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Benzo[*a*]pyrene: Kinetics of *In Vitro* Bioactivation in Relation to Inhibition of Viral Interferon Induction

NICHOLAS HAHON[1,2] and JAMES A. BOOTH[1]

ABSTRACT

The kinetics of benzo[*a*]pyrene (BaP) bioactivation by rat liver S9 fraction was characterized on the basis of inhibition of influenza virus induction of interferon-α/β (IFN-α/β) in mammalian LLC-MK$_2$ cell cultures. Both viral IFN induction and production phases were sensitive to the adverse effects of bioactivated BaP. The integral role of S9 for BaP bioactivation and the resultant inhibition of viral IFN induction was substantiated by dose-response relationships, time-dependency of effects, and reversibility of adverse reactivity. When preceded by the analog, benzo[*e*]pyrene (BeP), the inhibitive action of bioactivated BaP on IFN induction was abrogated.

That the ability of exogenous IFN to confer antiviral cellular resistance was unaffected by bioactivated BaP indicates that neither requisite cellular protein nor enzyme syntheses were impaired. In cells pretreated with bioactivated BaP, influenza virus multiplication reached a level that was more than twofold higher than in normal cells which was a reflection of decreased IFN production. These findings further imply that neither virus inducer–cell interactions (attachment and penetration) nor requisite viral protein and RNA syntheses were affected appreciably. BaP was selectively cyto-antagonistic to critical inducer-processing phases of IFN induction. Of 32 different mammalian cell cultures tested for indigenous metabolizing enzyme-bioactivation of BaP, based on $\approx$50% resultant inhibition of IFN induction, only 37.5% were responsive.

INTRODUCTION

BENZO[*a*]PYRENE (BaP) is representative of a class of compounds, the polycyclic aromatic hydrocarbons (PAH), and has been the focus of increasing public health concern and research because it is a widespread environmental pollutant with demonstrable mutagenic/carcinogenic activity.[1-3] Numerous demonstrations of the inhibitory effect of known carcinogens, including BaP, on interferon-α/β (IFN-α/β) induction in mammalian cell cultures and the corresponding absence of adverse activity by noncarcinogenic analogs have been reported.[4-11] Recently compiled data in-

[1]National Institute for Occupational Safety and Health, Division of Respiratory Disease Studies, ALOSH, 944 Chestnut Ridge Road, Morgantown, WV 26505, and [2]Department of Pediatrics, West Virginia University School of Medicine, Morgantown, WV 26505.

dicate an excellent correlation between mutagenic/carcinogenic potential of chemicals and inhibition of IFN-α/β induction.[12] That systemic administration of BaP can significantly depress the whole-animal IFN response to viral stimulation, as evidenced by dose-response and time-dependency of effect relationships, further supports the concept that this depression may be an early and unique expression of immunotoxicity.[13] It seems plausible, in view of the many biologic response modification properties of IFNs, *i.e.*, antiviral, anticellular, immunomodulatory,[14] with the totality incomplete, that adverse effects by BaP on the IFN system may be a factor, albeit limited, in the multistep or multigenic stages of BaP carcinogenesis.

BaP, in common with most PAHs, is inactive and requires enzymatic activation by the mixed-function oxidases (cytochrome P450) to form either proximate and ultimate metabolites of varied biologic activity or detoxified products, depending on the pathways of bioactivation.[15] That bioactivation-dependent chemicals may be metabolized, when introduced with liver S9 fraction into mammalian cell cultures with resultant inhibition of IFN-α/β induction, was demonstrated with azo dyes and derivatives.[16] Based on studies using glutathione or β-naphthoflavone, which can interfere with the bioactivation of BaP by mouse embryo fibroblast cultures, it was suggested that BaP requires bioactivation to inhibit IFN induction.[6] This was established factually with the introduction of an exogenous metabolizing system (liver S9 fraction) into cell cultures for bioactivation of BaP. It has facilitated studies for discriminating and correlating mutagenic and IFN induction inhibitory activities of major BaP metabolites,[17] multicausal relationships between BaP–asbestos and coinhibition of IFN induction,[11] and related PAH-bioactivation studies.[18-20] Understanding the interactions between BaP and biologic defense mechanisms adds to our overall comprehension of PAH-associated carcinogenesis.

This report describes further studies to characterize BaP bioactivation kinetics in mammalian cell cultures in relation to inhibition of viral IFN induction as well as associated effects of BaP on the IFN system.

MATERIALS AND METHODS

Viruses: The Ao/PR/8/34 influenza and parainfluenza (Sendai) virus strains employed in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Stock virus pools of each strain were prepared from embryonated chicken eggs in the manner described previously.[21] Influenza and Sendai virus pools contained 5×10^7 and 1×10^9 cell-infecting units of virus per milliliter, respectively, when assayed by the immunofluorescent cell-counting procedure.[21]

Cell Cultures: Rhesus monkey kidney (LLC-MK_2) and human Chang conjunctival (clone 1-5c-4) cell lines obtained from ATCC were used for induction and assay of IFN, respectively. Cell lines were propagated in plastic tissue culture flasks (75 cm^2) with Eagle's minimum essential medium fortified with 100× essential vitamin mixture (10 ml/liter), 200 m*M* solution L-glutamine (10 ml/liter) to which was added sodium bicarbonate (2.2 gram/liter), and fetal bovine serum to 10%. Cells were maintained with the aforementioned medium containing only 0.5% fetal bovine serum. Additional human, primate, and rodent (primary, diploid, continuous) cell lines which were used in IFN induction experiments were obtained from either ATCC, Whittaker M.A. Bioproducts, Inc. (Walkersville, MD), or Flow Laboratories, Inc. (McLean, VA) and are specified accordingly.

Reagents: Stock preparations of each chemical (3.96 μmoles/ml), benzo[*a*]pyrene (BaP) and benzo[*e*]pyrene (BeP) (Sigma Chemical Co., St. Louis, MO), were dissolved in dimethyl sulfoxide (DMSO) and, subsequently, diluted in maintenance medium to the desired concentration. The highest final concentration of DMSO used in experimental tests was 1.0%, which was neither detrimental to cell viability nor viral IFN induction.

Liver homogenate (9,000 × *g*) supernatant fraction (S9) was prepared from the livers of male Wistar/Lewis rats (225 grams/rat) after induction with Aroclor 1254 (i.p. 500 mg/kg body wt) as described by Ames *et al.*[22] For experimental use, 0.5% suspension of S9 homogenate was prepared in maintenance medium and then passed through a 0.45-μm Nalge Filter Unit (Nalge Co., Rochester, NY) to obtain sterile preparations. The suspension, after filtration, contained 77.5 μg of protein/ml.[23]

Anti-human IFN-α and IFN-β sera were obtained from Nutritional Biochemicals (Cleveland, OH).

