ference, and the first steps were taken at that conference to clear channels for radio astronomy. The very important band of frequencies near the hydrogen line (1400 to 1427 Mcy/sec) was cleared, as a result of almost complete agreement. Various other frequency bands were given less protection, generally by allowing radio astronomy to share the band with other users. Although the results of the 1959 conference obviously fall short of all that radio astronomers hope for, the conference represents to scientists a very valuable first step.

This article has attempted to show that further needs for clear frequency bands exist, and it has told of the work now going on in preparation for the next radio conference. There will be a special ITU conference in 1963 to allocate frequencies for radio communications for space research, and it is the hope of radio astronomers that their science and its needs may be further considered at that time.

References and Notes

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6. In this example we have to assume the effective area of the transmitting antenna (we have taken 10 m^2) and a receiver band width (5 Mcy/sec), and we have assumed an electron content of the ionosphere typical of a winter noon at Washington, D.C.

The Quest for Human Cancer Viruses

A new approach to an old problem reveals cancer induction in hamsters by human adenovirus.

John J. Trentin, Yoshiro Yabe, Grant Taylor

Many forms of cancer in many species of animals are now known to be caused by viruses (*1*). While all attempts to isolate causative viruses from human cancer have thus far been unsuccessful, it would appear to be biologically unusual if at least some forms of human cancer were not also caused by viruses. By far the most fruitful technique for the demonstration of tumor viruses in animals has been the inoculation of extracts of tumors, or of nontumorous tissues from tumor-bearing animals, into newborn animals of the same species. This technique is obviously denied to the searcher for human cancer viruses. Attempts to induce cancer in the young of other species by inoculation of human tumor extracts have thus far been negative or inconclusive.

The technique perhaps most widely used at present is the attempted propagation of human cancer viruses in vitro by inoculation of tissue cultures with human cancer materials. Yet this tissue culture technique has not resulted in the discovery of a single animal tumor virus, and only secondarily has it been possible to grow easily some exceptional animal tumor viruses in tissue culture. Because of the severe limitations imposed on the virologist concerned with human cancer, we must continue to use the tissue culture screening approach. But the need for new approaches is obvious. One such new approach is described in this article.

It was demonstrated by Duran-Reynals (*2*) that known animal tumor viruses can, under certain conditions of host susceptibility, produce acute noncancerous diseases. The production of tumors appeared to be a late manifestation of the slow growth of virus in a relatively resistant host. It therefore appeared possible that some human viruses already known for their acute disease manifestations might, under other conditions of host resistance or in a host surviving the acute disease, produce the late manifestation known as cancer. Some of the already known (or unknown) human viruses of acute diseases of childhood might therefore also be cancer viruses.

Another large group of already isolated viruses should also be suspect—the human "orphan" viruses. These are viruses that have been isolated from humans but whose disease manifestations are unknown—"viruses in search of disease." Cancer may well be the disease that some or many of these viruses are at present "orphan" to! With these concepts in mind, we initiated a program of testing known human viruses for cancer-inducing effect in newborn animals. The newborn Syrian hamster was selected as the test animal of choice because of its known relative susceptibility to tumor induction by tumor viruses of other species (*3*), and because of its relatively poor defense against the growth of normal-tissue and tumor-tissue transplants from other species, including the human (*4*). Newborn hamsters are actually more susceptible to tumor induction by polyoma virus of the mouse than are newborn mice (*5*). In spite of the many great advantages of inbred mice in other areas of cancer research, mice were excluded as test animals in this study because of the numerous tumor (and nontumor) viruses which they are known to harbor, and because of past experience with interference by such viruses in attempts to isolate human cancer viruses by other methods (*6*).

One of the first groups of human viruses chosen for extensive testing was the adeno group, because of similarities to some known animal tumor viruses, including polyoma virus, and because of the latency of adenoviruses in a large percentage of children. The adeno-

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Table 1. Tumors resulting from injection of human adenoviruses into newborn hamsters.

Virus type	Virus titer* ($MTCID_{100}$/0.1 ml)	Dose (ml)	Route of injection	Tumorous hamsters/ injected hamsters†	Tumor deaths (days)
2	10^2	0.1	Intraperitoneal	0/6	
		0.01	Intracranial	0/2	
3	10^2	0.1	Intraperitoneal	0/2	
		0.01	Intracranial	0/3	
7	10^2	0.05	Intrapulmonary	0/6	
7*a*	10^2	0.05	Intrapulmonary	0/8	
9	10^2	0.05	Intrapulmonary	0/2	
10	10^2	0.05	Intrapulmonary	0/5	
11	10^2	0.05	Intrapulmonary	0/4	
12	10^2‡	0.05	Intrapulmonary	8/10	33–90
	10^2§	0.05	Intrapulmonary	1/1	82
14	10^3	0.05	Intrapulmonary	0/5	

* All titers represent 1:10 dilution of the culture fluids sent from the American Type Culture Collection. † "Injected hamsters" signifies hamsters injected within 24 hours after birth and surviving longer than 3 weeks. Most nontumorous hamsters are 8 to 11 months old and still under observation at the time of writing. ‡ First shipment of type 12 adenovirus tissue culture fluid received from the American Type Culture Collection. § Second shipment of type 12 adenovirus tissue culture fluid, received from the American Type Culture Collection about 5 months after the first shipment.

viruses grow in the nucleus, and their nucleic acid is deoxyribonucleic acid. The virus particles occur in crystalline arrays comparable to those reported by Bunting in benign human skin papillomas (*7*), and by Banfield *et al.* (*8*) in tissue culture cells infected with polyoma virus.

