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A Mammalian Virus (SV40) Inductest for Putative Carcinogens

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A MAMMALIAN VIRUS (SV40) INDUCTEST FOR PUTATIVE CARCINOGENS

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A MAMMALIAN VIRUS (SV40) INDUCTEST FOR

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A MAMMALIAN VIRUS (SV40) INDUCTEST FOR PUTATIVE CARCINOGENS

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The *in vitro* mammalian virus inductest is based on the measurement of induced viral gene expression from Simian virus 40 (SV40) transformed weanling Syrian hamster kidney cells (clone E). Upon challenge by many DNA-damaging chemical agents, infectious virions were produced which were quantitated by assay on a cell line permissive to SV40, such as the highly contact inhibited African green monkey kidney cells, CV-1P. Some known carcinogens and non-carcinogens were tested in this research including mitomycin C, aflatoxins B1 and G1, sterigmatocystin and several polycyclic hydrocarbons.

Lysolecithin (a membrane permeabilizing agent) and S9 mix (a pre-metabolizing mixture) were used in conjunction with the potential carcinogen treatments. Several screening assays for carcinogenic and mutagenic agents are currently in use and the results obtained with these systems were used to determine the reliability of the SV40 inductest system for mammalian cells.

INTRODUCTION AND LITERATURE REVIEW

In recent years cancer and its effects in humans has been under intense investigation. As with any etiological disorder, the solution to the cancer phenomenon cannot be determined until the various causes have been identified. In 1911 Rous (1911) discovered that a virus could cause cancer. Since then, the presence of latent virus in mammalian cells, their periodic induction, and their subsequent expression have been of paramount concern. Interest in these oncogenic viruses increased when Sweet and Hilleman (1960) discovered that a simian vacuolating virus (SV40) was a contaminant in some of the Salk polio vaccines. Furthermore, it was demonstrated that the synthesis of infectious SV40 could be induced in SV40-transformed hamster kidney cell clones when challenged by various chemical agents (Rothschild and Black, 1970; Fogel, 1972; Gerber, 1964; Kaplan *et al*. 1975; Zamansky *et al*. 1976; Morris *et al*. 1977; Rakusanova *et al*. 1978).

The purpose of my research was to develop and investigate an *in vitro* mammalian virus induction test (inductest) system for determining the carcinogenic potential of various environmental chemical agents. The fact that various chemical agents in the environment enhance the incidence of cancer in humans has led to the development and employment of a number of testing systems to predict the carcinogenic potential of these agents. Those test systems based on studies using whole

animals are probably the most conclusive. However, animal carcinogenicity tests are costly, time-consuming, and may detect only a small proportion of potential human mutagens (Hollstein and McCann, 1979). Ideally, a test system employing humans could best be used to determine the carcinogenic effect of these agents, but this is understandably improper. Therefore, a number of *in vitro* test systems for detecting potential carcinogens have emerged using a) prokaryotic microorganisms, bacteriophages, etc.; b) eukaryotic microorganisms, mutagenesis and transformation tests in mammalian cells in culture, and c) various related biological endpoints (Hollstein and McCann, 1979).

Of the available *in vitro* test systems using prokaryotic organisms, the most widely known is the Ames/*Salmonella* microsome test (Ames *et al*. 1973a; Ames *et al*. 1973b; McCann *et al*. 1975; McCann and Ames, 1976). In this standard *Salmonella* mutagenicity test, bacteria, dilution of the test chemicals and microsomal activation mixture are incorporated into an agar overlay, and following 2 days of incubation the number of revertants to histidine-independence are enumerated (Ames *et al*. 1973a; McCann *et al*. 1975). This mutagen assay uses a set of four histidine mutant strains of *Salmonella typhimurium* which are specially constructed for the detection of frame shift or base-pair substitution reverse mutations (Hollstein and McCann, 1979). The usefulness of this test in screening for carcinogenicity is based on the premise that the cancer causing potential of most chemicals is based on its

mutagenicity. Investigators have tested with known carcinogens and shown a high correlation between carcinogenicity and mutagenicity.

In 1953 Lwoff (1953) suggested that inducible lysogenic bacteria may be useful testing mechanism for carcinogenic activity. The use of prophage induction as a method to detect mutagens and potential carcinogens has been investigated by a number of researchers. Moreau et al. (1976) used Escherichia coli K12 lysogenic for lambda phage for detecting carcinogenicity. This test system relies on the ability of a potential carcinogen to induce the latent virus. They modified this system by introducing into an E. coli K12 lysogen the envA mutation, that increased permeability to many mutagens, and the uvrB mutation, that inactivates the excision-repair system. A similar inductest has been developed using a prophage-containing strain of Micrococcus lysodiekticus (Sussmuth, 1980). Results of some investigators have led to the hypothesis that the inductest may in certain instances be a more reliable test for carcinogenicity than is the mutagenicity test (Moreau and Devoret, 1977; Elespuru and Yarmolinsky, 1979). The reasoning for this hypothesis is that lyosgenic induction is always a result of the error-prone pathway whereas mutagenesis may occur by two different pathways, one of which is not error-prone (Moreau and Devoret, 1977). The biochemical induction assay has several advantages, such as rapidity, simplicity, safety and the ability to test complex samples (Elespuru, 1981).

Several other prokaryotic test systems have also been developed. These include a forward mutation assay in Salmonella, a sporulation test in Bacillus subtilis, a differential growth inhibition test of which the pol test in E. coli K12 is most widely used, and other tests based on repair-deficient bacteria (Hollstein and McCann, 1979).

Several eukaryotic test systems for potentiating carcinogens are in use including some using mammalian cells. Eukaryotic cells can demonstrate some mutagenic effects not detectable in prokaryotes such as chromosomal anomalies. Examples of these are mitotic crossing over, gene conversion, and mitotic non-disjunction (Hollstein and McCann, 1979).

Mutagenesis tests in mammalian cells in culture generally uses established cell lines. These tests are sensitive to a number of variables inherent in the system, for example, cell density, media components, and mutation expression time (Hollstein and McCann, 1979) and are based on mutations at certain genes. The most commonly used loci in mutagenesis testing are the hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) loci (Hollstein and McCann, 1979). Other loci include the ouabain resistance locus, amino acid auxotrophs, temperature sensitive mutants, and cyclic AMP protein kinase (Hollstein and McCann, 1979). The most popular mammalian cell lines for mutagenesis testing are chinese hamster ovary (CHO) cells and mouse lymphoma cells.

An in vitro transformation test can also be valuable for screening potential carcinogens. This test system has a

unique endpoint that is directly related to the *in vivo* disease. Furthermore, some mammalian cells in culture retain the ability to activate metabolically many pro-carcinogens. *In vitro* transformation systems can detect a wide range of carcinogens (Hollstein and McCann, 1979).

