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Enhanced Transformation of Hamster Embryo Fibroblasts by SA7 Following Pre-treatment with Various Chemicals

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Abstract—*Four compounds (MCA, 1, 2, 3, 4 DBA, ICdR, and EtBr) were examined for their effect on SA7 induced transformation of hamster embryo fibroblasts. MCA enhanced transformation, its effect increasing with increasing dose to the optimum, beyond which a relative decrease in enhancement was found. 1, 2, 3, 4 DBA also enhanced transformation and its effect was dose related, but no relative decrease in enhancement was found at high doses. The DNA base analogue ICdR also significantly enhanced transformation at two of the doses tested and the intercalating agent EtBr was also found to enhance transformation. It is postulated that these compounds exert their effect by interacting with host cell DNA causing gaps which lead to increased integration of SA7 DNA into the host cell genome.*

INTRODUCTION

THE *in vitro* treatment of cells with agents such as X-rays [1] and ultraviolet light [2–4], has been shown to alter the frequency of cell transformation by oncogenic DNA viruses. The suggested mechanism for these observations is that cell transformation by DNA viruses requires integration of at least part of the viral genome into the host cell genome [5], and such integration has been demonstrated [6]; and compounds which are able to cause gaps in the cellular DNA would increase the frequency of viral transformation by providing additional areas for integration of viral DNA [7]. Quantitative data have always been obtained with DNA viruses [1–5].

In the present investigations, we have studied the transformation of hamster embryo fibroblasts by simian adenovirus 7 (SA7), as developed by Casto [3] to examine the effects of various compounds on virus transformation. Unlike many other systems transformation is both reproducible and quantitative; transformed foci can be counted 25–30 days following cell transfer and virus inoculation.

The effects on SA7 transformation of four different compounds which have different interactions with host DNA are reported. These were the polycyclic hydrocarbons 20 methylcholanthrene (MCA) and 1, 2, 3, 4 dibenzanthracene (DBA); the DNA base analogue 5-iodo2′-deoxycytidine (ICdR); ethidium bromide (2,3 diamino-5-ethyl-6-phenylphenanthridium bromide, EtBr).

MATERIALS AND METHODS

Cell cultures

Tissue cultures of primary hamster embryo fibroblasts (HEF) were prepared by trypsinization of eviscerated and decapitated embryos after approximately 14 days gestation, as described by Casto [9]. Ten millilitres of Dulbecco's modified Eagle's medium (DMEM) [10] containing 5×10^6 trypsinized cells were inoculated into 4 oz medical flats and incubated statically at 37°C; after 3 days incubation, the bottles contained $3.5–6.0 \times 10^6$ cells.

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A continuous cell line of African Green monkey kidney cells (VERO) were obtained from Flow Laboratories, Irvine, Scotland; these cells were cultured in medium 199 with antibiotics containing 5% inactivated foetal calf sera (Flow Labs. Inc.) and passaged at 5–6 day intervals.

Simian adenovirus 7

Virus (SA7), obtained from Dr. B. C. Casto (Institute for Biochemical Research, American Medical Association, Chicago, IL) was inoculated on to confluent VERO cells grown in 20 oz bottles at an input multiplicity of 2–5 $TCID_{50}$/cell. After adsorption for 2 hr, at 37°C, 50 ml of Eagle's minimal essential medium containing 2% foetal calf serum and antibiotics were added to each bottle, and the bottles incubated at 37°C. Cytopathic effects were normally complete after 72 hr incubation, when the infected cells were harvested by four cycles of freeze-thawing (−80°C /25°C). Virus was separated from cell debris by low speed centrifugation, and stored in 1–2 ml aliquots at −70°C. The pool of virus used in these studies had a titre of $10^{7.0}$ $TCID_{50}$/ml in VERO cells (21-day reading).

Chemicals

Stock solutions of 1, 2, 3, 4 dibenzanthracene (DBA) obtained from Koch Light Laboratories, Colinbrooke, Buckinghamshire, and 20 methylcholanthrene (MCA) obtained from Sigma Chemicals Co. Ltd. (London), Poole, Dorset, were prepared fresh by dissolving the chemical in ice-cold acetone. For each experiment, a bottle of confluent HEF cells was treated with each dilution of chemical diluted in DMEM 2% (v/v) final concentration of acetone for 18 hr prior to performing the transformation and survival assays. Controls consisted of cells treated with medium alone or with medium containing 2.0% (v/v) acetone.

