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Inactivation of Adenovirus and Simian Virus 40 Tumorigenicity in Hamsters by Vaccine Processing Methods

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The effectiveness of the adenovirus vaccine inactivation process in destroying the tumorigenic potential for hamsters of adenoviruses, simian virus 40 (SV-40), and adenovirus-SV-40 hybrids was studied. Baby hamsters injected with untreated virus and with samples subjected to the complete inactivation process and to portions of the process were observed for tumor development for periods in excess of 300 days. Over 20,000 hamsters were injected. From 1 to 7 hr of exposure to formaldehyde at a concentration of 0.031 M at 37 C was sufficient to destroy the tumorigenicity observed in the nontreated preparations. Since the inactivation process included 48 hr of exposure at 37 C to 0.031 M formaldehyde plus treatment with ultraviolet (UV) and with β-propiolactone (BPL), it was concluded that the process has a large margin of safety. Adenovirus isolates free from tumorigenic potential are difficult, if not impossible, to obtain. Therefore, a proven inactivation process appears to provide the best assurance for obtaining adenovirus vaccines free from such potential. Data presented suggest that the tumorigenic property of the viruses studied might be independent of the infectivity of the preparation. The tumorigenic property was found to be highly susceptible to formaldehyde, but less sensitive to BPL or UV treatment. In contrast, treatment with UV or BPL decreased viral infectivity more readily than tumorigenicity. The three-stage inactivation process (formaldehyde, UV, and BPL) inactivated both tumorigenicity and infectivity.

Preparation of the vaccines from virus grown in monkey kidney tissue cultures (MKTC) has been complicated by the occasional occurrence of contaminating simian viruses arising from the culture cells. Extensive testing and processing precautions have been followed regularly to detect and prevent the occurrence of any such contaminants in final vaccine preparations. Several investigators (6, 7, 12, 13, 32), however, demonstrated that one such simian virus contaminant (SV-40) was not detectable by the then routine test procedures and was not consistently inactivated by the standard processing methods for the vaccines (Poliovirus Vaccine and Adenovirus Vaccine) produced from MKTC-grown virus. Additional measures (38) were then instituted to assure that all materials used in the production of such vaccines were free from SV-40.

More recently, it has been reported (20, 24, 28, 30) that, although SV-40 itself had been eliminated from all adenovirus vaccine preparations, some adenoviruses demonstrated the potential to incorporate part of the SV-40 genome or to "hybridize" with SV-40 when cultured together with this virus contaminant. Because the growth and antigenic properties of the resulting hybrid were those of the parent adenovirus, the hybridization was not detected by routine test procedures (20).

The property of the SV-40 adenovirus hybrids causing concern, particularly in vaccine production, was the increased or acquired potential of these viruses to produce tumors in baby (less than 48 hr of age) hamsters.

In addition to the findings above, Trentin et al. (37) reported that adenovirus type 12 of human origin was tumorigenic for hamsters. These findings were confirmed shortly thereafter by Huebner et al. (21), who additionally described the hamster tumorigenicity of type 18 adenovirus, also of human origin. Of even greater public health significance was the finding that two human adenovirus serotypes (types 3 and 7 nonhybridized with SV-40) utilized in vaccine production also caused tumors in hamsters (10, 19). The finding of hamster tumorigenic potential asso-

ciated with non-SV-40 hybrid types 3 and 7 adenovirus demanded further considerations and decisions with respect to vaccine production, since the active, nonhybrid immunizing components of the vaccine were involved rather than a contaminant that might be eliminated. These findings prompted the Division of Biologics Standards in 1964 to suspend further release of vaccines containing adenovirus. Viral tumorigenicity in hamsters had not been associated with inactivated virus vaccines or with any known effect in humans. The purpose of these studies, therefore, was to ascertain the effects of recognized viral inactivating procedures on the tumorigenic property of adenoviruses.

The importance of adenoviruses as components of multivalent, upper respiratory viral vaccines for use in the general population and as the sole component of vaccines for military recruits is recognized (18, 19, 21, 39). Therefore, extensive studies were initiated to determine whether properly inactivated vaccine was entirely free from the hamster tumorigenic potential. The results reported here demonstrate the effectiveness of the vaccine inactivation method in destroying hamster tumorigenic potential that might be associated with adenoviruses involved in vaccine preparation.

MATERIALS AND METHODS

Viruses. In-process samples from eight type 3 and eight type 7 regular production lot adenovirus vaccine strain pools (40- to 80-liter volumes of active or inactivated monotypic virus) were available for study (samples supplied by the Production Division of Parke, Davis & Co.). The virus for these strain pools was grown in Cynomolgus monkey kidney (MK) cells free from SV-40. Freedom from SV-40 was demonstrated by testing a sample of the virus pool and pools of tissue culture fluids from corresponding control vessels in primary Cercopithecus tissue cultures in accordance with Section 73.132 of the Regulations (38). Retained samples of the 1- and 7-hr formaldehyde inactivation stages plus samples of the active virus and completely processed vaccine material were tested. The infectivity titers in Cynomolgus MK cells for the type 3 active virus samples ranged from $10^{-2.7}$ to $10^{-4.0}$ and the titers for the type 7 active samples from $10^{-3.1}$ to $10^{-4.0}$ per 0.5 ml. The 1-hr formaldehyde-treated samples of type 3 and type 7 contained minimal amounts of active adenovirus, but actual titers were not determined. The 7-hr formaldehyde-treated samples were negative for adenovirus cytopathic effects (CPE) on titration, but would not pass standard, large-volume, safety-testing procedures (500-ml sample tested in Cercopithecus MK cells, 250 ml in Cynomolgus MK cells, and 250 ml in HEp-2 cells). The postinactivation samples passed vaccine safety tests involving inoculation of monkeys and the testing of 500-ml samples for infectivity in sensitive cell cultures (38). The seed virus employed in preparing these samples was demonstrated to be free from all simian viruses, including SV-40, by testing 5-ml samples in tissue culture with specific adenovirus antiserum as described in the National Institutes of Health minimal requirements. Fluorescent-antibody tests for SV-40 hybridization on cells infected with these type 3 and type 7 seeds demonstrated that they induce SV-40 early-antigen. This demonstrates a genetic hybridization of the adenovirus with SV-40 (30).

