Repression of Benzo[*a*]pyrene Tumorigenesis by Agents Present in Cells Infected or Transformed by Type 12 Adenovirus* (33939)

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The literature dealing with the interaction of viruses and chemical agents in tumor induction and development was reviewed in 1963 by Southam (1). Early observations by Rous and his co-workers and by Francisco Duran-Reynals, showed potentiation of cutaneous papillomas in experimental animals by the combined action of viruses and chemical carcinogens.

Tanaka and Southam (2) found that the intraperitoneal injection of West Nile virus, which is not oncogenic, potentiated the development of methylcholanthrene (MCA)- and benzo[*a*]pyrene (BP)-induced skin papillomas in mice. Heated virus (60° for 0.5–2 hr) had no such effect; nor was the effect noted if virus injection was delayed until after the period of MCA application. These authors later reported (3) that herpes simplex virus, injected intradermally during a 5-day period of MCA application, enhanced the development of MCA-induced cutaneous tumors. The herpesvirus multiplied in the skin, but did not cause papillomas when given alone. They concluded that it was neither an inducer or promoter, and that it enhanced tumor development only when its activity coincided in time and place with that of the chemical inducer. Several other examples of synergistic or "cocarcinogenic" activity of chemicals and viruses are cited in the review by Southam (1).

In contrast to these synergistic effects, Sachs (4) noted a suppressive effect of polyoma virus (PV) injection on a transplantable BP-induced tumor in mice, when marginal numbers of cells (10^4 or 10^5) were subsequently injected. Similarly, Sjögren (5) reported that transplants of 2 out of 35 nonpolyoma-induced tumors (one spontaneous mammary and one MCA-induced) were somewhat susceptible to PV-induced resistance. One MCA-induced sarcoma, originally not susceptible, became susceptible when artifically contaminated with PV. He suggested that "the exceptional transplantation behavior of the 2 nonpolyoma tumors might be caused by an accidental viral contamination before they were tested."

During the past 2 years, we have studied the effect of pretreatment with type 12 adenovirus or Ad12-induced tumor cells on the development of BP-induced tumors in hamsters. Sufficient data have accumulated to indicate that the injection of either of these materials significantly retarded the development of the chemically-induced tumors.

Materials and Methods. Hamsters. Random-bred Syrian hamsters were obtained from a local dealer, or from the Lakeview Hamster Colony. Male and female animals were caged separately in groups of 2–6 and given Purina laboratory chow with water *ad libitum.*

Benzo[a]pyrene (BP) was obtained from Sigma Chemical or Nutritional Biochemicals. On each injection date, an appropriate amount was dissolved in high quality olive oil by heating to 60° in a water bath. The solutions remained clear during the injection period, and precautions were taken to ensure even distribution of BP between control and experimental animals. Each injection consisted of 0.25 ml of the solution, containing 3.0 mg of BP, drawn into a tuberculin syringe with a 26 gauge needle.

Viral materials. Ad12. Lytically infected KB cells were sonicated and frozen in multiple tubes to ensure a stable supply of stock virus. In recent tests, 8 of 24 newborn hamsters developed tumors when injected with 0.1 ml of this stock virus.

HET. Hamster embryo cells, transformed

* Supported by U. S. Public Health Service Grants CA-07972, and CA-10630, and National Cancer Institute contract PH 43-67-692.

TABLE I. Effect of Ad12 and Ad12-Transformed Cells on BP Tumor Development.[a]

