Vol. 88, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1979 Pages 688-695

INACTIVATION OF SV40 REPLICATION BY DERIVATIVES OF BENZO[a]PYRENE

George T. Chang*†, Ronald G. Harvey††, Wen-Tah Hsu*†, and Samuel B. Weiss*†

*The Franklin McLean Memorial Research Institute,
†The Departments of Biochemistry and Microbiology, and
††The Ben May Laboratory for Cancer Research
The University of Chicago
Chicago, Illinois 60637

Received April 17,1979

SUMMARY

When African green monkey kidney cell lines, infected with simian virus 40, were exposed to benzo[a]pyrene-7,8-dihydrodiol or anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide, inhibition of progeny virus formation was observed. Alkylation of SV40 DNA with anti-BPDE inhibits the infectivity of this viral DNA; however, the inactivation does not follow a single-hit mechanism. Studies on [^{3}H]thymidine incorporation indicate that SV40 DNA synthesis is markedly impaired for the first 12 hours following BPDE treatment; 24 to 36 hours later, however, SV40 DNA synthesis is almost normal. These data suggest that the inhibition of SV40 DNA synthesis by BP derivatives is reversible and that the observed reduction in viral titer requires some other explanation.

Reports from this laboratory and from others have shown that carcinogenic polycyclic aromatic hydrocarbons (PAH) inhibit the replication of both DNA- and RNA-containing bacterial viruses (1-4). Studies with trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE) indicate that this derivative of benzo[a]pyrene (BP) effectively alkylates ØX174 DNA and MS2 RNA, that it blocks replication of both viral genomes, and that a single alkylation event suffices for total inhibition of the capacity of either ØX DNA or MS2 RNA to induce new virus formation in Escherichia coli (3, 5, 6).

Since the phage nucleic acids used in the above studies are single-stranded, it seemed of interest to determine whether viruses containing double-stranded genomes would respond in a similar manner to PAH exposure. We chose to examine the effect of BP derivatives on the replication of simian virus 40 (SV40), because this virus contains infectious duplex DNA of known

0006-291X/79/100688-08$01.00/0

sequence (7), is of a size comparable to that of the small bacterial viruses, and replicates in animal rather than bacterial cell lines.

MATERIAL AND METHODS

Cell Growth, SV40 Preparation and Titer. Continuous African green monkey kidney cell lines (BSC-1 and CV-1) were propagated in modified Eagle medium (MEM) (Grand Island Biological Co.) containing 10% heat-inactivated fetal calf serum, 0.1% $NaHCO_3$, and 200 µg of streptomycin sulfate per ml. Confluent monolayer cultures were infected with SV40 (Strain 776) at a multiplicity of infection (MOI) of 0.001 to 0.1 plaque-forming units (PFU) per cell in 10 ml of MEM containing 2% fetal calf serum. After about 1-2 weeks, the cells were collected, frozen and thawed repeatedly, sonicated, and subjected to low-speed centrifugation (5000 x g). The collected supernatant was used as the virus stock. Cell lines and SV40 were obtained from Dr. K. Subramanian, the University of Illinois Medical Center, Chicago, Ill. SV40 was titered on monolayers of CV-1 cells according to the plaque assay procedure of Tevethia and Tevethia (8), modified as follows. After infection (30 minutes with SV40 DNA or 2 hours with intact virus), 9 ml of MEM containing 2% fetal calf serum, 0.9% agar, 0.03% glutamine, 10% tryptose phosphate broth, 0.1% $NaHCO_3$, and antibiotics (100 units penicillin, 200 µg streptomycin, and 20-50 units mycostatin per ml of medium) were supplied to each plate. Nine days after infection, MEM (5 ml) containing neutral red (50 µg/ml) was overlaid in each plate. Infection and incubation were carried out at 37° in an atmosphere of 5% CO_2. Plaques were counted 17 days after infection. Plaque assays for SV40 DNA were carried out by infection of CV-1 cell monolayers with TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing viral DNA and 500 µg/ml of DEAE Dextran (Pharmacia, Sweden).

