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Detection and Characterization of a Low Affinity Form of Cytosolic Ah Receptor in Livers of Mice Nonresponsive to Induction of Cytochrome P_1-450 by 3-Methylcholanthrene

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SUMMARY

Ah "nonresponsive" mice (prototype, DBA/2) show no significant increase in hepatic P_1-450 (P450IA1) when treated with 3-methylcholanthrene or other nonhalogenated polycyclic aromatic hydrocarbons. Potent halogenated aromatics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induce P_1-450 in liver of nonresponsive mice, but the dose required is ≈15-fold higher than in "responsive" mice (prototype, C57BL/6). It was postulated several years ago that the genetic basis of nonresponsiveness was a "defect" in the Ah receptor, which normally binds TCDD and other inducers and mediates the induction process. Cytosolic Ah receptor hitherto had not been detectable in hepatic cytosol from nonresponsive mice. Using a modified sucrose gradient assay that we developed in studies on human tissue [*Cancer Res.* **47**:4861–4868 (1987)], we now have detected cytosolic Ah receptor in nonresponsive mice. By saturation analysis, the concentration of specific binding sites for [^{3}H]TCDD in hepatic cytosol from DBA/2J mice was (mean ± SE) 55 ± 6.6 fmol/mg of cytosolic protein (n = 21) compared with 133 ± 7.1 fmol/mg (n = 21) in responsive C57BL/6J mice. Ah receptor also was detected in significant concentrations in other nonresponsive strains; SWR/J, AKR/J, RF/J, and DBA/2N. The sedimentation coefficient on sucrose gradients was the same (≈9 S) in nonresponsive as in responsive strains. The major difference in nonresponsive mice is that hepatic cytosolic Ah receptor has an apparent affinity for [^{3}H]TCDD that is about 10-fold lower than in responsive strains; K_d in DBA/2J mice = 16 ± 2.5 nM (n = 21) and K_d in C57BL/6J mice = 1.8 ± 0.2 nM (n = 21). Thus, nonresponsive mice do possess the cytosolic Ah receptor in liver. However, the receptor is present in reduced concentration and appears to be a low affinity form, possibly as the result of a mutation in the gene(s) coding for the receptor protein(s).

In mice, a genetic difference at the *Ah* locus, which governs inducibility of cytochrome P_1-450 (P450IA1), has provided a powerful model for studying early events in the induction mechanism (1). Certain strains of inbred mice such as C57BL/6 are termed genetically "responsive" because they exhibit high induction of P_1-450 and associated AHH activity in liver when they are treated with nonhalogenated polycyclic aromatic hydrocarbons such as MC, BP, or BA. Genetically "nonresponsive" strains such as DBA/2 do not show a significant increase in hepatic P_1-450 even when treated with very high doses of nonhalogenated PAHs (2, 3). The highly potent halogenated inducer TCDD does induce P_1-450 in nonresponsive strains of mice, but the dose required for half-maximal induction by TCDD is approximately 15-fold higher in DBA/2N mice than in C57BL/6 mice (2, 3).

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Thus, nonresponsive mice do, in fact, respond to high doses of TCDD. Moreover, [^{3}H]TCDD·Ah receptor complexes can be recovered from cell nuclei of liver, lung, and kidney from DBA/2 mice injected with [^{3}H]TCDD *in vivo* (4, 5). These pieces of evidence suggest that the cytosolic Ah receptor, which normally binds TCDD and other inducers and thereby initiates the induction process, is present in liver from nonresponsive strains. However, the cytosolic Ah receptor has not been detectable in any previous assays using nonresponsive mice (6–8).

It was postulated several years ago (9–11) that nonresponsiveness might be due to a mutation at the *Ah* locus, leading to production of a defective receptor that was unable to functionally bind inducers of the MC type. Because nonresponsive mice do respond (albeit with reduced sensitivity) to TCDD and because binding to the cytosolic Ah receptor is believed to be the essential first step in P_1-450 induction, it would be expected

ABBREVIATIONS: Ah, aromatic hydrocarbon; AHH, aryl hydrocarbon hydroxylase; BA, benz[a]anthracene; BP, benzo[a]pyrene; BSA, bovine serum albumin; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PCDBF, 2,3,4,7,8-pentachlorodibenzofuran; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

that binding of [^{3}H]TCDD to the cytosolic receptor should be detectable in nonresponsive mice.