Stock solutions of 5 iodo-2′-deoxycytidine (ICdR), and ethidium bromide (EtBr) obtained from Sigma Chemical Co. Ltd., were prepared by dissolving in DMEM and sterilised by membrane filtration. ICdR was added to the cells 24 hr after seeding into 4 oz bottles. For each experiment, one bottle of cells was treated with each dilution of ICdR for 48 hr during growth, prior to performing the transformation and survival assays. After analogue treatment, all subsequent procedures were performed in a semi-darkened room to minimise photolysis of the cell DNA. Control HEF cultures were treated with DMEM medium alone. Pre-treatment with EtBr was performed in the same way as for MCA and DBA.

Enhancement of virus transformation

Primary HEF cell cultures grown in 4 oz medical flat bottles were treated with one of the various chemicals described above. Following treatment the cells were washed once with phosphate buffered saline, pH 7.2 (PBS), trypsinized and resuspended in a modified DMEM containing 0.1 mM Ca^{2+}, 10% foetal calf serum and 2.2 g% $NaHCO_3$ (ERM). Aliquots of 0.2 ml of cell suspension containing 2×10^5 cells were distributed into 5 cm Petri dishes, and approximately 2×10^6 $TCID_{50}$ of SA7 contained in 1 ml ERM added to each plate. Five Petri dishes were inoculated for each dilution of chemical tested. After incubation at 37°C in a 5% CO_2 atmosphere for 2 hr with occasional mixing, 4 ml of ERM was added to each plate, and the plates reincubated. After 3 days incubation the medium was replaced with 5 ml of fresh ERM, and at 6 days the plates were overlayed with 3 ml of ERM containing 0.5% Difco Purified Agar (Difco Ltd.). At intervals of 4, 5 and 6 days after the first overlay, 3 ml of additional overlay medium was added to each plate. The final counts for transformed colonies were made 25–30 days after inoculation and cell transfer. The foci of transformed cells seen under agar were easily identifiable because of their unique cytomorphology [10], and the failure to find similar foci in cultures treated with medium or chemical alone.

To determine the proportion of cells which survived chemical treatment, aliquots were taken from the treated cell suspension described above containing $10^{6.0}$ cells/ml, and further diluted in DMEM to give 1000 cells/0.2 ml. Each of five 5 cm dishes was seeded with 1000 cells from either treated or control suspensions, and 3 ml of DMEM were added to each plate. After 5 days incubation at 37°C in 5% CO_2, an additional 3 ml of medium were added to each plate. Eight to nine days after seeding the cells were fixed in 10% buffered formalin, stained with a 1:30 dilution of stock Giemsa and the cell colonies counted. This procedure usually resulted in cloning efficiencies of 10% with untreated cells.

Determination of chemical enhancement of virus transformation

The fraction of cells surviving chemical treatment was determined from survival assays, as described above; the number of colonies in five plates each seeded with 1000 chemically-treated cells was divided by the number of colonies in five plates of control, untreated cells to give the surviving fraction of chemically-treated cells. The number of SA7 transformed foci per 10^6 plated cells not treated with chemical was taken as the transformation frequency for control cultures. The transformation frequency for chemically-treated cultures was calculated by multiplying the actual number of foci from 5 plates by the reciprocal of the surviving fraction of cells. Hence both treated and control transformation frequencies were expressed as the number of SA7 foci for 10^6 surviving cells (assuming 10^6 viable cells) in control cultures.

The enhancement ratio was determined by dividing the transformation frequency in treated cultures by that obtained in control cultures. Determination of the statistical significance was carried out as described by Casto *et al.* [3].

Isolation and growth of transformed cells

Lines of SA7 transformed cells were established by stabbing foci growing under agar with a Pasteur pipette and transferring the cells to a 5 cm Petri dish. Cells were then cultivated either in 5 cm Petri dishes or 4 oz medical flats as described by Casto [9].

Detection of T-antigen in transformed or tumour cells

Transformed cells or cells re-established *in vitro* from tumours were grown on coverslips on 10 cm Petri dishes and processed for indirect immunofluorescence according to the method of Pope and Rowe [11].

Tumourgenicity of transformed cells

Transformed cells at the first or second *in vitro* passage were removed from the culture vessel with trypsin–versene and resuspended in ERM. Groups of weanling hamsters were inoculated subcutaneously with 10^6 cells contained in 0.1 ml of medium. Animals were palpated at regular intervals for presence of tumours.

