IN VITRO CULTIVATION AND IMMUNOFLUORESCENT STUDIES OF TRANSPLANTABLE CARCINOMAS Vx2 AND Vx7

Persistence of a Shope Virus-Related Antigenic Substance in the Cells of Both Tumors*

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It has been well documented that, among the series of transplantable strains of carcinomas established by Rous and his associates (1, 2) from the malignant growths primarily originated from Shope virus–induced papillomas (3), the Vx7 carcinoma still possesses Shope papilloma viral antigen even after serial transplantation for over 15 yr while the Vx2 loses the antigen sometime during that period (1). The basis for this concept was provided by the fact that the neutralizing antibody against the etiological virus is demonstrable in sera of Vx7 carcinoma–bearing rabbits but not in animals with Vx2 carcinomas. Immunofluorescent technique applied to the tissue sections of the system also added supportive evidence by demonstrating viral antigen in Vx7 carcinomas but failing to do so in Vx2 (4). In a paper now in preparation (5), it has been shown that nucleic acid extracts endowed with tumorigenic capacity are consistently recovered from the tissues of Vx7 carcinomas but not from the Vx2 carcinomas. This finding also seems to agree with the current interpretation of the virus-tumor relationship in the Shope papilloma-carcinoma system. Most of these studies on the system to date, however, were carried out at the tissue level and little information has been available on the event taking place in the individual cells comprising the neoplasia.

The present study was designed primarily to investigate whether such difference in basic characteristics is demonstrable between these two neoplasia at the cellular level. For this purpose, both Vx2 and Vx7 tissues were cultivated in vitro and the cells derived from these explanted tissues were studied by immunofluorescent technique. Evidence is presented that not only Vx7 cells but also Vx2 cells are not completely free of antigenic substance possibly related to the etiological Shope papilloma virus.

Materials and Methods

Vx2 and Vx7 Tumors.—Two strains of transplantable carcinomas of rabbit, Vx2 and Vx7, were employed. Details of their origin and histological characteristics have been described in the paper already mentioned (5).

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Media.—Eagle's minimum essential medium containing 20% fetal calf serum or YLE medium (Earle's balanced salt solution with 0.1% yeast extract and 0.5% lactalbumin hydrolysate) containing 20% calf serum and 10% tryptose phosphate broth was used throughout the experiment.

Cell Culture.—Finely minced Vx2 and Vx7 tissue fragments were washed thoroughly with phosphate-buffered saline. For immunofluorescent studies and morphological observation with stained preparations, the small tissue fragments were placed on cover slips (10 × 35 mm) in Leighton tubes. To establish continuous cell lines, tissue fragments were placed directly on the surface of 50 mm glass Petri dishes or TD-40 flasks. After the tissue fragments were firmly attached onto the glass, the growth medium was added, and the incubation was carried out at 37°C in a stationary position in a regular incubator or in a humidified atmosphere containing 5% CO_2.

Immunofluorescent Staining Procedure.—The indirect staining method was used for immunofluorescent studies (6). Cover slips with grown cells were dried and fixed in carbon tetrachloride for 30 min at room temperature. Antisera to Shope papilloma virus were obtained from "regressor" cottontail rabbits. The serum specimens were kindly provided by Dr. C. A. Evans of The University of Washington, Seattle. For the staining of the cells, the serum was placed on cover slips and allowed to stand for 50 min at 37°C. The cover slips were then rinsed with phosphate-buffered saline and fluorescein isothiocyanate (FITC)–conjugated anti-rabbit γ-globulin goat serum was applied. After additional washing with phosphate-buffered saline, the cover slips were mounted in 20% buffered glycerin and were examined with a Nikon fluorescence microscope with a Corning No. 5840 or a Schott BG 12 exciter filter. The light source was an Osram HBO 200 lamp.

EXPERIMENTAL

Growth Characteristics of Vx2 and Vx7 Carcinoma Cells In Vitro.—In both Vx2 and Vx7 carcinomas, growth of primary cells from the explants of tumorous tissues attached on the cover slips and cultured in Leighton tubes was observed approximately 2 wk after incubation.

In primary Vx2 cultures, uniform, round and polygonal epithelial cells were predominant. The cells were small but had an increased nucleocytoplasmic ratio. These cells showed a tendency to disintegrate from the explants in the earlier stage of growth and then grew over one another, forming multilayered areas (Figs. 1 and 3).

In contrast to this, Vx7 cultures were characterized by pleomorphic, round and polygonal epithelial cells. The nuclei were also pleomorphic and had increased nucleocytoplasmic ratios. The nucleoli showed irregular outlines. Many vacuoles in various sizes were observed in the cytoplasm. The surface structure of cells which contained large vacuoles appeared to be thick and rigid in both stained and unstained preparations. Multinucleated giant cells were frequently present. These primary Vx7 cells showed a tendency to form a colonial growth and piled up over each other forming multilayered areas (Figs. 2 and 4).

