9. Joseph, N. R., Molimard, R., Bourliere, F., Gerontologia, 1957, v1, 18.
10. Catchpole, H. R., Engel, M. B., Joseph, N. R., Fed. Proc., 1961, v20, 143.
11. Knisely, M. H., Bloch, E. H., Eliot, T. S., Warner, L., Science, 1947, v106, 431.
12. Rothman, S., Adelson, E., Schwebel, A., Langdell, R. D., Vox Sanguinis, 1957, v2, 104.

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Studies on Induction of Virus from Adenovirus and SV_{40} Hamster Tumors 1. Chemical and Physical Agents.* (31355)

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Cells of various animal species rendered neoplastic *in vivo* or *in vitro* by viruses commonly exhibit properties which are presently believed to be indicative of continued presence in the cells of viral genetic material even though the presence of infectious virus is no longer demonstrable. These indicators of viral presence include a new transplantation antigen and "T" antigen (sometimes referred to as neo or ICFA antigen) which is demonstrable either by fluorescence microscopy or by complement-fixation using sera from tumor-bearing animals as the source of antibody (1-13). That these antigens are virus-specific rather than host species-specific is taken as support for the concept of continued presence in the cells of part or all of the viral genome.

The retention of viral genetic markers in neoplastic cells of animals is of interest in relation to pathogenesis of tumor and raises the possibility of retention of viral genetic material in human tumor for which viral etiology is presently unproved. Development of methods for inducing retained viral genome in animal tumor to release infectious virus might find ready application in attempts to induce virus from human neoplastic tissues.

Several workers(5,9,13-18) have recorded attempts by a variety of methods to induce or detect occasional infectious virus release from animal tumors initiated by DNA viruses, mostly with negative results. Gerber(19,20), however, claimed to have induced infectious virus from "virus-free" but "virogenic" SV_{40} tumor using chemical or physical procedures or by copropagation *in vitro* with susceptible cells. Additionally, Sabin and Koch(21,22) have presented evidence to indicate spontaneous release, on rare occasions, of infectious virus from SV_{40} tumors and have demonstrated increased release of virus by prolonged cultivation of tumor cells *in vitro*, by X-irradiation or by copropagation with indicator cells in culture.

Extensive studies carried out in our laboratories during the past 2 years have sought to ascertain, in a definitive way, whether infectious virus could be induced from virus-free SV_{40} or adenovirus hamster tumor cells propagated *in vitro*. The methods included exposure to chemical agents known to have metabolic significance, to X-ray, to increased oxygen, and to potential helper viruses with assay for virus by subculture into susceptible cells and by copropagation with susceptible indicator cells. Tumor cell cultures proved free of infectious virus were chosen for study since these seem to bear the closest analogy to human tumors, which, to the present, have not yielded infectious virus(1,23-25) of proved etiologic significance. The present report summarizes the work relating to attempts to induce infectious virus from tumor cells or from "superinfected" tumor cells employing chemical and physical procedures. A sec-

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ond report(26) will describe attempted use of helper viruses for this purpose.

Materials and methods. In the experiments to be described, the cell cultures were incubated in a stationary position at 36°C. All growth and maintenance media contained 100 units of penicillin and 100 μg of streptomycin per ml, sometimes with 100 μg per ml of kanamycin added. Cultures were examined microscopically at 3- to 7-day intervals or as often as necessary to detect toxic and viral cytopathic effects. Growth and maintenance media were changed weekly or as frequently as required to maintain the cell sheets in good condition. *Tumor cell cultures.* The F5-1 cell culture line of an SV_{40}-induced hamster tumor has been described(10). The Pinckney C1-B2 cell culture was initiated from a first passage hamster transplant tumor originally induced by Pinckney strain type 7 adenovirus(9). The Grider F1 cell line was derived from a primary hamster tumor induced by Grider strain type 7 adenovirus (9). The SV_{40} tumor cell line was routinely propagated in medium 199 containing 5% inactivated calf serum; the adenovirus lines were grown in basal medium Eagle (BME) with 10% agamma calf serum. Infectious SV_{40} virus was detected in the primary cell culture of the SV_{40} hamster tumor but not thereafter. Infectious adenovirus was never detected in either adenovirus tumor line. The SV_{40} tumor line was free of pleuropneumonia-like organisms (PPLO) throughout the study but the Pinckney and Grider cell lines were shown to contain PPLO when tested at passages 16 and 31, respectively. Tube cultures used in the induction experiments were prepared using from 2 to 6 $\times$ 10^5 cells and incubated 2 to 4 days prior to use. The cultures were maintained during induction in the same medium as used for growth except that the serum concentration was reduced to 2%.

