Factors Influencing Tumor Induction in Hamsters by Vacuolating Virus, SV_{40}.* (28133)

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The vacuolating agent, SV_{40}, was first discovered as an indigenous contaminant of primary cell cultures of rhesus and cynomolgus monkey kidney and of oral poliovirus vaccine (1,2). Subsequent findings revealed an oncogenic quality of the virus with induction of fibrosarcomatous neoplasms and ependymomas(3-5) in newborn hamsters and of papillary ependymomas(6) in newborn Mastomys. The oncogenic quality of the virus has become more understandable in the recent demonstration of its close biologic relationship to other tumorigenic agents including papilloma viruses of man and rabbits and of the mouse polyoma agent(7). The virus produces subclinical infection in human subjects following respiratory inoculation or oral ingestion and is excreted into the feces subsequent to inoculation by the latter route(8,9). In primary embryonic, neonatal or adult human cells in culture, the virus causes epithelioid transformation with consistent chromosomal aberration of a probable heritable nature(10-13).

The importance of SV_{40} virus as a malignant oncogenic agent prompted descriptive studies of the pathogenesis of the virus in the hamster model. The present report presents findings in investigations to measure the effect of host age, virus dose, and route of inoculation on oncogenic efficiency and to ascertain the transmissibility of the agent by contact.

Materials and methods. The detailed methods described previously(3) were generally followed. *Virus strain* VA 45-54 derived from uninoculated grivet monkey kidney (GMK) cell culture was used. Virus in first subculture passage was employed and exhaustive tests revealed that SV_{40} was the only detectable agent present. The infected cell cultures were frozen and thawed several times at time of harvest and the virus pool was clarified at 2000 rpm for 20 minutes in the horizontal centrifuge. The supernate used in the animal experiments titered 10^{-6} per 0.2 ml in GMK cell cultures (10 day reading) and was stored frozen in flame-sealed glass ampules in the dry ice refrigerator. *Control tissue culture fluid* (NTCF) was prepared in similar manner from uninoculated cell cultures of the same lot of GMK and was stored as described above. *Control medium* (CM), composed of medium 199 containing 2% of heat-inactivated chicken serum, pH 7.0, was the same lot used for maintenance of the cell cultures described above and was stored in similar fashion. *Hamsters* were from the Lakeview Hamster Colony, Lakeview, N. J., and were inoculated when newborn or 7, 30 or 90 days of age. The virus dose was 0.2 ml given subcutaneously unless otherwise indicated. The virus was titrated for infectivity in GMK each time it was used. When diluted for hamster inoculation, medium CM was employed. Animal care, clinical observations, and pathologic examinations were as described previously. *Serology.* Hamster sera were tested for neutralizing antibody using 30-300 TCD_{50} of strain 45-54 of SV_{40} virus. The sera were inactivated at 56°C for 45 minutes and incubation was at 37°C for 45 minutes and overnight at 4°C. The inoculum dose was 0.2 ml of the virus-serum mixtures and the cultures were read on the 20th day following inoculation. The titer was the highest initial dilution of serum which effected at least 50% suppression of viral cytopathology.

Results. Effect of age. Table I shows clearly the influence of age on tumor incidence following subcutaneous administration of SV_{40} virus. This ranged from 96% in the newborn animals to 60% in 7 day, 23% in 1 month and 0% in 3 month old hamsters. Similar influence of age on time of tumor appearance was shown, the averages being 201, 314 and 360 days for the newborn, 7 day and

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TABLE I. Influence of Age on Tumorigenesis of SV_{40} Virus in Hamsters.

