

INVESTIGATIVE OPHTHALMOLOGY

In vitro neoplastic transformation of uveal and retinal tissue by oncogenic DNA viruses

Daniel M. Albert,* Alan S. Rabson, and Albert J. Dalton

Explant cultures of adult hamster retina, choroid, and iris were infected with four oncogenic DNA viruses: (1) simian virus 40 (SV40); (2) the LLE46 strain of adenovirus 7; (3) polyoma virus; and (4) human adenovirus 12. Most cultures underwent transformation as evidenced by sustained rapid growth, development of an acid pH soon after instillation of fresh media, and formation of multiple layers of cells and clumps. After several in vitro passages, transformed cells injected subcutaneously into irradiated adult hamsters produced tumors at the injection sites. All ocular tissue transformed with SV40 and produced neoplasms composed of epithelioid and spindle-shaped cells with large nuclei; however, no pigmented tumors were observed. Choroid and iris transformed with LLE46 produced tumors histologically similar to the SV40-type growths. Retina infected with LLE46 produced tumors which were bimorphic, containing, in addition to the SV40-like component, areas of small hyperchromatic cells resembling an adenovirus-induced tumor. Polyoma virus transformed only retina, producing tumors with elongated cells with hyperchromatic pleomorphic nuclei and small amounts of basophilic cytoplasm. Adenovirus 12 also transformed only retina and gave rise to typical adenovirus-type neoplasms composed of layers of small, darkly staining cells. Although these tumors had some resemblance to retinoblastomas, no rosettes or neural elements could be identified.

In recent years viruses have been widely studied as a possible etiology of many types of human cancers. Their role in the development of malignant intraocular tumors has not been investigated. The pres-

ent experiments were designed to evaluate the effects of four oncogenic DNA viruses on hamster retina, choroid, and iris grown in tissue culture.

Such viruses cause changes referred to as "transformation" in a variety of mammalian cells grown in vitro. The cytologic appearance, rate of multiplication, colonial morphology, antigenic characteristics, and chromosome pattern are generally altered in the transformed cultures. When the injection of such transformed cells into an animal of the same species is followed by the development of a malignant tumor at the site of inoculation, the changes in the cells are referred to as "neoplastic transformation."

From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness; Pathological Anatomy Branch and Viral Biology Branch, National Cancer Institute, National Institutes of Health, Public Health Service, United States Department of Health, Education and Welfare, Bethesda, Md. 20014.

Reprints: Ophthalmology Branch, National Institute of Neurological Diseases and Blindness.

*Present address: Ophthalmic Pathology Branch, Armed Forces Institute of Pathology, Washington, D. C.

In the present studies, polyoma, SV40, adenovirus 12, and LLE46 were introduced to retina, choroid, and iris grown in vitro. Following infection, cells in tissue culture were observed for morphologic characteristics of transformation and in SV40-infected lines for the presence of intracellular SV40 "T" antigen. After several passages, cells were injected subcutaneously into adult hamsters and the resulting tumors were studied.

The viruses used in these experiments have been demonstrated to cause neoplastic transformation in a variety of non-ocular tissues. This effect was first observed with polyoma virus of mice in organ cultures of mouse salivary gland in 1959¹ and then with monolayer cultures of baby hamster kidney² and mouse and hamster embryo cells.³ In 1962, SV40, an agent found as a contaminant in rhesus monkey kidney culture, also was noted to induce neoplastic transformation in hamster kidney in vitro.⁴⁻⁶ Similar neoplastic changes have been described in other tissues, including hamster pineal gland⁷ and thyroid gland.⁸

The oncogenic activity of human adenovirus 12 has been clearly established in the hamster^{9, 10} and other species. While most reports concern tumors that have been produced by inoculation of newborn animals with virus, in vitro neoplastic transformation has been demonstrated.¹¹

