Interaction of polycyclic aromatic hydrocarbons with the aryl hydrocarbon hydroxylase enzyme system in normal and neoplastic C3H mouse embryo cell lines.

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INTERACTION OF POLYCYCLIC AROMATIC HYDROCARBONS WITH THE ARYL HYDROCARBON HYDROXYLASE ENZYME SYSTEM IN NORMAL AND NEOPLASTIC C3H MOUSE EMBRYO CELL LINES

by

Malcolm Mark Feeley

A Thesis Submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the requirements for the Degree of Master of Science at The University of Windsor

Windsor, Ontario, Canada
1983
to my parents
ABSTRACT

INTERACTION OF POLYCYCLIC AROMATIC HYDROCARBONS WITH THE ARYL HYDROCARBON HYDROXYLASE ENZYME SYSTEM IN NORMAL AND NEOPLASTIC C3H MOUSE EMBRYO CELL LINES

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Two mouse embryo cell lines, C3H/10T1/2 cl-8 and its polycyclic aromatic hydrocarbon (PAH)-transformed counterpart C3H/10T1/2 MCA cl-16, were analyzed with respect to their growth characteristics and aryl hydrocarbon hydroxylase (AHH) enzyme system (AHH activity and Ah receptor binding properties).

It was found that the malignant 10T1/2 MCA cl-16 had a longer generation time, a higher saturation density and quantitative polypeptide differences (as measured by sodium dodecyl sulphate/polyacrylamide gel electrophoresis) when compared to its non-neoplastic progenitor, 10T1/2 cl-8.

When the AHH enzyme system of the two cell lines was examined, it was discovered both cell lines possessed inducible AHH activity as measured by the standard fluorometric assay with cl-8 having approximately 1.7 times greater maximally-induced activity compared to cl-16 (5.0 vs. 2.8 picomoles 3-hydroxy benz(a)pyrene formed per milligram protein per minute respectively). Of four PAH tested, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benz(a)anthracene (BA) both induced AHH activity while 3-methylcholanthrene (MCA) and di-benz(a,h)anthracene (DBA), two potent carcinogens, did not.
In addition, the two non-inducing PAH had the ability to totally suppress AHH induction by TCDD and BA when co-administered in a dose-dependent manner.

When the regulatory gene product of the Ah locus, the Ah cytosolic receptor, was examined by sucrose density gradient centrifugation, it was found that both MCA and DBA could inhibit the binding of $^3$H-TCDD to the receptor, the initial step in the induction of AHH activity. Furthermore, neither $^3$H-MCA or $^3$H-DBA could specifically bind to the same receptor. This afforded a possible explanation for their inability to induce AHH activity in cl-8 and cl-16. In comparison, when the two latter PAH were tested in another cell line which possessed inducible AHH activity, Hepa cl-9, it was found that both MCA and DBA could induce AHH activity and specifically bind to the Ah receptor.

These results suggest that there is a possible defect in the Ah receptor found in the two 10T$^3$ cell lines which results in a decreased affinity towards normally-inducing PAH. This decreased affinity may result in the inability of certain PAH to bind specifically to the receptor thus preventing AHH induction. The reason(s) why the two non-inducing PAH still retain the ability to suppress AHH induction by other active PAH may involve unknown cellular controls over the entire AHH induction process that when resolved could provide further insight into the role of the AHH system in chemical carcinogenesis.
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LIST OF ABBREVIATIONS

AHH  aryl hydrocarbon hydroxylase
BA   benz(a)anthracene
B(a)P benzo(a)pyrene
BSA  bovine serum albumin
cl-8 C3H/10T1 cl-8
cl-16 C3H/10T2 MCA cl-16
DBA  dibenz(a,h)anthracene
DMSO dimethylsulfoxide
DNA  deoxyribonucleic acid
DPM disintegrations per minute
DTT  dithiothreitol
fmol femtomole (10^{-15} mole)
GPO_4 0.25 M potassium phosphate/ 30% glycerol pH 7.5
3-OH BP 3-hydroxy benzo(a)pyrene
MCA 3-methylcholanthrene
mg  milligram
mRNA messenger ribonucleic acid
NADPH nicotinamide adenine monophosphate-reduced
NEB nuclear extraction buffer
NM  nanomolar (10^{-9} molar)
PAH polycyclic aromatic hydrocarbons
PBS phosphate buffered saline, 0.8% NaCl pH 7.4
pmol picomole (10^{-12} mole)
SDG sucrose density gradients
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
Tris tris (hydroxymethyl)aminomethane
ug microgram
uL microlitre
uM micromolar (10⁻⁶ molar)
CHAPTER I
INTRODUCTION

It has recently been estimated that approximately 85% of all human cancers can be attributed to direct or indirect association with various physical and chemical environmental agents (Fishbein, 1980). Historically, polycyclic aromatic hydrocarbons (PAH) comprise one of the most extensively-studied groups of chemical carcinogens known to occur both naturally and artificially in the environment (Weisburger and Williams, 1980; Sall et al., 1940).

The majority of PAH occur as both incomplete pyrolysis products of organic material (i.e. forest fires) and as the transformation byproducts of the petroleum industry released directly or indirectly after mechanical processing (Stegeman, 1981). Common PAH generally found in the environment are shown in figure I.

Numerous epidemiological studies now relate dermal exposure, exposure through inhalation and exposure by ingestion as the three main routes of chemical contact in humans which may lead to occupational- or environmental-induced neoplasias (Wynder and Gori, 1977). Accordingly, PAH have been isolated from foods (Miller and Miller, 1976; Hirono, 1981), natural plant material (Emerole et al., 1982), water, sediments (Stegeman, 1981), polluted air (Nettesheim, 1981) and human tissues (Obana et al., 1981). With a relatively
FIGURE I

Benz(a)anthracene (BA)

Dibenz(a,h)anthracene (DBA)

Benzo(a)pyrene (BP)

3-Methylcholanthrene (MCA)

7,12-Dimethylbenz(a)-anthracene (DMBA)

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Examples of Polycyclic and Heterocyclic Aromatic Hydrocarbons
constant supply of PAH confronting most humans daily, it was inevitable that the carcinogenic risk posed by these chemicals be elucidated.

Benzo(a)pyrene (BP, Fig. I) represents the most widely studied member of the PAH group. Because BP is primarily found in association with numerous other PAH (primarily BA, DBA, DMBA and MCA, Fig. I), measurements of human exposure to PAH generally utilize BP as an index compound (Bidbord, 1976). BP, as with most PAH, is found in motor vehicle emissions, char-broiled foods, as a constituent of tobacco smoke and as an industrial contaminant. Because of its widespread occurrence, BP is considered omnipresent throughout all areas of the environment.

The initial studies into the metabolism of PAH showed that when these chemicals were applied to intact mouse skin or transformable rodent cells in culture, certain fractions became covalently bound to DNA, RNA and proteins (Boyland, 1950). This, coupled with the fact that metabolic activation of PAH was necessary before this covalent binding could occur, led to extensive studies into PAH metabolism by liver microsomal preparations. In 1964, Brookes and Lawley demonstrated that radiolabelled PAH when applied to mouse skin became covalently bound to nucleic acid fractions. But the focal point of their investigation was that the extent of covalent binding to DNA correlated directly with the carcinogenic potential of the hydrocarbon. These findings combined
with further metabolic studies led to the PAH electrophile theory as proposed by J.A. Miller in 1970 (see review by Weinstein et al., 1978). Briefly, the theory stated that the majority of PAH were found in a precarcinogenic state and underwent chemical decomposition or cellular metabolism to generate highly reactive electrophiles. This ultimate carcinogenic form of PAH could then establish covalent bonds with nucleophilic residues (DNA) and these covalent interactions were necessary events in the carcinogenic process.

Studies with NADPH-dependent microsomal enzyme systems led to the discovery that a monooxygenase was involved in the conversion of various PAH to metabolites that bound covalently to nucleic acids. Subsequently, various specific components of this monooxygenase enzyme system have been isolated and purified (Kouri et al., 1980). The postulated organization of this monooxygenase system responsible for the metabolism of PAH is illustrated in figure 2.

The functional components of this endoplasmic reticulum-located oxygenase system include: 1) the substrate and oxygen binding site, hemeprotein Cytochrome P₄₅₀ and 2) the electron-shuttling system, NADPH-Cytochrome c reductase. The ability for this system to accept a wide variety of endogenous and exogenous (xenobiotics) compounds led to the acronym MSMO (mixed function multi-substrate monooxygenase). A simplified reaction involving this enzyme system and PAH
Fig. 2.

Postulated scheme for NADPH-dependent monoxygenase(s) responsible for the metabolism of hydrophobic xenobiotics (PAH). Modified from Nebert et al., 1975.
can be summarized as follows: 1) the initial substrate, PAH, has one atom of molecular oxygen inserted across an aromatic double bond with reducing equivalents being supplied by NADPH; 2) following the formation of this epoxide-PAH, a hydration reaction can occur, catalyzed by epoxide hydrase, to form a dihydrodiol of the initial PAH; 3) the dihydrodiol-PAH can again be reoxygenated by the Cytochrome P₄₅₀-dependent monooxygenases resulting in the formation of the extremely electrophilic diol epoxide-PAH; 4) this compound, along with the epoxide-PAH, can then be either conjugated with glutathione and excreted (the reaction being catalyzed by glutathione S-transferase) or be covalently bound to cellular macromolecules, including DNA (Grover, 1982) (Fig. 3).

The specific enzyme system utilizing Cytochrome P₄₅₀ and metabolizing PAH is commonly referred to as the aryl hydrocarbon hydroxylase (AHH) system and has been found to be active in numerous animal and human tissues (Weinstein et al., 1978).

AHH, along with several other hepatic enzyme systems, has been found to be substrate inducible; that is, substrates for the enzyme system cause increases in enzyme activity levels (Conney et al., 1957). This "responsiveness" to induction by PAH has been observed in most tissues of a variety of inbred mouse strains while both basal and induced levels of AHH are decreased or altogether absent in other strains. This has
Fig. 3. The metabolic activation of BP by enzymes of the endoplasmic reticulum.

The aryl hydrocarbon hydroxylase (AHH) monooxygenase enzyme system inserts one atom of O₂ into the hydrophobic PAH, BP. The resulting epoxide then becomes a substrate for a hydration reaction catalyzed by epoxide hydrase. The newly formed dihydrodiol of BP can be once again re-oxygenated by the NADPH-dependent AHH system to form the electrophilic BP-diol epoxide. This reactive metabolite may then bind to various cellular macromolecules such as DNA, RNA and protein resulting in the initiation of the cell to a tumorigenic state.
prompted researchers to designate murine species responsive or nonresponsive in terms of their reaction to AHH induction (Nebert and Jensen, 1979).