IFN Induction: Experiments usually were performed in duplicate or triplicate. Although the procedure varied, depending on experimental design, the effect of BaP on IFN induction generally was carried out as follows: BaP (0.039 μmoles) in a 10-ml volume of maintenance medium with or without 0.5% S9 was added to 75-cm² plastic flasks containing confluent LLC-MK$_2$ cell monolayers (~ 2 × 10^7 cells) which were then incubated at 35°C for 20 h. Residual medium was decanted and 2 ml of influenza virus, which had been inactivated by ultraviolet irradiation for 45 s at a distance of 76.2 mm and wavelength of 253.7 nm. was added onto cell monolayers that were then incubated at 35°C for 2 h. The multiplicity of infection (moi) was approximately 2.0. Inoculum was removed and 10 ml of maintenance medium was added to each flask. After incubation, at 35°C for 20 h, supernatant fluid was decanted and centrifuged at 100,000 × *g* for 1 h and dialyzed against HCl-KCl buffer (pH 2.0), at 4°C for 24 h. Dialysis was continued against two changes of PBS (pH 7.1), at 4°C for 24 h. Fluids were passed through 0.22-μm Millex filters GV (Millipore Corporation, Bedford, MA) to obtain sterile preparations. Samples were stored at −80°C until they were assayed for IFN activity. Preparations with antiviral activity possessed the biological and physical properties ascribed to viral IFNs.[24] Controls consisting of cell monolayers that were not treated with chemical solutions were handled exactly as described above. Viral induced IFN from cell cultures contained both IFN-α and IFN-β as determined by the procedure of using constant antiserum and varied IFN concentrations.[25]

IFN Assay: An immunofluorescent cell-counting assay that has been described previously[26] was used to determine the IFN potency of test samples. IFN yields from human and primate cell cultures were assayed on 1-5c-4 cells while IFN yields from mouse and rat cell cultures were assayed on L-929 and RFL-6 cells, respectively. IFN-treated cell monolayers were challenged with 10^4 cell-infecting units of Sendai virus, and infected cells were visualized by direct fluorescent antibody staining. The reciprocal of the IFN dilution that reduced the number of infected cells to 50% of the control served as the measure of IFN activity, *i.e.*, 50% infected cell-depressing dilution ($ICDD_{50}$). With this assay, 0.89 IFN unit corresponds to 1.0 unit of National Institute of Health reference standard HuIFN-β (G-023-902-527). A 50% or greater decrease of IFN induction, which exceeds 98% confidence limits of the assay, was considered significant.[27]

Virus Growth Determination: Influenza virus replication concomitant with IFN production was measured in both untreated and BaP-treated LLC-MK$_2$ cells maintained in 75-cm² plastic flasks. Following adsorption of virus to cells at 35°C for 2 h (moi = 1.0), cell monolayers were rinsed with PBS and incubated at 35°C with 10 ml of maintenance medium. At designated time intervals, from 0 to 48 h, flasks were removed and stored at −80°C. Thereafter, each flask was thawed (25°C) and frozen (−80°C) twice to disrupt cells and the fluid content of each flask was divided into aliquots. One aliquot was assayed for virus content and the other processed for IFN assessment in the described manner.

RESULTS

Preliminary considerations

An experimental prerequisite, the quantity of BaP compatible with maximal cell viability, was determined previously.[11] Nondividing LLC-MK$_2$ cells (2 × 10^7) in monolayers maintained >95%

viability when exposed to 0.039 μmoles BaP for 24 h. Rat liver S9 fraction used for enzymatic activation of BaP was tested for its effect on viral IFN induction. Results (Table 1) show that S9 concentrations of 0.5% or less had no adverse effect on viral induction of IFN, however, S9 concentrations >0.5% markedly inhibited the IFN induction process.

S9 fractions of liver homogenates prepared from rats or hamsters pretreated with different cytochrome enzyme-inducing reagents, Aroclor 1243, 1254, or 3-methylcholanthrene, and those prepared from normal animals were tested for bioactivation of BaP. All S9 preparations were effective and comparable in their ability to bioactivate BaP as evidenced by resultant inhibition of viral IFN induction which ranged from 54 to 69% (data not shown).

IFN production in LLC-MK_2 cell monolayers that were exposed for 20 h to BaP, with and without rat liver S9, and then to influenza virus inducer, was monitored periodically for 48 h. Cell cultures pretreated with BaP alone produced IFN in a manner similar to that of the medium control with regard to rate, peak time (20 h), and magnitude of IFN yield (Fig. 1). In cell cultures pretreated with BaP bioactivated by S9, the production rate was similar but the peak IFN level was attained 10 h earlier and the IFN yield was fivefold less than that of cells reated with BaP alone or medium control.

Dose-response relationships

Concentration-dependent interactions relative to bioactivation of BaP by rat liver S9 fraction and the magnitude of inhibition of viral IFN induction involved exposure of LLC-MK_2 cell monolayers to the reactants for 20 h prior to addition of viral inducer. There was a dose-response relationship between S9 concentrations ranging from 0.01 to 0.5% to bioactivate 0.039 μmole BaP and inhibition of IFN induction (Fig. 2). The above quantity of BaP was bioactivated by 0.16% S9 to inhibit IFN induction by 50%. A dose-response relationship was also noted between BaP ranging in quantity from 0.0018 to 0.039 μmole bioactivated by 0.5% S9 and inhibition of viral IFN induction (Fig. 3). Under these conditions of bioactivation, 0.025 μmole BaP depressed IFN induction by 50%.

Kinetics of BaP bioactivation and IFN induction inhibition

The time required to bioactivate BaP by S9 extracellularly into metabolic forms capable of inhibiting viral IFN induction was determined as follows; preheated (35°C) BaP (0.039 μmole) and 10 ml of maintenance medium with 0.5% S9 were mixed in a series of tubes and incubated at 35°C for

TABLE 1. INFLUENCE OF RAT LIVER S9 CONCENTRATION ON VIRAL INDUCTION OF IFN IN LLC-MK_2 CELL MONOLAYERS

S9 (%)	*Protein content (μg/ml)*	*IFN yield ($ICDD_{50}$/ml)*[a]	*IFN inhibition*[b] *(% ± SEM)*
10.0	1,550	40	72.5 ± 2.3
5.0	798	58	60.0 ± 1.4
1.0	163	82	43.5 ± 6.3
0.5	77.5	150	0.0 ± 2.4
0.1	15	145	0.0 ± 1.7
0.0 (Control)	0	145	0.0

[a]Reciprocal of 50% infected cell-depressing dilution.
[b]Reciprocal of $ICDD_{50}$/ml of IFN yield/control $ICDD_{50}$/ml) − 1.0 × 100 (n = 2).

