THE EFFECT OF CHEMICAL CARCINOGENS AND ONCOGENIC VIRUS ON THE INDUCTION OF CELLULAR TRANSFORMATION IN VITRO

by

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by John Joseph Docherty

entitled The Effect of Chemical Carcinogens and Oncogenic Virus on the Induction of Cellular Transformation In Vitro.

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Peter P. Ludovici — Dissertation Director

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ABSTRACT

The possibility that two carcinogenic agents may have an effect on the neoplastic phenomenon was suggested by the "Two Stage Theory of Carcinogenesis." An in vitro study was initiated in which 7,12-dimethylbenz(a)anthracene (DMBA) or 3-methylcholanthrene (3MC) was applied separately to the 3T3 mouse fibroblastic-like cells treated with Simian Virus 40 (SV40). The effect of the chemicals on the "transformation" frequency by SV40 was studied.

First, authenticity studies of the SV40 were performed. Neonatal Syrian hamsters were injected with 0.1 ml of SV40. Approximately 150 days later all animals injected developed sarcomas. The BS-C-1 African Green Monkey Kidney cell line was infected with SV40 and tested for T-antigen. All cells infected with virus contained the T-antigen while non-infected cells were negative. The SV40 was successfully neutralized with commercially prepared SV40 antisera.

Because of the extreme toxicity of the two chemicals, plating efficiency studies of 3T3 cells were carried out in the presence of the chemicals to determine a satisfactory concentration to use. Such studies indicated that 0.0005 μg/ml of DMBA and 0.05 μg/ml of 3MC had no significant effect on the plating efficiency of 3T3 cells. In all "transformation" studies the chemicals were used at the above concentrations.

Both DMBA and 3MC were applied to the 3T3 cells before, with, and after SV40 inoculation. After fourteen days the "transformation"

frequency was determined on the basis of differences in morphology between clones. "Transformed" clones showed a loss of contact inhibition and a random pattern of cell growth.

Both DMBA and 3MC decreased the "transformation" frequency of the virus when applied to the system 24 hours after the virus. If the chemicals were applied before the virus, no effect was detected. Simultaneous application of chemical and virus to the system led to no effect with 3MC and an increase in "transformation" frequency with DMBA. Because spontaneous "transformation" occurred in control cultures, the results were not statistically significant.

Therefore, all "transformation" experiments were repeated except the frequency of conversion was calculated on the basis of the presence of the SV40 T-antigen in the "transformed" clones. When the chemicals were added before or with the virus, no change in the "transformation" frequency was observed. However, if the chemicals were added 24 hours after the virus, a statistically significant decrease in the "transformation" frequency was demonstrated.

The direct effect of DMBA or 3MC on SV40 was investigated. After incubating the virus and chemical at concentrations from 0.002 μg to 200 μg, the virus was titered according to standard viral procedures in the BS-C-1 cell line. No effect of the chemical on the virus was observed, suggesting that the chemicals reduced "transformation" through a mechanism which directly affects the 3T3 cells.

Advantage was taken of the inherent natural characteristic of the chemicals which causes them to fluoresce in the presence of

ultraviolet light to study their intracellular location. Both DMBA and 3MC accumulated within the cytoplasm of the cell.

INTRODUCTION

The malignant "transformation" of cells in vivo or in vitro by viruses or chemicals is well substantiated (8, 35, 43, 61, 62, 70). Whether one or the other can specifically be implicated as the primary cause of malignant "transformation" has as yet to be unequivocally established. The possibility of an interaction between the two "transforming" agents was postulated by Rous and Friedewald (54) in their "Two Stage Theory of Carcinogenesis." In vivo studies in which viruses or chemicals were used as initiators or promoters have lent a degree of credence to this theory (29, 51, 52, 55).

The results reported in this dissertation deal with the effect of two chemical carcinogens and an oncogenic virus on an established cell line. The following is a review of the various systems employed and also of the evolution of the problem reported in this dissertation.

Simian Virus 40

Simian Virus 40 (SV40) is a small virus approximately 30-50 mμ in diameter (39, 47) exhibiting an icosahedral symmetry with 42 capsomeres (38). It is a DNA virus (30, 39) that was first described by Sweet and Hilleman (64) in 1960 shortly after it had been observed during tests on the safety of poliomyelitis vaccines. These authors (64) noted that the virus appeared to be a common contaminant of rhesus (Macaca mulatta) monkey kidney cell cultures. In rhesus monkey kidney cell culture the virus developed to a high titer without apparent cell

damage. But in patas monkey (Erythrocebus patas) kidney cell culture, the virus multiplied and produced ballooning of the cells and multiple small cytoplasmic vacuoles. The specific cytopathogenic effect of the virus caused it to be named the "vacuolating" virus.

Simian Virus 40 has been shown to cause active infections in tissue cultures of cercopithecus monkey kidney, patas monkey kidney, baboon kidney, rhesus monkey testicle, and human cell cultures of kidney, intestine, brain, skin, muscle, lung, liver, heart, adrenal, spleen, and testes (25, 37, 42, 58, 64). Cultures of cells from rabbits, dogs, hogs, cattle, ducks, and chicks were reported as resistant to SV40 infection by Hsiung and Gaylord (39).

In 1962 Eddy et al. (26) injected newborn hamsters with SV40. Approximately five months later the animals developed subcutaneous neoplasms. Girardi et al. (32) confirmed the oncogenicity of SV40 as demonstrated by Eddy et al. (26). A correlation between amount of virus and route of injection with tumor incidence was also demonstrated by Eddy, Grubbs, and Young (25).

In vitro cell "transformation" by SV40 was first observed in adult and fetal human cell cultures by Koprowski et al. (42). This phenomenon was also observed by others at about the same time (59, 60). Rabson and Kirschstein (49) described the "transformation" of SV40 infected hamster kidney cells in vitro in 1962.

7,12-Dimethylbenz(a)anthracene (DMBA) and 3-Methylcholanthrene (3MC)

These two chemicals belong to a group of polycyclic aromatic hydrocarbons that may show strong carcinogenic activity. Both DMBA

and 3MC are considered to be strong carcinogens as measured by tumor induction after skin painting of animals. The yield of tumors with DMBA is not as great as with 3MC, but the DMBA induction period is less than that with 3MC.

In 1775 Sir Percival Pott called attention to the fact that chimney sweeps had a high incidence of scrotal cancer (40). Epidemiological study of various other industries using coal or coal tar products showed a high incidence of cancer.

In 1915 Yamagiwa and Ichikawa successfully induced tumors in the ears of rabbits with tar paintings (40).

It was Sir Ernest Kennaway and his group in the early 1930's that implicated and identified a polycyclic aromatic hydrocarbon in tar, benzo(a)pyrene, as the responsible agent for the carcinogenicity in tar (40). Since then considerable emphasis has been placed on the study of polycyclic aromatic hydrocarbons.

The chemical DMBA is a dimethylated tetracyclic compound while 3MC is a monomethylated pentacyclic compound. The resemblance of the skeletal structure of 3MC to that of the steroids has been recognized. The production of mammary tumors in rats by DMBA is accelerated by simultaneous administration of small amounts of diethylstilbestrol or estradiol (31).

Viral "Transformation" In Vitro

In 1941 Halberstaedter, Doljanski, and Tenenbaum (34) reported that when chicken fibroblasts growing in vitro were infected by Rous Sarcoma Virus (RSV) their appearance changed morphologically and they

resembled typical sarcoma cells. Manaker and Groupe (46) observed that following infection of chick embryo cells in vitro with RSV "transformed" cells appeared in discrete foci and that the number of these foci was proportional to the concentration of infecting virus. Rubin and Temin (56) utilizing cloning techniques demonstrated that the fibroblastic type of cell can be "transformed" into a monocyte-like cell after infection with RSV. Their results also suggested that some of the viral genetic material was integrated into the genome of the cell.

In 1960 Vogt and Dulbecco (73) reported the successful "transformation" of Swiss mouse or Golden hamster cells in vitro with polyoma, a DNA virus. They treated either type of secondary embryo cultures with polyoma virus and observed the development of "transformed" cells. These cells exhibited lack of contact inhibition and were able to produce tumors when inoculated into animals. Similar results were obtained using SV40 in hamster, human, rabbit, mouse, hog, and cattle cells (9, 20, 42, 49).

One of the major drawbacks of the in vitro system was the relatively low frequency of "transformation" by the DNA viruses. The proportion of "transformed" colonies to total colonies seldom exceeded one percent (45). Todaro, Green, and Goldberg (72) developed a Swiss mouse embryo cell line that was "transformed" by polyoma virus and SV40 at a high rate. The line was developed by successive transfer of the cells every three days at an inoculation density sufficiently low to insure that the cells did not attain confluence between transfers

(1, 67). The line possessed an extremely high degree of contact inhibition. When Green and Todaro (33, 70) infected this cell line with polyoma virus or SV40, they reported up to a 50% "transformation" frequency. Polyoma virus was less efficient than SV40 since cultures infected with polyoma virus supported considerable viral multiplication and there was extensive cell death. However, "transformed" cells emerged from among the survivors.

