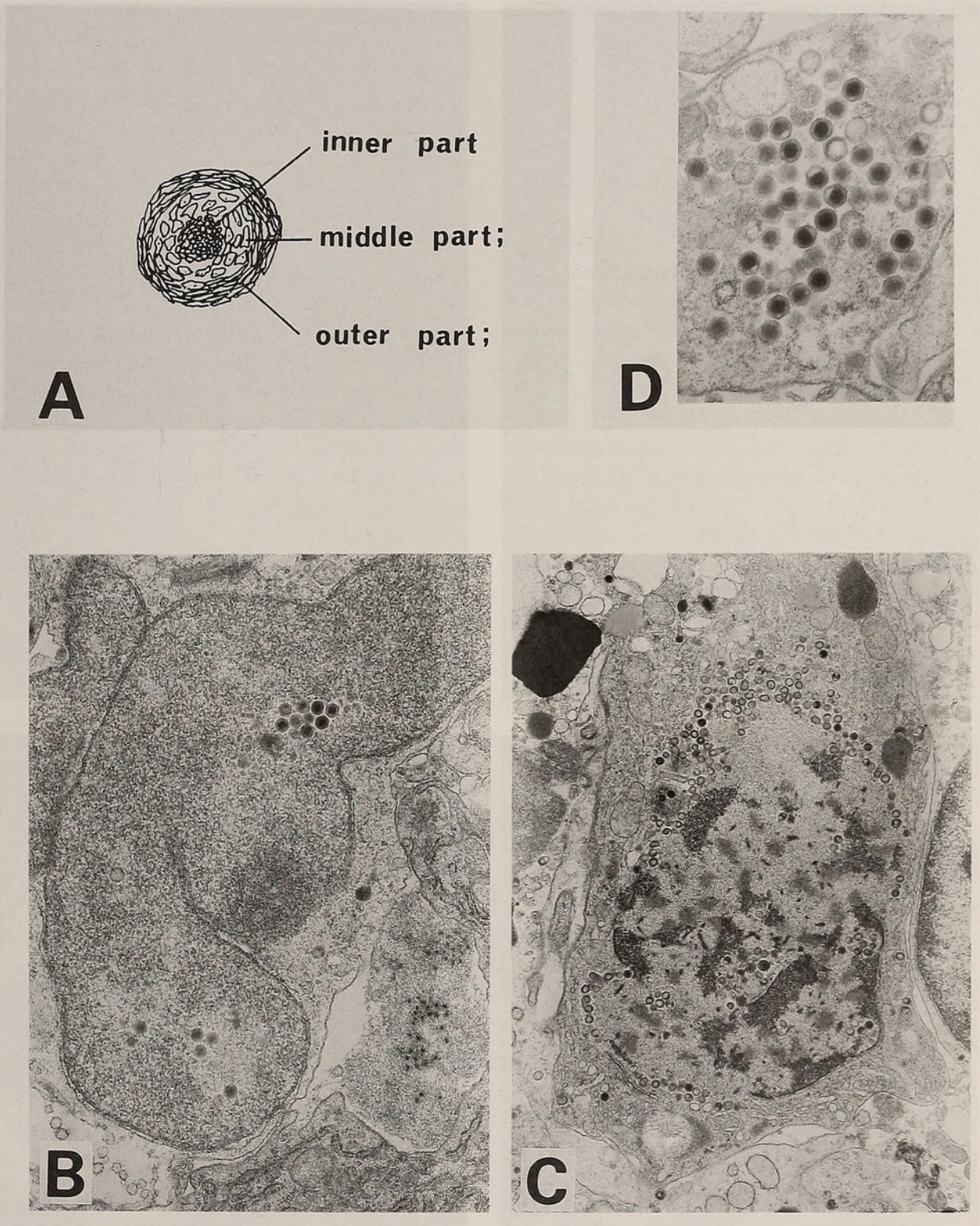
