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Influence of Herpes Simplex Virus Infection on Benzo(a)pyrene Metabolism in Monkey Kidney Cells

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Polycyclic aromatic hydrocarbons (PAH) have been detected in some petroleum products, coal tar, cigarette smoke, engine exhaust, atmospheric particles and several marine environments (Dunn and Fee 1979; Howard and Fazio 1980; Iosifidou et al. 1982). These compounds occur naturally or are generated by incomplete combustion (NAS 1972). Several PAH, including benzo(a)pyrene (BP), are potent carcinogens of animals and man (Levin 1978). Benzo(a)-pyrene has been detected in human tissues (Tomingas et al. 1976; Obana et al. 1981) and is actively metabolized to carcinogenic metabolites by human and animal cells (Levin 1978; Buening 1978). BP metabolites bind covalently to DNA, RNA, and proteins in the cell (Weinstein et al. 1976; Koreeda et al. 1978; Murakami et al. 1980).

Several recent investigations have shown that chemical promoters, environmental chemicals and physical agents interact synergistically with animal DNA viruses to enhance oncogenic transformation of mammalian cells (Howett et al. 1979; Casto et al. 1974; Pearson and Beneke 1977) and it has been suggested that intracellular interactions of multiple agents may be highly significant as inducers of mutations and tumors in nature (DePaolo and Casto 1978).

Current research in our laboratory is designed to investigate the intracellular interactions of BP with oncogenic DNA viruses of animals and humans (Hall and Stoming 1980). In this study, our purpose was to determine whether BP is metabolized in herpes simplex virus type 2 (HSV-2) infected cells and whether HSV-2 infection affects intracellular levels of the aryl hydrocarbon hydroxylase system necessary for BP metabolism.

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MATERIALS AND METHODS

Virus. Stock preparations of HSV-2 (strain E-304) were grown in CV-1 cells, a continuous line of African green monkey kidney cells. The titer of these preparations ranged from 1.0×10^6 to 3.15×10^7 plaque forming units/ml when assayed on CV-1 monolayers.

CV-1 cells were grown as monolayers in 100 mm petri dishes in 10.0 ml of Eagle medium containing 10% newborn calf serum. Cells were subcultured once a week by trypsinization with 0.25% trypsin and were used to seed new dishes at a concentration of approximately 5×10^{-5} cells/dish in fresh medium.

[^{3}H]BP metabolism was determined with live cells in culture. Labeled BP (10 uCi/ml) was added to mock infected or infected cells as freshly prepared solutions in DMSO. The final BP concentration was 10^{-5} M. Controls consisted of the selected concentrations of BP in petri dishes containing medium but no cells. After 10 hrs incubation at 37°C (10 hrs post infection), the medium (5.0 ml) and cells from triplicate 100 mm petri dishes were removed by scraping the cells into the medium with a rubber policeman and extracted 3 x with equal volumes of ethyl acetate. Ethyl acetate extracts were pooled, dried over anhydrous sodium sulfate, evaporated to dryness and the residue (metabolites) dissolved in 100 ul methanol for high pressure liquid chromatographic analysis (Selkirk 1974) (see HPLC methods). Estimates of the percentage of [^{3}H]benzo(a)pyrene metabolites remaining in the aqueous phase and those extracted into the organic phase were made by treating three petri dishes with 1.0 ml each of 1% SDS solution following removal of media at 10 hrs post infection and exposure to 10^{-5} M BP with radioactive label (10 uCi/ml). The SDS solution and cells were added to the original media and a 0.5 ml sample was removed, evaporated to dryness in a scintillation vial and counted by standard liquid scintillation techniques. A 1.0 ml sample of the remaining media and cells was removed and added to 9.0 ml of a 2:1 mixture of $ChCl_3$/MeOH. Separate 0.5 ml samples of the organic and aqueous phases of this extraction were removed, evaporated to dryness in scintillation vials and counted by standard techniques.

High pressure liquid chromatographic separation of BP metabolites was conducted on a Waters Associates Model 440 liquid chromatograph equipped with a 254 nm detector, model 6000-A solvent delivery system,

model U6K universal injection system, model 660 solvent programmer and a uBondapak C_{18} column (4 mm I.D. x 30 cm). The column was eluted with a linear gradient of methanol and water (60% methanol/water to 80% methanol/water over 40 min followed by 80% methanol/water to 100% methanol over 10 minutes). The flow rate was maintained at 1.0 ml/min and fractions were collected at 30 second intervals. The effluent was monitored by a 254 nm ultraviolet detector. Benzo(a)pyrene metabolite standards were added to each residue to assist in identification during HPLC analysis. Radioactivity was determined by pipeting a 50 ul aliquot of each sample into TT-21 scintillation fluid (Yorktown Research) and counting in a Beckman LS-100C Counter.

