Interruption of SV_{40} Virus Tumorigenesis Using Irradiated Homologous Tumor Antigen.* (29717)

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Conclusive evidence(1-4) has been presented to indicate that virus-induced tumors, including SV_{40}, possess new antigens. Certain of these antigens appear to be synthesized by the cell under the influence of the viral genetic code. Such difference in antigenic constitution of the cells provides an immunologic basis for prophylaxis or therapy in tumorigenesis of this sort.

Recently, our group(5) described the development of a method for measuring protection against oncogenesis in the SV_{40} and polyoma hamster systems. By this method, hamsters were infected with virus when newborn and were immunized with homologous tumor during the latent period between virus inoculation and appearance of tumor. First studies were carried out using homologous "Bjorklund-type"(6-9) lipoprotein tumor antigen in mineral oil adjuvant and aqueous formulations and no protection was afforded. These studies have been continued to include the testing, in the model system of X-irradiated homologous SV_{40} tumor cell antigen preparations made from whole tumor or from cell cultures of tumor. A definite protective effect against SV_{40} virus-induced tumorigenesis was shown and is recorded here.

Materials and methods. Virus and cell culture. Prototype strain VA 45-54 of SV_{40} virus(10) propagated in cell cultures of grivet monkey kidney (GMK) was used to induce tumors by inoculation into newborn hamsters. Methods for virus propagation and for preparation of GMK cell cultures were described earlier(11,12). *SV_{40} and polyoma transplant tumors.* Hamster SV_{40} tumor line F5-1(13) was transplanted *via* trocar into the dorsal subcutaneous space in 1- to 3-month-old hamsters. The tumors from this transplant line were free of detectable infectious virus. The polyoma tumor line was obtained initially from Dr. B. Eddy and was routinely transplanted in this laboratory by the same procedure as for SV_{40} tumor. All hamsters used in these studies were obtained from the Lakeview Hamster Colony, Newfield, N. J. *SV_{40} tumor cell culture.* Primary SV_{40} tumor F5-1 was propagated serially in monolayer cell cultures employing medium 199 with 5% calf serum. SV_{40} virus was detected in the fluid of the primary culture but all subsequent passages were free of detectable infectious virus. *Preparation of immunizing antigens.* These were prepared from hamster SV_{40} or polyoma transplant tumor or from the SV_{40} hamster tumor cell culture line. The SV_{40} and polyoma hamster tumors were excised, trimmed free of apparent normal or necrotic tissue, minced with a scissors, and passed through a stainless steel tumor press.§ The brei was diluted with Hanks' balanced salt solution (HBSS) in a ratio of 20 g of tumor per 100 ml volume of medium. This was irradiated in 30 to 40 ml amounts in plastic petri dishes of 100 mm diameter. The cell cultures of SV_{40} tumor were trypsinized and suspended in HBSS in a concentration of 5×10^6 cells per ml. Thirty to 40 ml volumes were distributed into plastic petri plates for irradiation. *X-irradiation.* This was performed at Wistar Institute‖ using a Keleket X-ray machine. Irradiation was carried out at 200 KV, 20 MA at a distance of 30 to 40 cm. No filters were used and irradiation was standardized in the usual procedure employing an ionization probe. *Viability tests.* Ir-

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TABLE I. Tumorigenic Quality for Hamsters of SV_{40} Hamster Tumor F5-1 Cultivated *in vitro*.

Inoculation of cells			Tumor occurrence, cumulative, according to time* in days after inoc						
Age of hamster	No. cells inoc	Route of inoc	25	50	75	100	150	300	%
<24 hr	10,000	Subcut	12/14†	13/14	14/14				(100)
	"	Intraper	1/20	14/17	17/17				(100)
	1,000	Subcut	5/9	6/9	8/9	9/9			(100)
	"	Intraper	—	4/16	8/14	11/14		12/14	(84)
	100	Subcut	—	3/20	9/20	14/20	17/20	17/20	(85)
	"	Intraper	1/17	3/17	5/15	9/15	11/12	11/12	(92)
	10	Subcut	—	—	—	1/10	4/10	4/9	(44)
	"	Intraper	—	—	1/16	5/15	6/14	9/13	(69)
1 mo	10,000	Subcut	—	5/5					
	"	Intraper	—	3/5	4/5	4/5	4/5	4/5	
	1,000	Subcut	—	3/5	3/5	3/5	4/5	4/5	
	"	Intraper	—	2/3	2/3	3/3			
	100	Subcut	—	1/5	1/5	2/5	2/4	2/3	
	"	Intraper	—	—	1/3	2/3	3/3		
	10	Subcut						0/4	
	"	Intraper						0/5	