Attempts to Establish Continuously Growing Cell Lines In Vitro from Vx2 and Vx7 Tumors.—

Both Vx2 and Vx7 tumor tissues were incubated in 50 mm glass Petri dishes with growth medium at 37°C in a humidified atmosphere containing 5% CO_2. The growth

of cells from explants of the tumorous tissues were observed approximately after 1 wk of incubation. Subcultivation of the primary growth of cells was carried out after 3 wk in the case of Vx2 cultures and after 5 wk in Vx7 cultures, respectively. Then, several transfers were made once a week. For subcultivation, each Petri dish culture was transferred to two Petri dishes by separating the cells with 0.2% trypsin. Figs. 5 and 6 show cells in the ninth and eighth passages of Vx2 and Vx7 cell cultures, respectively. These cultures are still characterized, as had been observed in the primary cultures, by pleomorphic, round and polygonal epithelial cells in Vx7 and by uniform, round and polygonal epithelial cells in Vx2. Unfortunately, however, both Vx2 and Vx7 cell cultures were lost at the tenth and ninth passage levels by an accident due to a mechanical difficulty in our CO_2 incubator.

Establishment of Vx7 In Vitro Cell Line.—

Subsequent trial was carried out to establish a Vx7 cell line in vitro after the unlucky event just mentioned. This time, finely minced tumor tissues were incubated in TD-40 flasks at 37°C in a regular incubator. After 6 wk of incubation, each flask culture was transferred to two flasks, again after it had been exposed to 0.2% trypsin. Succeeding transfers of a similar sort were then carried out every 10–20 days. Two cell lines, designated as Vx7a and Vx7b, respectively, were thus obtained. The morphology of the cells comprising the cultures of these two cell lines were essentially identical with that of the primary and subcultured Vx7 cells, as already described in the previous sections. The cell line Vx7a was frozen in its sixth passage and stored at −70°C. The second cell line, Vx7b, is at present (17 June 1967) in its fifteenth passage.

Pathogenicity of Established Cell Line Vx7b.—

To test whether the established Vx7b cells still retain their original neoplastic property, approximately 5×10^4 cells in the third passage, and approximately 5×10^5 cells in the seventh passage, were implanted intradermally in the ears of young domestic rabbits. One to four sites of inoculation were utilized on each ear. Tumorous growths were first evident at day 4, and they attained a diameter of 1.5–2 cm by the third week (Fig. 7). Histological examination of biopsied specimens from the growths exhibited proliferation of tumorous cells of characteristic Vx7 morphology. As listed in Table I, all 23 sites implanted with Vx7b cells gave rise to tumorous growth in 2 wk. These tumors showed a tendency to dwindle in size after 4–8 wk of growth and the majority of them retrogressed in 12 wk. After 12 wk of observation, only one animal among the total of six implanted with the Vx7b cells retained tumorous growths. All four tumors in this rabbit had become ulcerated at 5 wk (Fig. 8), and the tumors had extended through to the inner surface of the ears. Later, they caused big holes in the ear owing to destruction of the cartilage (Fig. 9). Histological examination of all specimens biopsied from the lesions after the appearance of ulceration showed massive growths of an almost anaplastic epidermal carcinoma (Figs. 10 and 11).

Immunofluorescence of Vx2 and Vx7 Carcinoma Cells In Vitro.—Cells of Vx2 and Vx7 carcinomas were examined for immunofluorescence in primary cultures, in third passage cultures, and in fifth passage cultures, respectively. In each examination, three cover slips with grown cells were used. They were fixed

according to the procedure previously described and the number of fluorescent cells were counted on a mechanical stage. A total of 10,000 cells on each cover slip of Vx2 cultures and 5000 cells on each cover slip of Vx7 cultures were examined and the ratio of fluorescent cells was calculated. The number of cultures thus examined were 12 primary cultures and two third and two fifth passage cultures in Vx2 and eight primary and three third and three fifth passage cultures in Vx7. These were cultures derived from different in vivo transplanted tumors.

In Vx7 cells, approximately 10–20% of primary passage, 5–10% of third passage, and 5–10% of fifth passage cells were specifically stained with the

TABLE I

The Results of Implanting Vx 7 Cells Cultivated In Vitro into the Ears of Domestic Rabbits

Cells*	No. of cells implanted per site	No. of rabbits implanted	No. of positive growths at implantation sites‡	Tumor incidence	Average period before a tumor was visible	No. of tumors that regressed per No. of positive growths		
						Weeks after inoculation		
						4	8	12
				%	*days*			
Vx7b(3)§	5×10^4	2	4/4	100	7	1/4	2/4	4/4
Vx7b(7)	5×10^5	4	19/19	100	5	2/19	6/19	15‖/19

* The cells were suspended in Eagle's minimum essential medium containing 20% fetal calf serum; 0.1 ml was inoculated by intradermal injection into the epidermis of the outer surface of the rabbits' ears.

‡ Results 2 wk after implantation.

§ Figures show the number of passages of the cells employed.