Hydroxylase (AHH) activity was determined by the method of Nebert and Gelboin, 1968. Samples included mock infected cells, infected cells, BP treated cells, BP treated cells with HSV-2 infection and growth medium alone. Assays were performed at 2, 4, 6, 8, 10, 12, 24, and 48 hrs after infection and/or BP treatment. All assays were performed on an Aminco-Bowman spectrophotofluorometer with activation at 396 mu and fluorescence at 522 mu.

The extent of binding of [^{3}H]BP metabolites to viral DNA was determined by extraction of genome DNA from viruses replicated in the presence of [H]BP. Intact HSV-2 DNA was prepared according to Skare et al. (1975) with the following exceptions. Virus pellets were suspended in 0.1 ml of 0.05 M Tris-HCl, pH 8.0 and 0.01 M EDTA then treated overnight with 0.2 ml 0.2 M EDTA, and 0.1 ml of 10% sarkosyl at 37^{o}C. The intact virus DNA was dialyzed overnight against 0.1 M Tris-HCl pH 7.5 then precipitated with six volumes of ethanol. The ethanol precipitate was pelleted at 20,000 rpm in a Beckman SW41 rotor for 20 min. The pellet was resuspended in 0.5 ml of 0.002 M Tris-HCl, pH 7.5, and 0.002 M EDTA and the DNA content determined by absorption at 260/280 nm on a Gilford spectrophotometer. The entire DNA sample was dried and counted in a Beckman LS-100C liquid scintillation counter.

RESULTS AND DISCUSSION

The initial separation of benzo(a)pyrene metabolites was based on their extractability from an aqueous solvent, growth medium, into the organic solvent ethyl acetate. Approximately 70% of the total radioactivity added could be extracted into the organic phase with samples from either infected or uninfected cell cultures. Furthermore, approxi-

mately Ø.5% of the total input radioactivity could be detected in the aqueous phase of samples from infected or uninfected cells. Analyses of the BP metabolites extracted into ethyl acetate from CV-1 cells and their spent culture medium were performed by high pressure liquid chromatography.

Figure 1 compares the metabolic profiles obtained with a synthetic mixture of metabolite standards (top), cultures of mock infected CV-1 cells (middle), and cultures of HSV-2 infected CV-1 cells (bottom). Although a synthetic metabolite mixture was added to all test metabolic extracts, they have been charted separately for clarity.

The several quinones produced by metabolism of BP are poorly resolved by the HPLC techniques utilized in this investigation and the same is true for phenols. Therefore, since individual metabolites within these two groups cannot be identified with certainty, the peaks of metabolites are identified only as diols, quinones, and phenols. Several radioactive peaks (Figure 1) emerging from the column early are within the fractions usually identified as BP diols, (Selkirk et al. 1974) with peak 7 in the mock infected profile and peak 5 in the HSV-2 infected profile chromatographing with the 7,8-diol standard. Radioactive peaks 8,9 and 1Ø in the mock infected samples and 6,7 and 8 in the HSV-2 infected samples chromatograph within the region of quinones. Peaks 13 and 14 from the mock infected and probably peaks 11 and 12 from the virus infected samples chromatograph with the phenol metabolite standards 9-OH-BP and 3-OH-BP. Also evident from the HPLC profiles (Figure 1) is the greater amount of radioactivity in the metabolites from mock infected cells than HSV-2 infected cells. Quantitative summaries of BP metabolism, obtained by determination of the areas under peaks in Figure 1, (Table 1) reveal that the over 25% higher production of total metabolites by mock infected cells is primarily due to increased production of diols and quinones.

One explanation for the higher rates of BP metabolism produced by mock infected cells relative to HSV-2 infected cells is that HSV-2 infection is known to decrease cellular protein production (Sydiskis and Roizman, 1976). Thus, decreased production of the AHH could lower metabolism of BP in HSV-2 infected cells. However, throughout the range of assays utilized at various times after addition of BP to cells and/or after infection, no differences in AHH levels could be detected.