Age of host	Inoculum: Kind	Inoculum: Infect titer (.2 ml)	Results (16 mo): No. weaned or inoc'd*	Results (16 mo): No. non-specific deaths	Results (16 mo): Tumors in survivors: No. without	Results (16 mo): Tumors in survivors: No. with	Results (16 mo): Time of tumor appearance (days): Earliest	Latest	Avg
Newborn	SV_{40} virus	$10^{-5.75}$	61	7	2	52 (96%)	115	403	201
	NTCF†	$<10^{0}$	61	28	33	0			
	Control medium	$<10^{0}$	46	22	24	0			
	Uninoculated	—	74	20	54	0			
7 days	SV_{40} virus	$10^{-5.5}$	69	29	16	24 (60%)	178	487	314
	NTCF	$<10^{0}$	40	20	20	0			
	Control medium	$<10^{0}$	47	30	17	0			
	Uninoculated	—	74	20	54	0			
1 mo	SV_{40} virus	$10^{-6.0}$	48	22	20	6 (23%)	188	494	360
	NTCF	$<10^{0}$	59	33	26	0			
	Control medium	$<10^{0}$	50	30	20	0			
	Uninoculated	—	50	37	13	0			
3 mo	SV_{40} virus	$10^{-6.0}$	60	25	35	0 (0%)	—	—	—
	NTCF	$<10^{0}$	60	27	33	0			
	Control medium	$<10^{0}$	60	18	42	0			
	Uninoculated	—	58	23	35	0			

* For 0 and 7 day animals, equals No. weaned; for 1 and 3 mo animals, equals No. inoculated.
† NTCF is fluid from uninfected grivet monkey kidney cell cultures; control medium is unused culture maintenance medium.

1 month groups, respectively. None of the animals in the control groups which received uninfected cell culture fluid (NTCF), cell culture maintenance medium (CM) or which were held uninoculated developed tumors. There was considerable variation in the numbers of non-specific deaths among the groups but this appeared to be unrelated to the inoculum given.

The fate of the tumor-bearing animals was similar irrespective of age at time of inoculation. Thus, all tumors grew rapidly, the hosts succumbed, and no regressions were observed. Histopathologic examination indicated a similar type neoplasia among tumors in animals of all 3 groups. "Metastases" to the lungs and/or kidneys were observed in 5/52 animals of the newborn group, 1/24 animals of the 7 day group, and 0/6 animals of the 1 month group. The numbers of tumor-bearing animals in the various groups are insufficient to assess definitively the significance of these differences.

Serologic findings. The cytopathic effect of SV_{40} virus, as measured in cell culture systems, is neutralized readily by homologous immune serum(1,2). The oncogenic quality of the virus for newborn hamsters was also readily neutralized by specific antiserum (Table II).

To ascertain whether presence or absence of serologic response to SV_{40} virus in inoculated hamsters could be correlated with tumor presence or absence, sera from tumor and

TABLE II. Neutralization by Homologous Antiserum of Oncogenic Quality of SV_{40} Virus for Newborn Hamsters.

Inoculum given*	Results obtained in hamsters (day 300): No. weaned	No. non-specific deaths	Tumors in survivors: No. without	No. with
SV_{40} virus + pre-immune serum	25	2	1	22 (96%)
SV_{40} virus + immune serum	31	7	24	0 (0%)

* Inoculum dose was 0.2 ml given subcutaneously and consisted of a mixture, in equal amounts, of SV_{40} virus and rabbit serum. SV_{40} virus titer was $10^{-6.0}$ per 0.1 ml and immune serum titer was 1:64.

The rabbit was immunized using SV_{40} virus grown in human embryo kidney cell culture. The virus-serum mixtures were incubated for 2 hr at 37°C prior to inoculation.

TABLE III. Occurrence of SV_{40} Neutralizing Antibody in Sera of Tumor and Non-Tumor Bearing Hamsters Inoculated at Various Ages and Bled 13 Months after Virus Was Given.

Age of host at time of inoculation	SV_{40} neutralizing antibody incidence*	
	Tumor-bearing animals	Non-tumor bearing animals
Newborn	4/9	0/2
7 days	0/8	2/22
1 mo	1/3	5/26
Total group	5/20 (25%)	7/50 (14%)

* Sera were tested diluted 1:3 against 100 TCD_{50} of SV_{40} virus.