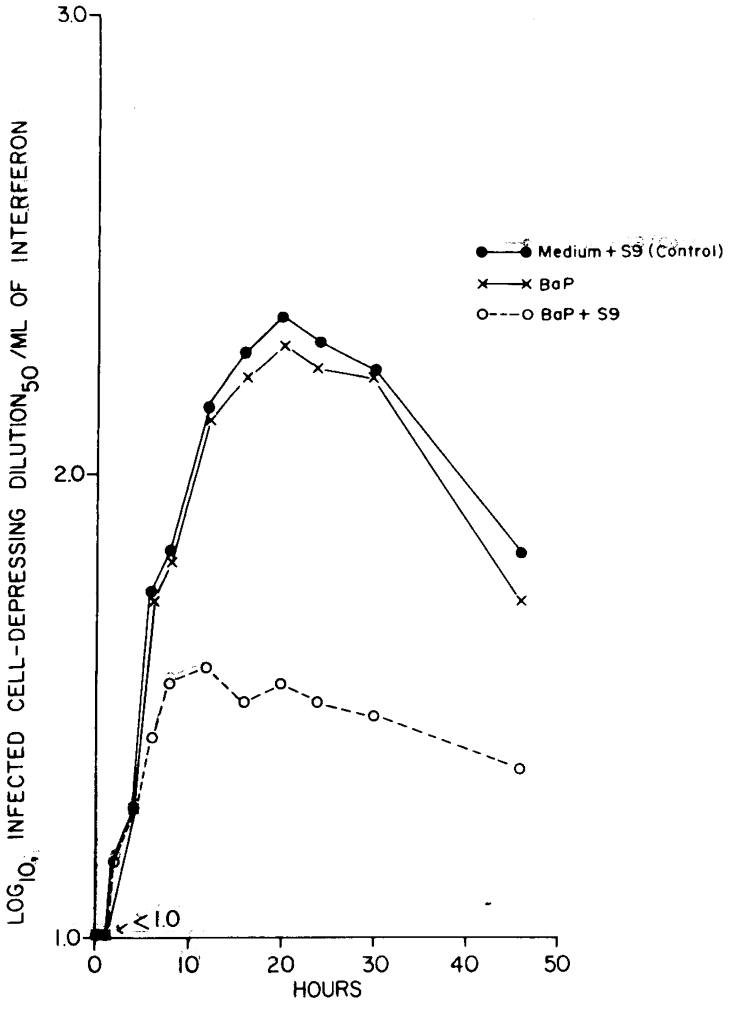

FIG. 1. IFN production induced by influenza virus in LLC-MK$_2$ cell monolayers (moi = 1.0) pretreated 20 h earlier to 0.039 μmole BaP without and with 0.5% rat liver S9 (mean of n = 3).

designated time periods. Reaction between BaP and S9 was terminated by heating time-related samples at 85°C for 5 min. Test samples and appropriate controls were then added to cell monolayers and incubated at 35°C for 20 h. Thereafter, viral induction of IFN was carried out as described earlier. Results (Table 2) show that maximal inhibition of IFN induction occurred when BaP interacted with S9 for 0.5 h in cell-free medium. Metabolites formed, whether proximate or ultimate, were biologically active. Succeeding time periods for bioactivation of BaP did not result in any further magnitude in depression of IFN induction by metabolites. To determine the optimal length of time required for bioactivation of BaP by S9 and concomitant interaction of metabolites with cells resulting in maximal inhibition of viral IFN induction, cell monolayers were exposed to BaP (0.039 μmole) in maintenance medium with 0.5% S9 for designated time periods. Cell cultures were rinsed, exposed to the viral IFN inducer for 2 h, and then incubated at 35°C for 20 h. Results (Fig. 4) show that the bioactivation of BaP, together with metabolite-cellular interaction, required from

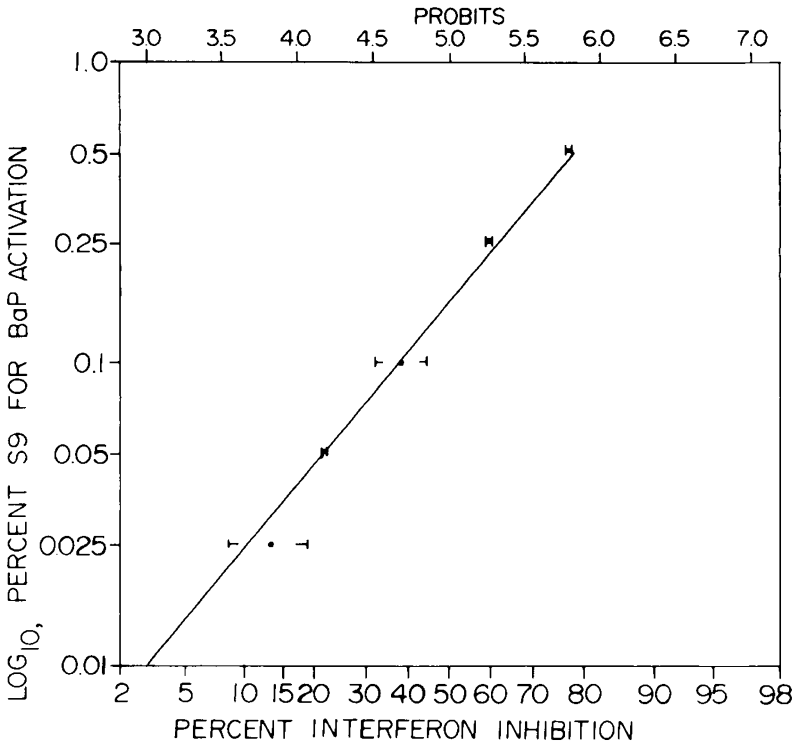

FIG. 2. Dose-response relationship between different concentrations of rat liver S9 used to bioactivate 0.039 μmole BaP and resultant inhibition of IFN induction by influenza virus in LLC-MK_2 cell monolayers at 20 h (mean of $n = 2$).

1 to 2 h to achieve more than 60% reduction of IFN induction under the designated conditions. The cellular output of IFN remained depressed at this level even with extended time periods of interaction.