Three preparations of active SV-40 derived from an isolate made in our laboratories from naturally infected MK cells were utilized during these studies. The preparation described in Table 7 had a titer of $10^{-6.6}$ per 0.5 ml before inactivation. The other two SV-40 preparations, included in Fig. 1, were tested only as active virus. One had a titer of $10^{-6.9}$ per 0.5 ml, and the other, $10^{-4.9}$ per 0.5 ml. All titrations were performed in African green monkey kidney cells.

Adenovirus type 12. Seed virus was obtained through the courtesy of J. J. Trentin of Baylor University, Houston, Tex. Virus was grown from this seed in SV-40-free primate kidney cells. The pooled, filtered harvest fluids from the third tissue culture passage used in the study had a titer of $10^{-5.1}$ per 0.5 ml before inactivation.

Adenovirus type 7, Pinckney strain. Seed virus was obtained through the courtesy of M. R. Hilleman, Merck Institute for Therapeutic Research, West Point, Pa. The seed virus was in fifth passage in human embryo kidney (HEK) and had a titer of $10^{-7.5}$ per 0.2 ml in HEK. The virus for inactivation was grown in SV-40-free primate kidney cells. The titer of the active virus was $10^{-3.5}$ per 0.5 ml.

Virus inactivation. The strain pool samples were obtained, as noted, from actual vaccine production processing steps. The adenovirus vaccine inactivation process is a three-phase process in which the filtered virus suspension is treated with formaldehyde at a concentration of 0.031 M, incubated at 37 C for 48 hr (in two steps), subjected to 25 incident watts of ultraviolet (UV) light in a Centrifilmer (25), and, finally, treated for another 4 hr at 37 C with β-propiolactone (BPL) at a final concentration of 0.014 M. This procedure and individual phases of it were duplicated experimentally and used on the type 12 and type 7 (Pinckney) adenovirus and SV-40 preparations. Specific parts of the inactivation procedure were evaluated by testing experimentally prepared samples.

Hamster testing. Special facilities to accommodate large numbers of baby hamsters were established. Hamsters, 12 to 13 days pregnant, purchased from the Con-Olson Co., Madison, Wis., were housed in a quiet, isolated "delivery" area. All baby hamsters were less than 24 hr old when injected. All injections were given subcutaneously in the nape of the neck with 0.2 ml of test material. Litters were randomly selected, and, when necessary or desirable, baby hamsters were transferred from one litter to another prior to inoculation. The baby hamsters remained with a mother until they were 21 days old, at which time they were weaned and separated by sex. Before weaning, the litters were housed in a "nursery" area,

separated from other holding areas. Handling of mother or baby hamsters, particularly through the first week, was avoided wherever possible. After weaning, the mother was killed and the weanlings were caged, two to three animals from a given test group per cage. All animal quarters were maintained at 75 to 80 F (23.9 to 26.7 C) with the humidity at a minimum of 20%. A 25 to 30% humidity enhanced the survival of very young (1 to 4 days) hamsters. Whenever possible, groups of approximately 100 animals were used. Control animals injected with phosphate-buffered saline (PBS) or 0.85% saline were used with each study, but are discussed as a group.

Immunofluorescence tests. A modification of the procedures described by Rapp (27) and by Pope et al. (26) was employed. The test was applied to detect and differentiate the following: (i) the early-antigen inducing capacity of the viruses, (ii) antibody to specific T-antigens in sera of tumor-bearing hamsters, and (iii) T-antigen in cultured tumor cells and in direct tumor impression smears. Cercopithecus MK cells were used as the host-cell system for the detection of early-antigen induced by the viruses, and adenovirus and SV-40 transformed cells were used for the detection of specific antibody in tumor-bearing hamsters.

Virus titrations and safety tests. Tissue culture titrations were performed in sensitive primate kidney cell tube cultures in 10-fold dilution steps, with five tubes per dilution. Final titers, expressed per 0.5 ml, were determined on the basis of the presence or absence of CPE at 13 days after tubes were inoculated. [The tube titrations used in these studies are less sensitive than plaque titration techniques presently available. However, a reference virus standard was used in all titrations, and all virus titers (within each type) were controlled by, and corrected to, this standard. Furthermore, the vaccines had been prepared to meet government regulatory standards, and the tube titration method is the one officially recognized.] Tissue culture safety tests were conducted by use of 500-ml samples in three cell types according to National Institutes of Health Minimum Requirements for Adenovirus Vaccine (38).

Evaluation of hamster data. Where applicable, the data were evaluated by use of the "Life Table" analysis for cumulative percentage of tumor development, as utilized by Gellhorn (8) and originally described by Merrell and Shulman (23). This procedure is demonstrated in Table 1 for tumor production by active type 7 adenovirus. All cumulative percentage values reported in this paper were calculated in this manner.

