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INDUCTION BY ADENOVIRUS TYPE 7 OF TUMORS IN HAMSTERS HAVING THE ANTIGENIC CHARACTERISTICS OF SV40 VIRUS

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Tumors having the virus-specific antigenic characteristics of those produced by SV40 virus[1-5] developed in 27 of 36 hamsters injected as newborns with adenovirus type 7, strain L. L., a strain isolated and grown continuously in monkey kidney tissue cultures. The antigenic character of these tumors was particularly interesting because the 28th passage inoculum which produced them contained no detectable SV40 virus, the latter having been eliminated from the L. L. strain at 23rd passage with the use of hyperimmune SV40 monkey serum.

The SV40 tumor antigens were demonstrated in the complement fixation (CF) test with the use of serums from tumorous hamsters which contained virus-specific antibodies to SV40 tumor[2] and to similar cell-associated antigens found in cells infected with SV40 virus;[6, 7] conversely, CF antibodies to SV40 tumor and the cell-associated antigens were demonstrated in the serums of hamsters carrying the L. L.-induced tumors.

Shortly after these findings were available, Rowe and Baum, and Rapp *et al.* (see accompanying papers in this issue of these PROCEEDINGS[8, 9]), using fluorescent antibody techniques, demonstrated similar virus-specific antigens in various tissue cultures infected with adenovirus 7, strain L. L.

More recent tests showed that although the primary tumors induced by the L. L. strain revealed no direct evidence of adenovirus type 7 tumor antigen, one hamster carrying a very large tumor developed complement-fixing antibodies to antigens in tumors induced by the Pinckney strain of adenovirus type 7.[10] Additional evidence for type 7 tumor antigens was also found in transplanted L. L. tumors passaged serially in newborn hamsters.

Materials and Methods.—Adenovirus 7, strain L. L.: This strain was obtained by inoculating rhesus kidney tissue cultures with throat washings[11] taken in 1955 at Fort Ord from a military recruit (L. L.) having a diagnosis of acute respiratory disease (ARD). For the next 5 years the L. L. strain was used in commercially produced adenovirus vaccines;[12] during this time the virus was carried through 22 serial passages in primary rhesus kidney cells. SV40 virus was demonstrated in the 22nd passage,[13] and retrospectively also in tissue culture fluids derived from the ninth serial passage.[14] In 1961, the 23rd and 24th passages, grown in green monkey kidney (GMK) tissue cultures, were treated with rabbit serum having antibodies to SV40 virus. The procedure was as follows: to 9 ml of $^1/_{10}$ dilution of 22nd passage virus was added 9 ml of a serum mixture, containing a $^1/_{30}$ dilution of the anti-SV40 serum and a $^1/_3$ dilution each of monkey "normal" and antifoamy virus serums. The serum-virus mixture was incubated at 37°C for 3 hr, followed by 18 hr at 4°C, and 0.2-ml aliquots of the mixture were inoculated per tube culture of primary GMK tissue culture cells. After 18 hr incubation at 36°C, the supernatant fluids were removed and the cultures refed with maintenance medium.

Subsequent passages were in GMK cultures, and all the seed viruses used for passages 25–29 were found to be free of infectious SV40 virus, except for 27th passage which was not tested.[13]

Tests for infectious SV40 virus: A large virus pool labeled E46 was accumulated in 28th passage. This passage, which induced the tumors in hamsters described herein, was found by three different laboratory units[13–15] to be negative for SV40 virus in GMK tissue cultures. Hyperimmune rabbit antiserums to the prototype strain (Gomen) of adenovirus 7 were used to neutralize the type 7 adenovirus in tests for SV40 virus; these serums were shown to be devoid of antibodies to SV40 virus.[14]

A large pool of L. L. virus (29th passage) derived from E46, designated as lot #2, was also exhaustively tested for virus in GMK tissue cultures. A total of 192 ml of this large lot was tested and found negative for SV40 virus;[13] the original cultures and subcultures were observed for a total of 42 days. In addition, the L. L. E46 stock was tested for its ability to produce antibodies in susceptible monkeys; neutralizing antibodies to SV40 virus were not produced.[13] Similarly, a rabbit hyperimmunized with the E46 stock developed serum neutralizing antibodies at dilutions greater than 1:32 to the prototype adenovirus type 7 (Gomen strain) and to the homologous L. L. strain; it was completely negative at a 1:2 dilution for neutralizing antibodies for SV40 virus.

