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Induction and Decay of Aryl Hydrocarbon Hydroxylase Activity in Mouse 3T3 Cells

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SUMMARY

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Aryl hydrocarbon hydroxylase activity in mouse 3T3 cells is induced by 24 hr of exposure to 9 μM benz[*a*]anthracene or to 1 μM 3-methylcholanthrene; however, maximal activity induced by 3-methylcholanthrene is only about 33% of that induced by benz[*a*]anthracene. Induction by benz[*a*]anthracene is inhibited by simultaneous administration of 3-methylcholanthrene. Inducible aryl hydrocarbon hydroxylase activity attains a maximum during the late log-early confluence stage of culture growth and is lower in confluent, nonfed cells. Feeding confluent cells results in another peak of inducibility, concomitant with an increase in DNA synthesis. However, blockade of DNA synthesis with cytosine arabinoside does not block the peak of aryl hydrocarbon hydroxylase activity which occurs after feeding. After removal of inducer from cultures with maximally induced activity, two apparent decay rates are observed, one with a $t_{1/2}$ of 15-30 min and another with a $t_{1/2}$ of 4-6 hr. Cultures with submaximally induced activity show only the slow rate of decay. Addition of inducer in fresh medium to washed, maximally induced cultures stabilizes induced activity. Readdition of benz[*a*]-anthracene to cultures deprived of inducer for 1 or 4 hr results in reinduction of aryl hydrocarbon hydroxylase. This second increase in activity is faster than the initial rate of induction and is prevented by cycloheximide or actinomycin D. These data indicate the importance of culture growth phase, feeding schedule, and duration of exposure to inducer in the determination of aryl hydrocarbon hydroxylase activity in cultured 3T3 cells. In addition, it was observed that both control and induced levels of this enzyme are decreased in cultures contaminated with mycoplasma before cytopathic effects of the contamination are evident.

INTRODUCTION

Aryl hydrocarbon hydroxylase is a microsomal oxygenase which catalyzes the oxidation of polycyclic aromatic hydrocarbons. The products of this reaction and/or their subsequent oxidative metabolites have been implicated as the actual carcinogenic intermediates of the polycyclic hydrocarbons (1-3). At least two nonlinked genetic loci control the inducibility of this enzyme by aromatic hydrocarbons in the liver and in several other tissues of the mouse (4, 5). Genetic variation in the inducibility of aryl hydrocarbon hydroxylase in human lymphocytes has also been reported (6). A relationship between the inducibility of this enzyme and susceptibility to methylcholanthrene-induced sarcomas

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has been shown in various strains of mice (7). In man, a correlation between aryl hydrocarbon hydroxylase inducibility in peripheral blood lymphocytes and bronchogenic carcinoma has also been suggested (8). However, tumorigenesis initiated by the polycyclic hydrocarbons 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]-pyrene does not appear to be associated with the genetic loci controlling aryl hydrocarbon hydroxylase induction (3, 9).

In order to investigate the control of aryl hydrocarbon hydroxylase activity in various cell types and thereby to elucidate its role in chemical carcinogenesis, a number of laboratories have turned to the use of cell culture systems. This enables one to study malignant transformation of cells by chemicals *in vitro* and to control many of the variables which cannot be controlled *in vivo*. However, a number of important variables exist in cell culture systems and must be taken into account for interpretation of data relating to aryl hydrocarbon hydroxylase induction, such as the effects of serum concentration; the content of hormones, biogenic amines, and other natural compounds which may be present in the medium (10); the proliferative state of the cells (11, 12); the feeding schedule (11, 13); and the duration of log phase growth of the cultures (13).

A number of studies characterizing aryl hydrocarbon hydroxylase induction have been done with primary or secondary cultures derived from fetal tissues (10, 11, 14–16). The use of established cell lines (17–22) to study the control of enzyme induction has certain advantages, among which are the ease of obtaining standard stock cultures for study and the reproducibility of induction with continued passage of the cells. We have characterized the induction of aryl hydrocarbon hydroxylase in an established line of murine 3T3 fibroblasts. In this report we emphasize some of the apparent differences between the 3T3 system and primary fetal cultures, as well as point out a major potential pitfall in the use of established cultures to study aryl hydrocarbon hydroxylase induction, namely, mycoplasmal contamination. In 3T3 cultures we find that (a) 3-methylcholanthrene is a very weak inducer of aryl hydrocarbon hydroxylase compared to benz[*a*]anthracene and inhibits induction of this enzyme by BA;[1] (b) the activity of aryl hydrocarbon hydroxylase induced by BA is maximal as the cells reach confluence, and a second peak of activity can be produced by refeeding confluent cultures; (c) neither the initial maximum (as the cells reach confluence) nor the additional peaks produced after refeeding are coupled to DNA synthesis; (d) there are two rates of decay for induced aryl hydrocarbon hydroxylase activity, and the more rapid rate of decay is seen only in maximally induced cultures; and (e) a low level of mycoplasmal contamination decreases both control and induced aryl hydrocarbon hydroxylase activity.