RESULTS

The preinactivation data for each of the eight type 3 and type 7 adenovirus strain pools are plotted in Fig. 1 as pooled samples (one curve for each), since these samples actually represent a single seed virus. Similarly, the curve plotted for SV-40 active virus (Fig. 1) represents the cumulative pooled data for three preparations, since these also represent a single seed virus sample.

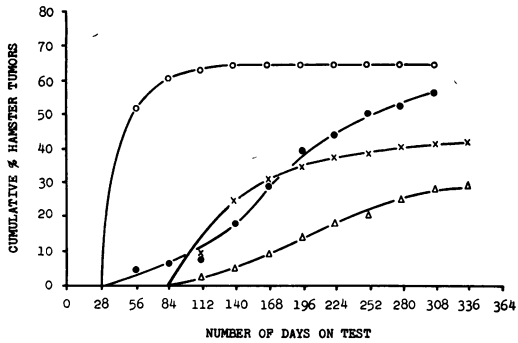

FIG. 1. *Comparative tumorigenicity of active adenovirus types 3, 7, and 12 and SV-40 for hamsters. Symbols:* ○, *type 12;* ●, *SV-40;* ×, *type 7;* △, *type 3.*

The preinactivation data summarized in Fig. 1 show that type 12 adenovirus was the most potent tumorigenic virus both on the basis of rate of tumor appearance and relative incidence.

The numbers of tumors developing in each 4-week period through 336 days after inoculation of the vaccine strain pool type 3 and 7 adenovirus-SV-40 hybrids are shown in Tables 3 and 5. The active type 7 samples were more tumorigenic than the active type 3 adenovirus samples. Tumors first appeared in animals injected with active type 7 adenovirus during the 57- to 84-day interval and continued to appear through the 309- to 336-day period. Maximal tumor production occurred during the interval between 85 and 140 days (Table 1 and Table 5), with a gradual reduction in number of new tumors appearing thereafter. Tumor production in hamsters injected with active type 3 adenovirus first occurred in the 85- to 112-day period and reached a maximum during the period between 141 and 224 days. Although a definite reduction of new tumors in this group (active adenovirus type 3) was observed from day 280 through day 336 (Table 3), some tumors continued to appear after that time.

Infectivity titers for each of the active virus samples of type 3 (Table 2) and type 7 (Table 4) are shown with the total number of tumors produced by each particular preparation. The infectivity titers of the active type 3 adenovirus samples ranged from $10^{-2.7}$ through $10^{-4.0}$ for the eight samples. The number of tumors produced by these samples ranged from 1 to 31 (Table 2). The results show that tumorigenic activity did not correlate directly with infectivity. For example, one preparation with a titer of $10^{-3.7}$ produced only 1 tumor (78 animals weaned with 67 surviving after 327 days on test), whereas a similar preparation with a titer of $10^{-3.5}$ produced 31 tumors (81 hamsters weaned with 70 surviving after 327 days on test). As noted previously, the

TABLE 1. *Hamster tumorigenicity[a] of adenovirus type 7 (vaccine strain) before inactivation*

Days	No. of hamsters[b]						
	1	2	3	4	5	6	7
1–28	1,370	723	1,008.5	0	100	100	0
29–56	647	12	641	0	100	100	0
57–84	635	8	631	2	99.68	99.68	0.32
85–112	625	6	622	56	91.00	90.71	9.29
113–140	563	6	560	92	83.57	75.81	24.19
141–168	465	3	463.5	39	91.59	69.43	30.57
169–196	423	0	423	26	93.85	65.16	34.84
197–224	397	5	394.5	14	96.46	62.85	37.15
225–252	378	0	378	6	98.41	61.85	38.15
253–280	372	4	370	12	96.76	59.84	40.16
281–308	356	7	352.5	7	98.01	58.65	41.35
309–336	342	0	342	3	99.72	58.13	41.87

[a] Analyzed by the "Life Table" technique of Merrell and Shulman (25) as adapted by Gellhorn (10).
[b] Results are as follows. Column 1: number of hamsters alive at beginning of period without tumors. Column 2: number of hamsters dying, or lost, during period without tumors. Column 3: effective number of hamsters at beginning of period; column 1 minus (½ × column 2). Column 4: number of hamsters developing tumors during period. Column 5: estimated per cent of hamsters not developing tumors during period; 100 minus (column 4/column 3). Column 6: cumulative per cent of hamsters not developing tumors; column 6 (day 196) = 93.85 × 69.43 = 65.16 and column 6 (day 224) = 96.46 × 65.16 = 62.85. Column 7: cumulative per cent of hamsters developing tumors; (100 minus column 6).

TABLE 2. *Tumorigenicity of individual adenovirus type 3 strain pool components prior to inactivation*

Strain pool no.	Infectivity titer ($\log_{10}$/0.5 ml)	No. of hamsters		
		Injected	Surviving	Tumors
025012	4.0	168	75	26
025912	3.5	167	70	31
025913	3.2	172	57	17
025914	3.3	170	60	23
025915	3.5	185	56	22
025916	3.7	175	67	1
025917	3.3	170	79	8
025918	2.7	168	79	18

type 7 preparations appeared to be more tumorigenic and, as seen in Table 4, produced a more uniform distribution of tumors. Here again, however, some discrepancy in correlation between infectivity and tumorigenicity was detected. Thus, a type 7 preparation with a titer of $10^{-3.6}$ produced 49 tumors (91 animals weaned with 80 surviving after 327 days), whereas another preparation which had a titer of $10^{-4.0}$ produced only 15 tumors (69 animals weaned with 64 surviving after 327 days). It is also noteworthy (Table 5) that the two active preparations producing the smallest number of tumors were the only ones still capable of producing tumors after 1 hr of formaldehyde treatment.