SV40 DNA Preparations. SV40 DNA was isolated from virus-infected cells by the procedure of Hirt (9). Hirt supernatants were extracted twice with phenol (equilibrated with 100 mM Tris, pH 7.9, 10 mM EDTA), and the DNA was precipitated with ethanol. Following centrifugation, the precipitate was dissolved in a buffered solution containing 100 mM NaCl, 2 mM EDTA, 20 mM Tris, pH 8.5, and was banded in CsCl-ethidium bromide. SV40 DNA corresponding to form 1 was collected and dialyzed against the above buffer, and its purity was determined by electrophoretic migration on agarose gels. The DNA concentration was estimated on the basis that 50 µg/ml give an absorbance of 1 at 260 nm in a 1 cm light path.

Treatment of Cells and DNA with Anti-BPDE. Anti-BPDE and BP-7,8-dihydrodiol (BPD) were synthesized according to published methods (10a, 11, 12). [^{3}H]Anti-BPDE (specific activity 344 cpm/ng) was synthesized under NCI Contract CP-033387 by the Midwest Research Institute, Kansas City, Mo., and further purified by Dr. Shen Yang, Uniformed Services University of the Health Sciences, Bethesda, Md. Stock solutions of anti-BPDE were made up in dimethylformamide (DMF) and added to infected confluent cell monolayers at 37° at the concentrations and times indicated in the text. The DMF concentration in the cell culture media did not exceed 0.14%. Infected control cells without anti-BPDE also contained DMF. The alkylation of SV40 DNA with [^{3}H]anti-BPDE and the determination of the molar ratio of BPDE bound to DNA were as previously described (5).

Determination of SV40 DNA Synthesis. Radioactive [^{3}H]thymidine (5-6.7 Ci/mM, Amersham and New England Nuclear Corporation) was added to confluent monolayers of infected CV-1 cells at an isotope concentration of

10-25 μCi/ml of medium. At the end of the labeling period, the medium was removed, and the monolayers were washed twice with 5 ml of cold phosphate-buffered saline. Viral and cellular DNAs were separated from the collected cells by the procedure of Hirt (9). The Hirt supernatant, which contained the viral DNA, was assayed for acid-precipitable counts either directly or precipitated with ethanol, resuspended in buffered-saline, and subjected to sucrose gradient centrifugation at 38,000 rpm in a Spinco SW50 rotor for 4 hours at 4°. The fractions collected were counted in a Nuclear Chicago Mark III scintillation counter. Linear 5-20% sucrose gradients (5.2 ml) were either neutral (1M NaCl, 10 mM Tris, pH 7.8, 5 mM EDTA) or alkaline (0.3N NaOH, 0.7M NaCl).

RESULTS AND DISCUSSION

Although the precise mechanism by which PAH induces mutagenesis and carcinogenesis is still unclear, it is generally believed that these biological responses derive from the metabolic activation of PAH to reactive metabolites which bind covalently to cellular macromolecules (10). For benzo[a]pyrene, anti-BPDE has been implicated as the active metabolite which complexes with DNA (10). Table 1 shows that, when SV40-infected cells are exposed to BPD or anti-BPDE, a significant reduction occurs in the titer of newly formed virus. Anti-BPDE is about 10 to 20 times more inhibitory than BPD.

Table 1. Effect of Benzo[a]pyrene Derivatives on SV40 Production

Compound	Hydrocarbon concentration μg/ml of medium	SV40 titer PFU x 10^{-5} per ml of lysate	SV40 titer % Inhibition
None	0	99	0
BPD	1	56	44
	2	26	74
BPDE	0.05	59	41
	0.10	44	56

Confluent CV-1 monolayer cultures were infected with SV40 at a MOI of 5. Two hours after infection, 7,8-BPD and anti-BPDE were added to the infected-cell media in the amounts shown above. Five days after infection, the cells were harvested, lysed, and titered for SV40 as described in Materials and Methods. The PFU values shown represent an average of four plates for each level of PAH examined from two separate experiments.

SV40 DNA-BPDE complexes were prepared at different molar ratios, free from unreacted hydrocarbon, as previously reported (5). Table 2 indicates that alkylation of SV40 DNA with the diolepoxide significantly lowers the specific infectivity of viral DNA. Although inactivation of viral DNA increases with increased binding of BPDE, complete inhibition of virus production was not observed even at a molar binding ratio of 10. These data suggest that the inactivation of SV40 DNA by BPDE in CV-1 host cells does not follow a single-hit mechanism and differs from that observed with infectious single-stranded nucleic acids of phage origin (3, 5, 6).