Exptl. group	Treatment	Tumor incidence	Av tumor wt (g/hamster)	Range	Treated/control
1	Ad12[b]	8/17	0.9	0.1– 6.0	0.10[c]
	None, contr.	7/9	10.1	1.3–24.2	
2	HET[d]	12/14	1.5	0.1– 6.9	0.47
	Saline contr.	13/13	3.2	0.1–10.2	
	HET (60°, 1 hr)	16/16	5.0	0.3–19.2	1.55
3	HET	13/14	1.6	0.1– 4.4	0.42
	Saline contr.	17/18	3.8	0.4–11.1	
4	HET ip	9/14	3.7	0.1–18.0	0.75
	Saline contr.	15/17	4.9	0.2–25.7	
5	Ad12	10/15	2.6	0.5–14.7	0.31
	Ad12 (60°, 1 hr)	8/13	2.6	0.5–13.7	0.31
	Saline contr.	12/24	8.3	0.2–33.8	
6[e]	Ad12 (60°, 1 hr)	19/23	2.2	0.1– 9.8	0.33
	Ad12	14/19	1.5	0.1–13.8	0.22
	Saline contr.	14/16	6.7	0.2–20.4	
7	HET[f]	8/9	2.6	0.1– 7.8	0.59
	None, contr.	13/14	4.5	0.3–35.0	

[a] All injections, unless otherwise indicated, were given subcutaneously in the lower dorsum. All hamsters were initially 3–4 weeks of age, except in Expt. 6.

[b] Stock virus, grown in KB cells, sonicated and frozen.

[c] Values in this column show ratio of average tumor weight in grams per treated hamster to average tumor weight per control hamster.

[d] Hamster embryo cells transformed by Ad12 and sonicated.

[e] Hamsters less than 24 hr old. Eight Ad12 tumors developed; all tumors identified histologically.

[f] Live HET cells (10^5/hamster). Five HET cell tumors developed and were excised. All tumors were identified histologically as well as by site of injection: Right axilla for HET; left inguinal region for BP.

in tissue culture by Ad12, were routinely grown in spinner cultures at the Institute of Molecular Virology. Injection into adult hamsters of 10^5 cells regularly caused tumors in 70–85% of the animals. Each HET-treated animal (except in group 7, where live cells were injected) received 1 or 2 injections of 0.5 ml of spinner culture medium, containing $4–7 \times 10^7$ cells, disrupted by sonication.

Active immunization. Hamsters in each group (see Table I) were given 1–3 (usually 2) subcutaneous injections of either stock virus (Ad12) or HET cells, the first injection usually 2–3 weeks before the first BP injection, and the second and third injections between 60 and 100 days after the first BP injection. In groups 5 and 6, active and heat-inactivated Ad12 were compared. In experimental groups 6 and 7, Ad12 tumors and HET tumors, respectively, were allowed to overlap in time, but not in position, with BP tumors. In such doubly exposed animals, BP-induced tumors were readily identified by histologic examination.

Approximately 2% of all hamsters died during the 30–45-day period of tumor growth. When tumors were not found postmortem, such animals were excluded from Table I, unless death occurred during the 5 days preceeding termination. When tumors were present, the weight they would have attained if they had lived to the termination date was estimated and recorded. (Deaths in this period were about equally distributed among control and experimental hamsters).

Maternal immunization. Twenty-four syn-

chronously impregnated hamsters, 7 weeks of age, were obtained from the lakeview Hamster Colony, and caged separately. On the third day of pregnancy, 8 were injected subcutaneously with 0.5 ml of active Ad12, in which 1.0 ml of Freund incomplete adjuvant was emulsified, to make a total inoculum of 1.5 ml. A second group of 8 animals were similarly injected, each receiving 0.5 ml of sonicated material, representing about 10^8 HET cells, with 1.0 ml of the adjuvant. The control group received 1.0 ml of adjuvant, emulsified in 0.5 ml of culture medium 199. On the tenth day of pregnancy, the Ad12 and HET injections were repeated without adjuvant, and controls received only culture medium.

Offspring. All hamsters were delivered on day 16. When the offspring were 21 days old, there were 24 survivors in the Ad12 group, 30 in the HET group, and 28 in the control group. At this time, males and females were separately caged in groups of 2–5, put on Purina laboratory chow and water, and given an initial injection of 3 mg of BP in 0.25 ml of olive oil. This BP injection was repeated on day 28 and again on day 35. All injections were given subcutaneously in the right lower back.

Termination procedures. Animals were examined by inspection and palpation at frequent intervals. Each experiment was terminated by etherizing all animals on arbitrarily chosen days, usually when no new tumors had appeared for 14 days, or when several tumors in the control animals became very large and necrotic. The skin was peeled from the back and hind legs, and the exposed tumors were carefully excised and weighed. Small tumors were distinguished from oil granulomas by histologic study, and all tumors less than 1 g in weight were similarly studied.