MATERIALS AND METHODS

Chemicals. Chemicals used in this study were obtained from the following sources: benz[*a*]anthracene, Eastman Organic Chemicals; benzo[*a*]pyrene and NADPH, Sigma Chemical Company; 3-methylcholanthrene and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Calbiochem; [^{3}H]thymidine (55 Ci/mmole) and [^{3}H]-uridine (20 Ci/mmole), Nuclear Dynamics; Protosol and [^{3}H]amino acid mixture, New England Nuclear; and [^{3}H]benzo[*a*]pyrene (25 Ci/mmole), Amersham/Searle. The 3-hydroxybenzo[*a*]pyrene used as a standard for aryl hydrocarbon hydroxylase determinations was the generous gift of Dr. Harry Gelboin, National Institutes of Health.

Determination of aryl hydrocarbon hydroxylase activity. Mouse 3T3 cells (Swiss) were obtained from Dr. Dale Oxender at the University of Michigan (originally from Dr. George Todaro, National Institutes of Health) and were grown as monolayer cultures in Blake bottles at 37° in 80 ml of Dulbecco's modified Eagle's medium (Grand Island Biological Company) supplemented with 10% calf serum (KC Bio-

[1] The abbreviations used are: BA, benz[*a*]anthracene; DMSO, dimethyl sulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MC, 3-methylcholanthrene; ara-C, cytosine arabinoside.

logical, Inc., Lenexa, Ks.) and 10 μg/ml of tylosin (Grand Island Biological Company). Cultures were free of mycoplasmal contamination, except where otherwise indicated, as determined by the absence of both uridine phosphorylase activity (23) and arginine deiminase activity (24). Cultures were routinely treated with 12 μM BA in DMSO (final concentration in medium, 0.5% DMSO). Control cultures received 0.5% DMSO only. This concentration of DMSO did not affect cell growth or morphology. After 24 hr of incubation with drugs, medium was removed and cells were rinsed twice with HEPES-buffered saline solution (pH 7.2) containing NaCl, 140 mM; KCl, 5.4 mM; $Na_2HPO \cdot 7H_2O$, 0.7 mM; dextrose, 5.6 mM; and HEPES, 10 mM. This was followed by an additional wash with EDTA solution containing NaCl, 140 mM; KCl, 2.7 mM; KH_2PO_4, 1.5 mM; Na_2HPO_4, 8.1 mM; and EDTA, 0.48 mM. The cells were then suspended in HEPES-buffered saline, centrifuged at 1000 × *g* for 10 min at 0°, resuspended in HEPES-saline, and centrifuged again. Duplicate cultures were harvested and pooled for each experimental point. The washed cell pellet was then frozen at −80°. Storage for several days at −80° did not affect aryl hydrocarbon hydroxylase activity.

Cell pellets were then thawed, suspended in 2.0–4.0 ml of a buffer (pH 7.5) containing Tris-HCl (10 mM), sucrose (250 mM), $MgCl_2$ (5 mM), and 2-mercaptoethanol (5 mM), and homogenized in a Dounce ball-type homogenizing tube (10 strokes with a loose and 60 strokes with a tight pestle). This produced greater than 90% cell breakage with minimal breakage of nuclei. Protein determinations were performed on the homogenates by a modification of the method of Lowry *et al.* (25). There was approximately 1 mg of protein per 10^6 3T3 cells.

