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Carcinogens enhance survival of UV-irradiated simian virus 40 in treated monkey kidney cells: Induction of a recovery pathway?

(chemical carcinogen/inducible DNA repair/UV-reactivation/carcinogen-reactivation)

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ABSTRACT **Treatment of monkey kidney cells with low doses of carcinogens enhances the survival of UV-irradiated simian virus 40 (SV40). This is true for compounds with UV-like effects (metabolites of aflatoxin B_1, *N*-acetoxyacetylaminofluorene) and compounds with x-ray-like effects (methyl methanesulfonate, ethyl methanesulfonate). This phenomenon resembles the UV-reactivation of viruses in eukaryotic cells. The carcinogen-induced enhancement of the survival of UV-irradiated SV40 is correlated with the inhibition of host-cell DNA synthesis, suggesting that the inhibition is an inducing agent.**

An enhancement of UV-irradiated SV40 survival is also obtained in cells treated with hydroxyurea or cycloheximide for long enough that there is still inhibition of host DNA synthesis during the early stage of SV40 infection.

We hypothesize that treatment of host cells with carcinogens induces a new recovery pathway that facilitates the replication of damaged DNA, bypassing the lesions and resulting in the enhanced survival of UV-irradiated SV40. This inducible process might represent the expression of "SOS repair" functions in eukaryotic cells analogous to the previously demonstrated induction of SOS repair in bacteria after UV or carcinogen treatment.

It is now recognized that there is a good correlation between chemical damage to DNA, measured essentially by mutagenic activity, and carcinogenesis in mammalian cells (1). Unrepaired DNA damage in man can lead to cancer, as suggested by the autosomal recessive hereditary disease, xeroderma pigmentosum, in which a deficiency in DNA repair correlates with a high susceptibility to sunlight-induced carcinomas and melanomas of the skin (2, 3). Other hereditary diseases such as Fanconi's anemia and ataxia telangiectasia also appear to involve deficiencies in DNA repair processes (4, 5). It is of interest to know whether there exist new DNA repair or recovery pathways that can be induced by the preconditioning of the organism, to possibly enhance the repair of potentially carcinogenic lesions. In bacteria the existence of an inducible, but "error-prone," DNA repair pathway is now reasonably well established (6). Treatment of bacterial host cells, before phage infection, with various agents such as UV or x-ray irradiation, mitomycin C, aflatoxin B_1 metabolites or deprivation of thymine in *thy*$^-$ hosts enhances the survival of UV-irradiated phage λ (7, 8). This phenomenon, which has been called "induced phage reactivation" involves the induction of a type of DNA repair that is highly mutagenic and is a part of the expression of the "SOS functions" observed in treated bacteria in response to certain kinds of inducing damage (9). Although its mode of action is not known, the inducing agent may be closely related to the inhibition of host-cell DNA synthesis that follows all these pretreatments (10).

It has been suggested that eukaryotic cells might show a similar induced repair pathway after carcinogen treatment (9–11). Such an error-prone process, which could be responsible for the mutagenic activity of various chemicals, might also enhance the probability of malignancy. In certain mammalian host cells, UV or x-ray irradiation prior to a viral infection enhances the survival of UV-irradiated virus such as herpes virus (12, 13), simian adenovirus (14), or Kilham rat virus (15). This protocol parallels the one used for bacterial studies, and we have used it as a probe to detect the expression of SOS functions in eukaryotic cells treated by carcinogens. We show in the present work that enhanced survival of UV-irradiated simian virus 40 (SV40) is obtained upon preconditioning the host monkey kidney CV-1P cells by carcinogens known to mimic either UV or x-ray irradiation. Moreover, a correlation has been found between the inhibition of host cell DNA synthesis and the induction of SV40 reactivation.

MATERIALS AND METHODS

Cells. The established CV-1P and MA-134 lines of African green monkey cells were obtained from P. Berg (Department of Biochemistry, Stanford University, CA) and grown on plastic dishes (Falcon) in Eagle's medium as modified by Dulbecco (Gibco) containing 5% fetal calf serum (Gibco) in a CO_2 incubator at 37°.