The LLE46 strain of human adenovirus was also found to cause tumors in hamsters.¹² The subcutaneous tumors produced by this virus contained "T" antigen of SV40 even though no infectious SV40 could be detected in the inocula or in the tumors.¹³ On the basis of this observation and studies of antigens produced in human and monkey kidney cells infected with LLE46 in vitro, it has been postulated that LLE46 preparations contain particles with a portion of the genome of SV40 incorporated within an adenovirus 7 capsid.¹²⁻¹⁴ In vitro transformation with this agent has been described with a number of tissues.^{7, 8, 15, 16}

Materials and methods

Viruses. The strain of polyoma virus used was isolated originally from a cell-free filtrate of an extract of a parotid gland tumor of a C₃H/Bi mouse. The details of the derivation of this strain of virus have been given by Dawe and associates.¹ The polyoma virus preparation in the present studies was grown in P388 D₁ cells maintained in medium No. 199 with 20 per cent fetal bovine serum and contained 10⁶ tissue culture infectious doses (TCID₅₀) per milliliter when titrated by limiting dilution in P388 D₁ cells.

SV40 strain VAC777L was grown and titrated in African green monkey kidney cell cultures maintained in medium No. 199 with 2 per cent fetal bovine serum. The pool we used contained 10^{7.94} TCID₅₀ per milliliter.

Adenovirus 12 (Huie strain) was obtained from the American-Type Culture Collection and passed in cultures of Hep-2 cells maintained in medium No. 199 with 2 per cent fetal bovine serum. The pool that we used titered 10^{8.5} TCID₅₀ per milliliter in primary human embryo kidney cells.

LLE46, the adenovirus 7-SV40 "hybrid," was passed in primary green monkey kidney cell cultures maintained in medium No. 199 with 2 per cent fetal bovine serum. The pool we used in the present experiments contained 10^{8.2} TCID₅₀ per milliliter when titrated in green monkey kidney cells.

Hamster ocular tissues. Eyes were obtained from Syrian hamsters (3 to 4 weeks old). The neural retina, choroid with attached pigment epithelium, and iris were aseptically removed by microscopic dissection.

Culture methods. Retina, choroid, and iris were cut into explants of approximately 1 mm.². Twenty-five pieces of tissue were inserted into a 2 ounce prescription bottle (Brockway Saniglas). Experiments were designed so that 20 cultures were prepared for each of the 3 ocular tissues studied. After 48 hours, 4 bottles of each tissue type were infected with one of the following viruses: SV40, LLE46, polyoma, and adenovirus 12. An additional 4 bottles each of retina, choroid, and iris were kept as virus-free controls. Thus a total of 60 cultures was studied.

Medium No. 199 with 20 per cent fetal bovine serum was used as growth medium; fluid was changed 3 times weekly. All bottles were incubated at 37° C. Thirty days after the cultures were started, they were trypsinized with 0.25 per cent trypsin in Dulbecco's tris buffer free of calcium and magnesium for 30 minutes at 37° C. The cells then were resuspended in growth medium and were passed to other bottles. Transformed cells were subcultured in a similar manner at one- to two-week intervals thereafter.

Injection of cell suspension into animals. Cell suspensions were prepared by scraping confluent

layers from glass bottles with rubber policemen. Three-week-old male hamsters which had received 400 R total body x-radiation 24 hours earlier were injected subcutaneously with 1.0 ml. of cell suspension which contained approximately 1×10^8 cells.

Animals that developed tumors after injection of cells were killed with ether and autopsied. Tissues were fixed in Zenkerformol solution and sections stained with hematoxylin and eosin.

Electron microscopy. Cells in tissue culture and tumors were examined. Tumor material was minced into fine pieces. Cells in tissue culture were scraped from the glass and formed into a pellet by centrifugation at 748 G for five minutes. Fixation of tumors and pellet was carried out in 1.5 per cent glutaraldehyde at 4° C. for one hour. The material was then postfixed in Dalton's chrome-osmium fixative.¹⁷ Tissue was dehydrated in graded ethyl alcohol and embedded in Epon-Araldite mixture.¹⁸ Ultra-thin sections were cut with an LKB microtome and double-stained in uranyl acetate¹⁹ followed by lead citrate.²⁰

Micrographs were taken with a Siemens Elmiskop I electron microscope using an 80 kv. accelerating voltage and a 50 U. objective aperture. They were optically enlarged for the final desired size.