‖ The four tumors that did not eventually regress were all in one of the two implanted rabbits. All were malignant carcinomas (see text and figures).

antiviral antisera from regressor cottontail rabbits. Fluorescence was more conspicuous in the cytoplasm, appearing as fine bright granules, than it was in the nucleus (Figs. 12 and 13).

In contrast to these findings, relatively few fluorescent cells were detectable in the Vx2 cell cultures. Approximately 20–30 cells of this sort per cover slip (less than 0.1% of the total cells it carried) exhibited positive immunofluorescent reaction in the primary, third, and fifth passage cultures, the ones examined. These Vx2 fluorescent cells were usually found as isolated clusters composed of several cells (Fig. 14).

As controls, normal rabbit kidney and skin cells were treated with the same antisera, and Vx2 and Vx7 cells were also treated with normal rabbit serum. All of these control sets gave negative results.

DISCUSSION

It has been reported by Rous and his coworkers that Vx2 tumors are much more anaplastic than Vx7 tumors, these still having the character of squamous

epidermal carcinomas (7) to some extent. Recent histopathological observations have revealed that this difference is still retained (5). The same difference is evident in vitro, not only in our primary cultures but also in later passages. The neoplastic character of Vx7 cells was plainly visible in the culture cells of Vx7b.

In previous immunofluorescent studies by Mellors (4) of Vx2 and Vx7 carcinomas in tissue sections, fluorescence could only be detected in the Vx7 tumors. This accorded with the results of studies on the presence of neutralizing antibody against Shope papilloma virus in the sera of Vx2 and Vx7 tumor-bearing rabbits (7). However, it is clearly shown in the present study with in vitro cultured cells that immunofluorescence is not only demonstrable in Vx7 cancers but also in Vx2 cancers, although the reactivity of cells of the latter very seldom occurs. These findings indicate that the Shope papilloma viral antigen is still persisting in certain Vx2 cultured cells but in a very small proportion compared with those of Vx7.

The immunofluorescent reaction in both Vx7 and Vx2 cells cultured in vitro was nearly all cytoplasmic fluorescence, though some nuclear fluorescence was also found. In contrast to these findings, the antisera used in this present study have been found to yield fluorescence in the nucleus, not in the cytoplasm, when applied to cultured cells that were procured from in vitro domestic rabbit papillomas that were induced with the Shope virus (8). The significance of this important difference is not understood at the present time.

From the observations here reported, the inference seems justified that the Vx2 and Vx7 carcinomas differ much in respect to the possession of viral antigen. The difference in incidence of fluorescent cells in the Vx2 and Vx7 remained much the same in different passages in vitro. It would seem that this difference in incidence was transferred to generation after generation of cells even in the case of Vx2 tumors.

SUMMARY

The Vx2 and Vx7 rabbit carcinomas, both of Shope papilloma derivation, were cultivated in vitro and studied for immunofluorescence indicative of the presence of viral antigen. The cultured Vx7 cells were characteristically pleomorphic, round or polygonal cells, and immunofluorescence was demonstrable in 5–20% of them as counted on cover slip cultures after exposure to rabbit sera containing anti-Shope papilloma virus antibody. The Vx2 cells in contrast to this were of almost uniform size and character, small and approximately round, and the specific fluorescence was found in less than 0.1% of the total number. These findings indicate that some antigenic derivation from the Shope papilloma virus is still persisting at least in some fractions of the cells, not only of Vx7 tumors but also of the Vx2 tumors, even after maintenance of the growths by transplantation for more than 20 yr. During the course of the study, two lines of the Vx7 were kept continually growing while the culture of Vx2

was discontinued at its tenth passage after an accident due to mechanical failure of the incubator. The neoplastic capacity of the Vx7 cells cultured in vitro was demonstrated by implanting these intradermally on the ears of domestic rabbits. The result was destructive carcinomas of the Vx7 sort.