Results and Discussion. Active immunization. The results, as well as some data omitted from the text, are seen in Table I. Tumors first appeared 115–140 days after the first injection of BP, the number and spacing of the injections apparently having little or no effect on the induction period or growth rate. Losses from fighting and from intercurrent infection (chiefly enteritis) ranged from 5 to 15% during the 11–20 weeks which elapsed before any tumors appeared. (Animals injected with sonicated tumor cells showed some initial reduction in weight gain, and thereafter averaged about 10 g lighter than the controls. Animals receiving virus were comparable in weight to controls. No correlation was found between the size of the hamsters and the weight of the tumors).

While the values for individual experiments (Table I) have relatively low significance, examination of the data from all 7 experiments, involving 154 treated and 111 control animals, indicates a significantly decreased growth rate of BP tumors in hamsters receiving Ad12 or HET. Apart from the overall values, certain characteristics of the data lend additional support to this statement. (i) There is a rather remarkable agreement among the values for each group, the only discordant value appearing in group 2, where heated HET cells resulted in more tumor growth than seen in the controls. (Whether this is due to chance, or to some attribute of the heated cells is not clear.) In group 4, where HET cells were given intraperitoneally, significance is obviously doubtful. (ii) There was a much greater incidence, in treated as compared to control hamsters, of nonpalpable tumors, discovered only at autopsy. (iii) Tumors appeared earlier in the control animals, so that on some examination dates the incidence of palpable tumors in controls was 3–4 times that in treated animals. (iv) Multiple tumors were much more common in control animals. (v) In group 7, where HET and BP tumors were allowed to overlap, 4 BP tumors, after reaching an estimated bulk of 1–2 ml, become necrotic and were rejected percutaneously, a phenomenon not previously seen in any BP tumors. (These BP tumors, identified histologically by finding typical cells at the margins of the necrotic areas, were entered in Table I at their estimated weight before rejection began). (vi) In each group (excepting animals in group 2 given heated HET) the largest tumor was always in the untreated group (Table I) and the average weight of the largest tumors in all treated animals was less

TABLE II. Effect of Maternal Immunization with Ad12 and HET or BP Tumorigenesis in the Mature Offspring.[a]

Treatment	Tumor incidence (palpation)	Tumor incidence (autopsy)	Av tumor wt (g/hamster)	Range	Treated/control
Ad12[b]	4/20	12/20	0.86	0.1– 5.5	0.3[c]
HET[d]	8/28	14/28	0.85	0.1–10.0	0.3
Adjuvant only	14/24	14/24	2.85	0.8–23.0	

[a] See Table I for footnotes *b*, *c*, and *d*.

than half that noted in the controls.

Though tumor incidence was 6% lower in the treated than in control animals, it is probable that additional tumors would eventually have developed in the treated group. Our results probably can be explained by delaying appearance and slower growth in treated hamsters.

Maternal immunization. At the time of the third BP injection, one control animal and 2 from the Ad12 group had been lost by combat. Between then and the appearance of the first 2 tumors in the controls, 3 more control animals, 2 from the Ad12 group, and 2 from the HET group, were similarly lost. Postmortem study of these 7 animals did not show tumor tissue at the site of injection or elsewhere, and there was no apparent cause of death other than trauma. (These 7 animals which died during the period of tumor induction are excluded from Table II).

Tumors were first noted in control animals, about 77 days after the first BP injection. This induction period was considerably shorter than that previously observed, probably because the three injections were given at weekly intervals. Also, the incidence of tumors (about 50%) was lower than that previously noted. The experiment was terminated 95 days after the first BP injection. At autopsy, the average weight of the control animals (tumors included) was 130 g; HET group, 120 g; Ad12 group, 135 g.