RESULTS

Effect of 20 *methylcholanthracene* (*MCA*)

Table 1 shows the effect of MCA on the transformation of HEF cells by SA7. HEF cell cultures were pre-treated for 18 hr with 5-fold dilutions of MCA, ranging from 10 μg/ml to 0.016 μg/ml. At the highest dose of carcinogen, there was a reduction in cell viability of 66%, but at the two lowest doses tested there was no reduction in cell viability compared to controls. As reported by Casto [3], there was a linear increase in transformation frequency compared to controls up to an optimal carcinogen concentration of 0.4 μg/ml in our experiments; at concentrations of MCA greater or lower than this concentration a relative decrease in the transformation frequency was found (Table 1).

Effect of 1, 2, 3, 4 *dibenzanthracene* (*DBA*)

The effect of SA7 transformation of pre-treating HEF cells for 18 hr with various concentrations of the carcinogenic hydrocarbon DBA are shown in Table 2; this com-

Table 1. *Enhancement of SA7 transformation of hamster embryo cells pre-treated with* 20 *methylcholanthrene*

MCA (μg/ml)	Surviving fraction	SA7 Foci*	Tranformation frequency	Enhancement Ratio†
10	0.34	12	35	0.35
2	0.34	47	138	1.37
0.4	0.52	117	225	2.23‡
0.08	0.95	129	136	1.35§
0.016	1.07	129	121	1.20
Control	1.00	101	101	1.00

*Per 10^6 plated cells.

†Enhancement ratio was determined by dividing the transformation frequency of treated cells by that obtained from control cells. Transformation frequency = SA7 × reciprocal or surviving fraction.

‡Underlined figures are significant ($P = <0.01$)

§Significant at $P \leqq 0.05$.

Table 2. Enhancement of SA7 transformation of hamster embryo cells pre-treated with 1, 2, 3, 4 dibenzanthracene

DBA (μg/ml)	Surviving fraction	SA7 Foci*	Transformation frequency	Enhancement ratio†
20	0.16	39	244	7.00‡
10	0.40	39	98	2.90
5	0.67	47	70	2.00
1	0.62	47	76	2.20
Control	1.00	35	35	1.00

*Per 10^6 plated cells.
†Determined as for Table 1.
‡Underlined figures are significant ($P = <0.01$).

Table 3. Enhancement of SA7 transformation of hamster embryo cells pre-treated with 5 iodo-2′-deoxycytidine

ICdR (μg/ml)	Surviving fraction	SA7 Foci*	Transformation frequency	Enhancement ratio†
25	0.24	46	192	4.90‡
12.5	0.32	27	84	2.20
6.25	0.55	18	33	0.80
3.125	0.60	23	38	0.98
1.56	0.99	35	35	0.90
Control	1.00	39	39	1.00

*Per 10^6 plated cells.
†Determined as in Table 1.
‡Underlined values are significant ($P = <0.01$).

pound was one of a series of polycyclic hydrocarbons studied by Casto [3]. Significant enhancement of SA7 transformation was found at all the doses tested; however, no decrease in transformation frequency was found at the highest doses tested. In neither the experiments with MCA nor DBA was cytotoxicity or effect on virus transformation frequency observed in control cultures treated with growth medium incorporating 2% acetone, as compared with growth medium alone. In contrast to the experiments reported by Casto [3] we found no absolute increase in the numbers of transformed foci arising from cells pretreated with either MCA or DBA.

Effect of DNA base analogues

It has previously been shown that the treatment of growing HEF cells with certain DNA analogues for 48 hr prior to exposure to SA7 greatly enhanced cell transformation [2]. In the present studies, the previously untested base analogue 5 iodo-2′-deoxycytidine (ICdR) significantly increased cell transformation of HEF cells pretreated with doses of 25 and 12.5 μg/ml, but lower concentrations of ICdR had no significant effect. (The results are shown in Table 3.) No evidence for a decrease in the virus-induced transformation frequency was found at the highest concentration of ICdR tested (25 μg/ml) which killed 76% of the cells; other base analogues have been reported to have decreased transformation at high concentrations [2]. In contrast to previous reports we found no increase in the absolute number of foci in treated cells.

Effect of ethidium bromide (EtBr)

Ethidium bromide is able to intercalate between the strands of double stranded DNA [15], and has been shown to be therapeutically active against several virus-induced tumours of laboratory animals [8]. The present results show that pretreatment of HEF cells for 18 hr with EtBr significantly enhanced transformation at a dose of 1 μg/ml although no increase in the absolute numbers of transformed foci arising from treated cells was found; lower concentrations of the compound had no significant effect. These results are shown in Table 4, and are the average of two separate experiments.