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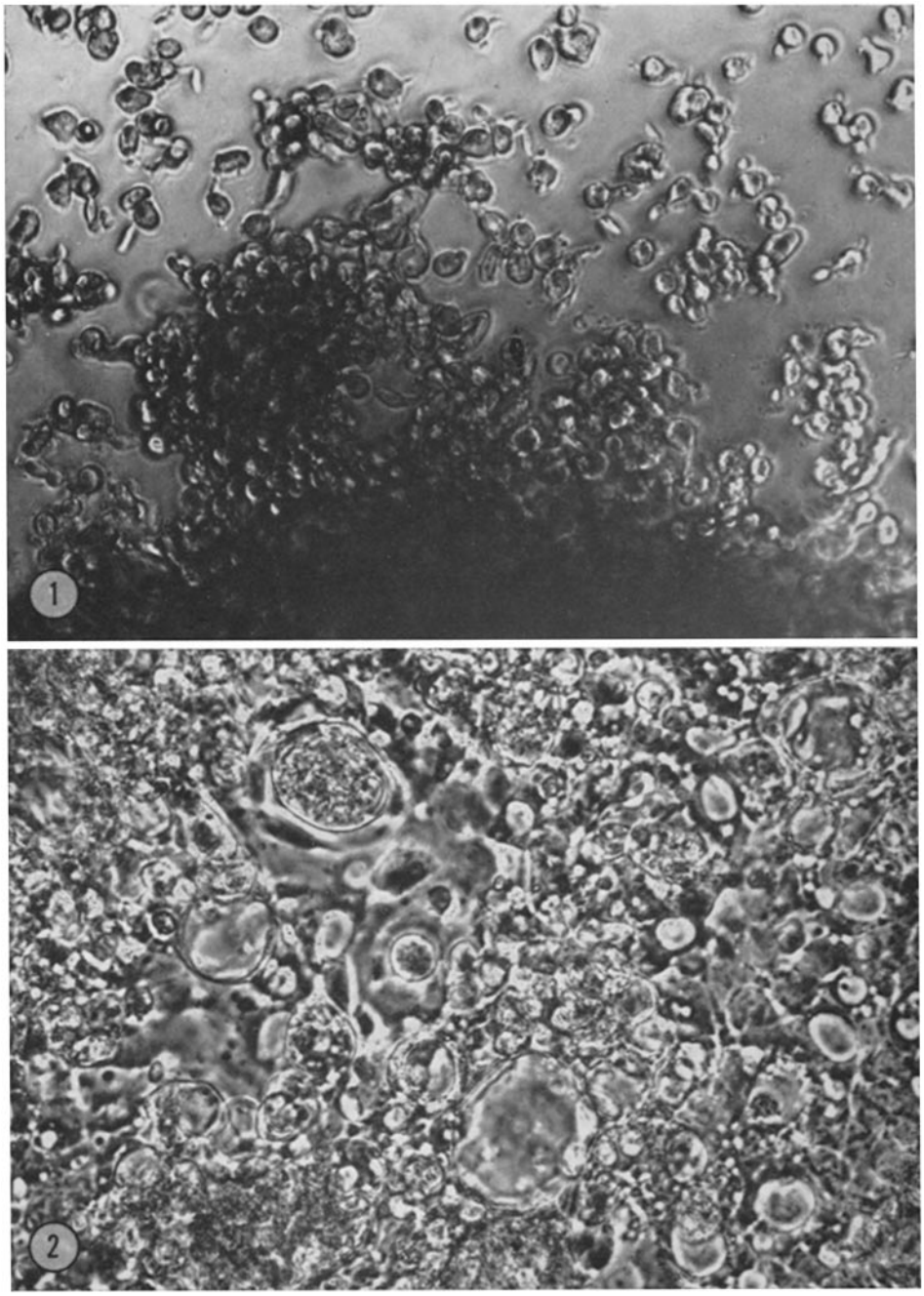
EXPLANATION OF PLATES

PLATE 63

FIG. 1. Primary culture of Vx2 carcinoma cells after 3 wk. Round and polygonal epithelial cells are predominant. They are uniform in morphology and relatively small in size. Soon after culture begins, the cells separate and spread out, but later they pile up, forming multilayered areas. These are shown in the darker part of the picture Phase-contrast. × 400.

FIG. 2. Primary culture of Vx7 carcinoma cells after 4 wk. It is composed of pleomorphic, epithelial cells, multilayered and irregular. Phase-contrast. × 400.