Serological methods and reagents: The complement fixation test was the microtechnique used previously for demonstrating virus-specific antigens in adenovirus 12, SV40, and Rous sarcoma tumor systems in the hamster.[1, 2, 7, 16] The fluorescent antibody procedures and reactions, also described previously,[4, 5] are considered in more detail in the accompanying paper.[8] Hamster tumor antigens and the apparently identical cell-associated CF antigens derived from viral preparations, also referred to as "T" antigens, and virus-specific hamster serum antibodies to the "T" antigens were prepared and used as described in other reports.[1, 2, 6–8]

Procedures for inoculating newborn hamsters and for transplanting the tumor cells to newborn hamsters have also been described.[17] The prototype (Gomen) strain[18] and the oncogenic Pinckney strain reported by Girardi *et al.*[10] were used in addition to the L. L. strain for preparing viral (virion) and cell-associated "T" antigens. We have induced tumors in hamsters with the Pinckney strain kindly supplied by Dr. Hilleman, and several hamster tumors became available for antigen analysis only very recently.

Results.—Tumors were first observed 71 days after injection of 28th passage (E46) of the L. L. strain, and 69 days after injection of the 29th tissue culture passage material (lot #2). By the 131st day, 27 of 36 hamsters injected with the L. L. E46 developed subcutaneous tumors. Explants of several of the tumors grew

rapidly when they were transplanted intraperitoneally and subcutaneously into newborn hamsters.

The primary tumors appeared grossly to resemble the tumors induced by SV40 virus. This was confirmed microscopically, although some tumors also showed islands of smaller cells similar to those regarded as characteristic for adenovirus type 12 tumor cells. Most of the tumors in second and third serial transplant passages in the subcutaneous tissues of newborn hamsters revealed similar microscopic appearances; however, many of these tumors also had areas similar to those seen in adenovirus-induced tumors. A report on the histology of the E46 tumors was prepared by Dr. Leonard D. Berman.[19] The following is an abstract of this report.

Histologically the tumors appeared to be basically dimorphic in appearance. Some of the tumors were spindle cell sarcomas exhibiting a moderate amount of pleomorphism and cellular variation. Out of 5 such tumors studied, the variation of histologic spectrum ranged from rather loose interwoven bundles of tumor cells to dense sheets of tightly packed tumor cells forming whorls and well-organized fasicles. In these tumors the cells were always spindly with homogeneous elongated nuclei and indistinct eosinophilic cytoplasm.

Other tumors were entirely different in morphology. These were small cell sarcomas of uniform cellularity growing in broad sheets pierced by numerous stromal strands and small blood vessels. Tumor cell nuclei were small, round, and vesicular, the cytoplasm scant, polygonal, and sometimes indistinct. Mitoses were frequent, and there was some tendency for tumor cells to orient themselves about small vessels and stromal strands.

The range of histologic variation in the spindle cell sarcomas, and the appearance of the giant cells are quite typical of SV40 tumors. The small cell tumors are characteristic adenovirus tumors. The atypical small cell tumors with giant cells might represent some sort of mixing of the two morphologic pictures. Certainly in one tumor histologic mixing of tumor morphology is clear-cut.

Serological Findings.—Complement-fixing antigens and antibodies: A majority of the hamsters carrying primary or first through third transplants of the L. L. tumors for periods of 6 days or more developed complement-fixing antibodies to SV40 hamster tumor antigens, to homologous (L. L.) tumor antigens, and to an SV40 tissue culture preparation containing nonvirion cell-associated "T" antigens (Table 1). The serums did not react with conventional SV40 viral antigen, adenovirus type 12, or polyoma hamster tumor antigens; similarly, none of the serums possessing SV 40 antibodies reacted with adenovirus type 7 viral antigen. The hamsters which

TABLE 1

DEVELOPMENT OF ANTIBODIES TO SV40 HAMSTER TUMOR OR "T" ANTIGENS IN HAMSTERS* CARRYING L. L.-INDUCED TUMORS

Sera from test animals passage in hamsters	Antigens Tested: SV 40 hamster tumor (8 units)	L. L. hamster tumor (4 units)	SV 40 cell-associated ("T") (8 units)	SV40 viral (8 units)
Primary	13/23†	11/22	10/23	0/20
1st Transplant	7/8	5/8	5/8	0/8
2nd Transplant	7/8‡	7/8	7/7	0/7
3rd Transplant	7/9	7/9	5/9	0/9

* Hamsters bearing tumors 5 mm or greater for 6 days of more.
† Number positive at 1:10 dilution or greater/number tested.
‡ The one animal with tumor which did not have reactive serums vs. SV40 hamster tumor antigen was not tested against SV40 "T" or viral antigen.

