0026-895X/91/050818-09$03.00/0

MOLECULAR PHARMACOLOGY, 40:818-826

Ah Receptor in Mice Genetically "Nonresponsive" for Cytochrome P4501A1 Induction: Cytosolic Ah Receptor, Transformation to the Nuclear Binding State, and Induction of Aryl Hydrocarbon Hydroxylase by Halogenated and Nonhalogenated Aromatic Hydrocarbons in Embryonic Tissues and Cells

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Received March 14, 1991; Accepted August 8, 1991

SUMMARY

The aromatic hydrocarbon (Ah) receptor mediates induction of cytochrome P4501A1 and associated aryl hydrocarbon hydroxylase (AHH) activity in tissues or cells exposed to polycyclic aromatic hydrocarbons. Strains of mice designated "nonresponsive" do not show increased hepatic AHH activity when exposed *in vivo* to nonhalogenated aromatic hydrocarbons such as 3-methylcholanthrene, benz[*a*]anthracene (BA), or benzo[*a*]pyrene and have reduced sensitivity to halogenated inducers such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Recently, with a modified assay, we detected Ah receptor in hepatic cytosols from adult nonresponsive mice [*Mol. Pharmacol.* 35:823–830 (1989)]; the receptor was present in reduced amount, and the apparent affinity for TCDD was lower than in hepatic cytosol from responsive C57BL/6J mice. Using the same assay procedure, we now report detection of Ah receptor in cytosols prepared from embryonic tissue and from cultured embryo cells of both responsive (C57BL/6J) and nonresponsive mice (DBA/2J, AKR/J, and SWR/J). Cytosolic receptor in embryonic cells from nonresponsive as well as responsive strains was detectable both with [^{3}H]TCDD and with [^{3}H]3-methylcholanthrene. In addition, the receptor-ligand complex could be extracted from nuclei of embryo cells exposed to [^{3}H]TCDD in culture. AHH activity was induced in embryo cell cultures incubated with either TCDD or BA. The EC_{50} values for AHH induction were virtually identical in cell cultures from nonresponsive (DBA/2J) and responsive (C57BL/6J) strains, using either TCDD or BA as the inducer. Moreover, the affinity with which [^{3}H]TCDD bound to cytosolic Ah receptor was much more similar in cytosols from cell cultures from the two strains than in cytosols prepared from adult liver. Thus, embryonic cell cultures differ in at least three respects from the adult liver, as follows: (i) Ah receptor can be detected with [^{3}H]3-methylcholanthrene in embryonic cell cytosols but not in cytosols from adult liver; (ii) the degree of difference between nonresponsive and responsive strains in the affinity with which [^{3}H]TCDD binds to receptor is only about 2-fold in cytosol from embryonic cells, whereas it is almost 10-fold in adult liver; and (iii) induction of AHH activity (by either TCDD or by the nonhalogenated inducer BA) shows no significant difference between strains in embryonic cell culture, whereas there is at least a 15-fold difference in responsiveness between C57BL/6J and DBA/2J mice in adult liver *in vivo*. The mechanistic reason for the diminished degree of difference between responsive and nonresponsive mice during embryonic cell culture (compared with adult tissues) is not yet known.

The most completely understood function of the Ah receptor is regulation of induction of P4501A1[1] (1–3). TCDD and related halogenated and nonhalogenated hydrocarbons bind to the Ah receptor in cytoplasm (1, 4–7), thereby triggering transformation of the ligand-receptor complex to a form having high affinity for dioxin-responsive enhancer elements in DNA (8–11). Binding of the ligand-receptor complex to these specific DNA sequences leads to increased synthesis of the mRNA encoding P4501A1, as well as increased expression of some other genes (3, 12, 13).

Strains of mice such as C57BL/6J are termed "responsive"

This work was supported by a grant from the Medical Research Council of Canada to A.B.O.

[1]P4501A1 was known previously as P_1-450 in mice and P-450c in rats.

ABBREVIATIONS: Ah, aromatic hydrocarbon; P450, cytochrome P450; AHH, aryl hydrocarbon hydroxylase; BA, benz[*a*]anthracene; BP, benzo[*a*]pyrene; BSA, bovine serum albumin; MC, 3-methylcholanthrene; MEM, minimal essential medium; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

because they exhibit high P4501A1 induction in liver when adults are treated with nonhalogenated polycyclic aromatic hydrocarbons such as MC, BP, or dibenz[*a*,*h*]anthracene or with halogenated aromatic hydrocarbons such as TCDD. "Nonresponsive" strains such as DBA/2J, AKR/J, and SWR/J show no significant increase in hepatic P4501A1, even when treated with high doses of nonhalogenated polycyclic aromatic hydrocarbons; moreover, the dose required for half-maximal induction by TCDD is approximately 15-fold higher in DBA/2J mice than in C57BL/6J mice (1, 6).

The difference in response to nonhalogenated polycyclic aromatic hydrocarbons between responsive and nonresponsive mice is not so distinct *in vitro*. When cell cultures established from nonresponsive mice are exposed to MC, there has been reported to be some increase in AHH activity (14, 15). The magnitude of the response, however, has been reported to be much less than that of cells from responsive mouse strains. Although cells from both responsive and nonresponsive mouse strains show induction of AHH activity, higher concentrations of MC (or TCDD) were reported to be necessary for induction in cell cultures from nonresponsive mice than in cells from responsive mice (14, 15). Because nonresponsive mice do respond (although with reduced sensitivity) to TCDD and because TCDD regulation of P4501A1 is mediated by the Ah receptor, it would be expected that [^{3}H]TCDD should bind Ah receptor in nonresponsive mice. It has been postulated that the nonresponsive phenotype is due to a defect in the Ah receptor resulting in an inability to bind effectively the appropriate ligands; thus, transformation to a DNA-binding protein and subsequent P4501A1 induction cannot occur (1, 16).

We recently modified the assay procedure for Ah receptor and were able to detect lower affinity forms of Ah receptor (17–19). In hepatic cytosols prepared from adult nonresponsive DBA/2J mice, the Ah receptor was found to have a K_d of about 16 nM for binding of [^{3}H]TCDD, compared with a K_d of about 1.8 nM in hepatic cytosols from responsive C57BL/6J mice (19).