Materials and Methods

An isolated building was designed and constructed for the study, with three separately air-conditioned hamster rooms, a tissue culture laboratory, a sterile room, and a wash room and sterilizing facility. One of the rooms was used exclusively for a hamster breeding colony. Newborn litters and their mothers were transferred to one of the other two hamster rooms as needed for inoculation. The other experimental hamster room was used for a concomitant study involving inoculation of newborn hamsters with human cancer tissue or extracts, or with fluids from tissue cultures inoculated with human cancer tissue. No animals were transferred back from the two experimental rooms to the breeding room. The breeding colony was established originally with Syrian hamsters from the polyoma-virus-free colony of the National Institutes of Health (*9*). Strict isolation procedures were instituted from the outset with respect to all other species of animals and to human visitors. This combined hamster colony and virus laboratory is referred to hereafter as the hamster laboratory.

FL amnion cells and HeLa cells (*10*) and lung cells of human embryos (*11*) were used. The FL amnion cells were cultured in medium 199 with 10 percent calf serum. The lung cells of human embryos were cultured in medium consisting of either medium 199 or Eagle's minimum essential medium with 10 percent calf serum. The HeLa cells were cultured in Eagle's minimum essential medium with 10 percent calf serum (*12*).

Human adenovirus types 2, 3, 7, 7*a*, 9, 10, 11, 12, and 14 were obtained from the American Type Culture Collection. The virus tissue culture fluids thus obtained were directly diluted to a concentration of 1:10 with the maintenance medium described later, injected into animals, and at the same time titrated in tissue culture tubes of the cells described in the preceding paragraph. Later, however, type 12 virus was propagated in HeLa cells in the tissue culture room of the hamster laboratory and used for further experiments.

Polyoma virus (*13*) was recultured in, and stored in, a building separate from the hamster-laboratory. No tissue culture work with polyoma virus was done during the period of the experiment under discussion.

The polyoma virus hemagglutination inhibition test was carried out according to the procedure of Rowe *et al.* (*14*) on sera collected by exsanguination.

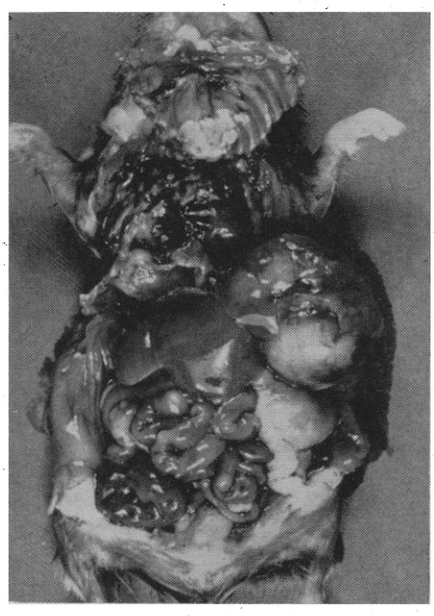
At first Earle's balanced salt solution, medium 199, or Eagle's basal medium with 5 percent horse serum was used as the maintenance medium for the tenfold serial dilutions of each adenovirus tissue culture fluid. Each dilution (0.1 ml) was inoculated into two tubes containing a monolayer of either FL amnion or human embryonic lung cells newly replenished with 1 milliliter of maintenance medium. Inoculated tubes were incubated at 36°C for 5 days, and the final reading was made on the 5th day of incubation. Later, however, HeLa cells and Eagle's basal medium with 10 percent horse serum were consistently used for the titration of type 12 virus, and the procedure was modified as follows. Maintenance medium was used for tenfold serial dilutions of virus tissue culture fluid; to 0.1 milliliter of each dilution, 1 milliliter of freshly prepared 0.1 percent HeLa cell suspension in maintenance medium was added; the tubes were incubated at 36°C for 5 days, and the final reading was made on the 5th day. The highest dilution which produced clear cytopathic effect (CPE) in both tubes was taken as the minimum 100 percent tissue culture infectious dose ($MTCID_{100}$). No very significant difference was observed between the $MTCID_{100}$ obtained by the two procedures, but the latter procedure was simpler and the effect was a little sharper. The 50 percent tissue culture infectious dose ($TCID_{50}$) was determined in a similar manner, with four tubes used for each dilution.

