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*POTENTIATION OF ONCOGENICITY OF ADENOVIRUS TYPE 12 GROWN IN AFRICAN GREEN MONKEY KIDNEY CELL CULTURES PREINFECTED WITH SV40 VIRUS: PERSISTENCE OF BOTH T ANTIGENS IN THE TUMORS AND EVIDENCE FOR POSSIBLE HYBRIDIZATION**

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Rabson *et al.*[1] reported enhancement of the growth of adenoviruses in African green monkey kidney cell cultures (AGMK) following preinfection with SV40 virus. We confirmed his findings and corroborated them with other serotypes of adenovirus (unpublished observations). In the course of investigating the oncogenicity of adenoviruses grown in the presence of SV40, we observed marked enhancement of oncogenicity as well, particularly when adenovirus type 12 and SV40 virus were passed serially together for five or more passages in AGMK. The purpose of this paper is to describe the development of tumors and the virus-specific antigens found in them. Evidence of possible "hybridization," or genetic mixing, between the two viruses is also discussed.

Materials and Methods.—Cell cultures were obtained from Microbiological Associates, Inc. African green monkey kidney (AGMK), human embryonic kidney (HEK), as well as BSC-1 (a continuous line derived from AGMK)[2] were maintained on Eagle's minimum essential medium (EMEM) in Earle's balanced salt solution containing 3% agamma calf serum,[3] 4 mM of glutamine, 100 units of penicillin, and 100 μg of streptomycin per ml. Medium changes were done every 4 days. KB cells were maintained on EMEM as above except that the agamma calf serum concentration was increased to 10%. Medium was changed every 2 days in the case of normal KB cultures; virus-infected cultures were changed as required depending on the development of CPE.

Virus: Adenovirus type 12, strain Huie, was obtained from Dr. R. R. Rafajko[4] and passed once at 34°C in KB cells inoculated with high multiplicity of infection. SV40 virus, strain #776, obtained from Dr. H. M. Meyer[5] was passed four times in AGMK cells and twice in BSC-1 cells. It was propagated at 34°C using minimum virus doses, i.e., 1–10 ID_{50} per 32 oz bottle culture.[6]

Virus assays were done at 37°C using HEK cell cultures as indicators for adenovirus and AGMK cell cultures for SV40 virus. In order to assay the SV40 infectious component in the virus mixture, titrations were done in the presence of adenovirus type 12 antiserum. Tenfold dilutions and two to three roller tubes per dilution were employed. The cultures were read twice weekly until there was no increase in titer (approximately 21–28 days). Titers were estimated according to Reed and Münch;[7] they are given as reciprocals adjusted to 1.0-ml volumes.

The nonvirion T or neoantigen preparations were produced as described earlier.[8–13] Adenovirus T antigens were produced in KB cell monolayers inoculated with virus multiplicities of 10 or greater and harvested at the earliest appearance of CPE or 72 hr after inoculation. The cells were scraped off the glass surface, centrifuged gently for 10 min at 1000 rpm, and resuspended in

1/20th the supernatant fluid (20× cell pack) equivalent to a 3–4% cell suspension. SV40 T antigen was produced in the same manner in AGMK cells. The antigens thus obtained were assayed against appropriate antibody-containing sera from hamsters bearing tumors induced by the respective viruses. HEK cells were infected and fixed on coverslips for analysis in fluorescent antibody (FA) tests as described previously.[12]

Tumor antigens were made by homogenizing tumors in four times their own weight of Eagle's basal medium at 4°C with mortar and pestle or in a TenBroeck grinder and clarifying for 15 min at 2000 rpm in a refrigerated centrifuge.[14]

Hamster sera: Tumor-bearing hamsters were bled from the inner canthus of the eye at 2-week intervals. The sera assayed for the antibodies were final bleedings of hamsters that had carried tumors for various periods of time. Previously defined standard pools of sera from tumor-bearing hamsters were used for antigen assays.