In order to establish the mammalian virus inductest system presented here it was necessary to develop an *in vitro* system which mimics some aspects of *in vivo* carcinogenesis. Cells of many animal species contain endogenous viral genetic information. Bockstahler and Hellman (1979) showed that when such cells are exposed to certain chemical and physical agents, viral induction can occur with the release of infectious virus and expression of viral gene products. These inducible mammalian viruses include both RNA-containing and DNA-containing viruses, some of which have been demonstrated to be oncogenic or associated with oncogenesis. Bockstahler and Hellman (1979) concluded that since the expression of endogenous viral genes is regulated by cellular control processes and since carcinogenesis involves alterations of normal cellular regulation, viral induction systems should yield information important for the understanding of carcinogenesis.

Although the relationship of viruses to carcinogenesis is not completely understood, several hypotheses have been presented to explain their widespread association with tumors. The viral oncogene hypothesis proposed by Todaro and Huebner (1972) suggests that the virus provides the information that

transforms the cell. Alternatively, the symptomatic hypothesis states that activated viruses, although present and capable of replication in the tumor cell, contribute no oncogenic information to the host cell. A third hypothesis, the cofactor hypothesis, postulates that during the course of carcinogenesis, latent viruses are activated and that the viral products contribute oncogenic information to the cell (Weinstein et al. 1976). This suggests that studies on the interaction of chemicals and viruses in the induction of cancer may prove fruitful in the development of more sensitive assays for potential chemical carcinogens.

The weanling Syrian hamster kidney cells, lysogenic for SV40, used in the mammalian inductest developed in this research, were cloned by Kaplan (1975). These cells have been shown to produce infectious virus when challenged with a number of carcinogenic agents, such as ultraviolet radiation (UV) (Coohill et al. 1982), mitomycin C (Kaplan et al. 1975), and 8-methoxypsoralen (8-MOP) plus long wavelength UV (Moore and Coohill, 1981). It has been suggested that photoinactivation of DNA by photodynamic treatment may unmask the oncogenic potential of tumor viruses and lead to induction of possible latent tumor viruses (Bockstahler et al. 1979). Presumably, other DNA-damaging agents could have a similar effect.

The SV40 mammalian inductest is based on the action of carcinogenic agents within the cell. In bacteria, DNA damage anywhere along the bacterial chromosome can initiate lysogenic induction (Elespuru and Yarmolinsky, 1979). Presumably, the

same is true for mammalian cells. In the bacterial inductest doses of carcinogens can be tested which would be cytotoxic in mutagenicity tests (Devoret, 1979). Evidence exists that this is also true for viral induction in mammalian cells (Rakusanova et al. 1978). Therefore, the mammalian inductest should detect a wider variety of types of DNA damage. The induction of SV40 from clone E cells appears to be similar to the induction of prophage lambda from E. coli, and induction is thought to occur by the error-prone repair system which has been linked to carcinogenesis and mutagenesis (Witkin, 1976). Even though the existence of an error-prone repair system in mammalian cells is still questionable (Radman, 1980), mammalian cells do exhibit many characteristics similar to those associated with the error-prone repair system in bacteria.

Virus induction from mammalian cells has been shown to result from treatment of the cells with a number of DNA-damaging agents (Bockstahler and Cantwell, 1979; Burns and Black, 1969; Coohill et al. 1982; Fogel and Sachs, 1970; Fogel, 1972; Kaplan et al. 1975; Moore and Coohill, 1981; Rothschild and Black, 1970; Zamansky et al. 1976). It is presently not known if induction is a direct result of the damage, an indirect result caused by the action of a repair system, or the result of the release of a viral repressor. It has been shown that viral induction is enhanced by inhibition of post-replication repair (Zamansky et al. 1976). Furthermore, it was shown that agents which cause DNA strand breakage are also potent inducers (Kaplan et al. 1975, Rakusanova et al. 1978). Therefore, it

appears that either direct or indirect DNA damage alone can lead to viral induction. Concomitantly, any chemical agent that damages DNA and induces the expression of latent viral genetic material has carcinogenic potential. If a system can be developed that will show latent viral expression as a result of contact with environmental mutagens, then mankind will benefit. Thus, the research presented is based on the measurement of induced viral gene expression from simian virus 40 (SV40) transformed weanling Syrian hamster kidney cells (clone E) in response to treatment with various environmental chemical agents.

MATERIALS AND METHODS

Cell Lines and Culture Media

Clone E cells, an SV40 transformed line of weanling inbred Syrian hamster kidney cells (Kaplan et al. 1975), were obtained from J. C. Kaplan and P. H. Black of Harvard Medical School. The cells were maintained in a growth medium containing four times the usual concentration of essential amino acids and vitamins (4X MEM), supplemented with 10% fetal bovine serum and an antibiotic-antimycotic solution containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. Fetal bovine serum was obtained from Microbiological Associates (Walkersville, Maryland) while all other media components were from Grand Island Biological Company, Grand Island, New York. The 4X MEM was buffered with 2.0 g/l sodium bicarbonate and 15 mM concentrations of N-2-hydroxyethylpiperazine-n-2-ethane sulfonic acid (HEPES), N-tris(Hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES), and Morpholinopropane sulfonic acid (MOPS). The final pH was adjusted to 6.8-7.2 with concentrated sodium hydroxide before filter sterilization with a 0.22 µm pore size millipore membrane filter. Stock cultures of clone E cells were maintained at 37 C in 150 cm^2 tissue culture flasks (Corning Co., Corning, New York) and split at a density of 10^6 cells per flask upon confluency.

CV-1P cells, a highly contact inhibited clone of African green monkey kidney cells isolated by Mertz and Berg (1974) and obtained from Dr. Paul Berg, Stanford University, were used to assay the induced virions. These cells were maintained in a growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose, supplemented with 10% newborn calf serum, the antibiotic-antimycotic mixture, and 200 mM/ml glutamine. The buffers, pH, and sterilization of DMEM were the same as those described above. The stock flasks were incubated at 37 C in 150 cm^2 tissue culture flasks (Corning) and split to a density no less than 10^6 cells per flask upon confluency.