Our present experiments provide direct evidence that the cytosolic Ah receptor is present in nonresponsive mice and that this receptor has reduced affinity for [^{3}H]TCDD.

Materials and Methods

Animals. C57BL/6J, DBA/2J, RF/J, SWR/J, and AKR/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DBA/2N mice were obtained from Charles River Breeding Laboratories of Canada (Montreal, Quebec). Mice were 3 to 6 months old at the time of study and were males unless otherwise indicated.

Chemicals. [^{3}H]TCDD (32 Ci/mmol), nonradioactive TCDBF, and PCDBF were synthesized and generously provided by Dr. S. Safe (Texas A & M University). [^{3}H]TCDD was repurified to greater than 95% radiochemical purity by high performance liquid chromatography, as previously described (12). [^{3}H]MC (generally labeled, 37 Ci/mmol), obtained from Amersham Corp. (Oakville, Ontario), and [^{3}H]BP (55 Ci/mmol), from New England Nuclear-Dupont Canada (Dorval, Quebec), were repurified in our laboratory by reverse phase high performance liquid chromatography to greater than 97% radiochemical purity. Sodium molybdate, dimethylsulfoxide, charcoal (Norit A), and EDTA were from Fisher Chemical Co. (Toronto, Ontario); BSA, catalase, and dithiothreitol were from Sigma Chemical Co. (St. Louis, MO).

Buffers. The standard buffer was HEDG (25 mM HEPES/1.5 mM EDTA/1 mM dithiothreitol/10% glycerol by volume, pH 7.6). In most experiments, sodium molybdate was added to this buffer to a final concentration of 20 mM; this is termed HEDGM buffer.

Cytosol preparation. Mice were killed by cervical dislocation and the livers were perfused with cold HEDG buffer via the hepatic portal vein. All subsequent procedures were performed at 0 to 4°. Livers were removed and homogenized either in HEDG buffer or in HEDGM buffer (3 ml/g of tissue), as indicated in the figure legends for particular experiments. In some experiments in which the effect of molybdate was to be tested, minced livers were pooled from two or three animals of the same strain, the pool was mixed, and then half the mix was homogenized in HEDG buffer and half in HEDGM buffer. Homogenates were centrifuged at 10,000 × g for 20 min. The resulting supernatant was filtered through glass wool to reduce lipid content and then recentrifuged at 105,000 × g for 1 hr. This supernatant (cytosol) was frozen in liquid nitrogen until use.

Incubation of cytosol with radioligands. Cytosol samples were diluted to 5 mg of protein/ml [as measured by the method of Bradford (13)] and then incubated at 4° with either [^{3}H]TCDD, [^{3}H]MC, or [^{3}H]BP. Specificity of binding was assessed using nonradioactive compounds known to be potent Ah receptor agonists, i.e., TCDBF or PCDBF. Radioligands and competitors were added to cytosol in dimethylsulfoxide (final dimethylsulfoxide volume = 10 μl/ml cytosol). In some cases, samples were treated with charcoal/dextran by adding the labeled cytosol to a charcoal/dextran pellet. Charcoal was resuspended in the sample, incubated for 15 min, then removed by centrifugation at 4000 × g for 15 min.

Velocity sedimentation on sucrose gradients. Samples were analyzed by density gradient centrifugation using the vertical-tube rotor technique described by Tsui and Okey (14). Aliquots (300 μl) of labeled cytosol were layered onto linear (10–30%) sucrose gradients that had been prepared in the same buffer used to homogenize and prepare that particular cytosol sample. Gradients were centrifuged at 2° for 2 hr at 372,000 × g_{av}. After centrifugation, 25 fractions (200 μl each) were collected from each gradient and radioactivity was determined by liquid scintillation counting and was corrected for counting efficiency. Sedimentation coefficients ($S_{20,w}$) for radioactive peaks were calculated by the method of Martin and Ames (15) relative to [^{14}C]formaldehyde-labeled BSA (4.4 S) and catalase (11.3 S), which were included in each gradient as internal sedimentation markers. BSA and catalase were labeled with [^{14}C]formaldehyde as previously described (12).