Data presented here from our current studies demonstrate that cytosolic Ah receptor is detectable in whole embryos and in embryo cell cultures from nonresponsive mouse strains. In addition, nuclear receptor-ligand complexes can be extracted from cultured embryonic cells from nonresponsive strains, and P4501A1 (as measured by AHH activity) can be induced in cells from all strains by both halogenated and nonhalogenated polycyclic aromatic hydrocarbons.

Materials and Methods

Chemicals. [^{3}H]TCDD (32 Ci/mmol) and nonradioactive TCDF (used as a competitor to check specificity of binding) were generous gifts from Dr. S. Safe (Texas A & M University). The purity of [^{3}H]TCDD, when analyzed in our laboratory by high performance liquid chromatography (20), was >95%. TCDD and TCDF are extremely toxic substances and should be handled with special care, as described by Poland and Glover (21). [^{3}H]MC (generally labeled, 37 Ci/mmol) was from Amersham Corporation (Oakville, Ontario). Sucrose (density gradient grade) was from Beckman Instruments (Toronto, Ontario); HEPES was from Calbiochem-Behring Corp. (La Jolla, CA); sodium molybdate, dithiothreitol, BSA, and catalase were obtained from Sigma Chemical Co. (St. Louis, MO); DMSO, glycerol, and EDTA were obtained from Fisher Scientific Co. (Toronto, Ontario). Nonradioactive BA, BP, and MC were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade and were obtained from Fisher Scientific Co. (Toronto, Ontario).

Buffers. Sodium molybdate (20 mM final concentration) was present in all buffers unless otherwise stated in the text. The main buffer for cytosol preparation was HEGDM (25 mM HEPES, 3 mM EDTA, 2 mM dithiothreitol, 10%, v/v, glycerol, 20 mM sodium molybdate). HEGDK buffer is HEGDM without molybdate but with the addition of 0.5 M KCl. HEDM is a hypotonic buffer consisting of HEGDM without the glycerol; HE2GDM is HEDM plus 20% (v/v) glycerol. All buffers were adjusted to pH 7.4 at room temperature.

Animals. Pregnant responsive C57BL/6J and nonresponsive DBA/2J, AKR/J, and SWR/J mice were obtained at 12–15 days of gestation from The Jackson Laboratory (Bar Harbor, ME).

Cell culture. Cell cultures were established from 15–19-day-old fetuses (minus head, feet, tail, and viscera) from C57BL/6J, DBA/2J, AKR/J, and SWR/J mice. The embryos were minced and incubated overnight at 4° with 0.1% trypsin (Difco Laboratories, Detroit, MI), in α-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin. The next morning the tissue suspension was warmed to 37° for 45 min, to obtain a suspension of single cells. The cells were grown in α-MEM, containing 20% fetal bovine serum and antibiotics, at 37° in 5% CO_2/95% air. Cultures were harvested by trypsinization, when the density on the culture dish appeared to reach 100%, and then washed in Dulbecco's phosphate-buffered saline before homogenization. Cells were routinely used at generation 4 to 5 (passage 2 to 3).

Tissue and cell fractionation. Cytosol was prepared from 15–19-day-old fetuses (minus head, feet, tail, and viscera) from C57BL/6J, DBA/2J, AKR/J, and SWR/J mice. Tissue, suspended in HEGDM with or without sodium molybdate (as indicated in the text), was homogenized with a Polytron fitted with a PT7 probe (Brinkmann Instruments, Rexdale, Ontario). The homogenate was centrifuged at 105,000 × *g* for 1 hr, and the resulting supernatant (cytosol) was stored in 1–2-ml aliquots in liquid nitrogen until analysis. Similarly, prewashed cells from embryonic cell cultures were suspended in the hypotonic HEDM buffer, at 1–2 × 10^8 cells/ml, and homogenized with a Polytron, fitted with a PT7 probe, until >90% of the cells were ruptured but the nuclei were intact. Cell breakage was assessed by microscopic examination. The homogenate was then diluted 1/1 with HE2GDM buffer, centrifuged, and processed as for tissue homogenates. Protein concentrations were determined by the method of Bradford (22), using BSA as the standard.

Preparation of nuclear extract. If a nuclear extract was to be prepared from cells exposed in culture to [^{3}H]TCDD, the cell homogenate was centrifuged for 4 min at 10,000 × *g* in a Beckman microfuge, and the resulting nuclear pellet was washed 2 or 3 times with HEGDM buffer, incubated for 1 hr in HEGDK buffer at 4° to extract the nuclear receptor, and then centrifuged at 105,000 × *g* for 1 hr. The resulting supernatant is termed the nuclear extract.

Preparation of cytosol for analysis by velocity sedimentation. Cytosols to be analyzed for Ah receptor were incubated with 10–20 nM [^{3}H]TCDD, in the absence or presence of a 100-fold molar excess of competitor, for 2 hr at 4°. After incubation, unbound [^{3}H]TCDD was not removed with dextran-coated charcoal; rather, the cytosol was immediately analyzed by density gradient centrifugation.

Velocity sedimentation on sucrose gradients. Samples were analyzed by density gradient centrifugation using a vertical tube rotor technique, as described by Tsui and Okey (23). Aliquots (300 μl) of cytosol were layered onto linear 10–30% sucrose gradients that had been prepared in the same buffer in which the cytosol or nuclear extract had been prepared. Gradients were centrifuged for 2 hr at 372,000 × *g*. After centrifugation, 25 fractions were collected, and the radioactivity in each fraction was determined by liquid scintillation counting and was corrected for counting efficiency. [^{14}C]Formaldehyde-labeled BSA and [^{14}C]formaldehyde-labeled catalase were included as internal sedimentation markers in each gradient. Marker proteins were labeled with [^{14}C]formaldehyde as previously described (20).