The development of cell lines such as these with an increased "transformation" frequency after exposure to oncogenic viruses has greatly enhanced research in this area. Quantitative studies are now more meaningful and it is relatively easy to isolate and secure a clone of virus "transformed" cells. The 3T3 cell line is the one used in the "transformation" experiments reported in this paper.

Chemical "Transformation" In Vitro

The induction of malignant "transformation" in vitro by chemicals has proven to be a difficult task with success reported by only a few. In 1939 Creech (14) reported an increase of cell proliferation and chromosome abnormalities in mouse embryo fibroblasts treated with dibenz(a,h)anthracene-choleic acid. Several years later Earle (23) and Earle, Schilling, and Shelton (24) found that 3MC could cause morphological "transformation" in a line of mouse embryo fibroblasts. However, the spontaneous changes that occurred precluded the direct implication of the carcinogen as the "transforming" agent. Ludovici, Ashford, and Miller (43, 44) in 1962 found a persistent change of human cells from fibroblast-like cells to epithelium-like cells after

treatment with a trypsin-antibiotic solution. They utilized normal and malignant tissues from the human female genital system and were able to show that the "transformation" which they observed was chemically induced and not spontaneous. One of the interesting aspects of their study was the fact that none of the chemicals required for the "transformation" was carcinogenic.

In 1963 and 1965 Berwald and Sachs (7, 8) reported successful malignant "transformation" of cells in vitro with carcinogenic hydrocarbons. They treated normal hamster embryo cells in vitro with benzo(a)pyrene, 3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene, and 10-methylbenz(a)anthracene. These cells showed(i) a hereditary random pattern of growth, (ii) the ability to grow continuously in culture, (iii) progressive growth as tumors after subcutaneous inoculation into adult hamsters, and (iv) a resistance to the toxic action of the chemicals when tested at a later stage of growth in culture. They reported a high "transformation" rate with a maximum of 25.6% "transformed" clones of benzo(a)pyrene treated cells. Their further studies indicated that a period of one to two days after addition of the carcinogen is required for expression of the "transformed" state.

For several years the work of Berwald and Sachs (7, 8) was not appreciated because many investigators were unable to duplicate their findings (19). Finally in 1969, DiPaolo, Nelson, and Donovan (21) confirmed Berwald and Sachs' work when they treated Syrian hamster embryo cell cultures with benzo(a)pyrene and were able to obtain "transformed" cells that exhibited a random pattern of growth and formed tumors when

injected into animals. They noted that certain concentrations of carcinogen, while producing "transformation," decreased the cloning efficiency of cells about 50%. This suggested that the "transformation" was due to induction rather than selection. They also noted that the frequency of "transformation" was related to the known potency of the compounds tested. Sivak and Van Duuren (62) obtained similar results by treating hamster embryo fibroblasts with benzo(a)pyrene or 7,12-dimethylbenz(a)anthracene. They developed a "transformed" established cell line with an abnormal karyotype which was oncogenic in hamsters.

Heidelberger and Iype (35) utilized a slightly different but successful approach to the "transformation" of cells in vitro with chemical carcinogens. They took pieces of ventral prostate from adult C3H mice and cultivated them in organ culture in the presence of 3MC or DMBA for 7 days. The pieces were pooled, dispersed with pronase, and plated as cell cultures. The cells treated with the chemicals formed established cell lines that produced tumors upon injection into C3H mice.

Two Stage Carcinogenesis In Vivo

Considerably more success has been obtained when the "Two Stage Concept of Carcinogenesis" has been applied to an in vivo rather than an in vitro system. Rous and Kidd (53, 54) in 1936 and 1938 reported that rabbits with tar-treated skin developed numerous tumors at these sites when injected intravenously with Shope Papilloma Virus (SPV). Control rabbits that did not receive chemical treatment did not develop tumors upon injection of SPV. Rous and Friedewald (52) applied SPV to

the ears of rabbits and then followed with coal tar or 3MC treatment. This led to the development of more malignant tumors than with SPV alone. The SPV gave rise to papillomas, but under the influence of coal tar or 3MC many of these became malignant. Carr (12) reported that inoculation of RSV caused virus-type tumors at the site of 3MC injection in a strain of inbred chickens not normally sensitive to tumor induction by the virus. Tanako and Southam (65) found that the intradermal inoculation of herpes simplex virus into mice during a 5-day course of application of 3MC to the skin gave rise to a faster appearance and higher incidence of skin papillomas than treatment with 3MC alone. In 1961 Rowson et al. (55) injected Swiss mice at birth with polyoma virus. From the age of 7 weeks on, DMBA, croton oil and DMBA, or croton oil alone were applied to the skin. In all groups that received chemical treatment the incidence of tumors was increased.

In contrast to these findings, Fiscus, Schloss, and Wertman (28) applied DMBA or 3MC by injection or skin painting to Balb/c mice infected with Friend or Rauscher virus. Their results revealed an inhibition of splenomegaly normally associated with Friend or Rauscher virus-induced leukemia. They suggest a possible interference of the chemicals with the leukemic process, but there was no evidence that the chemicals had an inhibiting effect on the virus itself.

Two Stage Carcinogenesis In Vitro

In order to elucidate a possible two-stage mechanism in carcinogenesis, it becomes expedient to utilize an in vitro model. Such a model offers the advantage of avoiding extraneous factors including

immunological or hormonal influence and permits study of the agents at the cellular level.

In 1965 Todaro and Green (69) produced a double "transformation" of the 3T3 cell line with polyoma virus and SV40. Utilizing clonal isolation techniques, they secured polyoma "transformed" 3T3 cells. They infected this altered line with SV40 and were able to isolate clones with properties characteristic of SV40 "transformed" cells.

Sivak and Van Duuren (61) successfully increased the "transformation" frequency of SV40 exposed 3T3 cells by exposing them to phorbol ester isolated from croton oil, a known carcinogenic promoter.

DiPaolo, Rabson, and Malmgren (22) utilized 3MC, SV40, and LLE46 (adeno-12 virus-SV40 hybrid) in their studies of viruses and chemicals in vitro. First they treated hamster embryo cells with 3MC. Such chemically treated cells maintained a normal morphology in vitro, but neoplasms developed when they were injected into homologous hosts. If these chemically treated cells were exposed to SV40 or LLE46, they underwent changes in morphology, growth rate, and chromosome pattern. These cells also exhibited SV40 T-antigen and when injected into homologous hosts produced tumors morphologically different from the cells exposed to 3MC alone.

Statement of the Problem

As indicated in the literature review, only a few studies have been done on "Two Stage Carcinogenesis" in vitro.

The object of this study was an attempt to formulate and investigate a two-stage system in vitro. The experimental approach was

to apply chemical carcinogens to a known virus "transforming" system and measure the effect on the "transformation" frequency. The "transforming" system chosen for study was the SV40-3T3 system of Todaro and Green (70). Two powerful carcinogens, 3MC and DMBA, were applied at various intervals to this system and the effect on the "transformation" frequency of SV40 was measured.

MATERIALS AND METHODS

Cell Lines

An aneuploid, fibroblast-like cell line, BS-C-1, derived from an African Green Monkey (Cercopithecus aethiops) kidney which is susceptible to productive infection by SV40 was employed. The growth medium used for the BS-C-1 cell line was Medium 199 with Earle's balanced salt solution containing 20% fetal calf serum (FCS), 0.1% yeastolate, supplemented with 50 units/ml of penicillin, and 50 µg/ml of streptomycin. Maintenance medium consisted of medium 199 with Earle's balanced salt solution containing 2% FCS, 50 units/ml of penicillin, and 50 µg/ml of streptomycin. The cells were grown on the glass surface of Brockway prescription bottles at 37 C. All experiments were carried out utilizing cells between the 45th and 80th subculture (37).

An aneuploid, fibroblast-like cell line, 3T3, derived from a Swiss mouse embryo was used. It is readily "transformed" by SV40. Growth as well as maintenance medium was Dulbecco-Vogt's modification of Eagle's Basal Medium (DMEBM) supplemented with 10% FCS, 25 units/ml of penicillin, and 25 µg/ml of streptomycin (hereafter designated as 3T3-growth medium). The cells were grown in 60X15 mm plastic tissue culture dishes incubated in 5% CO_2-95% air at 37 C with a humid atmosphere. In order to preserve the property of high contact sensitivity, the stock cells were never permitted to reach confluence. They were subcultured regularly at high dilutions. All experiments were carried out using cells between the 115th and 130th subculture.

Simian Virus 40

The SV40 strain 776 was inoculated onto monolayers of BS-C-1 grown in two 32 oz prescription bottles. The cultures were observed daily for cytopathogenic effect (CPE) and scored according to standard procedures. When 4+ CPE appeared (100% of the cells affected), the cells were frozen and thawed three times. The contents of the two bottles were pooled, centrifuged to remove cellular debris, and the supernatant fluid was dispensed in 5-ml portions and frozen at -60 C in a Revco freezer.

The titer of the stock virus was determined by diluting the virus from 10^{-1} to 10^{-12} and placing 0.1 ml of diluted virus into each of three tubes of BS-C-1. The cells were observed for 14 days and scored for CPE by standard viral procedures. The $TCID_{50}$/ml of the virus pool as determined by the method of Reed and Muench (50) was 10^{-10}. This virus pool was used in all experiments.