Saturation analysis and determination of binding affinity. Cytosol aliquots (5 mg of protein/ml, prepared in HEDGM buffer) were incubated at 4° for 2 hr with [^{3}H]TCDD, at concentrations ranging from 0.25 to 20 nM (C57BL/6J mice) or 2 to 40 nM (DBA/2J mice). Specific binding in the 9 S peak was determined for each sample by velocity sedimentation on sucrose gradients. Samples were not treated with charcoal/dextran. Data were analyzed both by Scatchard plots (16) and by Woolf plots (17). Because it is difficult to experimentally measure the "unbound" fraction for extremely hydrophobic ligands such as [^{3}H]TCDD, unbound [^{3}H]TCDD in the Scatchard plots and in the Woolf plots is defined as all [^{3}H]TCDD that is not specifically bound to the 9 S receptor peak. Note that our assay (which does not use charcoal adsorption) differs somewhat from an Ah receptor assay, in which a charcoal adsorption step is employed as the sole means of separating "bound from unbound radioligand; in the charcoal adsorption assay, unbound was computed by subtracting the radioligand in solution after charcoal treatment from the total radioligand in solution before charcoal treatment (18). Although the commonly used Scatchard plot is shown for purposes of illustration, our quantitative comparisons of binding affinity in C57BL/6J versus DBA/2J are based on data from the Woolf plots. Woolf plots have two major advantages in this analysis. First, the only measured parameter required is the amount of specifically bound ligand; unbound ("free") ligand constitutes all ligand that is unbound or that is bound to nonreceptor sites (17). Second, the Woolf plot is less sensitive than the Scatchard plot to outliers in the data points and to data that are less than perfectly linear (17).

Results

Stabilization by molybdate. Fig. 1 illustrates the importance of having molybdate in the homogenizing buffer in order to stabilize the Ah receptor in hepatic cytosol of nonresponsive DBA/2J mice. If molybdate is not included in the buffer, most or all of [^{3}H]TCDD binding in the specific 9 S peak is lost (Fig. 1). In a group of five DBA/2J mice, the concentration of specific [^{3}H]TCDD binding sites detected after incubation with 20 mM [^{3}H]TCDD was 29 ± 1.5 fmol/mg of cytosol protein (mean ± SE) in cytosol from portions of liver prepared with molybdate, whereas in cytosol prepared from the same livers without molybdate the specific binding was only 6.2 ± 3.8 fmol/mg. In three of the five mice, samples prepared without molybdate had no detectable binding, but specific binding was detected in all mice in the presence of molybdate. Molybdate was tested at various concentrations; although some molybdate was required for detection of receptor, there were no significant differences in the concentration of Ah receptor sites detected at molybdate concentrations between 10 and 100 mM (data not shown). In contrast to the importance of molybdate for DBA/2J mice, in liver from responsive C57BL/6J mice molybdate had no significant effect on the concentration of Ah receptor sites detected during the standard sucrose gradient assay (Fig. 1) (19).

Loss of specifically bound ligand after treatment with charcoal. The Ah receptor in DBA/2J hepatic cytosol was highly susceptible to loss of specifically bound [^{3}H]TCDD if charcoal/dextran was used to adsorb "nonspecifically bound" radioligand before the separations on sucrose gradients. As shown in Fig. 2, charcoal treatment (1 mg of charcoal/mg of cytosol protein) reduces the background radioactivity in sucrose gradients but also removes a significant amount of [^{3}H]TCDD from the specific 9 S peak in DBA/2J cytosol. In cytosol from C57BL/6J mice this amount of charcoal reduces background radioactivity in the gradient without having a deleterious effect