The aryl hydrocarbon hydroxylase activity was determined by a modification of the method of Nebert and Gelboin (26). The standard reaction mixture (1.0 ml) contained 38 μmoles of Tris-HCl (pH 7.5), 3.6 μmoles of $MgCl_2$, 75 mmoles of sucrose, 0.18 μmole of NADPH, and 0.75 mg of protein (whole cell homogenate). The reaction was started with the addition of 80 nmoles of benzo[*a*]pyrene in 0.05 ml of methanol; tissue blanks received 0.05 ml of methanol only. Samples were incubated in air for 30 min with shaking at 37°. The incubation and all subsequent steps were carried out in a darkened room, since the reaction product 3-hydroxybenzo[*a*]pyrene is extremely light-sensitive. The formation of product was linear for 60 min and was also linear with respect to protein concentrations up to 1 mg/ml of assay mixture. Saturating concentrations of NADPH and benzo[*a*]pyrene were 0.09 μmole/ml and 10 nmoles/ml, respectively, under these conditions. The reaction was stopped by the addition of 1.0 ml of cold acetone, followed by 5.0 ml of hexane. Samples were extracted for 10 min with shaking at 37°, then transferred to shaker bottles for further extraction, and centrifuged for 10 min at 1000 × *g* to improve separation of the organic and aqueous layers. A 4-ml aliquot of the hexane phase was then extracted twice with 3 ml of 1 N NaOH. Aliquots (2 ml) of the two hydroxide extracts were pooled, and the amount of 3-hydroxybenzo[*a*]pyrene was determined on an Aminco-Bowman spectrophotofluorometer with activation at 396 nm and fluorescence at 522 nm. Each experimental point represents triplicate assays (range, less than ±6%) from two or three pooled cultures (0.5–2.0 × 10^7 cells/culture). The major NaOH-extractable, polar metabolite formed by control and BA-treated cells from two different lines of 3T3 cells co-chromatographed with authentic 3-hydroxybenzo[*a*]pyrene in a thin-layer chromatography system as described by Kinoshita *et al.* (27).

DNA and protein synthesis. Cultures were pulsed with [^{3}H]thymidine (0.3 μCi/ml) for 3 hr, washed, and harvested in HEPES-buffered saline as described above. After thawing, the cell pellets were washed three times in 5% perchloric acid, then suspended in 5% perchloric acid and heated at 80° for 20 min. Samples were centrifuged, and aliquots of the supernatant were taken for determination of both DNA content, by the diphenylamine assay, and radioactivity. All assays were performed in duplicate on each sample.

Cultures were pulsed with [^{3}H]amino

acids (0.1 μCi/ml) for 2 hr and harvested as described above. Cell pellets were then homogenized in 0.25 M sucrose solution (as above), and aliquots were taken for protein and radioactivity determination. Protein was precipitated by the addition of an equal volume of cold 10% trichloroacetic acid. The samples were centrifuged, and the pellets were washed twice in 5% trichloroacetic acid, solubilized in 0.5–1.0 ml of Protosol, and counted on a liquid scintillation spectrometer.

Determination of mycoplasmal contamination. Cell cultures were assayed for mycoplasma based on the presence of uridine phosphorylase activity according to the method of Levine (23). The reaction mixture contained 1.4 ml of cell homogenate and 2–4 μCi of [^{3}H]uridine in 0.1 ml of sterile water. Aliquots were removed from the test samples and boiled controls after 30 and 180 min of incubation at 37° and then diluted with an equal volume of a solution containing 2 mM uracil and 2 mM uridine in water. Samples were then spotted on Whatman No. 1 filter paper and developed for 45 min in 4% boric acid–concentrated ammonium hydroxide–1-butanol (7:0.1:43 by volume) at 37°. Spots were visualized under ultraviolet light, and radioactivity was determined by liquid scintillation counting. The percentage conversion of uridine to uracil after 30 and 180 min of incubation was calculated, and the results were expressed as the difference between the two values. At each time point the percentage conversion in a boiled control was subtracted from the experimental value. For low rates of conversion, the increase in percentage conversion from 30 to 180 min is more significant than the amount of conversion at any one time point (23).

RESULTS

Characterization of aryl hydrocarbon hydroxylase induction and decay in 3T3 cultures. Figure 1 illustrates the kinetics of induction of aryl hydrocarbon hydroxylase after the addition of BA to 3T3 cells. The greatest activity occurred 24 hr after the addition of BA to cultures approaching confluence (day 3) as well as to those maintained in the confluent state (day 6). Re-

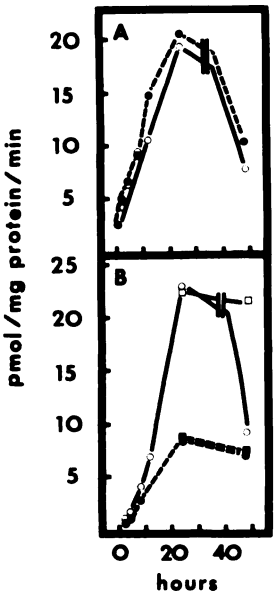

FIG. 1. *Time course of BA induction of aryl hydrocarbon hydroxylase activity during late log phase growth (day 3) and after confluence (day 6)*