TABLE IV. Occurrence of SV_{40} Neutralizing Antibody in Sera of Hamsters Inoculated at Various Ages and Bled 1 Month after Virus Was Given.

Age of host at time of inoculation	SV_{40} antibody incidence*
Newborn	0/19
7 days	8/20
14 "	10/12
21 "	14/16

* Sera were tested diluted 1:4 against 100 TCD_{50} of SV_{40} virus.

non-tumor bearing animals collected 13 months after virus inoculation were assayed for SV_{40} neutralizing antibody. The sera were diluted 1:3 and 100 TCD_{50} of SV_{40} were used. Table III shows that a detectable SV_{40} antibody response occurred among non-tumor as well as among tumor-bearing animals. The over-all incidence of antibody was roughly comparable considering the size of the 2 groups and tumor incidence appeared to be unrelated to whether there was or was not a concomitant SV_{40} antibody response.

In another experiment, the immunologic responsiveness of hamsters, according to age when inoculated with SV_{40} virus, was measured in tests in which the sera were collected 1 month after virus inoculation. Increasing responsiveness with increasing age was found (Table IV). These findings indicated that immunologic competence for SV_{40} virus was age-related. The high serologic conversion rate among older animals in Table IV contrasted with a lower rate among similar animals in Table III suggested that antibody titers in the latter group might have been decreased to non-detectable levels by the end of 13 months.

Effect of virus dose. Newborn hamsters were inoculated subcutaneously with 320,000, 3,200 or 32 TCD_{50} of unfiltered or with 1000 TCD_{50} of Selas (02)-filtered SV_{40} virus in 0.2 ml amount. Appropriate controls were included. Table V shows a definite relationship between virus dose and tumor occurrence. No tumors occurred when only 32 TCD_{50} of virus were given and it appeared that around 1000 TCD_{50} of virus were needed to induce neoplasia in these animals. Time of tumor appearance appeared to be related, also, to virus dose. Filtered as well as unfiltered virus was capable of oncogenesis, showing thereby the primary role of virus rather than infected kidney cells in the neoplastic process.

Effect of route of inoculation. Table VI compares tumor incidences among newborn hamsters given SV_{40} virus by the subcutaneous, intraperitoneal, intranasal or intrapulmonary route. Intranasal virus was given

TABLE V. Influence of SV_{40} Virus Dose on Tumorigenesis in Newborn Hamsters.

Inoculum	No. weaned	Results (16 mo)				
		No. non-specific deaths	Tumors in survivors		Time of tumor appearance (days)	
			No. without	No. with	Earliest	Latest	Avg
Unfiltered SV_{40} virus:							
320,000 TCD_{50}	61	7	2	52 (96%)	115	403	201
3,200 "	50	22	23	5 (18%)	262	416	344
32 "	52	27	25	0 (0%)	—	—	—
Filtered SV_{40} virus:							
1000 TCD_{50}	51	25	23	3 (12%)	242	375	298
NTCF	61	28	33	0	—	—	—
Control medium	46	22	24	0	—	—	—
Uninoculated	74	20	54	0	—	—	—

TABLE VI. Influence of Route of Inoculation on Tumorigenesis of SV_{40} Virus in Hamsters.

Inoculation		Results					
					Tumors in survivors		
Route	SV_{40} virus dose	No. inoculated	No. weaned	No. non-specific deaths	No. without	No. with	Observation period (mo)
Exp 1							
Subcutaneous	600,000 TCD_{50}	82	61	6	6	49 (89%)	10
	Control NTCF	76	61	19	42	0 (0%)	
Intraperitoneal	600,000 TCD_{50}	32	19	0	17	2 (11%)	
	Control NTCF	19	16	5	11	0 (0%)	
Intranasal	40,000 TCD_{50}	40	27	3	24	0 (0%)	
Exp 2							
Subcutaneous	10,000,000 TCD_{50}	—	25	3	5	17 (77%)	7
	Control NTCF	49	44	2	42	0 (0%)	
Intraperitoneal	10,000,000 TCD_{50}	41	27	2	25	0 (0%)	
	Control NTCF	31	28	4	24	0 (0%)	
Intrapulmonary	10,000,000 TCD_{50}	32	19	1	16	2 (11%)	
	Control NTCF	37	24	2	22	0 (0%)	

