Administration of Ethylnitrosourea to Neonate Hamsters Increases Growth and Frequency of SV40-Induced Fibrosarcomas (42958)

SUKHENDRA CHOUDHURY,[1] ANTHONY E. PEGG, JOHN W. KREIDER, DONALD R. MAYO,[2] AND MARY K. HOWETT[3]

Departments of Microbiology and Immunology, Physiology, and Pathology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Abstract. **The *in vivo* interaction between the chemical carcinogen ethylnitrosourea (ENU) and the oncogenic simian virus 40 (SV40) was studied. Inbred newborn Syrian golden hamsters were injected subcutaneously with SV40 (5×10^6 plaque-forming units), ENU (0.5% solution, 125 or 25 mg/kg body wt), or equal mixtures of the two. Animals that received SV40 and ENU developed more tumors (100% vs 52%) within a shorter latent period (10 weeks vs 18 weeks) than animals that received SV40 alone. Animals given SV40 and ENU showed increased mortality and increased metastatic tumors (54.2% vs 30.8%) compared with those given SV40 alone. The SV40 and ENU group also exhibited multiple (>10 nodules) pulmonary metastases (33.3% vs 7.7%) and metastases in multiple organs (12.5% vs 0%) compared with animals injected with SV40 alone. No difference in primary tumor size, histology, and SV40 T-antigen content was detected between SV40- and SV40/ENU-induced tumors. Four weeks after SV40 or SV40 plus ENU treatment, animals were challenged intradermally with 2.7×10^6 SV40-transformed hamster cells. Five weeks after challenge, 89.5% of the animals treated with SV40 and ENU and 45.4% of animals treated with SV40 developed tumors at the challenge site. Newborn animals given SV40 and ENU developed larger tumors at the challenge site ($P < 0.002$) than newborns treated with SV40 alone. Thus, administration of ENU to hamsters during the neonatal stage of development produced a long-lasting systemic effect that enhanced tumor development by transplanted SV40-transformed hamster cells.** [P.S.E.B.M. 1989, Vol 192]

Tumor formation in animal models has been established with a large number of chemical carcinogens and a number of animal viruses. Work on the synergism between viruses and chemicals in the oncogenic process *in vivo* or *in vitro*, however, has been less extensive. Rous and Kidd (1) first showed the cocarcinogenic effect of papillomavirus on the tarred skin of rabbits. Work by Casto and co-workers (2–7) and others (8–10) has shown that chemical and physical agents can enhance the frequency of *in vitro* transformation of hamster cells by DNA viruses. Past *in vivo* studies have shown cocarcinogenic effects of both tumor and nontumor viruses when coadministered with chemical carcinogens to animals. *In vivo* systems using tumor viruses include synergism between papillomavirus and tar (1, 11, 12), rabbit fibroma virus and tar (13), and herpes simplex virus (HSV) and 3-methylcholanthrene (14), as well as others. Hatch *et al.* (7) obtained transformed hamster cell lines by pretreatment with a chemical carcinogen and subsequent infection with simian adenovirus (SA7) that produced a higher incidence of tumors than cells transformed by

[1] Present address: Department of Microbiology, Georgetown University Medical Center, 3900 Reservoir Road, NW, Washington, DC 20007-2197.

[2] Present address: State Virology Laboratory, 10 Clinton Street, Hartford, CT 06144.

[3] To whom correspondence and reprint requests should be addressed at Department of Microbiology and Immunology, The Pennsylvania State University, College of Medicine, Hershey, PA 17033.

Received February 7, 1989. [P.S.E.B.M. 1989, Vol 192]
Accepted May 2, 1989.

0037-9727/89/1921-0072$2.00/0

SA7 alone. These data suggest that the chemical carcinogen influences transformation of the cells by the virus.

We examined the subcutaneous inoculation of the DNA tumor virus, simian virus 40 (SV40), and the *N*-nitroso compound, ethylnitrosourea (ENU) in newborn inbred hamsters in efforts to develop a system of cocarcinogenic interaction. The *N*-nitroso compounds themselves are important environmental carcinogens (for review, see 15), and have been shown to produce tumors in many species of animals and malignant transformation in a number of cell lines. One subclass of these carcinogens, the nitrosamides, is advantageous for cocarcinogenic studies because these compounds do not require metabolic activation and have a short half-life at physiologic pH. Because of these properties, accurate control of the time that the carcinogen is present relative to the addition of virus is possible. The nitrosamides include ENU.