Induction and assay of AHH. Monolayer cultures at 95% con-

fluency, growing on the culture dish in α-MEM containing 20% fetal bovine serum, were treated with various concentrations of BA or [^{3}H]TCDD dissolved in DMSO. Because the amount of TCDD used in the induction assays was very small, [^{3}H]TCDD was used to verify the actual concentration in the medium. The final concentration of DMSO was 1% in all samples. This concentration of DMSO was not toxic and did not induce AHH activity when added alone. After 24 hr, the cells were harvested by trypsinization and washed 2 or 3 times with phosphate-buffered saline. AHH activity was determined in whole cells by the method of Nebert and Gelboin (24), with protein concentrations of 0.5–0.75 mg/assay flask and incubation for 45 min at 37°. One unit of AHH activity is defined as the amount of hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[*a*]pyrene (recrystallized) standard. Activity is expressed as pmol/min/mg of protein. There was no fluorescent contribution by either TCDD or BA at any concentration, because parallel induced samples assayed in the absence of the added substrate BP, gave values that did not differ significantly from the uninduced control.

Results

Cytosolic Ah receptor can be detected and characterized in cytosols prepared either from tissue samples or from cells grown in culture. Cell cultures allow us to examine three of the major steps in Ah receptor-mediated function, namely, ligand binding to cytosolic receptor, identification of a nuclear-associated receptor-ligand complex, and the resulting induction of P4501A1-associated monooxygenase activity, as measured by AHH activity. Thus, cell cultures were established from fetuses of responsive C57BL/6J mice and nonresponsive DBA/2J, AKR/J, and SWR/J mice, to complement and extend studies on cytosols from embryonic tissues.

Ah receptor in cytosols from whole embryos. Initially, cytosols were prepared from whole embryo tissue (minus head, limbs, tail, and viscera) and assayed for Ah receptor. Cytosols from C57BL/6J embryos showed a large specific peak of [^{3}H]TCDD binding, which sedimented at approximately the 9-S region (near fraction 15) of the sucrose gradient. Binding in the 9-S region was completely inhibited by the presence of a 100-fold excess of nonradioactive TCDF (Fig. 1). This profile is very similar to profiles previously obtained for the Ah receptor in cytosols from tissues of adult responsive animals.

In cytosols prepared from embryos of the nonresponsive strain DBA/2J, binding in the 9-S region of the sucrose gradient also was detected. It was necessary, however, to include 20 mM sodium molybdate in the homogenization buffer (Fig. 1). Using these conditions, a specific binding peak was clearly defined in the 9-S region of the gradient, but the concentration of Ah receptor was lower in cytosols from nonresponsive strains than in cytosol prepared from the prototype responsive strain C57BL/6J. The concentration of specific binding in this assay was 256 fmol/mg of cytosol protein for C57BL/6J, versus 5 fmol/mg of protein for DBA/2J in the absence of molybdate and 21 fmol/mg of protein for DBA/2J in the presence of 20 mM sodium molybdate.

Similar results were obtained when whole-embryo cytosols from other nonresponsive mouse strains were analyzed. As with DBA/2J, cytosols prepared from both AKR/J and SWR/J mouse embryos showed a binding peak in the 9-S region of the sucrose gradient that was inhibitable by a 100-fold excess of TCDF (Fig. 2). The concentration of specific binding was 21 fmol/mg of cytosol protein for DBA/2J, 15 fmol/mg of cytosol protein for AKR/J, and 19 fmol/mg of cytosol protein for SWR/J The presence of 20 mM sodium molybdate was necessary to reliably demonstrate specific Ah receptor binding in all three nonresponsive mouse strains analyzed, but molybdate did not increase the the amount of Ah receptor detected in cytosols from the C57BL/6J mouse (data not shown).

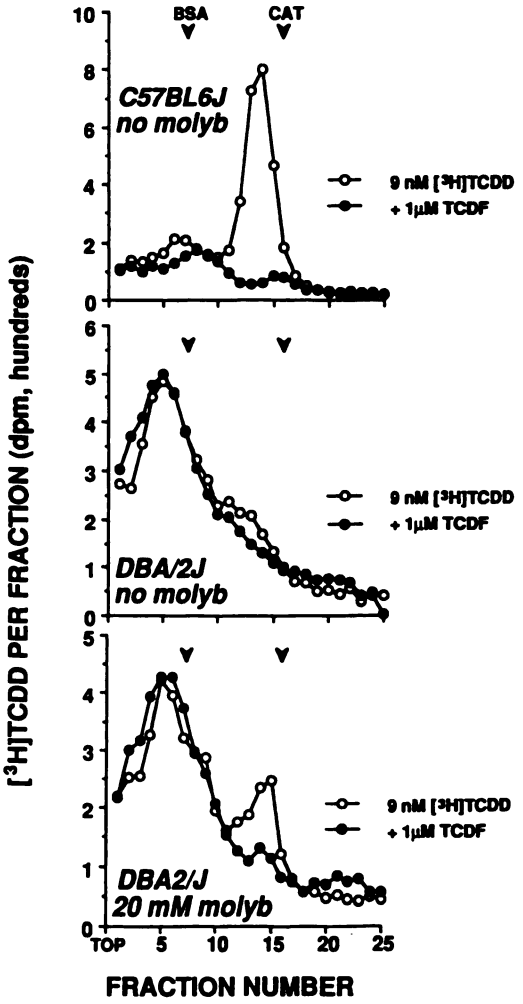

Fig. 1. Sucrose density gradient profiles comparing Ah receptor in cytosols from eviscerated embryos of C57BL/6J versus DBA/2J mice. Cytosols prepared in HEGD buffer, with or without 20 mM sodium molybdate (*molyb*) (5 mg of protein/ml for C57BL/6 cytosol and 2 mg/ml for both DBA/2 cytosols), were incubated with 9 nM [^{3}H]TCDD in the absence (○) or presence (●) of a 100-fold molar excess of the competitor TCDF. Samples were analyzed by velocity sedimentation on sucrose density gradients. [^{14}C]Formaldehyde-labeled BSA (4.4 S) and [^{14}C]formaldehyde-labeled catalase (*CAT*) (11.3 S) were added to the gradients as internal sedimentation markers (*arrowheads*).