Assay cell cultures and assay for infectious virus. Routine assay for presence of infectious SV_{40} virus was in the susceptible grivet renal cell line BSC-1; assay for adenovirus was made in primary cell cultures of human embryonic kidney (HEK) obtained commercially. The BSC-1 and HEK cultures were maintained with BME or Eagle's minimal essential medium (EMEM), respectively, containing 5% agamma calf serum. For detecting virus, the cultures to be tested were rapidly frozen and thawed 3 times and 0.1 ml amounts were inoculated into the appropriate cultures which were observed microscopically for cytopathic change for approximately 1 month. At this time, the cultures were freeze-thawed 3 times, subcultured and observed for an additional month for presence of viral cytopathic change. *Indicator cell cultures* employed in the *copropagation* experiments consisted either of BSC-1 cells for SV_{40} tumor cultures, or HEK for adenovirus tumor cultures. One to 3 $\times$ 10^5 indicator cells were added per SV_{40} or adenovirus tumor culture and these were maintained as described above. *Chemical reagents.* Actinomycin D, mitomycin C, puromycin, hydrocortisone, ACTH (porcine), and insulin were from the Merck chemical collection. Proflavine dihydrochloride and sodium thyroxine were obtained from Mann Research Laboratories, Inc., New York and hydrogen peroxide was purchased from Baker Chemical Co., Philadelphia. The stock solutions were made as follows: actinomycin D in 95% ethanol; hydrocortisone phosphate and puromycin dihydrochloride in Hanks' balanced salt solution (HBSS); proflavine in EBME or medium 199 with 2% agamma calf serum; mitomycin C and ACTH in distilled water with addition of the required amount of 1 N or 0.1 N HCl, respectively; sodium thyroxine in 0.05 N NaOH; insulin in distilled water made acidic with HCl and immediately diluted 1:10 in Earle's salt solution containing 0.14% Na HCO_3 or medium 199. All stock solutions contained 100 units penicillin and 100 μg streptomycin per ml and were stored at —20°C until used. Culture tubes to which proflavine or actinomycin were added were wrapped in aluminum foil. *Chemical induction experiments.* Dilutions of the stock chemical solutions were added to the tumor cell cultures and the latter were incubated for the periods shown in the text. After exposure, the cultures were washed once with HBSS and drug-free maintenance medium was added. The cultures were then incubated