Results

Within 24 hours after tissue was put into culture, cuboidal and elongated cells had migrated from the edges of some explants of choroid and iris. The earliest outgrowth of retinal cells occurred about 72 hours after the cultures were started; the cells were small and round.

Cultures infected with SV40 and LLE46 showed no cytopathic effect after virus was added. Polyoma virus caused severe cell damage while adenovirus 12 caused only limited cytotoxic effect, characterized by an adenovirus type of cell clumping and rounding.

Control cultures and those receiving SV40 and LLE46 initially showed vigorous growth and rapid acidification of freshly fed media. With successive passages control cultures grew poorly and contained decreasing numbers of viable cells; no control cultures transformed spontaneously. SV40 and LLE46 transformed cells continued to undergo active multiplication with formation of clumps and sheets of cells. Polyoma- and adenovirus 12-infected

tissue also grew well after recovering from the cytopathic effects.

The predominant cell type in cultures of retina, choroid, and iris infected with SV40 was a long slender cell with indistinct borders which grew in a whorllike pattern. Multinucleated giant cells were common. Pellets from successive passages of choroid and iris infected with SV40 were increasingly less pigmented. Electron microscope examination was carried out on the original explants (Fig. 1) and on cells harvested sequentially at one week intervals following SV40 infection (Fig. 2). A rapid decrease in the number of premelanosomes and melanosomes present in melanocytes and pigment epithelium cells was observed as *in vitro* growth progressed. By six weeks after infection (and following one trypsinization) no evidence of premelanosomes and melanosomes was found, although compound melanin granules were plentiful.

As in previous studies, SV40 virus was not observed in electron microscopic examination of the cells. Immunofluorescent studies done on SV40-transformed cells demonstrated SV40 "T" antigen in more than 90 per cent of cells.

All cultures transformed by LLE46 virus showed morphologic and growth characteristics similar to SV40-infected tissue, as did polyoma-transformed cells. Adenovirus 12 cultures contained sheets of small round cells.

All transformed cultures had been subcultured to 32 ounce bottles 40 to 60 days after infection. The cells in each bottle (approximately 1×10^8) were scraped and suspended in 1 ml. of No. 199 culture medium and injected subcutaneously into an irradiated hamster. Those animals in which tumors were produced had palpable subcutaneous nodules 6 to 12 weeks later.

All animals injected with SV40-transformed cells developed neoplasms. On gross examination these were firm and white with small areas of central necrosis. Microscopically the basic cell types were spindle and epithelioid. Both had either