Ah receptor in cytosols from cultured embryo cells. Cell cultures were established from embryos of all four mouse strains, to enable us to investigate some of the other steps involved in Ah receptor-mediated AHH induction. Cytosolic fractions were prepared from these cultures, to determine whether there was any change in Ah receptor expression as a result of the cell culture conditions. As shown in Fig. 3, Ah receptor was detectable in cytosols from both responsive and nonresponsive mouse strains. Ah receptor measured in the nonresponsive strains was low, compared with the responsive mouse strains; however, the concentration of specific binding sites in the 9-S region of the gradient was dramatically higher in the cytosols from cultured cells than in tissues obtained

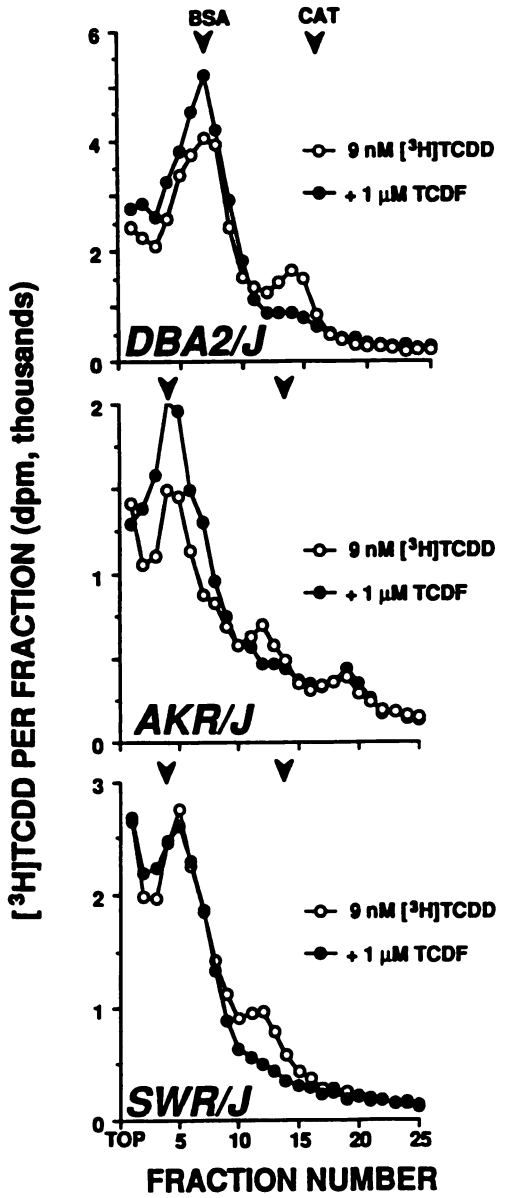

Fig. 2. Sucrose density gradient profiles comparing Ah receptor in cytosols prepared from eviscerated embryos of DBA/2J, AKR/J, and SWR/J mice. Cytosols from DBA/2J (7 mg of protein/ml), AKR/J (3 mg of protein/ml), and SWR/J (6 mg of protein/ml) mice, prepared in HEGDM containing 20 mM sodium molybdate, were incubated with 9 nM [^{3}H]TCDD in the absence (○) or presence (●) of 100-fold molar excess of the competitor TCDF. Samples were analyzed by velocity sedimentation on sucrose gradients. [^{14}C]Formaldehyde-labeled BSA (4.4 S) and [^{14}C]formaldehyde-labeled catalase (*CAT*) (11.3 S) were added to the gradients as internal sedimentation markers (*arrowheads*).

directly from embryos without culturing. The specific binding in these assays was 601 fmol/mg of protein for C57BL/6J, 154 fmol/mg for DBA/2J, 113 fmol/mg for AKR/J, and 35 fmol/mg for SWR/J cells. In all instances, 20 mM sodium molybdate was used in buffers.

The 9-S peak in the cytosol from cultured nonresponsive mouse embryo cells also bound specifically [^{3}H]MC, a nonhalogenated agonist for the Ah receptor. As shown in Fig. 4, incubation of cytosols from cultured cells of nonresponsive strains with [^{3}H]MC resulted in a small but detectable specific binding peaks in the 9-S region of the gradient. The concentration of specific binding sites detected with [^{3}H]MC was lower in all cases than the specific binding detected with [^{3}H]TCDD and was near the limit of detection in the nonresponsive strains.

Determination of the apparent binding affinity of [^{3}H]TCDD to cytosolic Ah receptor. The apparent K_d for the interaction of [^{3}H]TCDD with the Ah receptor was estimated by incubating cytosols from cultured cells with a wide variety of ligand concentrations and then determining specific binding in the 9-S region by density gradient analysis for each sample. The data are illustrated as a saturation plot in Fig. 5. Similar concentrations of [^{3}H]TCDD were used (0.25–20 nM) for cytosols prepared from C57BL/6J and DBA/2J. From the saturation plots, binding of [^{3}H]TCDD in C57BL/6J cell cytosol was half-saturated at ~2.5 nM, whereas half-maximal saturation of DBA/2J cell cytosolic receptor occurred at ~6 nM. These data were more rigorously analyzed by both Woolf and Scatchard plots. The kinetic parameter, apparent K_d, was computed from both plots for several litters from both C57BL/6J and DBA/2J strains. As can be seen (Table 1), there was only about a 2–3-fold difference between responsive and nonresponsive strains in the binding affinity of [^{3}H]TCDD in these cytosols, i.e., 2.4 ± 1.3 nM for C57BL/6J, compared with 6.7 ± 1.1 nM for DBA/2J, by Woolf plot analysis.

Nuclear ligand-receptor complex. Regulation by the Ah receptor involves binding of an Ah receptor-ligand complex to specific regions on DNA. Incubation of cells with [^{3}H]TCDD in culture at 37° results in the accumulation of ligand-receptor complexes in the nucleus. Under these conditions, the Ah receptor can be isolated from cell nuclei as a [^{3}H]TCDD-receptor complex, and no further *in vitro* labeling is required to identify the specific binding component. The nuclear ligand-receptor complex was extracted with 0.5 M KCl and analyzed directly on sucrose density gradients. Fig. 6 shows the gradient profiles obtained when nuclear extracts from C57BL/6J, DBA/2J, AKR/J, and SWR/J cells incubated in culture with [^{3}H]TCDD were analyzed by velocity sedimentation on sucrose gradients. Specific [^{3}H]TCDD binding peaks in the nuclear extracts sedimented in the 6-S region (near fraction 10) of the gradient, typical of nuclear-associated Ah receptor. The binding peak was specific for the Ah receptor, because nuclear extract from cells incubated with [^{3}H]TCDD in the presence of a 100-fold molar excess of the competitor TCDF showed no binding in this region of the gradient (data not shown).