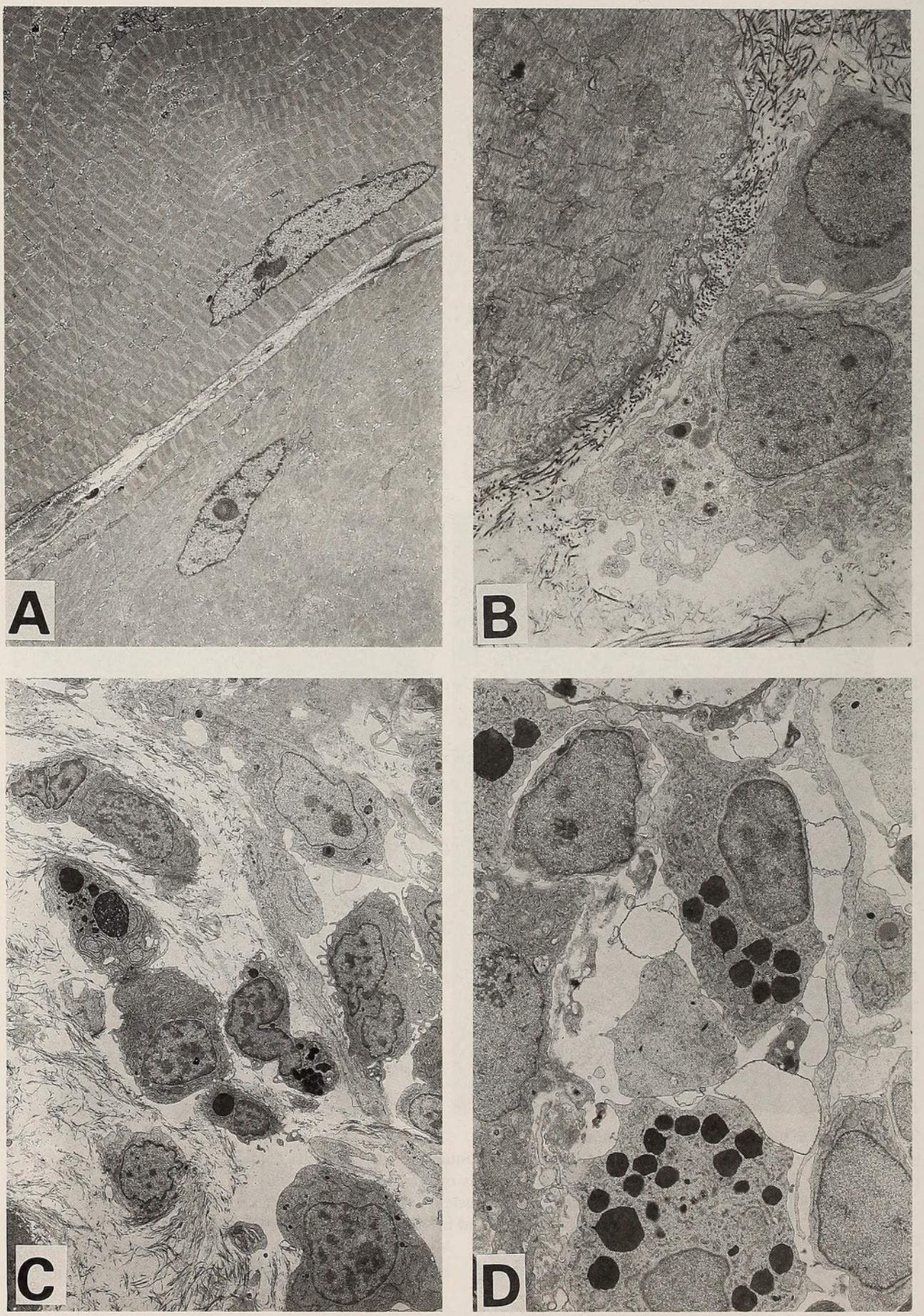