Our experiments showed that a decreased latent period for tumors that possessed the properties of virus-induced tumors occurred in animals given virus plus carcinogen compared with the latent period for tumor development in animals that received virus alone. Further investigation of this phenomenon has shown that administration of ENU to neonatal hamsters caused a long-lasting systemic effect that altered the growth of tumors arising from transplantation of SV40-transformed fibroblasts. One likely explanation of this phenomenon is a generalized suppression of immune development in animals exposed at an early age to ENU.

Materials and Methods

Virus and Carcinogens. SV40 (LP-4 large plaque variant of the RH 911 strain) was administered as a cell-free virus stock. Virus was grown in a continuous line of African green monkey (CV-1) cells as previously described (16), and virus titer was measured by plaque assay on primary African green monkey kidney cells as described previously (17). The titer of the stocks used was about 1×10^8 plaque-forming units (PFU)/ml. Animals that received SV40 received 0.05 ml of virus, i.e., 5×10^6 PFU.

Animals given ENU received either 0.05 (0.25 mg) or 0.01 ml (0.05 mg) of stock solution (a 0.5% solution made in 5 m*M* citrate buffer, pH 5). Carcinogen was stored at −20°C, wrapped in aluminum foil to protect it from light. Carcinogen concentration was routinely monitored by spectrophotometry. The average newborn weight of a hamster is 2 g; therefore, these doses correspond to about 125 mg/kg and 25 mg/kg, respectively. Syrian golden hamsters (LAK:LSH strain; Lakeview) received SV40, ENU, or mixtures of the two. Mixing was performed just before subcutaneous injection. Animals were injected within 72 hr of birth on the back between the shoulders. Control animals and animals receiving one member of the pair received admixtures that contained appropriate sham inoculation solutions. The injected volume per animal was kept constant at 0.1 ml. Animals were sexed and separated at 3–4 weeks of age. Each animal was individually marked by ear punch.

Tumor Measurement. Tumor size was measured twice weekly using slide calipers. Three measurements (height, width, and breadth) were used to calculate geometric mean diameter (GMD) of the tumor mass. In tumors with more than one nodule, each was measured separately and the results were summed.

Measurement of Metastases. Only grossly visible metastases were counted. Visualization of surface pulmonary metastases was assisted by transtracheal inflation of the lungs with 10% India ink in 10% neutral-buffered formalin and subsequent placement in Fekete's (18) solution (8.7% formaldehyde, 60.9% alcohol, and 4.4% glacial acetic acid). Because metastatic nodules do not share the alveolar airspace, the metastases remain white, whereas the normal lung tissue is blackened.

Growth of Tumor Cells for T-Antigen Staining. Portions of the tumors (approximately 2 cm^3 in size) were minced, washed, and trypsinized. The cells were seeded into 60-mm plates and incubated at 37°C. Clones of cells were evident in 7–10 days. The cells were passaged, and confluent monolayers of these cells were trypsinized and seeded onto coverslips. The coverslip cultures were incubated for 3 to 4 days at 37°C. The cells were fixed and stained for immunofluorescence or immunoperoxidase visualization at a point when they were still subconfluent on the coverslips.

Immunofluorescence Assays for SV40 T-Antigen. Coverslips containing cells were fixed in acetone and placed in Petri dishes; then, 100 μl of normal mouse serum (diluted 1/10), 100 μl of mouse anti-T serum (diluted 1/10) or anti-T mouse monoclonal antibody (diluted 1/100) was added to each coverslip. Anti-T antibody preparations were kindly provided by Dr. S. Tevethia (The Pennsylvania State University College of Medicine). Goat anti-mouse fluorescein isothiocyanate-conjugated serum (diluted 1/20) (100 μl) was added as the secondary antibody.

The positive control used for SV40 T-antigen immunofluorescence was an SV40-transformed cell line (C57BL6/WT-1; kindly provided by Dr. S. Tevethia) known to exhibit T-antigen.

Immunoperoxidase Assays for SV40 T-Antigen. Coverslips containing cells were washed twice with phosphate-buffered saline (PBS) and fixed with cold 95% ethanol at −70°C for 1 hr and allowed to dry. Dako Pap kit K550 (Dako Corp., Santa Barbara, CA) was used for immunoperoxidase staining as per the manufacturer's instructions. Normal mouse serum (diluted 1/300) and anti-T mouse monoclonal antibody (diluted 1/300) were used. Both positive and negative controls were used in every test run.

