On the Oncogenic Properties of Chicken Embryo Lethal Orphan Virus, an Avian Adenovirus

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SUMMARY

The oncogenicity of chicken embryo lethal orphan virus for newborn hamsters has been confirmed. Tumor incidence was found to be a function of the titer of the virus inoculum. Fifty-four % (7/13) of the hamsters given 10^3.3 50% lethal dose developed tumors, while only 8% (8/99) of the hamsters given 10^0.0 50% lethal dose developed tumors. With a single exception, lower virus doses were nononcogenic; one tumor developed among 26 hamsters given virus inactivated from 10^4.0 to 10^0.0 50% lethal dose by nitrous acid. Although all the tumors were of mesenchymal origin a variety of sarcomatous types were noted. The latent period varied from 14 to 52 weeks and all but one tumor appeared at the site of injection. Female hamsters appeared more susceptible than males. Indirect immunofluorescence and complement-fixation tests failed to demonstrate antibody in the sera of tumor-bearing hamsters to autologous tumor extracts or to chicken embryo lethal orphan virus-infected chick and hamster embryo cells.

INTRODUCTION

CELO virus was isolated from eggs of apparently normal chickens by Yates et al. in 1954 (25). A viral agent had been suspected when chicken embryos died without apparent cause. The virus cross-reacted with an agent previously found by Olson (12) in bobwhite quail with respiratory disease and was thought to be the same or a closely related virus. Yates et al. (22) found that CELO virus was regularly fatal for embryos within 3 to 5 days following inoculation, although when the eggs were infected prior to incubation there were no ill effects unless high titers of virus were injected. A mild respiratory disease was induced in young chicks, but adult chickens showed no clinical evidence of infection (23).

Dutta and Pomeroy (6) examined CELO virus by electron microscopy and found it morphologically identical with the adenovirus groups. Several strains of adenovirus identical with or closely related to CELO have been isolated from avian sources in Germany and Japan (4, 8).

In 1965, Sarma et al. (17) reported that 23 of 69 hamsters injected at birth with high-titer CELO virus, Phelps strain, developed well-differentiated fibrosarcomas within 88 to 195 days. These workers were not able to demonstrate a soluble T antigen in the CELO tumors by complement fixation tests with sera from tumor bearing hamsters. Similarly, they were unable to obtain reactions with sera from hamsters with tumors induced by avian leukemia and sarcoma agents, human adenovirus types 7, 12, and 18, SV40, and polyoma virus.

Since then, 2 reports (1, 16) of a specific CELO virus-induced T antigen have appeared. In the first, by Anderson et al. (1), hamster embryo cells were transformed in vitro by CELO virus. Hamsters were immunized with tumorogenic doses of the transformed cells grown in vitro. The sera of these hamsters contained antibody reactive with the transformed cells in immunofluorescent tests. Similarly, Potter and Oxford (16) grew CELO tumor cells in vitro and then induced transplantable tumors in weanling hamsters with the 51st in vitro passage. Sera from these hamsters contained CF antibody specific for an antigen in the transplantable CELO tumor cells. By immunofluorescence the antigen was reported to be intranuclear and to resemble the granular appearance of SV40 T antigen. The CELO T antigen was not present in early in vitro and in vivo tumor cell passages. These results indicate that phenotypic expression of CELO virus T antigen may require long-term cultivation in a favorable environment, such as in vitro. Such a requirement might explain the inability to demonstrate T antigen in primary CELO tumors and T antibody in the sera of hamsters bearing primary CELO tumors.

The present studies were undertaken initially to search for CELO T antibody in the sera of hamsters bearing primary tumors by the sensitive indirect immunofluorescent test (10). The experiments were designed to provide information on the relationship of virus dose to tumor incidence and the relationship of the sex of the animal and tumor incidence.

MATERIALS AND METHODS

Preparation of Virus

Stock Virus. CELO virus was received through the kindness
of Dr. V. J. Yates and Dr. Padma S. Sarma. The Phelps strain, obtained from Dr. Sarma, was used for these studies.

The strain used was found by Sarma et al. (17) to be free of avian leukemia viral contaminants. Virus from the 8th embryo passage was maintained in specific pathogen-free embryonated eggs (SPAFAS, Inc., Norwich, Conn.). Fertile eggs were inoculated on the 8th day of incubation with allantoic fluid containing 10^6 to 10^7 LD_50 in a volume of 0.1 ml. Allantoic-amnionic fluid was harvested after 48 hr, centrifuged at 800 X g for 15 min, pooled, and stored at —80° until needed. Each virus pool was titrated in 9- or 10-day-old embryonated eggs, with death of the embryo as the end point. Virus titer was calculated by the Reed-Muench method (9) and expressed as the LD_50/0.1 ml. Aliquots of the virus pools were centrifuged for 16 hr at 107,000 X g, and the pellets were resuspended in distilled water and examined by electron microscopy with negative staining techniques.