"Fed" cells were refed at the time of addition of BA, and duplicate cultures were harvested at the indicated times. Hash marks indicate discontinuity of aryl hydrocarbon hydroxylase measurements. In other experiments the enzyme activity remained maximal for 24 to 36 hr after addition of BA and then decreased. A. Cultures in late log phase growth (day 3): O——O, fed, 12 μM BA; ●- - -●, not fed, 12 μM BA. B. Cultures after confluence (day 6): O——O, fed, 12 μM BA; ●- - -●, not fed, 12 μM BA; □——□, fed, 24 μM BA; ■- - -■, not fed, 24 μM BA.

feeding confluent cultures increased the level of enzymatic activity achieved but did not alter the time to reach maximum. Moreover, if twice the usual amount of BA (i.e., 24 μM) was added to the cultures, maximal activity was maintained for an additional 24 hr in the refed, maximally induced cultures. Thus a 24-hr treatment with BA was used as an index of maximal induction in these studies.

Dose-response curves for the induction of aryl hydrocarbon hydroxylase activity by a 24-hr treatment of 3T3 cells with BA or MC are shown in Fig. 2. Maximal activity was achieved with 9 μM BA. MC was a weak inducer and inhibited enzyme induction when added together with BA to the cell cultures (Fig. 3). This result did not appear to be due to increased cytotoxicity, since the growth kinetics and maximal cell

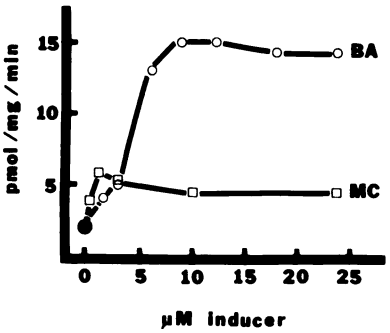

FIG. 2. *Dose-response curve of induction of aryl hydrocarbon hydroxylase activity in 3T3 cells by BA and MC*

Cultures were treated for 24 hr with the indicated dosage of hydrocarbon dissolved in DMSO, to a final concentration of 0.5% DMSO in the medium, harvested, and assayed for aryl hydrocarbon hydroxylase activity as described in MATERIALS AND METHODS. For each point, duplicate cultures were pooled and assayed in triplicate. O——O, BA; □——□, MC; ●, control (0.5% DMSO only).

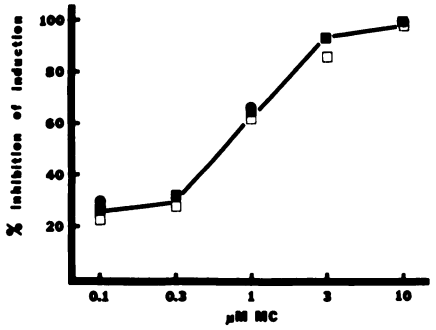

FIG. 3. *Inhibition of aryl hydrocarbon hydroxylase induction by BA due to simultaneous addition of MC*

Cultures were treated for 24 hr with BA and MC in DMSO. In calculating the percentage inhibition, the activity induced by each concentration of MC was subtracted from the activity achieved with MC plus BA; this number was then divided by maximal BA-induced activity. ●——●, cells induced with 12 μM BA for 24 hr, not fed at time of BA and MC addition; ■——■, cells induced with 9 μM BA, not fed; □——□, cells induced with 9 μM BA, refed at time of addition of BA and MC.

density achieved were the same whether or not MC was present. Although MC competitively inhibited the metabolism of benzo[*a*]pyrene when it was added directly to the aryl hydrocarbon hydroxylase assay mixture *in vitro*, the maximal concentration of MC (after addition *in vivo*) which could possibly have been carried through to the assay mixture was 100 times lower than that necessary to produce inhibition of benzo[*a*]pyrene metabolism.[2]

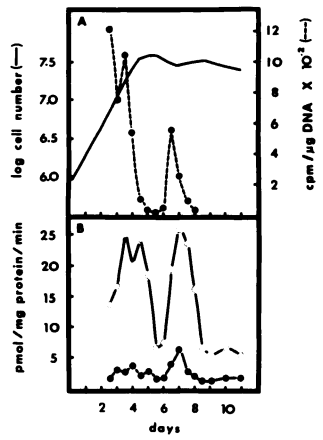

FIG. 4. *Relationship among refeeding, DNA synthesis, and induced aryl hydrocarbon hydroxylase activity*

All cultures were refed on days 3 and 6 after plating. Cultures were pulsed for 3 hr with 25 μCi of [^{3}H]thymidine. The cell number was determined by hemocytometer. Cultures were treated for 24 hr with 12 μM BA and harvested, and aryl hydrocarbon hydroxylase activity was assayed as described in MATERIALS AND METHODS. A. ——, log cell number; ●- - -●, counts per minute per microgram of DNA. B. ●——●, control enzyme activity; O——O, BA-induced enzyme activity.