Microscopic Examinations. Tumor samples as well as visceral organs from a number of animals were fixed in 10% neutral buffered formalin. The tissue pieces were embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin.

Challenge Experiment. Two groups of 2-day-old hamsters were injected subcutaneously with SV40 and ENU or SV40 and citrate buffer, as described above. Four weeks later, both groups of animals were injected intradermally over the rump with 2.7×10^6 syngeneic SV40-transformed hamster cells (F5-1). These cells were kindly provided by Dr. F. K. Huebner (The Wistar Institute, Philadelphia, PA). The body weights of each animal were recorded 4 weeks after subcutaneous injection. Tumor measurements at the challenge site were taken once a week.

Results

Effect of Carcinogen on Virus Titer. Because we were concerned that the ability of ENU to alkylate proteins and nucleic acids might affect the infectivity of our virus preparations, we titrated the SV40 stock after exposure to ENU. Stock SV40 (in cell culture medium) was mixed with high doses of carcinogen under conditions identical to those used for preparation of injectable virus. The final ENU concentration in two separate samples was 5 mg/ml and 1 mg/ml. Equal volumes of SV40 and ENU solutions were mixed, incubated for 1 hr at 37°C, and then titrated in our standard plaque assay. Table I shows that there was no significant effect on the plaque-forming ability of the virus after this treatment. While ability to form plaques was not affected, we cannot exclude other more subtle effects on virus viability.

Tumor Growth in Animals Injected with Virus, Carcinogen, or Virus Plus Carcinogen. Three experiments were performed. In each experiment, at least two litters (about 20 animals) were devoted to each experimental variable. The results of the three experiments were identical and indicated a drastic shortening of the latent period for tumor development in virus plus carcinogen-treated animals compared with animals infected with SV40 only. Animals given ENU alone did not develop any grossly visible tumors within the 6- to 9-month time frame of the experiments. Some of these animals developed nevi at the injection site.

In the first two experiments both ENU doses (125 mg/kg and 25 mg/kg) were admixed with virus. The most notable differences were seen in animals given the 125 mg/kg dose, although both doses demonstrated an effect. Only 125 mg/kg of ENU was used in the third experiment, the results of which are described below.

Table I. Effect of ENU on SV40 Infectivity

Conditions	Virus titer (PFU/ml)
SV40	7.8×10^8
SV40 + ENU (5 mg/ml)	5.3×10^7
SV40 + ENU (1 mg/ml)	4.5×10^7

Figure 1 demonstrates that tumor onset in the SV40 plus ENU group was earlier (10 weeks vs 18 weeks) than in the group given SV40 alone. In addition, more animals (100% (25/25) vs 52% (13/25)) developed tumors within the observed time period. Figure 2 demonstrates that the SV40 plus ENU group also exhibited dramatically increased mortality compared with the group receiving only SV40.

In addition to advanced onset and increased incidence of primary tumors, animals given SV40 plus ENU exhibited increased numbers of metastases (Table II). The metastatic lesions demonstrated the histology and distribution characteristic of metastases from an SV40-induced primary tumor. Such lesions never appeared in animals inoculated with ENU alone. In total, 54.2% (13 of 24) of animals receiving SV40 plus ENU vs 30.8% (4 of 13) of animals receiving only SV40 showed metastatic lesions, with the most common site for metastases being the lungs. Additionally, multiple

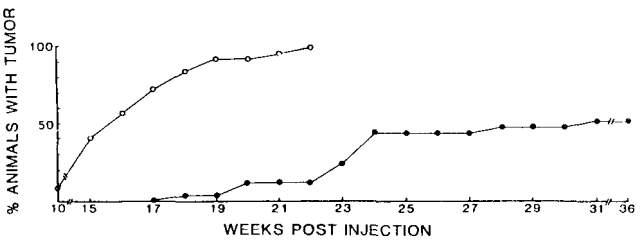

Figure 1. Incidence of tumors in hamsters after inoculation with SV40 (●) (25 animals), or SV40 plus ENU (○) (25 animals). Tumors appeared earlier (10 weeks after infection) in animals given SV40 plus ENU than in animals given SV40 (18 weeks after infection). One hundred percent (25 of 25) of animals given SV40 plus ENU developed tumors by week 22 after infection and 52% (13 of 25) of animals given SV40 developed tumors by termination of the experiment (36 weeks after infection). Statistical analysis using the Mann-Whitney *U* test indicated $P < 0.00003$.