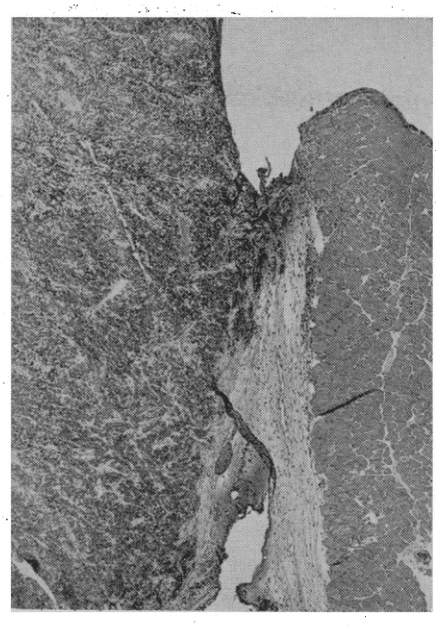
HeLa cells were infected with type 12 adenovirus. Tissue culture fluid was harvested 2 days after complete cytopathic effect was observed. It was centrifuged at 1500 to 2000 revolutions per minute for 10 minutes and filtered through Selas 02 filter candles. To test the completeness of filtration, 0.5 milliliter of fresh *Serratia marcescens* broth culture was mixed in the tissue culture

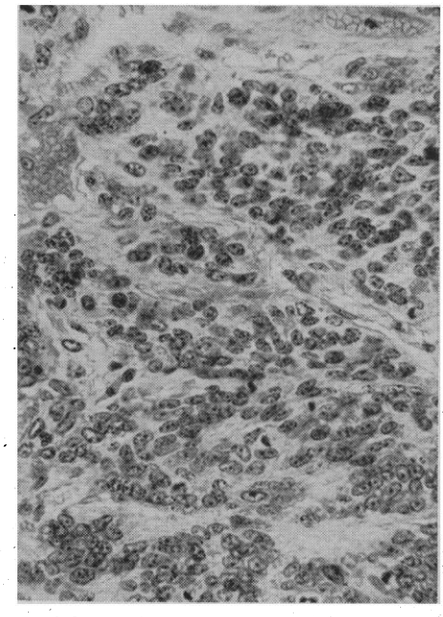
Table 2. Tumor induction by cell-free filtrate of human type 12 adenovirus tissue culture fluid injected intrapulmonarily (dose, 0.05 ml).

Tissue culture passage*	Virus titer ($MTCID_{100}$/ 0.1 ml)	Tumorous hamsters/ injected hamsters†	Tumor deaths (days)	Dead without tumors		Alive without tumors	
				No.	Days	No.	Days
1st	10^2	7/8	33–91			1	376
3rd	10^3	26/27‡	35–157	1	156		
8th	10^3	6/6	29–45				
Control culture§	None	0/7		2	321, 327	5	374–376

* The second shipment of type 12 adenovirus from American Type Culture Collection was used for tissue culture passage. † "Injected hamsters" signifies hamsters injected at birth and surviving over 3 weeks. ‡ Four hamsters of this group developed large tumors in the liver in addition to tumors in the thorax. § Noninoculated HeLa cell tissue culture fluid centrifuged at 1500 revolutions per minute for 10 minutes to remove cells.







Figs. 1–3. Fig. 1 (left). Tumors filling the right thoracic cavity and in the liver of a hamster injected intrapulmonarily on the right side, at birth, with 0.05 milliliter of type 12 adenovirus (10^8 $MTCID_{100}$/0.1 ml) (about × ½). Fig. 2 (middle). Intrathoracic sarcoma adherent to the diaphragm at the site of intrapulmonary injection, 45 days previously, of type 12 adenovirus into a newborn hamster (about × 17). Fig. 3 (right). Higher magnification of a portion of the sarcoma shown in Fig. 2 (about × 175).

fluid before filtration. About 0.5 milliliter of each of the fluids, before and after filtration, was inoculated into broth and tested for the growth of *Serratia marcescens*.

Newborn hamsters less than 24 hours old were moved with their mothers from the breeder room to the experimental room and inoculated with viruses intraperitoneally, intracerebrally, or intrapulmonarily. For intrapulmonary inoculation the virus was injected through the chest wall with a 30-gauge needle. The young were usually observed every day for the first week and thereafter at least three times a week; they were weaned at 3 to 4 weeks of age.

Healthy-looking whitish portions of tumor were taken aseptically and minced with scissors, and about 27 cubic millimeters of tumor tissue was transplanted subcutaneously or intraperitoneally, by trocar, into otherwise untreated (unconditioned) hamsters of different ages.

Human serum (stored at temperature of −70°C) was inactivated by heating at 56°C for 30 minutes and diluted in twofold series to a concentration of 1:64 with Eagle's basal medium containing 10 percent horse serum. To 0.1 milliliter of each serum dilution, 0.1 milliliter of virus fluid (10^8 $TCID_{50}$/0.1 ml) was added, and the mixture was kept at room temperature for 30 minutes. To each mixture, 1 milliliter of freshly prepared 0.1 percent HeLa cell suspension in Eagle's basal medium with 10 percent horse serum was added, and the resulting mixtures were incubated at 36° to 36.5°C. On the 5th day of incubation a reading was made, and the highest serum dilution which completely inhibited the cytopathic effect of adenovirus type 12 was taken as the neutralizing antibody titer.

Four human sera with type-12 adenovirus CPE-neutralizing antibody titers of 1:16 or higher (as a result of exposure in nature) and four negative human sera (with titers lower than 1:2) were otherwise randomly selected from among 700 sera tested at random from among 1870 patients admitted to the M. D. Anderson Hospital and Tumor Institute for all causes over a period of approximately 6 months. These eight sera were inactivated by heating at 56°C for 30 minutes, mixed with an equal volume of virus fluid ($10^{3.2}$ $TCID_{50}$/0.1 ml), and kept at room temperature for 30 minutes; then 0.05 milliliter of this mixture was injected into newborn hamsters less than 24 hours old.