To confirm the identity of the stock SV40, the following studies were performed.

1. Oncogenicity In Vivo - Outbred neonatal Syrian hamsters were inoculated subcutaneously in the suprascapular region with 0.1 ml of stock SV40. They received food and water ad libitum and were observed 6 months for tumor appearance.

2. Neutralization Test - Equal volumes of SV40 ($1000TCID_{50}$/0.1 ml) and calf SV40 antiserum (Baltimore Biological Laboratory lot #51093) diluted 1:2, 4, 8, 16, and 32 were mixed and incubated at 37 C for 30 minutes. Controls included mixtures of equal volumes of calf

SV40 antiserum and Medium 199 and also equal volumes of SV40 (1000 $TCID_{50}$/0.1 ml) and Medium 199 followed by incubation at 37 C for 30 minutes. The various mixtures were then placed into each of three tubes of BS-C-1 and observed 14 days for CPE.

3. The SV40 T-antigen - One-tenth ml of stock SV40 was placed on 9X22 mm coverslip monolayers of BS-C-1. After allowing 60 minutes for virus adsorption, 1 ml of growth medium was added. Thirty-six to 42 hours later, the coverslips were recovered, rinsed twice in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), and fixed 10 minutes in acetone at -60 C. The coverslips were air-dried, layered with 1:5 dilution of hamster anti-T antiserum, and incubated in a humid atmosphere at 37 C for 30 minutes. After being rinsed twice with CMF-PBS, they were layered with fluorescein-labeled rabbit anti-hamster serum (Microbiological Associates, Inc., lot #55879) and reincubated in a humid atmosphere at 37 C for 30 minutes. Following three rinses with CMF-PBS the coverslips were mounted on glass slides with buffered glycerol at a pH of 7.4. The cells were examined at a magnification of 200X with a Reichert Microscope equipped with a dark field condenser and an ultra-violet light source provided by a Hacker Model MDC-201. Cells with SV40 T-antigen showed diffuse nuclear fluorescence. Photographs were taken with an Exacta Model VX camera using Kodak Plus-X Pan film or Kodak Ektachrome Type B.

Chemical Effect on Plating Efficiency

Stock solutions of 3-methylcholanthrene (3MC, Eastman Organic Chemicals, lot #4383) or 7,12-dimethylbenz(a)anthracene (DMBA, Eastman

Organic Chemicals, lot #5149) were prepared by placing 4.0 mg of either chemical in 5 ml of concentrated dimethylsulfoxide (DMSO) and storing in an amber-colored bottle at room temperature. The stock chemicals were diluted in DMEBM to contain 2.0, 0.2, 0.02, 0.002 μg/0.5 ml.

Stock 3T3 cells were dissociated with 0.25% trypsin. Viable cell counts were performed with 0.5% trypan blue using an American Optical Spencer bright line hemocytometer. Only the cells not staining with trypan blue were considered viable. The cells were serially diluted in complete growth medium so that approximately 180 viable cells/0.1 ml could be placed into each of 60 plastic dishes. Four dilutions of each chemical were added in 0.5 ml volumes to each series of six dishes. Controls included six dishes with untreated cells as well as six dishes containing 0.5 ml of a 1:100 dilution of DMSO. All dishes received an additional 3.5 ml of 3T3 growth medium and were incubated in a humid atmosphere containing 5% CO_2 and 95% air at 37 C. Medium was replaced in all dishes on the 7th day of incubation. On day 14, the clones were fixed with 10% formaldehyde in CMF-PBS for 30 minutes, stained with crystal violet for 2 minutes, and the absolute plating efficiency (P.E.) was determined.

$$\text{P.E.} = \frac{\text{clones formed}}{\text{cells plated}} \times 100$$

Chemical Effect on SV40 "Transformation" of 3T3 Cells

The 3T3 cell line was treated with DMBA (0.0005 μg/ml) or 3MC (0.05 μg/ml) before, with, or after SV40 treatment.

The experimental design consisted of plating 3 or 5 X 10^4 viable cells in six 60 X 15 mm plastic tissue culture dishes, each containing 4 ml of 3T3 growth medium. In the series in which chemicals were added before virus, two dishes received DMBA and two dishes received 3MC. Two dishes without addition of chemicals served as controls. The cells were incubated in a 5% CO_2-humid atmosphere at 37 C for 48 hours. The medium was then removed from the plates and 0.5 ml of SV40 was plased in one DMBA plate, one 3MC plate, and one control plate. The other DMBA plate, 3MC plate, and control plate received 0.5 ml of DMEBM without virus. All plates were returned to the 5% CO_2-humid atmosphere at 37 C for 3 hours. The dishes were rotated every 15 to 20 minutes. The plates were rinsed twice with DMEBM and 4 ml of 3T3-growth medium were added to each plate. Incubation at 37 C was continued for 24 hours in the 5% CO_2-humid atmosphere. The cells were then released from the plastic dish with 0.25% trypsin and a viable cell count done. The cells were serially diluted so that 150 to 300 cells could be plated in groups of six to ten plates for each treatment. All plates received 4 ml of 3T3-growth medium, and they were incubated at 37 C for 14 days. The medium was changed on day 7.

In the second part of this series of experiments, all steps were the same as described above except that the chemicals and virus were applied simultaneously to the 3T3 cells. Similarly, in the third part of this series of experiments all steps remained the same except that the chemicals were added to the cells 24 hours after the virus.

After incubation at 37 C for 14 days the cells were fixed with 10% formaldehyde in CMF-PBS and stained with crystal violet. The clones were examined with a Leitz Wetzlar dissecting microscope at 12.5 and 50X and scored as normal or "transformed." The basis for judging a clone "transformed" was a random pattern of growth, loss of contact inhibition as shown by cellular overlap, and denser staining with crystal violet. Normal clones maintained a normal pattern of growth, remained sensitive to contact inhibition, and did not stain densely with crystal violet. "Transformation" frequency (T.F.) is the number of "transformed" clones per total clones formed.

SV40 T-antigen Studies

The 3T3 cell line was treated with DMBA (0.0005 μg/ml) or 3MC (0.05 μg/ml) before, with, or after SV40 in the same manner as outlined previously. This time instead of determining the "transformation" frequency by the number of "transformed" clones, relative to normal clones, it was calculated on the basis of the number of "transformed" clones containing the SV40 T-antigen. After chemical and viral treatment the cells were incubated 14 days in the 5% CO_2-humid atmosphere at 37 C. Growth medium was changed on day 7. After 14 days incubation all plates were rinsed twice in CMF-PBS, fixed with absolute ethanol for 10 minutes at -60 C, and air-dried at 4 C. The plates were examined with a Leitz Wetzlar inverted microscope at 21 or 36X and the location of clones marked and enumerated. One drop of a 1:5 dilution of hamster anti-T serum was placed on each clone. The plates were incubated in a humid atmosphere at 37 C for 30 minutes and then rinsed three times in CMF-PBS. Fluorescein-conjugated anti-hamster serum was

layered over each clone and the plates reincubated for 30 minutes. The plates were rinsed twice with CMF-PBS and coverslips were mounted over the clones using buffered glycerol at pH 7.4 for a mounting fluid. The clones were examined with a Reichert microscope equipped with a dark field condenser and ultraviolet light source. Positive clones were those which contained cells with fluorescing nuclei. Photographs were taken with an Exacta VX camera mounted on the Reichert microscope using Kodak Ektachrome Type B film ASA 125.

Effect of Chemicals on the Infectivity of SV40 for BS-C-1

Stock DMBA or 3MC was diluted in Medium 199 to contain 200, 20, 2, 0.2, 0.02, or 0.002 μg/0.1 ml. Stock SV40 was mixed with each of these concentrations. Chemical and viral controls were employed. The mixtures were incubated at 37 C for 30 minutes and logarithmic dilutions made with Medium 199. Then 0.1 ml of the 10^{-3} through 10^{-7} dilutions was placed on triplicate monolayer tube cultures of BS-C-1. The mixtures were permitted to adsorb at 37 C for 60 minutes. Then 1 ml of 199 maintenance medium was added and the tubes were incubated at 37 C for 14 days. All tubes were examined daily and scored as 0, 1, 2, 3, or 4+ CPE. At the end of 14 days the $TCID_{50}$/ml of each treatment was determined according to the method of Reed and Muench (50).

Localization of Chemicals in 3T3

Advantage was taken of the fact that DMBA and 3MC fluoresce naturally in the presence of ultraviolet light to study their intracellular localization. The 3T3 cells were dissociated with 0.25%

trypsin and 3 X 10^4 viable cells were placed in 12 plastic tissue culture plates. All plates received 4 ml of 3T3 growth medium. After incubation in a 5% CO_2-humid atmosphere at 37 C for 48 hours, the medium was replaced and DMBA or 3MC added at various concentrations (0.002 µg to 20 µg/0.1 ml).

The plates were reincubated for 24 hours. They were rinsed twice with DMEBM and coverslips mounted over the cells using the same medium. The cells were examined for localization of the chemical with a Reichert microscope equipped with a dark field condenser and an ultraviolet light source provided by a Hacker Model MDC-201. Photographs were taken with an Exacta Model VX camera containing Anscochrome film ASA 200.