Treatment of Cells. Confluent CV-1P cells on 60-mm plastic dishes were treated with one of the following agents. *UV*: After the plates had been washed with phosphate-buffered saline, cells were irradiated with UV light from a 15-W germicidal lamp at an incident dose rate of 0.9 J/m^2·s. *Aflatoxin B_1*: Cells were exposed to aflatoxin B_1 (Sigma) in the presence of rat liver microsomal enzymes for 30 min at 37° as previously described (16). N-*Acetoxyacetylaminofluorene* (AAAF; gift from J. A. Miller, University of Wisconsin, Madison, WI): Cells were treated for 1 hr at 37° by adding to the culture medium various amounts of stock solution of AAAF dissolved in dimethyl sulfoxide at 1 mg/ml. (*Methyl methanesulfonate (MMS) or ethyl methanesulfonate (EMS)*: Cells were treated with MMS (Aldrich) or EMS (Sigma) for 90 min at 37° by adding to the medium various amounts of pure compounds diluted by 100 or 10, respectively, in sterile water. After carcinogen treatment, cells were washed three times with phosphate-buffered saline. Culture medium containing 2% fetal calf serum was added until virus infection.

Virus. SV40 (strain SVS WT 830) obtained from P. Berg was grown on MA-134 cells at 0.01 plaque-forming unit (PFU) per

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Abbreviations: AAAF, *N*-acetoxyacetylaminofluorene; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; PFU, plaque-forming unit.

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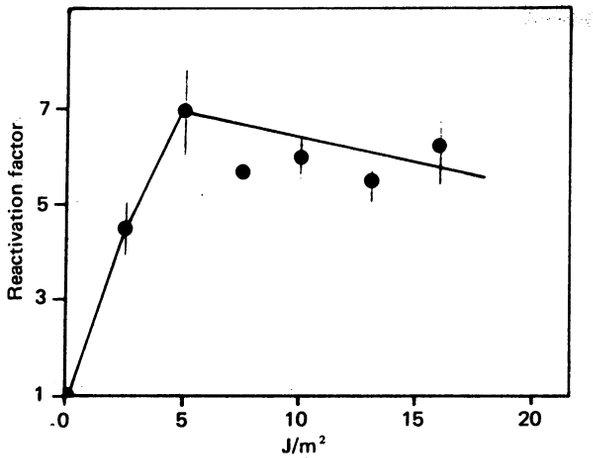

FIG. 1. Reactivation factor of UV-irradiated (1300 J/m²) SV40, calculated as described in *Results*, as a function of UV fluence on the host cell. Each point is the average of 4–10 values. The error bars correspond to the standard error of the mean.

cell and purified according to Estes *et al.* (17). UV irradiation of virus (1300 J/m²) was done on a 1-ml suspension (about 10^8 PFU/ml) in a 35-mm culture dish at room temperature with the same lamp that was used for cell irradiation.

Plaque Assay. Usually 24 hr after pretreatment, confluent CV-1P cells were washed with Tris/saline (25 mM Tris·HCl at pH 7.5/140 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/1 mM $MgCl_2$/1.8 mM $CaCl_2$), and 0.2 ml of virus dilution, in Tris/saline containing 2% serum, was added per dish. After a 2-hr absorption at 37° the cells were washed twice with Tris/saline and then an agar medium overlay was added (minimum Eagle's medium, 3.8% fetal calf serum, 1% Difco agar). A second overlay was added 5 days later and a third overlay containing neutral red (0.01%) was added 4 days later. Plaques were counted over a period of 2 days.

DNA Synthesis and DNA Repair Measurements. DNA repair replication was determined using the combined BrdUrd density label and [^{3}H]dThd radioisotope label method as previously described (16, 18). The neutral CsCl gradient used in that technique permits the determination of the amount of semiconservative DNA synthesis and the alkaline gradient facilitates the quantitation of the amount of DNA repair synthesis. Values for synthesis were expressed in ^{3}H cpm incorporated per μg of DNA determined by 254-nm absorbance.