Preparation of Chemicals

The potential carcinogens tested were aflatoxin B1, aflatoxin G1, sterigmatocystin, benz(a)anthracene, benzo(a) pyrene, 7,12 dimethylbenzanthracene, 20-methylcholanthrene, 6-azouracil, s-triazine, nitrosomorpholine, 0.05% para-amino-benzoic acid (PABA), saccharin and urethane. All were obtained from Sigma Chemical Company (St. Louis, Missouri) except for 6-azouracil and s-triazine which were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). Mitomycin C (Sigma), a known SV40 inducer (Kaplan et al. 1975), was used in each experiment as a chemical control to check the inducibility of the passage of cells being used. These chemicals were dissolved and diluted to appropriate concentrations in the growth medium used for clone E cells. Those chemicals insoluble in growth medium were dissolved in dilute reagent

grade ethyl alcohol (U.S. Industrial Chemicals Co., New York, New York). One milligram of each was dissolved in 10 ml of solvent for a 100 µg/ml concentration which was syringe filtered through a 0.22 µm membrane filter for sterilization. Further dilutions of the filtrate were made to obtain lower concentrations. The potentially carcinogenic chemicals were prepared at the beginning of each induction experiment.

An S9 mix, an activator system for metabolizing pre-carcinogens into ultimate carcinogens (Sarasin et al. 1977), was used in conjunction with the chemical agents in some experiments. This mixture contained homogenized rat liver microsomes (0.04 ml per ml of complete activator), magnesium chloride (8 µmoles/ml), potassium chloride (33 µmoles/ml), glucose-6-phosphate (5 µmoles/ml), NADP (4 µmoles/ml), glucose-6-phosphate dehydrogenase (0.002 units/ml) and sodium phosphate buffer pH 7.4 (100 µmoles/ml) consisting of monobasic and dibasic sodium phosphate. The rat liver microsomes were prepared by Dr. M. R. Houston, Western Kentucky University, and the homogenate was syringe filtered with a millipore AP20 prefilter for the removal of large particulate debris and mixed with the enzyme components. This mixture was then syringe filtered with a 0.22 µm pore size filter for sterilization. Other components of this mixture were obtained from Sigma Chemical Co. The S9 mix was prepared at the beginning of each induction experiment and maintained on ice throughout the procedure.

Lysolecithin (Sigma Chemical Co.), a membrane permeabilizing agent, was diluted in phosphate buffered saline (PBS) to a final concentration of 50 µg/ml and sterilized by filtration. Stock solutions were maintained at -40 C and then on an ice bath during use. Cells treated with lysolecithin were rinsed twice in cold PBS before receiving lysolecithin and maintained on ice during the two-minute treatment (Miller et al. 1979).

Viral Induction

Stock flasks of clone E cells were rinsed three times with trypsin in PBS, placed in a 37 C incubator for 5 to 10 min. to allow time for cell detachment, and the cells were transferred to 60 mm petri dishes (Falcon Co.) at a density of 8×10^5 cells per dish (Fig. 1). These dishes of clone E cells were maintained in an incubator at 37 C in a 5% CO_2 atmosphere until cell monolayers reached 80-90% confluency, after which the growth medium was removed, the cells were washed with PBS, and then treated with an appropriate dilution of a potential carcinogenic chemical. The concentration of the chemicals tested ranged from 0.0001 - 100 µg/ml. In conjunction with chemical agents, S9 activator mix (0.2 ml) was added to enzymatically metabolize the chemicals into their carcinogenic form. When used, lysolecithin was applied to the cells prior to treatment with chemical agents. As controls, the growth medium, S9 mix, and lysolecithin were all tested alone. Furthermore, mitomycin C, a known SV40 inducer, was used as a control to insure that the cell line

was inducible in each experiment. Additional replicate dishes were trypsinized, and the cells were counted in a hemacytometer to determine the number of cells per dish. After a 24-hr. induction period the chemical reagent mixtures were removed, replaced with 4 ml of growth medium, and the cells were incubated at 37 C for 96 hrs. to allow maximum viral expression (Kaplan et al. 1975). After incubation, the cells were quickly frozen in a -40 C Revco freezer to halt all metabolic activity and prevent viral degradation until harvested.

Viral Harvest

At the time of harvesting, the cells were removed from the -40 C freezer and allowed to thaw. The growth surfaces of the dishes were gently scraped with a rubber policeman to insure that all the cells were detached. The cell suspensions were transferred to 15 ml centrifuge tubes (Corning Co), centrifuged at 100 x g for 10 min. in an IEC PR-J centrifuge (Damon/IEC). After centrifugation the supernatants containing extracellular virions were transferred to tissue culture tubes (Corning Co.). The remaining cell pellets were externally sonicated at 60 watts for 45 seconds with a W140D Sonifier (Ultrasonics, Inc., Plainview, New York) to disrupt the cell membranes and release any intracellular virions. The corresponding sonicates and supernates were recombined and stored at -40 C until assayed.

Plaque Assay for Infectious SV40

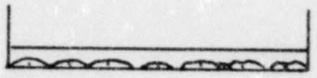
CV-1P cell stock cultures were trypsinized, and the cells were transferred to petri dishes (60 mm). These dishes of

cells were maintained at 37 C in a 5% CO_2 atmosphere until confluent cell monolayers formed. The growth medium was then removed, the cell monolayer was washed once in PBS and treated with an appropriate dilution of the harvested virus. Volumes of 0.05 or 0.5 ml of the harvested material were used to infect 2 petri dishes of CV-1P cells. Virions were allowed to adsorb for 2 hrs at 37 C. During the adsorption period the petri dishes were continuously agitated to insure equal distribution of viral inoculum over the monolayer surface. The viral inoculum was then removed and the monolayer overlaid with 10 ml of plaquing medium which consisted of equal portions of 2% DIFCO purified agar and 2X MEM, double strength Eagles's Minimum Essential Medium. The 2X MEM was buffered with 2.2 g/l sodium bicarbonate and 15 mM each of HEPES, TES, and MOPS with a final pH of 6.8-7.2. Plaquing medium was supplemented with 2% newborn calf serum, antibiotic-antimycotic mixture and 10^{-9} M dexamethasone. After the medium solidified, the dishes of cells were incubated at 37 C in 5% CO_2 for 8-10 days. The agar overlay confined the viruses to their original infection sites, thus producing distinct, quantitative plaques. As controls, some of the dishes of CV-1P cells were overlaid uninfected, while others were infected with a known titer of SV40 virus. Following the incubation period, the cells were overlaid with an additional 2 ml of plaquing medium containing 12.5% neutral red for staining. The cells were returned to the 37 C incubator for 24 hrs to allow time for the stain to diffuse through the agar and stain the

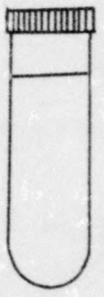
living cells leaving the areas of cell destruction, plaques, as clear unstained zones. Plaques were counted with the aid of a Quebec Darkfield Colony Counter (American Optics Corporation). The number of plaques in each dish was multiplied by the appropriate dilution factor and normalized based on the number of clone E cells per dish at the start of that experiment. This allowed induction to be expressed in plaque forming units per million clone E cells (PFU/10^6 cells). A brief summary of the SV40 mammalian virus inductest is provided in Figure 1.