FIG. 2. Virus particles in tumor cells.
(A) Diagram of three layers of tumor mass containing virus particles (×1.5).
(B) Virus particles are first seen in the nucleus of tumor cells (×13,700).
(C) The particles have spilled out from the nucleus into the cytoplasm (×9,100).
(D) High magnification of virus particles found in *Xenopus* tumor cells (×22,000).


A
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appeared in the nucleus (Fig. 2B). When they had filled the nucleus, they aggregated near the nuclear membrane and spilled out into the cytoplasm (Fig. 2C). The particles were about 0.15 μm in diameter (Fig. 2D). Based on these observations, the virus observed in all five toads (100%) was presumed to be of the herpes type.

Infiltration of tumor cells Gross observation revealed the five back tumors to have infiltrated the abdomen and thighs. The process of infiltration was observed in the muscles of the thigh. The regular arrangement of striated muscle fibers was preserved in that region as yet not reached by tumor cells (Fig. 3A). Destruction of muscle fibers began at the point of intrusion of tumor cells into the muscle (Fig. 3B). Tumor cells moved through the connective tissue and they infiltrated into other tissues (Fig. 3C). Finally, only irregularly shaped tumor cells containing electron dense granules could be seen (Fig. 3D). Based on these observations, the tumor was thought to be malignant.

DOPA Test

A previous study had indicated that the tumors were melanophoma-like because melanin granules had been observed in parts of the tumor mass. We therefore utilized the DOPA test to examine whether or not premelanophore cells which did not contain melanin granules, but had the ability to synthesize melanin, were present. Reaction-positive cells were detected as cell islands in tumor tissue when L-DOPA was used as a substrate (Fig. 4). Melanin granules were not seen in these cells. Some mature melanophores were seen near the positive cell islands, but such activity was not detected when L-tyrosine was used. In the control experiments, no reaction products were found.

Transplantation Study

Within eight months after tumor transplantation, swellings appeared on the backs of seven of the 15 toads. In four cases, the swellings grew to 0.5 cm in diameter during the first three months and to 0.9 cm within eight months. In the three other cases, the swellings grew to 0.5 cm during the first three months and then remained stable thereafter. With the transplants showing progressive growth in four toads, there was clear indica-

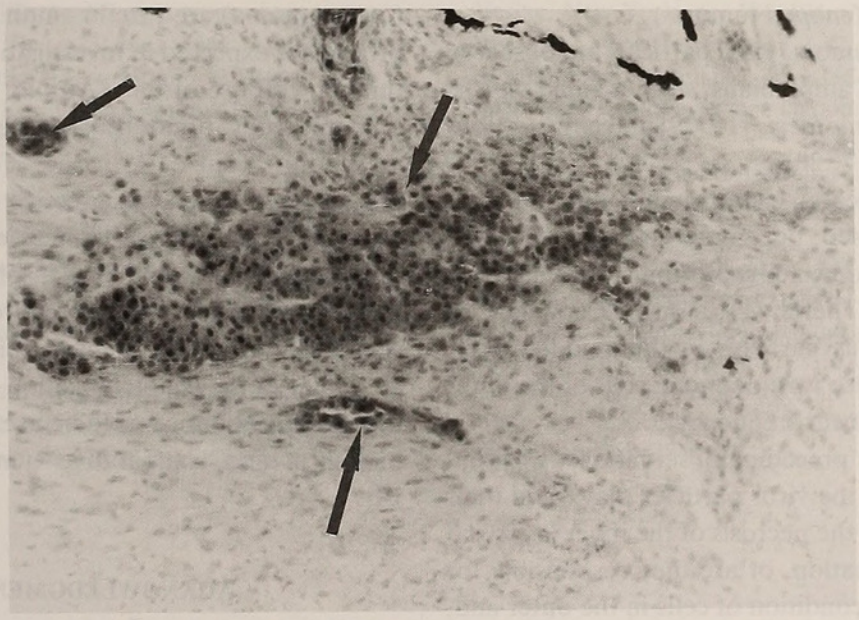

FIG. 4. Histological section of DOPA-positive cells (arrows) in tumor cells (×150).