The high incidence and early appearance of tumors (Fig. 1) from type 12 adenovirus confirmed results reported by Trentin et al. (37) and by Huebner et al. (21). In our experiments, tumors developed in 64% (Table 6) of the hamsters inoculated with type 12 (Baylor) adenovirus. Of these, 51% developed during the 29- to 56-day period postinoculation. Tumor production dropped off rapidly thereafter, and no tumors were found after the 140-day period; these hamsters were observed for more than 300 days.

Tumors induced by active SV-40 (Fig. 1 and Table 7) did not appear until the 141- to 168-day interval and continued to appear at a rather steady rate through 308 days of this study. The incidence of tumors reached 35% during the 281- to 308-day period.

The striking effect of formaldehyde on inactivation of the tumorigenic property of these viruses is apparent in Tables 3, 5, 6, 7, and 8 and in Fig. 2 and 3. Table 9 shows various steps in the inactivation process at which samples were tested. Only 1 hr of formaldehyde treatment was necessary to reduce the tumor rate (cumulative percentage as described under Materials and Methods and illustrated in Table 1) from 28.1 to 0 for the type 3 hybrid and from 41.87 to 0.43 (100-fold) for type 7. Equally important was the finding that no tumors were observed with either of these hybrid strains after inactivation for 7 hr with formaldehyde or with the vaccine after the full inactivation process (formaldehyde-UV-BPL).

TABLE 3. *Summary of effect of vaccine inactivation process on tumorigenicity of adenovirus type 3 strain pool components*[a]

Days on test	Active virus		Virus + HCHO[b] (1 hr)		Virus + HCHO[b] (7 hr)		Post-inactivation[c]	
	No. alive	No. with tumors	No. alive	No. with tumors	No. alive	No. with tumors	No. alive	No. with tumors
1–28	607	0	464	0	622	0	566	0
29–56	591	0	452	0	613	0	512	0
57–84	584	0	448	0	612	0	505	0
85–112	579	13	446	0	607	0	502	0
113–140	578	15	442	0	605	0	501	0
141–168	576	23	431	0	600	0	496	0
169–196	569	26	429	0	592	0	490	0
197–224	568	22	423	0	590	0	481	0
225–252	564	14	415	0	587	0	472	0
253–280	554	26	409	0	573	0	466	0
281–308	545	15	403	0	573	0	466	0
309–336	543	5	403	0	573	0	466	0

[a] Combined data for the eight strain pools listed in Table 2.
[b] Virus treated with HCHO (0.031 M) at 37 C for indicated time, and neutralized with sodium bisulfite for testing.
[c] Includes HCHO (37 C, 48 hr) plus UV (25 incident watts) plus BPL (0.014 M) at 37 C for 4 hr.

TABLE 4. *Tumorigenicity of individual adenovirus type 7 strain pool components prior to inactivation*

Strain pool no.	Infectivity titer (log_{10}/0.5 ml)	No. of hamsters		
		Injected	Surviving	Tumors
025881	4.0	171	64	15
025882	3.4	174	69	14
025883	3.1	159	68	48
025884	2.5	167	64	32
025885	3.5	165	71	37
025886	3.6	173	54	23
025887	3.4	169	66	27
025889	3.6	192	80	49

The effect of formaldehyde on the tumorigenic property of type 12 (Table 6) was even more dramatic than with the hybrid type 3 and type 7 adenoviruses. Hamster tumorigenicity of type 12 adenovirus was completely eliminated by 1-hr inactivation with formaldehyde. Formaldehyde also rapidly inactivated the tissue culture infectivity of type 12 virus. Treatment with UV alone inactivated 99% of the tissue culture infectivity, but produced only a relatively small decrease in tumorigenicity (Table 6 and Fig. 2). The lack of correlation between loss of infectivity and loss of tumorigenicity was even more striking when BPL was used as the sole inactivating agent. Although the tissue culture infectivity was reduced by more than 4 logs, the tumorigenicity level was reduced by only about one-third when compared with the active type 12 adenovirus. The active type 12 adenovirus produced tumors rapidly (Fig. 2). Treatment with UV alone or BPL alone reduced the number of tumors developed and caused some delay in their appearance. The estimated time required to produce tumors in 50% of the hamsters was 6.3, 14.8, and 54.1 weeks for active adenovirus, adenovirus treated with UV, and adenovirus treated with BPL, respectively. There was no overlap in the 95% confidence limits, and, therefore, the differences observed in the tumorigenic activity of the above three groups of adenovirus are statistically significant. Thus, the effect of UV alone and BPL alone on type 12 adenovirus appeared to exert a reproducible and definite reduction in tumorigenicity. However, it should be noted that consistent complete inactivation of infectivity required treatment with formaldehyde combined with either UV or BPL. In contrast, elimination of tumorigenicity was achieved with formaldehyde alone.

To ascertain more quantitatively the effect of formaldehyde on tumor induction, a rate inactivation study was performed with type 12 adenovirus. Complete inactivation of tumorigenicity was obtained when the virus was treated with formaldehyde at a final concentration of 0.103 M for as little as 5 min (Table 8). Similarly, total elimination of tumorigenesis was obtained with less than 15 min of exposure to a final concentration of 0.031 M formaldehyde, the concentration routinely used for vaccine preparations. The effect of formaldehyde dropped off at higher dilutions.