RESULTS

Authenticity of SV40

Oncogenicity

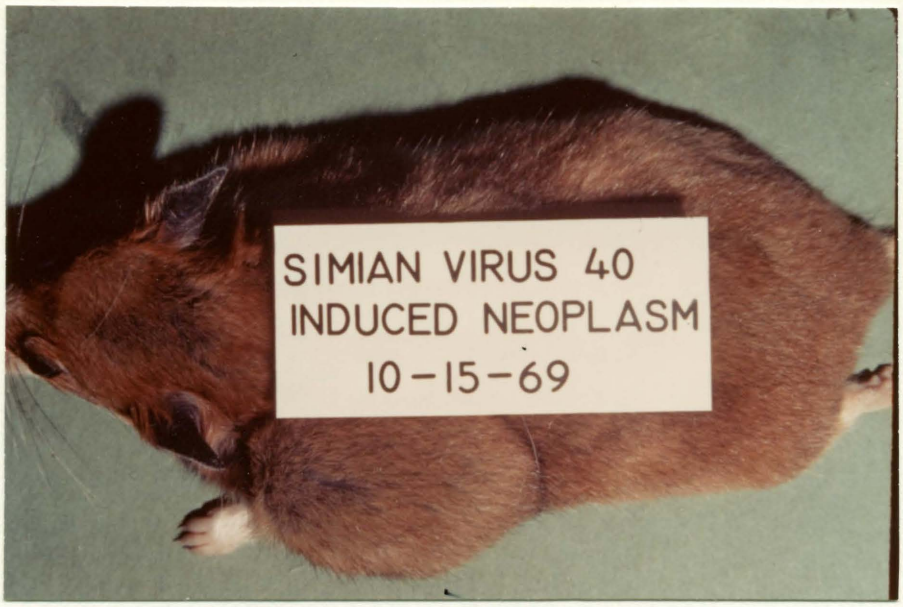
Four neonatal Syrian hamsters were injected subcutaneously with 0.1 ml of SV40 stock ($TCID_{50}10^{-9}$) and were observed six months for the appearance of tumors. After approximately 150 days small, firm nodules began to form in the areas of injection. Within a few weeks time the nodules progressed rapidly to large neoplasms (Fig. 1a). The tumors continued to grow rapidly and if permitted would attain a size larger than the hamster. All animals injected developed neoplasm. Figure 1b illustrates a histological section of the tumor and depicts a sarcoma showing a giant nucleus, a bizarre mitotic figure, and spindle-shaped nuclei.

Neutralization Test

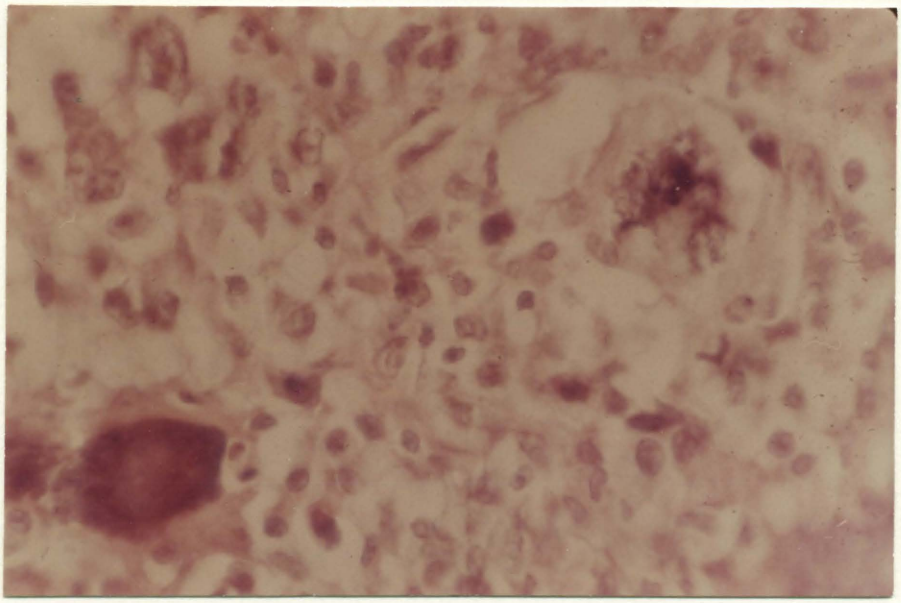
As indicated by Table 1, the cytopathogenicity of SV40 for BS-C-1 cells was successfully neutralized using commercially prepared antiserum. A toxic effect of the antiserum to the BS-C-1 cells was observed in the antiserum control.

SV40 T-antigen

Infected and non-infected BS-C-1 cells were examined for fluorescent nuclei after staining for T-antigen. Approximately 60-70% of the SV40 infected cells exhibited extensive nuclear fluorescence



a.



b.

Figure 1. Simian Virus 40 Induced Neoplasm.

a. Syrian hamster 171 days after a subcutaneous injection of 0.1 ml of SV40 ($TCID_{50}10^{-9}$) showing induced neoplasm.
b. Histological section of SV40 induced neoplasm.

Table 1. Neutralization of the cytopathogenicity (CPE) of SV40 for BS-C-1 cells by antiserum.[a]

Treatment	Result[b]
Antiserum Dilution	
1:2	-
1:4	-
1:8	-
1:16	-
1:32	-
Antiserum Control[c]	0
Normal Control[d]	-
Viral Control	+

a. SV40 (1000 $TCID_{50}$/0.1 ml) was incubated for 30 minutes with an equal volume of calf SV40 antiserum obtained from Baltimore Biological Laboratory (lot #51093) prior to addition to BS-C-1 monolayers.

b. Values are the average of three tubes expressed as -(No CPE), 0 (non-specific cellular destruction) or + (4+ CPE).

c. Antiserum dilution 1:2.

d. Tubes of BS-C-1 cells that were uninoculated.

(Fig. 2). None of the BS-C-1 cells non-infected with SV40 contained fluorescent nuclei.

Chemical Effect on Plating Efficiency

The P.E. represents the percentage of individual cells able to remain viable and undergo the process of mitosis to form a clone or colony. Table 2 presents data on the P.E. of 3T3 in the presence of various concentrations of DMBA or 3MC. It can be seen that DMBA was considerably more toxic than 3MC. Concentrations as low as 0.005 μg/ml of DMBA reduced the relative P.E. of the 3T3 cell line approximately 64%, whereas at a concentration of 0.5 μg/ml 3MC only reduced the relative P.E. 25%. Experiments were carried out with chemicals at the highest concentration that did not affect the P.E. (0.005 μg/ml for DMBA and 0.05 μg/ml for 3MC).

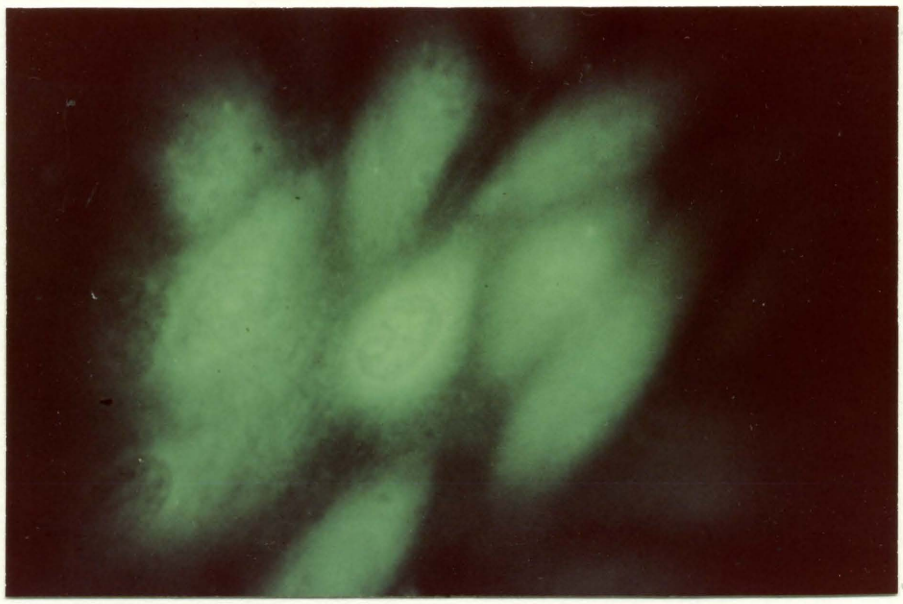
Chemical Effect on SV40 "Transformation" of 3T3 Cells

Figure 3 shows a "transformed" clone beside a normal clone. The basis for judging this clone "transformed" was loss of contact inhibition, random pattern of growth, and heavier staining.

When the chemicals were applied to the cells prior to the virus, there was no marked effect on the "transformation" frequency (Table 3).

If the chemicals were applied to the cells with the SV40, there appears to be an increase in the "transformation" frequency of the DMBA-SV40 treated cells (Table 4). The 3MC-SV40 treatment of the cells did not cause a significant variation from the SV40 control.

a. SV40-Treated BS-C-1 Cells



b. Normal BS-C-1 Cells

Figure 2. SV40 T-antigen in the BS-C-1 Cell Line.