Under the culture conditions utilized in these experiments, maximal inducibility of aryl hydrocarbon hydroxylase activity by BA was invariably obtained within 24–36 hr after the cultures had reached confluence (Fig. 4). It should be noted that each data point represents maximal induced activity obtained by exposure to BA

[2] M. A. Bittner and R. W. Ruddon, unpublished observations. We have observed that the addition of MC to the assay mixture *in vitro* competitively inhibits the metabolism of benzo[*a*]pyrene, with K_i = 1 μM. If one calculates the maximum amount of polycyclic hydrocarbon that could have been bound to the cell homogenate fraction, based on the observation that about 1% of radioactively labeled hydrocarbon remains bound to the cells after removal of the medium and extensive washing, it would be approximately 100 times lower than the concentration observed to inhibit benzpyrene metabolism *in vitro*.

for 24 hr prior to assay of aryl hydrocarbon hydroxylase activity. Inducibility of the enzyme subsequently declined as the cells were maintained in the confluent state, but induced activity returned to peak levels if the cultures were refed with fresh medium (Fig. 4). Concomitantly with the aryl hydrocarbon hydroxylase activity peaks, there was an increase in DNA synthesis. Both these events occured 12–24 hr after refeeding on days 3 and 6. The peak of aryl hydrocarbon hydroxylase activity observed as the cells reached confluence, however, was not dependent on the feeding schedule, since it also occurred whether the cultures were fed only on day 0, with no further feeding until after the cells reached confluence, or whether they were fed every day (data not shown).

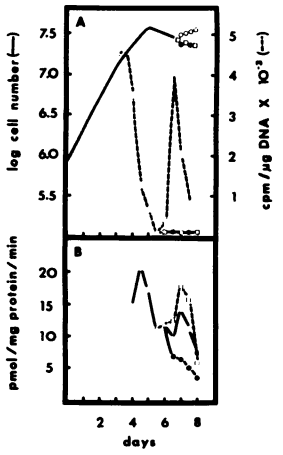

FIG. 5. *Failure of cytosine arabinoside to inhibit aryl hydrocarbon hydroxylase induction stimulated by feeding*

All cultures were fed on day 3, and on day 6 they were divided into three groups: group 1 was refed; group 2 was refed and treated with 5 μg/ml of ara-C (these cultures received an additional 5 μg/ml of ara-C after 24 hr); group 3 was not fed. Induced aryl hydrocarbon hydroxylase activity was determined after 24 hr of treatment with 12 μM BA. A. ——, log cell number; - - -, counts per minute per microgram of DNA; ○, group 1 (fed); □, group 2 (fed, ara-C); ●, group 3 (not fed). B. Aryl hydrocarbon hydroxylase activity: ○——○, group 1 (fed); □- - -□, group 2 (fed, ara-C); ●——●, group 3 (not fed).

In order to determine whether the inducibility of aryl hydrocarbon hydroxylase was coupled to DNA synthesis and cell proliferation, cytosine arabinoside was added to cultures at a dose (5 μg/ml) which blocked DNA synthesis by more than 95% (Fig. 5). When the cultures were refed on day 6 there was the expected increase in DNA synthesis, a small increase in cell number, and a second peak of induced aryl hydrocarbon hydroxylase activity. Neither the nonfed cultures nor the ara-C-treated, fed cultures exhibited an increase in DNA synthesis or cell proliferation. However, the ara-C-treated cultures exhibited the second peak of aryl hydrocarbon hydroxylase activity, whereas the nonfed cultures did not. Similar results were obtained if late log phase cultures were treated with ara-C just prior to the peak of enzyme inducibility (Table 1).

When BA was removed from maximally induced cultures, two decay rates were observed: a rapid phase with a $t_{1/2}$ of 15–30

TABLE 1

Failure of cytosine arabinoside to block aryl hydrocarbon hydroxylase induction in late log phase cultures of 3T3 cells

Cytosine arabinoside (5 μg/ml) was added to cultures on days 2 and 3 after plating. DNA synthesis and aryl hydrocarbon hydroxylase activity were determined as described in MATERIALS AND METHODS. The enzyme activity was measured 24 hr after the addition of BA.