into the nares under light ether anesthesia. Intrapulmonary virus was inoculated directly into the lungs through the intercostal spaces. Appropriate controls were included. Greatest tumor incidence occurred in hamsters given virus by the subcutaneous route. A small portion of animals developed tumors following intraperitoneal or intrapulmonary inoculation while no tumors followed intranasal instillation. All tumors which developed were located in the subcutaneous space even though the inoculum had been directed at the lung or peritoneal cavity.

Attempts at contact transmission. Studies of contact transmission of SV_{40} virus, as measured by oncogenesis, were undertaken in hamsters given the agent during the newborn or early post weaning period. In one experiment, one-half of each of 4 litters of hamsters was inoculated when newborn and the inoculated and control animals in each litter were housed together in the same cage. In a second experiment, the inoculated and uninoculated animals were housed separately until weaning, when they were mixed and kept in the same cage. All animals were observed for 17 months. No tumors developed in any of the uninoculated controls, even when housed in the same cage immediately following inoculation.

Discussion. These findings demonstrated quite remarkably the importance of both host and virus factors in conditioning tumor development by SV_{40} virus in hamsters. As for the polyoma and for certain of the avian leucosis virus systems(14), host age at time of virus inoculation was a prime factor in conditioning tumor development. Thus, animals which received virus as newborns developed tumors with near 100% frequency whereas this was diminished to a low level by one month and was nil by 3 months of age. The age at time of inoculation did not appear, however, to affect the malignant quality of the resultant tumor since neoplasms, once developed, were all rapidly progressive and no regressions occurred.

Tumor development in hamsters appeared to be unrelated to whether there was or was not a detectable SV_{40} neutralizing antibody response in the host. Thus, tumors occurred in animals which developed antibody as well as in those which did not. It was found, however, that there was a more uniform antibody response to the virus with increasing age at time of inoculation. Failure of antibody to occur in certain of the younger animals was unrelated to induction of immunologic tolerance for the agent since, as shown previously (3), all such animals are capable of response on injection of the virus in later life. Such findings with respect to antibody against the virus do not preclude a possible role of age influence on conditioning immunologic tolerance to SV_{40}-induced tumor cell antigens such as postulated for the polyoma system(15).

TABLE VII. Contact Transmission Studies with SV_{40} Virus in Newborn Hamsters.

Litter	No. weaned		No. with tumors/total survivors (17 mo)	
	SV_{40} inoc'd	Uninoc	SV_{40} inoc'd	Uninoc
Exp 1. Half of each litter inoculated at birth with SV_{40} virus				
A	5	4	3/4	0/2
B	5	4	5/5	0/3
C	4	2	3/3	0/2
D	5	5	5/5	0/5
Total	19	15	16/17 (94%)	0/12 (0%)
Exp. 2. Inoculated and uninoculated animals mixed at weaning				
Total	44	33	30/35 (86%)	0/21 (0%)

Studies relating to the role of antitumor antibody in tumorigenesis are in progress.

The amount of virus required to effect near 100% tumor induction in hamsters was large and was about 300,000 TCD_{50}. Only minimal tumor incidence was obtained when 1000 TCD_{50} were given. These findings suggested a lack of significant SV_{40} virus proliferation in the hamster host and a limited sensitivity of the hamster subcutaneous tissue to malignant transformation by the virus.

One of the more striking findings was the predilection for development of tumors in the subcutaneous tissues of the hamster, even when the virus was given by the intrapulmonary or intraperitoneal route. This might have resulted from inadvertent deposition of SV_{40} virus into the subcutaneous tissue during penetration of the needle into the thoracic or peritoneal cavity. Conversely, it might represent a high degree of specificity in the cell capable of malignant transformation, such susceptible cells being confined to the subcutaneous tissues. The latter possibility is given substance in the findings by Rabson *et al.*(6) who showed development of brain papillary ependymomas in animals of the genus *Rattus* following subcutaneous inoculation of SV_{40} virus.