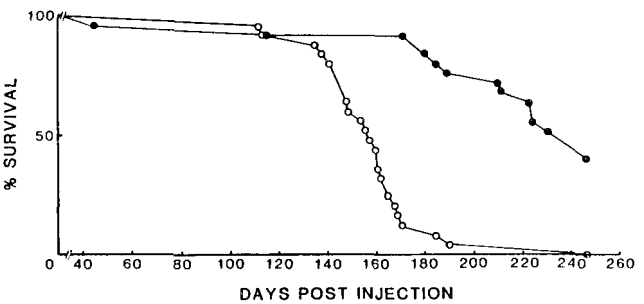

Figure 2. Animal survival after inoculation of newborns with SV40 (●) (25 animals) or SV40 plus ENU (○) (25 animals). Animals died as a result of tumors or were sacrificed when moribund. The cause of death was either metastatic tumor burden or overwhelming size of the primary tumor. The last point on each line represents termination of the experiment. Three tumor-bearing animals given SV40 and one tumor-bearing animal given SV40 plus ENU were sacrificed at this time. None of the animals in the SV40 plus ENU group survived, whereas 10 animals in the SV40 group survived without any development of tumors at termination of the experiment. Statistical analysis using the Mann-Whitney *U* test indicated $P < 0.00023$.

Table II. Incidence of Metastases in Tumor-bearing Hamsters Inoculated as Newborns with SV40 or SV40 Plus ENU

	SV40	SV40 + ENU
All animals	4/13 (30.8%)	13/24 (54.2%)
Animals with multiple (>10) lung metastases	1/13 (7.7%)	8/24 (33.3%)
Animals with metastases in multiple organs	0/13 (0%)	3/24 (12.5%)

(>10) pulmonary metastases were seen in 33% (8 of 24) of the SV40 plus ENU group but only in 7.7% (1 of 13) of the SV40 group. Metastases also were seen in other organs (kidney, liver, spleen), but only in animals with large numbers of pulmonary metastases and only in the SV40 plus ENU group (12.5%, 3 of 24). In summary, coadministration of carcinogen with virus increased the rate of metastatic disease, multiple pulmonary metastases, and metastases in multiple organs. The trend indicated a correlation between carcinogen exposure and increased risk for metastatic disease. Of the animals studied, relatively few developed metastatic disease, especially in the SV40 group. To specifically examine differences in metastatic disease between the two groups, a larger number of animals bearing primary tumors would need to be studied. There were no major differences in tumor size at time of death or sacrifice [44.7 ± 19.7 (n = 13) vs 46.9 ± 11.7 (n = 24)] between animals given SV40 and those given SV40 and ENU.

Morphologic Appearance and T-Antigen Content of Tumor Samples. The microscopic appearance of tumors found in all animals that received virus alone or admixtures of virus and carcinogen was typical of SV40 fibrosarcomas. Primary tumors contained spindle-shaped, multinucleated cells that grew in whorls. The cells in subcutaneous tumors were oriented along collagen fibers. The metastases exhibited a greater degree of pleomorphism and appeared far less differentiated. More mitotic cells were observed in these sections. The subcutaneous lesions that developed in the ENU-treated animals, however, presented with a totally different morphology. These lesions were diagnosed as benign pigmented cell clusters (nevi). The nevi grew in nests of cells and contained melanin as determined by microscopy.

Tumors from each experiment were sampled for T-antigen staining. All of the tumors examined from animals that were inoculated with only SV40 (16 of 16) or with SV40 and ENU (16 of 16) were T-antigen positive when tested with serum against SV40 T-antigen using either immunofluorescence or immunoperoxidase methods. These tumor cells did not stain when normal mouse serum was used. The "lesions" derived from animals that received only ENU were consistently T-antigen negative (0 of 6) and, in addition, it was difficult to establish these cells in culture. These lesions had a separate and distinct appearance when compared with the tumors of SV40 and/or SV40 plus ENU origin. Pulmonary metastatic tumor cells grown *in vitro* also were T-antigen positive, as expected.