The ability to induce AHH activity seems to be under genetic control, specifically the Ahh or Ah gene locus, with a single gene difference determining responsiveness (Heidelgerger, 1975; Grover, 1982; Kouri et al., 1980; Nebert et al., 1977). The actual mechanism of AHH induction by PAH has now been determined to be regulated by cytosolic receptor protein(s) designated the Ah receptor, which recognizes a wide variety of polycyclic (including halogenated) aromatic hydrocarbons (Legraverend et al., 1982; Hitchins et al., 1980) (Fig. 4).

After combining with PAH, the PAH-receptor complex has been shown to translocate from the cytoplasm to the nucleus where it apparently "stimulates" the transcription of structural genes of the Ah locus causing the appearance of AHH-specific messenger RNAs (Kahl et al., 1980; Okey et al., 1980; Legraverend et al., 1982; Tukey et al., 1982). The increase in AHH-specific mRNAs is then correlated to elevated monooxygenase activity which results in increased metabolism of PAH. Of all mouse strains tested, approximately 50% show AHH induction while nonresponsive strains seem to either quantitatively or qualitatively lack the regulatory gene product of the Ah locus, the Ah cytosolic receptor (Poland and Glover, 1975) (Fig. 5).
Fig. 5. Hypothetical scheme for the involvement of the Ah receptor molecule for non-responsiveness of AHH activity (or induction). Receptor sequence of structural gene(s) does not recognize Ah receptor alone.
Because of the role that the AHH enzyme system has in the metabolic activation of numerous PAH precarcinogens, there has been an increased effort to relate AHH activity levels to increased susceptibility to numerous chemical-induced cancers. The area of lung cancer and its link to cigarette smoking has received a great deal of interest within the past 10 years. Tobacco smoke has been found to contain numerous PAH, particularly BP and BA, which are present in microgram quantities (Nettlesheim et al., 1981). Consequently, AHH activity has been compared between smokers and non-smokers who have or have not developed various lung cancers. Preliminary results now suggest that smoking does induce AHH activity in pulmonary tissue and higher levels of AHH inducibility are associated with an increased susceptibility to bronchogenic carcinomas (Jett et al., 1978; Cantrell et al., 1973). Furthermore, the mere presence of the Ah receptor is now associated with various disease states associated with receptor ligands (TCDD and PAH) (Dencker and Pratt, 1981) and possibly an increased risk towards chemically-induced neoplasias. While a correlation between inducible AHH activity and increased risk to PAH tumorigenesis is generally accepted (Kouri, 1976), what factors, both intrinsic and extrinsic, affect the PAH-metabolizing system? It is now clear that humans are exposed daily to subthreshold concentrations of numerous chemicals and various PAH, few of which are or can be considered carcinogenic or mutagenic at such low single doses.
Because of the long latency or developmental periods needed for the majority of cancers (3–20 years) (Fishbein, 1976), it would seem that carcinogenesis can be considered a prolonged, multistage process involving a number of modifying factors. Early after the carcinogenic capability of PAH in experimental animals was ascertained, numerous modifiers of PAH-induced neoplasias were found that could both inhibit or increase PAH tumorigenicity (Falk et al., 1964). Specifically, PAH analogues, both natural and synthetic, were discovered that could alter the mutagenic potential of various known carcinogens. But it was unknown whether-or-not these PAH actually altered monooxygenase activity at the level of metabolism or at the level of gene expression (Weinstein, 1980). The potential for interaction of several different PAH at once within a single system is highly probable if the total number of weakly and non-carcinogenic compounds which are undetectable in current bioassays are taken into account.

One system, somatic cell culture, has proven invaluable in the study of the multiple interactions of both carcinogenic and non-carcinogenic chemicals and their role in the malignant transformation of cells. Since the initial discovery by Berwald and Sachs (1965) that PAH could oncogenically transform Syrian hamster embryo cells in culture, methods for the concise manipulation of the multiple stages of progression that are thought to occur after the initial exposure of a cell to a carcinogen have developed. The nature of the transformed
genotype as correlated to phenotypic expression could be compared to both the non-transformed cell and eventually to the whole animal system. Also, interactions of multiple carcinogens at sub-threshold doses could be examined both at the metabolic and genome levels and applied to research on the identification and quantitation of carcinogenic and/or mutagenic agents in the environment.

In particular, the C3H/10T\(\text{I}\) mouse embryo cell system has been beneficial in both the identification of environmental carcinogens, including PAH (Reznikoff et al., 1973), co-carcinogens (Mondal et al., 1976) and tumor promoters, substances which both alter the frequency and latency period of tumor formation (Nesnow et al., 1981).

In this present study, C3H/10T\(\text{I}\) embryo cell lines are used to investigate: 1) the phenotypic changes associated with the PAH-induced malignant transformation process and 2) interactions of PAH with the AHH enzyme system including the Ah receptor in normal versus neoplastic cells.
CHAPTER II

CELL CULTURE TECHNIQUES

A. Introduction

The initial phenomenon of cell culture malignant transformation by carcinogens (Sachs and Berwald, 1968) has allowed for the analysis of the different progressive stages thought to occur after the initial exposure of a cell to a cancer-causing agent. Individual transformed cells can be isolated after carcinogen treatment of the parent population and compared with respect to their various growth parameters and genetic composition in an attempt to understand the possible mechanisms of action of a particular carcinogen.

B. Materials and Methods

1) Cell Growth Conditions

The culture medium used for both 10T½ cl-8 and 10T½ MCA cl-16 was Basal Medium Eagle (BME) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 50 µg/ml gentamycin sulphate. Cells were seeded at varying concentrations per tissue culture flask or dish that allows for confluency to be reached within 72 hours after seeding. At or near confluency, the medium is removed, the cells washed with warm citrate saline and then incubated for 4 minutes with 0.05% Trypsin to collect the cells. Following trypsinization, the cells are pelleted by centrifugation at 1000 x g for 10 minutes; resuspended in fresh medium and split 1:4 into new
flasks or dishes. At regular intervals, healthy cultures of both cl-8 and cl-16 in the exponential growth phase were collected and cryopreserved in medium consisting of 80% BME, 10% heat-inactivated FCS and 10% dimethylsulfoxide (DMSO). Testing of cell viability following freeze-down periods revealed that a slow freeze at -20°C for two hours followed by a quick freeze to -195°C in liquid nitrogen gave the highest cell viability (20-35%).

2) Growth Curve Analysis

The generation times and saturation densities of cl-8 and cl-16 were studied by first obtaining a growth curve for each cell line. At time 0, a predetermined number of cells (1x10^5) were seeded in 60 mm tissue culture dishes. At 24 hour intervals, 2 plates from each cell type were collected and counted to determine the net increase of cells. This procedure was continued until the saturation density was reached and cell death started to occur (Fig. 6).

3) Karyotype analysis of 10T^6 cl-8 and 10T^6 MCA cl-16

Chromosomal studies were undertaken of both cl-8 and cl-16 to see if any gross cytogenetic changes could be attributed to the malignant transformation process. The basic technique for obtaining chromosome spreads employs the use of colcemide to arrest the cells in metaphase. Usually a small flask (25 cm^2) of cells in mid-exponential growth was incubated for 2-3 hours with 1.0 ug colcemide per ml of medium in Hank's balanced salt solution. Following the incubation
Fig. 6

Growth curves of $10T^4$ cl-8 (○-○) and $10T^4$ MCA cl-16 (●-●)

Cells were seeded at $1 \times 10^5/60$ mm tissue culture dish. At 24 hour intervals, two dishes per cell line were collected by trypsinization and counted in a haemacytometer. Each graph value represents the average of two dish counts.
period, the cells were collected by trypsinization and centrifugation and then placed in a 37°C hypotonic solution composed of distilled water and 0.075 M KCl for approximately 12 minutes. After centrifugation to collect the cells, the hypotonic solution was replaced with cold methanol:acetic acid (3:1) and the cells fixed for 20 minutes at room temperature. Air-dried slides were then prepared and stained with fresh Giemsa for 10 minutes (Fig. 7). As seen in Table 2, a fairly unstable chromosome number exists between both cell lines with only 25-35% of viewed spreads fitting the modal chromosome number. As with most established cell lines, both cl-8 and cl-16 have progressed to the hypertetraploid state when compared to the original C3H mice.

4) Densitometer tracings of Coomassie Blue-stained gels of 10× cl-8 and 10× MCA cl-16.

Quantitative polypeptide composition was compared between the two C3H cell lines by sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, cells to be subjected to SDS-PAGE were grown to near confluency, collected by scraping with a rubber policeman and washed three times with citrate saline. Following this, the cells were lysed by homogenization with sterile glass beads and the whole cellular protein sample collected by centrifugation. After protein determination, aliquots of protein sample were run on a SDS/PAGE buffer system as described by Laemmli (1970). At the
Table 1. Generation Times and Saturation Densities of 10T½ cl-8 and 10T½ MCA cl-16

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Generation Time² (hours)</th>
<th>Saturation Density³ (10⁴ cells/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10T½ cl-8</td>
<td>15.4 ± 0.5</td>
<td>0.85 ± 0.3</td>
</tr>
<tr>
<td>10T½ MCA cl-16</td>
<td>20.6 ± 0.5</td>
<td>1.13 ± 0.5</td>
</tr>
</tbody>
</table>

1. Calculated from growth curves (Fig. 6).
2. Taken from exponential phase of growth curve.
3. Averaged from 60 + 100 mm dishes.

Table 2. Karyotype analysis of 10T½ cl-8 and MCA cl-16

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Chromosome Range</th>
<th>Modal No.¹</th>
<th>% Spreads with² marker chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>10T½ cl-8</td>
<td>75-82</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>10T½ MCA cl-16</td>
<td>70-78</td>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>

1. Modal chromosome number calculated from 50 spreads.
2. Presence of large, acrocentric marker chromosome (Fig. 7).
end of the electrophoretic run, the gels were removed, fixed in acetic acid: methanol (3:1) and stained with Coomassie Blue. Individual gel lanes were then removed and scanned at 260 nm on a Gilford gel scanner (Fig. 8). Scanning procedures for both cell types were identical as was the protein concentration used in the SDS-PAGE (55 μg).

C. Establishment of Cell Lines

1) C3H/10T^5 cl-8 and C3H/10T^5 MCA cl-16

The two mouse embryo lines used for the majority of experiments, 10T^5 cl-8 and 10T^5 MCA cl-16, were initially developed by Reznikoff et al. in 1973. The original clone, cl-8, was initiated from a pool of C3H mouse (Heston strain) embryos collected after the thirteenth day of gestation (Reznikoff et al., 1973). After the primary culture period of 150 days (passage 20), the resulting cells were cloned following the schedule of transfer of Todaro and Aaronson for BALB/313 cells. One clone, cl-8, was selected and extensively characterized. The nomenclature for 10T^5 cl-8 was coined from the culture procedure used during the early developmental phase, namely, seeding the cells at $0.5 \times 10^5$ cells/60 mm tissue culture dish and subculturing every 10 days (10T^5).