The failure of development of tumors in uninoculated hamsters which were litter or cage mates of SV_{40}-infected animals was not unexpected in view of the previously demonstrated(3) failure of the virus to appear in the secretions or excretions of such infected animals. Lack of susceptibility to tumor development following nasal infection would also limit the routes by which effective contact transmission could be achieved.

The definition in the present study of the sharply limited conditions for oncogenesis by SV_{40} virus in the hamster, should provide worthwhile guidelines for investigations of the oncogenic potential of other viruses in animal host systems.

Summary. Descriptive studies of the pathogenesis of SV_{40} virus in the hamster model were carried out. Ninety-six per cent of animals given SV_{40} virus when newborn, 60% of 7 day old animals, 23% of 1 month old animals, and none of 3 month old animals developed tumors. Tumors developed earlier, on the average, in the younger animals. All tumors which did develop were highly malignant, irrespective of host age at time of inoculation. Development of SV_{40} tumors in hamsters was unrelated to appearance or absence of SV_{40} antibody although stimulation of such antibody was progressively enhanced with increase in age at time of inoculation. Tumorigenesis was also related to virus dose, 300.000 TCD_{50} being required to effect near 100% efficiency and 1000 TCD_{50} being necessary for minimal response. The subcutaneous route was most efficient for tumor induction. A few tumors followed intrapulmonary or intraperitoneal inoculation and these appeared in the subcutaneous tissue. Litter or cage mate contact transmission of tumorigenesis did not occur. The significance of the findings is discussed.

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A Function of the Eosinophil: Phagocytosis of Antigen-Antibody Complexes. (28134)

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Although it is known that eosinophilia can be readily produced by repeated injections of foreign protein(1) and can also be mediated by injection of immune complexes(2), the function of these cells in hypersensitivity states has heretofore not been demonstrated.

Previous studies have shown that antigen-antibody complexes can be visualized with the electron microscope when the electron dense protein ferritin is used as antigen(3). This technic was employed in the present investigation in an attempt to elucidate the function of eosinophils obtained in peritoneal exudates induced by injection of foreign proteins in previously sensitized animals.

Materials and methods. 1. Method of immunization. Horse spleen ferritin, Nutritional Biochemicals, Cleveland, Ohio, was used to sensitize 2-month-old Hartley strain male guinea pigs. Equal volumes of 0.1% ferritin in normal saline solution and Complete Adjuvant (Freund) Difco Labs, Detroit, Mich., were emulsified. The guinea pigs were immunized by injecting 1 cc of the emulsion into multiple sites in the skin and toe pads. Two weeks later the animals received similar injections with 1 cc of an emulsion of Incomplete Adjuvant (Freund) and ferritin.

2. Specificity of anti-sera for ferritin. This schedule of immunization results in formation of antibodies which are immunoelectrophoretically specific for ferritin(4). The anti-sera form complexes with ferritin which have a characteristic electron microscopic appearance. The adequacy of the present immunization was determined by the production of Arthus lesions in the skin of sensitized animals 2 weeks after injection of the Incomplete Adjuvant-Ferritin emulsion. Animals so tested were challenged by intradermal injection of 0.1 cc 1% ferritin solution. Classical Arthus reactions developed in all sensitized animals within 6-8 hours. Anti-sera obtained at the time of challenge were tested for precipitating antibody by layering 0.3 cc ferritin over 0.3 cc anti-sera in a narrow tube. In all of the tubes a precipitate formed at the interface between the antigen and anti-sera.

3. Production of peritoneal exudates. One week after the demonstration of Arthus sensitivity, 5 mg of ferritin in 1 cc normal saline solution was slowly injected into the peritoneal cavity with a 21 gauge needle. Twenty-four hours later the animals were rapidly killed with ether, their peritoneal cavities exposed by a mid-line incision in the anterior