To determine whether SV40 DNA synthesis is affected by BPDE, we pulsed infected cells with [^{3}H]thymidine in the presence or absence of the diolepoxide. We found that BPDE reduces the incorporation of labeled substrate into newly synthesized viral DNA, but that the extent of reduction is dependent upon the amount of diolepoxide present and on the time span between addition of BPDE and addition of [^{3}H]thymidine. Figure 1A illustrates the radioactive profiles obtained by neutral sucrose gradient centrifugation analysis of Hirt supernatants derived from infected cells pulsed with labeled thymidine 26 hours after BPDE treatment. Most of the ^{3}H-labeled radioactivity sedimented identically with SV40 [^{14}C]DNA used as marker (arrow) and was acid-precipitable. The results indicate that the levels of viral DNA synthesis in infected cells exposed to 0.1 μg and 0.2 μg of BPDE per ml of medium for 26 hours were approximately 75% and 25%, respectively, of that for control infected cells. When infected cells were pulsed with [^{3}H]thymidine 2 hours after BPDE treatment (0.1 μg per ml of medium), the labeled viral DNA synthesized was only 15% of that for untreated infected cells (Figure 1B). Similar results were observed when the pulse-labeled DNA was analyzed under alkaline centrifugation conditions.

The time-dependent recovery of viral DNA synthesis from PAH inhibition was examined more closely. CV-1 cells were infected with SV40 virus, and 26 hours after infection half of the cells were treated with BPDE. One hour

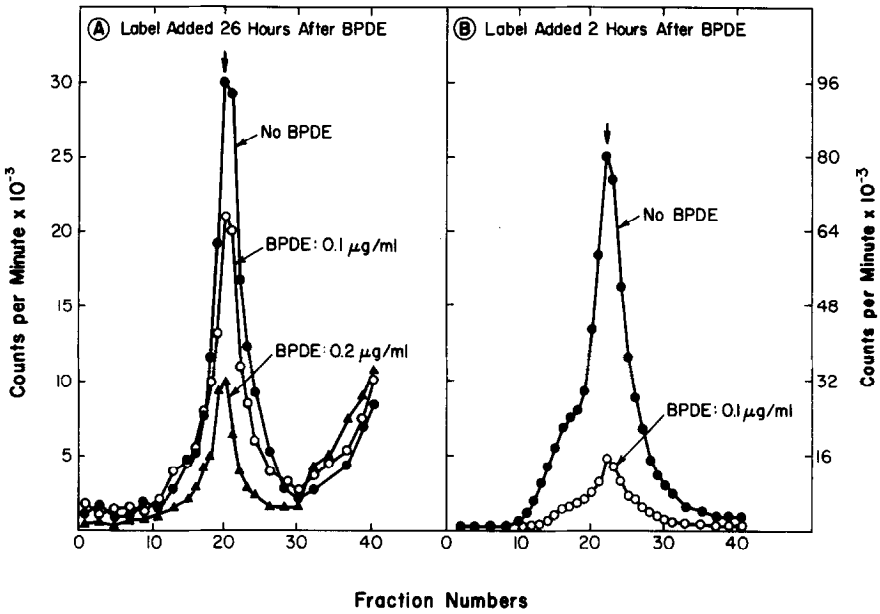

Figure 1. Effect of BPDE on SV40 DNA synthesis in infected cells. (A) Confluent CV-1 cells were infected with SV40 (MOI=16) and, 2 hours later, were treated with a given concentration of anti-BPDE, as shown in the figure above, in 10 ml of MEM containing 2% fetal calf serum. Twenty-six hours after BPDE exposure, 0.25 ml of [^{3}H]thymidine (1 mCi/ml at 6.7 Ci/mmol) was supplied to each infected monolayer, and the cells were harvested 2 hours later after being washed twice with 5 ml of cold phosphate-buffered saline. Hirt supernatants were prepared (see Materials and Methods) and subjected to neutral sucrose gradient centrifugation. Radioactivity was determined by direct counting of each fraction. (B) The experimental conditions were the same as in (A) except for the following. Infected cells were treated with anti-BPDE 26 hours after infection and, 2 hours later, were pulsed with 0.1 ml of [^{3}H]thymidine for 40 minutes. Prior to analysis by gradient centrifugation, two volumes of ethanol were added to the Hirt supernatants, and the precipitate which formed after standing at -20° overnight was collected and resuspended in buffered saline (100 mM NaCl, 2 mM EDTA, 20 mM Tris, pH 8.5).