RESULTS

UV Reactivation of UV-Irradiated SV40. UV irradiation of confluent CV-1P host cells enhances the survival of SV40 virus irradiated with 1300 J/m². The UV doses that give rise to UV-reactivation are rather low and fall in the range of 2.5–15 J/m² (Fig. 1). This result confirms the recent report of Bockstahler and Lytle (14), who reported UV-reactivation of UV-irradiated SV40 in another line of monkey host cells (CV-1 cells). However, the reactivation factor we observe is about 2 to 3 times higher than the value they published for identical UV fluences (14). We calculated the reactivation factor by dividing the surviving fraction of SV40 (ratio of the titer of UV-irradiated SV40 to that of unirradiated SV40) in pretreated cells by the surviving fraction of SV40 in nontreated cells. We determined the values for the UV-reactivation factor following UV fluence to the virus between 500 and 2500 J/m². The maximal reactivation was obtained for a dose of 1300 J/m² (data not shown). Therefore, we used this UV fluence for all the subsequent experiments described in this paper. The survival of 1300 J/m²-irradiated SV40 is about 10^{-2} in untreated cells.

Reactivation of UV-Irradiated SV40 by UV-Like Carcinogens. The treatment of prokaryotic or eukaryotic cells with the metabolites of the potent hepatocarcinogen aflatoxin B_1 gives rise to numerous responses similar to those obtained after UV irradiation (19). The pretreatment of CV-1P cells with activated aflatoxin B_1 enhances the survival of UV-irradiated SV40 (Fig. 2*A*) and the dose–response curve of the reactivation factor is similar to that obtained after UV treatment (Fig. 1). The effective carcinogen concentration is low and the capacity of treated cells to grow unirradiated virus is not impaired at aflatoxin B_1 concentrations below 5 μg/ml, while maximal DNA repair synthesis is observed at 40–60 μg/ml (16). The reactivation factor after aflatoxin B_1 treatment of host cells attains a maximum 3 days after treatment (Fig. 3), and its change with time is similar to that reported for herpes virus reactivation after UV treatment of the host cells (20). This increase in the survival of UV-irradiated viruses after 3 days has been interpreted as a delayed expression of Weigle reactivation (20).

The activation mixture alone, composed of rat liver microsomes and a NADPH-regenerating system (16), employed to activate aflatoxin B_1 enzymatically, produces no reactivation of UV-irradiated SV40. However, treatment of host cells with pure aflatoxin B_1 resulted in some enhancement of survival of UV-irradiated SV40 (data not shown). This is probably due to the endogenous activity of microsomes in the monkey kidney cells, because both mouse and rat kidney cells have been shown to be capable of activating aflatoxin B_1 *in vivo* (21). However, we continued to use the exogenous activation of aflatoxin B_1 by rat liver microsomes in order to easily compare this result with results using human cells, which are unable to activate the carcinogen (16).

Treatment of cells with another UV-like carcinogen, *N*-acetoxyacetylaminofluorene, also produces an enhancement of the survival of UV-irradiated SV40 (Fig. 2*B*). A low concentration of AAAF induces a high level of reactivation, yet does not affect the capacity of cells to grow unirradiated virus, indicating a high efficiency for this active metabolite of acetylaminofluorene to induce the reactivation process. The same dose range of this drug has been shown to increase the rate of postreplication DNA strand rejoining in UV-irradiated hamster cells (22).

Reactivation of UV-Irradiated SV40 by X-ray-Like Carcinogens. It has been shown that x-irradiation of CV1 cells enhances the survival of UV-irradiated herpes (13) or SV40 viruses (14). We found an increase in the survival of UV-irradiated SV40 in CV-1P cells pretreated with two carcinogens that mimic x-irradiation: MMS and EMS (Fig. 2 *C* and *D*). The values of the factors obtained after EMS or MMS treatment were similar to those seen after UV irradiation or treatment with UV-like carcinogens.