The effect of BaP with S9 on the induction-production phases of IFN was determined by adding the mixture to cell monolayers at 2, 4, and 8 h after the viral IFN inducer. The course of IFN production was followed for 48 h (Fig. 5). Cell monolayers exposed only to medium with S9 and the viral inducer served as the control. When BaP was added during the induction phase (2 h post-virus inducer), IFN production was severely depressed (99% of the control yield at 20 h). The result was similar when BaP was added at 4 h after the viral inducer. BaP added at 8 h into the production phase also resulted in a diminished yield of IFN as compared to the control, but it was not as severe as that noted with earlier additions of BaP. It would appear that both induction (inducer processing) and production (transcription and/or translation of IFN mRNA) phases of the IFN yield pattern are susceptible to the adverse effect of metabolized BaP.

The ability of cells to reverse the deleterious effect of metabolized BaP on IFN induction was determined. Cell monolayers were exposed to BaP plus S9 for 20 h and then washed, and fresh medium was added. At designated intervals of incubation after removal of BaP metabolites, cell monolayers were exposed to viral IFN inducer for 1 h, and incubated again for 20 h. Control cell cultures were only exposed to the viral IFN inducer. Results (Fig. 6) show that at approximately 7 h after removal of metabolized BaP, the IFN yield was 50% of the control. Thereafter, cellular recovery proceeded rapidly and was considered to be complete at 15 h. The IFN yield at this time was equivalent to that of the control.

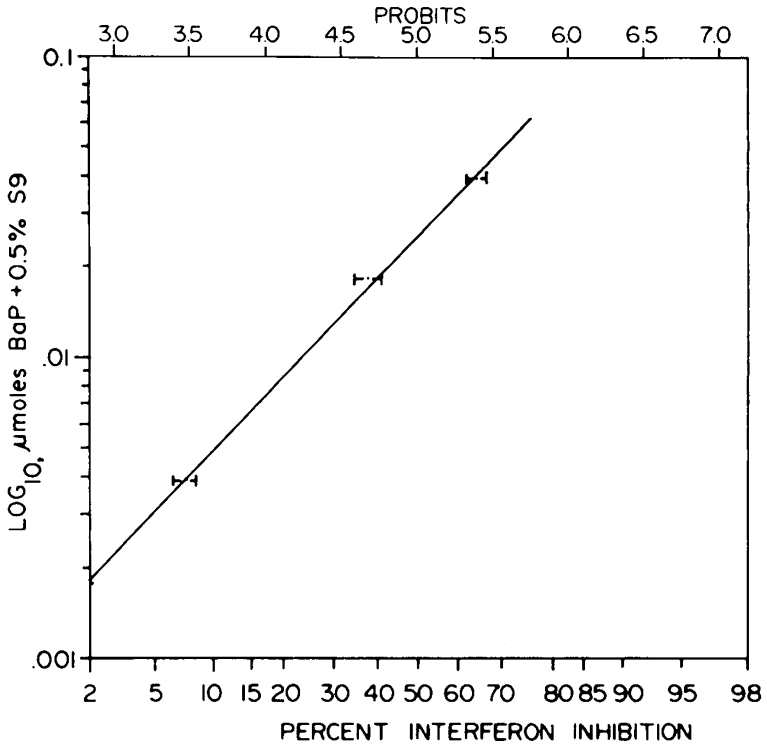

FIG. 3. Dose-response relationship between different amounts of BaP bioactivated with 0.5% rat liver S9 and inhibition of IFN induction by influenza virus in LLC-MK_2 cell monolayers at 20 h (mean of $n = 2$).

BaP, virus multiplication, and IFN-mediated antiviral activity

Influenza virus growth with concomitant IFN production were studied in LLC-MK_2 cell monolayers that were pretreated for 20 h with either BaP, BaP plus S9, or medium with S9 (control). The rates of virus multiplication were similar during the initial 8 h (Fig. 7), and the plateaus of virus growth were reached at 16 h in all pretreated cell cultures. In cells pretreated with BaP plus S9, however, the peak virus level attained was 2.5-fold higher than in either the control or BaP-treated cells. IFN production in both of these latter cell cultures was fourfold higher than that noted in cells pretreated with BaP plus S9. This suggests that the higher level of virus growth noted in cell cultures pretreated with bioactivated BaP than in other treated cells may be a reflection of the partial suppression of IFN synthesis by BaP metabolites.

The effect of bioactivated and nonbioactivated BaP on antiviral-mediated activity of IFN was determined in two ways: (i) an IFN preparation of known potency was assayed in the usual manner using 1-5c-4 cell monolayers that had been exposed to BaP or BaP plus S9 for 20 h previously followed by challenge with Sendai virus, and (ii) pretreating LLC-MK_2 cell monolayers with similar BaP preparations (20 h), then with IFN (20 h), and, subsequently, infecting cells with influenza virus. After incubation of cell cultures at 35°C for 20 h, cell medium was assayed for virus content. Results (Table 3) show that cell cultures pretreated with BaP or bioactivated with S9 did not alter IFN's antiviral potency. All IFN titers were comparable to the control. Influenza virus yields from cell cultures pretreated with different forms of BaP and then with IFN were equivalent to those of cells treated only with IFN. The virus yield was approximately sevenfold higher in cell cultures

TABLE 2. TIME REQUIRED FOR BIOACTIVATION OF BaP BY RAT LIVER S9 IN CELL-FREE MEDIUM AS MONITORED BY INHIBITION OF VIRAL IFN INDUCTION

Reactants[a]	*Reaction time*[b] *(h)*	*IFN* *($ICDD_{50}$/ml)*[c]	*IFN inhibition* *(% ± SEM)*[d]
	0.25	94	26.0 ± 0.7
	0.5	49	61.5 ± 8.3
BaP + S9	1.0	50	60.7 ± 1.1
	2.0	52	59.1 ± 6.3
	4.0	50	60.7 ± 7.1
	8.0	48	62.3 ± 4.9
Controls:			
MM[e] + S9	4.0	120	5.6 ± 2.3
MM + S9	4.0 (NH)[f]	115	9.5 ± 3.4
BaP + S9	4.0 (NH)	57	59.9 ± 5.2
BaP + MM	4.0	120	5.6 ± 3.5
MM	4.0 (NH)	127	0.0

[a]BaP (0.039 μmole) in 10 ml of MM with 0.5% S9.
[b]After designated reaction time at 35°C and heating (85°C, 5 min), mixtures were added to LLC-MK_2 cell monolayers incubated at 35°C for 20 h. Influenza virus was added for IFN induction as described in Methods, followed by incubation for 20 h with 10 ml of MM.
[c]Reciprocal of 50% infected cell-depressing dilution.
[d]See Table 1 for computation based on MM control (n = 2).
[e]Maintenance medium.
[f]Not heated at 85°C.

(control) that were not pretreated with either BaP reagents or IFN. That the ability of exogenous IFN to confer antiviral cellular resistance was unaffected by earlier exposure of cells to bioactivated or inactive BaP is evident from these findings.