Antivirion antisera: Adenovirus group reactive reference sera were obtained from patients and volunteers convalescent from infection with a variety of adenoviruses. SV40 antiserum was obtained from naturally infected monkeys. Specific neutralizing antisera were produced by hyperimmunizing rabbits with the respective viruses and, in some cases, prototype rabbit antisera were obtained from the National Institute of Allergy and Infectious Diseases' reagent program.[15]

Complement fixation (CF) tests were carried out in the microtechnique system[16] as described by Sever.[17] Titers were recorded as reciprocals of the highest dilution showing 3+ or 4+ fixation of 1.8 units of guinea pig complement. Four to eight units of standard antigen and antisera preparations were used for all assays of unknown materials.

Experimental: AGMK cell cultures were inoculated with a multiplicity of 1–10 of SV40 virus and 24 hr later were superinfected with the same multiplicity of adenovirus type 12. The cultures were harvested 48 and 72 hr after adenovirus superinfection and assayed for viral CF antigen and infectivity; the 72-hr harvest was then used to initiate subpassages in AGMK cells. The harvests from primary and sixth and seventh passages were inoculated into newborn hamsters.

For the purpose of determining the antigenic characteristics of tumors induced by these virus mixtures, tumor homogenates were assayed for complement-fixing antigens against sera from hamsters carrying tumors induced by SV40 and adenovirus type 12 viruses. In addition, a number of primary tumors were excised and transplanted to newborn and weanling hamsters. The antigens present in the transplants were determined in the same way. Sera from tumor-bearing hamsters were tested for antibodies binding complement with SV40 and adenovirus type 12 tumor extracts and their respective tissue-culture-grown T antigens.

Results.—African green monkey kidney cells preinfected with SV40 virus harvested 2 and 3 days after adenovirus inoculation contained approximately 100 times more adenovirus than did control cultures which were not preinfected. The adenovirus CF antigen levels were also increased by preinfection with SV40 virus (Table 1). SV40 infectivity titers were not perceptibly altered by adenovirus superinfection. It can be seen from Table 1 that the adenovirus/SV40 virus ratio was approximately one in the dually infected AGMK cultures (primary cultures). The ratio increased to more than 100,000 after five more passages in AGMK cells; the adenovirus infectivity was increased by 1–2 logs and SV40 infectivity was reduced by more than 4 logs.

The responses of newborn hamsters to the dually infected primary culture materials were compared with those obtained in hamsters given either adenovirus type 12 or SV40 virus. The SV40-infected cultures without adenovirus superinfection produced tumors in 15 out of 22 hamsters (68%); the median time until the first appearance of tumors (incubation period) was 117 days, which is typical for SV40 virus and the viral dose given. When examined in the CF test, these tumors contained only SV40 antigen and induced antibodies only to SV40 tumor and T antigens. Adenovirus grown alone in AGMK cells did not induce any tumors during the time of observation (300 days).

TABLE 1

INFECTIVE VIRUS AND CF VIRAL ANTIGEN PRODUCTION IN AFRICAN GREEN MONKEY KIDNEY CELLS INFECTED BY SV40 VIRUS AND ADENOVIRUS TYPE 12 IN SINGLE AND MIXED INFECTIONS

Virus inoculum	Harvest Time (Hr after Inoculation) SV40	Adeno 12	Viral CF Antigen vs: SV40 AS*	Adenovirus AS*	Infectious Virus SV40 Virus	Adenovirus
Adeno 12	—	48	—	<8†	—	5.2‡
	—	72	—	<8	—	5.2
SV40	72	—	<2	—	7.5§	—
	96	—	<2	—	8.0	—
Adeno 12 + SV40						
Primary culture						
48-hr harvest	72	48	<8	8	7.2	6.7
72-hr harvest	96	72	<8	16	7.7	7.7
Combined passage**						
Sixth passage	—	—	<8	16	3.2	9.2
Seventh passage	—	—	<8	8	2.7	8.2

* Antivirion antisera.
† Reciprocal of dilution at which there was 3+ or greater complement fixation with the respective antisera.
‡ Adenovirus infectivity titers expressed as $\log_{10}$ HEK ID_{50}/ml.
§ SV40 titers expressed as $\log_{10}$ AGMK ID_{50}/ml.
** Harvested 96 hr after inoculation.