Since formaldehyde was neutralized with sodium bisulfite in the 1- and 7-hr formaldehyde-

TABLE 5. *Summary of effect of vaccine inactivation process on tumorigenicity of adenovirus type 7 strain pool components*[a]

Days on test	Active virus		Virus + HCHO[b] (1 hr)		Virus + HCHO[b] (7hr)		Post-inactivation[c]	
	No. alive	No. with tumors	No. alive	No. with tumors	No. alive	No. with tumors	No. alive	No. with tumors
1–28	647	0	512	0	554	0	597	0
29–56	635	0	496	0	530	0	554	0
57–84	627	2	488	0	519	0	542	0
85–112	621	56	480	0	500	0	538	0
113–140	615	92	479	0	500	0	532	0
141–168	612	39	472	1	484	0	517	0
169–196	612	26	471	0	474	0	512	0
197–224	607	14	466	0	464	0	509	0
225–252	607	6	457	1	459	0	505	0
253–280	603	12	449	0	452	0	500	0
281–308	596	7	443	0	448	0	500	0
309–336	596	3	443	0	448	0	500	0

[a] Combined data for the eight strain pools listed in Table 4.
[b] Virus treated with HCHO (0.031 M) at 37 C for indicated time, and neutralized with sodium bisulfite for testing.
[c] Includes HCHO (37 C, 48 hr) plus UV (25 incident watts) plus BPL (0.014 M) at 37 C for 4 hr.

TABLE 6. *Effect of adenovirus vaccine inactivation process on the hamster tumorigenicity of type 12 adenovirus*

Stage of inactivation	Virus titer[a]	No. of hamsters			Per cent developing tumors[d]
		Injected	Alive[b]	With tumors[c]	
Active virus	$10^{-5.08}$	185	24	53	64.27
HCHO[e] only					
1 hr	0	153	70	0	0
7 hr	0	163	92	0	0
24 hr	0	138	81	0	0
48 hr	0	160	97	0	0
UV[f] only	$10^{-3.08}$	155	38	47	52.24
BPL[g] only	$10^{-0.75}$	155	48	40	42.72
HCHO (24 hr) + UV	0	137	98	0	0
HCHO (48 hr) + UV	0	162	63	0	0
HCHO (48 hr) + UV + BPL	0	163	86	0	0

[a] Infectivity titer per 0.5 ml.
[b] Number of hamsters alive without tumors 308 days after injection.
[c] Total number of tumors developed within group through 308 days.
[d] Calculated from "Life Table" analysis, cumulative per cent.
[e] Final concentration, 0.031 M at 37 C.
[f] Total of 25 incident watts.
[g] Final concentration, 0.014 M at 4 hr and 37 C.

treated samples from actual vaccine lots, a control sample was tested to show that bisulfite was not responsible for inactivation of the tumorigenic property. Type 12 adenovirus treated with sodium bisulfite alone produced tumors in 61% of the hamsters (Table 8), showing good agreement with the active type 12 adenovirus (Table 6). The untreated virus in both tests produced tumors in 65 to 70% (cumulative) of the hamsters injected.

The tumorigenic property of SV-40 was also susceptible to formaldehyde. Data summarizing the effectiveness of the vaccine inactivation process on SV-40 are shown in Table 7. In general, the results support those reported for the type 3 and type 7 adenovirus-SV-40 hybrids and for the nonhybrid type 12 adenovirus. No tumors were formed when the virus was treated with formaldehyde for only 1 hr. A 3-log decrease in

TABLE 7. *Effect of adenovirus vaccine inactivation process on the hamster tumorigenicity of SV-40*[a]

Stage of inactivation	Virus titer	No. of hamsters			Per cent developing tumors
		Injected	Alive	With tumors	
Active virus	$10^{-6.55}$	151	63	35	35.35
HCHO only					
1 hr	$10^{-3.55}$	157	81	0	0
7 hr	$10^{-2.4}$	153	97	0	0
24 hr	$10^{-1.05}$	152	99	0	0
48 hr	0	163	86	0	0
UV only	$10^{-2.35}$	154	63	50	43.87
BPL only	$10^{-2.2}$	150	34	82	70.07
HCHO (24 hr) + UV	$10^{-0.45}$	144	111	0	0
HCHO (48 hr) + UV	$10^{-0.58}$	147	106	0	0
HCHO (48 hr) + UV + BPL	0	160	80	0	0

[a] Explanation of column headings and description of HCHO, UV, and BPL are given in footnotes to Table 6.

TABLE 8. *Effect of formaldehyde concentration and time of inhibition of the tumorigenic property of adenovirus type 12*[a]

Exposure to formaldehyde	No. (cumulative per cent) of tumors appearing after treatment with formaldehyde[b] at a final concn (M × 10^{-3}) of					
	103	30.7	10.3	3.07	1.03	0.307
min						
5	0[c]	5 (71)	8 (53)	19 (66)	15 (44)	17 (80)
15	0	0	9 (50)	6 (41)	9 (51)	7 (47)
30	—	0	0	8 (42)	6 (34)	14 (66)
60	0	0	1 (4)	10 (89)	7 (51)	14 (82)
420	0	0	0	0	9 (64)	7 (64)

[a] Controls: percentage values are cumulative percentages of tumors developed (50 animals/group injected). (i) Untreated virus held at 4 C = 22 tumors (68%); (ii) untreated virus held at 37 C for 60 min = 25 tumors (67%); (iii) HCHO (37 C) for 60 min with no $NaHSO_3$ added = 0 tumors; (iv) virus treated with $NaHSO_3$ (final concentration, 0.35%) for 60 min at 37 C = 20 tumors (61%). Animals were tested 83 to 86 days.