Rabbit anti-simian-virus-40 serum was obtained from the National Institutes of Health. The neutralizing antibody titer of this serum, as determined by B. E. Eddy, was 1:1500 when tested against 100 $TCID_{50}$ of SV-40 and read in 10 days (*13*). Control rabbit serum was obtained from normal rabbits in our laboratory. One volume of undiluted serum was mixed with 3 volumes of type-12 adenovirus fluid ($10^{3.2}$ $TCID_{50}$/0.1 ml) and kept at 4°C overnight. Of this mixture, 0.05 milliliter was inoculated intraperitoneally into newborn hamsters and 0.1 milliliter was inoculated into 1 ml of HeLa cell tissue culture.

One-tenth milliliter of the particular dilution of simian virus 40 (SV-40) fluid (*13*) which showed cytopathic effect of 3+ to 4+ (vacuolation) on the 7th day of inoculation in green monkey kidney cell (*15*) was mixed with 0.1 milliliter each of a 1:4 dilution of rabbit anti-SV-40 serum and a 1:4 dilution of control rabbit serum or undiluted patient's serum. The mixture was kept at room temperature for 30 minutes, inoculated into 1 milliliter of green monkey kidney cell culture, and observed for 2 weeks. Eagle's basal medium with 2 percent horse serum was used as the maintenance medium for green monkey kidney cells.

Results

Of the first nine types of human adenovirus injected into newborn hamsters, none except type 12 has shown tumorigenic effects to date. Of ten hamsters injected intrapulmonarily with type 12 virus and surviving longer than 3 weeks, eight developed tumors in 33 to 90 days (Table 1). Tumors developed in the mediastinum, on the internal chest wall, or on the diaphragm. Most adhered to the surrounding organs or

Table 3. Trocar transplantation of tumors induced by human type 12 adenovirus.

Site of primary (induced) tumor	Transplant generation	Age of host (days)	Site of transplant	"Takes"/No. transplanted	Days to death from tumor growth
		Tumor A12, No. 15			
Thorax	T_1	<1	Intraperitoneal	1/1*	20
Thorax	T_1	<1	Subcutaneous	1/1*	34
Thorax	T_1	23–25	Intraperitoneal	4/4	26–50
Thorax	T_1	23–25	Subcutaneous	3/3	26–36
		Tumor A12, No. 14†			
Thorax	T_1	28–31	Intraperitoneal	3/4	35–62
Thorax	T_1	28–31	Subcutaneous	2/3	28‡–80
		Tumor A12, No. 6			
Thorax	T_1	25	Intraperitoneal	2/3	20, 24
Thorax	T_1	25	Subcutaneous	2/3	24, 30
Thorax	T_1	51–53	Intraperitoneal	2/2	28, 31
Thorax	T_1	51–53	Subcutaneous	1/2	63
		Tumor A12, No. 5			
Thorax	T_1	84	Intraperitoneal	2/2	117, 139
Thorax	T_1	84	Subcutaneous	0/1	
		Tumor A12, No. 20			
Thorax	T_1	24–25	Intraperitoneal	1/2	51
Thorax	T_1	24–25	Subcutaneous	1/2	36
Liver	T_1	20–21	Intraperitoneal	2/3	35, 87
Liver	T_1	20–21	Subcutaneous	1/3	45
		Tumor A12a			
Thorax	T_1	54, 55	Intraperitoneal	2/2	15, 27
Thorax	T_2	31	Intraperitoneal	3/3	16–20
Thorax	T_3	23	Intraperitoneal	3/3	25–27
Thorax	T_4	30	Intraperitoneal	3/3	19–21
Thorax	T_5	27–28	Intraperitoneal	3/3	22–42
Thorax	T_6	30	Intraperitoneal	2/3§	72§
Thorax	T_6	30	Subcutaneous	2/3§	72§, 73§
Thorax	T_7	29	Intraperitoneal	2/3§	
Thorax	T_7	29	Subcutaneous	2/3§	
		Tumor A12b			
Thorax	T_1	26–27	Intrapertioneal	4/4	17–30
Thorax	T_1	182	Intraperitoneal	2/3	58, 91
Thorax	T_2	28	Intraperitoneal	3/3	16–27
		Tumor A12c			
Thorax	T_1	26	Intraperitoneal	3/3	13–14
Thorax	T_2	37	Intraperitoneal	2/2	22, 22
Thorax	T_3	32–34	Intraperitoneal	3/3	20–26

* Animal transplanted with tumor within 24 hours after birth, which survived longer than 7 days. † This tumor was taken from a hamster which was found dead. ‡ This hamster showed a take, but actually died of submandibular abscess. § Smaller inoculum used to prolong host survival.

Table 4. Evidence against involvement of simian virus 40 in the tumor-inducing activity of type 12 adenovirus tissue culture fluids.