When 10T^5 cl-8 cells reach confluency, a flat monolayer of epithelioid-like cells is formed (Fig. 9) which are extremely sensitive to postconfluence inhibition of cell division. 10T^5 cl-8 cells are not tumorigenic when inoculated into immuno-
Fig. 8

Densitometer tracings of Coomassie Blue-stained Gels of 10T\(\frac{1}{2}\) cl-8 and 10T\(\frac{1}{2}\) MCA cl-16 cellular protein samples.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (PAGE) samples of whole cellular protein extracts were scanned at 260 nm on a Gilford gel scanner. The appearance of new peaks unique to one of the cell lines is indicated by an "X". Increased peak height corresponds to increased protein concentration at that particular area of the gel. Both PAGE samples were run with 55 ug of protein according to the procedure of Laemmli (1970).
10T\(^4\) cl-8

43,000 m.w.
(actin)

10T\(^4\) MCA cl-16
suppressed mice (Table 3) and there is no record of spontaneous transformation up to 150 passages in culture.

The second C3H mouse embryo cell line employed, 10T1/2 MCA cl-16 was developed by the process of malignant transformation. The establishment of this cell line resulted from the knowledge that numerous polycyclic aromatic hydrocarbons (PAH) could cause varying degrees of morphological transformation which were expressed as a change in the sensitivity to postconfluence inhibition of cell division. This change resulted in the development of transformed foci which can be described as tightly packed cells of numerous layers and densities which continue to divide after confluency has been reached (Fig. 11). The cell line in question, 10T1/2 MCA cl-16, was developed by the initiation of transformed foci by the PAH, 3-methylcholanthrene (MCA). Briefly, cl-8 cells were exposed to varying concentrations of MCA (15 ug/ml medium) for 24 hours. This ensures that each cell has undergone at least one complete cell cycle in the presence of the PAH. Following the exposure period, the medium containing the PAH was removed and replaced with fresh control medium twice a week or every three days. At confluency, cells from visible foci were isolated, cloned and checked for neoplastic growth potential by two criteria: 1) growth on soft agar (or loss of anchorage dependency [Green, 1980]) and 2) tumorigenicity or malignancy when inoculated into syngeneic mice (Table 3). For the sake of clarity, morphological transformation is equated to malignant,
Table 3. Growth of 10T\(\text{1/2}\) cl-8 and 3-Methylcholanthrene-transformed clones on soft agar and in isologous mice\(^1\).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Colonal Growth on(^2) soft agar</th>
<th>Mice with tumors(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10T(\text{1/2}) cl-8</td>
<td>0.5 ± 0.2</td>
<td>0/2</td>
</tr>
<tr>
<td>10T(\text{1/2}) MCA cl-15</td>
<td>1869 ± 147</td>
<td>2/2</td>
</tr>
<tr>
<td>10T(\text{1/2}) MCA cl-16</td>
<td>1753 ± 142</td>
<td>2/2</td>
</tr>
</tbody>
</table>

1. From Reznikoff et al., 1973 and Jones et al., 1976.
2. Colonies containing greater than 8 cells; 10\(^5\) cells seeded/60 mm dish on 0.3% agar.
3. 2\(\times10^6\) cells injected sub-cutaneously into preirradiated C3H male mice, 3-6 weeks old; latency period of 2 weeks to 6 months for fibrosarcoma development.
Fig. II. Developing transformed foci of $10T_2$ cl-8
neoplastic or oncogenic transformation.

Both cl-8 and cl-16 were generous gifts from Dr. C. Heidelberger of the U.S.C. Cancer Centre and were cloned upon receipt to give homogeneous populations. The morphological transformability of 10T^6 cl-8 was verified by the production of transformed foci upon treatment with the carcinogenic PAH, dimethylbenz(a)anthracene (DMBA) (Fig. 11).

Unlike cl-8 cells, 10T^6 MCA cl-16 does not readily form a flat monolayer of cells but tends to grow in irregular patterns with clumping (Fig. 10). When the generation times and saturation densities of the two cell lines were compared (Table 1), it was found that although cl-16 had a somewhat slower generation time when compared to cl-8 (20.6 vs. 15.5 hours respectively), its saturation density was appreciably higher (1.13 x 10^4 vs. 0.85 x 10^4 cells/mm tissue culture dish). The apparent loss of sensitivity to postconfluence inhibition of cell division not only results in a saturation density higher than that of the non-transformed cl-8 but it has been correlated to the development of cancer in vivo in other cell systems (Pollack and Teebor, 196y).

2) Hepa cl-9

The mouse hepatoma cell line used as a control for this study has been previously characterized with respect to AhH induction by PAH and receptor binding (Okey et al., 1980). Hepa was originally derived from a transplanted hepatoma from a C57L/J mouse and was a generous gift from Dr. O. Hankinson.
of U.C.L.A. Culture conditions for Hepa cl-9 were identical to those of cl-8 except minimal essential medium (MEM) was used.

D. DISCUSSION

Growth characteristics of 10T4 cl-8 and its MCA-transformed clone, 10T4 MCA cl-16, were examined as well as karyotyping analysis and quantitative polypeptide composition. It was found that the malignant-transformation process had created a cell line distinct from the original in 4 major areas. Under standard growth conditions, 10T4 MCA cl-16 had both a longer generation time and a higher saturation density than its non-transformed progenitor, 10T4 cl-8. The apparent loss of sensitivity to post-confluence inhibition of cell division exhibited by cl-16 resulted in its ability to form colonies on soft agar (anchorage-independent growth). This in turn has been correlated to the ability to form in vivo neoplasias (Jones et al., 1976; Kamei, 1982) as demonstrated by cl-16. Definite morphological changes are evident from Fig.10 and these changes can be correlated to reductions in cell-surface and cell-cell interactions which usually accompany neoplastic transformation (Vasiliev and Gelfand, 1982). Karyotype comparison between both cl-8 and cl-16 revealed both a difference in the modal chromosome number and the presence of a large acrocentric marker chromosome common to all cl-16 spreads but totally absent from cl-8 spreads (Table 2). Whereas this chromosome study is superficial,
previous results with PAH-induced morphological transformation have shown various chromosomal aberrations and sister chromatid exchanges to occur (Gehly et al., 1982). Also, the progression to the malignantly-transformed state may involve just a single gene change or mutation which then leads to a magnitude of phenotypic changes (Shih and Weinberg, 1982).

Finally, quantitative polypeptide composition differences were examined between the two cell lines. While certain minor changes are evident from the gel scans (Fig. 8), the major difference seems to be the almost uniformly higher protein concentrations associated with corresponding gel bands. These results are consistent with previous results (Leavitt et al., 1980) which have shown at least 32% of the genes expressing abundant polypeptides are modulated quantitatively as a consequence of neoplastic transformation.

In conclusion, treatment of 10T½ cl-8 with the PAH, 3-methylcholanthrene (MCA), has resulted in the formation of an entirely different cell line which is considered neoplastic by two criteria, growth on soft agar (anchorage independent growth) and the ability to form in vivo malignancies. Also, 10T½·MCA cl-16 shows various phenotypic and genotypic changes which can be associated with the morphological transformation process.
CHAPTER III

ARYL HYDROCARBON HYDROXYLASE ENZYME ASSAY

Previous results with animal studies have shown that the majority of PAH exist in a precarcinogenic state and rely on metabolic activation by a substrate-inducible monooxygenase, the aryl hydrocarbon hydroxylase (AHH) enzyme system, for both their detoxification and excretion from biological systems and to obtain the ultimate carcinogenic form of PAH, a diol epoxide (Fig. 3) (Lagonbach et al., 1981; Weisburger and Williams, 1980; Selkirk, 1977; Grover, 1974). The formation of this highly mutagenic proximate carcinogen of PAH can then lead to covalent interactions by the diol epoxide with DNA, RNA and chromatic proteins (Zythovicz et al., 1980) resulting in a block in DNA synthesis in vivo (Rinaldy et al., 1982) and the initiation of cells to a malignant or transformed state which may ultimately lead to the development of neoplasias (Ashurst and Cohen, 1981). Accordingly so, the metabolites of PAH are always more carcinogenic than the parent compounds (Slaga et al., 1978).

A. Materials and Methods

Since its initial development (Nebert and Gelboin, 1968), the fluorometric AHH assay has proven to be a sensitive and reliable method to show the responsiveness of mammals to PAH inducers. Extraction of the metabolites from the index
PAH, benzo(a)pyrene (BP), has shown that levels of one metabolite, 3-hydroxy BP (3-OH BP), reflect total BP metabolism levels by the AHH system. Utilizing the fluorescent properties of 3-OH BP, the current assay offers a feasible method for the study of PAH metabolism by whole animals, humans and cells in culture (Gielash and Nebert, 1971).

Cells to be assayed for AHH activity were initially seeded at $1 \times 10^6$ cells/100 mm tissue culture dish. When the dishes neared confluency (72 hours after seeding), the medium was removed, the cells washed with warm citrate saline and supplied with fresh medium containing the appropriate PAH dissolved in dimethylsulfoxide (DMSO). Concentrations of DMSO up to 0.1% did not significantly effect AHH induction or cell viability. The cells were exposed to the PAH inducers for 18 hours as previous results have shown that AHH activity in Hepa cl-9, 10T1/2 cl-8 and 10T1/2 MCA cl-16 is linear up to that time period. After the 18 hour incubation period, the medium with the PAH was decanted off and the cells collected by washing and scraping with cold phosphate-buffered saline (PBS, pH 7.4, 0.8% NaCl). This and all subsequent procedures were performed at 4°C. Following 3 washes with cold PBS to remove any traces of medium and PAH-inducer, the cells were pelleted by centrifugation at $1000 \times g$ for 10 minutes and resuspended in cold glycerol phosphate buffer (pH 7.5). Approximately 1.25 mg of the resuspended total cellular protein sample (100 uL) was
then added to 900 µL of prechilled assay buffer (AHH assay buffer: 0.1 M MgCl₂, 0.2 M Tris buffer (pH 7.5), 3.6 × 10⁻⁴ M NADH, 3.6 × 10⁻⁴ M NADPH, 10 µM BSA). The enzymatic reaction was then initiated by the addition of 2 mM BP to each flask, vortexing the samples to distribute the cells and placing the samples in a 37°C shaker bath for 20 minutes. After the incubation period, the reaction was terminated by the addition of 3.0 ml cold hexane:acetone (3:25:1) to each flask. The reaction flasks were then reincubated for an additional 10 minutes in the shaker bath to facilitate the extraction of the major fluorescent products (3-OH BP). After 10 minutes, the organic phase of each flask was then removed and added to 3.0 ml 1 N NaOH, which after vortexing should contain the phenolic reaction metabolites. The 3.0 ml of NaOH was then transferred to cuvettes and read at 396 nm excitation and 522 emission in a Turner model 430 spectrofluorometer which had previously been calibrated with a purified 3-OH BP standard (Fig. 12). One unit of AHH activity is defined as that amount of enzyme catalyzing per minute at 37°C the formation of hydroxylated product causing the fluorescence equivalent to 1 pmol of 3-hydroxy benzo(a)pyrene. All PAH concentrations tested were in duplicate with blanks consisting of cells exposed to solvent alone (DMSO) and reaction flasks determining fluorescence caused by BP alone. Protein concentration was determined by the method of Bradford (1976) using the BioRad protein assay kit.
B. 3-Hydroxy Benz(a)pyrene Standard Curve

In order to quantify the F units obtained from the spectrofluorometer readings, a 3-OH BP standard curve was prepared (Fig. 12).