On the other hand, virus harvests obtained in AGMK cells that had been preinfected with SV40 virus and then superinfected with adenovirus type 12 induced tumors readily. The 48-hr harvests from primary dually infected cultures induced tumors within 36 days with an over-all incidence of 60 per cent (9/15) and a median incubation time of 56 days. The 72-hr harvest, which contained 10 times more adenovirus, was also more oncogenic; the first tumors appeared within 29 days, and eight of the nine hamsters inoculated had developed tumors by day 68 with a median incubation time of 42 days, thus providing an oncogenic pattern similar to that exhibited by adenovirus type 12 grown in KB cultures. Figure 1 (*upper graph*) shows the responses of newborn hamsters inoculated with various doses of adenovirus type 12 grown in KB cells. The lower graph on this figure illustrates the tumor response of newborn hamsters to inoculation with virus from AGMK cell cultures dually infected with these viruses.

Although each of the eight tested tumors induced by the primary dual culture materials contained adenovirus type 12 tumor CF antigen, only three of these tumors contained demonstrable SV40 tumor antigen (Table 2).

Tumors induced by the 48- and 72-hr virus dual cultures were minced and subpassed to 27 suckling hamsters. In none of 15 transplant tumors tested could we demonstrate SV40 antigen (Table 3), even though they had been produced by primary tumors containing considerable amounts of SV40 antigen. Furthermore, antibodies to SV40 tumor and tissue-culture-grown T antigens were not demonstrated in any serum taken from hamsters transplanted with these tumors, although the sera did contain antibodies to both tumor and T antigen preparations of adenovirus type 12. However, data which will be the subject of another report indicated that SV40 tumor antigen may be less predictable in eliciting complement-fixing antibodies in hamsters than the tumor antigens of adenovirus type 12.[18]

Tumor response in newborn hamsters following inoculation with material of sixth and seventh tissue culture passages of adenovirus type 12 with SV40: As mentioned above, the adenovirus type 12/SV40 virus ratio increased in the course of six tissue culture passages so that there was over 100,000 times more adenovirus than SV40 virus (Table 1).