Approx. titer ($TCID_{50}$/0.1 ml)	Treatment	*Cercopithecus* kidney cells		HeLa cells		Observation period before cell deterioration (days)	Tumor induction in newborn hamsters by 54 days (No. with tumors/No. injected)
		Vacuolation	Adenotype CPE	Vacuolation	Adenotype CPE		
		Simian virus 40					
10^9	None	+	−	−	−		
10^9	Heat (60°C, 30 min)	+	−	−	−		
10^8	Rabbit anti-SV-40 serum	−	−	−	−	14	
10^7	Rabbit anti-SV-40 serum	−	−	−	−	14	
		Human adenovirus type 12					
10^1	None	−	+	−	+		
10^{-3}	None	−	−	−	−	14	
Subculture at 14 days	None	−	−			9	
10^1	Heat (60°, 30 min)	−	−	−	−	14	
10^{3}*	Heat (60°, 30 min)	−	−	−	−	14	
$10^{3.3}$	Rabbit anti-SV-40 serum	−	+	−	+		3/4†
10^2	Rabbit anti-SV-40 serum	−	+	−	+		
$10^{3.3}$	Normal rabbit serum	−	+	−	+		4/5†
10^2	Normal rabbit serum	−	+	−	+		
		None					
	Control tissue culture	−	−	−	−		

* The same dilution before heat inactivation gave 6/6 tumors in 45 days (see Table 2). † One animal was still alive and tumor-free at 54 days of age.

tissues, and bloody pleural fluid was present. In gross appearance the tumors were whitish with red hemorrhagic areas. In some hamsters metastases to the mediastinal lymph nodes were observed. No extrathoracic tumors were observed in these first eight tumorous hamsters.

A second shipment of human adenovirus, type 12, obtained from the American Type Culture Collection about 5 months after the first shipment induced a similar tumor in a hamster intrapulmonarily injected within 24 hours after birth.

To determine whether the tumors arising in hamsters injected with type 12 adenovirus might possibly be the result of transplantation of malignant tissue culture cells, cell-free filtrates of this second shipment of type 12 adenovirus tissue culture fluid on HeLa cells were prepared, by the method described earlier. There was a very high incidence of intrathoracic tumors, similar to those observed earlier, 1 to 3 months after injection of cell-free filtrates of tissue cultures infected with type 12 adenovirus but not after the injection of filtrates of control tissue culture (Table 2). These tumors developed in or on the mediastinum, the internal chest wall, the diaphragm, the lung, and the heart. In many hamsters multiple intrathoracic tumors were found. In four of 26 tumorous hamsters which had been injected with the virus fluid of the 3rd passage, one to three large tumors were found in the liver in addition to the tumors in the thorax (Fig. 1). Active propagation of the virus in HeLa cells is indicated by increased tumor-inducing ability and shorter period of latency after eight tissue-culture passages.

Histological sections of several of the tumors at the site of injection and in the liver were seen by four pathologists, who agreed that the tumors were undifferentiated sarcomas (Figs. 2 and 3). While the liver tumors may be metastatic, the following isolated observation indicates the possibility that some of them may be primary. Of three hamsters injected intravenously at birth with type 12 adenovirus, one is still alive, one died at 60 days with a subcutaneous tumor at the site of injection, and one died at 40 days with a large liver tumor but no other detectable tumors.

All of eight tumors induced in the thorax by type 12 adenovirus and one of the liver tumors, when transplanted intraperitoneally or subcutaneously into unconditioned hamsters from 1 to 182

days old, grew and killed a high percentage of the host animals in 13 to 139 days (Table 3).

Thus far, 220 and 950 untreated hamsters in the breeder room have been observed over periods of 12 and 6 months, respectively. Of these, one developed a spontaneous tumor involving the intestine and mesenteric lymph nodes in the duodenal region at 14 months of age. The rest are nontumorous.

The following facts indicate that the tumors observed are not attributable to contamination of animals or cultures by polyoma virus.

1) Sera of 30 untreated hamsters and of 14 hamsters that developed tumors after injection of type 12 adenovirus were tested for polyoma (hemagglutination inhibiting) antibodies at dilutions of 1 to 10 and higher. All were negative.

2) The tumor-inducing activity of the type 12 cultures was readily propagable in HeLa cells, whereas polyoma virus is not.

3) The tumors induced by type 12 adenovirus do not resemble those induced in the hamster by polyoma virus, which produces primarily kidney sarcomas, of which we saw none. The type 12 adenovirus tumor is, by contrast, typically at the site of injection.

Hamsters have been shown to develop sarcomas at the site of injection, in the newborn animal, of cell extracts of frozen and ground primary cultures of rhesus monkey kidney (*16*). The oncogenic agent involved has more recently been shown to be simian virus 40 (SV-40) (*17*). The occurrence of sarcomas at the site of injection in the experiments under discussion therefore suggests involvement of simian virus 40. However, the tumor-inducing capacity of the type 12 adenovirus cultures is readily propagable in HeLa cells, whereas simian virus 40 is not. In our experiments tumors began to appear by 29 days (the 8th HeLa passage filtrate), and almost all injected animals had tumors by 90 days, whereas tumors induced by simian virus 40 usually do not appear until after 3½ months (*17*). Nevertheless, experiments were performed as follows with *Cercopithecus* monkey kidney cells, simian virus 40, and rabbit anti-SV-40 serum. These experiments were performed in a building separate from the hamster isolation building and tissue culture laboratory, since only human viruses and human tissue culture cell types are permitted in the latter.