Briefly, a crystallized amount of purified 3-OH BP (a gift from Dr. A. Okey) was dissolved in acetone and methanol to give a final concentration of $2 \times 10^{-4}$ M. From this stock solution, dilutions ranging from $2 \times 10^{-4}$ to $2 \times 10^{-8}$ M were prepared and added to reaction flasks containing AH activity assay buffer and heat-inactivated cell protein samples. The samples were then treated as normal AH assay reaction mixtures, extracted and read in 1 N NaOH.

As shown by Fig. 12, a straight line is obtained but with a lack of linearity at low 3-OH BP concentrations. Because of the difficulty in extraction of low 3-OH BP quantities, the lower limits of the AH assay were established as 0.1 pmol 3-OH BP/mg protein/minute.

C. Results

1) Hepa cl-9

The mouse hepatoma cell line was tested with a variety of PAH at different concentrations. As shown by Table 4, all PAH tested induced AH activity to some degree. In fact, even weakly-carcinogenic PAH (benzo(e)pyrene) were also able to induce AH activity (unreported results). The chlorinated PAH, TCDD, induced maximal levels of AH activity at the lowest
Fig. 12

3-Hydroxy Benzo(a)pyrene Standard Curve

The relative fluorescence (F Units) of 3-hydroxy benzo(a)-pyrene as measured by a Turner Model 430 Spectrofluorometer was plotted against 3-hydroxy benzo(a)pyrene concentration in picomoles (10^{-12}). See "Aryl Hydrocarbon Hydroxylase Assay-Materials and Methods". Standard Error (S.E.) = ± 2.5 F Units.
Table 4. Aryl Hydrocarbon Hydroxylase (AHH) activity in Hepa cl-9

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Inducer (^1)</th>
<th>Concentration</th>
<th>Specific Activity (^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepa clone 9</td>
<td>BA</td>
<td>10 (\mu)M</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 (\mu)M</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>10 (\mu)M</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>1 (\mu)M</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>0.1 (\mu)M</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>1 nM</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>0.1 (\mu)M</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0.1 %</td>
<td>0.3</td>
</tr>
</tbody>
</table>

1. BA = Benz(a)anthracene  
DBA = Dibenz(a)anthracene  
MCA = 3-Methylcholanthrene  
TCDD = 2,3,7,8-Tetrachlorodibenzo-p-dioxin  
DMSO = Dimethylsulfoxide (control)

2. Expressed as picomoles 3-hydroxy Benzo(a)pyrene formed per milligram protein per minute.
concentration, 1 nM, while other PAH such as BA and DBA showed inducing optimas at or near 1 nM. TCDD has been shown to be an extremely stable compound in vivo with an approximate half life of up to 17 days in rats and it is known to have a $30,000 \times$ greater AHH inducing capability than MCA when tested in mice at the above mentioned concentrations. The Hepa cell line's response to a wide variety of PAH may stem from the fact that its organ of initiation, liver, is the main source a PAH detoxification in vivo and thus would be expected to have high inducible AHH activity in responsive animals (Niwa et al., 1975).

2) 10T1/2 cl-8

The non-transformed embryo cell line, 10T1/2 cl-8, had been previously shown to be sensitive to malignant transformation by PAH. As seen by Table 5, cl-8 does possess inducible AHH activity towards TCDD and BA but not DBA and MCA. Once again, TCDD induced maximal activity and at the same concentration as in Hepa cl-9. The fold-inducibility, however, differs by almost 6 times with Hepa having the greater inducible activity. On the basis of AHH activity, the lack of responsiveness towards MCA and DBA illustrated by 10T1/2 cl-8 could not be explained at this point in the investigation. Both PAH have been shown to be capable of malignantly transforming cl-8 and are known inducers of AHH activity (in vivo and in vitro) in other murine species (Reznikoff et al., 1973).

The low total AHH activity levels as seen in 10T1/2 cl-8 cells
Table 5.  AHH activity in 10T<sub>1/2</sub> cl-8 and 10T<sub>1/2</sub> MCA cl-16

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Inducer</th>
<th>Concentration</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10T&lt;sub&gt;1/2&lt;/sub&gt; cl-8</td>
<td>BA</td>
<td>10 uM</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>10 nM</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>10 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>1 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>100 nM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>10 nM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>1 nM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>10 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>1 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>100 nM</td>
<td>n.a.</td>
</tr>
<tr>
<td>10T&lt;sub&gt;1/2&lt;/sub&gt; MCA cl-16</td>
<td>BA</td>
<td>10 uM</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>1 uM</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>10 nM</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>1 nM</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>10 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>1 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>100 nM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>10 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>MCA</td>
<td>1 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>10 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>1 uM</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>DBA</td>
<td>100 nM</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

1. All specific activity values (pmole 3-OH BP/mg protein/min.) expressed minus solvent (DMSO) controls; n.a. = no activity detected over solvent control.
may in fact reflect the lack of optimal or peak levels of AHH induction as seen in fetal or pre-partum animals. Cytochrome P₂₄₅₀ content, and therefore specific AHH activity levels, do not reach a maximum in most laboratory animal species until 1-2 weeks post-partum (Kahl et al., 1980) while human AHH induction levels do not peak until 6-8 months after birth (Neims, 1982). Whether or not we are involved with an ontogenetic explanation to the quantitative differences as seen between fold-inducibility levels of AHH activity in the Hepa versus 10T½ cell lines may only be explained by following the developmental course of Ah locus gene expression in the C3H mouse. It would seem that even low levels of AHH activity are capable of contributing to the morphological transformation process as seen in 10T½ cl-8 as repression of AHH induction in this cell line leads to a decrease in malignant transformation caused by PAH (Marquardt et al., 1974). Also, a point of interest is the clonal stability in AHH activity levels as exhibited by 10T½ cl-8 (unreported results) which has previously been shown to be absent in the Hepa cell line (Legraverend et al., 1982).

3) 10T½ MCA cl-16

The rationale for investigating the AHH activity properties of 10T½ MCA cl-16 were two-fold; first, as AHH induction is intimately involved with the PAH-morphological transformation process, it was desirable to know whether-or-
not an altered response to PAH would be detected following
initiation of the cell line to the tumorigenic state; second,
as the transforming agent, MCA, did not induce AHH activity in
the parent cl-8, it was wondered if 10T1/2 MCA cl-16 still or
once possessed a responsiveness to MCA. Results in Table 3 show
that cl-16 responded identically to the 4 PAH tested as did cl-8;
TCDD and BA were able to induce AHH activity while DBA and MCA
did not. The lack of response to MCA by cl-16 was unexpected as
metabolism of PAH is thought to be necessary for both cyto-
toxicity and mutagenicity (Brookes et al., 1975). The approx-
imate two-fold lower AHH activity as shown by cl-16 may be
either as a result of quantitative changes in gene expression
which accompanied neoplastic transformation or Ah locus changes
involving AHH induction partially observed in the cl-8 cell
line. It may be possible that the latter explanation is more
plausible as it has been observed that less than one percent of
gene transcription products are altered during transformation in
other similar cell lines (Leavitt and Moyzis, 1978). The total
lack of response by either cl-8 or cl-16 to MCA has also been
observed in Swiss mouse 3T3 cells which progressively lose their
responsiveness as a function of both cell age and culture
conditions (Gehly et al., 1979).

4) Suppression of AHH Induction

As the simultaneous administration of both carcino-
genic and weak or non-carcinogenic PAH to whole animals is re-
ported to have a differing effect on the overall tumor response (either a tumor promoting or a tumor inhibiting effect) (Slaga et al., 1979), the possibility of a similar response in terms of induced AH activity by MCA and DBA in both 10T1/2 cl-8 and cl-16 was investigated. This was accomplished by simultaneously administering both an inducing PAH (TCDD or BA) along with a non-inducing PAH (MCA or DBA) to the cells and monitoring either the possible potentiating or inhibitory effect on AH activity induction. Table 6 and 7 illustrate the outcome of the co-administration of non-inducing PAH with TCDD or BA. Equimolar concentrations of both MCA and DBA when applied simultaneously with the maximum inducing concentration of BA, were able to suppress almost 100% of control specific activities in cl-8 and cl-16 (10 μM BA alone). The data with TCDD induction may suggest that this particular compound has a much greater affinity for AH-specific components than BA as a 10,000-fold greater concentration of either competitor still does not completely suppress TCDD-induced AH activity. Whereas in the majority of animal systems it is unclear whether PAH modifiers of PAH carcinogenesis do so by altering the metabolism of the carcinogens or by competitive interaction or antagonism (DiGiovanni and Slaga, 1981), it would seem clear that in this system, the non-inducing PAH are acting at the level of induction or gene expression. Weak or non-carcinogenic PAH have been previously shown to have an inhibitory effect on the overall tumor
| Inducer and competitor added simultaneously to solvent DMSO to fresh medium | Solvent controls (basal activity) subtracted from specific activity values |
|---|---|---|
| 0.6 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 2.7 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 3.8 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 5.9 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 7.0 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 8.1 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 9.2 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |
| 10.3 | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA | 0.1 mM DBA |

Table 6: Suppression of Vfl activity induction by non-inducing PAH
<table>
<thead>
<tr>
<th>Solvent controls (basal activity)</th>
<th>Inducer and competitor added simultaneously to solvent DMSO to fresh medium</th>
<th>Specific activity</th>
<th>Concentration</th>
<th>Competitor</th>
<th>Concentration</th>
<th>Inducer</th>
<th>Concentration</th>
<th>Competitor</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>10 μM</td>
<td>DBA</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
<td>BC</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
</tr>
<tr>
<td>100 μM</td>
<td></td>
<td>DBA</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
<td>BC</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
</tr>
<tr>
<td>10 μM</td>
<td></td>
<td>MCA</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
<td>BC</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
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<tr>
<td>1 μM</td>
<td></td>
<td>MCA</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
<td>BC</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
</tr>
<tr>
<td>0.1 μM</td>
<td></td>
<td>MCA</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
<td>BC</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
</tr>
<tr>
<td>0.01 μM</td>
<td></td>
<td>MCA</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
<td>BC</td>
<td>10 μM</td>
<td>BA</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

Table 7. Suppression of NRII activity induction by non-inductive P4H
response in whole animal systems when applied before or at the same time as known carcinogens (Gelboin et al., 1970) while the synthetic PAH, 7,8-benzoflavone, is known to suppress AHH induction both in vivo and in vitro (Baird and Diamond, 1976). This inhibition of AHH induction has then led to a decrease in the binding of reactive metabolites to DNA and therefore a reduction in PAH tumorigenesis (Coombs et al., 1981).