Reactivation of UV-Irradiated SV40 after Treatment with Inhibitors of DNA Synthesis. Because the current model for the induction of the SOS functions in bacteria implies that inhibition of host-cell DNA synthesis might be an inducing agent (6, 10, 23), we determined the effects of various inhibitors of host-cell DNA synthesis on the survival of UV-irradiated SV40. When CV-1P cells were treated for 18 hr with hydroxyurea, a very potent inhibitor of semiconservative DNA synthesis in eukaryotic cells (24), enhanced survival of UV-irradiated SV40 was observed for 1, 2, and 3 mM concentrations of the drug. Reactivation factors of 2.8, 5.7, and 3.5 were obtained, respectively.

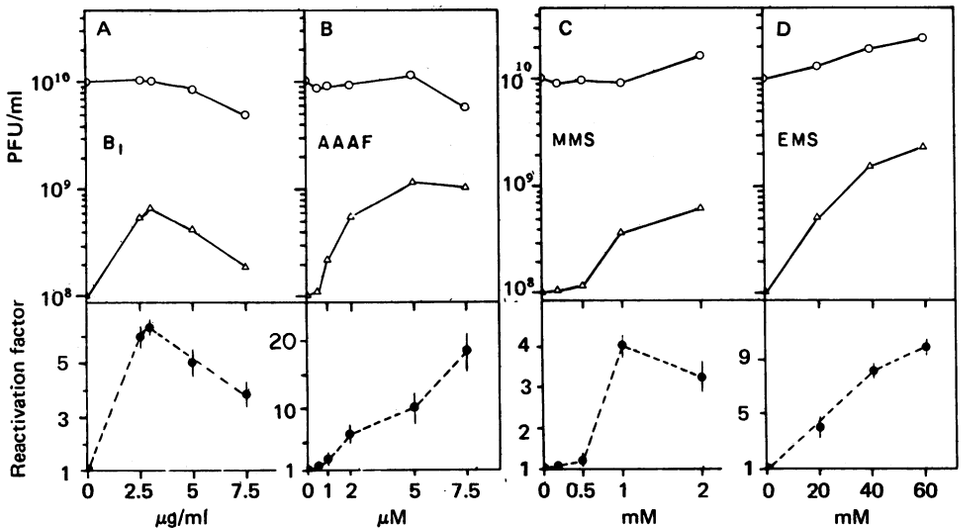

FIG. 2. Reactivation factor of UV-irradiated (1300 J/m^2) SV40 after treatment of host cells with various carcinogens. Top panels represent the survival of non-irradiated (○) or UV-irradiated (△) SV40 in CV-1P cells treated by increasing amounts of different carcinogens. Bottom panels represent the reactivation factor. CV-1P host cells were treated 24 hr before virus infection with (*A*) metabolites of aflatoxin B_1, (*B*) *N*-acetoxyacetylaminofluorene, (*C*) methyl methanesulfonate, (*D*) ethyl methanesulfonate. Each point is the average of four to eight values. The error bars correspond to the standard error of the mean.

In eukaryotic cells, DNA synthesis is inhibited rapidly and almost completely by inhibition of protein synthesis (see ref. 25 for review). However, DNA repair synthesis is not inhibited during protein synthesis inhibition (26) and a drug like cycloheximide, which inhibits protein synthesis and then DNA synthesis, does not produce detectable lesions in DNA, or at least does not promote DNA repair replication (Table 1).

We inhibited host-cell DNA replication more than 90% with cycloheximide at 10 μg/ml for periods of time ranging from 3–72 hr. After removing the drug we added [^{3}H]thymidine to cells and measured host-cell DNA synthesis over a period of 18 hr, the time necessary to begin replication of UV-irradiated SV40 after infection. The results show that enhancement of UV-irradiated SV40 survival is obtained in cells treated with cycloheximide for a long enough time that there is still inhibition of host-cell DNA synthesis during the early stage of SV40 infection (Table 1).