**Partially Purified Virus.** Pooled frozen stock virus in allantoic-amnionic fluid was thawed at 37°, diluted with an equal part of PBS, and centrifuged for 16 hr at 75,000 X g. The supernatants were discarded and the pellets were resuspended in PBS to 1/2 the original volume and sonically dispersed for 10 sec with the Branson Model 8125 sonifier. The pooled, partially purified virus suspension was stored in ampuls at —80°.

**Nitrous Acid Inactivation.** Partially purified virus was treated with nitrous acid by the method of Benjamin (2). Equal volumes 2 M NaNO_2, 1 M acetate buffer at pH 4.5, and lightly sonically extracted virus suspension were allowed to react at room temperature for 10 min while being mixed with a magnetic stirrer. The reaction was stopped by the addition of cold 5 M Tris buffer at pH 8.5. The mixture was then dialyzed overnight at 4° in small dialysis tubing against large volumes of 0.9% NaCl solution-citrate buffer to remove excess nitrous acid. The treated virus suspension was stored in ampuls at —80° for later titration and use.

**UV Inactivation.** Partially purified virus suspension was thawed at room temperature, lightly sonically extracted (10 sec), and mixed thoroughly. One ml was spread evenly over lamps at a distance of 5 cm for 1 or 3 min. The UV-treated virus suspension was then placed in ice and titrated the same day.

**Hamster Inoculations.**

Golden Syrian hamsters were given injections s.c. of 1 ml virus on the dorsum within 24 hr of birth. The animals were separated by sex at weaning and were examined semiweekly for palpable tumors. Each hamster that developed a palpable tumor was separated from its littermates and was observed until the tumor reached a size adequate for the necessary studies. Each tumor-bearing animal was bled by intracardiac puncture on the day of sacrifice and autopsied.

**Preparation of Tumors.** The tumors were sectioned and aliquots were taken for hematoxylin and eosin staining and for fixation in osmium tetroxide for electron microscopy; the remainder was stored at —80° for later use in immunological studies.

**Antigen Preparation.** Tumor to be used in complement fixation tests was trimmed of fat and necrotic tissue, washed with PBS, and homogenized in a Sorval Omnimixer with an equal volume of Eagle's balanced salt solution buffered at pH 7.2. The homogenate was centrifuged at 2000 X g for 15 min, resuspended in additional diluent and centrifuged; the combined supernatant fractions were pooled and stored at —80°.

**Tests for Antitumor Antibody.**

**Infected Hamster Cells.** Blood from tumor-bearing hamsters was allowed to clot and was centrifuged at 800 X g; the serum was removed and stored at —80°. Sera from un inoculated hamsters were collected similarly. Indirect immunofluorescence was used with pooled serum from tumor-bearing animals, hamster embryo fibroblasts infected with CELO virus, and monkey antihamster Ig-globulin. Hamster embryo fibroblast monolayers were grown on glass coverslips and infected with CELO virus at multiplicities of infection of 10, 100, and 1000 LD_50/cell. After an incubation period of 8 or 24 hr at 37°, cytosine arabinoside (15 μg/ml) was added and incubation was continued an additional 16 or 24 hr. The monolayers were then fixed with 1% buffered formalin and cold acetone (26). The coverslips were overlaid with serum from tumors bearing hamsters (primary reagent), rinsed, and stained with monkey antihamster Ig-globulin previously conjugated with fluorescein isothiocyanate (secondary reagent). Control slides were prepared with sera from nontumor-bearing hamsters or unconjugated secondary reagent prior to staining with conjugated secondary reagent.

**Infected Chick Embryo Cells.** Chick embryo fibroblast monolayers were infected with CELO virus at a multiplicity of infection of 100 LD_50/cell. The cultures were maintained in minimum essential medium with 10% decomplemented fetal calf serum. Twenty hr after infection, cytosine arabinoside was added to give a final concentration of 15 μg/ml and incubation was continued for another 48 hr. The monolayers were then fixed as described above and indirect immunofluorescence was done with pooled tumor-bearing hamster serum as the primary reagent and fluorescein-conjugated goat antihamster Ig-globulin as the secondary reagent.

**CELO-induced Tumor Slices.** Two tumors were examined by slicing frozen tumor segments 5 μ thick. The slices were then fixed and stained as described for infected chick embryo cells.