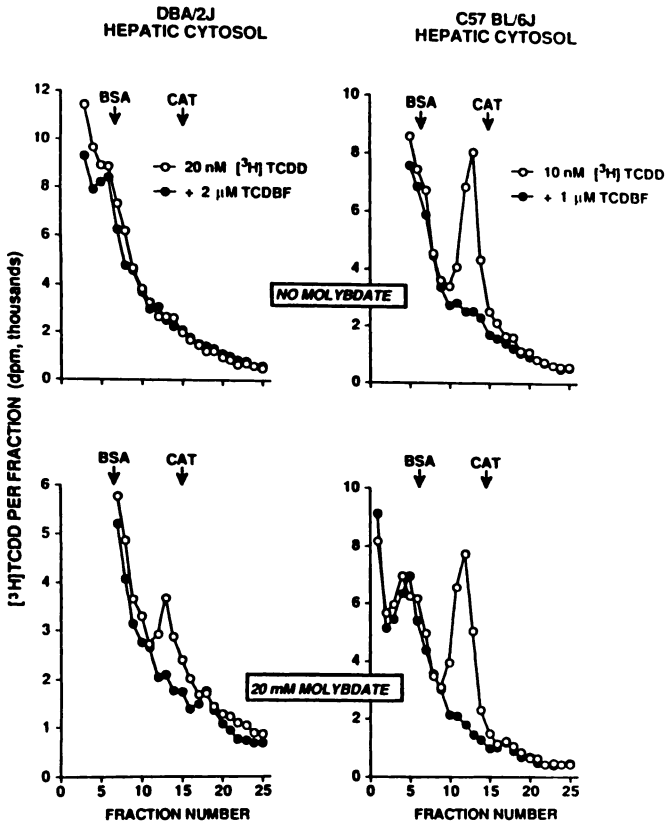

Fig. 1. Sucrose density gradient profiles illustrating the importance of molybdate for detecting the Ah receptor in hepatic cytosol from nonresponsive DBA/2J mice. Portions of livers from each mouse strain were homogenized either in HEDG buffer (without molybdate) or in HEDGM buffer (containing 20 mM sodium molybdate). Cytosol aliquots (5 mg of protein/ml) from each preparation then were incubated for 2 hr at 4° either with 20 nM [^{3}H]TCDD (for DBA/2J cytosol) or with 10 nM [^{3}H]TCDD (for C57BL/6J cytosol) in the absence of competitor (○) or in the presence of a 100-fold molar excess of TCDBF (●). Samples were analyzed, as described in Materials and Methods, by velocity sedimentation on sucrose gradients prepared in the same buffer system in which each particular tissue had been homogenized. None of the samples were treated with charcoal/dextran. The concentration of specific binding sites sedimenting in 9 S region (near fraction 15) is 28 fmol/mg of cytosol protein for the DBA/2J sample prepared in buffer containing 20 mM sodium molybdate; no specific binding was detectable in the DBA/2J sample prepared without molybdate. In the C57BL/6J cytosol, specific binding was 101 fmol/mg in the absence of molybdate and 110 fmol/mg with molybdate present. *Arrows at the tops of the profiles* indicate the sedimentation positions of [^{14}C]formaldehyde-labeled BSA (4.4 S) and [^{14}C]formaldehyde-labeled catalase (*CAT*, 11.3 S), which were added to each gradient as internal sedimentation markers.