AHH induction by a halogenated and a nonhalogenated polycyclic aromatic hydrocarbon. Binding of the ligand-receptor complex to DNA increases synthesis of specific mRNAs, resulting in an increase of P4501A1 mRNA (among others). AHH induction is the most well characterized biological response in cells exposed to Ah receptor agonists and has been taken as indirect evidence that the Ah receptor is present in a given tissue or cell line. Therefore, if the Ah receptor detected in the nonresponsive mouse cell cytosols is functional, it should be possible to measure increased AHH activity upon incubation of the cells with Ah receptor agonists.

AHH induction was examined by determining the dose-response curves for AHH induction by [^{3}H]TCDD and BA. The data in Fig. 7 are mean values of AHH activity detected in independent cultures from four litters of C57BL/6J mice and four litters of DBA/2J mice. For induction by [^{3}H]TCDD, the EC_{50} in cells from DBA/2J mice was 8×10^{-10} M, compared with 7×10^{-10} M in C57BL/6J mice; maximal activity was 1.5 pmol/min/mg of protein in cells from DBA/2J mice versus 2.0

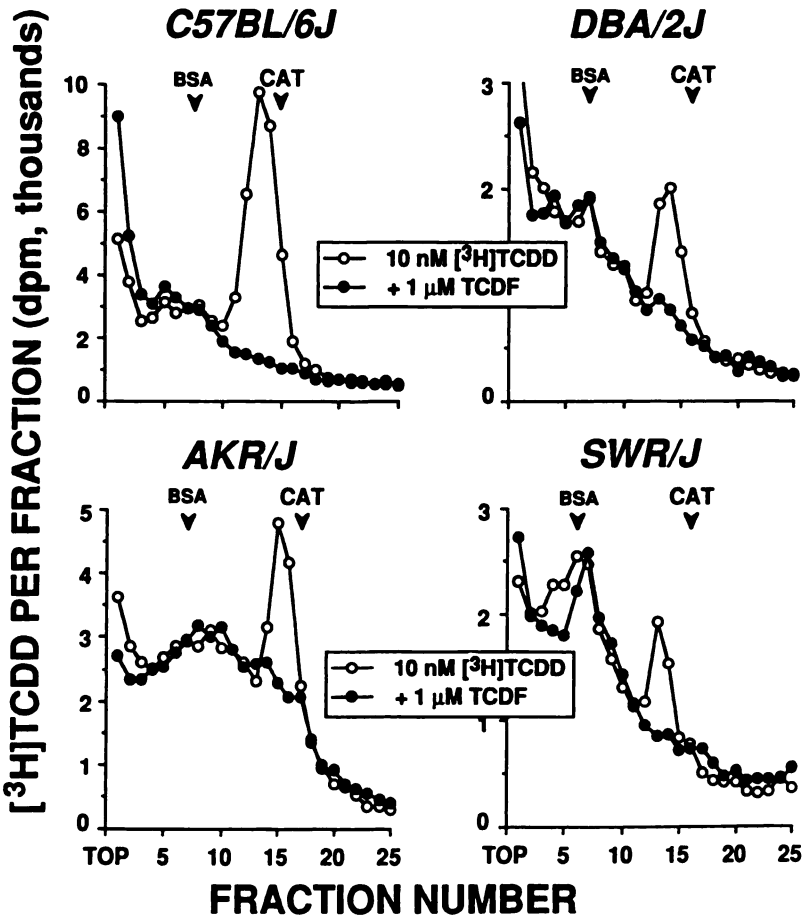

Fig. 3. Sucrose density gradient profiles comparing Ah receptor in cytosols prepared from cultured cells from C57BL/6J, DBA/2J, AKR/J, and SWR/J embryos. Cytosols prepared in HEGDM (20 mM sodium molybdate) were incubated with 10 nM [^{3}H]TCDD in the absence (○) or presence (●) of 100-fold molar excess of the competitor TCDF. Protein concentrations were 5 mg/ml for C57BL/6J, 1 mg/ml for DBA/2J, 3 mg/ml for AKR/J, and 3 mg/ml for SWR/J. Samples were analyzed by velocity sedimentation on sucrose gradients. [^{14}C]Formaldehyde-labeled BSA (4.4 S) and [^{14}C]formaldehyde-labeled catalase (*CAT*) (11.3 S) were added to the gradients as internal sedimentation markers (*arrowheads*).

pmol/min/mg of protein in C57BL/6J mice. For induction by BA, the EC_{50} in cells from DBA/2J mice was 4×10^{-6} M versus 1×10^{-5} M in cells from C57BL/6J mice; the maximal activity induced by BA was 2.0 pmol/min/mg of protein in cells from DBA/2J versus 4.5 pmol/min/mg of protein for C57BL/6J. In all instances, noninduced levels were 0–0.17 pmol/min/mg above background fluorescence. The aggregate induction data indicate no significant difference between responsive and nonresponsive mouse strains in the EC_{50} for AHH induction in cultured cells.

Toxicity was apparent at high concentrations of both TCDD and BA, as seen by the drop in activity at 1×10^{-7} M TCDD and 5×10^{-5} M BA. This was not due to toxicity of the solvent, because the DMSO concentration was kept constant throughout, at 1%. Because both TCDD and BA are toxic, our estimated maximal AHH activity may be lower than the true potential of these cells.

Discussion

The original designation (1, 25) of "nonresponsive" was applied to those mouse strains that did not exhibit increased hepatic AHH activity when treated with nonhalogenated compounds such as MC, although Wiebel *et al.* (26) reported that AHH activities in lung and kidney from both C57BL/6 and DBA/2 mice were induced severalfold after BA administration. It has been presumed that there is a defect or mutation in nonresponsive strains of mice, resulting in a lower affinity (or, possibly, a complete absence) of binding of agonists to the Ah receptor. Thus, BA and MC have insufficient affinity to elicit a response in nonresponsive strains, whereas TCDD, which is several orders of magnitude more potent than BA and MC, elicits responses only at relatively high concentrations.