Table 4. *Enhancement of SA7 transformation of hamster embryo cells pre-treated for* 18 hr *with ethidium bromide*

EtBr (μg/ml)	Surviving fraction	SA7 Foci*	Transformation frequency	Enhancement ratio†
1.0	0.47	42	89	2.34‡
0.5	0.71	38	54	1.42
0.25	0.71	28	39	1.03
0.125	0.95	31	33	0.87
Control	1.00	38	38	1.00

*Per 10^6 plated cells.
†Determined as for Table 1.
‡Underlined figures are significant ($P = < 0.01$).

Tumourgenicity of transformed cells for weanling hamsters

Groups of hamsters which had been inoculated with 10^6 transformed cells derived from single transformed foci developed palpable tumours at or near the site of inoculation within 30 days. These tumours were found to be undifferentiated sarcomas as has been previously reported [9]. Tumours produced by cells which had originally been isolated from cells pretreated with chemical were identical histologically to those produced by cells isolated from control cultures treated with virus alone.

Detection of T antigen in transformed cell lines and cell-induced tumours

Sera from hamsters bearing large (>3 cm) primary cell induced tumours were used in the indirect immunofluorescence procedure. Strong intranuclear flourescence in the form of flecks and dots was found in 95–100% of nuclei. No difference was found between cells originally pretreated with chemical or cells treated with virus alone. As a test of specificity cells and sera derived from an SV40 induced tumour of hamsters maintained in this laboratory were included in the test. No cross-reactivity between SA7 and SV40 induced T-antigens was found.

DISCUSSION

Focus assay systems have been widely used to study the effect of various chemicals on virus induced cell transformation [1–5]. It is possible that such systems will allow identification of compounds which may enhance transformation but cannot transform cells themselves.

In the present investigations the SA7–HEF system developed by Casto [9] has been used to test four compounds which are capable of interacting with hamster cell DNA. The transformed foci are easily recognised by their characteristic morphology [10] which is distinct from that obtained by chemical treatment alone. The transformed cells are tumourgenic in weanling hamsters and contain an intranuclear T-antigen which can be demonstrated by indirect immunofluorescence using sera from tumour bearing hamsters.

The effect of MCA on transformation was similar to that reported in [3] showing an increase in transformation frequency with increasing doses of carcinogen to an optimum dose. A relative decrease in the transformation frequency was found at doses greater than the optimum. It has been postulated that this effect is due to a decrease in the capacity of cells to achieve normal rates of DNA synthesis in the period immediately following infection by virus; it is at this time that the primary events in transformation are though to occur. It has also been reported that a period of DNA synthesis was necessary shortly after infection for transformation of cells both by SV40 [12] and adenoviruses [9, 13].

The carcinogen DBA also enhanced SA7 transformation; the degree of enhancement was linearly related to the carcinogen concentration but no relative decrease in enhancement was found at high doses. This suggests that DBA does not severely impair DNA synthesis.

Pretreatment of HEF with the thymidine analogue ICdR was also found to enhance transformation; similar enhanced transformation in cells pretreated with DNA base analogues has been found with SV40 [12] and other adenoviruses [2]. Although base analogues do cause sedimentation changes when

incorporated into cell DNA; they may not cause fragmentation of DNA in mammalian cells [14] and it seems likely that such lesions would produce additional sites for integration of viral DNA into cellular DNA. We have found that 5 bromouracil, a DNA base analogue not incorporated into DNA by mammalian cells, had no effect on SA7 transformation (data not shown) which also suggests that other base analogues enhance transformation by their effect on host DNA.

Ethidium bromide is known to be active against some C type viruses *in vitro* [15–17] and virus induced tumours of laboratory animals *in vivo* [8]. The *in vitro* activity against C type viruses is probably due to inhibition of closed circular viral DNA formation [18] which could prevent integration of viral DNA into host cell DNA. It is unlikely that EtBr could affect SA7 DNA in the present experiments since virus was added after removal of EtBr and any EtBr remaining associated with cells would be complexed with the cell DNA. However EtBr may cause gaps in cell DNA by stabilising mispaired regions [19] thus creating gaps during subsequent rounds of scheduled synthesis and leading to an increase in the number of transforming events. This possibility poses the more general question that some compounds which are active as antiviral or antitumour agents may predispose towards another type of tumour.

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