Figure 1. Procedure for the SV40 mammalian virus inductest.

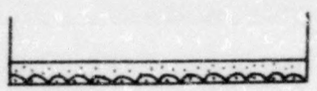
PROCEDURE



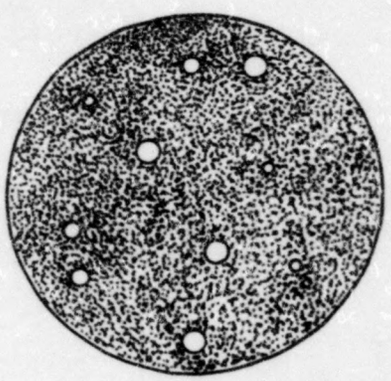
EXPOSE CLONE E CELLS TO CARCINOGEN.
RE-FEED. INCUBATE 37 C FOR 96 HRS.



HARVEST CELLS AND MEDIA
STORE -40 C



INFECT AFRICAN GREEN MONKEY KIDNEY
CELLS WITH HARVESTED MATERIAL.
INCUBATE 8-10 DAYS. STAIN WITH
NEUTRAL RED.

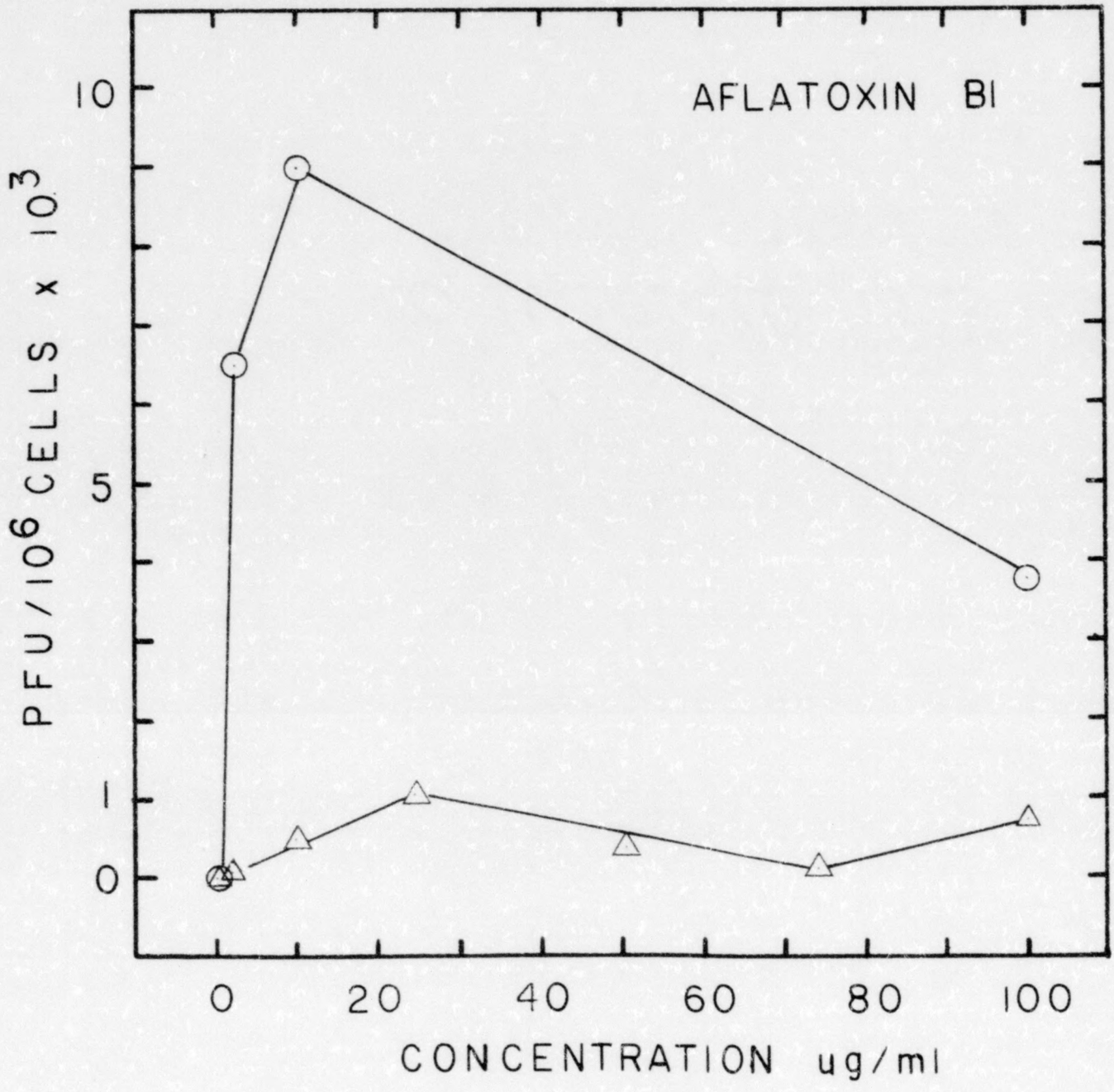


COUNT PLAQUES.

RESULTS

In developing the mammalian virus inductest the effects of certain variables were considered. Of major concern was how those variables would alter the inducing potential of the various chemical agents that were tested. The effect of two different solvents, PBS and growth medium (4X MEM), on the induction level of aflatoxin B1 is shown in Figure 2. This is a graph of the number of plaque forming units per million clone E cells (PFU/10^6 cells) produced as a function of the reagent concentration. The concentration of the chemical is plotted on the axis of abscissas while the PFU/10^6 cells is on the axis of ordinates. When dissolved in PBS, aflatoxin B1 showed significantly greater induction at all concentrations than it did in 4X MEM. At a concentration of 1 µg/ml aflatoxin B1 in PBS induced 6500 PFU/10^6 cells compared to only 100 PFU/10^6 cells induced by this chemical in 4X MEM. Similar results were observed when a 10 µg/ml concentration was used, in that aflatoxin B1 in PBS induced 9,000 PFU/10^6 cells while in 4X MEM only 500 PFU/10^6 cells were induced. At the 100 µg concentration the difference is not as drastic, but aflatoxin in PBS produced a greater number of PFU/10^6 cells. Furthermore, the peak of the aflatoxin in 4X MEM is shifted more to the right with maximum induction occurring at 25 µg/ml while aflatoxin in PBS peaked at 10 µg/ml and showed a gradual decline due to its cytotoxic effect. When tested alone PBS did not induce viral gene expression.

Figure 2. Effect of solvent on the level of induction of aflatoxin B1. PBS solvent (⊙); 4X MEM solvent (△).