* Approximate, within a few days of time period shown.
† Cumulative total with tumor/total survivors, including those with tumor.

radiated tumor or cell culture preparations were tested for viability by either dorsal subcutaneous or intraperitoneal inoculation of hamsters 1 to 3 months of age. The animals were observed for the time periods shown in the text for development of tumor.

Experimental design. Newborn hamsters less than 24 hours of age were each inoculated subcutaneously into the interscapular space with 10^6 to 10^7 TCD_{50} of SV_{40} virus in 0.2 ml volume. At indicated time intervals, the animals in *each litter* were divided into antigen and control groups, marked individually for identification, and given a single dose of immunizing antigen or appropriate control material. Cell culture irradiated antigen consisted of 5×10^6 cells in 0.2 ml or 1.0 ml volume and the irradiated tumor brei was in 1.0 ml volume. Such materials were injected into the peritoneal cavity. Each litter was housed in separate isolation filter cages from birth until the time of being given immunizing antigen. Thereafter, they were housed, as littermates, in open cages. *Adult hamsters* 4 to 6 weeks of age, which had not received virus when newborn, were given immunizing antigen or control material in 1.0 ml volume *via* the intraperitoneal route and were challenged later with SV_{40} tumor cell culture suspension or with freshly minced SV_{40} hamster tumor tissue *via* trocar. All transplant challenge material was given subcutaneously into the interscapular space. *Observations.* Animal care, clinical observations and pathologic examinations were carried out as described previously(5,11,12). All animals were palpated at least one time each week for presence of masses. In certain instances, the X-irradiation of immunizing antigen was insufficient to destroy the tumorigenicity. It was possible to differentiate such "immunization" tumors from virus-induced tumors by noting location. Thus, the virus-induced tumors appeared in the dorsal subcutaneous space while the transplant tumors resulting from the incompletely inactivated antigen appeared in the peritoneal cavity. All animals which died in the tests were autopsied to look for vaccine-induced peritoneal tumors.

Results. Tumorigenicity of SV_{40} tumor F5-1. Hamster SV_{40} tumor F5-1, either as transplant tumor or as tissue culture, was used to prepare all SV_{40} immunizing antigens and for challenge purpose. Findings in tests to measure the tumorigenic quality of F5-1 tumor grown in cell culture and tested in newborn and 1-month-old hamsters are summarized in Table I. This line proved very tumorigenic

TABLE II. X-ray Sensitivity of Early and Late Passage of SV_{40} Hamster Tumor F5-1 Propagated in Cell Culture.

X-ray dose (r)	F5-1 tumor passed 9 times *in vitro* — Tumor occurrence according to day post-inoc 20	42	66	F5-1 tumor passed 28 times *in vitro* — Tumor occurrence according to day post-inoc 20	42	66	Challenge‡ of survivors on day 44 — Tumor occurrence according to day post-challenge 40	85	233	%
10,000	0/4*	0/4	0/4	0/5	0/5	0/5	3/9	5/8	6/8	(75)
8,000	0/4	0/4	0/4	0/5	0/5	0/5	3/9	7/9	7/9	(78)
6,000	—	—	—	0/5	0/5	0/5	0/5	3/5	3/5	(60)
4,000	0/5	0/5	0/5	0/5	0/5	0/5	4/10	5/10	5/10	(50)
2,000	0/5	0/5	0/5†	0/5	0/5	0/5†	0/5	1/10	2/10	(20)
1,000	1/5	5/5	5/5	2/5	5/5	5/5	—	—	—	
500	2/5	5/5	5/5	3/5	4/5	5/5	—	—	—	
0	2/5	5/5	5/5	—	—	—	—	—	—	
Challenge controls	—	—	—	—	—	—	8/10	10/10	10/10	(100)

* Cumulative number with tumor/total survivors including those with tumor.