Days after plating	Cell number		[³H]Thymidine incorporation		BA-induced enzyme activity	
	Control	Ara-C	Control	Ara-C	Control	Ara-C
	log No. cells/culture		*cpm/μg DNA*		*pmoles/mg/min*	
2	6.61	6.61	13,100	1,000		
2.5	6.74	6.76	9,940	500	11.6	8.4
3.0	6.99	6.72	8,500	180	21.4	18.2
3.5	7.10	6.74			21.0	17.4
4	7.02	6.76			26.7	19.7

min, and a slower phase with a $t_{1/2}$ of 4–6 hr (Fig. 6). Submaximally induced activity exhibited only the slower rate. This was true for both confluent, nonfed cultures induced for 24 hr (Fig. 6) and for log phase cultures induced for only 8 hr (data not shown). Maximal activity could be maintained if 12 μM BA was added back to the fresh medium (Fig. 7). It was also observed that maximal activity could be reinduced if BA was added back to cultures at a time when aryl hydrocarbon hydroxylase activity had been allowed to decay partially, i.e., 1 and 4 hr after exposure of the cells to fresh medium without drug. The rate at which maximal activity could be reinduced was more rapid in the latter case than when BA was initially added to untreated cultures. The reinduction of aryl hydrocarbon hydroxylase after decay required protein and RNA synthesis, since it was blocked if either cycloheximide (5 μg/ml) or actinomycin D (1 or 2 μg/ml) was added to the cultures prior to the addition of BA at 1 and 4 hr (data not shown).

Effect of mycoplasmal contamination on aryl hydrocarbon hydroxylase induction in 3T3 cells. These experiments were prompted by the observation of a gradual, 10-fold decrease in both control and BA-induced aryl hydrocarbon hydroxylase levels in one of our 3T3 cell lines (3T3-M) over a period of 3 months. This did not appear to be due to any marked change in cell growth kinetics or technical problems with the enzyme assay. We therefore considered the possibility of mycoplasmal contamination.

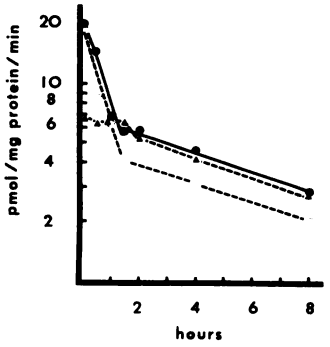

FIG. 6. *Decay of maximally induced and submaximally induced aryl hydrocarbon hydroxylase activity*

Cultures were grown for 3 days (late log phase) or for 6 days (confluent). At the time of induction half the 6-day-old cultures were refed. All cultures were induced for 24 hr with 12 μM BA, after which medium containing inducer was replaced with fresh medium without drug. ●——●, log phase cells, not fed; O- - -O, confluent cells, fed; ▲- - -▲, confluent cells, not fed.

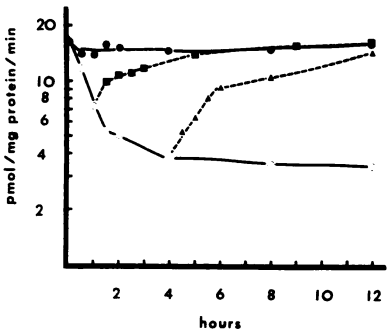

FIG. 7. *Time course of decay and reinduction of aryl hydrocarbon hydroxylase activity*

Cultures in late log phase were induced for 24 hr with 12 μM BA, after which medium containing inducer was replaced with fresh control medium. At zero time, or after 1 or 4 hr of decay, 12 μM BA was added back to some of the cultures. O——O, control medium only; ●——●, BA added back at zero time; ■- - -■, BA added back after 1 hr; ▲- - -▲, BA added back after 4 hr.

In a series of initially uncontaminated 3T3 cultures, we observed a gradual increase in uridine phosphorylase activity concomitant with a decrease in aryl hydrocarbon hydroxylase inducibility (Table 2). Treatment with tetracycline (25 or 50 μg/ml) did not eliminate the contamination. Treatment with tylosin (150 or 300 μg/ml) prevented an increase in uridine phosphorylase activity but did not decrease it; neither did it restore aryl hydrocarbon hydroxylase activity. Qualitatively similar results were observed when we purposely contaminated another cell line with cell-free medium from contaminated cells (Table 3). Again both control and BA-induced enzyme levels decreased as the mycoplasma "titer" increased. Attempts to isolate the mycoplasma were unsuccessful. An independent analysis done in the laboratory of Dr. George Kenny (University of Washington, Seattle) also indicated the presence of a nonculturable prokaryote based on tests for conversion of uridine to

TABLE 2

Correlation between increasing uridine phosphorylase activity and decreasing aryl hydrocarbon hydroxylase activity

Swiss 3T3-M cells (obtained from Dr. Gail Wertz, University of Michigan) were grown as monolayer cultures in Blake bottles at 37° in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% calf serum (KC Biological, Inc.), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml). Uridine phosphorylase and aryl hydrocarbon hydroxylase activities were determined as described in MATERIALS AND METHODS.