[b] All samples neutralized with sodium bisulfite (final concentration, 0.35%) after indicated time of reaction.

[c] A total of 25 animals/group injected.

infectivity titer was observed (Table 7), and titers dropped proportionately with longer exposure to formaldehyde. Only after 48 hr was infectivity no longer detectable by titration. Previous experience has shown that 48 hr (or longer) of treatment with formaldehyde does not consistently inactivate all traces of residual infectious SV-40. These traces of viruses are detected only when full safety test volumes (> 500 ml) are tested. Safety tests on the preparations studied here confirmed this.

A differential susceptibility to inactivation of the infectivity and tumorigenicity similar to that reported for type 12 adenovirus was observed with SV-40. Moreover, different inactivation procedures which produced similar degrees of effect on infectivity had markedly different effects on tumorigenicity (Table 7). Treatment for 7 hr with formaldehyde produced about the same titer reduction (4 to 4.5 logs) as treatment with UV or BPL, leaving a residual infectivity titer slightly greater than $10^{-2.0}$ in all three cases. However, tumor incidence was 0, 43.87, and 70.07% for the three preparations, respectively.

An apparently anomalous result was seen in the SV-40 study when treatment with UV only and BPL only were compared with the untreated active SV-40. In addition to a higher tumor incidence with the BPL- or UV-treated preparations, the data showed that the estimated time required for 50% of the hamsters to develop tumors was 67.3, 50.6, and 38.6 weeks for the active virus, UV-treated sample, and BPL-treated sample, respectively. Statistical analysis of these data

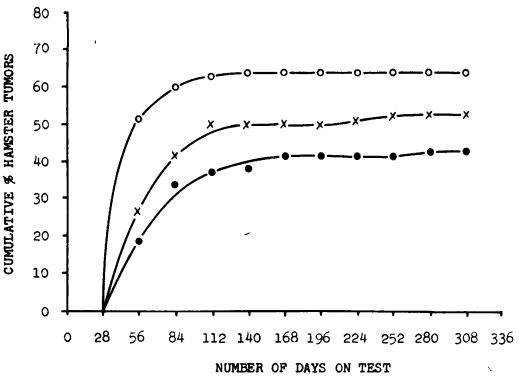

FIG. 2. *Effect of formaldehyde, ultraviolet light, and β-propiolactone on the tumorigenicity of adenovirus type 12. No tumors were produced by virus treated with HCHO for 1, 7, 24, and 48 hr, or after 24 hr of treatment with HCHO + UV, 48 hr with HCHO + UV, and 48 hr with HCHO + UV + BPL. Symbols: ○, active ($10^{-5.1}$ per 0.5 ml); ×, UV only ($10^{-3.1}$ per 0.5 ml); ●, BPL only ($10^{-0.75}$ per 0.5 ml.).*

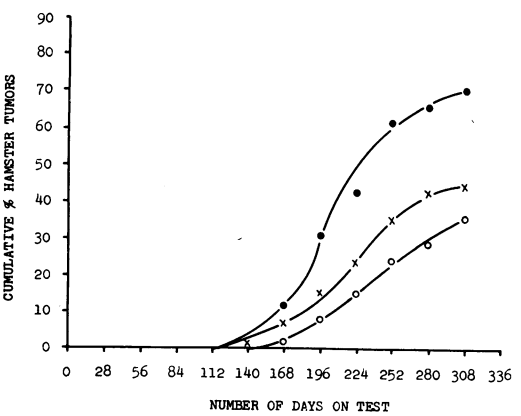

FIG. 3. *Effect of formaldehyde, ultraviolet light, and β-propiolactone on the tumorigenicity of SV-40 virus. No tumors were produced by virus treated with HCHO for 1, 7, 24, and 48 hr, or after treatment for 24 hr with HCHO + UV, 48 hr with HCHO + UV, and 48 hr with HCHO + UV + BPL. Symbols: ●, BPL only ($10^{-2.2}$ per 0.5 ml); ×, UV only ($10^{-2.4}$ per 0.5 ml); ○, active, ($10^{-6.6}$ per 0.5 ml).*

revealed, however, that the upper and lower 95% confidence limits show a marked overlapping, indicating that the differences observed are not statistically significant. Therefore, further studies will be required to determine significance of this observation.

No baby hamsters from the same litters used in the virus studies, but receiving control (saline) injections, developed tumors. Of the 981 hamsters surviving the 21-day weaning period, all remained tumor-free and 843 survived after being on test for 329 days.

As additional controls, and to insure that some unknown factor contributing to tumorigenicity had not been introduced from the MK cells or by their action on media constituents, other groups of hamsters were injected with tissue culture passage control fluids. Of 298 animals weaned, all were completely devoid of tumors through the 300 days of this study (233 survivors). These materials were from the same cell lots and comparable in every way to the test virus preparations except for the inoculation of virus.

The fluorescent-antibody results demonstrated that the vaccine strain pool viruses were adenovirus-SV-40 hybrids and that the other viruses were not hybridized. Tumors uniformly contained T-antigens of the inducing-virus type, and further confirmation was obtained by the presence of anti-early-antigen antibody in the serum of tumor-bearing hamsters.