Whether BeP, an analog of BaP, can block the adverse activity of the latter on viral IFN induction was determined by pretreating cell monolayers interchangeably and for different time sequences with both hydrocarbons with and without the presence of S9. Results (Table 4) show that prior treatment of cells with BeP negated the inhibitory activity of BaP on IFN induction. BeP *per se* or with S9 did not affect the IFN induction process. When cell monolayers were first treated with bioactivated BaP and then BeP, the former was still capable of depressing IFN induction but only in a metabolized form. These findings suggest that BeP may have the same apparent affinity for critical cellular elements as BaP and may either block or modify cell receptors, or act substitutively to sustain the IFN induction process from the depressive activity of BaP.

BaP bioactivation by different cell lines

Representative mammalian cell cultures of different animal origin, tissue, and propagation status were tested for their inherent ability to bioactivate BaP, as measured by the magnitude of inhibition of viral IFN induction. Different cell cultures were exposed to BaP with and without S9 fraction for 20 h prior to viral IFN induction. Cell monolayers exposed to medium plus S9 served as the control. Results (Table 5) reveal that of the five primary cell cultures tested, only one, Swiss mouse embryo cells, inhibited IFN induction, presumably the consequence of inherent or inducible enzymes that activated BaP. The remaining primary cell cultures required exogenous activating en-

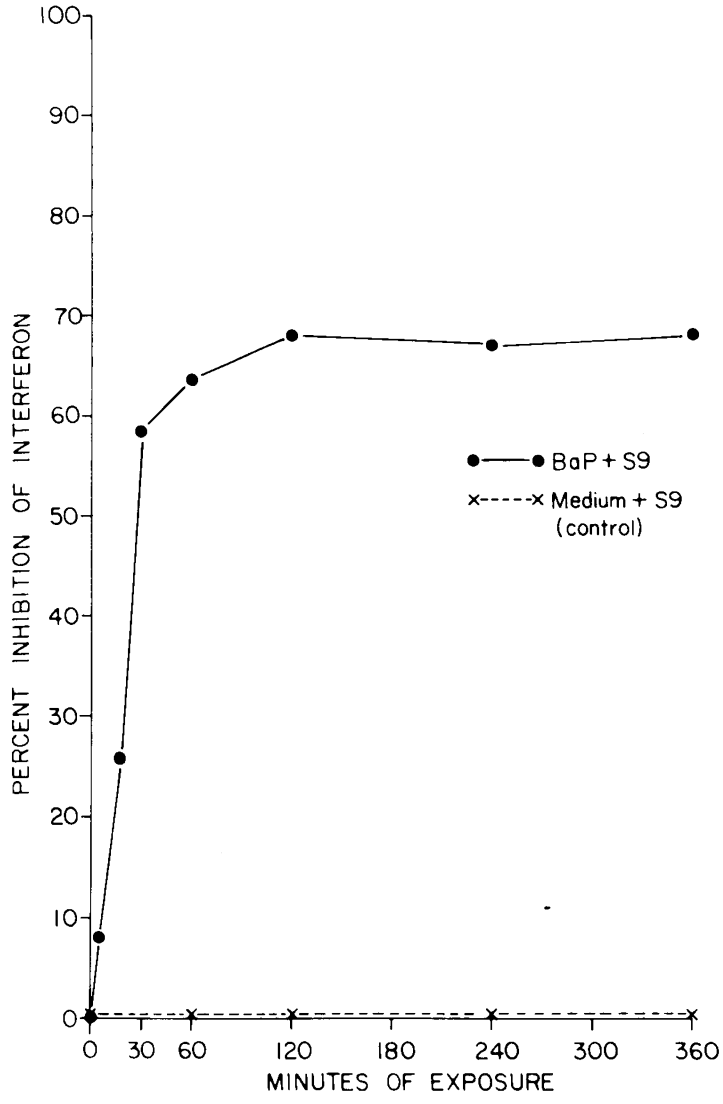

FIG. 4. Length of time required for BaP bioactivation by rat liver S9 and concomitant interaction with LLC-MK_2 cell monolayers for maximal inhibition of IFN induction by influenza virus. Cell monolayers first exposed to BaP (0.039 μmole) with 0.5% S9 for designated time periods, then influenza virus inducer, and incubated at 35°C for an additional 20 h (mean of $n = 2$).

zymes. None of the diploid cells *per se* depressed the IFN induction process without the presence of S9. Overall, IFN yields were highest from the control group of primary and diploid cell cultures. Of the 23 continuous cell cultures tested, all liver cells were capable of metabolizing BaP, resulting in marked depression of IFN induction with the exception of mouse embryo liver cells (BNL-SVA8). Six nonliver cell cultures, MRHF, RFL-6, C3H10T1/2 clone 8, TCMK-1, and NCTC clones 2472 and 2555, were also capable of metabolizing BaP without the addition of exogenous enzymes. When BaP was bioactivated by S9, IFN induction was depressed in magnitude from 32% to 86% in all cell cultures regardless of tissue origin, species, or propagation status.

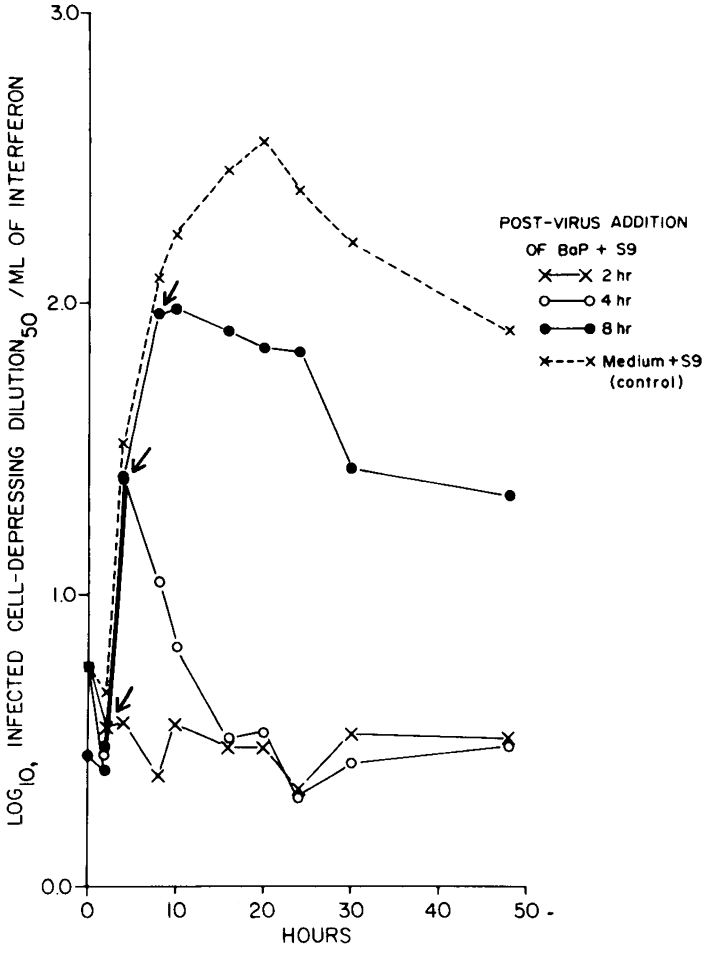

FIG. 5. IFN production in LLC-MK_2 cell monolayers induced by influenza virus with post-virus addition of 0.039 μmole BaP plus 0.5% S9 at 2, 4, and 8 h (mean of $n = 2$).