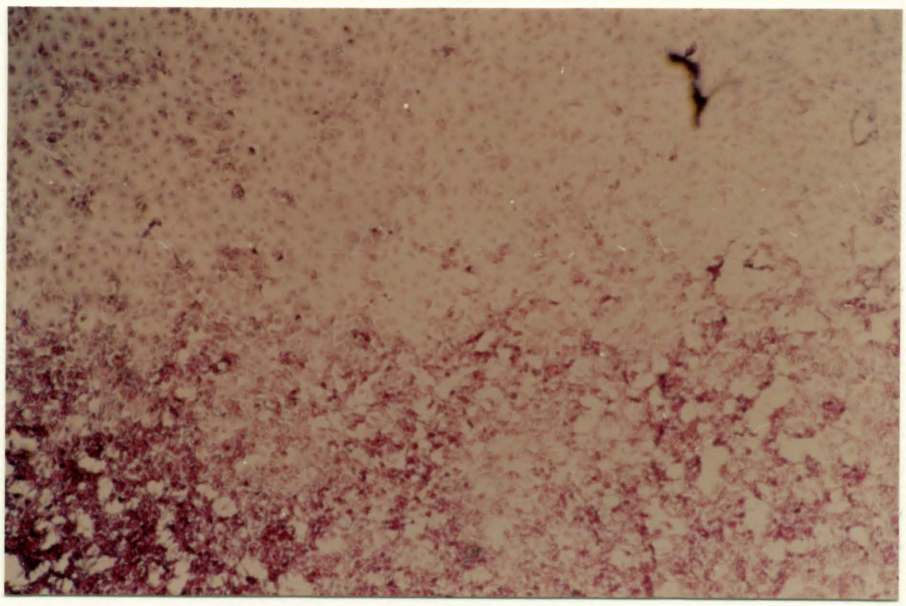
a. Positive cells indicated by fluorescent nuclei.
b. Negative cells lack the fluorescing nuclei.

Table 2. The effect of DMBA and 3MC on the plating efficiency of 3T3 cells.

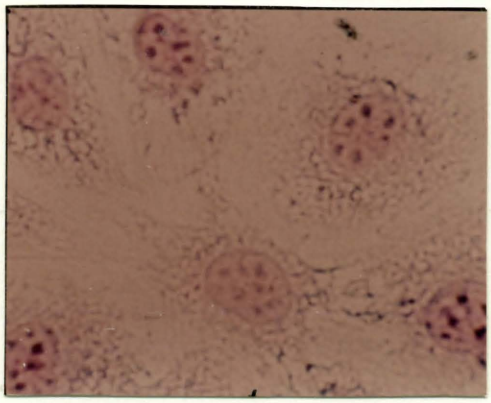
	Chemical Concentrations (μg/ml)	Total Clones in Six Plates (No.)	Absolute[a] P.E. (%)	Relative[b] P.E. (%)
DMBA	0.5	3	0.3	1.2
	0.05	22	2.1	8.6
	0.005	95	8.8	36.1
	0.005	242	22.4	92.0
3MC	0.5	199	18.2	74.8
	0.05	252	23.3	96.0
	0.005	240	22.2	91.0
	0.0005	264	24.4	100.0
DMSO Control		259	24.0	98.8
Normal Control		263	24.4	100.0

a. Absolute P.E. - absolute plating efficiency is the percentage of viable cells plated with the ability to form a clone.

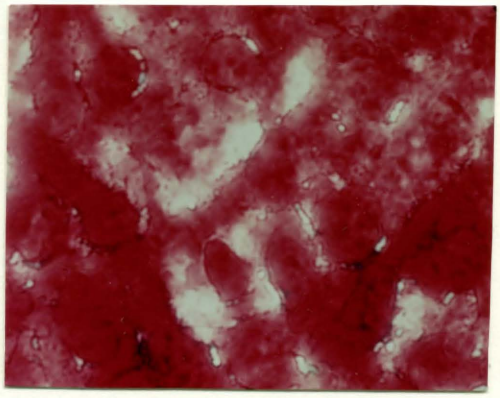
b. Relative P.E. - relative plating efficiency is the percentage of cells plated with the ability to form a clone relative to the non-chemically treated controls.



a.



b.



c.

Figure 3. Normal and "Transformed" 3T3 Clones.

a. A normal 3T3 clone beside a "transformed" 3T3 clone. Note the heavier appearance and darker staining of the "transformed" clone due to cellular overlap and random orientation of cell growth.
b. High magnification of normal clone.
c. High magnification of "transformed" clone.

Table 3. The effect on SV40 "transformation" of 3T3 cells when DMBA or 3MC was applied before SV40 to the cells.

	Total Clones (No.)	"Transformed" Clones (No.)	"Transformation" Frequency[a] (%)	Corrected "Transformation" Frequency[b] (%)
Normal Control	299	4	1.4	0
SV40 Control	381	12	3.2	1.8
SV40 DMBA	286	6	2.1	0.7
SV40 3MC	293	11	3.7	2.3
DMBA	198	4	2.0	0.6
3MC	141	2	1.4	0

a. The % of "transformed" clones relative to the total clones formed.

b. The "transformation" frequency corrected for spontaneous "transformation" of the normal controls.

Table 4. The effect on SV40 "transformation" of 3T3 cells when DMBA or 3MC was applied simultaneously with SV40 to the cells.

	Total Clones (No.)	"Transformed" Clones (No.)	"Transformation" Frequency[a] (%)	Corrected "Transformation" Frequency[b] (%)
Normal Control	271	13	4.8	0
SV40 Control	327	22	6.8	2
SV40 DMBA	242	23	9.5	4.7
SV40 3MC	275	20	7.3	2.5
DMBA	66	1	1.5	-3.3
3MC	73	1	1.3	-3.4

a. The % of "transformed" clones relative to the total clones formed.

b. The "transformation" frequency corrected for spontaneous "transformation" of the normal controls.

When the chemicals were applied to the cells 24 hours after the SV40, there appeared to be a reduction in the "transformation" frequency of DMBA and 3MC treated cells (Table 5). Also the normal background "transformation" that was observed was decreased by DMBA. Because of the frequency of spontaneous "transformation" observed, however, it was impossible to establish a statistically significant difference between treatments.

Chemical Effect on SV40 "Transformation" of 3T3 Cells as Measured by Clones Containing SV40 T-antigen

In order to avoid the consideration of spontaneous "transformation" which masked the significance of previous data, all experiments were repeated, but only colonies containing the SV40 T-antigen were used to calculate the "transformation" frequency (Fig. 4).

When the chemicals were applied to the cells before or with the virus, there was no effect on the "transformation" frequency (Tables 6 and 7).

If the chemicals were applied to the cells 24 hours after the virus, there was a significant reduction in the "transformation" frequency (Table 8).

The Effect of Chemicals on SV40 Infectivity for the BS-C-1 Cell Line

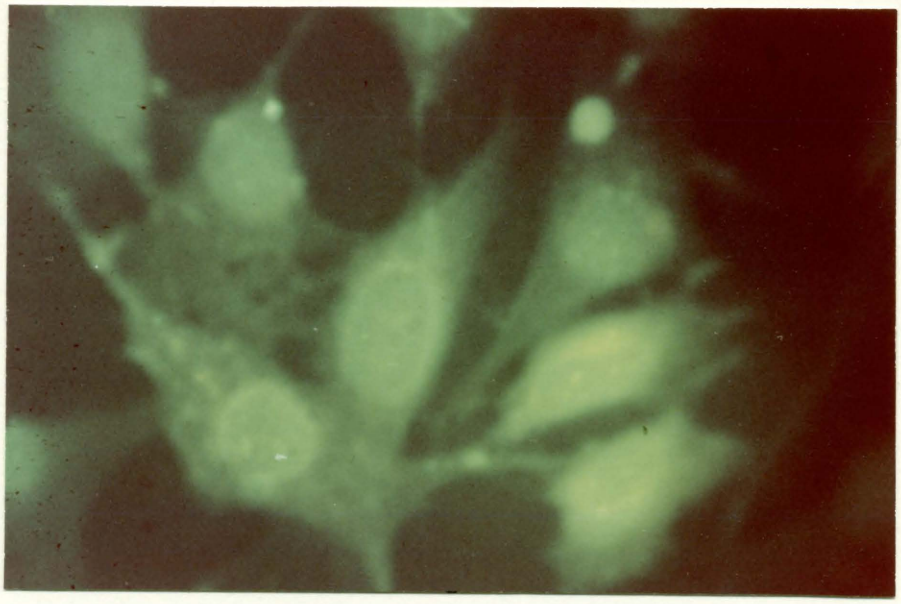
There was no effect of DMBA or 3MC on the ability of SV40 to actively infect the BS-C-1 cell line (Figs. 5 and 6). Chemical concentrations as high as 200 μg/0.1 ml had no observable effect on SV40

Table 5. The effect on SV40 "transformation" of 3T3 cells when DMBA or 3MC was applied to the cells 24 hours after the virus.