later, [^{3}H]thymidine was added to the culture media of both the treated and untreated infected cells. Figure 2A compares the total acid-precipitable radioactivity found in the Hirt supernatants of both types of cells at specific time intervals following diolepoxide treatment. Figure 2B shows the percent inhibition of SV40 DNA synthesis by BPDE at the same time intervals. The data indicate that label incorporation into viral DNA is suppressed by about 70% for the first 12 hours after exposure to diolepoxide. Within 24 hours after BPDE treatment, SV40 DNA synthesis has largely recovered (27%

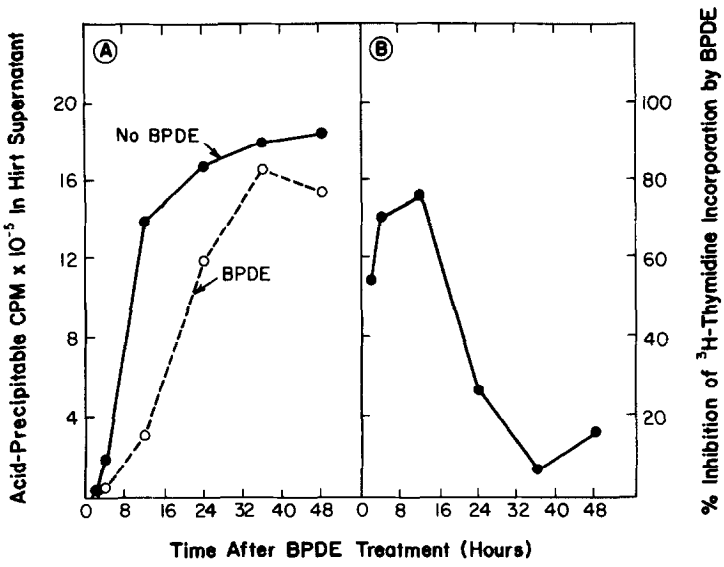

Figure 2. Time course of SV40 DNA synthesis following BPDE treatment. (A) Confluent CV-1 cells were infected with SV40 (MOI=15); 26 hours later, BPDE was added (as shown), and 60 minutes after BPDE, 0.1 ml of [^{3}H]thymidine was supplied to each culture as described in Figure 1A. At the times after BPDE treatment indicated in the figure above, cells were washed, Hirt supernatants were prepared as described in Figure 1B, and acid-insoluble counts were determined by precipitation with trichloroacetic acid. (B) The percent inhibition shown for [^{3}H]thymidine incorporation was derived from the difference between the acid-insoluble counts found for the untreated and BPDE-treated infected cells at the times shown in (A).

inhibition), and after 36 hours, synthesis is almost at the same level as that of control infected cells (10% inhibition).

The capacity of carcinogenic hydrocarbons to inhibit the replication of animal and bacterial viruses was initially demonstrated by DeMaeyer and DeMaeyer-Guignard (13), and by Hsu, Moohr, and Weiss (1). More recently, diol and diolepoxide derivatives of BP were shown to inactivate infectious ØX174 DNA, QB and MS2 RNA (4-6), and adenovirus 5 (14a). The inhibition of SV40 production by reactive BP metabolites reported here is consistent with the observations of the above investigators.

Our studies, as well as others, suggest that reactive PAH derivatives inhibit virus production by binding to viral DNA and blocking its replication. In infected CV-1 cells, SV40 DNA synthesis is markedly impaired shortly after BPDE treatment, but 24 to 36 hours later viral DNA synthesis appears to be

Table 2. Effect of SV40 DNA Alkylation with BPDE on Infectivity

Moles of BPDE bound per mole of SV40 DNA	SV40 titer	
	PFU/ng DNA	% Inhibition
0	230	0
4.6	90	59
9.1	60	74
10.1	57	75