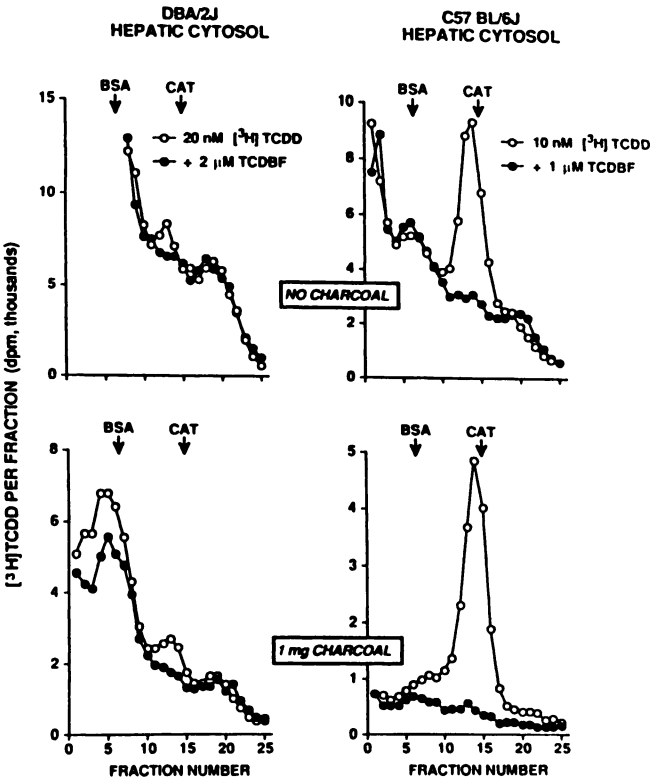

Fig. 2. Reduction of [^{3}H]TCDD binding to the specific 9 S peak in DBA/2J hepatic cytosol treated with charcoal/dextran. Hepatic cytosol (5 mg of protein/ml) from DBA/2J and C57BL/6J mice were prepared in HEDGM buffer, incubated with either 20 nM [^{3}H]TCDD (DBA/2J cytosol) or 10 nM [^{3}H]TCDD (C57BL/6J cytosol) for 2 hr at 4°. One portion of cytosol from each strain then was layered directly onto a sucrose gradient without being treated with charcoal/dextran (*top*). Another cytosol portion from each strain was incubated with charcoal/dextran (1 mg of charcoal/mg of cytosol protein) for 15 min at 4° (*bottom*); the charcoal was removed by centrifugation at 4000 × *g* for 15 min before the charcoal-treated sample was applied to a sucrose gradient. Samples then were analyzed by velocity sedimentation, as described in the legend to Fig. 1. In cytosol from DBA/2J mice the concentration of specific binding sites detected in the 9 S region was 27 fmol/mg of cytosol protein without charcoal treatment and 15 fmol/mg in the sample treated with charcoal. In C57BL/6J cytosol, specific binding was 147 fmol/mg without charcoal treatment and 149 fmol/mg in the sample treated with charcoal. Note that the *ordinate scales* are different for each *panel*. *CAT*, catalase.

on the amount of [^{3}H]TCDD specifically bound in the 9 S peak. Data in Fig. 3 indicate that the Ah receptor in DBA/2J hepatic cytosol is approximately 10-fold more susceptible to loss of ligand during charcoal treatment than is the receptor in C57BL/6J mice.

Attempts to detect Ah receptor in nonresponsive mice using nonhalogenated radioligands. In responsive strains of mice such as C57BL/6J, the 9 S form of cytosolic Ah receptor can be detected not only with the potent halogenated ligand [^{3}H]TCDD but also with the nonhalogenated P_1-450 inducer [^{3}H]MC (Fig. 4, *right*). In cytosol from DBA/2J mice, no specific binding of [^{3}H]MC could be detected in the 9 S region (Fig. 4, *left*) nor was specific binding of [^{3}H]MC detectable in the 9 S region in cytosol from SWR/J, AKR/J, or RF/J nonresponsive mice (data not shown). With another nonhalogenated P_1-450 inducer, [^{3}H]BP, specific binding could not be detected in the 9 S region of the gradient in cytosol from either C57BL/6J or DBA/2J mice (data not shown).

In C57BL/6J cytosol and in DBA/2J cytosol, both [^{3}H]MC and [^{3}H]BP bound extensively to a component in the 4–5 S region of the gradient. Binding of [^{3}H]MC and [^{3}H]BP in the 4–5 S region was not inhibited by incubation in the presence of a 100-fold molar excess of PCDBF (Fig. 4; data not shown for [^{3}H]BP) or in the presence of a 100-fold molar excess of nonradioactive TCDD (data not shown).

Nonradioactive MC, BP, dibenz[*a*,*h*]anthracene, and BA at 100-fold to 1000-fold molar excesses all were able to inhibit binding of [^{3}H]TCDD to the 9 S Ah receptor in hepatic cytosol from both C57BL/6J and DBA/2J mice (data not shown). Thus, although specific binding of [^{3}H]MC and [^{3}H]BP to the Ah receptor in DBA/2J cytosol could not be demonstrated directly by sucrose gradient analysis, the competition studies