While it is evident that MCA and DBA are inhibiting the induction of AHH activity by TCDD and BA, the actual mechanism by which these PAH can both not induce AHH activity and compete for induction is thought to involve some other aspect other than Cytochrome P₄₅₀ interaction.

D. Discussion

The AHH activity in three cell lines was examined by a standard fluorometric assay utilizing the major metabolite of benzo(a)pyrene as a standard. Hepa cl-9, which had previously been shown to possess inducible AHH activity towards TCDD (Okey et al., 1980), has now been shown to be similarly responsive to BA, DBA and MCA. The two mouse embryo cell lines, 10T¹ cl-8 and its malignantly-transformed counterpart, 10T¹ MCA cl-16, also possess inducible AHH activity but only towards TCDD and BA. Also the fold-inducibility levels differ between the Hepa and 10T¹ cell lines with Hepa cl-9 showing up to 6 times higher activity. The two non-inducing PAH in the


10T¹/² cells, MCA and DBA, still however retain the ability to suppress AhH induction by both TCDD and BA. If the actual mechanism of suppression is hypothesized to involve the regulatory gene product of the Ah locus, the Ah-cytosolic receptor, a detailed study of PAH binding to this receptor in 10T¹/² cl-8 and 10T¹/² MCA cl-16 (as compared to Heps cl-9) may explain this phenomenon. Moreover, the results from these studies may provide insight into the mechanism(s) of PAH inhibition of tumorigenesis in whole animal systems.
CHAPTER IV
Cytosolic Receptor Assay

A. Introduction

The ability for certain microsomal (endoplasmic reticulum) monooxygenases to undergo induction by PAH has been established for over 20 years (Nebert et al., 1981). But the molecular mechanisms leading to AH induction after a PAH enters a cell remained relatively unclear. Drawing on the biochemical model which explains the interaction between steroid hormones with target tissues through a receptor molecule, Guenthner and Nebert (1977) have hypothesized a similar sequence of events to explain enzyme induction (aryl hydrocarbon hydroxylase) by small molecular weight compounds of extracellular origin (PAH).

Their theory briefly states that: 1) an inducer, PAH, enters a cell and binds with high affinity to a specific cytosolic receptor protein; 2) this PAH-receptor complex can then undergo nuclear translocation where it may interact with one or more nuclear sites; 3) following this DNA-receptor interaction, specific mRNA synthesis is stimulated and protein synthesis results. Experimental evidence from genetically "responsive" and "non-responsive" rodent species now clearly supports this hypothesis and further states that AH induction is regulated by an Ah cytosolic receptor (Okey et al., 1979). The initial studies on whole animals have now expanded to cell
culture systems. Work in this laboratory in collaboration with Dr. A. Okey has established a definite correlation between AHH induction and the temperature-dependent cytosol-to-nucleus translocation of the receptor complex (Okey et al., 1980; Heintz et al., 1981).

It now appears that in the locus of genes controlling AHH induction (Ah locus), there is at least one regulatory gene and 5 structural gene products involved in the induction sequence of events (Lang and Nebert, 1981). Induction of AHH activity has been shown to involve de novo synthesis of Cytochrome P450, a critical part of the NADPH-dependent monooxygenase system (Fig. 2) crucially involved in the binding and metabolism of PAH (Kitchin and Woods, 1978; Imai, 1982). The regulatory gene product of the Ah locus is now identified as the Ah cytosolic receptor and "responsiveness" to AHH (Cytochrome P450) induction by PAH is regulated by its presence (Hannah et al., 1981). Finally, it is now accepted that transcriptional control and quantity of mRNA rather than gene duplication or rearrangement or some enhanced polypeptide initiation or elongation is the mechanism of AHH induction (Tukey et al., 1981).

B. Materials and Methods

The cytosolic receptor protein associated with AHH induction has been examined by a number of different techniques including Sephadex G-100 gel permeation chromatography (Mukhtar et al., 1982), isoelectric focusing in polyacrylamide
gels and DNA-cellulose chromatography (Gustafsson et al., 1980), activated dextran charcoal binding assay (Nabert and Guenthner, 1977) and sucrose density gradient centrifugation following dextran charcoal adsorption (Okey et al., 1980). Of these procedures, our lab has found the latter to be a rapid and sensitive method to test for both the presence and follow the cytosol-to-nucleus translocation of the Ah receptor in a variety of established cell lines.

Cell lines to be examined for the presence of the Ah receptor were grown under identical culture conditions as described for the AhH assay (see AhH assay, Materials and Methods). Following the standard growth period of 72 hours after seeding or when the cells neared confluency, the cells were washed with cold PBS and fresh serum-less medium containing the appropriate concentrations of radioligand and/or competitor supplied. Unless otherwise indicated, the standard treatment to show specific binding of a PAH to the receptor was incubation at 4°C for 1 hour in the presence of the radioligand and 100-1000-fold excess of unlabelled PAH. This temperature treatment prevents the temperature-dependent nuclear translocation of the receptor-PAH complex (Okey et al., 1980) and slows the metabolism of the PAH inducer.

Previous investigations with various PAH inducers have shown that there is no requirement for the metabolism of the PAH inducer during AhH induction, which simply suggests that the parent PAH is the best ligand to use for binding studies (Kano et al., 1977). When the detection of specific binding
and nuclear translocation is desired, the cells are once again incubated for 1 hour at 4°C then transferred to a 37°C incubator for 20 minutes. This time period at 37°C allows for detectable translocation to occur with minimal metabolism or receptor complex dissociation. Cell viability is not affected by the medium or temperature conditions used.

Following the prescibed temperature treatments, the cells are collected by scraping the plates with a rubber policeman and washed 3 times with PBS by centrifuging the cell suspension at 1000×g for 10 minutes. As with the AHV assay, this and all subsequent procedures were performed at 4°C. For Hepa c1-9 receptor assays, the cells from 20-100mm plates were combined to form one sample while 10T$	ext{r}$ cell line assays required the cells from 30-100mm plates.

Cell pellets obtained were then resuspended in 2 ml of HEDG buffer (standard homogenization buffer, HEDG: 25 mM Hepes, 1.5 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol and 10% glycerol by volume, pH 7.6) and homogenized with a Polytron PT-10 at setting no. 4 for 3-20 second bursts. Microscopic examination during the homogenization was used to ensure optimal cellular disruption. The total cellular homogenate was then centrifuged at 1000×g for 10 minutes. The pellet from this centrifugation constituted the nuclear fraction and was either stored or discarded, depending on the temperature treatment, while the supernatant was removed and
centrifuged at 105,000×g in a Beckman 60 Ti rotor. The supernatant from this fraction represented the cytosolic sample which was run on sucrose density gradients.

Nuclear Fractions

When nuclear translocation was allowed to occur, the pellet resulting from the 1000×g centrifugation of the cellular homogenization was washed 3 times by resuspending in 2 ml of HEDG buffer and centrifuging at 1000×g for 10 minutes. The pellet from the final wash was then resuspended in 1 ml nuclear extraction buffer (NEB: HEDG buffer + 0.4 M KCl), homogenized with the Polytron at setting no. 5 for 2-20 second bursts and allowed to incubate for 1 hour on ice to extract the nuclear receptor. The total extract was then centrifuged under the same conditions as the cytosolic extract (105,000×g for 60 minutes) with the resulting supernatant comprising the nuclear fraction. The total extraction procedure for both the cytosol and nuclear fractions is summarized in Fig. 13 (as well as in vitro labelling procedure).

Sucrose Density Gradient (SDG) Analysis

Following the preparation of the nuclear and cytosolic extracts, non- or loosely-bound radioligand was removed prior to SDG analysis by treatment with dextran charcoal. One ml of cytosolic or nuclear extract was added to the charcoal pellet obtained from centrifugation of 1 ml of dextran-charcoal-HEDG buffer solution, vortexed and incubated for 15 minutes on ice.
Fig. 13  Summarized Procedure for Ah Receptor Assay
(In vivo labelling)

Expose cells from 20-30 plates to label +/- competitors in culture

collect cells

Wash 3 x PBS
Centrifuge @ 1000xg 10 min.

Pellet

Resuspend in 2 ml HEDG buffer.
Homogenize with Polytron
Centrifuge @ 1000xg, 10 min.

Supernatent (cytosol)

Centrifuge @ 105,000xg, 1 hr.

Pellet (nucleus)

Wash 3 x with HEDG buffer.
Centrifuge @ 1000xg, 10 min.
Resuspend in 1 ml NEB buffer
Homogenize with Polytron
Incubate 1 hr. @ 4°C.
Centrifuge @ 105,000xg for 1 hr.

In vitro labelling

In vivo = cells exposed to label in culture.
In vitro = cytosolic and nuclear fractions from cells previously unexposed to label.
The resulting solution was then centrifuged at 4000×g for 10 minutes to remove the dextran charcoal and unbound label and 300 μL of the cytosol or nuclear fraction applied to pre-chilled 5-20% linear sucrose gradients (total volume = 4 ml). The sucrose gradients were prepared by layering 1 ml of 5-20% sucrose made with either HEDG buffer (cytosol samples) or HEDG buffer plus 0.4 M KCl (nuclear samples) in ascending order (1 ml 20% sucrose + 1 ml 15% sucrose etc.) onto chilled Beckman nitrocellulose tubes. The total gradient sample plus cytosol or nuclear aliquot comprised 4.3 ml. The gradients were then centrifuged at 48,000 rpm in a Beckman SW 60 rotor (εw = 235,000) for 16 hours at 2°C. After centrifugation, 20 200-μL fractions were then collected from each gradient tube on an ISCO model 640 gradient fractionator and the radioactivity per fraction (DPM) determined by liquid scintillation counting. An internal sedimentation marker, (14C) methylated bovine serum albumin (BSA)(4.6S), was added to sample gradients to determine approximate sedimentation values for radioactive peaks (Martin and Ames, 1961). Protein concentrations were determined by the method of Bradford (1976) using bovine gamma globulin as a protein standard.