FIG. 3. Degradation of muscle fibers by invading tumor cells.
(A) to (D) show progressive advancement of invasion by tumor cells. Normal regular microfilaments are destroyed in this penetration by tumor cells. magnification:
(A) ×2,200 (B) ×5,6000 (C) ×2,200 (D) ×5,800

tion that the tumors could be transplanted in normal toads.

DISCUSSION

There is a low frequency of spontaneous tumors in *Xenopus laevis,* only eight being found among 40,000 adult toads. Of the eight, five toads bearing tumors on their backs were subjected to biopsies. The tumors were found to be highly infiltrative, intruding from the primary region on the back into the muscles of the abdomen and the thighs. The process of tumor-cell infiltration into the muscles, resulting in destruction of muscle fibers, was observed. The muscle fibers dissolved into fragments and only their residue remained. Based on these observations, there was no doubt the tumor was malignant.

There have been no reports of such infiltration of tumors in amphibians. Lucké renal carcinoma cells proliferate and result in so-called renal adenocarcinoma [4, 5]. Epithelioma has been observed in the newt, *Cynops pyrrhogaster* [6, 8]. The cells of these tumors grow in the primary region of the tumor and do not infiltrate other tissues as in the manner of the *Xenopus* tumor.

In regard to tumors, virus particles are observed in some kinds of amphibians, such as *Rana pipiens* [4, 5, 18], *Xenopus laevis* [16, 19] and newt *Cynops pyrrhogaster* [6, 7, 20].

The inner layer of each primary tumor in our study was necrosed, but tumor cells in the middle layer appeared to be active, forming fibroblastic or moving cells. There was a high frequency of discovery of virus particles in this middle layer. Virus particles also were found in cells of the outer layer, but at a lower frequency.

In view of the preceding observations, we wondered what role the virus particles play. Did their presence lead to the necrosis of the inner layer and their own elimination, or are they responsible for the activity and condition of cells in the outer and, especially, middle layers? We do not know, but either could be possible.

The virus particles were observed to have first appeared in the nucleus. When the nucleus was filled, virus particles aggregated near the nuclear membrane and spilled out into the cytoplasm. Virus particles were about 0.15 μm in diameter. The movements, size and shape of the particles indicate the virus to likely be of the herpes type, resembling the herpes virus of Lucké renal adenocarcinoma [4, 5, 18].

We previously [16] found melanophores containing melanin granules in *Xenopus* tumor tissue, leading us to believe *Xenopus* tumors were similar to melanophoma. In this study, the tyrosinase activity of cells which did not contain melanin granules was examined by means of the DOPA test. Reaction products were detected in such cells. Hence, premelanophore cells with tyrosinase activity were involved in the tumor. Using the same type of tumor mass from *Xenopus,* Asashima *et al.* [15] have cultured cells for over 2 years and found four cell lines deriving from one of the *Xenopus* tumors.

Cells synthesizing melanin granules were found in only one of these cell lines. In a culture of these cells, the property of synthesizing melanin granules appeared after several weeks of subculture. The *Xenopus* tumors appeared to be multiple melanophomas which contained tumor cells in different stages of differentiation. Melanophoma (or melanoma) are rare in amphibians [21–23], so progress is expected in investigations of amphibian tumors with use of the *Xenopus* tumor.

In this study, tumor transplantation was partially successful: 7 out of 15 cases. Four of the transplants have been progressive for over eight months. This progressive nature of the *Xenopus* tumor may enable tumor transplantation and cell culture and prove to be a more satisfactory research material than the newt epithelioma. Further studies of the characterization and properties of the *Xenopus* tumor would be useful in the fields of biological and comparative tumors research [3, 24].

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