fected(18). The lemur kidney cells used in this study were infected at phase II. The question of whether these transformed cells will also enter into a crisis stage is under investigation.

Summary. Cultured kidney cells from a prosimian, mongoose lemur were infected with SV40, which induced a typical cytopathic effect after an extensive period of incubation. On the 119th day after infection, transformed cells developed from a focus of cells showing a certain degree of degeneration. The new growth of cells grew rapidly, lacked contact inhibition, formed multilayered cells, and synthesized SV40 and a soluble complement-fixing tumor antigen.

The authors thank Miss Beverly Cole for her technical assistance.

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Received February 8, 1966. P.S.E.B.M., 1966, v122.

Occurrence of SV40 Neoplastic and Antigenic Information in Vaccine Strains of Adenovirus Type 3.* (31225)

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Adenovirus type 7 strain LL, which has been employed since 1957 for the production of adenovirus vaccine, was shown to induce tumors in hamsters that contained SV40 complement fixing tumor antigen. This antigen, in turn, elicited antibodies to SV40 T (cell associated) and tumor antigens(1-3). This property of the virus was attributed to incorporation of part of the SV40 genome into the capsid of strain LL during passage of the adenovirus in cultures of rhesus monkey kidney cells infected with SV40 virus(1-3). A similar phenomenon was described by Lewis *et al*(4), as occurring in the JF strain of adenovirus type 3. It is the purpose of this note to record and discuss the oncogenicity of 2 lots of adenovirus type 3 strain JF which were employed as seed stock by 2 separate laboratories for the production of adenovirus vaccines and to corroborate the evidence that SV40 genetic material was encapsidated by the adenovirus particles.

* This work was partially supported by the Nat. Cancer Inst. Field Studies.

Materials and methods. Viruses. Adenovirus type 3 strain JF was isolated in cultures of rhesus monkey kidney (RhMK) cells from throat washings of a child with pharyngo-

conjunctival fever in 1955(5). It was passed in RhMK cell culture at the National Institutes of Health 9 times before it was sent to vaccine manufacturers for use in the production of seed virus for adenovirus vaccines. In the laboratories of manufacturer 1, the virus was carried through 14 additional passages in RhMK cell cultures in the presence of SV40 antiserum. Manufacturer 2 carried the virus for 26 passages through RhMK cell cultures before passing it 2 times in African green monkey kidney (AGMK) cell cultures containing SV40 antiserum. After propagation in the presence of SV40 antiserum the virus was passed in the laboratories of each of the manufacturers 4 more times in AGMK cell cultures with serum-free medium. The harvests from the 4th passage level which constituted the virus seed pools for use in vaccine production were used in the current work. They were designated JF-1 and JF-2 to indicate their sources. Their infectivity titers in AGMK cell cultures observed for 10 days were $10^{3.0}$/0.2 ml and $10^{3.5}$/0.2 ml, respectively.

Detection of SV40. Tests to detect infectious SV40 were carried out in 32-oz bottle cultures of AGMK cells maintained in Eagle's medium containing 1% guinea pig adenovirus type 3 antiserum. The adenovirus type 3 antiserum employed in these tests was obtained from guinea pigs following immunization with inactivated adenovirus type 3 monovalent vaccine. When used undiluted, this antiserum failed to neutralize 10 $TCID_{50}$ of SV40 virus in tests performed as previously described(6). Five ml of each of the virus seed pools were inoculated into each of 5 bottle cultures, and the cultures were observed for 28 days for the appearance of characteristic SV40 cytopathic effects. On the 28th day the cells and fluids were harvested and centrifuged at 300 rpm for 30 minutes; the supernatant fluid was decanted and set aside; the sedimented cells were triturated in a TenBroeck grinder and the ground cells were added to the original cell culture fluid which had been saved for this purpose. After clarification by slow speed centrifugation (3000 rpm/30 minutes), 5 ml of the supernatant fluid was inoculated into each of five 32-oz bottle cultures of AGMK cells. These cultures were observed for 28 days for the occurrence of characteristic SV40 cytopathic change.

