The Oncogenicity of Human Adenoviruses in Hamsters* (32773)

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In 1962 we reported that human adenovirus type 12 induces undifferentiated sarcomas at the site of injection into newborn hamsters in high incidence and after a very short latent period (1,2). Extensive attempts to recover virus from such tumors have been negative in this and several other laboratories (3,4), although Connor *et al.* have made a preliminary report of successful virus isolation (5). Huebner and others have shown such tumors to contain small amounts of the viral structural C antigen (6,7) and larger amounts of type 12 specific tumor antigens demonstrable by complement fixation (CF) (8,9), immunofluorescence (10,11) and immunodiffusion (7) with serum of adeno-12-tumor bearing hamsters. Such sera also react with type 12 neoantigens induced in virus infected cell cultures (7,8,11). Adeno-12 tumors of hamsters and mice also contain transplantation neoantigens as revealed by induction of transplantation immunity by prior immunization of adult hamsters or isogenic mice with live adenovirus type 12 (12) or with irradiated adeno-12 tumor cells (unpublished). A significant portion of the messenger ribonucleic acid of the polyribosomes of adeno-12 tumor cells and transformed cells is virus specific (13). Adeno-12 has been shown to be oncogenic also in newborn mice (14), rats (3), and mastomys (15), and to transform rabbit embryo cells *in vitro* (11). Human adenovirus types 18, 7, 31, and 3 have also been reported to induce undifferentiated tumors, lymphomas, and lymphosarcomas in newborn hamsters (16–19). Green *et al.* (personal communication) have unpublished data indicating that adenovirus types 14, 16 and 21 are also weakly oncogenic. Hull *et al.* have reported that 6 of 18 simian adenoviruses tested were also oncogenic in newborn hamsters, some of them highly oncogenic (20). The tumors induced were undifferentiated with some characteristics of lymphomas of the reticulum cell type. Sarma *et al.* induced fibrosarcomas in high frequency at the site of injection of an avian adenovirus (CELO) into newborn hamsters (21). Darbyshire reported a high incidence of undifferentiated sarcomas arising within 2 months at the site of injection of bovine adenovirus type 3 into newborn hamsters (22). Both the bovine and the avian adenovirus-induced tumors were either lacking in virus specific tumor antigenicity or had so little such antigenicity that positive results were only rarely encountered. We have reported that tumors induced in mice by adenovirus type 12 elicits CF antibodies in only a small percentage of tumor bearing mice, whereas a high percentage of the tumors can be shown to contain some antigen by reactivity with adeno-12 tumor bearing hamster serum (23). A specific positive CF tumor antigen or antibody finding is therefore much more meaningful than a negative finding. Tumors have also been induced in newborn hamsters by a canine adenovirus, namely, infectious canine hepatitis (Huebner, personal communication).

The present report deals with the results of long-term testing of 27 human adenoviruses for oncogenicity in newborn hamsters. These are types 1 through 30, with the exception of types 2, 10, and 11 previously reported (2).

Materials. For initial studies 1 ml of the prototype strain of each adenovirus used was received from the American Type Culture Collection. At the time of animal inoculation the virus was thawed and diluted sufficiently in tissue culture medium (1:10 in most instances) to inoculate 20–30 newborn hamsters. When additional studies were performed, prototype strains were propagated in KB, HEp 2, or primary human embryonic kidney

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cells (HEK).[2] Virus titers were determined by cytopathic effect in continuous human epithelial cells, i.e., HeLa, KB, or HEp2, held for 5 days, and/or in HEK cells observed for at least 21 days. Titers determined both in continuous cells lines and in HEK were invariably higher in the latter, as indicated in Tables II, III, IV, and V. All Syrian hamsters used were from a closed, isolated colony, the establishment of which has been previously described (2). Animals less than 24 hours old were inoculated with 0.1 ml by the intraperitoneal (ip) route and occasionally by the subcutaneous route (sc). Inoculated animals were weaned and separated by sex at 21–28 days. They were maintained in separate rooms for different groups of viruses, e.g., adenovirus-inoculated animals in a room separate from enterovirus-inoculated animals, etc. Inoculated hamsters and their mothers, and appropriate groups of controls were observed for tumor formation until death from whatever cause. Only animals coming to adequate autopsy are included in the tabulations. Animals eaten, missing, or unaccountable are not included in the tumor statistics. Autopsies were performed on all animals when found dead or when sacrificed by exsanguination or euthanasia when moribund. Tissues grossly suspicious of being neoplastic were transplanted by trocar to other hamsters (weanlings and/or newborn). Tissues grossly abnormal were preserved in formalin, sectioned, and stained with hematoxylin and eosin.