**Complement Fixation Tests.** The micro complement fixation test was used as described in the manual from the Laboratory Branch, USPHS, Communicable Disease Center, Atlanta, Ga. (20). NaCl solution (0.9%) tumor extracts (antigen) were used at the next highest dilution above that concentration which failed to fix complement in the absence of antibody. Tumor extracts were not pooled; each was used as a separate antigen. Pooled and individual sera from normal and tumor-bearing hamsters were tested. Complement was used at a concentration of four 50% hemolytic units.
RESULTS

**CELO Virus.** Electron microscopic examination of pelletized virus from the same stock used for inoculation of hamsters contained numerous adenovirus particles compatible with the morphology of CELO virus; no other type of virus particles was noted. The virus stock was known not to contain COFAL antigen as tested by Sarma et al. (17).

**CELO-induced Tumors.** Tumors developed in 8 of 99 hamsters given injections at birth of $10^{9.0}$ LD$_{50}$ and in 7 of 13 given injections of $10^{9.3}$ LD$_{50}$. Twelve tumors grew progressively, while 3 other palpable tumors regressed within 3 to 25 days.

Only 1 tumor appeared in the 26 hamsters injected with virus partially inactivated with nitrous acid (titers, $10^{4.0}$ and $10^{6.5}$ LD$_{50}$), while none developed in the 40 hamsters injected with UV-treated virus (titers, $10^{4.2}$ and $10^{6.5}$ LD$_{50}$).

Tumor incidences are summarized in Table 1. The time from inoculation to tumor palpability varied from 14 to 52 weeks. Thirteen of the total of 16 tumors occurred in female hamsters, although an approximately equal number of each sex had been injected.

Histological tumor types (Table 2) included 1 each of fibrosarcoma, leiomyosarcoma, osteogenic sarcoma (Fig. 1), and reticulum cell sarcoma and 7 undifferentiated sarcomas. In the animal with reticulum cell sarcoma, tumor masses were in the liver and in mediastinal and cervical lymph nodes, but there were none at the site of inoculation (Figs. 2 and 3). All other tumors occurred at the site of inoculation. The fibrosarcoma, which appeared subcutaneously 365 days after inoculation, metastasized to the lung and the peritoneum.

**T Antigen and T Antibody.** Attempts to demonstrate antigen (T) in the tumor homogenates by complement fixation tests were unsuccessful (Table 2). Variations in serum dilutions and antigen concentration also failed to give positive findings with the complement fixation tests.

Similarly, indirect immunofluorescent tests on CELO-infected chick cells with sera from the tumor-bearing hamsters were negative. Variations of virus dosages, length of incubation before and after the addition of cytosine arabinoside, and variation in dilutions of primary and secondary reagents failed to yield positive results by immunofluorescence. Attempts to demonstrate T antigen in CELO-infected hamster cells treated with cytosine arabinoside and in sections of the 2 tumors tested were also unsuccessful.

In other studies in this laboratory, adenovirus 12 T antigen was clearly demonstrated in adenovirus 21 tumors cell with the same techniques and reagents (except that the test serum was taken from hamsters with adenovirus 12-induced tumors).

No infectious virus could be recovered from any of the 8 CELO-induced tumors so tested.

Four tumor extracts kindly tested in COFAL tests by Dr. P. S. Sarma with pigeon antisera to Schmidt-Ruppin RSV were negative. None of the tumor extracts were reactive in CF tests with autologous sera, a further indication of the lack of COFAL antigens in the tumors.

**DISCUSSION**

In recent years it has been accepted that a virus is

<table>
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<tr>
<th>Tumor type</th>
<th>Tumor latent period (wk)</th>
<th>No. of animals with tumor</th>
<th>Sex</th>
<th>Complement fixation</th>
<th>Immunofluorescence</th>
<th>CELO T antigen in tumor</th>
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<tr>
<td>Fibrosarcoma</td>
<td>14</td>
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<td>NT</td>
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<td>F</td>
<td>0</td>
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<td>NT</td>
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<tr>
<td>Reticulum sarcoma</td>
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<td>1</td>
<td>F</td>
<td>0</td>
<td>0</td>
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<td>Myxosarcoma</td>
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<td>Mean, 17.5</td>
<td>4</td>
<td>F</td>
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<td>NT</td>
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<td>Range, 11–27</td>
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</table>

$^a$NT, not tested.