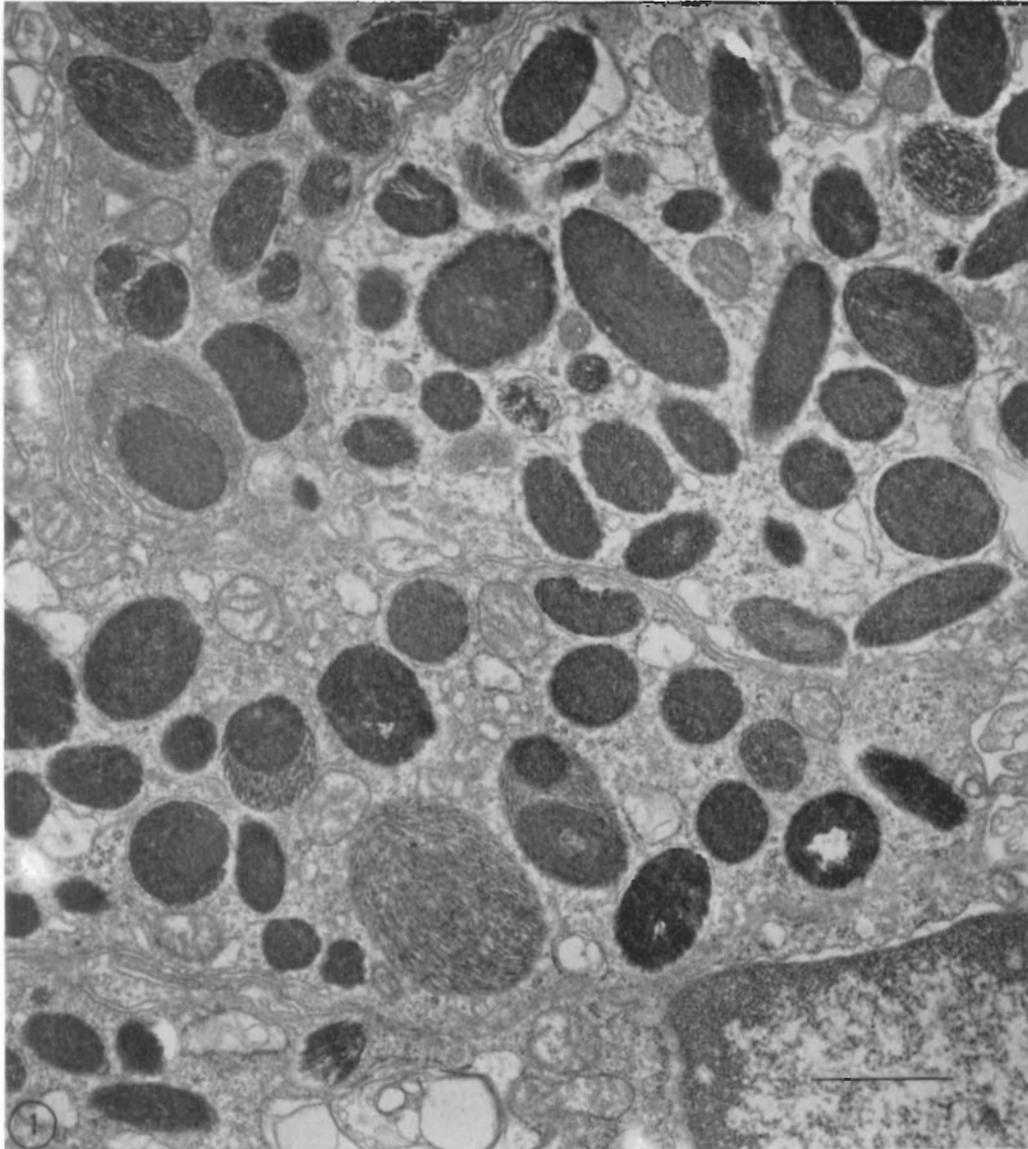


Fig. 1. Parts of several typical stromal melanocytes from iris explant used for tissue culture. Premelanosomes and melanosomes are visible in various stages of development. (Horizontal line represents $1\ \mu$; approximately $\times 21,000$.)

round or elongated vesicular nuclei, clumped chromatin, and light-staining, tapering, eosinophilic cytoplasm (Fig. 3). Mitoses were numerous. Many giant cells were present; these were round and often multinucleate (Fig. 4). In some sections of tumors derived from iris and choroid, epithelioid cells were arranged in an alveolar pattern. The histologic appearance of SV40 tumors derived from retina, iris, and choroid was very similar.