for 9 to 24 days at which time they were freeze-thawed 3 times and tested for presence of virus in the assay cell cultures. *Increased oxygen.* Approximately 2 to 6 $\times$ 10^5 tumor cells were added to monolayer cell cultures of HEK (for adenovirus tumor) and to BSC-1 (for SV_{40} tumor) or to empty tubes. After establishment of the tumor cells, the cultures were washed, appropriate maintenance medium added, and incubated at 37°C for 24 hours with loosened caps in a pressure tank at 10 lb p.s.i. of 95% O_2-5% CO_2. After 7 to 11 days further incubation under ordinary conditions, the cultures were freeze-thawed 3 times and assayed for presence of virus. *Irradiation.* Facilities were kindly provided by Dr. J. Gershon-Cohen at Albert Einstein Medical Center, Philadelphia, Pa. A model e Caesatron with cesium^{137} was used as the γ ray source. Tumor cell populations were irradiated in Leighton tubes placed 22 cm from the source using a 10 $\times$ 10 cm cone. The dose rate was 115 r/minute corrected for passage through glass. After irradiation, the supernatant fluid was replaced with fresh medium. The irradiated cultures were observed for 15 to 22 days after which they were freeze-thawed and tested for virus. *Super-infection with SV_{40} virus.* Strain 45-54 (27) of SV_{40} virus grown in grivet monkey kidney (GMK) and SV_{40}-induced F5-1 hamster tumor cell line were used. The tube cultures in Experiment 3 were inoculated with 2 $\times$ 10^4 or 2 $\times$ 10^6 50% tissue culture doses ($TCID_{50}$) of SV_{40} virus. After 18 hours' incubation, the cultures were washed 3 times with HBSS and reincubated with maintenance medium for 4 days. The cultures were treated with mitomycin C for 6 or 24 hours, washed, maintenance medium added, and observed for 18 days. At this time the cultures were freeze-thawed 3 times and titrations of infectious virus were performed in BSC-1 cultures. In Experiment 4, tumor cell cultures in milk dilution bottles were inoculated with 3.9 $\times$ 10^4 or 3.9 $\times$ 10^6 $TCID_{50}$ of SV_{40} virus. Eighteen hours later, the cultures were washed, refed and incubated for 5 days. Thereafter, 7 serial passages (1:3 split) of the cells were made by ordinary procedures following treatment with trypsin. Uninfected control cultures were carried in a similar manner. Infected and control cultures at 7th passage were planted in tubes and 2 days later treated with mitomycin C, proflavine or H_2O_2 by the same general procedure as the chemical induction experiments described above. After chemical exposure, BSC-1 indicator cells were added to half the cultures in the manner described above. Two days after removal of the chemicals, supernatant fluids were collected from all tubes and assayed for infectious virus. Both sets of cultures, *i.e.,* with or without BSC cells, were freeze-thawed on day 13-16 and tested for presence of infectious virus in GMK cell cultures.

TABLE I. Demonstration of Complement-Fixing "T" Antigen in Hamster Tumor Cell Lines.

Tumor cell line	No. of times passed in cell culture Passage levels used for induction exp	Passage level tested for "T" antigen Level	CF titer
Adenovirus 7			
Pinckney C1-B2	47-108	133	1:8
Grider F1	37-92	117	1:4
SV_{40} F5-1	15-49	67	1:16

Results. Evidence for presence of viral genome in tumor cell cultures. The tumor cell cultures employed in the induction experiments represented diverse passage levels as shown in Table I. It was necessary, therefore, to demonstrate that the viral genetic material had been retained on serial cell culture passage and was available for induction. Retention of viral genome was indicated by continued presence of "T" antigen in the cultures at passage levels greater than those employed in the experiments. Tests for "T" antigen were carried out by the complement-fixation method using appropriate antisera from tumor-bearing hamsters.

Induction experiments using cell cultures of hamster tumor. General and specific details concerning the conduct of all the experiments are presented in the section on *Materials and methods.* An abbreviated outline relating specifically to each experiment is given in Table II and should be followed for purpose of clarity. The capital letters (A), (B), (C), etc., in Tables III to VI refer to the

TABLE II. Summaries of Procedures Employed in Induction Experiments.

Exp No.	Procedure
1 (Table III)	(A) Tumor cell cultures treated with chemical or physical agents followed by freeze-thaw to disrupt the cells.
	(B) Freeze-thaw mixture tested in assay cell cultures for presence of infection virus.
2 (Table IV)	(A) Tumor cell cultures treated with chemical or physical agents.
	(B) Indicator cells added to treated cell cultures and observed for development of cytopathology.
	(C) Tumor-indicator cell mixtures were freeze-thawed to disrupt the cells and tested in assay cell cultures for presence of infectious virus.
3 (Table V)	(A) Tumor cell cultures treated with homologous SV_{40} virus for 18 hr to attempt superinfection. Residual free virus was washed from SV_{40}-treated cultures.
	(B) Cultures were exposed to mitomycin C 4 days after virus-treatment. After removal of the drug, cultures were observed for 18 days.
	(C) Cultures were freeze-thawed and tested for infectious virus on subpassage in BSC-1 cultures.
4 (Table VI)	(A) Tumor cell cultures treated with homologous SV_{40} virus for 18 hr to attempt superinfection. Residual virus was removed by washing.
	(B) Tumor cell cultures passed serially and tested for SV_{40} virus in GMK cells at each passage.
	(C) Tumor cell cultures in passage 8 (following virus) were treated with chemical agents after which the culture fluid was replaced.
	(D) BSC indicator cells were added to half of the chemically-treated cultures. These were tested on day 2 (tissue culture fluid) and on day 13-16 (freeze-thawed whole cultures) for infectious virus by subpassage in GMK cell cultures.
	(E) The remainder of the chemically treated cultures without added BSC-1 indicator cells were assayed for infectious virus in the same manner.

appropriate sections of the experiments so designated in Table II.