Uridine phosphorylase activity[a]	Aryl hydrocarbon hydroxylase activity[b]	
	Control	Induced
Δ% conversion	*pmoles/mg/min*	
3	5.0	30.2
6	1.8	16.0
12	1.1	7.6

[a] Expressed as the difference in percentage conversion between 30 and 180 min. For a set of eight duplicate experiments, the variability was ±1%.

[b] Expressed as pmoles of 3-hydroxybenzpyrene formed per milligram of protein per minute. For a set of four duplicate experiments, the variability was less than ±0.4 pmoles/mg/min.

uracil. We observed no change in growth rate or morphology, and no cytopathic effects, until the mycoplasmal contamination was sufficient to cause more than a 12% increase in conversion of uridine to uracil. A marked decrease in aryl hydrocarbon hydroxylase activity was observed, however, even at a 6% increase in conversion. In one cell line tested (3T6, CCl 96, American Type Culture Collection), there was a 20% increase in conversion of uridine to uracil. In this case there was no aryl hydrocarbon hydroxylase induction; both control and induced levels of the enzyme were 0.7 pmole/mg of protein per minute. A cytopathic effect was evident in these cultures and may explain the complete lack of enzyme induction. In another series of experiments, addition of *Mycoplasma bovigenitalium* (American Type Culture Collection) to 3T3 cultures produced marked cytopathology as well as increased uridine phosphorylase activity and decreased aryl hydrocarbon hydroxylase levels (data not shown).

TABLE 3

Effect of addition of mycoplasma-contaminated medium on uridine phosphorylase and aryl hydrocarbon hydroxylase activities

Swiss 3T3 cells (obtained from Dr. Dale Oxender, University of Michigan) were grown as described in Table 2 without antibiotics. On day 0, stock cultures were assayed; the cells were then plated and divided into two groups. 3T3 cells were carried as usual; 3T3c cells were exposed for 3 days to medium which had been incubated for 24 hr with contaminated cells. Cells were replated on days 3 and 6, then assayed for uridine phosphorylase on days 4 and 10, and for aryl hydrocarbon hydroxylase on day 10.

Day	Cells	Uridine phosphorylase activity[a]	Aryl hydrocarbon hydroxylase activity[b]	
			Control	Induced
		Δ% conversion	*pmoles/mg/min*	
0	3T3	3	2.2	14.2
4	3T3	3		
	3T3c	6		
10	3T3	3	2.7	13.1
	3T3c	11	0.9	4.4

[a] Expressed as the difference in percentage conversion between 30 and 180 min; variability was ±1%.

[b] Expressed as pmoles of 3-hydroxybenzpyrene formed per milligram of protein per minute; variability was no greater than 0.1 pmoles/mg/min.

DISCUSSION

The induction of aryl hydrocarbon hydroxylase by BA in cultured 3T3 cells provides a useful model for the study of polycyclic hydrocarbon-induced aryl hydrocarbon hydroxylase activity. The findings that MC is a weaker inducer than BA and that an equimolar concentration of MC completely inhibits BA-induced activity are somewhat surprising. The unresponsiveness of this line of 3T3 cells to MC might be due to an altered "receptor" which does not bind MC efficiently. However, Guenthner *et al.* (28) found no difference in the affinity of tetrachlorodibenzo-*p*-dioxin, a potent aryl hydrocarbon hydroxylase inducer, for a cytosol "receptor" in their "responsive" and "nonresponsive" cell lines. The possibility that MC might induce the formation of metabolic products not detected in our assay has not been evaluated.

The results of the present study confirm our earlier report (12), in that the induction of aryl hydrocarbon hydroxylase is greater in cultures of proliferating 3T3 cells than in cultures maintained in the confluent or nonproliferating state. However, it now appears that the inducibility of this enzyme is more closely related to a metabolic factor than to cell proliferation, since, in cultures treated with ara-C, levels of BA-induced activity equivalent to those of proliferating cells could be achieved. The peak of induced aryl hydrocarbon hydroxylase activity obtained after refeeding confluent cultures may be related to the ability of refed cells to generate factors needed for maximal activation of the microsomal oxygenase system. For example, it has been shown that the ratio of NADH to NAD is 3–4 times lower in stationary than in log phase 3T3 cells (29). Conversely, an inhibitory factor may be removed by refeeding the cells.