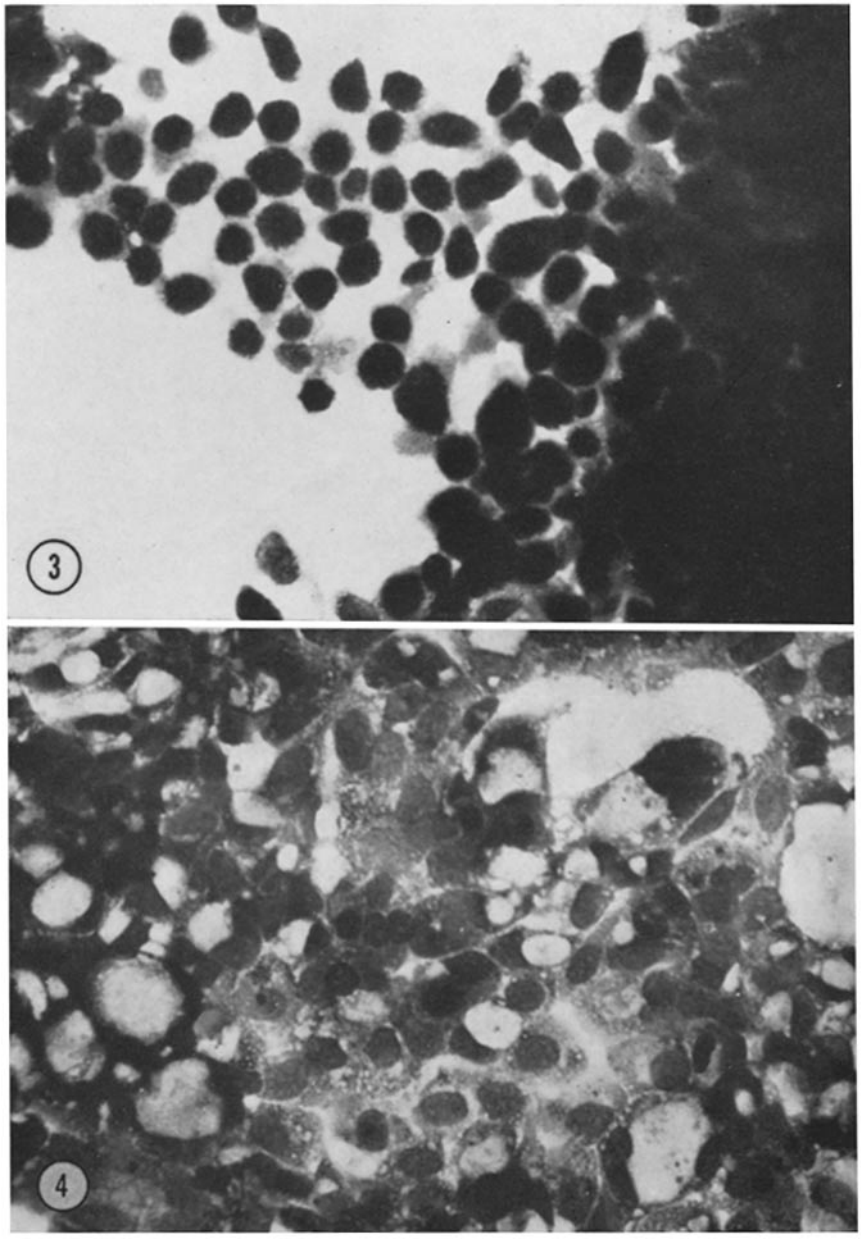


(Osato and Ito: In vitro studies of Vx carcinomas)

PLATE 64

FIG. 3. Stained preparation of a primary culture of Vx2 carcinoma cells after 3 wk. It shows essentially the same growth characteristics as are seen in Fig. 1. May-Grünwald Giemsa stain. × 700.

FIG. 4. Stained preparation of a primary culture of Vx7 carcinoma cells after 4 wk. It shows the same characteristics as are present in Fig. 2. May-Grünwald Giemsa stain. × 350.