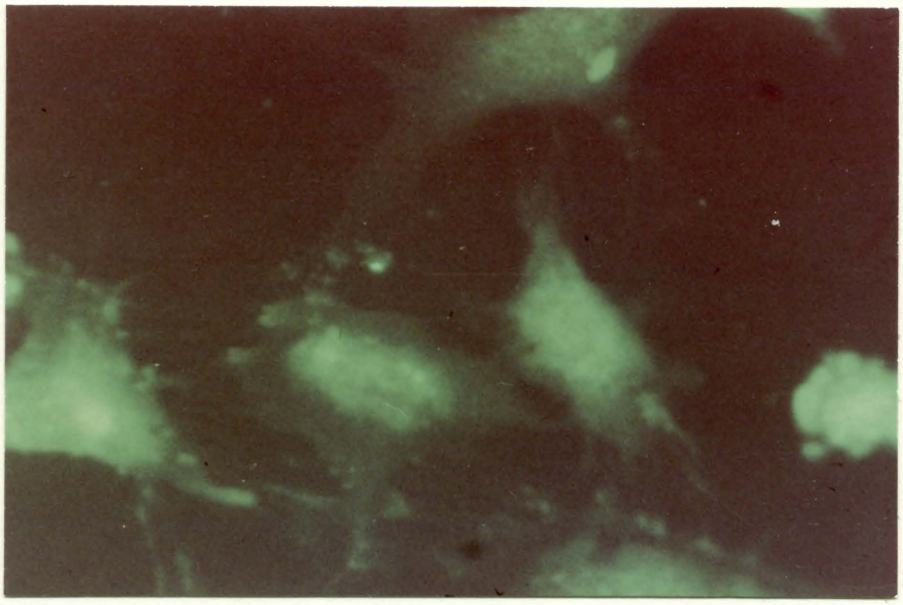
	Total Clones (No.)	"Transformed" Clones (No.)	"Transformation" Frequency[a] (%)	Corrected "Transformation" Frequency[b] (%)
Normal Control	313	17	5.5	0
SV40 Control	282	26	9.4	3.9
SV40 DMBA	306	19	6.2	0.7
SV40 3MC	335	22	6.7	1.2
DMBA	323	15	4.7	-0.8
3MC	330	19	5.8	0.3

a. The % of "transformed" clones relative to the total clones formed.

b. The "transformation" frequency corrected for spontaneous "transformation" of the normal controls.



a.



b.

Figure 4. Study of T-antigen of Viral and Spontaneously "Transformed" 3T3 Clones.

a. SV40 T-antigen exhibited by diffuse nuclear fluorescence in cells of SV40 "transformed" clone.

b. Cells from spontaneously "transformed" clone lacking nuclear fluorescence.

Table 6. The effect of DMBA or 3MC added 48 hours before SV40 on the "transformation" frequency of 3T3 cells measured by the presence of SV40 T-antigen.

Treatment	"Transformation" Frequency (%)	Confidence Limits of "Transformation" Frequency
SV40	3.8	1.138 (±) .17)
SV40 DMBA	3.1	0.965 (± .17)
SV40 3MC	3.2	0.939 (± .17)
None	0	0.707 (± .17)
DMBA	0	0.707 (± .17)
3MC	0	0.707 (± .17)

Table 7. The effect of DMBA or 3MC added simultaneously with SV40 on the "transformation" frequency of 3T3 cells measured by the presence of SV40 T-antigen.

Treatment	"Transformation" Frequency (%)	Confidence Limits of "Transformation" Frequency
SV40	2.8	1.052 (± .05)
SV40 DMBA	3.0	0.966 (± .05)
SV40 3MC	1.8	0.879 (± .05)
None	0	0.707 (± .05)
DMBA	0	0.707 (± .05)
3MC	0	0.707 (± .05)

Table 8. The effect of DMBA or 3MC added 24 hours after SV40 on the "transformation" frequency of 3T3 cells measured by the presence of SV40 T-antigen.

Treatment	"Transformation" Frequency (%)	Confidence Limits of "Transformation" Frequency
SV40	4.5	1.123 (± .08)
SV40 DMBA	2.0	0.879 (± .08)
SV40 3MC	1.0	0.793 (± .08)
None	0	0.707 (± .08)
DMBA	0	0.707 (± .08)
3MC	0	0.707 (± .08)

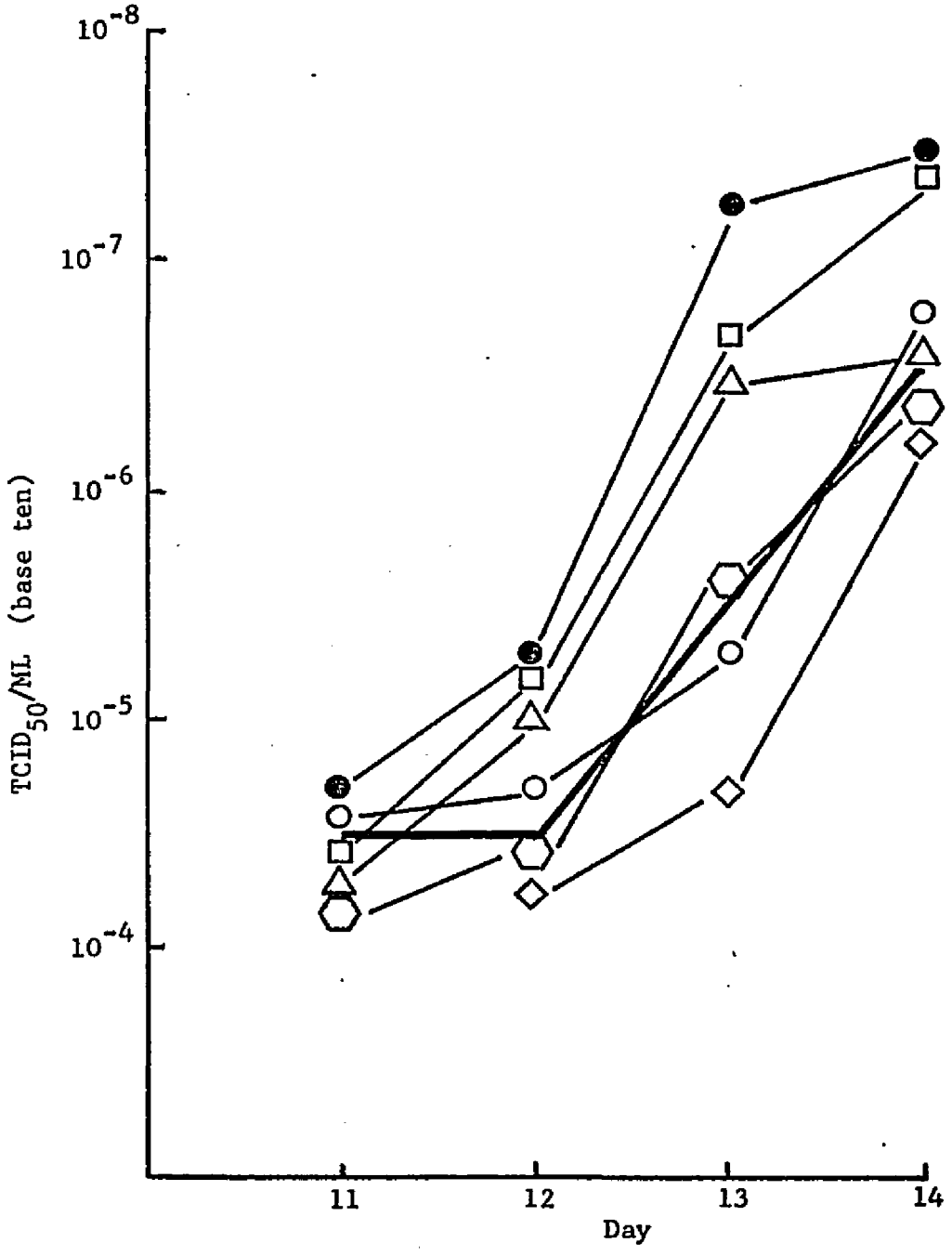


Figure 5. The Effect of DMBA on SV40 Infectivity in the BS-C-1 Cell Line.

● - 200 µg ○ - 20 µg □ - 2 µg
△ - 0.2 µg ⬡ - 0.02 µg ◇ - 0.002 µg
Viral control
All chemical controls were negative.