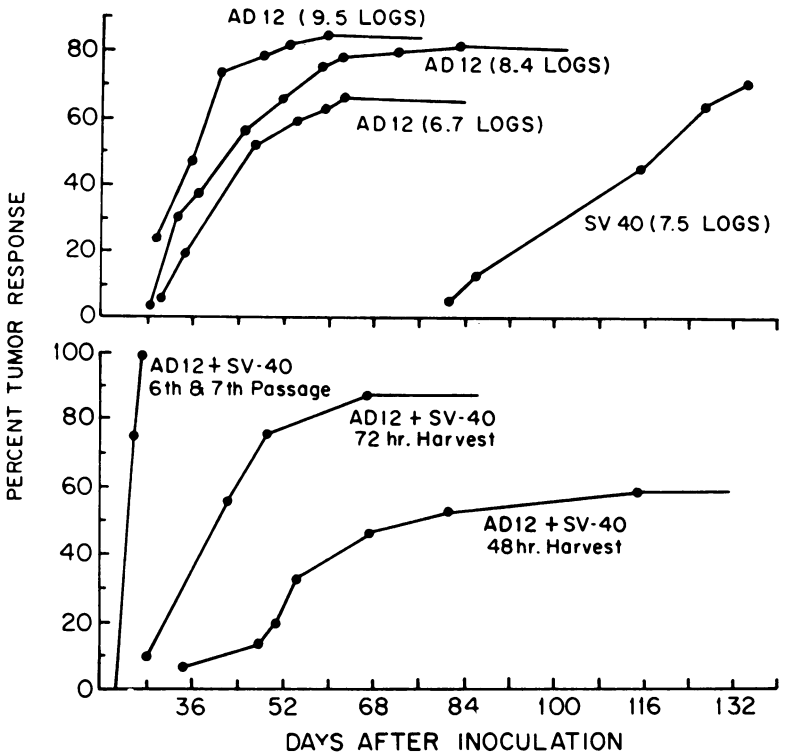

FIG. 1.—Development of tumors in newborn hamsters inoculated with adenovirus type 12, Huie, grown in KB cells, and with SV40 virus, #776, grown in AGMK cells (*upper graph*) compared to the tumor response of newborn hamsters inoculated with the progeny of the two viruses grown together in AGMK cell culture (*lower graph*). Tumor response characteristics to adenovirus type 12, Huie, (*upper graph*) are based on observations on more than 100 hamsters. Adenovirus infectivity titers expressed as $\log_{10}$ HEK ID_{50}/ml. SV40 infectivity titers expressed as $\log_{10}$ AGMK ID_{50}/ml (*upper graph*). For infectivity titers ($TCID_{50}$'s) of the adenovirus type 12 + SV40 dual passage materials (*lower graph*), see Table 1.

TABLE 2

ANTIGENIC COMPOSITION OF PRIMARY HAMSTER TUMORS INDUCED BY AGMK TISSUE CULTURES DUALLY INFECTED WITH ADENOVIRUS TYPE 12 AND SV40 VIRUS

Inoculum		Incubation period (days)	Titer vs. Hamster Sera* Indicated	
AGMK passage	Harvest time (hr)		Ad. 12	SV40
		49	16†	16
		52	>32	<4
1‡	48	56	>32	<8
		56	16	8
		68	>32	<4
		42	>32	16
1‡	72	42	>32	<4
		68	>32	<4
		27	16	16
		27	32	32
		27	32	32
6	96	27	32	32
		27	8	16
		27	>32	>32
		28	<4	8
		29	>32	16

* Pooled sera from tumored hamsters used at 4–8 units.
† Reciprocal of dilution.
‡ Primary cultures.

TABLE 3

SPECIFIC COMPLEMENT-FIXING ANTIGENS* IN HAMSTER TUMORS INDUCED BY AGMK TISSUE CULTURES DUALLY INFECTED WITH ADENOVIRUS TYPE 12 AND SV40 VIRUS

Inoculum		Primary Tumors		First Transplant		Second Transplant	
AGMK passage	Harvest time (hr)	Ad. 12	SV40	Ad. 12	SV40	Ad. 12	SV40
1‡	48	5/5†	2/5	5/5	0/5	NT	NT
1‡	72	3/3	1/3	10/10	0/10	NT	NT
6	96	7/8	8/8	24/24	24/24	12/12	12/12

NT: none tested.
* Complement fixation 3+ or greater with the indicated tumored hamster sera pools used at 4–8 units.
† No. positive at 1:4 dil./no. tumors tested.
‡ Primary cultures.

The sixth combined passage material produced tumors which were fatal within 27 days in every one of 13 hamsters; subsequent inoculation of newborn hamsters with sixth and seventh passage culture fluids gave similar results (Fig. 1). Unlike the tumors resulting from the initial tissue cultures of the adenovirus type 12-SV40 mixture (*vide supra*), these tumors contained large amounts of both adenovirus type 12 and SV40 tumor antigens (Tables 2 and 3) which have persisted through the fifth serial transplantation. Moreover, these antigens elicited high titered antibodies to the tumor and T antigen preparations of both adenovirus type 12 and SV40.