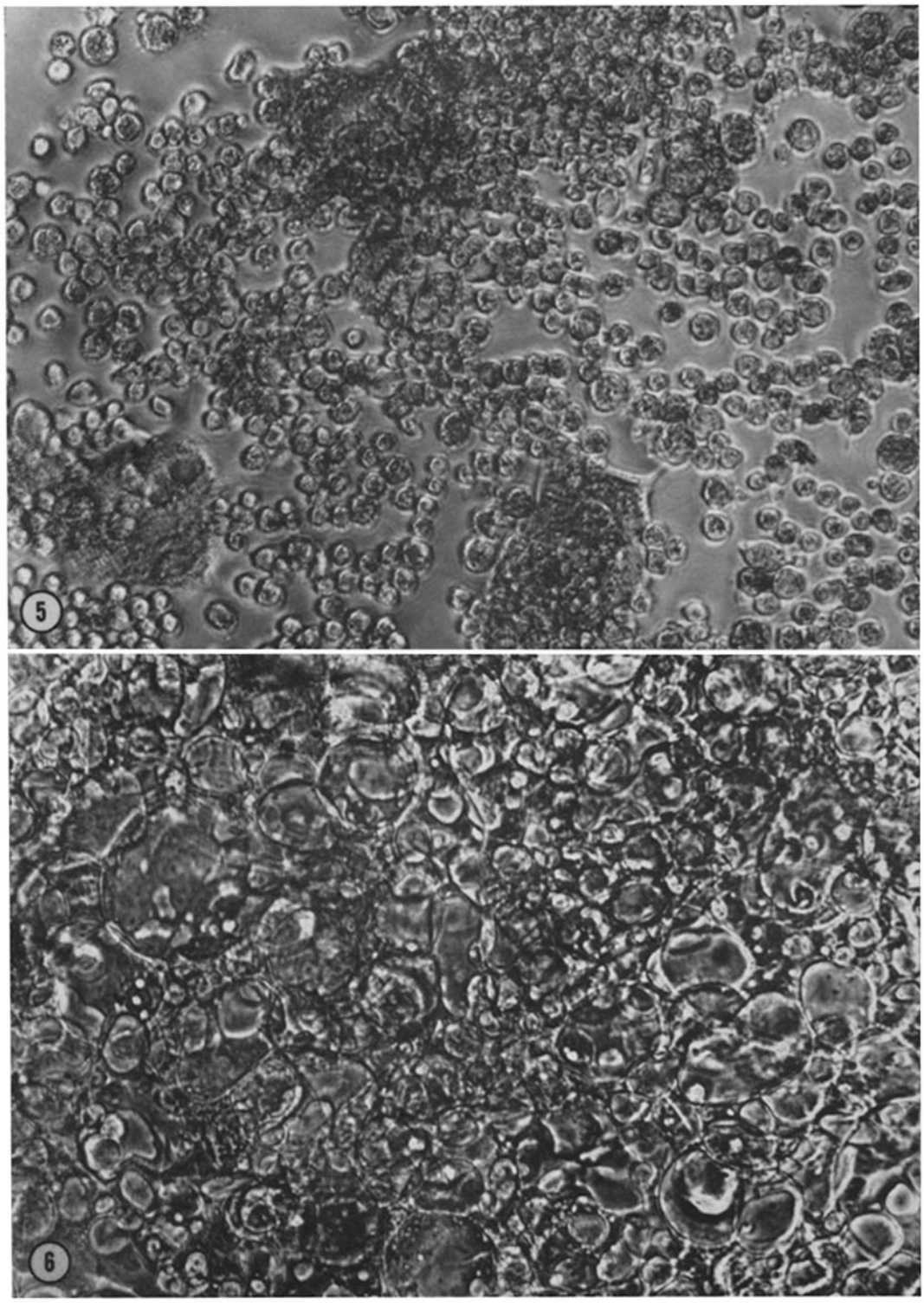


(Osato and Ito: In vitro studies of Vx carcinomas)

PLATE 65

FIG. 5. Vx2 carcinoma cells in the ninth culture passage. The culture consists of uniform, round and polygonal epithelial cells like those formed in the primary culture (Fig. 1). The tendency to spread out on the glass surface is also evident. Phase-contrast. × 480.

FIG. 6. Vx7 carcinoma cells in the eighth passage culture. The culture still consists of pleomorphic, epithelial cells of irregular growth pattern, like those observed in the primary culture shown (Fig. 2). Phase-contrast. × 480.



(Osato and Ito: In vitro studies of Vx carcinomas)

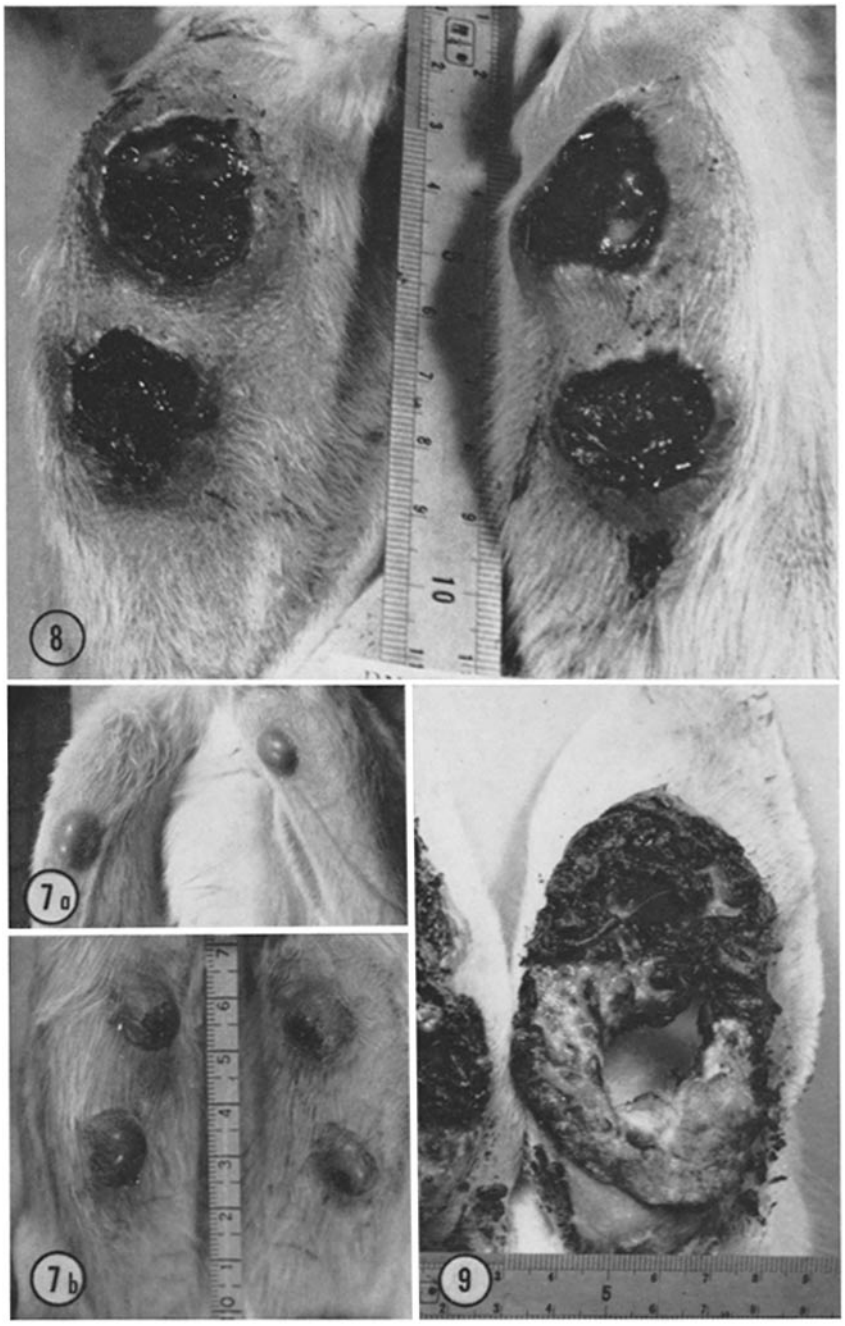
PLATE 66

Scale marks for Figs. 7–9 are in centimeters.