Hamster and hamster inoculation. Pregnant and weanling hamsters obtained from the Animal Production Unit of the National Institutes of Health were maintained in isolated rooms free of polyoma and SV40 viruses. Newborn hamsters were inoculated subcutaneously and intraperitoneally with 0.02 ml JF-1 seed virus to each site or intrathoracically with 0.1 ml of JF-2 seed virus. In transplantation studies, newborn and weanling hamsters were inoculated subcutaneously with 0.1-0.2 ml of finely minced pieces of fresh tumor tissue in saline suspension.

Serum collection. Hamsters developing tumors after inoculation with seed virus were bled for serum at the time of tumor harvest which varied from 144 to 395 days after virus inoculation. Most of the hamsters carrying transplanted tumors were bled for serum at the time of tumor harvest which varied from 28 to 67 days after transplantation. In both instances the sera were collected 3 to 68 days after the appearance of palpable tumors.

Complement fixation tests. The microtechnique described by Sever(7) employing overnight fixation with 4 to 8 units of standard antigens and 1.8 units of complement was used to examine hamster sera for presence of antibodies directed against hamster tumor antigens(8,9) and against T antigens(10). Tumors were examined in similar tests for the presence of tumor antigens in tests with standard pools of antisera obtained from tumor bearing hamsters(8,9). Virus strains employed in the preparation of these reagents were adenovirus type 7 strain Pinckney and SV40 strain 776. It was shown by Huebner *et al*(11) that adenovirus type 7 strain Pinckney induces T antigen in cell cultures and tumor antigens in hamsters that cross react in complement fixation tests with sera from hamsters bearing adenovirus type 3 tumors and, conversely, hamster tumors induced by adenovirus type 7 elicit antibodies which cross react with the T and tumor antigens of adenovirus type 3. Accordingly, adenovirus type 7 strain Pinckney tumor antigen was

used to detect antibody directed against adenovirus type 3-type 7 subgroup antigen. This antigen does not cross react with sera from hamsters carrying SV40 tumors(11). Furthermore, it is known that SV40 T and tumor antigens do not react with sera from hamsters carrying tumors induced by adenovirus type 3 or by adenovirus type 7(1,11).

Neutralizing antibody tests for detection of SV40 virion antibody in tumorous hamster serum were carried out by conventional methods which have been described(6). A serum was considered free of SV40 neutralizing antibody if it failed to neutralize completely 10 $TCID_{50}$ of SV40 virus in tests employing a 1:2 serum dilution.

Tests for induction of SV40 fluorescent T antigen were carried out employing methods described by Pope and Rowe(12). Briefly, plastic Petri dishes containing 2 or 3 coverslips were seeded with approximately 2.5×10^5 human embryo kidney (HEK) cells suspended in 4 ml nutrient fluid consisting of Eagle's basal medium, 2% agammaglobulinemic calf serum, 1% glutamine, streptomycin and penicillin. When the HEK cell sheet became confluent on the coverslips, the dishes were inoculated with 0.3 ml of a mixture containing 0.1 ml of either adenovirus type 3 lot JF-1 or adenovirus type 3 lot JF-2 which had been incubated at room temperature for 30 minutes with one or the other of the indicated volumes of the following heat (56°C/30 minutes) inactivated rabbit sera:normal, 0.2 ml; adenovirus type 3 antiserum diluted 1:2 with normal rabbit serum, 0.2 ml; or SV40 antiserum diluted 1:2 with normal rabbit serum, 0.2 ml. Control dishes with coverslips were left uninoculated. Coverslips were removed from the inoculated dishes at the first sign of cytopathic change (24 hours for lot JF-1; 48 hours for JF-2) and from uninoculated control dishes after the elapse of the same time periods. The cells attached to the coverslips were fixed in cold acetone and treated with specific antiserum obtained from an SV40 tumor bearing hamster(12). The antiserum was diluted 1:10 with a 20% extract of normal hamster brain tissue. After removal of the hamster serum, the cells were stained with goat anti-hamster serum conjugated with fluorescein-isothiocyanate, and counter-stained with rhodamine. The number of cells which contained fluorescent stainable antigen in a total of 2000 cells per coverslip was determined.