DISCUSSION

No association has been demonstrated between the tumorigenicity of adenoviruses or SV-40 for hamsters and any similar effect in man. The natural high incidence of adenovirus infections without demonstrated tumorigenicity in humans suggests, in fact, that such an association does not exist. Attempts to isolate viruses from human tumors have generally failed or produced misleading results (3, 4, 11, 14–17, 33). Serological techniques, such as the complement fixation and fluorescent-antibody tests currently providing highly significant results with respect to the hamster tumorigenicity of SV-40 and adenoviruses, have not been similarly useful in characterizing human tumors (29). Thus, it is difficult, if not impossible, to rule out the possibility of a relationship between tumorigenicity in hamsters and some detrimental effect in humans. On the other hand, the importance of adenoviruses in clinical disease is well recognized (18, 19, 31, 39). Further, the need for and effectiveness of adenovirus vaccines have been established (18, 31, 39).

With the knowledge that tumorigenicity for hamsters had been reported only for preparations containing significant levels of live virus, it was considered important to determine whether vaccines prepared from fully inactivated adenovirus retained any such potential. Limited studies to evaluate the vaccine inactivation process were conducted in our laboratories in 1961 and 1962 after reports that SV-40 was tumorigenic for hamsters. These studies attempted to determine whether SV-40 by itself, or as a contaminant of adenovirus vaccine, retained any tumorigenic

TABLE 9. *Tumorigenic activity of vaccine samples taken at various stages of the inactivation process*[a]

Virus treatment	Adenovirus type 3 samples		Adenovirus type 7 samples	
	No. tumors/ no. injected	Per cent with tumors[b]	No. tumors/ no. injected	Per cent with tumors[b]
Untreated	159/1375	28.10	257/1370	41.87
HCHO (0.031 M) for 1 hr at 37 C[c]	0/808	0	2/827	0.43
HCHO (0.031 M) for 7 hr at 37 C[c]	0/873	0	0/739	0
Final vaccine after additional treatment with HCHO, UV, and BPL	0/751	0	0/756	0

[a] Accumulated data on the individual component strain pools indicated in Tables 2 and 4.

[b] Cumulative percentage of hamsters developing tumors (see Table 1 for calculations) through 336 days.

[c] Formaldehyde action stopped by neutralization with 35% sodium bisulfite.

potential after inactivation by the then standard, combined formaldehyde-UV procedure. Results with these inactivated preparations were entirely negative. Although the studies were terminated after 120 to 158 days, 41% of the control group, which received untreated SV-40 virus, developed tumors.

The development of the formaldehyde-UV inactivation procedure as utilized in our laboratories has been extensively reviewed (22, 34–36). The addition of β-propiolactone resulted from an exhaustive search for an agent capable of inactivating residual traces of live SV-40 in formaldehyde-inactivated vaccines. β-Propiolactone was found to completely eliminate any SV-40 infectivity surviving the formaldehyde-UV process without reducing antigenic potency or adding undesirable side effects to the vaccine. The remarkable stability of such residual traces of infectious SV-40 to inactivation procedures has been reported (5, 9, 32). The effectiveness of β-propiolactone therefore was unique. Even though vaccines subsequently were produced completely free from SV-40, the three-phase inactivation process was retained to limit the possibility that other unknown and undetected adventitious viruses might contaminate vaccine preparations and survive standard processing.

The type 3 and type 7 adenovirus vaccine strain pools included in these studies were the most pertinent, and are directly related to the question of effectiveness of the vaccine process and safety of the vaccine since they were actual production preparations. It was assumed in the design of the study and later confirmed by the fluorescent-antibody tests that these adenovirus preparations were hybridized with SV-40. The retained samples of virus from which these strain pools were prepared showed that, before inactivation, these preparations did possess tumorigenicity for hamsters. This tumorigenic potential was destroyed by the vaccine processing method. In fact, no tumors developed in the 739 hamsters receiving retained type 3 and 7 vaccine samples that had been exposed to only 7 hr of formaldehyde treatment, a small fraction of the total process. Moreover, only 2 hamsters developed tumors of those 827 that had received material treated with formaldehyde for only 1 hr. These findings show that an adequate margin of safety is built into the described processing method. Vaccines processed in this manner, therefore, should be considered safe for clinical use.

The SV-40 and type 12 and 7 (Pinckney) strains of adenoviruses were included in this study to provide positive controls with established tumorigenic potential. These viruses, of known, high tumor-inducing potential, constituted a more rigorous challenge to inactivation and, therefore, a better basis for evaluation of the effectiveness of the process. Additionally, the relatively short period of time required for appearance of type 12 tumors provided a convenient model for study. With the peak production of tumors occurring early after injection, a long period would remain in the life span of the hamster, during which time comparisons of differences in tumor incidence resulting from active and inactivated virus of the same preparation would become more meaningful. With type 12 adenovirus, peak tumor production occurred in the 6th to 8th week after injection. Failure of tumors to develop during a period of over 250 days beyond this peak in those hamsters receiving inactivated samples is significant and suggests elimination of the tumorigenic potential rather than inhibition. The SV-40 and type 7 (Pinckney strain) adenovirus had added pertinence since they represented the specific parent gene pools inducing T-antigen and tumor formation concerned in vaccine considerations (SV-40, adenovirus 3, 4, or 7, and hydrids of these).

The data presented show the effectiveness of the vaccine inactivation process in inactivating the relatively high level of tumorigenicity of SV-40 for hamsters. The procedure was thus shown to be effective against the tumorigenicity of all three categories of virus that might be of concern in the production of adenovirus vaccine; i.e., SV-40 adenovirus hybrids (vaccine strain pools), nonhybrid simian virus (SV-40), and nonhybrid adenovirus of human origin (type 12). For all three of these categories the data also show that a significant margin of safety is provided by this process. With only one exception (the two tumors observed with the type 7 hybrid), formaldehyde treatment for only 1 hr completely inactivated the tumorigenic potential of the viruses studied. Since the inactivation process provides two 24-hr periods of formaldehyde treatment as well as exposure to UV and BPL, the procedure should assure the destruction of residual tumorigenic potential.