Experiment 1. (Table III). The tumor cell cultures were exposed to the chemical or physical agents listed in the range of concentrations and for the time periods shown (A). Except for increased O_2, tests with each agent included levels which were not toxic for the cells on the basis of morphology. Toxicity was defined as destruction of at least 25% of the cells. After exposure to chemical or physical agents, the cultures were maintained as long as possible (9-24 days). When the cell sheets began to deteriorate, passage was initiated in the assay cells (B). None of the tumor cells which were treated with the agent showed cytopathic change indicative of infectious virus and virus was not detected on passage in the assay cultures.

Experiment 2. (Table IV). The tumor cell cultures were exposed to 5 selected agents in the range of concentrations and for the time periods shown (A). Indicator cells were copropagated with the tumor cells following removal of the inducing agent. None of the indicator cells showed viral cytopathic change during the observation periods (B) shown. Serial subculture was made at the end of the observation period (C). None of the assay cultures showed cytopathic change indicative of presence of infectious virus.

Experiment 3. (Table V). It was considered possible that SV_{40} tumor cell cultures shown by others(19-22) to release infectious virus after various treatments might be persistently infected with virus and that treatment with chemical or physical agents may have increased the release of infectious virus to readily detectable levels. An attempt was made to develop superinfected cells artificially and to reveal infectious virus on treatment with mitomycin C. Tumor cell cultures exposed to high concentration of SV_{40} virus (2×10^6 $TCID_{50}$) showed the presence of minimal detectable virus whether treated with mitomycin C or not. The virus which was found was interpreted to be residual virus from the original inoculum. Cell cultures exposed to lesser concentration of SV_{40} virus (2×10^4) failed to show virus presence, even after treatment with mitomycin C. There was no evidence for establishment of superinfec-

tion with SV_{40} virus. If superinfection were established, treatment with mitomycin C did not make it apparent.

Experiment 4. (Table VI). This experiment attempted to establish persistently infected cultures by superinfection of tumor cell cultures with SV_{40} virus followed by serial passage. Cultures were assayed for infectious virus after treatment of the cell cultures with mitomycin C, proflavine or H_2O_2. Virus was sought in cultures copropagated with and without BSC-1 cells. SV_{40} virus was shown to be present in the second serial tumor cell passage but not in the remaining 5 passages. The chemical agents in the concentrations and time periods shown did not

TABLE III. Attempt to Induce Infectious Virus from Adenovirus and SV_{40} Hamster Tumor Cell Cultures by Chemical and Physical Procedures with Test for Induced Virus by Subpassage into Assay Cells.

Tumor cell treatment (A)				Observed minimal toxic dose for cells*			Assay result on cell culture passage (all tumor lines) † (B)	
	Concentrations			Adenovirus 7				
Agent	Range	No. tested	Exposure	Pinckney	Grider	SV_{40} F5–1	Days observed (range) ‡	Result (cytopathology)
Actinomycin D	.05– 10 μg	7	4 hr	0.10 μg	0.10 μg	0.10 μg	41–63	Negative
	”	”	24 ”	”	”	”	”	”
Mitomycin C	.10– 10 ”	7	4 hr	>10 ”	>10 ”	>10 ”	49–56	”
	”	”	72 ”	”	”	”	”	”
Puromycin	.01– 10 ”	6	4 hr	0.05 ”	>10 ”	10 ”	55–56	”
	”	”	24 ”	”	”	1 ”	”	”
Proflavine	.5 – 8 ”	5	6 hr	2 ”	4 ”	2 ”	56–57	”
	”	”	24 ”	1 ”	2 ”	1 ”	”	”
Hydrocortisone	.5 –200 ”	7	1 day	>200 ”	>200 ”	>200 ”	56–63	”
	”	”	4 ”	”	”	”	”	”
ACTH	.5 –100 ”	5	4 hr	100 ”	100 ”	100 ”	55–56	”
	”	”	24 ”	5 ”	1 ”	”	”	”
Thyroxine	.5 –100 ”	5	4 hr	>100 ”	>100 ”	>100 ”	50–56	”
	”	”	24 ”	”	100 ”	100 ”	”	”
Insulin	.5 –100 ”	5	4 hr	>100 ”	>100 ”	>100 ”	55–56	”
	”	”	24 ”	”	”	”	”	”
Hydrogen peroxide	4.3–9.8 × 10^{-5}M	4	6– 7 days	8.6×10^{-5}M	4.3×10^{-6}M	>9.8×10^{-5}M	50–57	”
	”	”	8–10 ”	”	”	”	”	”
Oxygen (95%)	10 p.s.i.§	1	24 hr	Toxic	Toxic	Toxic	56	”
γ rays (Cesium^{137})	250–3000 r	4	—	1000 r	>3000 r	3000 r	53–56	”