Alkylation of DNA with various amounts of [^{3}H]anti-BPDE was carried out as described previously (5). The binding reaction mixture (0.1 ml) contained 5 µg of SV40 DNA and different amounts of [^{3}H]anti-BPDE. After 10 minutes incubation at 25°, DNA was precipitated with ethanol, washed with acetone, air-dried, and dissolved in Tris-EDTA. Molar ratios were determined from the amount of label and A_{260} material in the resuspended DNA solution following precipitation and washing. Confluent CV-1 cells were exposed to untreated and modified SV40 DNA (0.8-1.6 ng), and the plaques formed after 17 days were counted. The PFU values shown represent an average of four plates for each level of modified DNA from two separate experiments.

almost normal. The resumption of near-normal levels of SV40 DNA synthesis can be explained by "repair" of PAH-alkylated sites in viral DNA (14) as well as other mechanisms, i.e., by-pass replication. This could also explain why single-hit inactivation was not found for CV-1 cells infected with BPDE-bound SV40 DNA (Table 2). If the inhibition of viral DNA synthesis is only temporary following BPDE treatment, how does one explain the significant reduction in viral titer by this same BP derivative? It is possible that PAH metabolites affect multiple steps in the viral replicative process; it is also possible that the viral DNA synthesized in the presence of BPDE is partially defective and therefore less infectious. These possibilities remain to be examined. In addition to providing information on the mechanism of action of BP metabolites on SV40 replication, the viral system described here may be suitable as a convenient and rapid assay for the detection of potentially toxic PAH agents in an animal cell environment.

ACKNOWLEDGMENTS

The Franklin McLean Memorial Research Institute is operated by The University of Chicago for the United States Department of Energy under Contract EY-76-C-02-0069. This study was partly supported by a grant from the American Cancer Society (BC-132C) and by funds from the Environmental Protection Agency, Subagreement 77BAN.

REFERENCES

1. Hsu, W.-T., Moohr, J. W., and Weiss, S. B. (1965) Proc. Natl. Acad. Sci. U.S.A. 53, 517-524.
2. Hsu, W.-T., Moohr, J. W., Tsai, A. Y. M., and Weiss, S. B. (1966) Proc. Natl. Acad. Sci. U.S.A. 55, 1475-1482.
3. Dipple, A., and Shooter, K. V. (1974) Biochim. Biophys. Acta 374, 392-399.
4. Hsu, W.-T., Harvey, R. G., Lin, E. J. S., and Weiss, S. B. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1378-1382.
5. Hsu, W.-T., Lin, E. J. S., Harvey, R. G., and Weiss, S. B. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3335-3339.
6. Sagher, D., Harvey, R. G., Hsu, W.-T., and Weiss, S. B. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 620-624.
7. Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L., and Weissman, S. M. (1978) Science 200, 494-502.
8. Tevethia, M. J., and Tevethia, S. S. (1976) Virology 69, 474-489.
9. Hirt, B. (1967) J. Mol. Biol. 26, 365-369.
10. Reviews: (a) Harvey, R. G., and Fu, P. P. (1978) Polycyclic Hydrocarbons and Cancer: Environment, Chemistry and Metabolism, Vol. 1, Gelboin, H. V., and Ts'o, P. O. P., Eds., pp. 133-165, Academic Press, New York; (b) Yang, S. K., Deutsch, J., and Gelboin, H. V., Ibid., pp. 205-231; (c) Miller, E. C. (1978) Cancer Res. 38, 1479-1496.
11. Beland, F. A., and Harvey, R. G. (1976) J. Chem. Soc. Chem. Commun., 84-85.
12. Fu, P. P., and Harvey, R. G. (1977) Tetrahedron Lett. 2059-2062.
13. DeMaeyer, E., and DeMaeyer-Guignard, J. (1964) Science 146, 650-651.
14. (a) Day III, R. S., Scudiero, D., and Dimattina, M. (1978) Polycyclic Hydrocarbons and Cancer: Molecular and Cell Biology, Vol. 2, Gelboin, H. V., and Ts'o, P. O. P., Eds., pp. 213-219, Academic Press, New York; (b) Strauss, B., Tatsumi, K., Karran, P., Higgins, N. S., Ben-Asher, E., Altamirano-Dimas, M., Rosenblatt, L., and Bose, K., Ibid., pp. 177-201; (c) Curutti, P., Shinohara, K., Ide, M.-L., and Remsen, J., Ibid., pp. 203-212; (d) McCormick, J. J., and Maher, V. M., Ibid., pp. 221-232.