Results. SV40 virus isolation attempts. Aliquots of the seed pools of adenovirus type 3 JF-1 and JF-2 were examined for the presence of infectious SV40 virus in tests in which the infectivity of the adenovirus type 3 component was suppressed by adenovirus type 3 guinea pig antiserum. The materials tested represented the 4th passage level in AGMK cells with serum-free medium after the strains had been propagated in the presence of SV40 antiserum. Infectious SV40 virus was not detected in either of the seed pools.

Oncogenic and serologic behavior in hamsters of adenovirus type 3 lot JF-1. Adenovirus type 3 lot JF-1 was inoculated subcutaneously and intraperitoneally into each of 14 newborn hamsters. The first tumor appeared on post-inoculation day 144. When the experiment was terminated 364 days after virus inoculation, 8 of the 14 hamsters had developed tumors. It is seen in Table I, part I, that antigens prepared by saline extraction of 2 of the 8 tumors fixed complement in titers of 1:4 and 1:8 in the presence of serum obtained from SV40 tumor bearing hamsters, but did not fix complement in the presence of serum obtained from adenovirus 7 tumor bearing animals. Transplantation studies were carried out with the tumor from one of these 2 animals. Tumors at the second and fourth transplantation level were examined for adenovirus and SV40 tumor antigens. Results presented in Table I, part II, show that at the second passage level, 2 of 4 tumors contained adenovirus tumor antigen at titers of 1:4 and all 4 tumors contained SV40 tumor antigen at titers greater than 1:16. At the 4th passage level, adenovirus tumor antigen was not detectable in any of 8 tumors examined, but all of these tumors contained SV40 antigen at titers of 1:16.

Sera were collected from 8 hamsters 10 to 68 days after primary tumors were first noted, *i.e.,* 144 to 364 days after the animals were injected with seed virus. From data presented in Table I, part I, it is seen that 3 of the 8

TABLE I. Results Obtained in CF Tests with Serum and Tumor Antigen from Hamsters with Tumors Induced by Adenovirus Type 3 Strain JF-1.

Part I. Primary tumors

	Serum							
	Days between		Reciprocal of antibody titer in tests with				Tumor antigen titer in tests with	
			Adeno 7 antigens		SV_{40} antigens			
Hamster	Inoculation and appearance of first tumor	Tumor appearance and serum harvest	T	Tumor	T	Tumor	Adeno 7 serum	SV_{40} serum
1	144	24	80	0*	>80	>80	ND	ND
2	144	58	0	0	>80	>80	<4	4
3	159	41	0	0	0	0	ND	ND
4	166	68	0	0	>80	>80	ND	ND
5	189	10	0	0	0	0	ND	ND
6	189	11	0	0	0	0	<4	8
7	265	50	20	0	20	40	ND	ND
8	364	16	>80	80	>80	>80	ND	ND

* 0 = <1:10

Part II. Transplanted tumors

	Number hamsters positive/Number hamsters examined					
	For tumor antigen reactive in tests with		For serum antibody reactive in tests with			
			Adeno 7 antigens		SV_{40} antigens	
Transplantation level	Adeno 7 serum	SV_{40} serum	T	Tumor	T	Tumor
2nd	2/4 (titers 1:4 for both)	4/4 (>1:16 for all 4)	1/6 (1:80)	1/6 (1:10)	5/6 (1:10 to 1:80)	5/6 (1:40 to >1:80)
4th	0/8	8/8 (1:16 for all 8)	ND	ND	ND	ND

ND = not done

sera contained antibody which fixed complement in tests with adenovirus type 7 T antigen (titers 1:20 to >1:80). Five of the 8 sera gave positive tests with SV40 T and tumor antigens (titers 1:20 to >1:80); one of these sera fixed complement in tests with adenovirus tumor antigen (1:80). In Table I, part II, it is seen that of the 6 sera obtained at the 2nd transplantation level, only 1 contained antibodies against adenovirus type 7 T and tumor antigens; while 5 of the 6 contained antibodies against SV40 T and tumor antigens. None of the sera from the 8 hamsters with primary tumors and none of the sera from 4 hamsters with tumors at the 2nd transplantation level contained SV40 neutralizing antibody.