FIG. 7. Tumorous growth in rabbit ears as result of the implantation of cells of the in vitro cultured Vx7b line. The growths are shown at 12 days after implantation of the cells (*a*) and after 34 days respectively (*b*).

FIG. 8. The four growths shown on the two ears of Fig. 7 *b* are now ulcerated carcinomas. Photographed at 91 days after inoculation.

FIG. 9. Later stage of one of the cancers shown in Fig. 8. It has caused great destruction of cartilage. Photographed 156 days after the implantation.

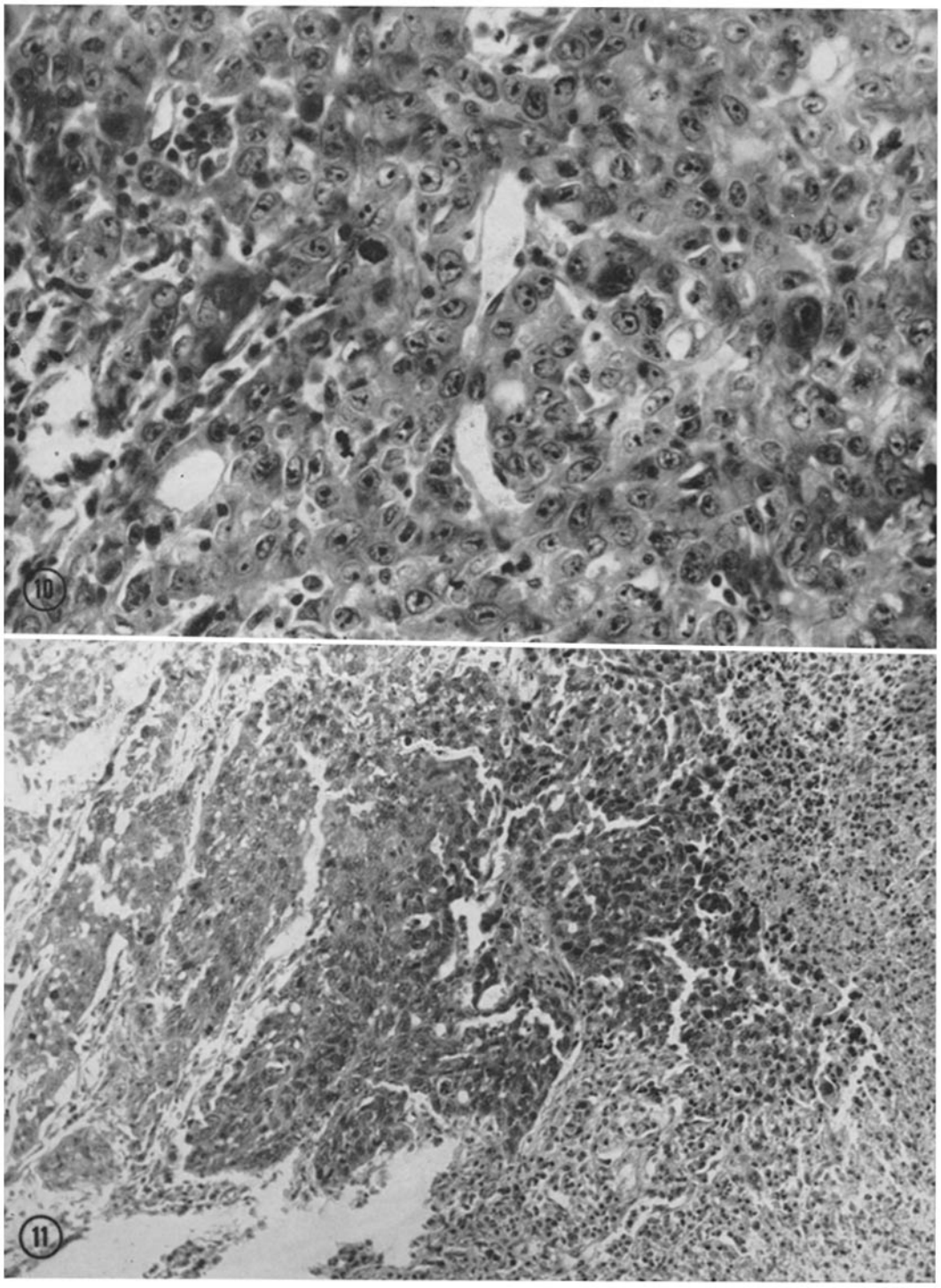


(Osato and Ito: In vitro studies of Vx carcinomas)

PLATE 67

FIG. 10. Section of one of the carcinomatous growths shown in Fig. 8. It is a typical squamous cell carcinoma with marked pleomorphism. Hematoxylin and eosin stain. × 420.