AFLATOXIN B1
PFU/10^6 CELLS x 10^3
10
5
1
0
0
20
40
60
80
100
CONCENTRATION ug/ml

The induction potential of two aflatoxins is plotted in Figure 3. PFU/10^6 cells is plotted as ordinates and the concentration as abscissas. At 1 µg/ml and 10 µg/ml aflatoxin B1 shows much more induction than does aflatoxin G1. However, at 100 µg/ml, aflatoxin G1 induces more than aflatoxin B1. As was shown in Figure 2, aflatoxin B1 peaked at 10 µg and decreased as the concentration was increased beyond 10 µg. But for aflatoxin G1, the induction level was minimal at 1 µg, increased at 10 µg and even shows a gradual increase up to 100 µg. Thus, for the concentrations tested, aflatoxin G1 showed an increase in the number of PFU/10^6 cells produced as the concentration was increased while aflatoxin B1 showed increased induction only up to 10 µg. Therefore, it is apparent that the concentration of the potential carcinogen affects the level of induction, in that some chemicals are cytotoxic to the cells at higher concentrations.

A positive correlation between the concentration of chemical and the level of induction is better exemplified in Figure 4. The number of PFU/10^6 cells is plotted on the ordinate as a function of the concentration of sterigmatocystin on the abscissa. The induction level of sterigmatocystin was minimal at 1 µg, increased to 4200 PFU/10^6 cells at 10 µg and 13,700 PFU/10^6 cells at 100 µg.

Figure 5 compares the effect of two different volumes of harvested viral material on SV40 induction using the number of PFU/10^6 clone E cells as ordinates as a function

Figure 3. Comparison of induction potential of aflatoxin B1 (⊙) and aflatoxin G1 (△).

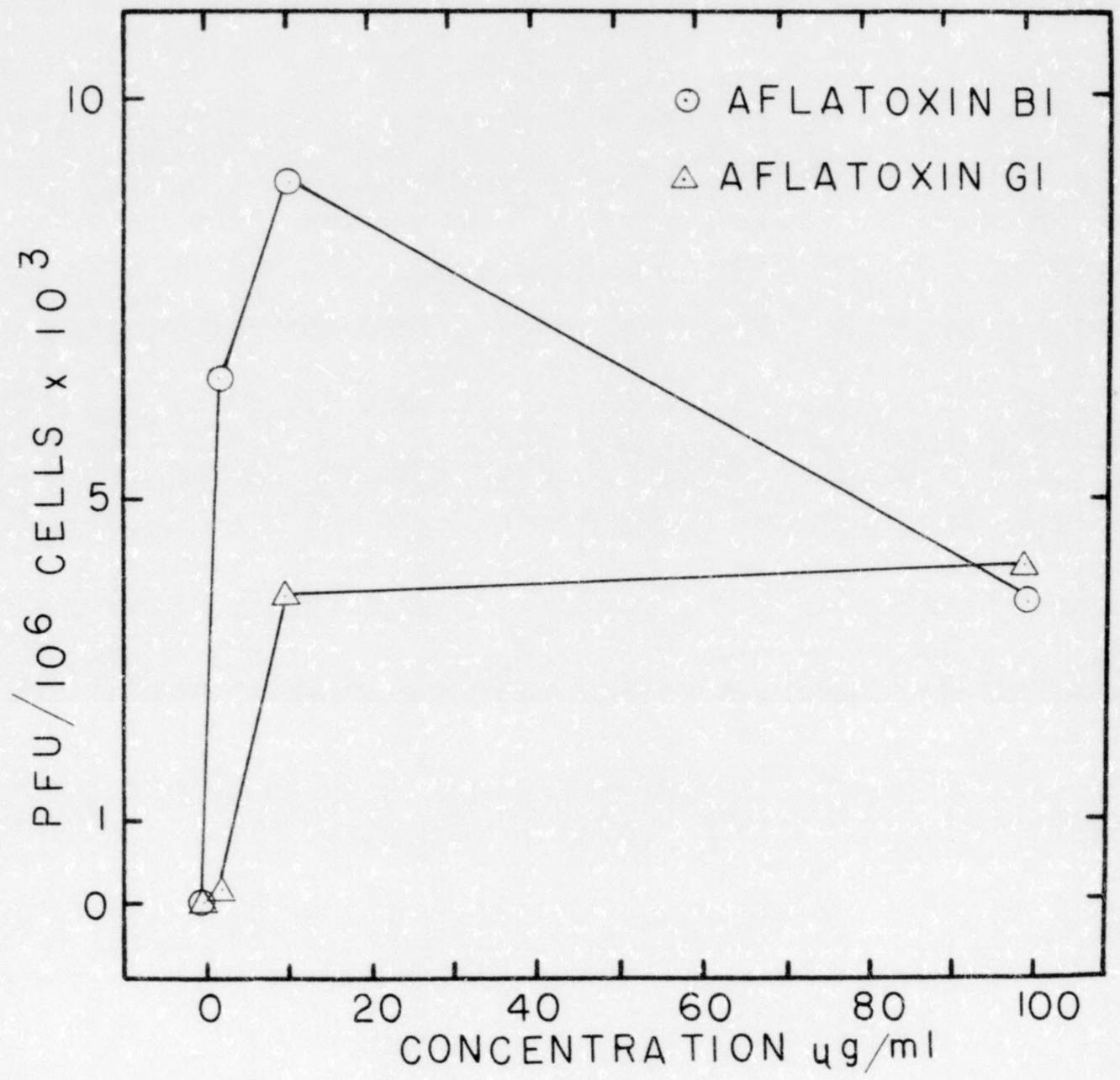

AFLATOXIN B1
AFLATOXIN G1
PFU/10^6 CELLS x 10^3
10
5
0
0
20
40
60
80
100
CONCENTRATION µg/ml

Figure 4. Effect of concentration on the induction of sterigmatocystin.