ENU Exerts a Systemic Effect on SV40 Tumor Growth. We wanted to determine whether the observed differences were related to a direct effect on virus transformation or to an indirect effect, such as a systemic action on the hamster physiology. We measured the ability of neonates injected with SV40 alone or in combination with ENU to respond to a challenge with SV40-transformed cells (F5-1). This experiment was designed to examine the systemic reactivity (presumably immunity) against a specific target, i.e., SV40-transformed hamster cells. Newborn hamsters received injections of SV40 or SV40 and ENU within 72 hr of birth. The tumor challenge consisted of 2.7×10^6 F5-1 cells injected intradermally over the rump 4 weeks later. An intradermal inoculation was chosen because tumor cells are confined to a much more circumscribed area than in a subcutaneous inoculation. This allows more precise measurement of tumor size. We observed that the body weight of animals 4 weeks after inoculation with SV40 and ENU was significantly lower ($P < 0.001$, Student's t test) than that of animals inoculated with SV40, suggesting that ENU might have some systemic effect on the physiology of hamsters. Animals that received SV40 and ENU, however, were not grossly underweight and did not appear sickly. The tumor challenge results (Fig. 3) demonstrated that, 5 weeks after challenge, 45.4% (5 of 11) of the group given SV40 alone and 89.5% (17 of 19) of the group given SV40 and ENU developed measurable tumors at the challenge site. In addition, the tumor masses in the virus/carcinogen group were significantly larger ($P < 0.002$, Student's t test) than in the virus-only group (Table III). The challenge experiment which measured SV40 tumor

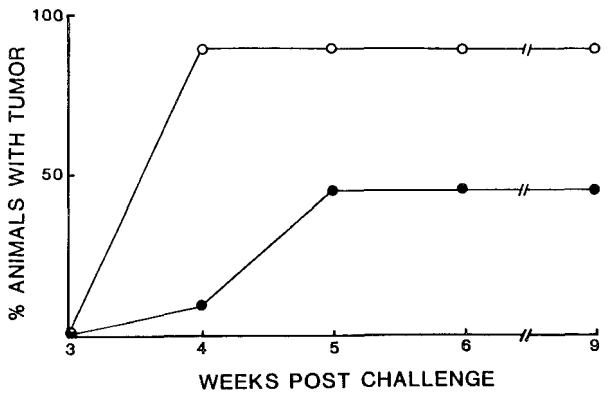

Figure 3. Development of tumors at the challenge site (over the rump) following intradermal inoculation of SV40-transformed hamster cells in SV40 (●), or SV40 plus ENU-treated animals (○). Five weeks after intradermal challenge, 45.4% (5 of 11) of animals given SV40 and 89.5% (17 of 19) of animals given SV40 plus ENU developed tumors at the challenge site. Statistical analysis using the Mann-Whitney U test indicated $P < 0.00003$.

Table III. GMD of SV40 Tumors at the Challenge Site, 8 Weeks after Challenge

SV40 (GMD ± SD)	SV40 + ENU (GMD ± SD)
4.3 ± 1.7 (n = 4)	9.2 ± 2.4 (n = 17)

growth at a site distant from the primary site (both in location and time) reflected the same result seen in the admixture experiments. This experiment indicated that advanced onset of tumors, increased incidence of tumors, and increased tumor size were mediated systemically rather than locally.

Discussion

N-Nitroso compounds are potent carcinogens (19, 20). We used a combination of SV40, a DNA tumor virus, and an *N*-nitroso compound, ENU, to study the interaction between the two. The use of the nitrosamide ENU in our experiment is well suited as this compound breaks down spontaneously at pH 7.0 or above to form reactive alkylating compounds (21). Previous experiments have shown that *in vitro* exposure of cells to nitrosamides can influence their ability to be transformed by the thymidine kinase gene of HSV (22).

Newborn hamsters that received virus plus carcinogen developed more tumors (100%) with a reduced latent period (10 weeks) and increased mortality compared with animals that received virus alone (Figs. 1 and 2). Animals given SV40 and ENU exhibited increased incidence of tumor metastases compared with those given SV40 alone (Table II). The histology of tumors that arose after inoculation with SV40 or SV40 and ENU was consistent with the SV40-induced hamster tumors, i.e., poorly differentiated fibrosarcomas. Cell lines established from SV40- and SV40 plus ENU-induced tumors, including metastases, demonstrated T-antigen at the same frequency and apparent intensity.

DNA extracted from tumor cell lines of SV40- and SV40 plus ENU-induced tumors was probed with a ^{32}P-labeled plasmid DNA containing an intact copy of the SV40 genome (WT-2). Probe was kindly provided by Dr. M. J. Tevethia (The Pennsylvania State University College of Medicine). There was no indication that SV40 plus ENU treatment consistently increased the SV40 copy number in these tumors relative to those arising from SV40 alone.