† In a repeat exp 4/30 and 16/20, respectively, developed tumor following irradiation at this level.

‡ With live tumor tissue culture cells F5-1; 5×10^3.

when implanted *via* the subcutaneous or intraperitoneal routes in animals of either age. As few as 10 cells sufficed to establish tumor in newborn animals while 100 cells were required for the older animals.

X-ray sensitivity of F5-1 tumor used to prepare immunizing antigens. The radiosensitivity of the oncogenicity of 9th and 28th cell culture passage of F5-1 tumor was measured in tests in 4- to 6-week-old hamsters. Table II shows that 2,000 r sufficed to destroy the tumorigenic quality of both passage levels of the tumor in cell culture when 5×10^6 cells were injected into the animals. This value was not absolute since, in other experiments, tumors developed in animals inoculated with tumor cells given the same amount of irradiation.

Animals which survived for 44 days after inoculation with irradiated cells were challenged with live tumor tissue culture cells. There was an indication of decreasing protection against tumor formation with increasing irradiation of the original cells (Table II).

The data presented in Table III showed that minced F5-1 transplant tumor required more irradiation to effect loss of oncogenicity than did the tumor cells grown *in vitro*. Three thousand r sufficed to inactivate most cells but even 4,000 r resulted in failure to inactivate in one instance.

Minimal irradiation was used to prepare immunizing antigen so as to minimize possible destruction of antigen. Two thousand or 3,000 r were routinely employed for this purpose. Additional experiments appear necessary to establish that level of irradiation which will guarantee inactivation but will permit retention of immunizing ability.

Immunization of hamsters against SV_{40} virus tumorigenesis using X-irradiated tumor cell antigens. The protective effect of irradiated cell culture or whole tumor antigen against SV_{40} virus tumorigenesis was measured in tests in which the antigen was given 34, 48 or 76 days following virus. Protective efficacy was measured in terms of rate of tu-

TABLE III. X-ray Sensitivity of SV_{40} Hamster Transplant Tumor F5-1.

X-ray dose (r)	Exp 18—0.2 ml dose given intraperitoneally — Tumor occurrence according to day post-inoc 11	70	Exp 23—1.0 ml dose given intraperitoneally — Tumor occurrence according to day post-inoc 11	70
4,000	0/10*	1/7	0/10	0/10
3,500	—	—	0/10	0/10
3,000	0/10	1/7	0/10	0/8
2,000	0/10	7/7	—	—
1,000	0/10	10/10	—	—
0	10/10	10/10	2/10	10/10

* Cumulative total with tumor/total survivors including those with tumor.

TABLE IV. Protective Efficacy of Administration of X-irradiated SV_{40} Hamster Tumor Cell Culture Antigen Given on 76th Day Following Administration of SV_{40} Virus to Newborn Hamsters. The SV_{40} tumor antigen was prepared from cell culture passage 38 of F5-1 tumor.

	Cumulative occurrence of tumors				
	SV_{40} inoculated animals given				Cell antigen control
Days after SV_{40} virus was given	X-ray treated tumor cell antigen No. tumors /total*	Adj %†	X-ray treated Hanks' BSS (control) No. tumors /total*	Adj %†	Animals given X-ray treated tumor cell antigen only
1	0/22		0/21		0/8
97	1/22		3/20		0/8
152	3/22		12/20		0/8
180	4/21		16/19		0/8
328	5/18		17/19		0/8
375	6/18	30	18/19	94	—
487	8/16	42	19/19	98	—