DISCUSSION

The initial findings of this study reaffirm earlier observations[11,17,20] that viral induction of IFN was significantly inhibited in mammalian cell monolayers by the addition of BaP together with exogenous metabolizing enzymes inherent in rat liver S9 homogenate. Although numerous metabolites, proximate and ultimate, resulting from the bioactivation of BaP have been incriminated in this phenomenon,[17] both BaP and S9 alone can also adversely affect the IFN induction process. Excessive amounts of BaP may be cytocidal, thereby limiting and diminishing the potential total cellular output of inducible IFN, while excessive concentrations of S9 can depress IFN induction directly. Determining the tolerance of cell cultures for BaP and S9 individually is a prerequisite for experimental studies involving IFN induction.

Dose-response relationships were realized between inhibition of viral IFN induction and different concentrations of S9 used to bioactivate a designated quantity of BaP (Fig. 2) as well as different amounts of BaP bioactivated by a constant concentration of S9 (Fig. 3). The extent of linearity was curtailed by cytocidal/cytotoxic effects of both BaP (>0.039 μmole) and S9 (>0.5%) quantities.

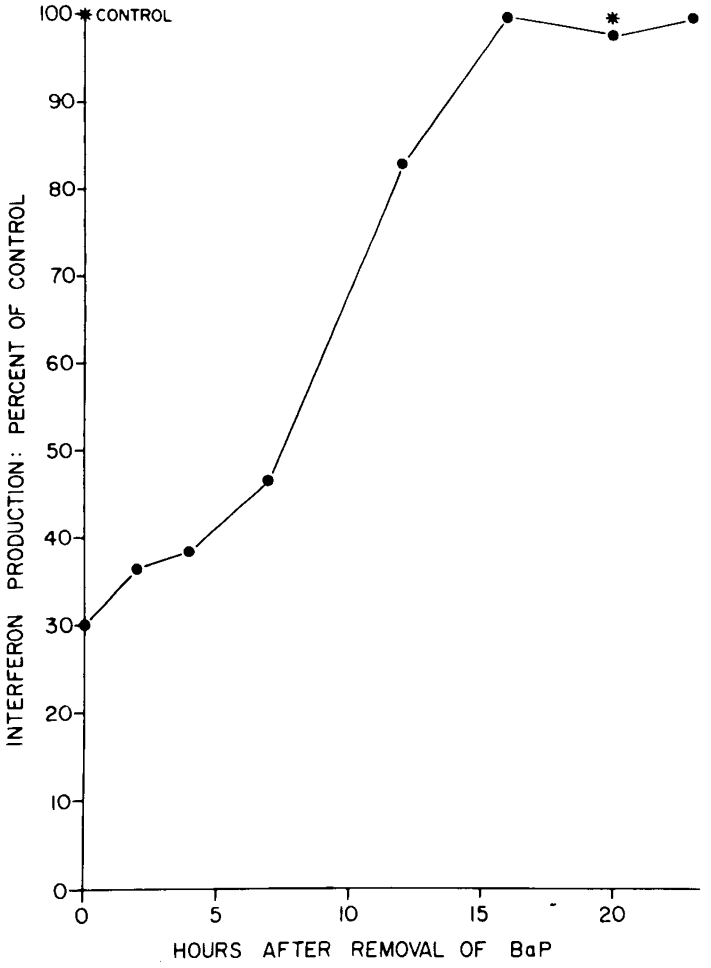

FIG. 6. Reversibility of inhibitory effect of bioactivated BaP on IFN production in LLC-MK_2 cell monolayers. Cells exposed to 0.039 μmole BaP and 0.5% S9 for 20 h. At designated hourly intervals of incubation at 35°C after removal of bioactivated BaP, influenza virus inducer was added for 1 h and cell monolayers were again incubated at 35°C for 20 h. Control consists of cells exposed only to viral inducer (mean of $n = 2$).

High S9 concentrations have also been noted to increase inhibition levels of IFN induction in L-929 cells.[19]

A time-dependency of effect was also noted in the interaction of BaP, S9, and cell cultures. The time interval of 0.5 h was sufficient for bioactivation of BaP by S9 in cell-free medium to attain maximal inhibition of viral IFN induction under the described conditions (Table 2). This short time interval was consistent with previous findings.[28,29] The combined time, inclusive of BaP bioactivation by S9 and interaction of resulting metabolites with proximate cell monolayers, was also 0.5 h but IFN induction was only partially depressed. This indicates that during the first half hour the variety of BaP metabolites formed are capable of depressing the subsequent viral induction of IFN.[17] Maximal IFN inhibition, however, was achieved between 1 and 2 h. This extended reaction time is related to the completion of interactions between BaP metabolites and cells. Others have noted that the time-dependent effect of BaP bioactivation with abrogation of IFN induction was 3 h, however, different cell cultures, reagent doses, and a nonviral inducer were involved.[19]