In our laboratory, we recently developed techniques suited to studying low affinity Ah receptors. We found that certain modifications in procedure are necessary in order to detect cytosolic Ah receptor. In particular, it is necessary to reduce the amount of charcoal used to adsorb nonspecifically bound radioligand before layering onto sucrose gradients. In early studies with rodent hepatic cytosol, approximately 2 mg of charcoal/mg of cytosolic protein was used. This charcoal concentration will remove [^{3}H]TCDD that has been specifically bound to the Ah receptor in DBA/2J hepatic cytosol (19), as well as nonspecifically bound radioligand. In our present study, therefore, we omitted charcoal treatments.

We also routinely included sodium molybdate in all buffers. Sodium molybdate has been effective in stabilizing several steroid receptors (27, 28) and, more recently, has been shown to stabilize the low affinity human Ah receptor (17). This was also the case for Ah receptor in cytosols prepared from nonresponsive mouse tissue. The inclusion of 20 mM sodium molybdate greatly enhanced the amount of detectable receptor. Using this modified assay, Okey *et al.* (19) detected and characterized a low affinity Ah receptor in hepatic cytosol from adult DBA/2J mice. They found that DBA/2J mice have receptor concentrations in hepatic cytosols that are about one third the levels in C57BL/6J mice and that there is about a 10-fold weaker binding of [^{3}H]TCDD. Similarly, sodium molybdate facilitated detection by photoaffinity labeling of the ligand-binding sub-

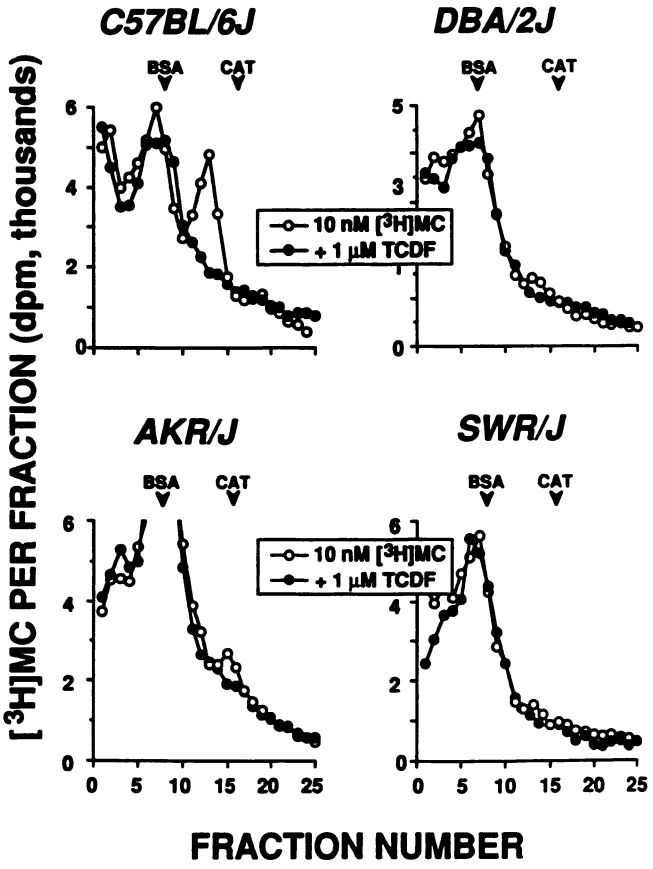

Fig. 4. Sucrose density gradient profiles comparing [^{3}H]MC binding to Ah receptor in cytosols prepared from cultured embryo cells established from C57BL/6J, DBA/2J, AKR/J, and SWR/J mice. Cytosols (protein concentrations were 3 mg/ml for C57BL/6J, 1 mg/ml for DBA/2J, 3 mg/ml for AKR/J, and 3 mg/ml for SWR/J) prepared in HEGDM (20 mM sodium molybdate) were incubated with 10 nM [^{3}H]MC in the absence (○) or presence (●) of 100-fold molar excess of the competitor TCDF. Samples were analyzed by velocity sedimentation on sucrose gradients. [^{14}C]Formaldehyde-labeled BSA (4.4 S) and [^{14}C]formaldehyde-labeled catalase (*CAT*) (11.3 S) were added to the gradients as internal sedimentation markers (*arrowheads*).

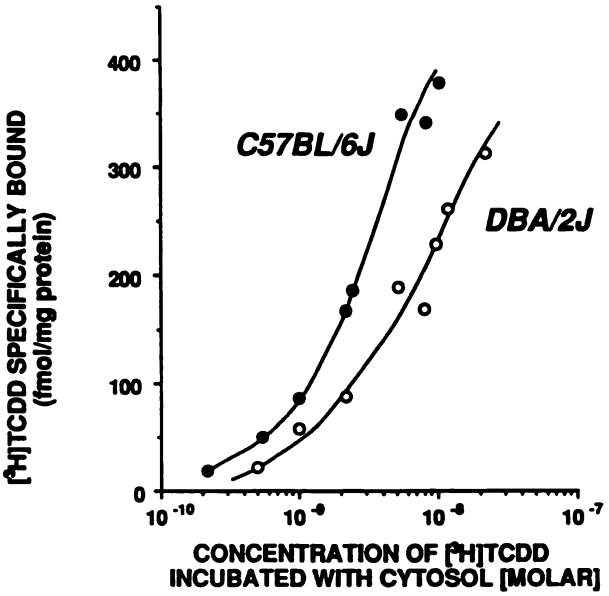

Fig. 5. Saturation analysis of [^{3}H]TCDD binding to cytosolic Ah receptor in cytosols from C57BL/6J versus DBA/2J embryonic cells. Cytosols from C57BL/6J (3 mg of protein/ml) and DBA/2J (3 mg of protein/ml) cell cultures, prepared in HEGDM buffer, were incubated for 2 hr at 4° with concentrations of [^{3}H]TCDD from 0.25 to 20 nM. Specific binding in the 9-S region was determined for each sample by sucrose gradient analysis.

TABLE 1

Determination of the apparent affinity of [^{3}H]TCDD for cytosolic Ah receptor obtained from embryo cells in culture

Cytosols (2–3 mg of protein/ml) from cells cultured from C57BL/6J and DBA/2J embryos were incubated for 2 hr at 4° with concentrations of [^{3}H]TCDD between 0.25 and 20 nM. Specific binding in the 9-S region was determined for each sample by sucrose gradient analysis. The apparent K_d was computed from Scatchard and Woolf plots. Each sample was prepared from an independently established cell culture from five different litters of C57BL/6J and five different litters of DBA/2J mice; values shown represent the mean ± standard deviation for the five different replicates from each strain.