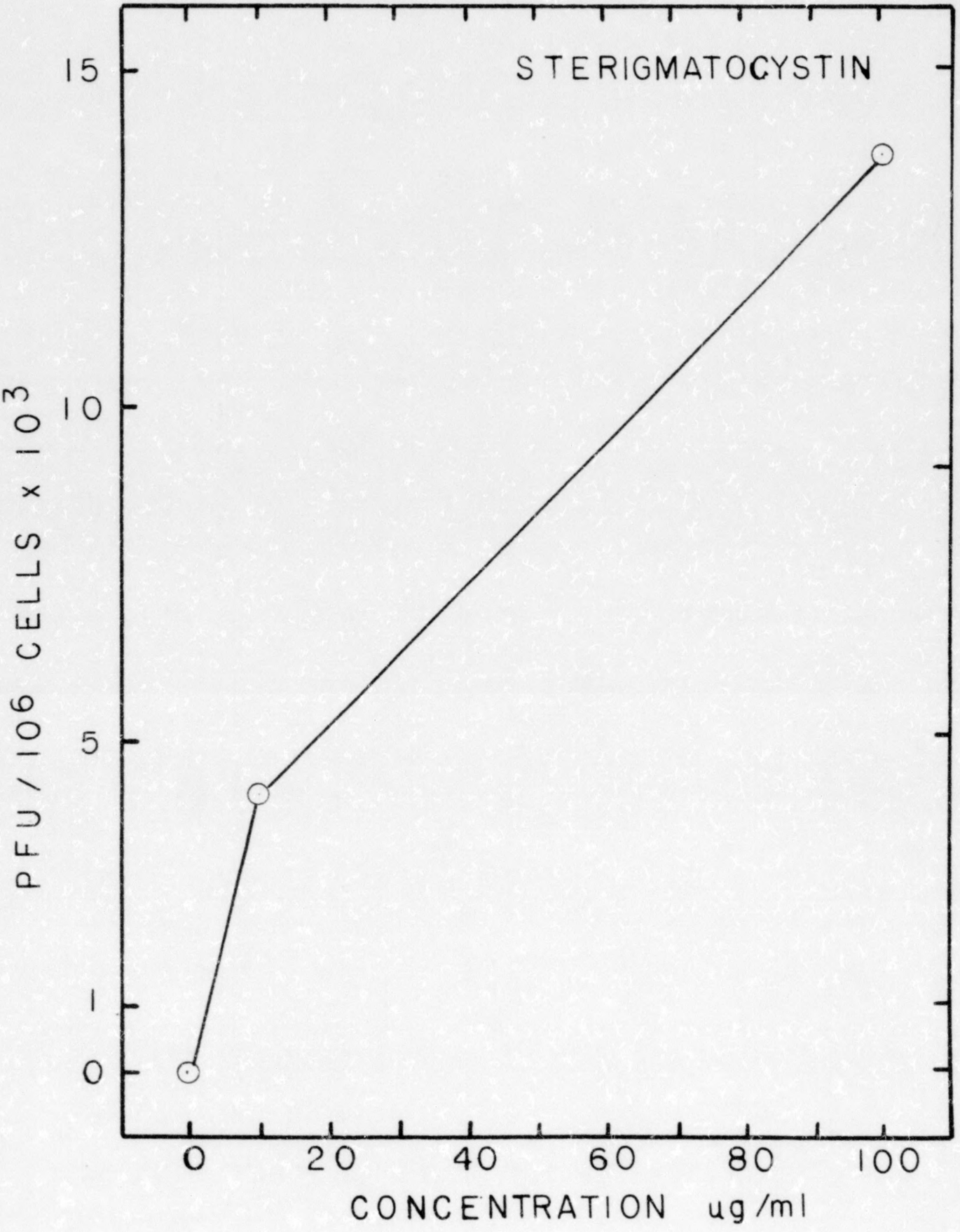
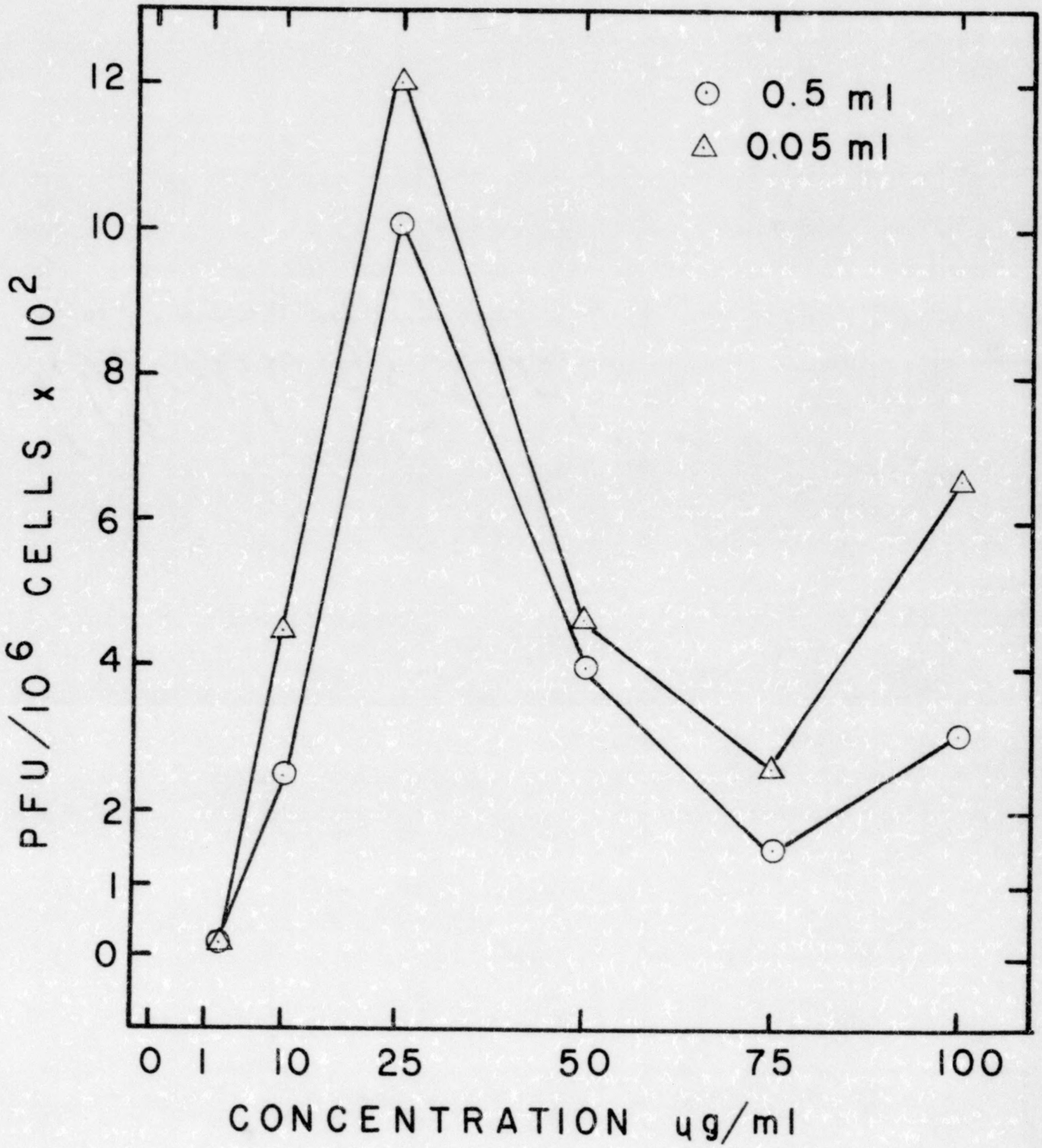

STERIGMATOCYSTIN
15
10
5
1
0
PFU / 106 CELLS x 10^3
0
20
40
60
80
100
CONCENTRATION ug/ml

Figure 5. Comparison of the induction level obtained with 0.5 (⊙) and 0.05 ml (△) volumes of harvested virus material for assay.