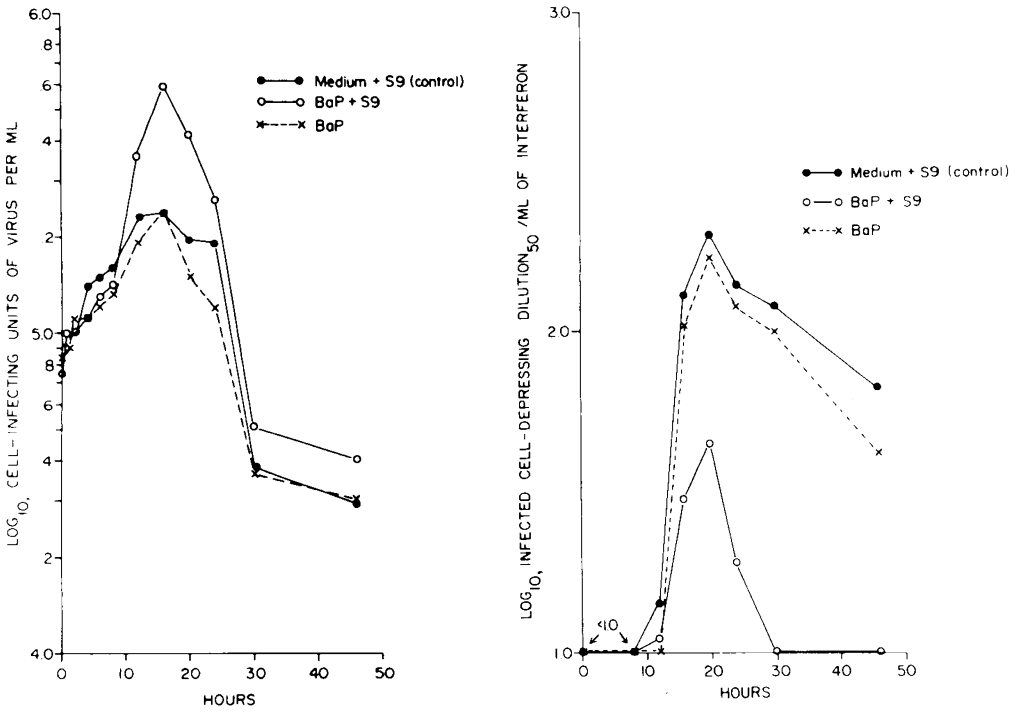

FIG. 7. Influenza virus growth at 35°C concomitant with IFN production in LLC-MK$_2$ cell monolayers (moi = 1.0) pretreated for 20 h with 0.039 μmole BaP without and with 0.5% rat liver S9 (mean of n = 2).

TABLE 3. BaP EFFECT ON ANTIVIRAL ACTIVITY OF IFN ASSESSED BY VIRUS YIELDS AND IFN TITRATION

	IFN antiviral assessment		
Cell treatment prior to IFN exposure	*IFN treatment*	*Influenza virus yield*[a] *(CIU/ml ± SEM) × 10²*	*IFN titration*[b] *($ICDD_{50}$/ml ± SEM)*
BaP[c]	+	6.2 ± 0.7	140 ± 5
BaP + S9	+	8.2 ± 0.2	142 ± 7
MM + S9[d]	+	8.0 ± 1.0	147 ± 2
MM (Control)	+	7.2 ± 0.7	147 ± 2
MM (Control)	−	47.0 ± 3.0	−

[a]After pretreatment with BaP for 2 h and IFN (50 $ICDD_{50}$) for 16 h, LLC-MK$_2$ cells monolayers were infected with virus, incubated for 24 h, and harvested medium was assayed for cell-infecting units of virus (n = 2).

[b]Coverslip 1-5c-4 cell monolayers were pretreated with BaP for 2 h, then a known IFN preparation was assayed in these cell cultures by the usual manner (see Materials and Methods). IFN potency is expressed as reciprocal of 50% infected cell-depressing dilution (n = 2).

[c]0.039 μmole.

[d]Maintenance medium + 0.5% rat liver homogenate.

TABLE 4. BLOCKING EFFECT OF BeP ON BaP INHIBITION OF IFN INDUCTION BY INFLUENCE VIRUS IN LLC-MK$_2$ CELL MONOLAYERS

Pretreatment of cells					
Chemical[a] *(4 h)*	*S9*[b]	*Chemical (16 h)*	*S9*	*IFN ($ICDD_{50}$/ml)*[c]	*IFN inhibition*[d] *(%)*
BeP	–	BaP	+	145	3.4
BeP	+	BaP	+	140	6.7
BeP	+	–	–	150	0.0
–	–	BeP	–	143	4.7
–	–	BeP	+	148	0.8
BaP	+	BeP	+	68	54.7
BaP	–	–	–	150	0.0
BaP	+	–	–	71	52.7
–	–	BaP	+	47	68.7
–	–	BaP	–	138	8.0
Controls					
–	–	–	–	150	0.0
–	+	–	+	150	0.0

[a]Concentrations; BaP (0.039 μmole), or BeP (0.0039 μmole)/10 ml medium.
[b]Rat liver S9, 0.5% in 10 ml of maintenance medium.
[c]Reciprocal of 50% infected cell-depressing dilution.
[d]See Table 1.

Influenza virus replication attained a level that was 2.5-fold higher in cell cultures pretreated with BaP plus S9 than with either BaP *per se* or medium with S9 (control). Conversely, IFN levels in cell cultures treated with the latter two reagents were fourfold higher than in cells pretreated with BaP plus S9. The high virus level is probably a reflection of the adverse effect of metabolized BaP on IFN induction. These findings imply, moreover, that neither the early stages of virus inducer-cell interactions (attachment and penetration) nor the requirement of viral protein and nucleic acid syntheses for virus replication were affected by bioactivated BaP. Metabolized BaP, therefore, may be selectively impairing some other inducer-processing phase required for IFN induction.

Besides IFN induction, the IFN production phase was susceptible to the adverse effect of metabolized BaP. When bioactivated BaP was added to cell cultures at either 2, 4, or 8 h post-viral inducer, IFN induction (2 h) was aborted and production (4 and 8 h) was severely depressed in comparison to that of controls. In view of this and preceding findings regarding virus growth in BaP-pretreated cells, the impairment to IFN formation at the induction stage may involve inducer processing/derepression rather than inducer uptake or binding and, at the production stage, either mRNA transcription or translation.

Metabolized BaP did not impair the ability of IFN to confer antiviral cellular resistance which concurred with findings involving other chemicals and particulates that exhibit adverse effects on IFN induction.[20,30-33] This implies, therefore, that no apparent effect occurred to preformed IFN-α/β or to that required aspect of cellular protein and enzyme (2.5A synthetase, protein kinase) syntheses which is an integral part of IFN-mediated antiviral activity.[34,35]

The inhibitive effect of metabolized BaP on IFN induction is reversible. Cells regained their capacity to produce IFN yields by 50% and 100% at 7 and 15 h, respectively, after removal of hydrocarbon metabolites. This rapid recovery is indicative of a transitory effect of BaP metabolites on

TABLE 5. INDIGENOUS AND LIVER S9 BIOACTIVATION OF BaP IN MAMMALIAN CELL CULTURES OF DIFFERENT ANIMAL ORIGIN, TISSUE AND PROPAGATION STATUS MONITORED BY INHIBITION OF INFLUENZA VIRUS INDUCTION OF IFN