C. Ah Cytosolic Receptor Binding in Hepa cl-9

The binding of (3H)-TCDD in Hepa-1 cells to a cytosolic receptor which then undergoes cytosol-to-nucleus translocation has previously been reported (Okey et al., 1980).
This receptor, now correlated to AH induction, was found to be sensitive to proteolytic enzymes, such as trypsin and chymotrypsin, but not to RNases or DNases. Similar results are shown by figures 14 and 15 for Hepa cl-9. As this clone of the original Hepa culture exhibits AH inducibility (Table 4), it was expected that this cell line would possess the regulatory gene product of the Ah locus, the Ah cytosolic receptor. Similar investigations into other Hepa clones (mutant) that have exhibited varying degrees of AH inducibility (total lack of response or a decrease in fold-inducibility levels) have shown a breakdown had occurred in some stage of the induction process (decreased or no detectable Ah receptor levels, defective nuclear translocation of the inducer-receptor complex, non-recognition of receptor complex by nucleus etc.) (Legraverend et al., 1982).

As demonstrated by figure 14, the 7-8 S peak on the cytosol gradient is completely eliminated by a 100-fold excess concentration of TCDD, a known inducer of AH activity in Hepa cl-9. This is one of the establishing criteria for specific binding to the Ah receptor; only known inducers of AH activity will compete for binding. In figure 15, no peak was detected. This was expected since the translocation process is temperature dependent. Figures 16 and 17 also illustrate this point; when the cells were allowed to incubate for 20 minutes at 37°C, a 6 S peak was detected in the nucleus. The difference in sedimentation coefficients between the cytosol
Fig. 14

- - - 1 nM ($^3$H)-TCDD.

0-0 1 nM ($^3$H)-TCDD + 0.1 μM TCDD.

Cells were labelled in culture for 1 hour at 4°C.

Fig. 15

Nuclear extracts of above cells
Fig. 16

- - 1 nM $^{3}H$-TCDD.
0-0 1 nM $^{3}H$-TCDD + 0.1 nM TCDD.

Cells were labelled in culture for 1 hour at 4°C then transferred to a 37°C-incubator for 20 minutes.

Fig. 17

Nuclear extracts of above cells.
and nuclear peaks is attributed to the presence of 0.4 M KCl in the nuclear gradients. When cytosolic extracts are run on nuclear gradients (cytosolic gradients + 0.4 M KCl), the main peak of radioactivity sediments at approximately 6 S.

The ability for only known inducers of Ah activity to eliminate the $^{3}$H-TCDD binding peak was also established (figures 18 and 19). TCDD, DBA and MCA, which are all capable of inducing Ah activity in Hepa cl-9 (Table 4), eliminate the specific binding peaks in both the cytosol and nucleus. The possibility therefore existed that both DBA and MCA would bind specifically to the Ah receptor and translocate to the nucleus, a prerequisite for inducing Ah activity. As seen in figures 20 and 21, $^{3}$H-MCA did bind specifically to the Ah receptor and translocate to the nucleus. Figure 20 also shows a non-specific peak in the 4-6 S region of the gradient (fractions 5-7) which did not translocate. This same non-specific region has been found in rat liver cytosol (Tierney et al., 1980) and is thought to be an area of high capacity, low affinity binding not associated with Ah induction.

Figures 22 and 23 also show that $^{3}$H-DBA binds specifically to the same receptor as TCDD and MCA and undergoes nuclear translocation. Also, a 2,500-fold excess concentration of the corticosteroid, dexamethasone, had no effect on binding or the translocation event. This indicates that Ah induction is not mediated through a glucocorticoid receptor which are known to occur in hepatoma-derived cell lines (Okoy et al., 1980).
Fig. 18

- - 1 nM $^3$H-TCDD
O--O 1 nM $^3$H-TCDD + 0.1 uM TCDD
X--X 1 nM $^3$H-TCDD + 0.1 uM MCA
Δ--Δ 1 nM $^3$H-TCDD + 0.1 uM DBA

Cells labelled in vivo for 1 hr. @ 4° then placed in an incubator @ 37° for 20 minutes.

Fig. 19

Nuclear extract of above cells; same conditions.
Fig. 20
-• 4 nM $^{3}H$-MCA
0-0 4 nM $^{3}H$-MCA + 0.1 uM TCDD
X-X 4 nM $^{3}H$-MCA + 0.1 uM MCA

Cells incubated in vivo for 1 hr. @ 4° then placed in an incubator @ 37° for 20 minutes.

Fig. 21 Nuclear extract of above cells; identical conditions.
Fig. 22

- - 4 nM $[^3]H$-DBA
0-0 4 nM $[^3]H$-DBA + 10 uM Dexamethasone
X-X 4 nM $[^3]H$-DBA + 0.1 uM MCA

Cells labelled in vivo for 1 hr. @ 4°C then placed in an incubator @ 37°C for 20 minutes.

Fig. 23
Nuclear extract of above cells; same conditions.
Also, phenobarbital and pregnenolone, which induce forms of Cytochrome P-450 distinct from P1-450, had no effect on either cytosolic or nuclear \((^{3}H)\)-PAH binding peaks (results not reported).

Discussion

TCDD, DBA and MCA were all able to compete for the binding of \((^{3}H)\)-TCDD to the Ah receptor in Hepa cl-9. As a consequence, both DBA and MCA were also tested for their ability to specifically bind to the receptor and translocate into the nucleus. The results obtained suggest all three PAH bind to the same receptor and can undergo nuclear translocation. These findings are supported by previous results which have shown \((^{3}H)\)-TCDD and \((^{3}H)\)-MCA both bind to the same cytosolic Ah receptor in mouse and rat hepatic cytosols and induce AHH activity through this receptor (Okey and Vella, 1982). An importance should be placed on the fact that we have shown that other PAH follow the same route of interaction with the Ah receptor as TCDD. The majority of binding studies involve the binding of \((^{3}H)\)-TCDD alone and as a consequence, the Ah receptor has not been correctly identified when binding studies with PAH other than TCDD are undertaken (Zytkovicz, 1982).
D. Ah Cytosolic Receptor Binding in 10T\textsubscript{1/2} cl-8 and 10T\textsubscript{1/2} MCA cl-16.

The aryl hydrocarbon hydroxylase and Ah receptor assay results from experimentation with the highly-inducible Hepa cl-9 cell line suggested that any PAH capable of displacing \textsuperscript{3}H-TCDD from the Ah receptor would also induce AHH activity through a specific interaction with the same receptor. This ability to displace \textsuperscript{3}H-TCDD from the Ah receptor should then result in the suppression of AHH induction by TCDD. This event is not measurable in Hepa at the AHH assay level due to the concomitant induction of AHH activity by the displacing agent. In the transformable C\textsuperscript{3}H/10T\textsubscript{1/2} cell line, cl-8, we have discovered the situation where two PAH which do not induce AHH activity, DBA and MCA, still retain the ability to suppress TCDD- and BA-induced AHH activity. As only known inducers of AHH activity were considered capable of displacing \textsuperscript{3}H-TCDD from the Ah receptor (Bigelow and Nebert, 1982) and therefore suppressing TCDD-induced AHH activity, it was decided that the binding characteristics of the Ah cytosolic receptor in C\textsuperscript{3}H/10T\textsubscript{1/2} cl-8 and 10T\textsubscript{1/2} MCA cl-16 be investigated.

Procedures for the in vivo labelling of the Ah receptor in cl-8 and cl-16 were identical to those for Hepa cl-9. The saturable peak femtomole values were calculated by first obtaining the DPM specifically bound per milligram protein and converting to femtomoles by specific activity values of the label (see Appendices, III).
The initial receptor assays with the two C3H cell lines were designed to show whether-or-not (3H)-TCDD would bind specifically to the same receptor as in Hepa. Figures 24 to 27 illustrate the time- and temperature-dependent movement of specifically bound label (DPM/mg protein) from the cytoplasm to the nucleus in both cl-8 and cl-16. In vitro labelling of nuclear extracts of either cell line, as well as in Hepa, detected no native nuclear receptor. Also, if nuclear samples from cells labelled in culture, where translocation had been allowed, were reincubated with extra amounts of (3H)-TCDD, no increase in the specific peak size was detected (results unreported). In cl-8 (figures 24 and 25), maximum binding occurred in the cytoplasm when the cells were held at 0°C for 1 hour to prevent any translocation. When the incubation temperature was raised to 37°C, an approximately 6 S peak was observed in the nucleus within the first 10 minutes. Within 40 minutes, the majority of the label in the cells was associated with the nuclear fraction. The reason why greater DPM values are found in the nucleus than observed at any time in the cytoplasm may be explained by two theories. First, we may be observing receptor recycling within the 40 minute 37°C period or second, maximum binding to the cytosolic receptor requires the temperature activation of the receptor. As in the case of the estrogen receptor, active substrate and physiological temperatures are required before translocation
Fig. 24

- 1 nM ($^3$H)-TCDD 0° - 1 hr.

- 0 1 nM ($^3$H)-TCDD 0° - 1 hr. + 37° - 40 min.

- 0 1 nM ($^3$H)-TCDD 0° - 1 hr. + 37° - 10 min.

- 0 1 nM ($^3$H)-TCDD + 1.0 uM TCDD 0° - 1 hr.

Cells were labelled for the indicated times and temperatures before receptor was extracted.

**Saturable-peak femtomole values per mg protein**

- 0-0 = 128

- 0-0 = 80

- 0 = 37

- 0 = 0

Fig. 25

Nuclear extracts of above cells.

**Saturable-peak femtomole values per mg protein**

- 0-0 = 0

- 0-0 = 149

- 0 = 42

- 0 = 0
Fig. 26

- - 1 nM \( \left( ^{3}H \right) \)-TCDD 0° - 1 hr.
0-0 1 nM \( \left( ^{3}H \right) \)-TCDD 0° - 1 hr. + 37° - 40 min.
X-X 1 nM \( \left( ^{3}H \right) \)-TCDD 0° - 1 hr. + 37° - 10 min.
Δ-Δ 1 nM \( \left( ^{3}H \right) \)-TCDD + 1.0 uM TCDD 0° - 1 hr.

Cells were labelled for the indicated times and temperatures before receptor was extracted.

Saturable-peak femtomole values per mg protein

- - 82
0-0 101
X-X 23
Δ-Δ 0

Fig. 27

Nuclear extracts of above cells.

Saturable-peak femtomole values per mg protein

- - 0°
0-0 127
X-X 29
Δ-Δ 0
can occur (de Boer and Notides, 1981). However, no measurable change in the sedimentation values of the Ah receptor—which may have indicated a transformation step—were observed. As the receptor is detectable in the nucleus within 5 minutes at 37°C (unreported results), it is more likely we are seeing some form of receptor recycling in its active form under these non-physiological doses of TCDD. Previously, nuclear binding was found to decrease after 1 hour at 37°C in Hepa-1 cl-7 (Okey et al., 1980), suggesting a possible movement of the receptor out of the nucleus in an active or inactive form.