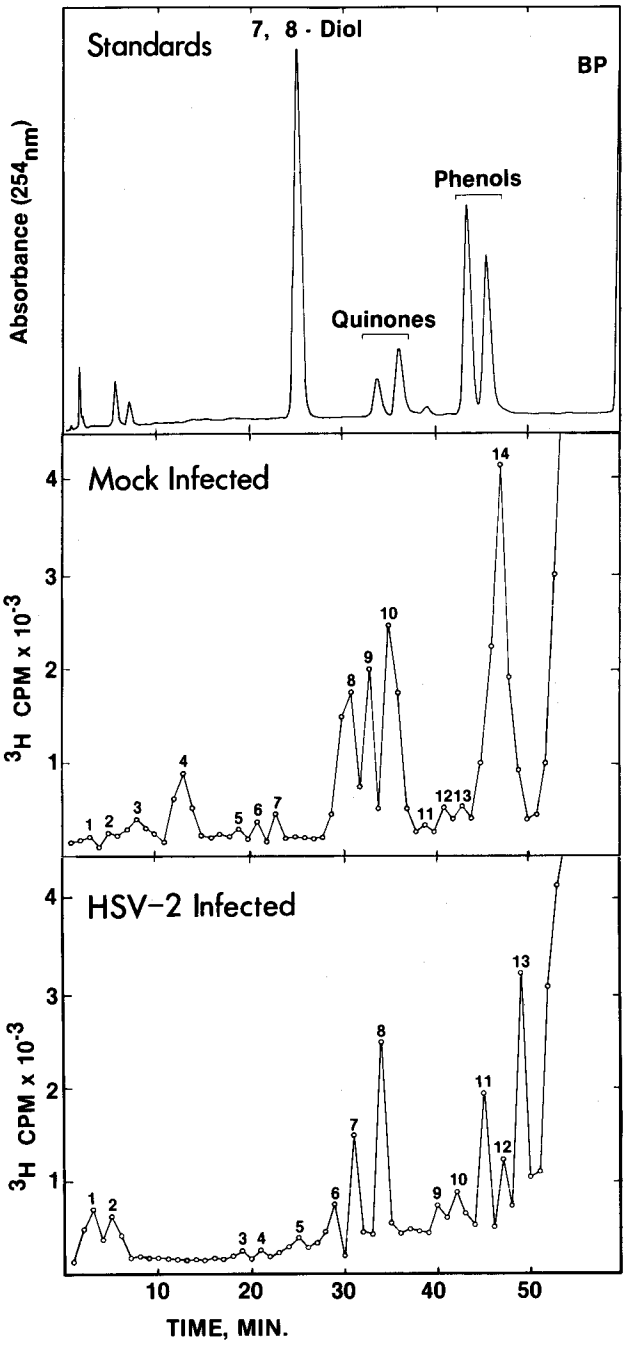

Figure 1. HPLC profiles of a synthetic mixture of BP and some of its metabolites (top), BP metabolites formed by mock infected cultures of CV-1 cells (middle) and BP metabolites formed by HSV-2 infected CV-1 cells (bottom).

Table 1. Quantitation of the metabolic rates of synthesis of the major metabolites of [^{3}H]BP in CV-1 cells.

Products	Metabolic Rates (n moles/10^7cells/hr)	
	Mock Infection	HSV-2 Infection
Diols	7.49×10^{-3}	3.5×10^{-3}
Quinones	1.83×10^{-2}	1.2×10^{-2}
Phenols	1.75×10^{-2}	1.6×10^{-2}

The data summarized in this table are derived from the metabolic profiles shown in figure 1.

[^{3}H]BP binding to intact HSV-2 DNA was determined in triplicate and averaged 78.7 DPM/mg or 42 BP molecules per HSV-2 genome.

The results of this study demonstrate that CV-1 cells infected with HSV-2 are capable of metabolizing the polycyclic hydrocarbon benzo(a)pyrene. Metabolism of BP by uninfected cells has been confirmed in previous work by Diamond (1971) with CV-1 and other primate cell lines and by Hall and Stoming, (1980). Several aspects of the HPLC profiles from both infected and uninfected cells are of particular interest. The peaks in the 7,8-diol region of the chromatogram are of interest since the 7-8 diol is an intermediate in the metabolic formation of the potent carcinogen 7,8-dihydroxybenzo(a)-pyrene-9,10-oxide (Thakker et al, 1976).

Also noteworthy is the markedly increased proportion of total metabolites represented by phenols in the metabolites from HSV-2 infected cells relative to that of uninfected cells. Although the 4 phenols known to be formed metabolically from BP are either noncarcinogenic or very weak carcinogens (Cohen et al, 1980), it has been proposed that most phenols are non-enzymatically derived from epoxides which are precursors to the carcinogenic and mutagenic BP metabolites (Thakker et al, 1976; Cohen et al, 1980).

The interaction between BP, its carcinogenic metabolites and HSV-2 has been a target of speculation for several years. The ability of the electrophilic BP metabolites to covalently bind to nucleophilic groups in proteins and nucleic acids is the probable method of interference by BP in viral and/or cellular metabolism (Hall and Stoming 1980; Cohen et al, 1980). The interaction of BP with DNA of HSV-2 could lead to a mutagenic effect on HSV-2 which

results in inhibition of its lytic replication and enhancement of its transforming potential. The findings of Duff (1971) exhibiting the increased transforming abilities of HSV-2 following inactivation by UV radiation lend support to this hypothesis.

An additional interaction between cells, virus and BP may involve an interference in cellular metabolism of BP by the infecting virus, influencing the production of carcinogenic metabolites and encouraging mutagenic events. Two aspects of our result support this possibility. First, as described above, phenols represent a higher proportion of total metabolites in extracts from HSV-2 infected cells than in extracts from uninfected cells. Second, metabolism of BP is reduced overall in HSV-2 infected cells. The probable explanation for decreased BP metabolism in HSV-2 infected cells is that host cell protein synthesis is suppressed soon after initiation of HSV-2 infection (Sydiskis and Roizman, 1976). Thus, inhibition of protein synthesis resulting in decreased enzymatic activity by the host cell could account for the observed reduction in BP metabolism in HSV-2 infected cells.

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