Cell cultures			*Chemical pretreatment of cells IFN inhibition*[a] *($ICDD_{50}$/ml)*[b]		
Propagation status	*Animal*	*Tissue*	*BaP*[c]	*BaP + S9*	*MM + S9*[d]
Primary	Primate:				
	Rhesus[e]	Kidney	0.0 (350)	56.6 (150)	0.0 (345)
	African Green[e]	Kidney	0.0 (450)	54.6 (200)	0.0 (440)
	Cynomolgus[f]	Kidney	2.1 (235)	59.6 (97)	0.0 (240)
	Mouse:				
	Swiss[e]	Embryo	67.5 (26)	66.3 (27)	0.0 (80)
	Human[e]	Embryonic kidney	6.9 (270)	63.8 (105)	0.0 (290)
Diploid	Human:				
	WI-38	Embryonic lung	2.5 (390)	50.0 (200)	0.0 (400)
	MRC-5	Embryonic lung	4.5 (430)	53.4 (210)	0.0 (450)
	IMR-90	Embryonic lung	0.0 (280)	32.2 (190)	0.0 (280)
	HEL[e]	Embryonic lung	2.5 (120)	78.1 (27)	0.0 (123)
Continuous	Primate:				
	Rhesus LLC-MK_2	Kidney	3.0 (165)	73.0 (46)	0.0 (170)
	African Green Vero[f]	Kidney	3.6 (54)	51.8 (27)	0.0 (156)
	African Green BS-C-1[f]	Kidney	5.5 (35)	46.0 (20)	0.0 (37)
	Human:				
	HeLa	Epithelioid carcinoma	0.0 (50)	75.0 (12)	0.0 (48)
	HEp-2	Epithelioid carcinoma	13.9 (31)	66.7 (12)	0.0 (36)
	L-132	Embryonic lung	23.7 (42)	78.2 (12)	0.0 (55)
	Chang	Liver	49.2 (94)	65.5 (64)	0.0 (185)
	WRL-68	Embryo liver	53.6 (58)	56.0 (55)	0.0 (125)
	MRHF[e]	Foreskin, newborn	66.0 (37)	66.0 (37)	0.0 (84)
	Mouse:				
	TCMK-1	Kidney	70.0 (36)	80.0 (24)	0.0 (120)
	BALB/3T12-3	Embryo	8.9 (41)	42.3 (26)	0.0 (45)
	NCTC4093	Embryo	0.0 (36)	45.5 (18)	0.0 (33)
	$P388D_1$	Lymphoid neoplasm	9.1 (80)	71.6 (25)	0.0 (33)
	BNL CL-2	Normal embryo liver	72.0 (21)	73.4 (20)	0.0 (75)
	BNL SV A8	Embryo liver	0.0 (100)	64.0 (36)	0.0 (100)
	3T3, Swiss[a]	Embryo	0.0 (44)	83.8 (7)	0.0 (43)
	NCTC clone 1469	Liver	56.8 (16)	86.5 (5)	0.0 (37)
	L-929	Connective	0.0 (76)	66.8 (25)	0.0 (75)
	C3H10T1/2 clone 8	Embryo	43.7 (31)	61.9 (21)	0.0 (55)
	NCTC clone 2472	Connective	64.3 (30)	72.7 (23)	0.0 (84)
	NCTC clone 2555	Connective	70.6 (23)	77.0 (18)	0.0 (78)
	Rat:				
	RFL-6	Lung	75.7 (10)	78.1 (9)	0.0 (41)
	Clone 9	Normal liver	56.5 (41)	62.3 (34)	0.0 (90)

[a]See Table 1 for computation method.
[b]Reciprocal of 50% infected-cell depressing dilution of IFN/ml.
[c]0.039 μmoles in 10 ml maintenance medium.
[d]Maintenance medium with 0.5% rat liver S9 fraction (control).
[e]Whittaker M.A. Bioproducts, Walkersville, MD.
[f]Flow Laboratories, Inc., McLean, VA. All other undesignated cell cultures obtained from ATTC, Rockville, MD.

critical cellular elements involved in IFN induction and is suggestive of a translation block.[36] To sustain the inhibitory effect of metabolized BaP on IFN induction, periodic or continual cellular exposure is probably required.

BaP and its analog BeP are both widespread atmospheric contaminates, with the former having a high and the latter a low mutacarcinogenic potential.[37] BeP is often cited to point out how structurally similar PAHs can have vastly different biological activities.[38] BeP or its metabolites are capable of altering the inhibitory activity of BaP metabolites on IFN induction. This adverse effect of metabolized BaP on IFN induction was abrogated when cell cultures were first pretreated with BeP. BeP appears to have an affinity equal to that of metabolized BaP for critical cellular elements involved in IFN induction, but the former does not subvert the process. The inability of rat liver microsomes to form BeP 9,10-dihydrodiol metabolically, and convert it to diol epoxides, may be a factor to account for BeP's lack of or weak mutagenicity/carcinogenicity.[38] This is reflected by BeP's innocuous influence on IFN induction. BeP and its metabolites under these circumstances could act by blocking cell receptors or act substitutively to protect intracellular target mechanisms involved in IFN induction from the adverse effect of metabolized BaP. As expected, pretreatment of cell cultures with metabolized BaP, followed by BeP, did not affect the capacity of the former to inhibit viral IFN induction. BaP metabolites resulting from enzymatic activation, *i.e.*, phenols, dihydrodiols, and ultimate compounds, diol epoxides I and II, are active inhibitors of viral IFN induction.[17]

Of 32 mammalian cell cultures of different animal origin, tissue, and propagation status tested for their indigenous ability to bioactivate BaP, as measured by inhibition of viral IFN induction, only 37.5% were responsive. Depending on their background, cells in culture have varied inherent levels of and capacities to produce drug-metabolizing enzymes[15,39,40] with the full spectrum of enzyme activity being influenced by the chemical inducer.[41] Consequently, the extent of xenobiotic bioactivation may be restricted to the formation of certain metabolites which may or may not be biologically reactive to alter specific cell responses. By compensation with S9 fraction, all 32 cell cultures were capable of bioactivating BaP with resultant inhibition of IFN induction. The ability to characterize BaP bioactivation in mammalian cell cultures on the basis of inhibition of IFN-α/β induction, as demonstrated in the study herein, provides an augmentative means for assessing other potential metabolic-dependent mutagens/carcinogens, their multiplex interactions in relation to the IFN defense mechanism, and conversely, the role of IFN in drug-metabolizing enzyme systems.[42]

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Address reprint requests to:
Dr. Nicholas Hahon
National Institute for Occupational Safety and Health
ALOSH, 944 Chestnut Ridge Road
Morgantown, WV 26505-2888

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