Oncogenic and serologic properties of adenovirus type 3 lot JF-2. Adenovirus type 3 lot JF-2 was inoculated intrathoracically into each of 10 newborn hamsters. Three of the 10 hamsters developed tumors. In Table II, part I, it is seen that the first tumor was noted on day 330; and in the ensuing 65 days, 2 other hamsters developed tumors. The remaining 7 hamsters had not developed tumors by day 570 when the experiment was discontinued. Twelve to 66 days after the tumors were first noted, the tumorous hamsters were bled for serum for use in serologic tests, and the tumors were removed for use in transplantation experiments. Antigens were not prepared from these tumors. All 34 hamsters to which these tumors were transplanted developed palpable tumors by day 67. Thirty-one of these tumors were examined in serologic tests; and as is seen in Table II, part II, all 31 tumors contained antigen which fixed complement with serum obtained from SV40 tumor bearing hamsters (titer 1:8 to >1:64), but only one of them fixed complement with serum from adenovirus type 7 tumor bearing hamsters (titer 1:8). The remaining 3 tumors were not examined in serologic tests. At the second transplantation level, tumors obtained from 7 hamsters contained SV40 tumor antigen (titers 1:8 and 1:16), but they did not contain detectable levels of the adenovirus type 7 tumor antigen. Thus, in this transplantation series,

TABLE II. Results Obtained in CF Tests with Serum and Tumor Antigen from Hamsters with Tumors Induced by Adenovirus Type 3 Strain JF-2.

Part I. Serum obtained from hamsters with primary tumors

Hamster	Days between: Inoculation and appearance of first tumor	Days between: Tumor appearance and serum harvest	Reciprocal of antibody titer in tests with: Adenovirus type 7 antigens: T	Adenovirus type 7 antigens: Tumor	SV_{40} antigens: T	SV_{40} antigens: Tumor
9	330	12	80	10	0*	10
10	347	66	80	20	10	20
11	395	50	80	40	>80	>80

* 0 = <1:10

Part II. Serum and tumor antigen obtained from hamsters with transplanted tumors

Number hamsters positive/Number hamsters examined

Transplantation level	For tumor antigen reactive in tests with: Adeno 7 serum	For tumor antigen reactive in tests with: SV_{40} serum	For serum antibody reactive in tests with: Adeno 7 antigens: T	Adeno 7 antigens: Tumor	SV_{40} antigens: T	SV_{40} antigens: Tumor
1st	1/31 (titer 1:8)	31/31 (1:8 to >1:64)	0/37	0/37	19/37 (1:10 to 1:20)	19/37 (1:10 to 1:20)
2nd	0/7	7/7 (1:8 to 1:16)	ND	ND	ND	ND

ND = not done

SV40 antigen was detectable in all tumors examined; whereas, the adenovirus tumor antigen was found in only one.

Sera from the 3 hamsters bearing primary tumors were examined in complement fixation tests with adenovirus type 7 and with SV40 T and tumor antigens. All 3 of these sera fixed complement in titers of 1:10 to >1:80 with both adenovirus 7 and SV40 tumor antigens. However, of 37 terminal sera obtained from hamsters at the first transplantation level, 19 contained antibodies against SV40 T and tumor antigens (titers 1:10 and 1:20), but none of the 37 sera contained antibodies against adenovirus type 7 T and tumor antigens. As observed in tests with sera obtained from hamsters employed in the study of lot JF-1, none of the sera from the 3 hamsters with primary tumors and none of the sera examined from 6 hamsters with tumors at the first transplantation level contained SV40 neutralizing antibody.