* The cultures were observed for toxicity and viral cytopathology for 9 to 24 days.
† All tumor cell lines included. Cytopathology on subculture in primary HEK (adenovirus) or BSC-1 (SV_{40}) cells.
‡ Usually one subpassage was made in each instance and each passage was observed for approximately 1 month.
§ Pounds/square inch pressure.

reveal infectious virus either in the presence or absence of indicator cells.

Attempted induction from human tumor. Experiments were carried out with human tumor in cell culture along the lines described above. *Human breast carcinoma (#4)* was propagated in cell culture for 10 passages. Twelve supernatant fluids collected during passages 1 through 4 and representing days 3 to 134 *in vitro* were tested for presence of infectious virus in primary HEK, GMK, and in the WI-38 strain of diploid human embryonic cell culture. No virus was detected during 52-58 days of observation in these cells. Human embryonic kidney cells were copropagated with passage 3 tumor cells representing day 113 *in vitro* and WI-38 cells with passage 4 tumor cells representing day 138 *in vitro*. These cultures were observed 77 and 118 days respectively without detection of virus. Passage 10 human tumor cells were treated with 0.1 and 0.5 μg actinomycin D for 4 hours, the culture fluid changed, and the cultures incubated for 7-14 days. Subpassage of supernatant fluid collected on days 3 and 7 and freeze-thawed whole cell culture from day 14 was made in HEK. No virus was detected during 56 days in subculture. *Human melanoma (#7)* was carried *in vitro* for 1 passage. Eleven culture fluids collected between days 6 and 117 failed to show presence of virus on passage to primary HEK, GMK or WI-38 cell cultures during 52 to 58 days' observation. *Human ovarian tumor (#11)* was carried *in vitro* for 1 passage. Three culture fluids collected between days 4 and 38 were tested in primary HEK and GMK and in WI-38 cell cultures in the same manner as the human melanoma, above. No virus was detected.

TABLE IV. Attempt to Induce Infectious Virus from Adenovirus and SV_{40} Hamster Tumor in Cell Cultures by Selected Chemical and Physical Procedures with Test for Virus by Copropagation with Indicator Cells.

Tumor cell treatment (A)				Observation for viral cytopathology after adding indicator cells (B)				Assay result on cell culture passage* (C) (all tumor lines)	
	Concentrations			Adenovirus 7		SV_{40} F5–1	Result (all lines)	Days observed (range)	Result
Agent	Range	No. tested	Exposure	Pinckney	Grider				
Mitomycin C	.1– 10 μg	5	4 hr	29 days	29 days	24 days	Negative	55–56	Negative
	"	"	72 "	26 "	26 "	21 "	"	"	"
Hydrocortisone	.5–200 "	5	1 day	17 "	12 "	17 "	"	54–56	"
	"	"	4 "	10 "	10 "	10 "	"	"	"
Hydrogen peroxide	4.3–9.8 × 10^{-6}M	4	6 "	17 "	12 "	19 "	"	56	"
	"	"	8 "	17 "	13 "	17 "	"	"	"
Oxygen (95%)	10 p.s.i.†	1	24 hr	11 "	9 "	12 "	"	56	"
γ rays ($Cesium^{137}$)	250–3000 r	4	—	21 "	15 "	22 "	"	53–56	"

* Usually one subpassage was made in each instance and each passage was observed for approximately 1 month.
† Pounds/square inch pressure.