0.5 ml
0.05 ml
PFU/10^6 CELLS x 10^2
12
10
8
6
4
2
1
0
0 1 10 25 50 75 100
CONCENTRATION µg/ml

of carcinogen concentration as abscissas. There was little induction by 1 µg/ml of aflatoxin B1 for either 0.05 or 0.5-ml inoculum volume. At all other concentrations the level of induction with the 0.05 volume was slightly higher than that of the 0.5 volume. The greatest differences were observed at 10 µg and 25 µg concentrations. There was very little difference between the volumes at 50 µg and 75 µg. At 100 µg the difference in induction level of the two volumes is great and the number of PFU/10^6 cells is greater than those at 75 µg, which contradicts the general pattern of decreasing induction with increasing concentration demonstrated by aflatoxin B1 at concentrations higher than 25 µg.

Table 1 is a list of the chemical agents that were positive in the mammalian virus inductest system emphasizing the conditions under which induction occurred. The conditions include the time of exposure (2 hr and 24 hr), solvent (PBS and 4X MEM), chemical concentration (0.0001 µg to 100 µg) prematabolization with S9 mix, and permeabilization of the cell membrane with lysolecithin. Of the chemicals tested, aflatoxin B1, aflatoxin G1, sterigmatocystin, and the mitomycin C control induced the virus at exposure times of 2 and 24 hrs in either PBS or 4X MEM solvents. These chemicals induced at each concentration tested and with or without S9 mix and lysolecithin. The time of exposure for the induction of the other chemicals was 24 hrs in a 4X MEM solvent. S-triazine also induced at all concentrations. The concentrations at which

TABLE 1

CONDITIONS FOR VIRUS INDUCTION

Chemical	Time of Exposure (in hrs)	Solvent	Concentration	S9 Mix	Lysolecithin
Aflatoxin B1	2	PBS	All	(-)	(-)
Aflatoxin B1	24	4X MEM	All	(+/-)	(+/-)
Aflatoxin G1	2	PBS	All	(-)	(-)
Aflatoxin G1	24	4X MEM	All	(+/-)	(+/-)
Sterigmatocystin	2	PBS	All	(-)	(-)
Sterigmatocystin	24	4X MEM	All	(+/-)	(+/-)
Mitomycin C	2	PBS	All	(-)	(-)
Mitomycin C	24	4X MEM	All	(+/-)	(+/-)
Benzo(a)pyrene	24	4X MEM	1-100 µg	(+)	(+/-)
DMBA*	24	4X MEM	1-100 µg	(+)	(+/-)
Nitrosomorpholine	24	4X MEM	1-100 µg	(+)	(+/-)
S-triazine	24	4X MEM	All	(+)	(+/-)
6-Azouracil	24	4X MEM	0.0001 µg	(-)	(+/-)
Saccharin	24	4X MEM	0.0001 µg	(-)	(+/-)
Urethane	24	4X MEM	100 µg	(-)	(+/-)
PABA**	24	4X MEM	0.05%	(-)	(+/-)

*Dimethylbenzanthracene

**para-Aminobenzoic Acid

the other chemicals showed induction varied. Benzo(a)pyrene, 7,12 dimethylbenzanthracene and nitrosomorpholine induced at concentrations ranging from 1 to 100 µg. 6-Azouracil and saccharin induced at the 0.0001 µg concentration while urethane induced at 100 µg and .05% PABA induced.

Not all chemicals required premetabolization with S9 mix. Examples of these are 6-azouracil, .05% PABA, saccharin and urethane (Table 1). Furthermore, there were some that would only induce with premetabolization, such as benzo(a)pyrene, 7,12 dimethylbenzanthracene, nitrosomorpholine and S-triazine. All other chemicals induced with or without premetabolization. Each chemical induced with or without cell membrane permeabilization with lysolecithin.

Those chemicals that showed no induction in this test system are listed in Table 2. These include benzanthracene, and 20 methylcholanthrene, along with the controls which include PBS, 4X MEM, lysolecithin, S9 mix and S9 mix with lysolecithin treatment. Also included in Table 2 are those chemicals that did induce along with results of other testing systems and the classification of these chemicals as mutagenic and/or carcinogenic agents. Table 2 will be discussed in the next section.

TABLE 2

COMPARISON OF RESULTS WITH OTHER TEST SYSTEMS

Chemical	Salmonella/ Ames	Mutagenicity	Bacterial Inductest	SV40 Inductest	Mutagen	Carcinogen
Aflatoxin Bl	+	+	+	+	+	+
Aflatoxin Gl	+	+	+	+	+	+
Sterigmatocystin			+	+	+	+
Dimethylbenzanthracene	+	+	+	+	+	+
Benzanthracene	+	+	0	0	+	?
Benzo(a)pyrene	+	+	+	+	+	+
Methylcholanthrene	+	+	+	0		
Nitrosomorpholine				+	+	+
PABA				+		
Saccharin				+		
S-triazine				+	?	+
Urethane	0			+		
Controls						
Mitomycin C	+	+	+	+	?	+
PBS				0	-	-
4X MEM				0	-	-
Lysolecithin				0	-	-
S9 Mix				0	-	-
S9 + Lysolecithin				0	-	-

+=induction occurred; 0=no induction; ?=questionable results.

References:

1. McCann et al. 1975.
2. Moreau et al. 1976.
3. Hollstein and McCann, 1979.

DISCUSSION

The mammalian virus inductest was developed as an analogue to the bacterial inductest. Both tests are based on the induction of latent virus when challenged with DNA damaging chemical agents. Bockstahler and Hellman (1979) concluded that since the expression of endogenous viral genes is regulated by cellular control processes and since carcinogenesis involves alterations of normal cellular regulation, viral induction systems would yield information important for the understanding of carcinogenesis. Based on this hypothesis and the results obtained with the bacterial inductest, the mammalian inductest was developed as a system for predicting the carcinogenic potential of chemical agents in man. Since mammalian cells are more closely related to man than are bacterial cells, this system should provide for more reliable prediction of human risk.

In early experiments clone E cells were exposed for a period of 2 hrs to varying concentrations of the chemical agents dissolved in PBS. This presented a problem in that the polycyclic aromatic hydrocarbons would not remain in solution. Casto (1973) suggests that growth medium, 4X MEM, be used as a solvent because the chemical would bind to the protein molecules of the calf serum and remain in solution. The difference in level of induction of a chemical agent in the two different solvents (Figure 2) can be attributed to a stress factor.