The data presented in Table II show no difference in eventual tumor incidence in the treated, as compared with the control animals. There was, however, significant retardation of tumor development in both treated groups, the average tumor weights in grams per hamster being less than ⅓ that noted in the controls. This degree of repression was about equal to that seen when animals were pretreated with Ad12 or HET. It should be noted that all tumors occurring in the control animals were visible and palpable on the termination date, while about half of the tumors in the treated animals were discovered only by postmortem study, and several required histological confirmation. The smallest tumor in the control group weighed 0.8 g, while 14 of the 26 tumors found in treated animals weighed only 0.1 g or less.

Maternal immunization with several oncogenic viruses protected the newborn offspring against tumor formation by the homologous virus (6). We found no previous report of suppression of unrelated tumors by the injection of viruses or virus-transformed cells, except as noted above (4, 5) with PV.

The mechanism of suppression is unknown. Heterogenization, at least by active virus, can be ruled out because heated material was often suppressive, and because HET cells contain no recoverable virus. A logical assumption might be that the phenomenon is related to viral neoantigens. Ad12 is rich in serologic (T) antigen, while HET contains both T and transplantation antigens. (It should also be noted that, although Ad12 does not multiply in hamster cells, abortive infection results in the formation of T antigen). Tumors induced by DNA viruses show immunologic cross-reaction within each group, but cross-reaction between chemical tumors, even when caused by the same chemical, occurs only very rarely (7). Reiner and Southam (8) however, working with 5 MCA-induced tumors, found that immunization with 4 of these sometimes protected against the omitted one. They postulated that points

of identity could occur in the antigenic profiles of some MCA-induced tumors. Similarly, there may be some overlapping between BP-induced tumor antigens and the antigens in Ad12 and HET cells.

Summary and Conclusions. A significant retardation in the appearance and growth of benzo[*a*]pyrene-induced tumors was noted in hamsters pretreated with unpurified type 12 adenovirus (active or heated-inactivated) and also with sonicated virus-free Ad12-transformed hamster embryo cells (HET). Maternal immunization with active Ad12 or sonicated HET similarly retarded the development of benzypyrene-induced tumors in the mature offspring, when the first of three injections of the chemical was given at the age of 3 weeks.

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Received Dec. 20, 1968. P.S.E.B.M., 1969, Vol. 131.

Susceptibility of the *Aedes albopictus* and *A. aegypti* Cell Lines to Infection with Arboviruses (33940)

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In 1967, Singh (1) successfully established cell lines from larvae of *Aedes albopictus* and *A. aegypti,* using a growth-promoting medium that Mitsuhashi and Maramorosch (2) described in 1964 for the propagation of leafhopper tissue culture.

Subsequently, Singh and Paul (3, 4) investigated the susceptibility of these *Aedes* cell lines to infection with various arboviruses. They observed a cytopathic effect (CPE) with Japanese B, West Nile, and dengue types 1, 2, and 4 viruses in the *A. albopictus* but not the *A. aegypti* cell line. Mosquito-borne viruses multiplied in at least one of the two cell lines; two tick-borne viruses, Kyasanur Forest disease and Kaisodi, failed to multiply in either.

The purpose of the present study was to determine whether the *A. albopictus* and *A. aegypti* cell lines could efficiently serve as *in vitro* assay systems for arboviruses. Twenty-three different arboviruses were screened for their ability to produce CPE in or to infect these two cell systems. As a base line for the study, the same viruses were simultaneously tested in two mammalian cell lines, baby hamster kidney (BHK-21) (5) and Vero, both employed routinely in this laboratory for the propagation of arboviruses.

Materials and Methods. Tissue culture. The BHK-21 cell line was maintained according to procedures already described (6). Transfers 12–22 were used in the experiments. The Vero cell line used was obtained as transfer 100 from Dr. N. Wiebenga in 1965, through the courtesy of the Laboratory of Tropical Virology, National Institutes of Health, Bethesda, Maryland. This continuous line of African green monkey (*Cercopithecus aethiops*) kidney cells was originally propagated by Dr. Y. Yasamura at Chiba University, Japan. Confluent monolayers in Roux bottles were dispersed with a mixture of

[1] The Yale Arbovirus Research Unit is supported in part by The Rockefeller Foundation.