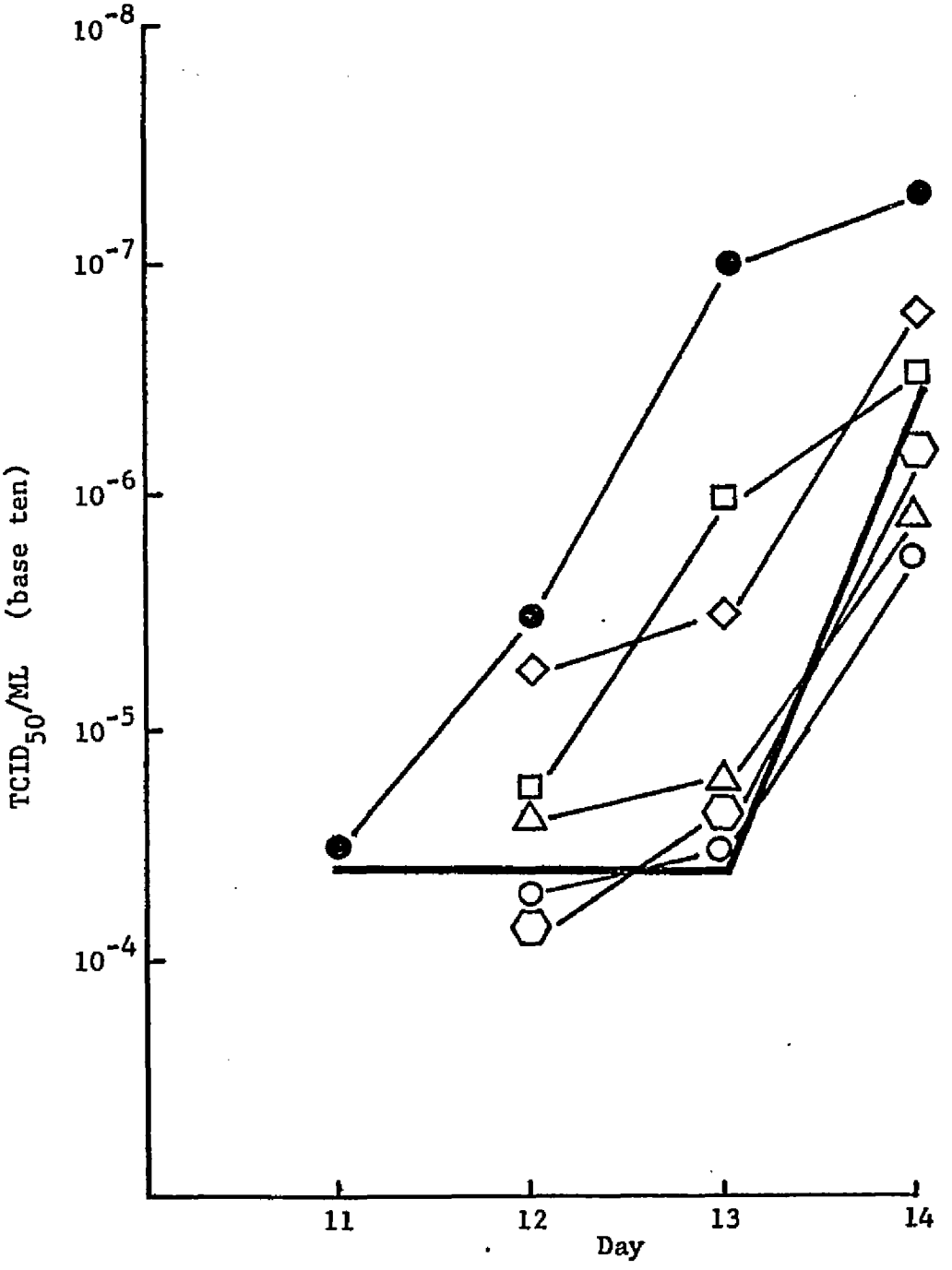


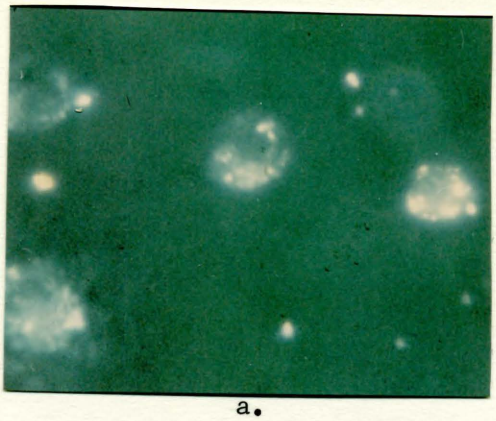
Figure 6. The Effect of 3MC on SV40 Infectivity in the BS-C-1 Cell Line.

● - 200 µg ○ - 20 µg □ - 2 µg
△ - 0.2 µg ⬡ - 0.02 µg ◇ - 0.002 µg
Viral control
All chemical controls were negative.

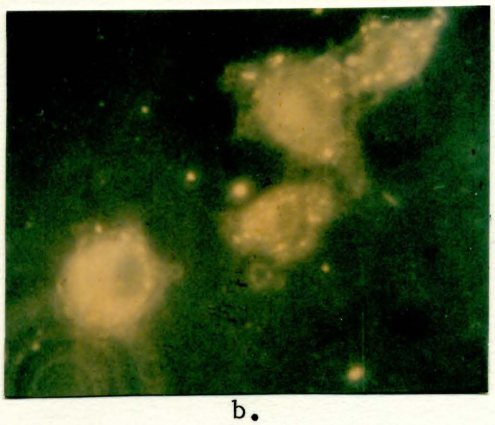
infectivity. Generally the titers of the virus on day 14 were within one log (base 10) dilution of each other.

Localization of Chemicals in the 3T3 Cell Line

Both DMBA and 3MC localized within the cytoplasm of the 3T3 cell (Fig. 7). The photograph of Figure 7 is of cells from a plate that received 20 μg of either chemical. At chemical concentrations lower than this, the fluorescence was too minimal to photograph or was non-existent.



a.



b.

c.

Figure 7. Localization of DMBA or 3MC Within 3T3 Cells.

a. DMBA. b. 3MC. c. Normal.

Fluorescent areas within the cytoplasm represent the chemicals.

DISCUSSION

When I began the study of "Two Stage Carcinogenesis" in vitro, I elected to study the combined effect of two known neoplastic agents at the cellular level. I chose two polycyclic aromatic hydrocarbons which are carcinogenic by themselves or in combination with viruses in vivo. The "transforming" system of choice was the SV40-3T3 system because of its high frequency of "transformation." The object of the study was to treat the 3T3 cells with SV40 and DMBA or 3MC and observe the effect on the "transformation" frequency. I expected one of three things to occur: an increase in "transformation" frequency, a decrease in "transformation" frequency, or no change in "transformation" frequency.

The "transformation" studies which were based on morphological changes, such as loss of contact inhibition, indicated that if the chemicals were applied to the cells before the virus, the "transformation" frequency did not vary significantly. If the chemicals were applied with the virus, little effect was observed with 3MC while an increase in "transformation" frequency occurred with DMBA treatment. By applying DMBA or 3MC to the cells 24 hours after SV40, there was a reduction in the "transformation" frequency. Although the chemicals were causing an effect, this effect apparently was being masked by the spontaneous "transformation" frequency of the 3T3 cells.

In order to evaluate the significance of the results from a statistical standpoint, it was necessary to rule out variations in the

data caused by spontaneous "transformation." Therefore, I repeated the experiments but expressed the "transformation" frequency in terms of the presence of SV40 T-antigen. Since spontaneously "transformed" clones lacked the SV40 T-antigen, the background problem was eliminated. These T-antigen studies agree in part with my original findings. When the chemicals were applied to the cells 48 hours before the virus, there was no effect on the "transformation" frequency. If applied to the cells 24 hours after the virus, a significant reduction in the "transformation" frequency occurred. The increase in "transformation" frequency which was previously observed when DMBA and SV40 were applied simultaneously was not confirmed by the T-antigen study. In this case, there was no effect on the "transformation" frequency when DMBA or 3MC was applied simultaneously with the SV40.

Little in vitro research using two distinct agents has been done. Much of the work has been directed toward enhancing viral "transformation" in some manner rather than inhibiting it. Since the "transformation" frequency is low in most systems, enhancing the frequency would make data on "transformation" more meaningful and permit the study of larger populations of "transformed" cells.

Some of the most successful work done with reference to increasing "transformation" frequency was carried out using radiation. In no way am I implying that polycyclic aromatic hydrocarbons are radiomemetic, but it is interesting to compare the two since it may give some insight into the mechanism of action.

Coggin (13) exposed hamster embryo cells to radiation prior to SV40. By doing this he was able to increase the "transformation" frequency of his system by 25%.

Defendi and Jensen (16) took a different approach and exposed SV40, polyoma virus, and LLE46 to ultraviolet and gamma radiation (cobalt-60). By doing this they were able to increase the oncogenicity of the viruses and decrease their ability to actively infect and destroy cells. They speculate that cancer may be caused by defective virus particles, and by radiation they merely increased the number of defective particles relative to normal particles. When I exposed SV40 to various concentrations of DMBA or 3MC, I found no effect of these chemicals on the infectivity of the virus for BS-C-1 cells (Figs. 5 and 6). Thus, the chemicals did not mimic radiation. More important, the findings indicate that the antagonistic effect of the chemicals on SV40 "transformation" is not mediated through the virus but apparently through the cell or some part of the cell.

Todaro and Green (68) treated 3T3 cells with 5-bromo-2'-deoxyuridine (BUDR) and 5-iodo-2'-deoxyuridine (IUDR) which are thymidine analogs. This treatment at least doubled the "transformation" of 3T3 cells by SV40. They imply that the chemicals do not modify the virus or select for "transformed" clones but have a general effect on the cells that renders them more susceptible to "transformation." My findings, although opposite of enhancement, may have a generalized effect on the cell which renders them resistant to viral "transformation."