$^b$Three tumors regressed; 1 animal escaped. No biopsies were obtained.
The Oncogenic Properties of CELO Virus

oncogenic in the absence of virus isolation from the induced tumor if the tumor is shown to contain neoantigens specifically induced by the virus (7, 15). Generally, the tumor-bearing host produces circulating antibodies to these antigens which are utilized in the serodiagnosis. However, with CELO virus-induced tumors, Sarma et al. (17) were unable to demonstrate specific CF tumor antibody in the sera of tumor-bearing hamsters. We have confirmed this observation and have also shown that sera from CELO-induced tumor-bearing hamsters are not reactive in indirect immunofluorescence with chick embryo fibroblasts infected with CELO virus in vitro, as well as with CELO-infected hamster embryo cells. Occasionally, parallel findings have been reported with other oncogenic DNA viruses. For example, Petursson et al. (14) were not able to demonstrate T-antigen in hamster embryonic kidney cells transformed by adenovirus 12 or in tumors induced by implantation of the transformed cells into adult and weanling hamsters. Diamandopoulos and Enders (5) previously had reported a lack of T antigen in 11 hamster liver and lung cell lines derived from SV40-transformed cultures. Black and Rowe (3) found SV40 T antigen in only 3 of 22 cell lines from SV40-DNA-treated hamster fibroblasts, although the cell lines which did not contain T antigen produced tumors when transplanted to hamsters as readily as did cells which contained T antigen.

In the present study, the primary evidence that CELO virus is oncogenic in hamsters was the dose dependency of tumor incidence. The greatest tumor incidence (54%) was obtained in 13 hamsters given injections of \(10^{9.3} \text{LD}_{50}\). A lower dose, \(10^{9.0} \text{LD}_{50}\) yielded only 8 tumors in 99 hamsters. Virus reduced in titer from \(10^{9.3}\) to \(10^{4.6} \text{LD}_{50}\) by nitrous acid treatment yielded a single tumor in 26 hamsters (4.0%), while virus reduced in titer from \(10^{4.5} \text{LD}_{50}\) or more from \(10^{9.0}\) by UV irradiation was not oncogenic in 40 hamsters.

Other indirect evidence of CELO oncogenicity was by exclusion of the participation of other viruses. The original virus stock used in these studies was known to be free of avian leukemia virus. All inocula were prepared in SPFAS eggs. Electron microscopy with negative staining techniques showed heavy concentration of adenovirus and no virions of other morphological types. Sarma et al. (17) had previously found the sera of CELO tumor-bearing hamsters to be free of COFAL antibody. Similar results have been reported by Anderson et al. (1) and Potter and Oxford (16). The complete lack of serological reactions with autologous sera and tumor extract in the present study also rules out the presence of COFAL antigen.

It has thus been demonstrated in 4 different laboratories (1, 16, 17), including ours, that hamsters bearing CELO virus-induced tumors do not produce demonstrable humoral antibodies reactive with their autologous tumor. This appears to reflect the absence of T antigen in the primary tumor cells. As shown by Anderson et al. (1) and Potter and Oxford (16), CELO T antigen is expressed by CELO-transformed hamster cells (1) and by CELO-induced hamster tumor cells (16) after multiple serial passages in vitro. Apparently, in vitro passage may permit expression of a viral coded function repressed during cellular replication in vivo. The implications of these findings to studies on the viral etiology of human neoplasia are evident.

No differences in tumor incidences between sexes were reported in previous studies with CELO virus. In our study, 12 of the 16 tumors occurred in females, although the ratio of females to males given injections of virus was 1:1. The mechanism for the sex differences is believed to involve estrogenic enhancement of adenovirus oncogenesis (27).

A striking finding in the present study is the variability of histological appearance of the tumors. Most tumors induced by adenoviruses in hamsters have been described as undifferentiated sarcomas, or fibrosarcomas, although malignant lymphomas have been reported (19). Although our tumors all appeared to be mesenchymal in origin, there were several specific sarcomatous types noted. One tumor contained elements of bone formation and was called an osteogenic sarcoma (Fig. 1). Other tumor types included myxosarcoma, leiomyosarcoma, and fibrosarcoma (fibroxanthoma type). One unusual tumor, which appeared 17 weeks after injections, was described as a reticulum cell sarcoma; this tumor did not appear at the site of inoculation, but involved the liver in multiple discrete nodules, as well as lymph nodes in the neck and mediastinum (Figs. 2 and 3). No other tumors with this histological appearance occurred. It is possible that this tumor may not have been induced by CELO virus, since it did not appear at the site of inoculation; however, dissemination of the inoculum followed by induction of a metastasizing tumor cannot be excluded. Metastasis to the lung and peritoneum occurred with the fibrosarcoma.

ACKNOWLEDGMENTS

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REFERENCES

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Fig. 1. Extraosseous osteogenic sarcoma. The tumor was s.c. at the site of injection.

Fig. 2. Reticulum cell sarcoma with multiple hepatic nodules. There was tumor in cervical and mediastinal lymph nodes, but none at the site of injection.

Fig. 3. Reticulum cell sarcoma in a cervical lymph node.
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