SV40 antigen induction studied by fluorescent antibody tests. Adenovirus type 3 lots JF-1 and JF-2 were tested for their ability to induce in HEK cells the formation of antigen reactive in fluorescent antibody tests with SV40 tumored hamster sera (T antigen). The data presented in Table III show that inoculation of either lot into HEK cell cultures was followed by the induction of immunofluorescent SV40 T antigen in a proportion of the cells(*2,12*). The induction of this antigen was not prevented by incubation of the virus with SV40 rabbit antiserum prior to inoculation of the HEK cell cultures. On the other hand, the fluorescent SV40 T antigen was not detected in cells inoculated with JF strain virus which had been incubated with adenovirus type 3 rabbit antiserum or in uninoculated control cells.

Discussion. It has been postulated that adenovirus type 7 strain LL became "hybrid-

TABLE III. Fluorescent Antibody Test Results with HEK Cells Infected with Adenovirus Type 3 Strains JF-1 or JF-2.

Rabbit serum in cell culture fluid	% of cells stained with SV_{40} tumored hamster serum: JF-1*	JF-2†
Normal	2.9‡	1.3
Anti-SV_{40}	2.8	1.1
Anti-adenovirus type 3	0	0

* Cells harvested 24 hr after infection.
† Cells harvested 48 hr after infection.
‡ Percentage of approximately 2000 cells which contained specific immunofluorescent antigen.
HEK = human embryo kidney.

ized" with at least a portion of SV40 viral genome as a consequence of prolonged passage in RhMK cell cultures contaminated with SV40 virus(1-3). The demonstration of SV40 tumor antigen-inducing information in adenovirus type 3 JF strain which contained no infectious SV40 emphasizes the need to test exhaustively adenovirus vaccines not only for intact SV40 virus but also to detect the presence of adenovirus particles which may have incorporated SV40 genetic material. The observation that induction of SV40 T antigen in cultured HEK cells was prevented by treatment with adenovirus type 3 antiserum but not SV40 antiserum, is direct evidence that SV40 antigen inducing genetic material was protected from the action of SV40 antiserum by being enclosed within an adenovirus type 3 capsid(1-4,13,14).

Information is not available on which to base a determination of the importance of the current findings so far as use of adenovirus vaccines for immunization of man is concerned. While it was demonstrated that oral (6) or intranasal(15) administration of infectious SV40 to man was followed by virus multiplication and the production of SV40 neutralizing antibodies, preliminary findings in our laboratory (Morris, J. A. and Jackson, G. G., in preparation) indicated that parenteral inoculation of man with inactivated virus vaccines prepared from adenovirus types 3 and 7 containing SV40 genetic material, but no infectious SV40, did not elicit neutralizing antibodies to SV40. On the other hand, the present studies and others(1) showed that these same adenovirus materials induced tumors in hamsters which did not result in antivirion antibodies to SV40. Therefore, the absence of neutralizing antibodies to SV40 in man may prove to have little relevance to the question on oncogenesis.

Summary. Adenovirus type 3 strain JF was propagated in its early passage history in rhesus monkey kidney cell cultures. Upon discovery of infectious SV40 in the adenovirus, further passages were carried out in African green monkey kidney cell cultures in the presence of SV40 antiserum. Following this procedure, two separate passage lines of the adenovirus were free of detectable infectious SV40, but vaccine seeds prepared from these lines (JF-1 and JF-2) induced in hamsters tumors which contained CF antigens identical to those found in tumors induced by SV40. These tumors, in turn, elicited serum antibodies that fixed complement with SV40 tumor and T antigens. In addition, both lots of adenovirus 3 strain JF induced the formation in human embryo kidney cells of SV40 antigen which was detected by the immunofluorescent technique. The induction of fluorescent-stainable SV40 T antigen in tissue culture was prevented by treatment of the virus with adenovirus type 3 neutralizing antiserum but not by treatment with SV40 neutralizing antiserum. This SV40 genetic material was presumably enclosed within adenovirus capsids. The findings are discussed as they related to the use of adenovirus vaccines in man.

The authors are indebted to Mr. Horace C. Turner for the performance of the complement fixation tests and to Mrs. Lena Wetherell for technical assistance.

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Received February 8, 1966. P.S.E.B.M., 1966, v122.