Tumors arose in all animals injected with LLE46-transformed cells. Those neoplasms obtained from iris and choroid infected with LLE46 were identical in both gross and microscopic appearance to SV40 tumors. Tumors produced by LLE46-transformed retina were soft and white with areas of hemorrhagic necrosis. Microscopically they were composed of two distinct histological types (Fig. 5): one characterized by epithelioid and spindle-shaped

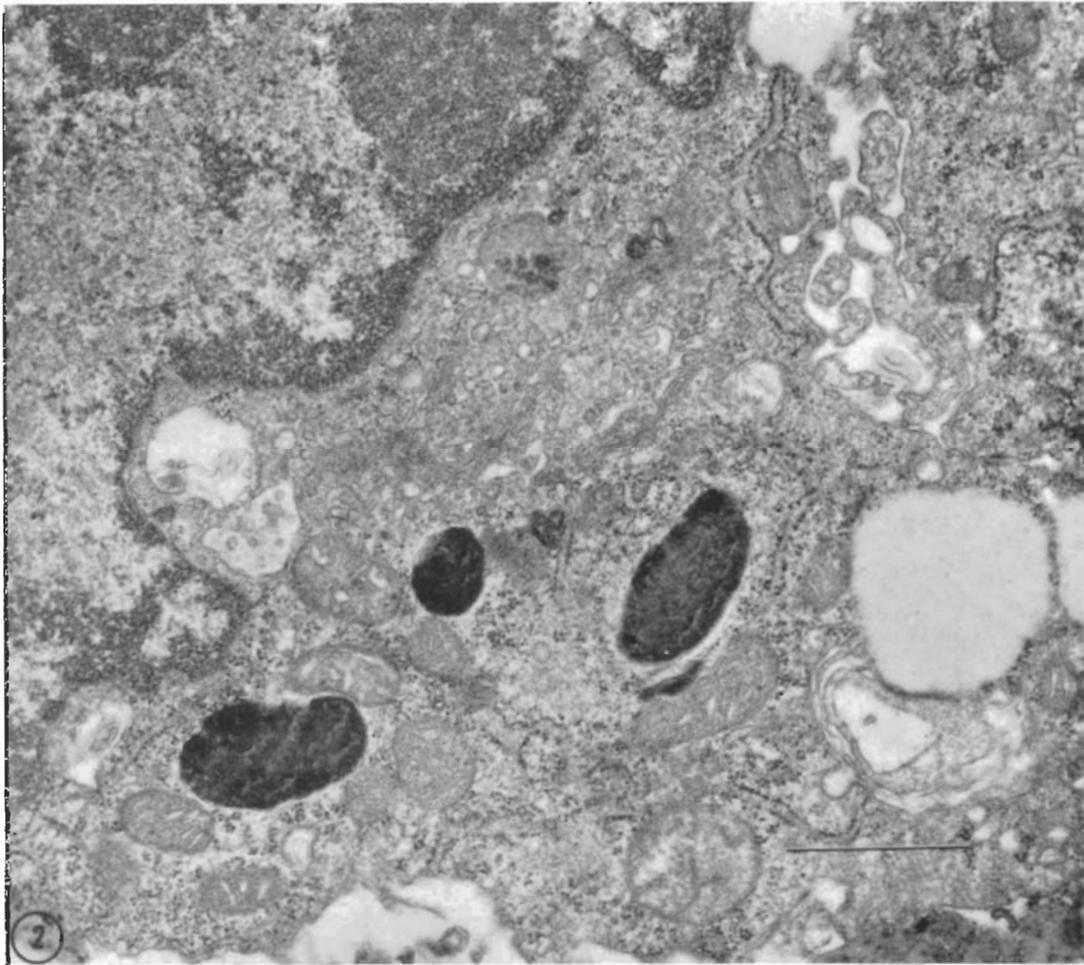


Fig. 2. Part of cell taken from SV40-iris culture 33 days after virus was added. Culture had undergone one passage in vitro. Fewer melanosomes are present than in original explant. (Horizontal line represents 1 μ ; approximately $\times 24,500$.)

cells with vesicular nuclei that resembled SV40-induced tumors, the other component composed of very small, closely packed cells with darkly stained nuclei similar in appearance to the adenovirus 12-induced tumors.