	K_d	
	Woolf plot	Scatchard plot
	nM	
C57BL/6J	2.4 ± 1.3	3.8 ± 1.4
DBA/2J	6.7 ± 1.1	6.4 ± 1.1

unit of the Ah receptor by 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin in nonresponsive mouse strains (29). In our current study, 20 mM sodium molybdate included in cytosol preparations from embryonic tissue and from cultured cells also greatly enhanced the detectability of Ah receptor in the nonresponsive mouse strains. In previous experiments, molybdate did not increase the level of receptor detected in hepatic cytosol from adult responsive mice (19, 30).

We established cell cultures so that three major events in Ah receptor-mediated induction could be analyzed. The amount of receptor detectable in cytosols prepared from the cell cultures was dramatically increased, compared with the tissues of origin. The most significant result of this was that it enabled us to detect a very small amount of [^{3}H]MC binding to the Ah receptor in cytosols prepared from cultured cells of nonresponsive mouse. Our data suggest that MC, at achievable radioligand concentrations, is bound only to a small fraction of the total pool of Ah receptor. With the higher concentration of Ah receptor in cells in culture, this small fraction of MC binding in the nonresponsive animals now is detectable. Thus, MC does bind to the Ah receptor in nonresponsive mice but to a much lesser degree than TCDD.

When we incubated the cell cultures with several Ah receptor agonists, including both nonhalogenated agonists (BA and BP) and halogenated agonists (TCDD and TCDF), all were found to induce AHH activity in cells from nonresponsive as well as responsive strains (data not shown for BP and TCDF). In addition, we compared the dose-response curves for AHH induction by the halogenated hydrocarbon TCDD and the nonhalogenated hydrocarbon BA. Cells from DBA/2J embryos required approximately the same concentration of TCDD or BA to elicit half-maximal response as did cells from C57BL/6J embryos. It appears that cell culture conditions diminish the phenotypic difference between responsive and nonresponsive mouse strains, such that DBA/2J and C57BL/6J mouse strains are essentially equally responsive; this is true with either TCDD or BA as the inducer. Thus, in cell culture nonhalogenated hydrocarbons such as BA do cause an increase in AHH activity from nonresponsive mice. This is in contrast to the data from whole animal studies, where BA is ineffective in liver. This phenomenon has been observed by others; Hosomi *et al.* (31)

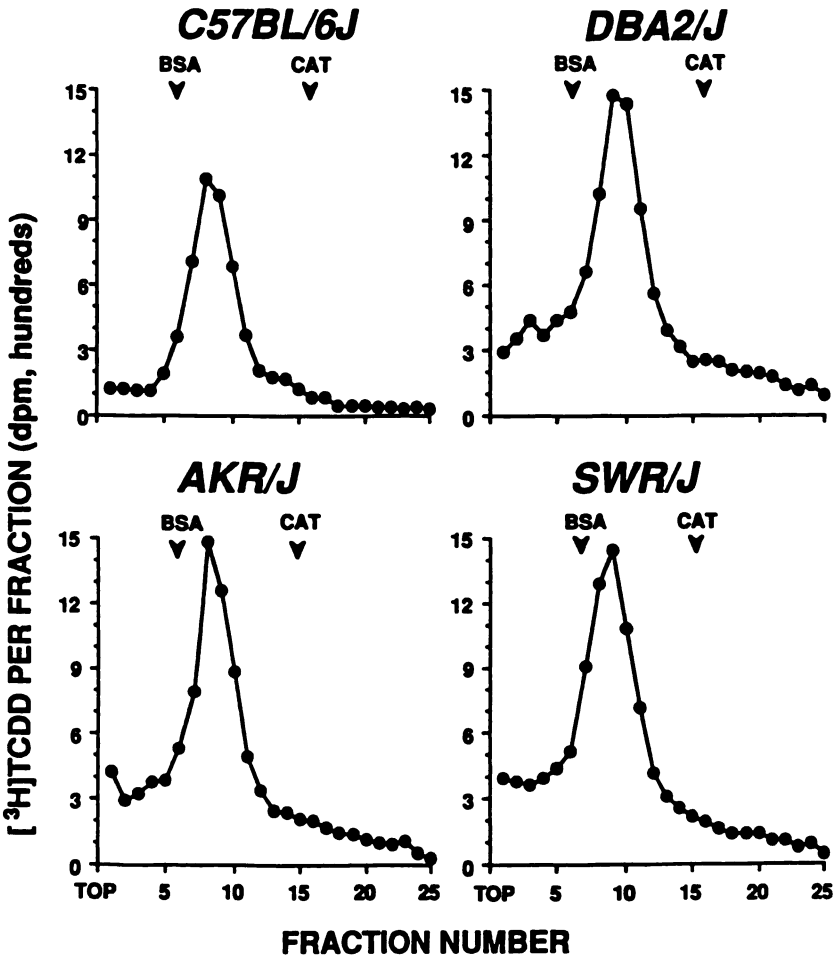

Fig. 6. Demonstration of nuclear Ah receptor-ligand complex. Cultured embryonic cells from responsive (C57BL/6J) and nonresponsive (DBA/2J, AKR/J, and SWR/J) mice were incubated in culture with 2 nM [^{3}H] TCDD for 2 hr at 37°. Nuclear extracts were prepared (1–2 mg of protein/ml of extract) as described in Materials and Methods and analyzed by velocity sedimentation on sucrose gradients containing 0.5 M KCl. [^{14}C] Formaldehyde-labeled BSA (4.4 S) and [^{14}C]formaldehyde-labeled catalase (*CAT*) (11.3 S) were added to the gradients as internal sedimentation markers (*arrowheads*).