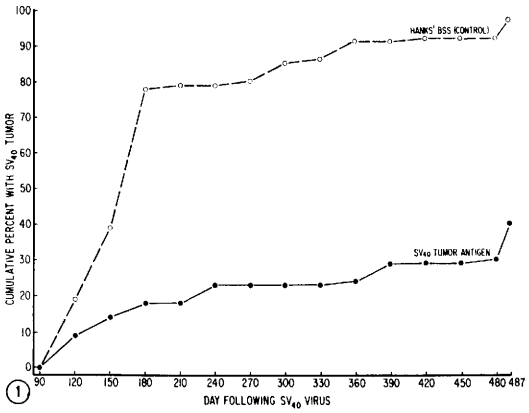
Differences (95% confidence limits): Day 375, 64 ± 24%; Day 487, 56 ± 23%

* Number with tumor/total survivors at time period shown including all animals with tumor.
† Percentage of animals which developed tumor. Values adjusted for nonspecific deaths based on standard life table procedures.

mor development and in absolute numbers of tumors which appeared in immunized compared with control groups of animals. The denominators in the fractions in the Tables show the reduction in population due to death from nonspecific cause (noncancer). In the analyses in Tables and Figures, the percentages were adjusted for nonspecific deaths according to standard life table procedures. Percentage values shown in the Tables were derived from calculations of a small number of points and are approximately the same as the percentages based on the more complete data from which the curves shown in the Figures were derived.

Table IV shows a 2- or 3-fold reduction in tumor incidence resulting from immunization with cell culture material on day 76 following virus. None of the animals developed tumor resulting from injection of irradiated cells. These same findings are presented in greater detail in Fig. 1 in which data relating to a greater number of time periods of observation are shown. The differences were highly significant.

Irradiated SV_{40} tumor cell culture antigen given on day 34 following virus was also highly protective as shown in Table V and in Fig. 2. The immunizing cell preparation was not totally inactivated. Three tumors ap-



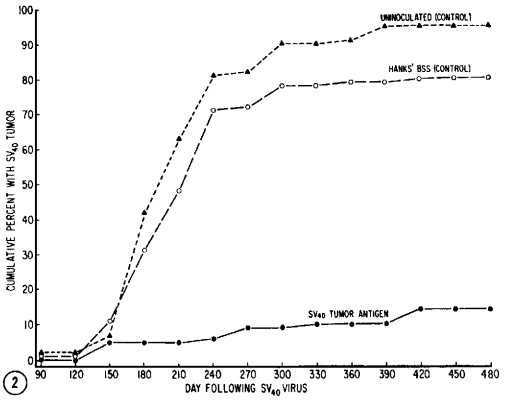


FIG. 1. See Table IV. Protective efficacy of SV_{40} tumor antigen administered on day 76 following SV_{40} virus.
FIG. 2. See Table V. Protective efficacy of SV_{40} tumor antigen administered on day 34 following SV_{40} virus.

TABLE V. Protective Efficacy of Administration of X-irradiated SV_{40} Hamster Tumor Cell Culture Antigen Given on 34th Day Following Administration of SV_{40} Virus to Newborn Hamsters. SV_{40} tumor antigen was prepared from cell culture passage 9 of F5-1 tumor.

	Cumulative occurrence of tumors						
	SV_{40} inoculated animals given						Cell antigen control
Days after SV_{40} virus was given	X-ray treated tumor cell antigen		X-ray treated Hanks' BSS (control)		Nothing (control)		Animals given X-ray treated tumor cell antigen only
	No. tumors /total*	Adj %†	No. tumors /total*	Adj %†	No. tumors /total*	Adj %†	
1	0/39		0/37		0/29		0/31
98	1/39		0/34		0/29		0/27
151	4/34		7/34		2/29		1/27
186	4/34		11/34		15/28		1/25
375	6/18		27/32		26/26		1/17
480	7/18	18	28/30	88	26/26	96	2/15
Differences (95% confidence limits): Tumor cell antigen: Hanks' BSS, 70 ± 20% Tumor cell antigen: Nothing (control), 78 ± 19%							

* Number with tumor/total survivors at time period shown including all animals with tumor.
† Percentage of animals which developed tumor. Values adjusted for nonspecific deaths based on standard life table procedures.
Corrected for 3 cell antigen transplant tumors which developed at peritoneal site on days 98, 145 and 242.