The same cytosol-to-nucleus translocation was seen in 10T^1 MCA cl-16 (figures 26 and 27). Increased cytosolic binding after 40 minutes at 37°C when compared to 1 hour at 0°C may once again suggest receptor recycling or an inherent error in reporting fmol/mg protein rather than fmol/μg DNA.

The approximate two-fold difference in maximally induced AHH activity levels between the two C3H cell lines was not explained by the initial receptor data. It appears under similar conditions, both cl-8 and cl-16 translocate almost equal amounts of (3H)-TCDD into the nucleus. However, adequate levels of cytosolic receptor with nuclear translocation does not ensure inducible AHH activity, as demonstrated with another hepatoma-derived cell line, HTC (Okey et al., 1980). The possibility exists that in the 3-methylcholanganthrene-altered cl-16 the inducer-
receptor complex in cl-16 is not fully "recognized" by the genome regions responsible for AHH induction. This may be a result of some unknown ontogenetical repression or more likely as a result of the malignant transformation process which may have altered AHH induction in cl-16 at the pre-transcriptional level.

As reported earlier (Tables 6 and 7), MCA and DBA were able to suppress TCDD and/or BA induction of AHH activity in both cl-8 and cl-16. When the ability of these two non-inducing PAH to displace \(^{3}H\)-TCDD from the A\(h\) receptor was examined, a suitable explanation for the above-mentioned phenomenon was discovered (Figures 28-31). 1000-fold excess concentrations of the non-inducing PAH, as well as TCDD, seemed to quantitatively eliminate \(^{3}H\)-TCDD binding to the A\(h\) receptor. These concentrations of competitors needed to compete for binding correspond approximately to the amounts of DBA and MCA required to suppress AHH activity in cl-8 or cl-16.

The ability for DBA and MCA to displace \(^{3}H\)-TCDD from the A\(h\) receptor suggested that they in turn should be able to induce AHH activity. As it had been demonstrated that they in fact do not, it was decided to investigate their ability, or lack of, to bind to the receptor and hopefully discover why they were no longer able to induce AHH activity. Figures 32 to 35 show a lack of detectable receptor in the nucleus of both cl-8 and cl-16 cells following treatment with \(^{3}H\)-DBA.
Fig. 28

- - 1 nM ($^3$H)-TCDD
0-0 1 nM ($^3$H)-TCDD + 1.0 uM TCDD
X-X 1 nM ($^3$H)-TCDD + 1.0 uM MCA
Δ-Δ 1 nM ($^3$H)-TCDD + 1.0 uM DBA

Cells were exposed to label and competitors for 1 hour at 0°C and then transferred to a 37°C incubator for 20 minutes to allow for translocation.

Fig. 29

Nuclear extracts of above cells.
Fig. 30

- 1 nM (3H)-TCDD
- 0-0 1 nM (3H)-TCDD + 1.0 uM TCDD
- X-X 1 nM (3H)-TCDD + 1.0 uM MCA
- Δ-Δ 1 nM (3H)-TCDD + 1.0 uM DBA

Cells were labelled in vivo for 1 hour @ 0° and then transferred to a 37° incubator for 20 minutes to allow for translocation.

Fig. 31

Nuclear extracts of above cells.
Fig. 32

- -  10 nM $^{3}$H-DBA
O-O  10 nM $^{3}$H-DBA + 1.0 uM TCDD
X-X  10 nM $^{3}$H-DBA + 1.0 uM MCA

Cells were labelled in vivo for 1 hr. @ 4°C and then placed in an incubator at 37°C for 20 min.

Fig. 33

Nuclear extracts of above cells; identical conditions.
Fig. 34

- 10 nM \(^3\text{H}\)-DBA
- 0-0 10 nM \(^3\text{H}\)-DBA + 1.0 μM TCDD
- X-X 10 nM \(^3\text{H}\)-DBA + 1.0 μM MCA

Cells labelled in vivo for 1 hr. @ 4\(^\circ\) then transferred to a 37\(^\circ\) incubator for 20 min.

Fig. 35

Nuclear extracts of above cells.
This would explain why DBA would not induce AHH activity but not how it was still able to successfully displace \((^3\text{H})\)-TCDD from the Ah receptor. Then \((^3\text{H})\)-MCA was tested in both cell lines (figures 36-37), again no receptor was detected in the nucleus. \(^3\text{H}\)-MCA did however bind with high capacity to an approximately 5 S region of the cytosolic gradients. This binding had been previously discussed in Hepa cell results and is considered non-specific or not associated with the AHH induction process due to the inability of known inducers of AHH activity to effectively compete for this binding. Further investigation of this peak (figures 40 and 41) revealed that unlike the Ah receptor, it was sensitive to neither heat or proteolytic enzyme denaturation.

Discussion

The inability for either MCA or DBA to induce AHH activity in 10T\(^6\) cl-8 and 10T\(^6\) MCA cl-16 was explained by their lack of specific binding to the Ah receptor. However, both non-inducing PAH still retained the ability to displace \((^3\text{H})\)-TCDD from the Ah receptor and thus suppress AHH induction by TCDD. Two possible explanations for this behavior are: 1) the Ah receptor in cl-8 and cl-16 is modified or mutated so recognition of normally inducing PAH such as DBA and MCA does not take place, or 2) binding does occur to some area of the receptor by DBA and MCA and is not detected by our assay procedure. Various unknown endogenous factors which regulate
Fig. 36

- - 4 nM \( ^3H \)-MCA
  O-O 4 nM \( ^3H \)-MCA + 0.4 \( \mu \)M TCDD
  X-X 4 nM \( ^3H \)-MCA + 0.4 \( \mu \)M MCA

Cells were labelled in vivo for 1 hr. @ 4°C then transferred to a 37°C incubator for 20 min.

Fig. 37

Nuclear extracts of above cells under identical conditions.
Fig. 38

- - 4 nM (3H)-MCA
0-0 4 nM (3H)-MCA + 0.4 mM TCDD
×-× 4 nM (3H)-MCA + 0.4 mM MCA

Cells were labelled for 1 hour @ 4°C and then transferred to a 37°C incubator for 20 minutes.

Fig. 39

Nuclear extracts of above cells.
Fig. 40  ●-●  4 nM (3)H-MCA, control.
      O-O  4 nM (3)H-MCA, 450°-20 min.
      X-X  4 nM (3)H-MCA, Trypsin.
Cells were incubated with 4 nM (3)H-MCA in culture for 1
hour at 4°C, following which cytosolic extracts were pre-
pared and either placed in a water bath at 450°C for 20
minutes or treated with 100 μg Trypsin for 1 hour at 4°C.
Control and treated portions were then applied to sucrose
gradients as described under "Receptor Assay - Materials
and Methods".

Fig. 41  ●-●  1 nM (3)H-TCDD, control.
      O-O  1 nM (3)H-TCDD, 450°-20 min.
      X-X  1 nM (3)H-TCDD, Trypsin.
Identical procedure as in fig. 40 but cells initially
incubated in culture for 1 hour at 4°C with 1 nM (3)H-TCDD.
the overall Ah induction process and other sustained pleiotropic responses associated with the Ah gene locus (induction of ornithine decarboxylase activity, UDP-glucuronosyltransferase activity and glutathione-S-transferase activity) (Poland and Knutson, 1982) may, in fact, regulate the ultimate binding of (3H)-PAH to the Ah receptor.

E. Scatchard Analysis of 3H-TCDD binding to the Ah receptor in 10T1/2 cl-6 and 10T1/2 MCA cl-16.

Originally designed for the analysis of steroid hormone receptors (Scatchard, 1949), the Scatchard plot analysis has been expanded to include the Ah cytosolic receptor and other cytosolic proteins capable of binding PAH (Schaeffer et al., 1981; Bresnick et al., 1980; Zykovicz, 1982). (3H)-PAH binding to a saturable receptor can be summarized by a graph showing the total amount of specifically-bound receptor divided by the total unbound PAH (B/U) on the ordinate axis plotted against the bound receptor (B) on the abscissa. In endocrine terminology, B/U is usually expressed as bound over free or B/F (Clark and Peck, 1977).

Cells to be subjected to Scatchard analysis were grown identical to those to be analyzed for the Ah receptor. The only difference in procedure is that the cells are previously not exposed to radiolabel in culture and are not subjected to any temperature treatments to limit or allow translocation.
Following the preparation of the cytosolic extract as per receptor assay procedures, aliquots of the cytosolic protein (1.5-2 mg protein/ml) were then incubated with concentrations of (3H)-TCDD ranging from 0.1-5.0 nM for 1 hour at 4°C with gentle mixing every 15 minutes. Following the incubation period, 50-μL samples of each group were added to 5 ml of cocktail and counted to determine the total radioactivity or binding (T) before dextran charcoal treatment. This total binding (T) was expressed as femtomeses ligand bound/mg cytosolic protein. After dextran charcoal adsorption to remove any unbound ligand, the samples were then applied to sucrose gradients in the same manner as described previously (see Ah receptor assay methods).

Specifically-bound ligand was calculated as the difference between (3H)-TCDD binding alone and (3H)-TCDD binding in the presence of 100-fold excess unlabelled TCDD. From before charcoal readings and the calculation of total specific binding (B), the amount of unbound ligand or that quantity removed by dextran-charcoal treatment can be calculated.

\[ T = \text{total binding before charcoal (fmol/mg protein)} \]
\[ B = \text{total specifically-bound ligand (fmol/mg protein)} \]
\[ U = \text{total unbound ligand (T-B) (fmol/mg protein)} \]

Figures 42 and 43 illustrate the saturability of (3H)-TCDD binding to a cytosolic fraction of cl-8 and cl-16. Maximum binding was recorded as 130.3 and 10.9 fmol/mg protein for
Fig. 42
Saturability of $^3$H-TCDD binding to Ah receptor of 10T$^4$ cl-8 and 10T$^4$ MCA cl-16.

Fixed amounts of cytosolic protein (1.5 mg protein) from cells previously unexposed to label were incubated in vitro with varying concentrations of $^3$H-TCDD. Specific binding, B, was calculated as the difference between total and nonspecific binding and plotted vs. $^3$H-TCDD concentration.

Fig. 43
Saturability of $^3$H-TCDD binding to Ah receptor of 10T$^4$ MCA cl-16.
Fig. 44

Scatchard analysis of $^3$H-TCDD binding to the Ah cytosolic receptor of 10T½ cl-8.