established primary cultures of epidermal cells from newborn mice. After BA exposure for 24 hr, AHH activity was found to be increased in all cultures, irrespective of the responsiveness *in vivo* of the original animal; similarly Hitchins *et al.* (15) and Niwa *et al.* (14) found BA to be an effective inducer in cultured embryonic cells from both responsive and nonresponsive mice. More recently, Nemoto *et al.* (32) reported that hepatocytes cultured from nonresponsive strains acquire AHH inducibility after prolonged exposure in tissue culture. Unlike our findings with cultured embryo cells, however, the agonist concentration required for maximum induction in cultures from nonresponsive strains in all previous studies was greater than the concentration required for maximum induction in responsive strains. A possible explanation has been suggested by Puhvel and Sakamoto (33). In earlier experiments, Knutson and Poland (34) demonstrated that TCDD-induced cutaneous toxicity in mice is related to an interaction between the *Ah* locus and the *hr* locus. Puhvel and Sakamoto (33) demonstrated that epidermal keratinocytes from homozygous newborn HRS/J *hr/hr* and HRS/J +/+ mice respond in culture to TCDD, suggesting that the potential to react to TCDD is present in +/+ as well as *hr/hr* cells but is suppressed in the haired +/+ animals. A similar interpretation of gene interactions could explain the results that we and others have found comparing cells in culture from responsive and nonresponsive strains; it should be noted, however, that the difference in responsiveness to halogenated hydrocarbons between DBA/2 and C57BL/6 mice is localized to only the *Ah* locus (35).

Recent reports from Prokipcak and Okey (36), Henry and Gasiewicz (37), and Hoffman *et al.* (38) demonstrate that an additional protein must associate with the Ah receptor-ligand complex in order for the receptor-ligand complex to bind to its specific enhancer sequence 5′ to the promoter of *CYP1A1*. Hepatic cytosol from adult DBA/2 mice is capable of supplying this factor to Ah receptor-ligand complexes from C57BL/6 cytosol stripped of this protein, thereby reconstituting specific DNA binding. These results suggest that it is the ligand-binding subunit of the Ah receptor that is defective in adult DBA/2 mice (37). Poland and Glover (29) have characterized the strain distribution of Ah receptor proteins specified by the various Ah receptor alleles and report that 18 strains of nonresponsive mice carrying the Ah^d allele express a M_r 104,000 protein, in contrast to the M_r 95,000 protein of the $Ah^{b\text{-}1}$ allele found in the responsive C57BL/6 family (allele $Ah^{b\text{-}2}$ is M_r 104,000 and allele $Ah^{b\text{-}3}$ is M_r 105,000). Whether this difference in molecular mass results in an Ah receptor with altered ligand affinity remains to be determined.

The data presented here are consistent with the hypothesis that nonresponsive mice have a mechanism similar to that in responsive mice for Ah receptor-mediated enzyme induction but that the ability of the receptor to bind nonhalogenated compounds such as MC is considerably less than in tissues or cells of responsive strains of mice.

Our studies provide the first direct evidence that nonhalogenated inducers such as [^{3}H]MC can bind the Ah receptor in

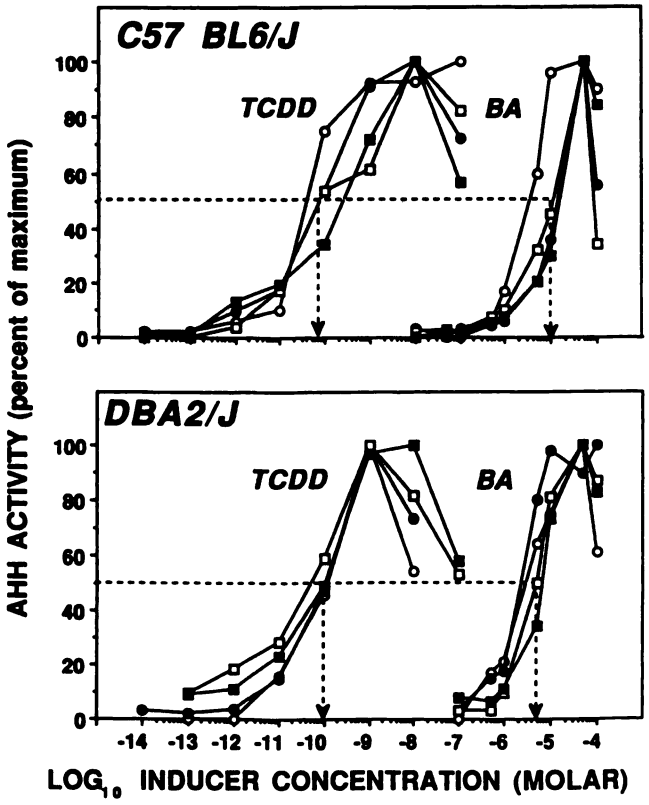

Fig. 7. Log dose-response curves for AHH induction by [^{3}H]TCDD and BA in culture: comparison of C57BL/6J and DBA/2J cultured embryo cells. C57BL/6J and DBA/2J cultured embryo cells were incubated in culture for 24 hr with concentrations of [^{3}H]TCDD and BA indicated in the *abscissa*. AHH activity was determined by the method of Nebert and Gelboin (24), as described in the text. *Points* are the mean of duplicate determinations for one experiment from each of four independent litters of C57BL/6J and four independent litters of DBA/2J mice. Because the absolute AHH activity varied somewhat among experiments with different cell cultures, the *ordinate* scale has been normalized to show AHH activity as a percentage of the maximum activity observed within each experiment for each cell type. For induction by [^{3}H]TCDD, the maximal activity was 1.5 pmol/min/mg of protein in cells from DBA/2J mice versus 2.0 pmol/min/mg of protein for C57BL/6J mice. The maximal activity induced by BA was 2.0 pmol/min/mg of protein in cells from DBA/2J mice versus 4.5 pmol/min/mg of protein for C57BL/6J mice. Noninduced levels were 0–0.17 pmol/min/mg.

cytosol from nonresponsive strains of mice. These studies also point to significant functional differences between embryonic cells in culture versus tissues *in vivo*. In cytosols obtained from cultured embryonic cells, the affinity for binding of [^{3}H]TCDD to cytosolic Ah receptor is only about 2–3-fold different between C57BL/6J (≈3 nM) and DBA/2 (≈6 nM), whereas in adult hepatic cytosol the difference is almost 10-fold (1.8 nM for C57BL/6 versus 16 nM for DBA/2J) (19). The reasons for this diminished degree of difference between responsive and nonresponsive mice in culture (compared with the more dramatic difference *in vivo* in adult animals) are as yet unknown.

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