Chemical carcinogens have been shown to reduce both the humoral and cell-mediated immune response of the host (23–26). Neonatal thymectomy, which induces severe impairment of the immunologic capacity of the host, has been shown to increase the susceptibility of rats (27, 28) hamsters (29), and mice (30, 31) to tumor induction by polyoma/SV40 viruses. A single sublethal dose of ENU in adult mice has been shown to cause a significant decrease in bone marrow cell numbers and weight decrease of thymus and spleen (32). The suppression of natural killer cell activity in ENU-treated animals could lead to decreased tumor latency and increased tumor incidence (26).

Because we did not observe any phenotypic differences in the SV40-induced tumors compared with the SV40 plus ENU-induced tumors, and because several investigators have suggested that carcinogens can enhance tumor induction by viruses by immunosuppression of the host (27–31), we tested the hypothesis that ENU can systemically alter the host in a specific way that is beneficial to the growth of transplanted SV40-transformed syngeneic tumor cells.

The role of the immune response in SV40 oncogenesis in the nonpermissive host has been well established (33). We observed that 4 weeks after challenge with SV40-transformed hamster cells (F5-1), palpable tumors were seen in 90% of animals that received SV40 and ENU, compared with 10–15% of animals that received only SV40. Since ENU reacts very rapidly at neutral pH and under physiologic conditions, and since the challenge inoculation was at a site (over the rump) distant both in time (4 weeks) and place from the carcinogen inoculation (over the shoulder), we postulate that inoculation of newborn hamsters with ENU resulted in a systemic effect on the host rather than a local effect that favored growth of transplanted SV40-transformed cells. One possible explanation of this phenomenon is that ENU ablates some aspect of the immune system of the host that is involved in suppression of SV40-induced tumor growth. ENU may be inducing chemothymectomy of the hamsters since it is well established that T cell function is involved in the immune response to SV40. Specific testing of T cell function will be necessary to fully understand the mechanism of this effect. We conclude that the enhancement of SV40 oncogenesis by ENU was a nonspecific, rather than a specific, effect on the virus interaction with the transformed cells.

This work was supported in part by Public Health Service Grants CA-25305, CA-09124, and CA-18137 awarded by the National Cancer Institute, National Institutes of Health; SIG-II, awarded by The American Cancer Society, and the Jake Gittlen Memorial Golf Tournament.

The authors are grateful to Dr. Gerald Bartlett (The Pennsylvania State University College of Medicine) for assistance with the statistical analyses. The authors thank Ms. Melissa C. Hill for careful editing of the manuscript and Ms. Elaine K. Neidigh for typing.

1. Rous P, Kidd JG. The carcinogenic effect of a papilloma virus on the tarred skin of rabbits. I. Description of the phenomenon. J Exp Med **67**:399–428, 1938.
2. Casto BC. Enhancement of adenovirus transformation by treatment of hamster cells with ultraviolet irradiation, DNA base analogs, and dibenz(a,h)-anthracene. Cancer Res **33**:402–407, 1973.
3. Casto BC. Enhancement of viral oncogenesis by chemical carcin-