Three of the four retinal cultures infected with adenovirus 12 underwent neoplastic transformation and produced tumors when injected into hamsters. These were soft, white growths with a large central area of necrosis and hemorrhage. Microscopically they contained densely packed masses of small, uniform cells; the nuclei were rounded or elliptical with clumped chromatin (Fig. 6). No tumors

were produced by cells from choroid and iris cultures morphologically transformed with adenovirus 12.

A tumor developed in only one of 12 animals injected with a polyoma-transformed cell line; this was derived from retina. The basic pattern was of a uniform spindle-cell tumor. The cytoplasm was eosinophilic and boundaries were sometimes indistinct. Nuclei were elongated, mitoses were numerous, and many giant cells were seen.

Portions of the initial tumors described above were injected subcutaneously into other hamsters and transplantable tumor lines were easily established. Neoplasms

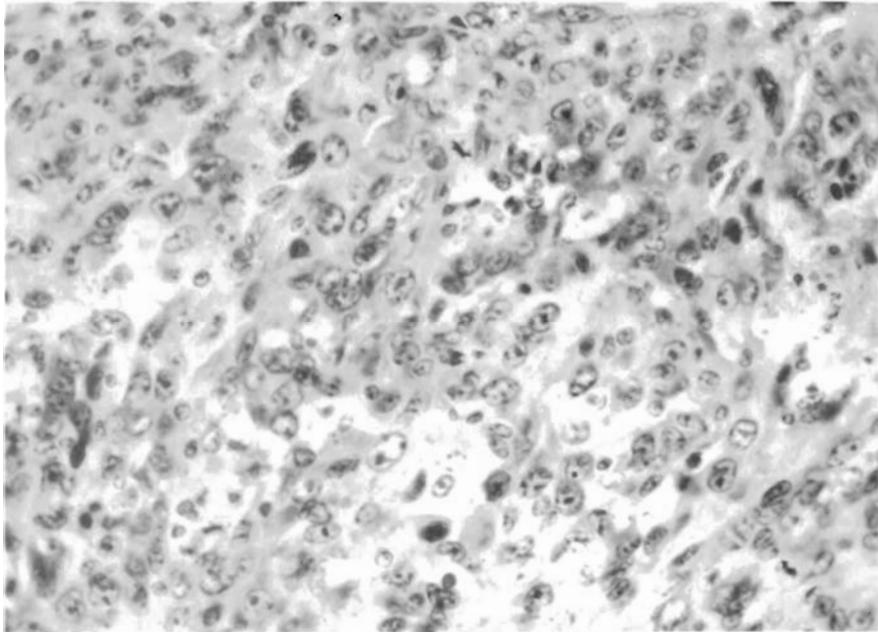


Fig. 3. Tumor produced in irradiated adult hamster by subcutaneous injection of transformed iris cells. The tumor is composed of elongated and epithelioid cells with light, tapering, eosinophilic cytoplasm. (H and E, $\times 400$.)