Table 5. Neutralization of both cytopathic effect and tumor-inducing activity of human type 12 adenovirus by human antisera (convalescent).

Human serum donor (No.)	Anti-SV-40 titer (vacuolation on *Cercopithecus* kidney)	Anti-type-12 adenovirus titer (CPE on HeLa)	Ratio of serum to adenovirus type 12 ($10^{3.3}$ $TCID_{50}$/0.1 ml)	No. of tumors/ No. hamsters injected	Age at death of hamster with tumor (days)	Age of hamster alive and tumor-free (days)
None*			1:1	6/6	29–45	
38478	<1:1	<1:2	1:1	7/7	35–79	
40003	<1:1	<1:2	1:1	1/1	42	
40186	<1:1	<1:2	1:1	4/4	32–155	
40146	Not tested	<1:2	1:1	5/7	45–83	170
40378	<1:1	1:32	1:1	0/8		167–175†
39835	<1:1	1:32	1:1	0/4		167
39528	<1:1	1:16	1:1	0/3		166–172
40523	<1:1	1:16	1:1	0/3		171

* Tissue culture medium was used instead of human serum. † Two were dead, tumor-free, at 41 and 80 days.

In retrospect it appears indeed fortunate that these tests for simian virus 40 were performed. Whereas the catalogue of the American Type Culture Collection states in the passage history of prototype 12 adenovirus that only human tissue culture cell types (KB and HeLa) are involved, an announcement made on 13 April 1962 amended the passage history to include monkey kidney cells, as follows: "Isolated in and passed for an unknown number of passages in human embryonic kidney cells. It then was passed one or more times in monkey kidney cells. After receipt in Huebner's laboratory, it was passed twice in KB cells after 12 passages in KB or HeLa cells." From the results of our experiments, however, it appears improbable that SV-40 contamination could have been responsible for the tumors induced (Table 4).

Simian virus 40 produced typical vacuolation in *Cercopithecus* kidney cell cultures, not in HeLa cells. Tumor-inducing type 12 adenovirus cultures produced typical adenovirus-type cytopathic effect (rounding up and clumping of cells) on both HeLa and *Cercopithecus* cells, but no vacuolation.

Simian virus 40 grows poorly if at all on HeLa cells. In order to test the possibility that slight contamination with simian virus 40 was being obscured by the adenovirus-type cytopathic effect, type 12 adenovirus cultures were heated to 60°C for 30 minutes. This treatment is known to inactivate adenovirus but not simian virus 40. This treatment did inactivate the adenovirus-type cytopathic effect of the type 12 adenovirus cultures and did not inactivate the vacuolating effect of the simian virus 40 cultures, yet it did not "unmask" a vacuolating effect in the type 12 cultures. Likewise, dilution of type 12 adenovirus to the point where it no longer destroyed the tissue culture cells by adenovirus-type cytopathic effect (dilution of 1:10,000) failed to reveal vacuolation even after subculture.

Rabbit anti-SV-40 serum inactivated the vacuolating effect of the SV-40 cultures but did not inactivate the adenovirus-type cytopathic effect of the type 12 cultures. In vivo testing of the type 12 cultures treated with anti-SV-40 serum has progressed for 54 days at the time of writing. Simian-virus-40 antiserum did not inactivate the tumor-inducing ability of our type 12 adenovirus cultures or prolong the period of latency; the first tumor arose in 31 days (Table 4).

Granted that the tumor-inducing activity is not attributable to polyoma virus or to contamination with simian virus 40, is it indeed due to the type 12 adenovirus rather than to yet a third, unrecognized, contaminant virus? Electron micrographs of HeLa cells infected with type 12 adenovirus reveal abundant virus particles of a single type concordant in location, size, arrangement, and morphology with the adenoviruses (Fig. 4). They occur as crystalline arrays in the nucleus, have a nucleoid surrounded by an outer limiting membrane, and have an average particle size of about 56 millimicrons. This finding, together with the other tissue culture characteristics mentioned and the adenovirus-type cytopathic effect, leaves little doubt that the predominant if not the only virus is an adenovirus. The data of Table 5 indicate a strong positive association between the adenovirus-type cytopathic effect and the tumor-inducing activity.

Sera of 700 randomly selected patients from the M. D. Anderson Hospital were tested for neutralizing anti-

bodies against the adenovirus-type cytopathic effect of our type 12 cultures. Approximately 26 percent showed neutralization at a dilution of 1:4 or higher. Approximately 6 percent showed neutralization at a dilution of 1:8 or higher. (Another 20 percent showed neutralization only at a dilution of 1:2.)

Four such positive "convalescent" antisera, each with a titer of 1:16 or higher, and four negative human sera were randomly selected. Aliquots of type 12 adenovirus cultures were incubated with each of these eight human sera and with tissue culture medium as a control. It may be seen that the four negative sera (those that did not neutralize the adenovirus-type cytopathic effect) did not neutralize the tumor-inducing effect, whereas the four positive sera (those that neutralize the adenovirus-type cytopathic effect) completely neutralized the tumor-inducing effect. None of the four positive sera contained SV-40 antibodies. These data provide strong indication that the tumor-inducing activity is indeed attributable to the type 12 adenovirus rather than to an unrecognized passenger virus.