TABLE VI. Protective Efficacy of Administration of X-irradiated SV_{40} Hamster Transplant Tumor Antigen Given on 48th Day Following Administration of SV_{40} Virus to Newborn Hamsters. SV_{40} tumor antigen was prepared from transplant passage 20 tumor.

	Cumulative occurrence of tumors							
	SV_{40} inoculated animals given						Antigen control	
Days after SV_{40} virus was given	X-ray treated SV_{40} tumor antigen		X-ray treated polyoma tumor antigen		Nothing (control)		Animals given tumor antigen only	
	No. tumors /total*	Adj %†	No. tumors /total*	Adj %†	No. tumors /total*	Adj %†	SV_{40}	Polyoma
20	0/28		0/27		0/17		0/7	0/9
97	1/28		0/25		0/17		0/7	0/9
151	2/28		4/23		1/16		0/7	0/9
186	2/28		10/23		8/14		0/7	0/8
291	5/21	13	16/21	74	14/14	93	0/3	0/4
Differences (95% confidence limits): SV_{40} tumor cell antigen: polyoma tumor antigen 61 ± 23% SV_{40} tumor cell antigen: nothing (control) 80 ± 19%								

* Number with tumor/total survivors at time period shown including all animals with tumor.
† Percentage of animals which developed tumor. Values adjusted for nonspecific deaths based on standard life table procedures.
Corrected for 2 SV_{40} cell antigen transplant tumors which developed at peritoneal site on days 97 and 111.

peared in the SV_{40}-inoculated animals at the peritoneal site of injection of the immunizing preparation. Percentage values given in the Table and in the Figure were corrected to include only SV_{40} virus-induced tumor.

Table VI and Fig. 3 summarize findings obtained in studies in which hamsters were immunized on day 48 with transplant tumor antigen and in which irradiated transplant polyoma tumor antigen was included for control purpose. SV_{40} transplant tumor antigen, like cell culture antigen, was protective. Heterologous polyoma tumor antigen did not afford immunity against development of virus-induced SV_{40} tumor. The percentage values given in Table and in Figure were corrected to exclude 2 tumors which were derived from the tumor antigen.

Immunization of adult hamsters against SV_{40} transplant tumor using X-irradiated F5-1 cell culture antigen. Table VII shows that the immunization procedure was ineffective

TABLE VII. Protective Efficacy of Administration of X-irradiated SV_{40} Hamster Tumor F5-1 Cell Culture Antigen Given 35 Days Prior to Transplant of F5-1 SV_{40} Tumor Mince or Tumor Cells Grown in Culture.

Days after transplant challenge	Cumulative occurrence of tumors, according to challenge					
	Tumor mince		5,000 cultivated cells		1,000 cultivated cells	
	Received antigen	Control (no antigen)	Received antigen	Control (no antigen)	Received antigen	Control (no antigen)
14	10/10*	0/10	1/9	0/9	0/10	0/9
26	"	10/10	3/7	9/9	0/8	6/9
46	"	"	4/7	"	0/6	6/9
84	"	"	6/7	"	0/5	6/9
189	"	"	6/7	"	1/5	7/8

Difference, Antigen:Control (95% confidence limits)

5,000 cells—Day 26	63 ± 35%
Day 189	16 ± 30%
1,000 cells—Day 26	67 ± 33%
Day 189	67 ± 37%

* Number with tumor/total survivors including those with tumor.

in protecting against transplant whole minced tumor. There was a slight delay in time of tumor appearance in animals given immunizing antigen and challenged with 5,000 tumor tissue culture cells. A significant protective effect (day 189) was obtained when only 1,000 cells were used for challenge.