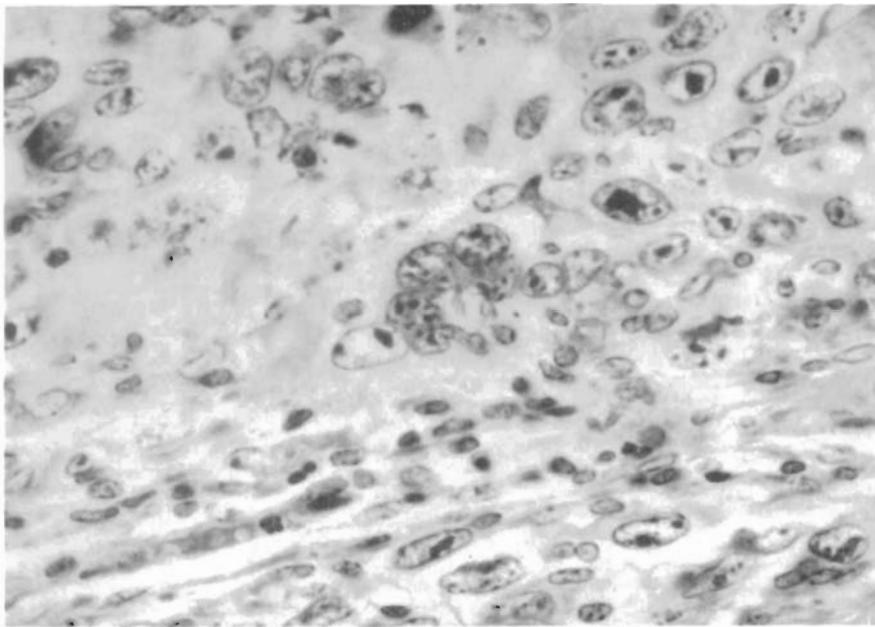


Fig. 4. Another portion of tumor in Fig. 3. In the center of the field is a round multinucleated SV40 giant cell. (H and E, $\times 750$.)

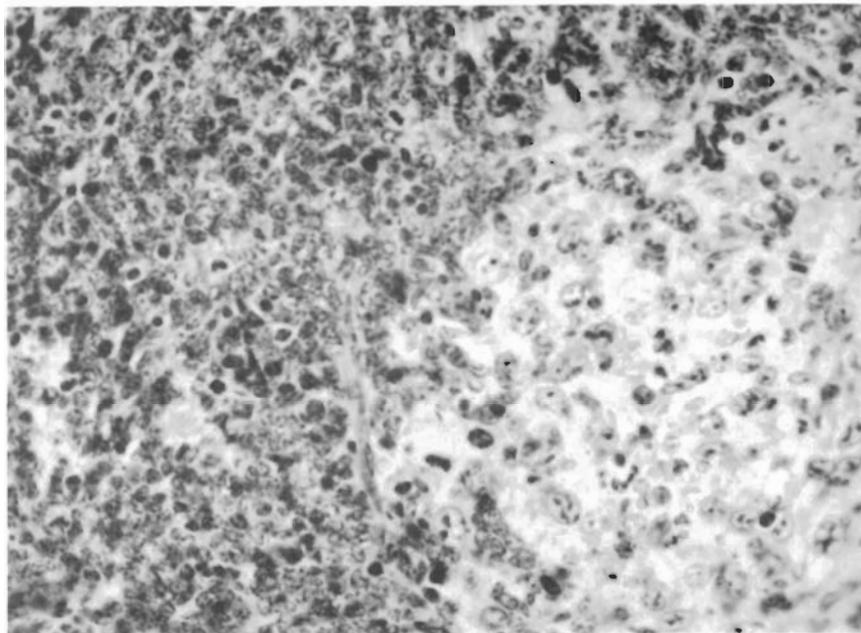


Fig. 5. Tumor produced in irradiated adult hamster by the subcutaneous injection of LLE46-transformed retinal cells. The tumor is composed of two distinct histological elements, one composed of small hyperchromatic cells (left) resembling an adenovirus-type tumor. The other component resembles an SV40-induced tumor. (H and E, $\times 400$.)