The differential susceptibility of tumor potential and infectivity (particularly evident with SV-40) suggests that the tumorigenic and infectious properties of these viruses are distinct independent entities, or at least are properties of different parts of the virion. With SV-40, inactivation of infectivity was not complete until the final stage of the process (BPL treatment). In contrast, the tumorigenic potential was inactivated within the first hour of formaldehyde treatment. Similarly, the infectivity loss produced by UV or BPL alone was not accompanied by a corresponding loss of tumorigenicity. Further emphasizing the lack of correlation between loss of infectivity and tumorigenicity are data from two additional studies with active SV-40, to be reported fully at a later date. Briefly, however, little or no correlation can be detected between infectivity titer and tumorigenicity of different tissue culture passages of the same virus (SV-40). A similar lack of correlation between infectivity and tumorigenicity has been observed repeatedly after physical attempts to concentrate SV-40.

As noted earlier, a similar lack of correlation was observed with type 12 adenovirus. Over 90% loss of infectivity was accomplished by treating with either BPL or UV alone (greater than 4-log decrease with BPL). In spite of this, the BPL-treated virus retained over 65% of its original tumorigenicity, whereas the UV-treated virus retained over 80%.

The relative differences of effectiveness on tumorigenic potential and infectivity observed among the different inactivation procedures indicate that: (i) the viral population is nonhomogeneous; (ii) tumorigenicity is not associated with the infectious unit; or (iii) the two viral effects are associated with different loci or combinations of loci on the viral genome. The results reported here do not provide sufficient information to decide among these hypotheses. It is not possible to explain the results on "target size" alone unless the "target" for Formalin inactivation is different from that for UV or BPL. If there is a "tumorigenicity gene" that is not essential to viral replication but is carried by the viral particle, treatment with formaldehyde may selectively inactivate this locus. The differences in susceptibility of infectivity and tumorigenicity demonstrated in this study are in accord with the reported findings of Benjamin (2) and of Basilico and di Mayorca (1). Working independently, these investigators demonstrated that the infectivity of polyoma virus was more susceptible than the tumorigenic property to the inactivating effect of X rays. Similar effects were observed when the polyoma virus was treated with either UV or nitrous oxide (2).

In apparent contrast to our results with SV-40, it has been reported (5) that 2 of 19 hamsters developed SV-40-type tumors 302 and 502 days after injection with formaldehyde-inactivated, SV-40-contaminated adenovirus vaccine concentrated 10 times by ultracentrifugation. It is our experience that concentration by ultracentrifugation has not proved an effective means of increasing virus tumorigenicity. Further, over 600 hamsters injected with SV-40 preparations treated with formaldehyde alone (1, 7, 24, and 48 hr with a final concentration of 0.031 M) have failed to show any evidence of tumors in over 300 days. It must be assumed, therefore, that these tumors were nonspecific. In view of the age of the animals and the lack of serological confirmation of the T-antigen content of these tumors, the relation to the administered small dose of SV-40 seems doubtful.

Rate inactivation studies, still in progress, show that the incidence and time of appearance of SV-40 tumors is directly proportional to the time of exposure of the virus to formaldehyde. To date (125 days), no tumors have been observed in the animals injected with virus treated with formaldehyde for more than 30 min, although 3 of 46 hamsters showed tumors as early as 98 days when the virus was treated for only 5 min. With type 12 adenovirus, more than 5 but less than 15 min of exposure to 0.031 M formaldehyde was required to inactivate tumorigenicity (Table 8). The concentration of formaldehyde was, as one might expect, an important factor influencing the effectiveness of the formaldehyde treatment in inactivating tumorigenic potential.

The results of all of our studies indicate that the tumorigenic potential of adenoviruses, of SV-40, and of adenovirus-SV-40 hybrids is inactivated

by relatively short exposure to the concentration of formaldehyde used for vaccine production. Treatment with UV or with BPL demonstrates some effect on tumorigenicity, but not in proportion to their effect on virus infectivity. Formaldehyde is used in the vaccine inactivation process at a concentration and for an exposure period greatly in excess of that shown to be required. Since BPL and UV treatments are included, vaccine prepared by this combined process is free from tumorigenic hazard beyond a reasonable doubt. Results indicate that this should be true, even if the adenovirus from which the vaccine is prepared contains inherently tumorigenic adenovirus, contaminating SV-40, or adenovirus-SV-40 hybrids.

Our experience in attempting to isolate and prepare new seed virus, free from SV-40 genome and adenovirus tumorigenic potential for hamsters, lends further preference for reliance on a proven inactivation procedure. All of the new isolates of types 3, 4, and 7 adenovirus we have tested induce formation of the type 7 (Pinckney) early-antigen in infected host cells. Hamster studies in progress show that six of the type 3 and three of the type 7 nonhybrid isolates are tumorigenic. These findings suggest that assurance of nontumorigenicity in the active adenovirus from which vaccine is produced may be an unattainable goal. Our experience indicates that approximately 8,000 hamsters would be required to establish nontumorigenicity with greater than 90% confidence on any new isolate, and similar tests requiring over 1 year of observation would have to be made with each passage level.

It would seem, therefore, that the only reliable route to a safe, potent vaccine free from tumorigenic potential is a proven inactivation process.

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