ogens. Chemical carcinogenesis. In: Tso POP, Dipaolo JA, Eds. The Biochemistry of Disease. New York: Marcell Dekker, Vol **4:** pp 607–618, 1974.
4. Casto BC, Dipaolo JA. Virus, chemicals and cancer. Prog Med Virol **16:**1–47, 1973.
5. Casto BC, Pieczynski WJ, DiPaolo JA. Enhancement of adenovirus transformation by pretreatment of hamster cells with carcinogenic polycyclic hydrocarbons. Cancer Res **33:**819–824, 1973.
6. Casto BC, Pieczynski WJ, DiPaolo JA. Enhancement of adenovirus transformation by treatment of hamster embryo cells with diverse chemical carcinogens. Cancer Res **34:**72–78, 1974.
7. Hatch GG, Balwierz PJ, Casto BC, DiPaolo JA. Characteristics of hamster cells transformed by the combined action of chemical and virus. Int J Cancer **21:**121–127, 1978.
8. Coggin JH. Enhanced virus transformation of hamster embryo cells *in vitro.* J Virol **3:**458–462, 1969.
9. Hirai K, Defendi V, Diamond L. Enhancement of simian virus 40 transformation and integration by 4-nitroquinoline 1-oxide. Cancer Res **34:**3497–3500, 1974.
10. Ledinko N, Evans M. Enhancement of adenovirus transformation of hamster cells by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, caffeine and hydroxylamine. Cancer Res **33:**2936–2938, 1973.
11. Rogers S, Rous P. Joint action of a chemical carcinogen and a neoplastic virus to induce cancer in rabbits: Results of exposing epidermal cells to a carcinogenic hydrocarbon at time of infection with Shope papilloma virus. J Exp Med **93:**459–488, 1951.
12. Rous P, Kidd JG. The carcinogenic effect of a virus upon tarred skin. Science **83:**468–469, 1936.
13. Ahlström CG, Andrewes CH. Fibroma virus infection in tarred rabbits. J Pathol Bacteriol **47:**65–86, 1938.
14. Tanaka S, Southam CM. Joint action of herpes simplex virus and 3-methyl-cholanthrene in production of papillomas in mice. JNCI **34:**441–451, 1965.
15. IARC monograph on the evaluation of the carcinogenic risk of chemicals to humans: Some *N*-Nitroso compounds. Lyon, France: IARC, Vol **17**, 1978.
16. Tan KB, Sokol F. Structural proteins of simian virus 40: Phosphoproteins. J Virol **10:**985–994, 1972.
17. Howett MK. Protein synthesis in African green monkey kidney cells infected by simian virus 40. Doctoral dissertation, University of Pennsylvania, Philadelphia, pp39–41, 1976.
18. Fekete E. A comparative morphological study of the mammary gland in a high and a low tumor strain of mice. Am J Pathol **14:**557–578, 1938.
19. Magee PN, Montesano R, Preussman R. *N*-Nitroso compounds and related carcinogens. ACS Monogr **173:**491–625, 1976.
20. Montesano R, Bartsch H. Mutagenic and carcinogenic *N*-Nitroso compounds: Possible environmental hazards. Mutat Res **32:**179–228, 1976.
21. Druckrey H, Preussman R, Ivanković S, Schmähl D. Organotrope carcinogene wirkungen bei 65 verschiedenen *N*-Nitrosoverbindungen an BD-Ratten. Z Krebsforsch **69:**103–201, 1967.
22. Howett MK, Pegg AE, Rapp F. Enhancement of biochemical transformation of mammalian cells by herpes simplex virus following nitrosomethylurea treatment. Cancer Res **39:**1041–1045, 1979.
23. Parmiani G, Colnaghi MI, Della Porta G. Immunodepressive and leukemogenic effects of urethan in C3Hf and SWR mice. Proc Soc Exp Biol Med **130:**828–830, 1969.
24. Parmiani G, Colnaghi MI, Della Porta G. Immunodepression during urethane and *N*-Nitrosomethylurea leukaemogenesis in mice. Brit J Cancer **25:**354–364, 1971.
25. Stjernswärd J. Immunosuppression by carcinogens. Antibiot Chemother **15:**213–233, 1969.
26. Talcott PA, Exon JH, Koller LD. Alteration of natural killer cell-mediated cytotoxicity in rats treated with selenium, diethylnitrosamine and ethylnitrosourea. Cancer Lett **23:**313–322, 1984.
27. Allison AC, Taylor RB. Observations on thymectomy and carcinogenesis. Cancer Res **27:**703–707, 1967.
28. Vandeputte M, Denys P, Jr, Leyten R, De Somer P. The oncogenic activity of the polyoma virus in thymectomized rats. Life Sci **2:**475–478, 1963.
29. Defendi V, Roosa RA. The role of the thymus in carcinogenesis. In: Defendi V, Metcalf D, Eds. The Thymus. Philadelphia: Wistar Institute Press, pp121–135, 1964.
30. Law LW. Neoplasms in thymectomized mice following room infection with polyoma virus. Nature **205:**672–673, 1965.
31. Miller JFAP, Ting RC, Law LW. Influence of thymectomy on tumor induction by polyoma virus in C57BL mice. Proc Soc Exp Biol Med **116:**323–327, 1964.
32. Frei JV, Maitra SC. Bone marrow and thymus regeneration is a condition for thymoma development. Chem Biol Interact **9:**65–69, 1974.
33. Tevethia SS. Immunology of simian virus 40. In: Klein G., Ed. Viral Oncology. New York: Raven Press, pp 581–601, 1980.