Dr. Howard Igel[19] examined the histology of the tumors produced by these virus mixtures. He commented as follows: "The microscopic appearance of the hamster tumors induced by both primary passage and later passage tissue culture materials was similar. The tumors were composed of fairly uniform undifferentiated small cells with occasional rosette formations typical of adenovirus-induced tumors. None had definite morphologic characteristics of the typical spindle cell sarcomas induced by SV40 virus." The tumor response in this case is in contrast to the tumors produced by the LL strain of adenovirus type 7 which carries a portion of the SV40 genome and which produces tumors having the cytologic as well as the antigenic characteristics of tumors produced by SV40.[11, 12]

Prevention of tumors by adenovirus type 12 hyperimmune rabbit serum: Hyperimmune rabbit sera containing high levels of neutralizing antibodies to adenovirus type 12 completely prevented the seventh tissue culture passage of virus from inducing tumors in newborn hamsters during an observation period of 100 days, whereas a normal rabbit serum and a hyperimmune serum versus SV40 virus did not prevent such tumors. The tumors produced in each case contained CF antigens characteristic of both adenovirus type 12 and SV-40-induced tumors.[18]

Prevention of T antigen induction by adenovirus type 12 hyperimmune rabbit serum: Rowe *et al.*[12] and Rapp *et al.*,[20] using the fluorescent antibody (FA) test, demonstrated SV40 T antigen in the nuclei of simian and human tissue cells 24 hr after infection with the LL strain of adenovirus type 7 (E46+ passage). They also reported that the induction of SV40 T antigen by this virus was completely suppressed by adenovirus type 7 hyperimmune rabbit serum but was unaffected by SV40 hyperimmune serum.

Observations in the FA test by Dr. Rowe of HEK cells infected with the adenovirus type 12-SV40 combination (sixth passage) virus materials gave results similar to those obtained with the adenovirus type 7 LL strain. Five to ten per cent of the cells exhibited SV40 T antigen at 24 hr. Hyperimmune rabbit sera prepared

against adenovirus type 12 mixed with the virus suppressed the development of SV40 T antigens, while SV40 hyperimmune rabbit serum and normal rabbit serum had no suppressive effect on the development of this antigen.

Persistence of SV40 infectious genome in the adenovirus hybrid particles: A 1/50 dilution of hyperimmune rabbit serum incubated for 90 min at 37°C with clarified sixth dual passage virus did not succeed in eliminating the SV40 virus component. Although no SV40 cytopathic effect developed in the cultures protected by SV40 antiserum when the culture fluids were subpassed at 72 hr to AGMK cell cultures, SV40 virus was demonstrated at a titer of 10^{-3} per 0.2 ml inoculum. A total of eight additional subpassages under SV40 antiserum cover failed to eliminate SV40 virus infectivity from the virus preparation which (as described above for earlier passages) was completely neutralized by adenovirus type 12 antiserum. In contrast, the same SV40 antiserum, diluted to more than 1/3200, completely suppressed 100–1000/ID_{50} of SV40 virus from nonhybrid SV40 stock preparations.

It seems likely, as has been reported for other adenovirus-SV40 hybrid systems,[21–23] that complete infectious genomes of SV40 virus were enveloped in a proportion of the adenovirus type 12 capsids where they were protected from the neutralizing action of the SV40 antiserum. Studies to clarify this question are under way.

Discussion.—Preinfection with SV40 virus is important in the multiplication of adenovirus type 12 in AGMK cells, an observation similar to that reported by Rabson *et al.* for adenovirus type 5.[1] The oncogenic activity of the virus mixture produced in the initial tissue culture passage reflected the ordinary behavior expected of adenovirus type 12 grown in human cells.[14] The tumors induced had the gross and microscopic characteristics of adenovirus type 12 tumors and the antigenic characteristics as well. Several tumors also contained SV40 CF antigen but the latter did not persist when these tumors were transplanted to other hamsters. When the two viruses were grown together in AGMK cells for six tissue culture passages, the oncogenic activity was both increased and accelerated; the "combined passage" virus induced tumors in 100 per cent of newborn hamsters with an incubation period much shorter than previously observed for either virus alone.