Results and Discussion. The frequency of spontaneous tumor formation in control hamsters autopsied in our closed colony is very low.[3] The only 7 spontaneous tumors observed were 2 cheek pouch tumors, 2 subcutaneous sarcomas, 2 lymphomas, and one carcinoma, among 1322 breeder hamsters coming to autopsy at or older than 188 days, the age of the earliest spontaneous tumor detected. The average age at autopsy of this group was 343 days. No additional tumors were detected among 218 uninoculated mothers of inoculated newborn, autopsied at an average age of 561 days, and among a group of 17 newborn inoculated with tissue culture medium and autopsied at an average age of 665 days. The overall tumor frequency of these control hamsters autopsied at 188 days or older (mean of 377 days) was thus 7 out of 1557, or 0.45%. Of these 7 tumors, 3 were of types (2 cheek pouch tumors and one carcinoma) not observed among the adenovirus-induced tumors. If all control hamsters autopsied earlier than 1 year of age are excluded from the series, giving a mean autopsy age of 542 days, the spontaneous tumor frequency was 5 out of 643 hamsters, or 0.77%. Many additional thousands of breeder hamsters have been discarded without autopsy and without obvious tumors.

Table I presents the data on 19 different

TABLE I. Human Adenovirus Types That Yielded No Tumors among Hamsters Inoculated at Birth.

Adenovirus type	No. weaned /no. inoculated	No. autopsied	Days at autopsy	
			Mean	Range
3[a]	36/55	29	377	46– 706[b]
4	13/58	7	546	456– 647
5	13/42	10	499	78– 780
6	14/31	8	575	228– 921
9	18/20	13	601	50– 868
13	17/39	8	556	46– 878
15	20/44	17	622	70– 921
16	10/25	8	640	411– 743
17	12/34	10	508	39– 717
19	20/28	12	719	407– 869
20	17/33	13	631	315– 925
22	13/19	11	691	512– 966
23	14/26	11	599	287–1000
25	6/13	4	676	498– 739
26	15/27	11	673	282– 956
27	12/26	10	730	511–1038
28	15/32	12	426	64– 875
29	19/28	13	580	48– 915
30	12/28	8	482	129– 700
Totals	296/608	215	569	39–1038

[a] The titer of adenovirus type 3 was 10^8 $TCID_{50}$/1.0 ml in HEK cells. The titer of the other types was not determined. They were used as a 1:10 dilution of the virus as received from the American Type Culture Collection.

[b] One animal still alive.

[2] Human embryonic kidney cells were supplied by the Human Tissue Procurement Program of the National Cancer Institute.

[3] Van Hoosier, G. L., Jr. and Trentin, J. J., in preparation.

adenovirus types that yielded no tumors in a total of 215 inocules autopsied from 39 to 1038 days postinoculation with a mean survival of 569 days. All inoculated hamsters autopsied beyond weaning age are included, since death from induced tumor has occurred as early as 29 days postinoculation at birth with adenovirus type 12 (2). A very low incidence of tumors has been observed by Huebner *et al.* for type 3 (19) and by Green *et al.* for type 16 (personal communication), with finding of type-specific CF tumor antigenicity. Our negative result with these two types is possibly related to one or more of several factors such as (a) low numbers of inoculated animals (only 8 inoculees coming to autopsy for type 16), (b) possibly low titer virus (type 16 was inoculated as a 1:10 dilution of the virus as received from American Type Culture Collection), (c) possible presence of adenovirus-associated virus (AAV), which is said to suppress malignant transformation by adenoviruses both *in vitro* (B. Casto, personal communication; K. O. Smith, personal communication) and *in vivo* (K. O. Smith, personal communication). The titer of adenovirus type 3 was reasonably high, i.e., 10^8 $TCID_{50}$/1.0 ml in human embryonic kidney cells. Twenty-nine inoculees came to autopsy at an average age approximately equal to that of the 1557 control hamsters. While the virus which was inoculated was not tested for AAV, a subsequent tissue culture passage of type 3 was tested for AAV by Dr. Kendall O. Smith and found to be negative by immunofluorescence for AAV types 1, 2, 3, and 4.