Discussion. The present experiments were undertaken to attempt induction *in vitro* of infectious virus from virus-free tumor cells of hamsters carried in cell culture. Adenovirus 7 tumor cell lines Pinckney C1-B2 and Grider F1 and SV_{40} line F5-1 were chosen because of failure to yield infectious virus during a long and exhaustive test period. The cultures did, however, carry the "T" antigen marker which is considered indicative of the continued presence of viral genetic material. These cultures were different from the SV_{40} tumor cell line of Sabin and Koch (*21,22*) which released SV_{40} virus spontaneously on rare occasions. The choice of virus-free tumor in the present experiments was purposeful in attempting to simulate the hypothetical situation in human tumor.

The methods chosen for testing for infectious virus were aimed at maximizing the chance for demonstrating virus presence, espe-

TABLE V. Attempt to Superinfect SV_{40} F5–1 Hamster Tumor Cells with SV_{40} Virus. Influence of mitomycin C upon recovery of infectious virus.

$TCID_{50}$ of SV_{40} virus added per tumor cell culture (A)	Mitomycin C (B)		SV_{40} titer on subpassage in BSC-1 cells (C)	
	Concentrations	Exposure (hr)	Days observed	Titer ($TCID_{50}$/0.1 ml)
2×10^6	None	6	56–60	$10^{0.2}$
	"	24	"	$10^{0.4}$
	.2 μg	6	"	10^0
	"	24	"	$10^{0.2}$
	.4 μg	6	"	$10^{0.3}$
	"	24	"	10^0
2×10^4	Same above	Same above	56	all $<10^0$
None (control)	" "	" "	"	"

cially through employment of long incubation periods. Tests in this laboratory have shown, *e.g.*, that infected cultures may not reveal the presence of SV_{40} virus until held for 30 to 60 days. The agents employed to attempt induction of virus were chosen based either on previous reports of success in inducing animal virus(19,20) or bacteriophage(28) or because of their known metabolic activities in cells. Actinomycin D(29-31) binds with cellular DNA and inhibits host cell DNA-dependent RNA synthesis. It inhibits interferon synthesis and antiviral activity of interferon. Mitomycin C(32,33) inhibits DNA biosynthesis in mammalian and bacterial cells and has been reported to release bacteriophage from the lysogenic state. Puromycin(30,34, 35) inhibits protein synthesis and has been reported to prevent the action of interferon. Proflavine(36) is a mutagenic agent. Hydrocortisone has been demonstrated to inhibit the synthesis and action of interferon(37). Thyroxine, ACTH, and insulin are hormonal regulators of cellular metabolism. Segre(38) reported production of cytopathic effects by hog cholera and transmissible gastroenteritis of swine in cell culture in the presence of 95% O_2-5% CO_2 at 10 lb p.s.i. Hydrogen peroxide may induce development of phage (28,39). Gamma rays are known to have profound effects on host cell nucleic acid and to induce(28) bacteriophage. Proflavine, H_2O_2 and mitomycin C were recorded by Gerber(20) to induce virus production in virus-free "virogenic" ependymal tumors of hamsters caused by SV_{40} virus. Gerber(19) also reported detection of infectious SV_{40} in the "virogenic" cells by copropagation with susceptible indicator cells. The findings in the present studies present convincing evidence for the failure of a variety of methods to detect minimal amounts or to induce infectious virus in virus-free cell cultures of SV_{40} or adenovirus 7 tumors of hamsters. The present data support the concept that the viral genetic material in the cell which was sufficient to induce "T" antigen was not sufficient to replicate the complete infectious virus. The possibility was considered that SV_{40} tumors might produce less than detectable amounts of infectious virus if superinfected with the agent and that such virus might be increased to detectable levels by treatment with the inducing agents. All evidence, however, indicated that superinfection could not be achieved. The attempts in the present studies to induce infectious virus from cell cultures of human tumor also resulted in failure and this is consistent with the failure in tests of human cells transformed *in vitro* by SV_{40} virus reported by Gilden *et al* (8).