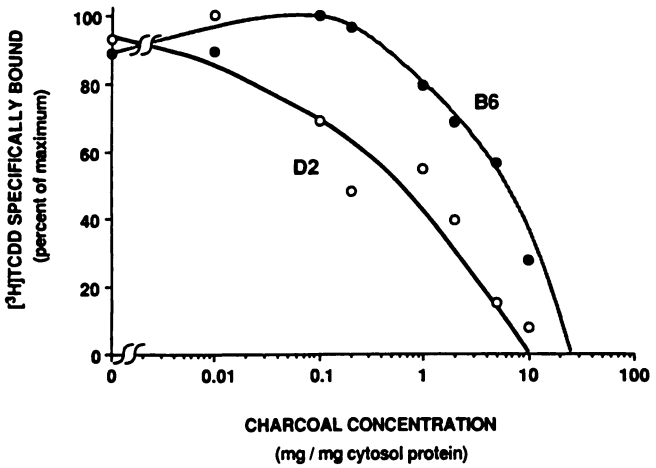

Fig. 3. Quantitation of the susceptibility of DBA/2J mouse Ah receptor to stripping of specifically bound [^{3}H]TCDD when treated with charcoal. Aliquots of hepatic cytosol (5 mg of protein/ml) were incubated with [^{3}H]TCDD [20 nM for DBA/2J (*D2*) mice; 10 nM for C57BL/6J (*B6*) mice] for 2 hr at 4°. Cytosol samples then were added to varied amounts of charcoal/dextran ranging from 0 to 10 mg of charcoal/mg of cytosol protein and incubated for 15 min at 4°. Charcoal/dextran was removed by centrifugation at 4000 × *g* for 15 min before the charcoal-treated cytosol was analyzed on sucrose gradients.

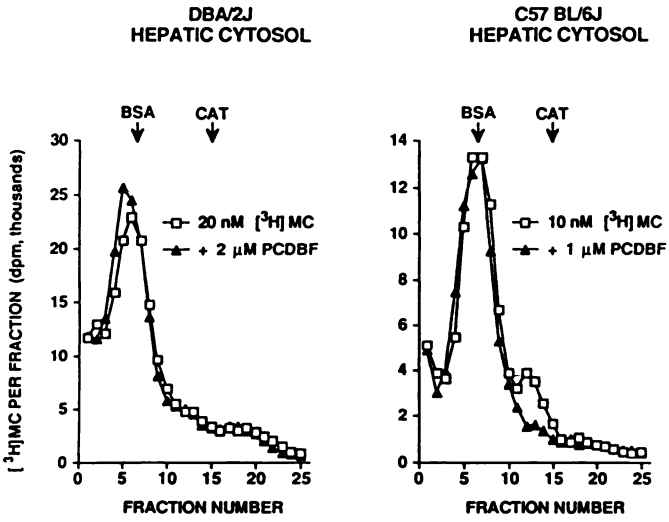

Fig. 4. Sedimentation analysis of cytosol labeled with [^{3}H]MC. Hepatic cytosol (5 mg of protein/ml) was incubated for 2 hr at 4° either with 20 nM [^{3}H]MC (DBA/2J) or with 10 nM [^{3}H]MC (C57BL/6J) in the absence of competitor (□) or in the presence of a 100-fold molar excess of PCDBF (▲). Samples were not treated with charcoal/dextran before analysis by velocity sedimentation. The concentration of specific binding sites detected with [^{3}H]MC in the 9 S region for C57BL/6J cytosol is 26 fmol/mg of cytosol protein. No specific binding peak for [^{3}H]MC was detectable in the 9 S region of cytosol from DBA/2J mice. *CAT*, catalase.

clearly indicate the ability of nonhalogenated PAHs, at high concentrations, to interact with Ah receptor in nonresponsive as well as responsive mice. The potency of nonhalogenated PAHs in competing with [^{3}H]TCDD for Ah receptors in DBA/2J mice, relative to their potency in C57BL/6J mice, has not yet been determined.

Ah receptor in DBA/2N mice and other nonresponsive mouse strains. In addition to detecting specific binding of [^{3}H]TCDD to the Ah receptor in DBA/2J mouse hepatic cytosol, we also detected specific 9 S peaks in hepatic cytosol from four additional nonresponsive strains; DBA/2N, RF/J, AKR/J, and SWR/J (Table 1; Fig. 5). The concentration of Ah receptor sites in DBA/2N hepatic cytosol was the same as that in DBA/2J hepatic cytosol (Table 1). However, as previously observed (7), in the responsive C57BL/6 strain the concentration of hepatic Ah receptor was significantly higher in C57BL/6J mice than in C57BL/6J mice; the higher concentration of receptor in C57BL/6N versus C57BL/6J was present both in males and in females (Table 1). In all strains tested the concentration of Ah receptor in hepatic cytosol was higher in males than in females in the same age group (Table 1).