The dose of virus inoculated is an important determinant of the frequency and latent period of tumor formation (24) (Tables II, III). The negative data of Table I cannot therefore be regarded as definitive for the detection of a low level of oncogenicity in hamsters. High-titered strain Huie of type 12 induced 64% tumors, at an average of 103 days (Table II). Lower-titered strain D39 (received from Dr. R. J. Huebner), which was a fresh isolate of type 12 from a different patient, gave 22% tumors. Still lower titered strain D44 gave no tumors in over 500 days. The D44 was a fresh isolate of the Huie strain made by Hilleman, from the frozen original Huie stool specimen. Freshly isolated strains of type 12 are invariably of low tissue culture titer and of correspondingly low oncogenicity. More recently we also have reisolated the Huie strain of type 12 from the frozen original Huie stool specimen, by culture in HEK cells (D53). After 2 tissue culture passages in HEK it achieved a good titer and gave tumors in 45% of inoculees. We are grateful to Dr. Sidney Kibrick for making available to us the D44 reisolate, and the frozen Huie stool specimen from which we reisolated (D53) the Huie strain of type 12. We are grateful to Dr. W. H. Murphy for the D49 strain of adeno-12. It was isolated from the bone marrow of a child with acute stem cell leukemia and passed 7 times in Chang's liver and in HeLa cells. It was passed in this laboratory on HEK cells and gave good titer and a high frequency of early tumors. Both the D49 and D53 strains were confirmed in our laboratory as adenovirus type 12 by neutralization with rabbit antiserum made against prototype 12 adenovirus from the American Type Culture Collection.

Four different tissue culture batches of prototype 18 adenovirus were tested. The tumor incidence obtained was only 5 or 9% with low-titered virus, but was 28 or 32% with high-titered virus (Table III). The tumors were mostly undifferentiated sarcomas with, however, a few lymphomas. Of possible significance is the fact that of 2 tumor transplant lines tested, one induced by high-titered adenovirus type 18 had good CF tumor antigen reactivity with tumor bearing hamster serum, whereas one induced by low-titered virus had little or no CF reactivity.

A total of eight adenovirus types have yielded tumors to date (Table IV). These are types 1, 7, 8, 12, 14, 18, 21, and 24. The tumor incidence varies from 4% for type 7, to 62% for type 12. Of these, types 7, 12, 14, 18, and 21 have been observed by others also to be oncogenic and to have specific CF tumor antigenicity. Except for the undifferentiated sarcomas induced by types 12 and 18, the predominant histologic pattern of the tumors induced is that of lymphoma or

TABLE II. Oncogenicity of Different Strains and Different Titers of Adenovirus Type 12.

Adenovirus Type 12 (log_{10} $TCID_{50}$/1.0 ml)								
Strain	Continuous cell lines	HEK	No. weaned/ no. inoculated	No. tumors/ no. autopsied	Tumors (%)	Mean days of tumor autopsies	Mean days of all autopsies	Tumor type
Huie (ATCC)[a]	3.5 (HeLa)	6.5[b]	135/174	76/118	64	103	240	undifferentiated sarcomas.
D 39	1.5 (HeLa)	NA[a]	34/62	6/27	22	110	239	undifferentiated sarcomas.
D 44 (Huie reisolate)	NA	3.0	13/13	0/12	0		501	
D 53 (Huie reisolate)	3.5 (KB)	NA	14/22	5/11	45	85	425	undifferentiated sarcomas.
D 49	2.5 (HEp 2)	6.7	27/33	13/15	87[c]	45	48	undifferentiated sarcomas.
	Totals		223/304	100/183	62	95	253	

[a] ATCC, American Type Culture Collection, prototype strain; NA, not available.
[b] Titers of adenovirus type 12 determined on primary human embryonic kidney cells are usually 3 or 4 logs higher than on HeLa, KB, or HEp 2 cells.
[c] 12 inoculees still alive at 128 days.