Discussion. Other investigators have established the presence of unique antigen in virus-induced tumor(1-4, 14-16), the retention of antigenicity of tumor antigen upon X-irradiation(17-19), and the protective effect of immunization with homologous tumor antigen or of early virus infection against subsequent homologous tumor transplant(1, 3,4,14-16). The present studies demonstrated protection against SV_{40} virus-induced tumor in hamsters by irradiated homologous tumor antigen administered subsequent to virus but prior to first appearance of virus-induced tumor. The studies also confirmed the earlier findings of others(1,3,4,14-16) who showed a protective effect of immunization with homologous tumor against a small, but not a large dose of homologous transplant tumor challenge.

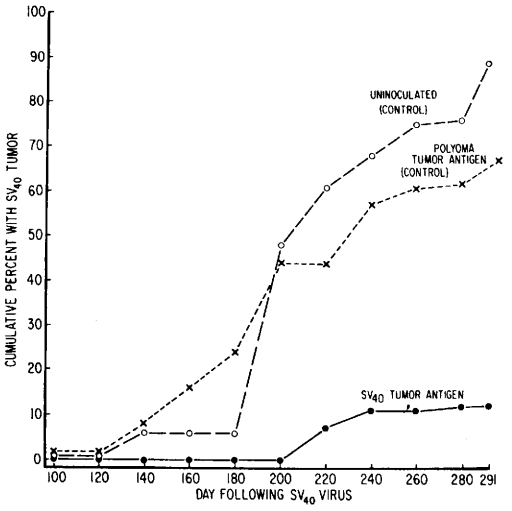


FIG. 3. See Table VI. Protective efficacy of SV_{40} tumor antigen administered on day 48 following SV_{40} virus.

The model system used made possible the measurement of potential for immunization against the consequence of oncogenic virus infection established early in life and is judged to provide a parallel for hypothetical virus-induced tumor as it might occur in the human species. A special advantage was achieved in the model system in that the immunizing antigen was given when the virus-induced tumor mass must have been quite small and vulnerable to the immunologic mechanism. The hamster system was considered to be immunologically isologous for all practical purposes since these animals are uniformly and completely histocompatible with respect to transplantation antigens(20).

The high degree of specificity shown in the protection afforded by homologous SV_{40} but not by heterologous polyoma tumor antigen was consistent with the findings of others(3, 21,22). The immunizing antigen in SV_{40} hamster tumor was retained for at least 20 trans-

plant passages in hamsters and in cell culture for at least 38 passages. The antigen was effective in preventing virus-induced tumor when given in a single dose as early as 34 days or as late as 76 days following SV_{40} virus. First virus-induced tumor usually occurred about the 90th day after SV_{40} virus was given.

The precise level of X-irradiation required to destroy *uniformly* the oncogenicity of SV_{40} tumor cells was not established. The amount of irradiation employed in the present investigation was either just sufficient to destroy tumorigenic capacity of the cells completely or slightly less than the minimal quantity needed. Tumors derived by transplant from the immunizing antigen were, however, few in number and were readily distinguished from the virus-induced tumor by the peritoneal location of the former. The limit of sensitivity of the immunizing antigen to destruction was not measured, except in one preliminary experiment, which seemed to indicate that increasing levels of irradiation resulted in less protection of hamsters after challenge with live tumor cells. The sensitivity of the immunizing antigen to excessive irradiation might explain the lack of resistance induced by X-irradiated polyoma tumor cells in Sjogren studies where 8,000 and 15,000 r were employed(23,24). Whether tumor cells receiving sufficiently heavy irradiation to guarantee uniform total destruction of viability are effective for immunization remains to be determined, but seems likely from these results.

Summary. X-irradiated SV_{40} transplant tumor or cell culture antigens were highly effective in preventing occurrence of homologous tumor in hamsters inoculated with SV_{40} virus when newborn. The X-irradiated immunizing tumor antigen was effective when given in a single dose as early as 34 days or as late as 76 days after SV_{40} virus was administered. The immunizing antigen was retained in tumor transplants in hamster for at least 20 passages and in cell culture of tumor for at least 38 passages. The significance of the findings in relation to protection against the hypothetical virus-induced tumor in man is discussed.

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