In RF/J, AKR/J, and SWR/J strains the level of specific binding sites was about 15 fmol/mg of cytosol protein (Fig. 5); however, the concentration of [^{3}H]TCDD we used in screening these samples (10 nM) probably was not sufficient to saturate the receptor population. Nevertheless, the Ah receptor clearly was present in hepatic cytosol from all five nonresponsive strains tested; DBA/2J, DBA/2N, RF/J, AKR/J, and SWR/J (Table 1; Figs. 1 and 5).

Nonhepatic tissues. The majority of our studies focused on the Ah receptor in liver of nonresponsive mice. We attempted to detect the Ah receptor in cytosol prepared from some nonhepatic tissues as well. No specific binding of [^{3}H]TCDD could be detected in cytosol from thymus, small intestine, urinary bladder, or lung of DBA/2J mice; specific binding of [^{3}H]TCDD to Ah receptors was detected in cytosol from each of these tissues in C57BL/6J mice (data not shown). In nonresponsive SWR/J mice we detected a small amount (10–15 fmol/mg) of specific binding in lung cytosol.

Estimation of [^{3}H]TCDD binding affinity in DBA/2J versus C57BL/6J cytosol. The apparent K_d for the interaction of [^{3}H]TCDD with the 9 S Ah receptor was estimated by incubating hepatic cytosols with a wide range of [^{3}H]TCDD concentrations and then determining specific binding in the 9 S region by sucrose gradient analysis for each sample. The duration of incubation with [^{3}H]TCDD was 2 hr in these studies; significantly more specific binding in DBA/2J cytosol was detected at 2 hr than at 1 hr (data not shown). We also tested incubation times up to 20 hr and found no significant changes in specific 9 S binding at 20 hr over that detected after 2 hr of incubation with [^{3}H]TCDD (data not shown). It also was of interest that the amount of specific binding of [^{3}H]

TABLE 1

Comparison of the concentration of Ah receptor in hepatic cytosol from male and female C57BL/6J, DBA/2J, C57BL/6N, and DBA/2N strains of mice

C57BL/6J and DBA/2J mice were from The Jackson Laboratory; C57BL/6N and DBA/2N mice were from Charles River Breeding Laboratories. The level of specific binding to the 9 S peak in hepatic cytosol from each animal was determined by sucrose gradient analysis after incubation with a single concentration of [^{3}H]TCDD (10 nM for C57BL/6J and C57BL/6N; 20 nM for DBA/2J and DBA/2N). Cytosol was prepared in HEDGM buffer and no charcoal was used in these assays. There were six animals in each group. Values shown are fmol of [^{3}H]TCDD specifically bound in the 9 S peak/mg of cytosol protein (mean ± standard error).

	Specific Binding	
	Male	Female
	fmol/mg	
C57BL/6J	141 ± 6.2[a]	112 ± 7.6
DBA/2J	34 ± 2.5[a]	26 ± 1.8
C57BL/6N	300 ± 38[b,c]	162 ± 11[c]
DBA/2N	40 ± 3.7[a]	25 ± 3.4

[a] Significantly greater than mean for female mice, $p < 0.05$.
[b] Significantly greater than mean for female mice, $p < 0.01$.
[c] Significantly greater than mean for C57BL/6J mice, $p < 0.01$.