Since the inducibility of aryl hydrocarbon hydroxylase is not strictly dependent on cell proliferation, it is difficult to explain the observation that peak inducibility always occurred at about the time the cultures reached confluence. It may be that the cells require a period of time to become established after replating or that they have to go through one or two division cycles before they can become maximally inducible. In these experiments the cells were harvested with EDTA rather than proteolytic enzymes, but some cell surface perturbation may occur and may need to be repaired before the cells become fully responsive.

The very rapid rate of decay for maximally induced aryl hydrocarbon hydroxylase has not been observed in the primary or secondary cultures from fetal tissues. The $t_{1/2}$ of this enzyme in cultured fetal hamster cells is 3.3 ± 1.2 hr (11) and the $t_{1/2}$ in cultured fetal rat hepatocytes is 10.5 ± 3.6 hr (30), whereas the $t_{1/2}$ of the rapid component in 3T3 cells is 15–30 min (Fig. 6). Moreover, Nebert and Gelboin (11) reported a 2–3-hr lag in the onset of decay of aryl hydrocarbon hydroxylase activity, which is not seen in maximally induced 3T3 cells. Thus our data suggest that control of degradation of aryl hydrocarbon hydroxylase components may be different in the two systems and that stabilization of the enzyme activity may play a greater role in its induction in 3T3 cells than in the cell lines studied by Nebert and his colleagues. They have suggested (30) that, since the rate of degradation of aryl hydrocarbon hydroxylase in fetal rat hepatocytes is slow compared to the rate of doubling of the enzyme activity (3 hr), modification of the rate of synthesis is the critical parameter during microsomal oxygenase induction. In 3T3 cells aryl hydrocarbon hydroxylase activity is also doubled about 2–3 hr after addition of BA, but decay of the enzyme is a much more rapid process.

The biphasic nature of the decay curve suggests that components of the system are degraded at different rates. There is precedent for the speculation that components of the microsomal oxygenase system have biphasic decay rates. For example, Levin and Kuntzman (31) have shown a fast-phase and a slow-phase rate of decay for hemoprotein from liver microsomal CO-binding particles. There is also evidence that different forms of the cytochrome P-450 system (i.e., P-450 and P_1-450) contribute to the level of aryl hydrocarbon hydroxylase activity *in vivo* (5). However, the predominant form of CO-binding cytochrome in fetal hamster secondary cultures, fetal rat liver primary cells, and hepatoma-derived cell lines appears to be P_1-450 (22). It remains to be seen whether cultured 3T3 fibroblasts contain both forms of CO-binding cytochrome, and which form(s) is induced by BA in these cells.

The "stabilization" of induced aryl hydrocarbon hydroxylase activity by the continued presence of inducer (Fig. 7) is not a constant feature of cultured cell systems. It has been reported for fetal rat hepatocyte cultures (15) but was not observed in hamster fetal cell cultures (16). The decay of aryl hydrocarbon hydroxylase in 3T3 cells in the absence of BA appears to involve protein degradation, and reinduction requires both RNA and protein synthesis. The time necessary to reachieve maximal aryl hydrocarbon hydroxylase activity

after readdition of BA to cultures in which activity has decayed is much less than that required for the initial induction (Fig. 3).

There are a number of possible mechanisms by which mycoplasmal contamination could cause a decreased enzyme activity in cell cultures. Cytotoxicity would be an obvious explanation. However, we observed a decrement in aryl hydrocarbon hydroxylase activity before any cytopathic effect was apparent. Other possible mechanisms for the inhibition of aryl hydrocarbon hydroxylase induction by mycoplasmal contamination include (a) mycoplasmal binding or metabolism of the inducer, (b) a decreased rate of protein synthesis due to amino acid depletion (32), (c) an increased rate of protein degradation caused by mycoplasmal proteases (33), (d) an alteration of DNA and RNA metabolism due to competitive utilization of intracellular nucleotide pools and the action of mycoplasmal deoxyribonucleases and ribonucleases (34, 35), (e) the formation of injurious metabolic products, such as hydrogen peroxide (36), or (f) an effect on other metabolic processes in the cell, such as energy-generating systems.

The results of this study have important implications for studies on the induction of aryl hydrocarbon hydroxylase activity in nonhepatic cells. If fibroblast cultures from human and animal sources are to be used to investigate aryl hydrocarbon hydroxylase activity and its susceptibility to chemical carcinogens, it will clearly be necessary to control the variability introduced by differing culture conditions.

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