TABLE III. Oncogenicity of Four Tissue Culture Batches of Prototype 18 Adenovirus in Newborn Hamsters.

Adenovirus prototype 18 (log_{10} $TCID_{50}$/1.0 ml)							
HeLa	HEK	No. weaned/ no. inoculated	No. tumors/ no. autopsied	Tumors (%)	Mean days of tumor autopsies	Mean days of all autopsies	Tumor type
1.5	NA[a]	27/58	2/22	9	a) 677[b] b) 536[b]	571	a) undiff. sarcoma in peritoneal cavity. b) undiff. sarcoma in liver, spleen, lung.
1.5	2.0[c]	50/65	2/38	5	766	626	undiff. sarcoma, possibly lymphoma; undiff. sarcoma, metastatic.
3.5	5.0	24/30	6/19	32	104	427	undiff. sarcoma, spleen; undiff. sarcoma, spleen, liver, lungs; undiff. sarcoma and lymphoma.
4.3	6.5	20/36	5/18	28	131	196	undiff. sarcoma.
Totals		121/189	15/97	15	268	495	

[a] NA = not available.
[b] Individual values rather than mean value.
[c] Titers of adenovirus type 18 determined on primary human embryonic kidney cells are usually .5 to 2 logs higher than on HeLa cells.

TABLE IV. Adenovirus Types That Yielded Tumors among Hamsters Inoculated at Birth.

Adenovirus (log_{10} $TCID_{50}$/1.0 ml)			No. weaned/ no. inoculated	No. tumors/ no. autopsied	Tumors (%)	Mean days of tumor autopsies	Mean days of all autopsies	Tumor type
Type	Strain	Cell line						
1	See Table V		69/271	4/60	7	506	553	lymphoma (benign?); undiff. sarcoma; malignant lymphoma.
7	Pinckney	4.6–5.3 (KB), 7.0 (HEK)	29/42	1/23[a]	4[a]	466	570	malignant lymphoma (reticular cell type).
8	ATCC[b]	NA[b]	10/25	1/9	11	684	702	lymphoma (autolysed).
12	See Table II		223/304	100/183	62	95	253	undiff. sarcomas.
14	ATCC	5.0 (KB)	24/55	1/15	7[c]	394	509	malignant lymphoma.
18	See Table III		121/189	15/97	15	268	495	undiff. sarcomas.
21	ATCC	7.5 (HEK)	17/20	2/13[a]	15[a]	651	631	undiff. sarcoma; malignant lymphoma.
24	ATCC	5.3 (KB)	15/27	1/11	9	587	577	malignant lymphoma.
None (spontaneous tumor incidence)								
Autopsied at 188 days or older				7/1557	0.45	503	377	see text
Autopsied at 1 year or older				5/643	0.77	616	542	see text

[a] Eleven and 4 additional tumors have been induced by adenovirus types 7 and 21, respectively, in thymectomized hamsters (see text).
[b] ATCC, American Type Culture Collection, prototype strain; NA, not available.
[c] 6 inoculees still alive.

TABLE V. Oncogenicity of Three Different Strains of Adenovirus Type 1 in Newborn Hamsters.

Adenovirus (log_{10} $TCID_{50}$/1.0 ml)				No. weaned/ no. inoculated	No. tumors/ no. autopsied	Tumors (%)	Days of tumor autopsies	Mean days of all autopsies	Tumor type
Type	Strain	HeLa	HEK						
1	ATCC[a]	NA[a]	NA[a]	22/34	1/16	6	699	680	lymphoma or reactive hyperplasia of mesenteric lymph node.
1	D 42	5.5	9.0[b]	24/111	2/22	9	a) 349 b) 445	465	a) autolysed. b) undiff. sarcoma.
1	D 43	5.5	9.0	23/126	1/22	5	530	548	malignant lymphoma invading liver and diaphragm.
		Totals		69/271	4/60	7	506	553	

[a] ATCC, American Type Culture Collection, prototype strain; NA, not available.
[b] As for adenovirus type 12, titers of adenovirus type 1 determined on HEK cells are usually 3 to 4 logs higher than on HeLa cells.

lymphosarcoma or reticulum cell sarcoma. For type 7, only 1 lymphoma was obtained in 466 days among 23 intact inoculees. However, 11 additional tumors have been induced among 39 thymectomized inoculees (25). For type 21, 2 tumors were obtained among intact inoculees and 4 additional tumors among thymectomized inoculees (25).