Specific binding was plotted according to Scatchard (1949) with amount of ligand bound divided by free ligand (B/F) plotted vs. amount of specific binding (B). The amount of binding sites per mg cytosolic protein, $B_{max}$, was calculated from the intercept abscissa. Slope = $1/K_d$, $K_d = 1.45$ nM and $B_{max} = 127.01$ fmol/mg protein.

Total number of receptor sites per cell = 903.

Fig. 45

Scatchard analysis of $^3$H-TCDD binding to the Ah cytosolic receptor of 10T½ MCA cl-16.

Similar procedure as above. $K_d = 1.01$ nM and $B_{max} = 74.78$ fmol/mg protein.

Total number of receptor sites per cell = 800.
cl-8 and cl-16 respectively. The corresponding values for B and U were then plotted according to Scatchard (1949) as illustrated by figures 44 and 45. From the Scatchard plots, an additional value for $B_{\text{max}}$ or maximum binding can be calculated from the abscissa intercept as well as obtaining a value for $K_d$, the dissociation constant. The $K_d$ value will tell the relative affinity of the receptor for the ligand in use.

By least-squares linear regression analysis, we obtained $B_{\text{max}}$ values close to previous maximum binding values from figures 42 and 43 for both cl-8 and cl-16 (127.01 and 74.78 fmol/mg protein respectively). The $K_d$ values were both within the expected range as had been reported for the Ah receptor in whole animals (1.45 nM-cl-8 and 1.01 nM-cl-16). Also, $B_{\text{max}}$ figures were quantitated to total receptor number per cell by direct cell count prior to cellular homogenization, assuming one binding site per ($^3$H)-TCDD molecule on each receptor.

Discussion

The actual relevance of the information gained by the Scatchard analysis is limited to our overall outlook on the Ah receptor. From specific binding data, we had obtained saturation values ($B_{\text{max}}$) and the fact that the receptor was responsive to ligand concentrations as low as 0.1 nM had been estimated from AHH induction results. A criteria for cytosolic receptors is their ability to bind endogenous and exogenous ligands at physiological concentrations (nM-pM). Our reported values for
$K_d$ are comparable to previous results (Gustaffson et al., 1982; Okey and Vella, 1982; Zytkovicz, 1982) which suggest that rodent species which are "responsive" to AHH induction have a common Ah receptor.

The low $B_{\text{max}}$ values obtained for cl-16 may be partially due to our straight line extrapolation by the least-squares analysis (Klotz, 1982). The approximate two-fold difference in AHH activity between cl-8 and cl-16 were at first thought to be due to lower $B_{\text{max}}$ values exhibited by cl-16 but when total receptor number per cell was examined, less of a difference was evident (903 receptors/cl-8 cell vs. 800 receptors/cl-16 cell). Also, it is possible that due to known chromosomal alterations as a result of the transformation process, nuclear binding sites for the receptor complex may be lost or modified. Without actual isolation of the receptor molecule as in the case of the estrogen molecule, we can only hypothesize to the total binding and/or functional sites on the Ah receptor.
CHAPTER V
CONCLUSION

The interaction of carcinogenic PAH with the aryl hydrocarbon hydroxylase enzyme system was examined in C3H mouse embryo cells. The PAH, 3-methylcholanthrene (MCA), was able to malignantly transform C3H/10T	extsuperscript{1} cl-8 to a cell line which differed in various growth parameters. This morphologically-altered clone of cl-8, 10T	extsuperscript{1} MCA cl-16, exhibited two of the standard characteristics of malignant cell lines; 1) the ability to form colonies on soft agar (loss of anchorage dependency) and 2) production of neoplasics upon inoculation into host animals.

Then the AHH enzyme system of the two C3H cell lines was examined, it was found that both possessed inducible AHH activity but at a much lower level than a control cell line, Heps cl-9. Also, while the Heps cell line responded to all PAH tested, only TCDD and BA were able to induce AHH activity in either C3H cell line. When two of the non-inducing PAH were examined for their effects on the overall AHH system, it was discovered that both MCA and DBA were still capable of interacting with AHH components in a way which prevented the induction of AHH activity by either TCDD or BA. Besides not inducing AHH activity, MCA and DBA also did not bind specifically to the Ah' cytosolic receptor, a prerequisite for the
induction of AHH activity. Recent investigations (Ho et al., 1983) have suggested that MCA under normal circumstances would induce AHH activity in 10T^1 cl-8. But MCA is somehow converted to cytotoxic derivatives (?) which interfere with the normal induction response of the cell. This explanation however does not interpret how the cytotoxic metabolites are formed or how MCA is still capable of competing for (3H)-TCDD to the Ah receptor in cell-free extracts. More probable is the theory that the Ah receptor in 10T^1 cl-8 and 10T^1 MCA cl-16 has undergone a mutation which is expressed as a decreased affinity for normally inducing PAH such as MCA and DBA.

Speculation has been raised that there exists an unknown endogenous ligand for the Ah receptor which regulates a much wider spectrum of events than AHH activity (cell division, cellular differentiation, cell toxicity) (Poland and Knutson, 1982). This may explain how MCA can still contribute to the PAH-mediated transformation process in 10T^1 cl-8 (co-carcinogenic) while not inducing AHH activity (Besnow and Heidelberger, 1976). Finally, an explanation may be offered by the system that AHH enzyme induction is patterned after, steroid hormones and the estrogen receptor. The estrogen receptor requires the presence of an "active" ligand before undergoing modification to a form which is recognized by DNA hormone receptor sequences (de Boer and Notides, 1981). It is possible that the Ah cytosolic receptor is only activated to a recognizable form
by PAH capable of inducing AHH activity. Non-inducing PAH can still occupy receptor binding sites but not in a specific manner. This explanation is plausible except no sedimentation differences were detected between cytosolic and nuclear forms of the receptor. However, a change in the final configuration of the receptor before translocation would not be detectable by S value comparisons and could possibly account for the recognition factor previously mentioned.

AHH activity has previously been associated with PAH-induced tumorigenesis in animals and man. However, our results with C3H cell lines indicate that PAH-induced AHH activity may not be a prerequisite for the malignant transformation process, as in the case of 3-methylcholanthrene and 10T1/2 cl-8. Whereas 3-methylcholanthrene did not induce AHH activity in cl-8, it still was able to interact in some unknown manner with the binding of normally-inducing PAH (TCDD) to the Ah receptor. This interaction is hypothesized to be a contributing factor in the malignant transformation process of cl-8 to 10T1/2 MCA cl-16 by 3-methylcholanthrene. The large regulatory nature of the Ah receptor warrants additional investigations which may ultimately lead to a more precise understanding into gene regulation and the role of the AHH enzyme system into PAH-and environmentally-induced tumorigenesis.
APPENDICES

I. BUFFERS

**Phosphate Buffered Saline (PBS)** pH 7.4

- KCl 0.2 g/L
- KH₂PO₄ 0.2 g/L
- Na₂HPO₄ 1.14 g/L
- NaCl 8.0 g/L

**Glycerol Phosphate Buffer** (GPO₄) pH 7.5

- K₂HPO₄ 44 g/L
- KH₂PO₄ 9.2 g/L
- Dissolve above chemicals in 700 ml dH₂O; add 300 ml glycerol.

**AHF assay buffer**

Each reaction flask contained: (final volume of 1 ml)

- 0.2M Tris 0.25 ml
- 0.1M MgCl₂ 0.03 ml
- dH₂O 0.62 ml
- NADH 0.3 mg
- NADPH 0.3 mg
- B₃A 0.7 mg

**HEG Buffer** pH 7.6

- HEPES 11.92 g
- EDTA 0.88 g
- Dissolve above salts in 1800 ml dH₂O; add 200 ml glycerol.

**HEG Buffer**

- HEG buffer 100 ml
- DTT 15.40 mg
NEB Buffer pH 8.5 (nuclear extraction buffer)

HEDG buffer 1000 ml
KCl 37.25 g

Dextran Coated Charcoal (DCC)

HEG 100 ml
Norit A 1 g
Dextran 0.1 g

Scintillation Fluid (Cocktail)

Toluene 2000 ml
Omnifluor 12.5 g
Triton X-100 1000 ml

- 5 ml of above cocktail was added to each collected fraction from the SDG.

II. Statistics

A. Least Square Linear Regression

Least square linear regression analysis was performed on Scatchard plots for IOP12 cl-8 and IOP14 MCA cl-16. Input data points, x and y, were applied to the following formulas to calculate slope and intercept values:

\[
\text{slope} = m = \frac{n \sum xy - (\sum x)(\sum y)}{n \sum x^2 - (\sum x)^2}
\]

\[
\text{intercept} = b = \frac{\sum y - m \sum x}{n}
\]
III. Radiolabel DPM to moles conversions

Specifically-bound DPM per peak were converted to femtomoles bound per mg protein by first calculating the moles of label per one DPM from the specific activity of the label. Using the two set variables of manufacturers specific activity (curies/mmol) and the fact that 1 millicurie = $2.22 \times 10^9$ DPM, a DPM to moles conversion factor was obtained.

\[
\begin{align*}
(\text{H})_{-}\text{TCDD} & & 1 \text{ DPM} = 8.58 \times 10^{-18} \text{ moles (50 Ci/mmol)} \\
(\text{H})_{-}\text{DBA} & & 1 \text{ DPM} = 1.21 \times 10^{-17} \text{ moles (37 Ci/mmol)} \\
(\text{H})_{-}\text{MCA} & & 1 \text{ DPM} = 1.28 \times 10^{-17} \text{ moles (35 Ci/mmol)}
\end{align*}
\]

The specifically-bound DPM values were obtained by calculation of total binding values with label as compared to DPM bound with label plus 100-fold radioinert competitor.

IV. Major Chemical Suppliers

Acetone Sargent-Welch
\'BA Sigma
BME Gibco
BSA Sigma
DB(a,h)A Sigma
\(3^\text{H})_{-}\text{DB(a,h)A NEN}
DMSO Fisher
DTT Sigma
EDTA BDH
FCS Gibco
Glycerol Sigma
Hepes Gibco
Hexane Sargent-Welch
MCA  
(3H)-MCA  
MEM  
NADH  
NADPH  
NaOH  
Omnifluor  
Sucrose  
TCDD  
(3H)-TCDD  
Tissue Culture Plastic Ware  
Tris  
Triton X-100  
Trypsin  

Sigma  
MEM  
Gibco  
Sigma  
Sigma  
Fisher  
NEN  
Beckman  
KOR  
KOR  
Gibco  
Sigma  
Amersham  
Gibco

Sigma Chemicals, St. Louis, Missouri.  
New England Nuclear (NEN), Boston, Mass.  
Amersham, Arlington Heights, Illinois.  
British Drug House (BDH), Toronto, Ontario.  
KOR Isotopes, Cambridge, Mass.  
Beckman Instruments Inc., Toronto, Ontario.  
Sargent Welch Scientific, Weston, Ontario.
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