In 1967 Sivak and Van Duuren (61) did a study which utilized normal and SV40 "transformed" 3T3 cellsand a phorbol ester of croton oil and DMBA. They applied croton oil or DMBA to normal 3T3 cells or SV40 "transformed" cells. They found that croton oil was an effective promotor in that it increased the spontaneous "transformation" of 3T3 cells above the normal and also increased the plating efficiency of 3T3 cells previously "transformed" by SV40. They also observed that by exposing the 3T3 cells to a toxic dose (0.1 µg/ml) of DMBA they obtained a reduction of 50% in the number of "transformed" clones. Their DMBA studies are in agreement with some of my results. They observed that spontaneous "transformation" of 3T3 cells was reduced after treatment with DMBA. I also noted a similar effect (Tables 4 and 5) of DMBA on spontaneous "transformation" although I employed a lower concentration of DMBA in my system.

Since there is very little work on "Two Stage Carcinogenesis" in vitro, it is difficult to compare my work with others. My study appears to be a new finding and considerable work on the mechanism remains to be done. The ensuing discussion will attempt to elaborate on the possible mechanisms involved in the antagonistic effect of DMBA or 3MC on the "transformation" of 3T3 cells by SV40.

One of the first topics that should be considered is the possible role of interferon. Whenever a virus is inhibited in its action, interferon immediately becomes suspect as a possible inhibiting agent. The synthesis of interferon may be stimulated by many agents such as viruses, bacteria, and synthetic polymers (27, 48). It is reasonable

to assume that DMBA and 3MC could initiate the production of interferon which would in turn inhibit the SV40 from exerting its presence in the manner of a "transformed" cell. Indeed, interferon is an effective inhibitor of viral "transformation" when it is added to cultures before or shortly after viral infection (33, 71). Todaro and Baron (66) infected exponentially growing 3T3 cells with SV40, exposed them to exogeneous interferon at various times thereafter, and then replated the cells for scoring of "transformation" frequency. Immediately after infection, the addition of interferon suppressed "transformation" quite effectively, but its ability to do so was lost one day later when the cells had completed one doubling. The "transformation" process is therefore fixed and past the interferon-sensitive stage by one generation after infection. If non-growing cells were infected and kept in the stationary state for four days, interferon added, and the cells replated, the interferon was fully effective in preventing "transformation." It is therefore unlikely that interferon would assume the primary role of inhibition in my system since the chemicals are applied to the cells 24 hours after the virus. Since I infect exponentially growing cells, the "transformed" state is fixed and beyond any effect interferon may play at the time the chemicals are added. It is also pertinent to note that DeMaeyer and DeMaeyer-Guignard (17) have reported a decrease in interferon production after 3MC treatment of rat cells.

The basic substance involved in the genetic control of cellular protein synthesis is DNA, including enzymes which control metabolic

events within the cell. "Transformed" cells have an accelerated growth and mitotic rate which are are under the control of the DNA. Therefore a "transformed" cell must have a fully functioning DNA system in order to keep up with its rapid rate of growth. If DMBA or 3MC become associated with DNA or pathways of DNA synthesis, it could suppress the expression of the viral "transformed" cell. It would not have to be specifically associated with viral DNA since the virus is still dependent upon the metabolic systems of the cell. By suppressing a function of the DNA or DNA synthesis, the chemicals in effect could suppress the "transformed" state initiated by the virus.

Alfred and DiPaolo (4) were able to show that DMBA binds to DNA (0.7 molecules/200,000 nucleotide units) and suppress DNA synthesis and mitotic activity. Their results suggested either a direct effect of DMBA on cellular DNA or the development of a biochemical lesion at some point in the pathway of DNA synthesis. Other investigators (3, 41) showed that DMBA induced a marked suppression on the rate of thymidine C^{14} uptake but no effect on uridine incorporation. The chemicals have been shown to remain bound in cells for up to 30 days and that there was a positive correlation between the binding of polycyclic aromatic hydrocarbons with DNA and their carcinogenic potency (11).

Further evidence for the binding of polycyclic aromatic hydrocarbons to DNA was shown by DeMaeyer and DeMaeyer-Guignard (18) using a biological system. Their work suggested that DMBA and benzo(a)pyrene interact with DNA and impair the expression of information contained in the DNA. They showed that the two chemicals inhibit plaque formation

by herpes virus and vaccinia (both DNA viruses). The chemicals had no effect on Sindbis virus, an RNA virus. They suggest the chemicals act like actinomycin D and prevent the expression of genetic information by combining with DNA. They do not suggest that the chemicals preferably bind with viral DNA and use the viruses merely as biological markers.

The carcinogenic polycyclic aromatic hydrocarbons have been shown to bind to proteins by Heidelberger and Moldenhauer (36). They applied C^{14}-labeled polycyclic aromatic hydrocarbons to the skin of mice and determined the degree to which chemicals bound to the protein. But they also found that 1,2,3,4-dibenzanthracene, which is not carcinogenic, was extensively bound but not at the same sites as the carcinogenic hydrocarbons. Other investigators have confirmed their work with similar studies (2, 15).

The "Protein Deletion Theory" of carcinogenesis has its foundation in the ability of chemicals to bind to proteins (57). The Protein Deletion Theory" states that the combination between carcinogen and protein leads to the eventual loss of function or deletion of proteins involved as enzymes for the control of growth. Studies have shown that the protein to which the carcinogenic compound is bound is deleted from the tumors subsequently induced (2, 63). Although this evidence has been presented in support of the "Protein Deletion Theory," it still remains difficult to visualize a mechanism where protein binding could ultimately result in a state that could be perpetuated in subsequent generations of cancer cells.

It is easy to visualize that by binding to ribosomal protein or DNA synthesizing enzymes, an immediate state of repression would be initiated. But my experiment lasted for 14 days and since the cells were in a dynamic state with continuing turnover of cellular products, it is difficult to imagine how this state of repression could be passed on to daughter cells.

If the chemicals were able to remain in the cell so as to bind with and inactivate SV40 induced enzymes as they were formed, the cell would remain essentially normal. The chemicals have been shown to remain within the cells for up to 30 days (11). Potentially the chemicals could remain with the cells throughout the "transformation" assay period. They may not be preferably associated with and inactivate SV40 enzymes but they may inactivate enzymes of DNA synthesis through a mechanism such as allosteric inhibition. If the DNA synthesizing enzymes are partially suppressed, the synthesis of DNA would not be able to keep up with the rate required of a "transformed" cell. Therefore, the cell would not be able to exhibit itself as "transformed."

Data presented (Fig. 7) has shown the chemicals to accumulate in the cytoplasm of the 3T3 cells. This is in agreement with Allison and Mallucci (5) who have shown that polycyclic aromatic hydrocarbons including DMBA and 3MC concentrate within cellular lysosomes. They added the chemicals to the cells and noted, by ultraviolet microscopy, that the chemicals concentrated in granules of the cytoplasm. The distribution pattern of the granules was similar to that of lysosomes. Further radioautography studies confirmed this finding.

The involvement of the lysosomes in cell division has been noted (6, 10). The rearrangement and loss of lysosomes precedes cell division. The release of lysosomal material into the cytoplasm is thought to act as an initiating mechanism to cell division. Since the nuclear membrane breaks down during cell division, the lysosomal enzymes may be involved.

My findings of reduced viral "transformation" attributable to chemicals in the cytoplasm may be explained in two ways. First, if the lysosomes are involved in initiating mitosis, chemical association with lysosomes could suppress the phenomenon. At the chemical concentration I used, the effect may be such that normal mitosis took place but accelerated divisions characteristic of "transformed" clones was inhibited.

The second possibility is actual damage to the lysosomal membrane by the chemicals, so that leakage occurs. The lysosomes contain deoxyribonucleases and ribonucleases. If these were released in small amounts, the possibility of direct damage to nuclear material is very real. This would include the DNA of SV40. Once damaged the SV40 would be unable to cause the cell to "transform."

Obviously this discussion on the mechanism of chemical inhibition of "transformation" has been conjecture. Considerably more work must be done before any of the mentioned or unmentioned mechanisms can be specifically implicated. An understanding of this inhibitory phenomenon may be important because it may provide the key that unlocks

how a cell undergoes "transformation" after viral infection. A determination of what system is inhibited should lead to an understanding of viral "transformation."

SUMMARY

The possibility that two carcinogenic agents might have an effect on the neoplastic phenomenon was suggested by the "Two Stage Theory of Carcinogenesis." An in vitro study was carried out in which 7,12-dimethylbenz(a)anthracene (DMBA) or 3-methylcholanthrene (3MC) was applied separately to the Simian Virus 40 (SV40)-3T3 "transforming" system. Various effects on the "transformation" frequency were investigated.

Both DMBA and 3MC decreased the "transformation" frequency of the virus when applied to the system 24 hours after the virus. If the chemicals were applied before the virus, no effect was detected. Simultaneous application of chemical and virus to the system led to no observed effect with 3MC and an increase in "transformation" frequency with DMBA.

Studies of "transformation" frequency in which the SV40 T-antigen in transformed clones was used as the "transformation" marker did not agree with the increase in "transformation" frequency that occurred when virus and DMBA were applied simultaneously. All other T-antigen studies agreed with the original findings.

The direct effect of DMBA or 3MC on the virus was investigated. After incubating the virus and chemical, the virus was titered according to standard viral procedures. No direct effect of the chemicals on the virus was observed.

Advantage was taken of the inherent natural characteristics of the chemicals which causes them to fluoresce in the presence of ultraviolet light to study their intracellular location. Both DMBA and 3MC accumulated within the cytoplasm of the cell.

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