Summary. Cell culture lines of virus-free hamster tumors initiated by SV_{40} virus or by the Pinckney and Grider strains of adenovirus 7 were tested exhaustively by procedures which might be hoped to induce the virus or to increase its chance for detection. This included treatment with actinomycin D, mitomycin C, puromycin, proflavine, hydrocortisone, ACTH, thyroxine, insulin, H_2O_2, increased O_2, γ-radiation and copropagation

TABLE VI. Attempt to Persistently Infect SV_{40} F5-1 Hamster Tumor Cells with SV_{40} Virus and to Recover Infectious Viruses After Treatment with Various Agents.

$TCID_{50}$ of SV_{40} virus added per culture (A)	Presence of SV_{40} virus during serial cell culture (B) Passage No. 1	2	3-7	Tumor cell treatment (C) Agent	Concentrations tested	Exposure	Tests for SV_{40} virus following addition of chemical agents: Plus BSC-1 cells (D) (Supt. fluid) Days	Result	Subpassage on day 13-16* (Disrupted whole cultures) Days	Result	No indicator cells (E) Subpassage on day 2 (Supt. fluid) Days	Result	13-16 (Disrupted whole cultures) Days	Result
3.9×10^4	0	+	0	Mitomycin C	.2 & .4 μg	24 hr	55	Neg.	55	Neg.	55	Neg.	55	Neg.
				Proflavine	1.0, 2.5, 5.0 μg	6 "	"	"	"	"	"	"	"	"
				H_2O_2	4.5, 9.8×10^{-6}M	8 days	"	"	"	"	"	"	"	"
3.9×10^6	+	+	0	Mitomycin C	.2 & .4 μg	24 hr	"	"	"	"	"	"	"	"
				Proflavine	1.0, 2.5, 5.0 μg	6 "	"	"	"	"	"	"	"	"
				H_2O_2	4.5, 9.8×10^{-6}M	8 days	"	"	"	"	"	"	"	"

* Additionally, no direct cytopathic effect was observed in the indicator cells during the 13 to 16 day period.

with indicator cells. In no case was infectious virus recovered. Superinfection of SV_{40} hamster tumor with SV_{40} virus could not be established and hypothetical inapparent superinfection was not demonstrated following treatment with mitomycin C, proflavine or H_2O_2. No virus was detected in the cell cultures of any of 3 human tumors tested. The findings supported the concept that viral genetic materials in the animal tumors which are sufficient to code for "T" antigen may be insufficient to replicate the complete infectious virus.

1. Habel, K., Yale J. Biol. & Med., 1965, v37, 473.
2. Sjögren, H. O., Hellström, I., Klein, G., Cancer Res., 1961, v21, 329.
3. Habel, K., Proc. Soc. Exp. Biol. and Med., 1961, v106, 722.
4. Huebner, R. J., Rowe, W. P., Turner, H. C., Lane, W. T., Proc. Nat. Acad. Sci., 1963, v50, 379.
5. Habel, K., Virology, 1965, v25, 55.
6. Black, P. H., Rowe, W. P., ibid., 1965, v27, 436.
7. Tevethia, S. S., Katz, M., Rapp, F., Proc. Soc. Exp. Biol. and Med., 1965, v119, 896.
8. Gilden, R. V., Carp, R. I., Taguchi, F., Defendi, V., Proc. Nat. Acad. Sci., 1965, v53, 684.
9. Larson, V. M., Girardi, A. J., Hilleman, M. R., Zwickey, R. E., Proc. Soc. Exp. Biol. and Med., 1965, v118, 15.
10. Goldner, H., Girardi, A. J., Larson, V. M., Hilleman, M. R., ibid., 1964, v117, 851.
11. Black, P. H., Rowe, W. P., Turner, H. C., Huebner, R. J., Proc. Nat. Acad. Sci., 1963, v50, 1148.
12. Pope, J. H., Rowe, W. P., J. Exp. Med., 1964, v120, 577.
13. Melnick, J. L., Khera, K. S., Rapp, F., Virology, 1964, v23, 430.
14 Strohl, W. A., Rouse, H. C., Schlesinger, R. W., ibid., 1963, v21, 513.
15. Kitamura, I., Van Hoosier, G., Jr., Samper, L., Taylor, G., Trentin, J. J., Proc. Soc. Exp. Biol. and Med., 1964, v116, 563.
16. Black, P. H., Rowe, W. P., Proc. Nat. Acad. Sci., 1963, v50, 606.
17. Habel, K., Silverberg, R. J., Virology, 1960, v12, 463.
18. Vogt, M., Dulbecco, R., ibid., 1962, v16, 41.
19. Gerber, P., Science, 1963, v140, 889.
20. ———, ibid., 1964, v145, 833.
21. Sabin, A. B., Koch, M. A., Proc. Nat. Acad. Sci., 1963, v49, 304.
22. ———, ibid., 1963, v50, 407.