This synergistic action of the two viruses in potentiating oncogenicity cannot be explained by assuming that a very potent adenovirus preparation resulted from an enhancement of adenovirus multiplication by SV40 virus-induced enzymes or by the two viruses acting independently but simultaneously. In either instance, the primary passage materials should have produced tumors with at least the same efficiency as sixth and seventh passage preparations, since it contained larger amounts of SV40 virus. This was not the case; the sixth and seventh passage tissue culture fluids which contained 10,000 times less infectious SV40 virus than the initial passage materials were much more efficient in producing tumors. Interestingly, all of the tumors resulting from the sixth passage tissue culture materials contained large amounts of SV40 tumor antigen as well as adenovirus type 12 tumor antigen. Since hyperimmune adenovirus type 12 specific antiserum prevented the seventh passage virus from producing tumors while hyperimmune SV40 antiserum did not, it appears that the combined passage material may have

contained hybridized virus such as was described earlier for the LL strain of adenovirus type 7.[11, 12, 20] This hypothesis was further supported by the large amounts of both antigens found in the tumors that developed in hamsters injected with the seventh passage virus in which the SV40 virus component was neutralized by SV40 antiserum.[18] Additional and even more direct support for this hypothesis was the fact that the induction of SV40 T antigen in HEK cells by the sixth passage virus was suppressed by adenovirus type 12 antiserum but not by SV40 antiserum. It may be postulated, therefore, that in the serially passed tissue culture materials SV40 genetic material was incorporated into adenovirus type 12 particles. The persistence of the entire infectious genome of SV40 in a proportion of the adenovirus particles despite serial passages under potent SV40 antiserum confirms similar observations by others[21-23] and the accelerated oncogenic activity of the combined, or hybrid, passage materials merits further investigation.

Summary.—The growth of adenovirus type 12 in African green monkey kidney was significantly enhanced by SV40 virus preinfection as indicated by the development of increased virus infectivity and CF antigen. After six tissue culture subpassages, the oncogenicity of the resulting virus for newborn hamsters was also remarkably potentiated and accelerated.

The potentiation of oncogenicity was not due to a mere mixing of SV40 virus and adenovirus particles but developed only after additional growth of the two viruses together for several subcultures. Tumor antigens characteristic of both viruses were demonstrated in all primary tumors induced by the postulated hybrid virus and remained present in tumors carried through five transplant passages. The oncogenic and T antigen determinants were eliminated by adenovirus type 12 antiserum but not by antiserum to SV40 virus, thus suggesting that SV40 genetic information was contained in some of the adenovirus capsids.

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*TOWARD A QUANTITATIVE VIEW OF THE ENGRAM**

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The qualitative nature of memory remains unknown, but common experience and laboratory observations attest to its quantitative character. Memories may be "strong" or "weak," as judged subjectively or as inferred from animal experiments. The typical experimental measure of a memory is the probability of emission of a learned behavioral response, the observable indicant of the memory. Each indicant is considered to reflect a corresponding memory trace in the brain, the so-called engram. The indicant is quantified, but the corresponding engram is not. This dichotomy adds a conceptual gap to the physiological gap that separates an engram from its indicant. There is a need for language to describe the engram in a quantitative sense, in order to link it more definitely with its measured indicant. It seems timely to introduce a quantitative unit of memory, even though such a unit must as yet be hypothetical, speculative, and tentative.

The proposed unit is defined as the minimal physical change in the nervous system that encodes one memory. The unit cannot be assigned physical dimensions until the nature of the engram is known; for the present, it may be thought of as a single facilitated neural pathway, or a single encoded macromolecule, or a single unit of whatever the engram is. The "strength" of a memory is a function of the number of units that encode the memory. In the limiting case, the "weakest" engram consists of one unit. The name proposed for the unit is the "mnemon" (mneme = memory; -on = suffix denoting a fundamental particle).[1] The potential usefulness of the mnemon concept is illustrated by its application to two current problems.

Controversy about the Memory Consolidation Hypothesis.—Long-term memory