TABLE 2

PRODUCTION OF COMPLEMENT-FIXING ANTIBODIES BY NEWBORN HAMSTERS CARRYING SUBCUTANEOUS TRANSPLANTS OF ADENOVIRUS TYPE 7 STRAIN L. L. TUMORS

Test H-1874 2nd transplant animal no.	First Serum, 33 Days After Transplant — CF Antibody Titer — Tumor size (mm)	SV40* tumor antigen	SV40† viral antigen	SV40 cell-assoc. "T" antigen	Adeno 7‡ L. L. tumor antigen	Second Serum, 40 Days After Transplant — CF Antibody Titer — Tumor size (mm)	SV40 tumor antigen	SV40 viral antigen	SV40 cell-assoc. "T" antigen	Adeno 7 L. L. tumor antgien	Third Serum, 47–49 Days After Transplant — CF Antibody Titer — Tumor size (mm)	SV40 tumor antigen	SV40 viral antigen	SV40 cell-assoc. "T" antigen	Adeno 7 L. L. tumor antgien
L-1 TC-3	5	0§	—	—	0	10	0	—	—	0	20	0	—	—	0
						15					20				
TC-4	15	0	—	—	0	10	20	0	10	10	40	40	0	40	40
						35									
TC-5	5	0	—	—	0	30	10	0	0	0	30	40	0	20	20
	10					30					30				
TC-6	0	0	—	—	0	0	0	—	—	0	0	0	—	—	0
TC-7	3	0	—	—	0	10	10	—	—	10	10	20	0	10	10
L-2 TC-4	10	0	—	—	0	?	20	0	10	20	15	40	0	40	40
TC-6	0	0	—	—	0	10	20	0	20	10	35	20	0	20	40
L-3 TC-4	15	0	—	—	0	20	10	0	10	10	20	40	0	40	80
	20					30					40				
TC-5	10	0	—	—	0	15	10	0	0	0	15	20	0	40	40

* SV40 tumor and "T" cell-associated antigens contained 8 units based on titration with SV40 hamster serums.
† SV40 viral antigen contained 8 units as tested with convalescent monkey serums.
‡ Adenovirus type 7 L. L. tumor antigen contained 4 units based on titration with adenovirus type 7 strain L. L. hamster serums.
§ No reaction at serum dilutions of 1:10 or greater.
Dash = not tested; question mark = no record of tumor size.

failed to develop tumors did not develop antibodies to tumor or "T" antigens. One hamster, carrying a primary tumor, developed a 1:80 titer to the "T" or cell-associated antigen of the type 7 prototype adenovirus (Gomen), and to tumor antigen present in a hamster tumor induced by the Pinckney strain of type 7. The antibody developed 20 days after the hamster tumor reached a size of 40 mm in diameter. Several hamsters carrying serial transplants of the L. L. tumor also developed serum antibodies to adenovirus type 7 tumor and cell-associated antigens.

Table 2 summarizes the serum antibody data on 10 hamsters carrying second passage transplants. All but one of the hamsters carrying tumors developed complement-fixing antibodies to SV40 hamster and tissue culture "T" antigens and the homologous L. L. virus-induced tumors; the antibody titers increased in relation to the size of the tumor and the period of exposure to tumors.[1, 2] The one hamster failing to develop a tumor also failed to develop complement-fixing antibodies. As was observed previously,[1, 2, 7, 20] in hamsters carrying transplants of virus-free SV40 tumors, the L. L. tumors did not produce antibodies to SV40 viral antigen. Although not shown on the table, none of these serums shown in Table 2 reacted with adenovirus type 7 viral or "T" antigens.

Neutralization tests: Thirty-one serums from 10 hamsters with primary tumors, having complement-fixing antibody to SV40 tumor and tissue culture cell antigens, were tested at a 1/5 dilution for neutralizing antibody to SV40;[21] all were completely negative. Of these serums 18 were also tested for neutralizing antibody to adenovirus type 7, with consistently negative results.

Hamster tumor antigens: Nearly all of the antigen preparations made from primary and transplanted tumors induced by the L. L. strain reacted to significant titers with several standard positive serum pools from SV40 tumor-bearing hamsters, and with serums from hamsters carrying L. L. tumors. They did not react with SV40 antiviral antiserum (convalescent monkey serum), or with standard hamster serums containing antibodies to polyoma, adenoviruses 12 and 18 "T" antigens. Suspensions of L. L. hamster tumor cells grown in tissue cultures also demonstrated complement-fixing antigens when tested with standard SV40 hamster serums.