23. Dmochowski, L., Proc. Fifth National Cancer Conference, Philadelphia, 1964, 57.
24. McAllister, R. M., Calif. Med., 1965, v102, 344.
25. Girardi, A. J., Slotnick, V. B., Hilleman, M. R., Proc. Soc. Exp. Biol. and Med., 1962, v110, 776.
26. Larson, V. M., Gosnell, P. A., Hilleman, M. R., to be published.
27. Sweet, B. H., Hilleman, M. R., Proc. Soc. Exp. Biol. and Med., 1960, v105, 420.
28. Lwoff, A., Bact. Rev., 1953, v17, 269.
29. Goldberg, I. H., Reich, E., Fed. Proc., 1964, v23, 958.
30. Lockart, R. Z., Jr., Biochem. Biophys. Res. Comm., 1964, v15, 513.
31. Taylor, J., ibid., 1964, v14, 447.
32. Iyer, V. N., Szybalski, W., Proc. Nat. Acad. Sci., 1963, v50, 355.
33. Reich, E., Franklin, R. M., ibid., 1961, v47, 1212.
34. Yarmolinsky, M. B., de la Haba, G. L., ibid., 1959, v45, 1721.
35. Friedman, R. M., Sonnabend, J. A., J. Immunol., 1965, v95, 696.
36. Drake, J. W., J. Cell. Comp. Physiol., 1964, v64, 19.
37. Smart, K. M., Kilbourne, E. D., J. Exp. Med., 1966, v123, 299.
38. Segre, D., Proc. Soc. Exp. Biol. and Med., 1964, v117, 567.
39. Tokunaga, T., Mizuguchi, Y., Murohashi, T., Am. Rev. Resp. Dis., 1964, v90, 431.

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Studies on Induction of Virus from Adenovirus and SV_{40} Hamster Tumors 2. "Helper" Viruses.* (31356)

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Cells of various animal species which have been rendered neoplastic *in vivo* or *in vitro* by DNA viruses and which are free of infectious virus appear to retain viral genetic material based on continued presence in the cells of new transplantation antigens and of "T" antigens which are demonstrable serologically(1-12). Cells rendered neoplastic by the RNA Rous virus may also fail to yield infectious virus but retention of viral genetic material is indicated by the apparent continued presence in the cells of a DNA precursor of Rous sarcoma virus(13) and by release of infectious virus from nonproducer cells by infection with another virus of the avian leukosis group which serves as a helper (14). The demonstration of retained viral genetic material in neoplastic cells is of considerable interest in the pathogenesis of cancer and in the development of an approach to elucidating a viral role in human tumor in which infectious virus of etiologic significance has not been demonstrated.

Extensive investigations carried out in our laboratory have sought to ascertain, in a definitive way, whether infectious virus could be induced or recovered from virus-free SV_{40} or adenovirus tumor cells of hamsters propagated *in vitro*. The first report(15) in the present series dealt with attempts to induce infectious virus by application of chemical or physical agents and by copropagation with indicator cells. The present report relates attempts to release infectious virus from virus-free adenovirus tumor cells in culture by use of potential "helper" viruses including SV_{40}, respiratory syncytial virus and reovirus type 1.

Materials and methods. In the experiments to be described, all cell cultures were incubated in a stationary position at 36°C. Growth and maintenance media contained 100 units of penicillin and 100 μg of streptomycin per ml and were changed at weekly intervals or as frequently as required for maintenance of the cells. Cultures were ex-

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