Lacking any nutrients, PBS added stress to the cells resulting in increased metabolic activity and,therefore, increased viral replication. Furthermore, when combined with a protein molecule, the chemical-protein complex formed is considerably larger than the pure chemical and smaller quantities may actually be penetrating the cell.

Some of the chemicals tested exhibited a cytotoxic effect. This effect is illustrated in Figure 3 for aflatoxin B1 and aflatoxin G1. Maximum induction was obtained using a 25 µg/ml concentration for aflatoxin G1 and 10 µg/ml for aflatoxin B1. At higher concentrations the level decreased due to cellular damage elicited by the toxic chemicals. It appears that the more potent inducers were cytotoxic at lower concentrations than lesser ones like sterigmatocystin (Figure 4), which showed increased levels of induction up to 100 µg/ml.

The reliability of this test system was determined by several means. First, each chemical was tested in no less than four experiments for reproducibility. Second, two volumes of each chemically induced harvested virus were used to infect the SV40 permissive CV-1P cells (Figure 5). The fact that the lesser volume (0.05) produced more PFU/10^6 cells may be attributed to the fewer virus particles contained in the 0.05 ml volume that must compete for combining sites on the plasma membrane of the CV-1P cells. Furthermore, in determining the number of PFU/10^6 cells the average number of plaques per dish at the 0.05 assay volume was multiplied by a normalizing

factor ten times that of the 0.5 volume. Thus the 0.05 volume produced fewer plaques than the 0.5 assay volume, but the number of PFU/10^6 cells was greater. The consistency obtained with replicate experiments and varying infection volumes, along with the third and most definitive criterion--the results of other test systems, indicates that the mammalian SV40 inductest is a reliable system for screening putative carcinogens. Table 2 shows a comparison of SV40 mammalian inductest results to the results of other test systems for potential carcinogens. These include the bacterial inductest, the Salmonella/Ames test and the mutagenicity test in mammalian cells in culture.

In the mammalian inductest, aflatoxin B1 and mitomycin C were effective viral inducers. Viral induction was also obtained with aflatoxin G1 and sterigmatocystin, but either gave lower induction or required greater concentrations of the inducing agent to elicit induction at a level comparable to that of aflatoxin B1 and mitomycin C. These results agree with those that Moreau et al. (1976) obtained with their bacterial inductest. With the exception of sterigmatocystin, these chemicals were also positive in the Salmonella/Ames test and the mutagenicity test in mammalian cells in culture.

Certain chemical require enzymatic conversion in order to exhibit mutagenic or carcinogenic activity. Such in vitro conversions are justified by similar events occurring in vivo. Benzo(a)pyrene, dimethylbenzanthracene and benzanthracene showed no induction without pre-metabolization with S9 mix. However, when tested in conjunction with S9 activator mix

benzo(a)pyrene and 7,12 dimethylbenzanthracene induced, while benzanthracene did not. The same was observed by Moreau et al. (1979) with their bacterial inductest. In contrast, benzanthracene was positive in the Salmonella/Ames test and the mutagenicity test in mammalian cells in culture. But these tests rely on the mutagenic potential of chemical agents and are restricted to mutations of specific gene loci. Although benzanthracene is a known mutagen, this chemical has a questionable carcinogenic activity (Moreau et al. 1976). This hypothesis is presently accepted by many investigators.

Liver homogenates are incorporated as a crucial aspect of some tests such as the Salmonella/Ames test (Hollstein and McCann, 1979) and the prophage λ inductest (Moreau et al. 1976). Since mammalian cells are used as the source of premetabolizing enzymes, the use of mammalian cells in this test, in some cases, eliminated the need for premetabolization. For example, when aflatoxins are tested in the Salmonella/Ames test or in the prophage λ inductest, premetabolizing enzymes must be used to obtain a positive result. However, in the SV40 mammalian inductest significant SV40 induction was obtained with aflatoxin B1 and aflatoxin G1 without premetabolization. Furthermore, urethane, 6-azouracil, 0.05% PABA + light and saccharin induced SV40 without premetabolization in this system. Of these, only urethane was tested in the Salmonella/Ames system and gave negative results. Additional chemicals tested include s-triazine and nitrosomorpholine, all of which tested positive for SV40 induction. 20-methylcholanthrene

tested negative in this test system, while a similar compound, 3-methylcholanthrene, tested positive in both the Salmonella/ Ames test and the bacterial inductest.

The mammalian virus inductest for screening putative carcinogens in mammalian cells is based on viral induction rather than mutagenesis. Unlike mutagenesis tests in mammalian cells in culture, this test requires 8-10 days while mutagenesis tests require 2-3 weeks. In addition, it is believed that damage anywhere along the host cell genome will induce latent virus (Elespuru and Yarmolinsky, 1979) while mutagenesis tests are restricted to damage at certain gene loci. Furthermore, results with the SV40 mammalian inductest show a many fold increase over the background level while in mutagenesis tests, even very low levels above background are considered positive. Finally, this system can be used to test a large number of chemical agents. This test may be considered as the mammalian cell analogue to the bacterial inductest (Moreau et al. 1976) which is based on λ induction from E. coli. The mammalian virus inductest for putative carcinogens may be a valuable tool for several reasons: a) Some cancers in humans may eventually be shown to be caused by the induction of a latent virus; there is evidence that latent viruses cause leukemia, in many animal species (Kaplan, 1978) and the same is believed to be true in humans, the mammalian virus inductest indicates agents which are capable of latent virus induction, b) some cancers may be caused by the induction of an oncogene which may be integrated into the host's genome in a manner similar

to the integration of a viral genome, consequently, the induction of a non-viral oncogene may mimic the induction of a latent virus, and c) in some cases, mammalian cells are capable of metabolizing pre-carcinogens into ultimate carcinogens, avoiding pretreatment by an exogenous enzymatic activator system. This condition may closely resemble the *in vivo* situation.

In detecting carcinogens, a battery of *in vitro* screening tests should be used to determine the carcinogenic agents worthy of *in vivo* testing. First prokaryotic systems can be used since they are rapid, reliable and relatively inexpensive. These should include a system based on mutagenicity, as the *Salmonella*/Ames test, and a system based on viral induction as the prophage λ inductest. Then eukaryotic systems can be used since eukaryotic cells can demonstrate some mutagenic effects not detectable in prokaryotes such as chromosomal anomalies. The eukaryotic tests should include a mutagenicity test in mammalian cells in culture and a system based on viral induction. The results of this research indicates that the mammalian virus inductest is a suitable addition to the battery of screening assays for determining putative carcinogens.

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