Discussion

That human type 12 adenovirus is highly tumorigenic for the hamster is indicated by the following results.

1) Each of two separate shipments of type 12 adenovirus received 5 months apart from the American Type Culture Collection produced fast-growing malignant tumors at the site of intrapulmonary injection into newborn hamsters.

2) Tumor-inducing activity was retained or increased after at least eight tissue culture passages in HeLa cells and cell-free filtration.

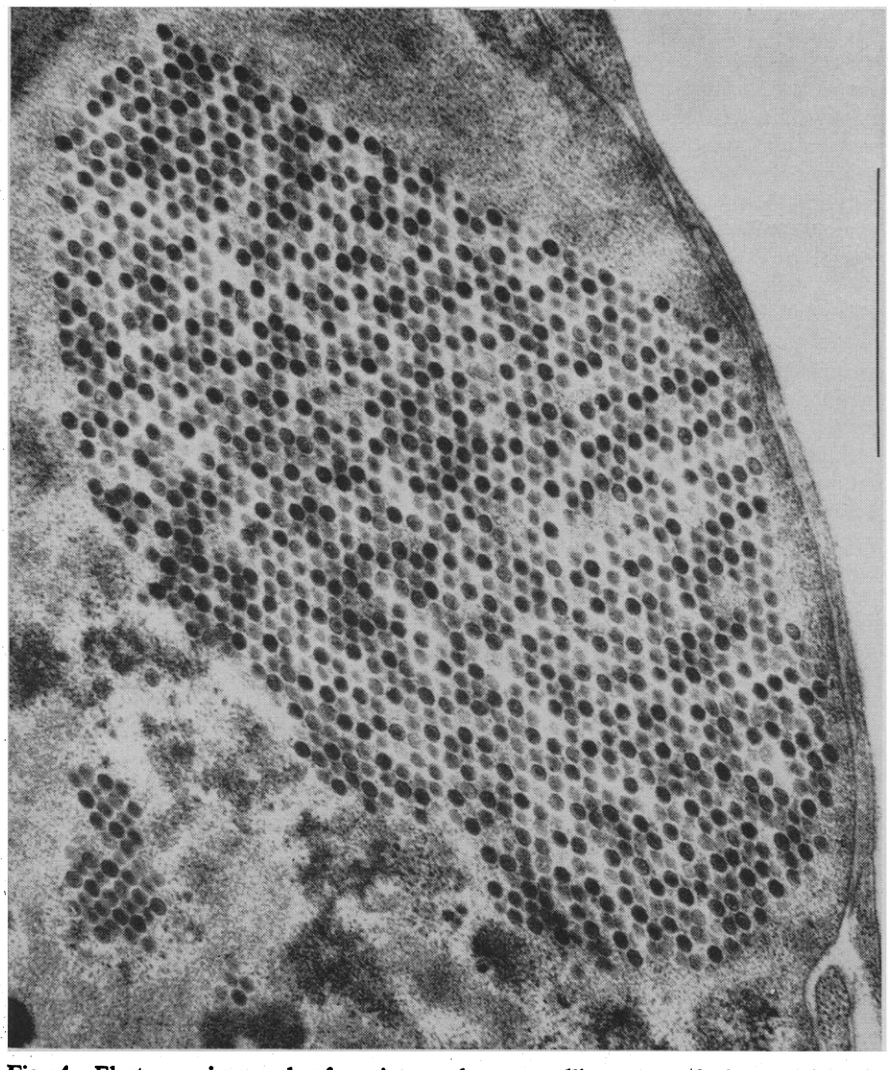


Fig. 4. Electron micrograph of an intranuclear crystalline array of virus particles in HeLa cell tissue culture infected with type 12 adenovirus (about × 39,000).

3) The incidence of the tumors was high, and the periods of latency were short.

4) The tumors grew progressively in, and killed, a high percentage of the unconditioned young adult hamsters into which they were transplanted.

5) No such tumors occurred in hamsters injected with control tissue culture fluid or with the other viruses tested, or in control breeder hamsters.

6) The possibility that polyoma virus or simian virus 40 (the only other viruses at present known to cause tumors when injected into newborn hamsters) might be responsible for the tumors observed was specifically excluded by a variety of tests.

7) The possible involvement of still other, as yet unknown, contaminant viruses was excluded by a positive association of the tumor-inducing ability with the adenovirus content. Of human sera tested, all those, and only those, which neutralized the adenovirus-type cytopathic effect also neutralized the tumor-inducing effect. This latter result indicates that tumor induction is the result of the type 12 adenovirus, and that newly isolated strains of the same virus should therefore also have tumor-inducing effect.

Whereas most of the tumors arose at the site of intrathoracic injection, four hamsters also developed from one to three large tumors of the same histological type in the liver. The question of whether these are metastatic or primary is under investigation.

All animal tumor viruses that are now known to produce tumors in a heterologous species, and that have been adequately tested in the species of origin, also produce tumors in the species of origin. Simian virus 40, which has recently been found to have a tumorigenic effect in hamsters, has not yet been adequately tested in newborn monkeys. It is yet to be determined what tumors, if any, may be produced by type 12 adenovirus in humans. While tumor induction studies in subhuman primates and in human cells in vitro (malignant transformation) may be helpful, the direct approach via tumor induction studies in the species of origin has obvious drawbacks in the case of human tumor viruses.