Types 1, 8, and 24 have not previously been reported to be oncogenic. Type 8 gave 1 tumor among 9 animals coming to autopsy. This animal was found already dead. The tumor was partially autolyzed and did not transplant successfully. Further attempts are indicated to obtain additional tumors with type 8. Type 24 gave 1 malignant lymphoma among 11 survivors coming to autopsy. This tumor was successfully transplanted. A small percentage of the transplanted tumors gave low CF antigen reactivity with tumor-bearing hamster sera, to be presented in a subsequent paper.[4] Additional investigations of CF antigen and of other viral markers are in progress for tumors of hamsters inoculated with adenovirus type 24 and type 1.

One or more tumors were obtained with each of 3 different strains of adenovirus type 1. Prototype 1 gave one tumor in 699 days among 16 survivors (Table V). This tumor was diagnosed histologically by one pathologist as a lymphoma, and by another pathologist as reactive hyperplasia of the mesenteric lymph node. D42 (Mont strain) and D43 (Kitt strain) are of special interest, for they were isolated by McAllister *et al.* from tumors of children (26). The D42 was isolated from a malignant abdominal lymphoma of a 5.5-year-old child. The D43 was isolated from an embryonal carcinoma of a 20-month-old child. We are grateful to Dr. Robert McAllister for making these two isolates of type 1 available to us. Each of these strains of type 1, on injection into newborn hamsters, yielded a tumor that was successfully transplantable. Another tumor arising in a D42-injected hamster was found postmortem and too autolyzed for successful transplantation. The tumor types induced were an undifferentiated sarcoma (D42) and a malignant lymphoma (D43). McAllister (personal communication) has induced transformation of rat embryo cells *in vitro* with the Mont strain of adeno-1 (D42). In view of the 4 tumors obtained in hamsters with adenovirus type 1 of 3 different strains, and the transformation of rat cells *in vitro,* type 1 must be regarded as an oncogenic adenovirus.

It is of interest that the tumors induced by adenovirus type 1 appeared to originate in the mesenteric lymphoid tissue and to have the histology of lymphoma or undifferentiated sarcoma. Acute infection with adenovirus types 1, 2, and 5 (and less frequently types 3, 6, and 7), with mesenteric adenitis, has been implicated in the etiology of intestinal intussusception of human infants (27). The D42 strain of type 1 was isolated from an abdominal lymphoma of a child. Whereas the known murine and avian leukemia viruses are of the ribonucleic acid type, the possible role of viruses of deoxyribonucleic acid type in leukemia and lymphoma of other species should not be categorically excluded.

Pina *et al.* have classified the human adenoviruses on the basis of low, medium, and high guanine plus cytosine content, into strongly, weakly, and nononcogenic, respectively (28). Since adenovirus types 1 and 24 fall into the high guanine plus cytosine content group of adenoviruses, yet gave tumors in the present study, it is possible that a high guanine plus cytosine content is an inadequate criterion for nononcogenicity.

The induction of tumors in experimental animals by three strains of human adenoviruses (D42, D43, D49) isolated from tumor tissue of children is of interest. The question remains, of course, as to whether the viruses were simply passengers in the human tumors from which they were isolated. This question will be difficult to resolve definitively, even by the detection of viral markers, for tumor virus markers may be superimposed on tumor cells of other origin (29, 30).

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Neutrophil Releasing Activity in Plasma of Normal Human Subjects Injected with Endotoxin* (32774)

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In dogs injected with endotoxin[2] or with vinblastine sulfate or nitrogen mustard (1) neutrophilia-inducing activity was demon-

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