FIG. 11. Section of the carcinomatous lesion shown in Fig. 9. The growth is now almost anaplastic and there is much necrosis. Hematoxylin and eosin stain. × 210.



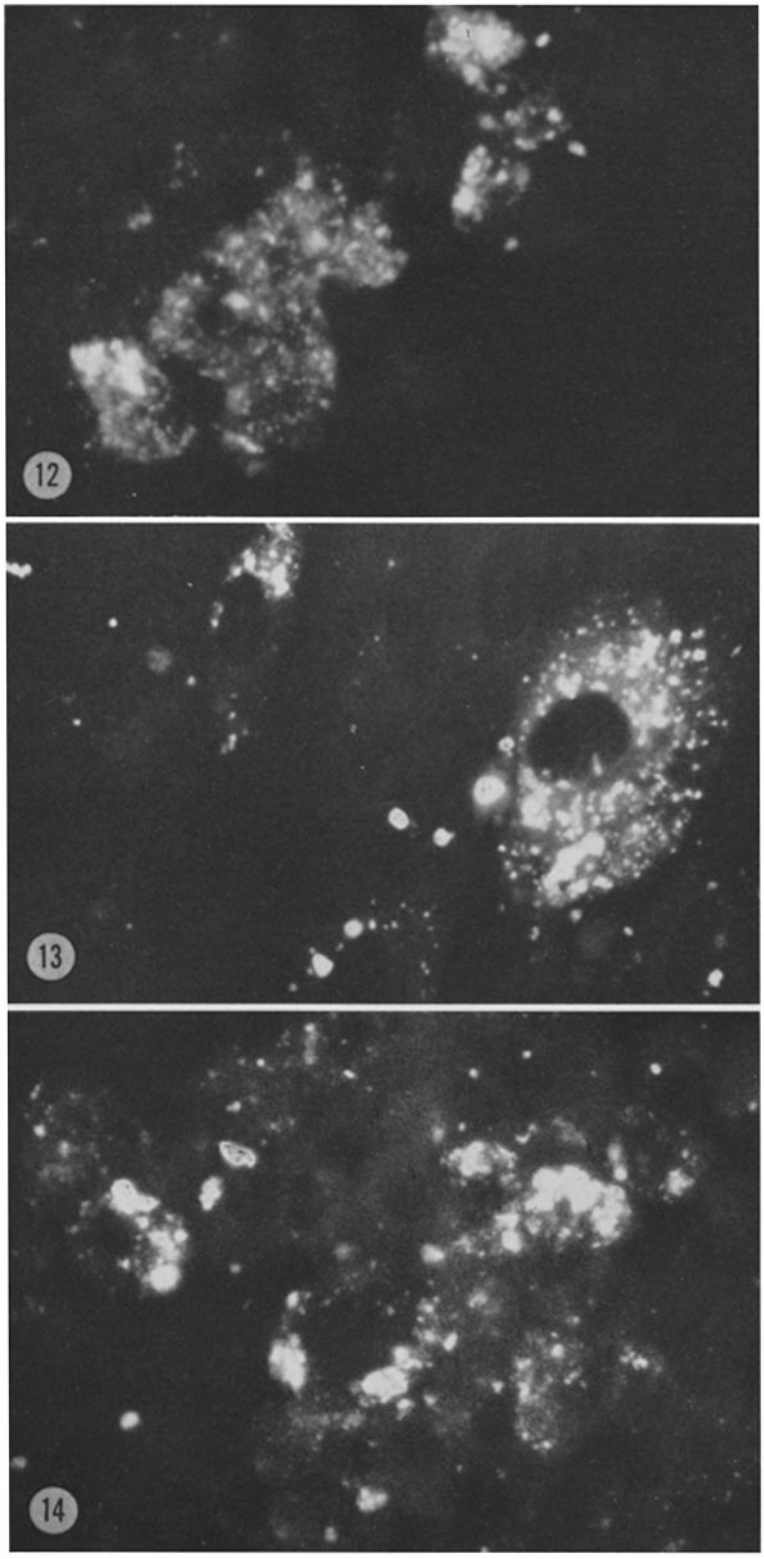
(Osato and Ito: In vitro studies of Vx carcinomas)

PLATE 68

FIG. 12. Fluorescence in the primary in vitro culture of a Vx7 carcinoma cells after 4 wk. Note the intense cytoplasmic and nuclear fluorescence. It appears granular. × 720.

FIG. 13. Fluorescence photomicrograph of a fifth passage culture of Vx7 carcinoma cells. Fluorescent granules vary much in size and are mostly, if not all, in the cytoplasm. × 720.

FIG. 14. Fluorescence photomicrograph of a primary culture of Vx2 carcinoma cells. Several fluorescent cells are shown lying near one another. Fluorescent granules are mostly in the cytoplasm. × 720.



(Osato and Ito: In vitro studies of Vx carcinomas)