Tests for SV40 virus in tumors: Five E46-induced hamster tumors, including one primary, 2 first transplant passage, and 2 second transplant passage, were tested for retrievable SV40 virus by overlaying trypsin-dispersed tumor cells on GMK or BSC-1 tissue cultures and blind-passing the fluids after 3–4 weeks.[22] In 14 separate tests, none of the 5 tumors yielded any evidence of infectious SV40 virus. This result is in contrast to the ready recoverability of virus from most other SV40-induced primary hamster tumors.[2, 3, 22] Tests for adenovirus type 7 were also negative.

Fluorescent antibody studies of L. L.-induced tumors: Tissue culture-grown cells from tumors induced by SV40 virus demonstrate nuclear fluorescence when tested in the indirect fluorescent antibody test with serums of SV40 tumorous hamsters;[4] entirely similar reactions with SV40 hamster serums were demonstrated in cells derived from the L. L.-induced hamster tumors. Six tumors (3 primary, 2 first transplant, and one second transplant passage) were grown in culture and tested for fluorescent stainable antigen. In all 6, the great majority of tumor cells showed intense nuclear fluorescence when tested with SV40 tumored hamster serum; of those tested against other serums, none showed fluorescence with adenovirus 12 tumored hamster serum, adenovirus 7 rabbit antiserum, or monkey SV40 antiserum. Additional studies of the L. L. hamster tumor cells induced in our laboratory are given in the accompanying paper.[8]

Prevention of tumors by adenovirus type 7 antiserum: Studies described in accompanying reports[8, 9] revealed that SV40 "T" antigens induced in tissue cultures by the L. L. strain were prevented by specific antiserum for adenovirus type 7, but not by antiserum for SV40 virus. Preliminary results available 280 days after injection of newborn hamsters show that L. L. strain tumors were prevented by adenovirus type 7 antiserum but not by SV40 antiserum. Thus it appears that the hamster tumors and the antigens found in tumor cells, as well as the SV40 virus-specific antigens found in infected tissue cultures, were wholly dependent on the adenovirus type 7 virions.

Discussion.—The viral specificities of the CF antigens found in hamster tumor cells induced by adenovirus types 12 and 18, SV40, polyoma, and Rous sarcoma have been well documented in a number of laboratories.[1–3, 6, 7, 16, 23] Reports by Pope and Rowe,[4] and Rapp *et al.*,[5] indicate that the reactions of serums of tumorous hamsters with SV40 and adenovirus-induced tumor cell antigens in the fluorescent antibody test are also virus-specific (see accompanying reports[8, 9]).

It seems most unlikely that the L. L. hamster tumors could have been caused by undetectably small amounts of infectious SV40 virus in the E46 or lot #2 preparations, since extensive tests in three laboratories were without exception negative for SV40 virus. In addition, the first tumors induced by both inocula occurred nearly 50 days earlier than we have observed with our highest titered ($>10^7$ $1D_{50}$) SV40 virus preparations. Also, over 25 per cent of the hamsters injected with the E46 and lot #2 stocks had tumors by 110 days, whereas our SV40 virus preparations seldom produced any tumors earlier than 120 days. In the accompanying report, Rowe and Baum furnish additional evidence concerning the absence of the SV40 virion not only in the E46 passage but in subsequent passages as well.[8]

Since all SV40 strains tested and several adenovirus type 7 strains have been shown to have oncogenic capabilities in hamsters, the neoplastic effects produced by the L. L. strain could have been produced (*a*) by adenovirus 7 information alone, but somehow potentiated by the linkage with SV40 virus information, or (*b*) by SV40 virus information alone but carried into the cell by adenovirus particles, or (*c*) by information derived from both genomes.

The first possibility seems least likely, since the tumors show morphological and biological similarities to SV40 tumors and since most of the tumors carry the virus-specific "T" antigen associated only with SV40 virus. However, it is not impossible that the neoplastic and specific antigenic potentials are associated but wholly independent properties of these viruses. In this case, the cells presumed to be transformed by adenovirus 7 in nearly every one of many instances would have had to acquire the virus-specific antigens associated only with SV40 virus.

The second hypothesis (*b*) seems more likely than the first (*a*), but this would not account for the several instances in which tumorous hamsters finally developed CF antibodies to adenovirus type 7 "T" antigens. Current data, therefore, would seem to favor the third hypothesis (*c*).