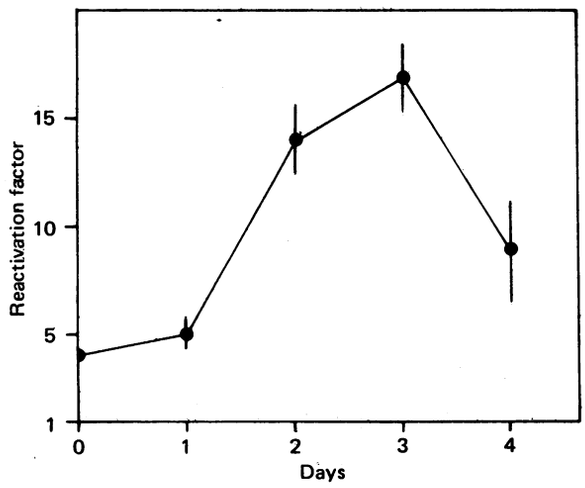

FIG. 3. Reactivation factor, after pretreatment of CV-1P cells with aflatoxin B_1 at 3 μg/ml, in presence of the activating enzymes, as a function of time between the pretreatment and virus infections. Each point is the average of four values. The error bars correspond to the standard error of the mean.

DISCUSSION

In this paper we show that an enhancement of the survival of UV-irradiated SV40 is obtained in CV-1P monkey kidney cells after pretreatment with carcinogens, including aflatoxin B_1 metabolites, AAAF, MMS, and EMS. The general conditions for this carcinogen-induced reactivation are similar to those observed after UV irradiation of the cells (Fig. 1). The inducing dose is low compared to the doses necessary to produce other measurable effects such as DNA repair or cell toxicity. The delayed reactivation observed by increasing the time lag between carcinogen treatment and virus infection shows the same time dependence for UV irradiation and aflatoxin B_1 treatment (Fig. 3).

Although the mechanism of UV- or x-ray-reactivation is not understood in mammalian cells, this phenomenon appears different from host-cell reactivation or multiplicity reactivation. For example, UV-irradiated SV40 (27), human adenovirus 2 (28), or herpes simplex virus (29) have a much lower survival in xeroderma pigmentosum cells compared to that in normal fibroblasts. These differences are attributed to the excision repair deficiency of xeroderma pigmentosum cells. Lytle *et al.*

Table 1. Effect of a cycloheximide pretreatment of CV-1P host cells on the survival of UV-irradiated SV40

Cycloheximide treatment,* hr	Reactivation factor	Inhibition of host-cell DNA synthesis, %†	DNA repair replication, cpm/μg DNA†
0	1	0	15
3	0.95	0	15
24	1.7 ± 0.4	44	7
48	1.8	58	6
72	7.8 ± 0.9	87	4

* After treatment of the host cell with cycloheximide at 10 μg/ml for the periods of time indicated, the compound was removed. Then either semiconservative DNA replication and DNA repair replication were measured over a period of 18 hr or infection with UV-irradiated SV40 was carried out. Each number represents the average of two to four experiments.

† The errors involved in these measurements are roughly 10% (16).

(30) showed that UV irradiation of xeroderma pigmentosum cells enhances the survival of UV-irradiated herpes simplex viruses when the infection occurs 4 days after irradiation. The reactivation is similar to that which occurs with normal human fibroblasts, suggesting that the UV-reactivation phenomenon is different from host-cell reactivation (15). Multiplicity reactivation has been reported for UV-irradiated human adenovirus 12 and SV40 during infection in nontreated cells and was detected by measuring virus survival at different dilutions of the infecting suspensions (31). Using this dilution technique, we were unable to detect any multiplicity reactivation after carcinogen treatment (data not shown). Thus, our enhanced SV40 survival cannot be explained in part by an enhanced multiplicity reactivation.