If human type 12 adenovirus were injected into newborn infants one might confidently expect, from what is known of animal tumor viruses, that it would induce tumors. Yet such a result would by no means prove that in nature this virus is responsible for even a single

"spontaneous" human tumor. Polyoma virus of the mouse produces a high incidence of tumors when injected into newborn mice or newborn hamsters. Polyoma virus is enzootic in most laboratory and wild mouse populations (*18*). Yet all of the evidence at present indicates that polyoma virus is responsible for very few, if any, "spontaneous" tumors in wild or laboratory mice. On the other hand there is strong evidence that certain other animal tumor viruses, such as the mouse mammary tumor milk agent, the mouse leukemia virus (or viruses), and the avian leukosis viruses are responsible for a high incidence of "spontaneous" breast cancer and leukemia in several strains of mice and of diverse "spontaneous" leukemias, lymphomas, and sarcomas in chickens. Whether type 12 adenovirus will be found to resemble polyoma virus or these other animal tumor viruses in this regard remains to be determined. The more feasible line of investigation in the human population would appear to be careful and extensive studies of the epidemiology of this and other adenoviruses, with special reference to cancer. Such studies would include (i) attempts to isolate this and other adenoviruses from the cancer tissue, body fluids, and excreta of cancer patients, and (ii) study of the occurrence of antibodies against this and other adenoviruses in the human population, with special reference to cancer and to individuals in young age groups.

Type 12 adenovirus was originally isolated by Kibrick *et al.* from the stool of a case of suspected nonparalytic poliomyelitis (*19*). It was classified as a new antigenic type of adenovirus by Rowe *et al.* (*20*), designated type 12, and placed in the American Type Culture Collection as a prototype, though its clinical and pathological significance has been obscure.

A large proportion of adenovirus infections occur in early childhood. Infection with types 1, 2, and 5 is prevalent chiefly in infancy and early childhood (*21*). Adenovirus respiratory disease epidemics in the general population have most often yielded types 3 and 7; epidemics among military recruits have generally yielded types 4 and 7. Adenovirus vaccines have been effective for the control of acute respiratory illness caused by the adenoviruses (*22*), and protective antibodies produced by adenovirus infections persist for many years (*21*). This offers real hope of prophylactic vaccination against other illnesses with which adenoviruses may be associated in the human population, including perhaps cancer.

Ginsberg has pointed out (*22*) that the properties of the adenoviruses should render them ideal as potential latent agents. They are very stable with respect to changes in temperature or *p*H; the principal site of propagation is in the nucleus; they do not dissociate readily from the cells that they infect; and they apparently do not kill the cells in which they multiply, although they can damage them markedly. These properties could explain their ability to persist in human tissues for long periods. Ginsberg goes on to state, "It is not unreasonable to believe that adenoviruses, like the herpes simplex virus, could then, by a still-unknown mechanism, be provoked to cause another disease episode, albeit the proof of such etiology by our present techniques may not be possible. If the hypothesis presented were true, this might explain the major role of adenoviruses as etiological agents in man."

The results presented here focus attention on the human adenoviruses as potential tumorigenic viruses etiologically related to cancer in man. The possibility that other human orphan viruses, or viruses of acute diseases, may have a delayed tumorigenic manifestation in man should be seriously considered and investigated. The described method of testing such already isolated and classified viruses for tumorigenic activity in newborn laboratory animals may prove to be an important tool in the armamentarium of the cancer researcher.

Summary

A new approach to the important but difficult task of revealing possible human tumor viruses has been presented in this article. By systematic testing of already known human viruses for oncogenic properties, it was found that in hamsters injected intrapulmonarily with tissue culture fluid of human type 12 adenovirus within 24 hours after birth there was a very high incidence of malignant tumors at the site of injection in from 1 to 3 months. The tumor-inducing activity was not lost by filtration through Selas 02 filters or by tissue culture passages in HeLa cells. Tumors thus induced grew in, and killed, a high percentage of the unconditioned young adult hamsters into which they were transplanted. No such tumors occurred in hamsters injected with control tissue culture fluid or with culture fluids of the other viruses tested, or in control breeder hamsters. The possibility that contamination with polyoma virus and simian virus 40 might be responsible for the tumors induced was specifically excluded by a variety of tests. The possible involvement of still other, as yet unknown, contaminant viruses was excluded by a positive association of the tumor-inducing ability with the adenovirus content. Of eight human sera tested, only those four which neutralized the adenovirus-type cytopathic effect also neutralized the tumor-inducing effect. Of 700 human sera tested, 26 percent contained CPE-neutralizing antibodies for type 12 adenovirus at titers of 1:4 and higher (*23*).

References and Notes

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11. This material was kindly supplied by Drs. Koprowski and Hayflick of Wistar Institute.
12. These materials were obtained from Microbiological Associates, Inc.
13. The polyoma virus, the simian virus 40 (strain A 426), and the rabbit anti SV-40 serum were kindly supplied by Dr. B. E. Eddy of the National Institutes of Health.
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