Significance of the L. L. strain phenomenon: The theoretical implications of the phenomenon produced by the L. L. strain of adenovirus type 7 hinge on the nature of the linkage which exists between the adenovirus 7 virions and the SV40 virus information responsible for the hamster tumors and for the "T" antigens in the tumor cells and in tissue culture cells infected with the L. L. strain. Should this linkage represent a hybridization of genetic material of these viruses incorporated in an infectious adenovirus type 7 virion, the implications are exciting indeed. Additional evidence favoring this hypothesis is considered in detail in the accompanying papers.[8, 9]

The observations of Rabson *et al.*[24] concerning the potentiating effects of SV40 virus contamination on the growth of certain adenoviruses in monkey tissue culture cells are also relevant to considerations of the mechanisms by which the SV40 virus neoplastic and antigenic information might be incorporated as a detectable component in the virion of adenovirus type 7. It is possible that selective growth advantages accrue in monkey kidney cultures to those adenovirus virions which have effected a tight bond or hybridization with SV40 virus genetic information. Evidence presented by Rowe and Baum in the accompanying article suggests that such a mechanism may best explain the differing growth potentials of several tissue culture lines of the L. L. strain derived from L. L. E46 lot of adenovirus type 7.[8]

The practical implications of the L. L. strain phenomenon are no less interesting. The possibility that one animal virus might serve as a vector of the antigenic and/or oncogenic potentials of a second quite unrelated virus is a startlingly new concept. This would be particularly true if such newly acquired capabilities cannot be detected by the most sensitive conventional procedures available for demonstrating infectious virus. From this point of view, questions concerning the precise nature of the linkage between the two viruses are perhaps less important than the question concerning the frequency with which this phenomenon might take place and, of course, the conditions which might make it possible. Both the theoretical and practical implications would be greatly magnified should the adenovirus-SV40 type of linkage described herein prove not to be unique, but a more general phenomenon likely to occur with some frequency in nature as well as in the laboratory.

It is perhaps worth while pointing out that the oncogenic potential of the SV40 information and possibly also of the adenovirus 7 virions in the L. L. strain appeared to be enhanced, since 28th and 29th passage lots of the L. L. strain of adenovirus type 7 produced tumors in hamsters 50–100 days earlier, respectively, than any we have observed in tests of our highest titered SV40 and adenovirus type 7 (Pinckney strain) preparations.

Should it be possible to reproduce a similar linkage of SV40 oncogenic information with additional possibly nononcogenic strains of adenovirus type 7 or with other adenovirus serotypes, then the deliberate or accidental conversion of nononcogenic viruses into agents capable of causing cancer in certain animals becomes a distinct possibility.

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EVIDENCE FOR A POSSIBLE GENETIC HYBRID BETWEEN ADENOVIRUS TYPE 7 AND SV40 VIRUSES

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The accompanying report[1] described an unusual pattern of hamster tumor induction by a monkey kidney adapted strain (L. L.) of adenovirus type 7 (Ad. 7). The tumors contained an antigen (referred to herein as SV40 T antigen) reactive with SV40 tumored hamster sera in complement fixation (CF) and fluorescent antibody (FA) tests, but no infectious SV40 virus could be demonstrated in the inoculum or tumors, and the tumored hamsters did not develop neutralizing antibody to SV40 virus. Since the Ad. 7 strain had been contaminated with SV40 in earlier passages, the contaminant being eliminated by passage with SV40 antiserum,[1] it appeared that there might be a previously unrecognized type of interaction between the Ad. 7 and SV40 viruses.

When it was found that the preparation (E46) of L. L. strain Ad. 7 which induced the hamster tumors also induced FA-stainable SV40 T antigen in acutely infected tissue culture cells, studies were carried out in this system to delineate the specificity of the reactions observed and the nature of the relationship between the Ad. 7 and SV40 genetic material.

Materials and Methods.—Tissue cultures: Fluorescent antibody tests were done with cells grown on 11 × 22 mm coverslips in 60-mm plastic Petri dishes. Primary cultures of human embryonic kidney (HEK) and African green monkey kidney (AGMK) were obtained from Microbiological Associates, Inc., and Flow Laboratories. The cells were dispersed by trypsinization and planted in the Petri dishes using 3.5×10^5 cells in 3.5 ml of growth medium consisting of 10% unheated fetal bovine serum in Eagle's basal medium (BME). Prior to virus inoculation the cultures were washed twice with BME, and 2% heated (56° for 30 min) agammaglobulinic calf serum (Hyland Laboratories) in BME was added as maintenance medium. All media contained penicillin, streptomycin, and glutamine. Plates were held at 37°C in humidified 5% CO_2 in air.

For FA staining of tumor cells, tumors were cultivated in tissue culture as described previously.[2]

Viruses: The origin and characterization of the E46 preparation of the L.L. strain of Ad. 7 have been described in detail.[1] In this report, the term L. L.-E46 will be used to refer to the line