The enhancement of survival of UV-irradiated SV40 after carcinogen treatment is comparable to the reactivation of UV-irradiated phage λ infecting UV-irradiated or aflatoxin B_1-treated *Escherichia coli* (19). These treatments induce in bacteria a group of processes (termed "SOS functions") such as lysogenic induction, cell filamentation, and mutagenesis (6, 9, 10, 19). By analogy, we hypothesize that treatment of mammalian cells with UV light or carcinogens induces a type of response similar to the SOS functions induced in *E. coli*. The carcinogen-induced reactivation of UV-irradiated SV40 could be the expression of some of these induced SOS functions in mammalian cells. Some other responses of mammalian cells to carcinogen treatment may belong to the same group of SOS functions. For example, low doses of carcinogens increase the frequencies of cell transformation induced by SV40, polyoma, or adenoviruses (32) or induce infectious viruses from transformed cell lines (33). Furthermore, inhibitors of protein synthesis, such as cycloheximide, have also been shown to induce infectious virus from SV40-transformed cells (34).

The importance of the concept of an induced virus reactivation comes from the fact that in bacteria the enhancement of the survival of UV-irradiated phage λ is accompanied by a high rate of mutation. The SOS repair that occurs during reactivation is highly mutagenic, perhaps due to the inhibition of the "proofreading" activity of one of the DNA polymerases (cf. ref. 6). However, mammalian DNA polymerases have not been shown to contain proofreading activity. Nevertheless, some induced protein could more directly alter the fidelity of replication. Noy and Weissbach (35) showed that treatment of HeLa cells with cycloheximide strongly inhibits the three known DNA polymerases but induces a new α polymerase, which represents more than 50% of total DNA polymerase activity 24 hr after cycloheximide treatment. Although nothing is known about the template requirement of this enzyme, it is tempting to hypothesize that this new activity could be responsible for the enhanced survival of UV-irradiated SV40 in our experiments.

D'Ambrosio and Setlow (22) reported an enhancement of postreplicative repair in Chinese hamster cells conditioned by UV radiation or by AAAF treatment. The effective doses were similar to those that induce SV40 reactivation. Induction of this enhancement was inhibited by cycloheximide and thus is thought to require *de novo* protein synthesis (22). Similarly, Lytle has reported that UV-reactivation of herpes virus is inhibited by cycloheximide (15). Our experiments with cycloheximide combined with UV irradiation of the host cells (not shown) do not indicate any inhibition of virus reactivation, perhaps because in our protocol we removed the drug before SV40 infection. Therefore, protein synthesis had resumed a long time before the onset of SV40 DNA replication, which starts between 14 and 18 hr after infection, compared to 4 hr for herpes virus (36). From these data we conclude that UV irradition or carcinogen treatment of eukaryotic cells could induce a new recovery pathway that permits DNA replication to bypass lesions on viral DNA leading to higher survival of UV-irradiated SV40. Such a recovery mode that does not remove damage from parental DNA would be expected to be highly mutagenic.

The use of various mutants and various chemicals in bacteria has led to the conclusion that disruptions of DNA metabolism (such as inhibition of DNA synthesis, degradation of DNA, and/or structural aberrations in DNA) are responsible for the induction of the SOS functions (23, 37). In our experiments, all of the agents that enhance the survival of UV-irradiated SV40 also block DNA synthesis in the host cell; the amount of SV40 reactivation appears to be correlated inversely with the rate of recovery from inhibition of host-cell DNA synthesis during the early phase of SV40 infection (Table 1). MMS and EMS, which do not inhibit DNA synthesis in bacteria (38, 39), do not induce the SOS functions in bacteria (40). However, these compounds do inhibit DNA synthesis in eukaryotic cells (unpublished results) and *do* induce the virus-reactivation response. In bacteria some chemicals, such as 5-fluorouracil or trimethroprim, induced SOS functions, inhibiting DNA replication without producing any detectable primary lesions in DNA (41). In like manner some compounds, such as hydroxyurea or cycloheximide, induce SV40 reactivation while not resulting in detectable DNA repair activity in the host cells. Thus, for both bacteria and mammalian systems the inducing agent need not produce repairable damage in the DNA.

If the phenomenon described does indeed represent a new inducible recovery pathway in mammalian cells, then there are several very broad and important questions that deserve study. First, is this induced process error-prone? Second, does the induction of this pathway enhance or reduce the probability of carcinogenesis? Finally, does malignant transformation result from the presence of the lesion in the DNA or is it a consequence of the error-prone bypass of that lesion?

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