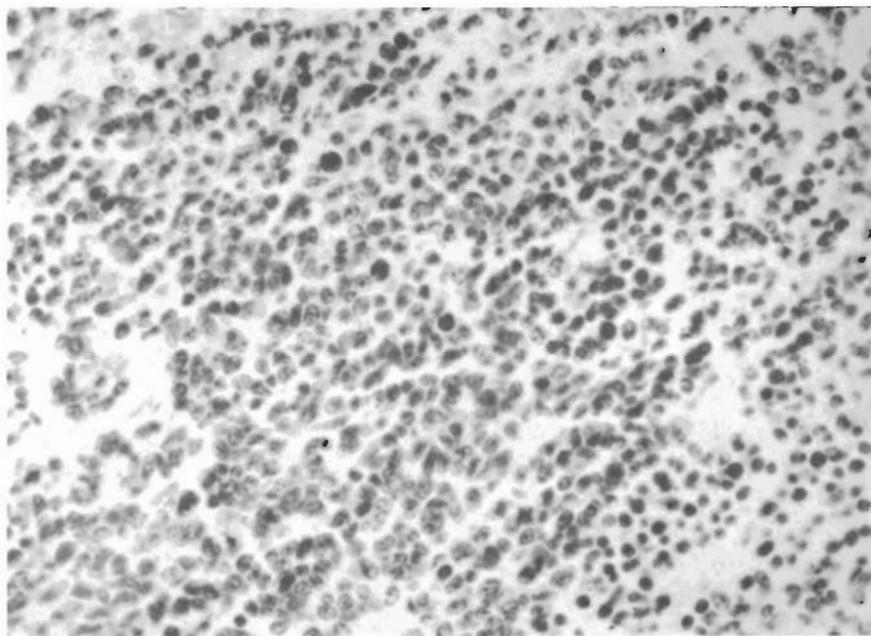


Fig. 6. Area of a tumor resulting from the subcutaneous injection of retina transformed with adenovirus 12 into an irradiated adult hamster. This section shows the typical adenovirus-type morphology. (H and E, $\times 400$.)

of each cell and virus type were transplanted through at least six generations. Alterations in morphology did not occur with succeeding generations. In no instance was there spontaneous regression of the tumors. Tumor-bearing animals which were not sacrificed died 3 to 6 weeks after nodules were first observed as a result of tumor invasion and metastasis.

Discussion

Experimental ocular tumors have been produced with carcinogenic agents. Systemic administration of certain of these substances to rats resulted in neoplasms of the eyelids²¹ and iris.^{22, 23} A combination of carcinogens and ultraviolet light produced corneal tumors in rats.²⁴ Direct introduction of methylcholanthrene into the eyes of rats was followed by malignancies of the Harderian gland, orbit, conjunctiva, iris, and choroid.²⁵ Rainbow trout maintained on a thioacetamide diet showed invasive proliferation of the lens epithelium having the characteristics of neoplastic growth.²⁶ The experiments described here are, we believe, the first report of neoplastic transformation of ocular tissue by viruses.

It is known that an oncogenic virus may produce tumors with similar morphology in many different tissues. In some cases the morphology of the tumor may actually be determined by the virus.²⁷ Small cell sarcomas are characteristic of adenovirus transformation and spindle-cell tumors containing giant cells commonly are produced by papovaviruses such as SV40. The close resemblance of neoplasms occurring from retina, choroid, and iris following infection with SV40 was therefore not unexpected.

LLE46 tumors derived from choroid and iris had no adenovirus type of component. In our experiments these tissues did not undergo neoplastic transformation with adenovirus 12. Retina infected with LLE46, in contrast, produced a bimorphic tumor containing small, round hyperchromatic cells, and in three of four experiments underwent neoplastic transformation with adenovirus 12. These findings suggest that in the hamster the neural retina may be more susceptible to transformation by oncogenic adenoviruses than iris, choroid, or pigment epithelium.

In spite of a common histopathologic appearance in tumors derived from different cell types after viral transformation, certain biochemical characteristics of the original tissue may persist. Neoplasms produced from hamster pineal gland contained hydroxyindole-O-methyl transferase, an enzyme found exclusively in pineal cells.⁷ Hamster prostate transformed with SV40 produced tartrate-inhibited acid phosphatase, a type of acid phosphatase associated with prostatic carcinoma in man.²⁸

It would be of interest to have a comparable marker to follow in ocular tissues. A striking feature of the hamster eye is the dense pigmentation of the iris, choroid, and retinal pigment epithelium. On electron microscopic examination of these layers, melanocytes and pigment epithelium cells contained numerous premelanosomes and melanosomes. When transformed *in vitro*, however, these structures rapidly disappeared and were not present in subsequent tumors. The point of interruption in the metabolic pathway of tyrosine to melanin was not determined.

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