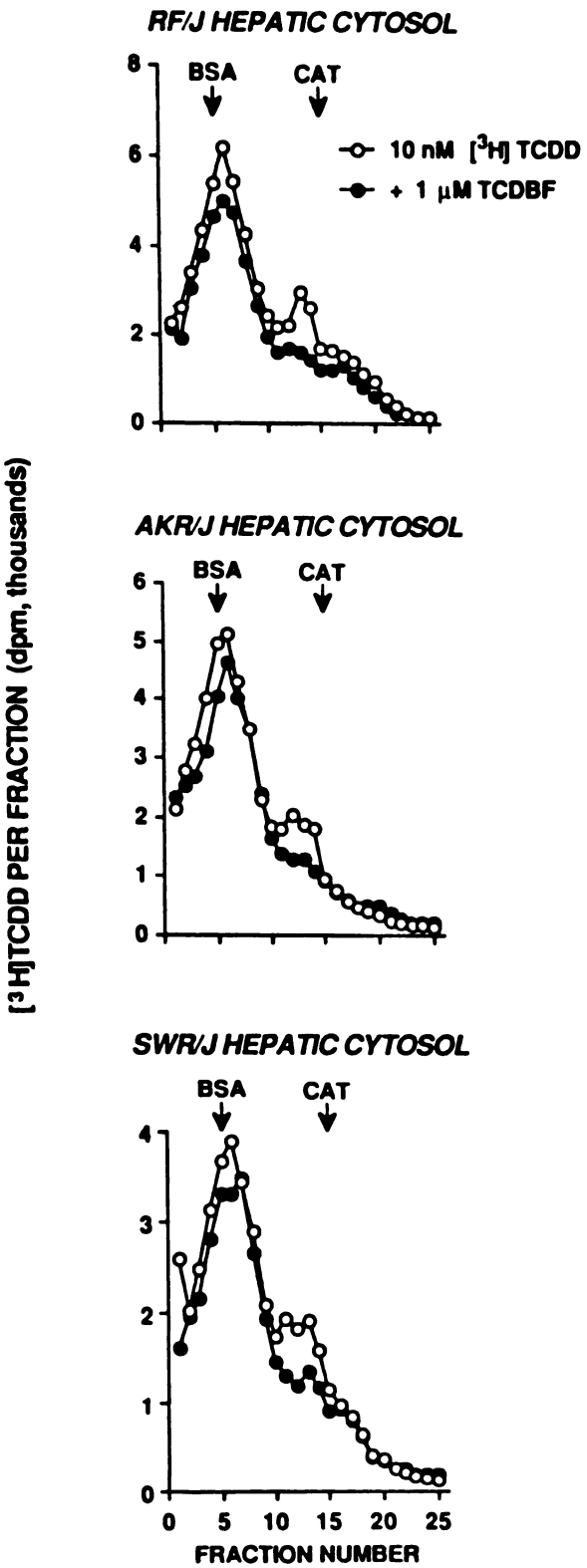

Fig. 5. Detection of Ah receptor in hepatic cytosol from the nonresponsive mouse strains RF/J, AKR/J, and SWR/J. Hepatic cytosol (5 mg of protein/ml) from each strain was prepared in HEDGM buffer and then incubated for 2 hr at 4° with 10 nM [^{3}H]TCDD in the absence of competitor (○) or in the presence of a 100-fold molar excess of TCDBF (●). Samples were treated with charcoal/dextran (0.1 mg of charcoal/mg of cytosol protein) before analysis by velocity sedimentation. The concentrations of specific binding sites detected in these samples (fmol/mg of cytosol protein) were RF/J = 14; AKR/J = 16; SWR/J = 13. It should be noted that the 10 nM [^{3}H]TCDD concentration used in this assay probably is not sufficient to saturate the Ah receptor in nonresponsive strains; hence, the values shown here are likely to substantially underestimate the true concentration of receptor sites in hepatic cytosol from each of these strains. *CAT*, catalase.

TCDD in DBA/2J cytosol did not *decrease* during prolonged incubation. In the presence of ligand and in the presence of molybdate, the Ah receptor in DBA/2J hepatic cytosol appears stable at least up to 24 hr at 4° (data not shown); thus, the [^{3}H]TCDD·receptor complex is not disrupted during the 2-hr separation on sucrose gradients and the amount of specific binding detected by this assay should accurately reflect the concentration of receptor sites present. The association rate for the [^{3}H]TCDD·Ah receptor interaction could not be determined due to the length of time required to separate samples on the sucrose gradients (2 hr) and the fact that the binding reaction cannot be terminated with charcoal without stripping specifically bound ligand from the receptor.

As illustrated in Fig. 6 (top), saturation of Ah receptors in cytosol from DBA/2J mice requires higher concentrations of [^{3}H]TCDD than are necessary to saturate receptors in cytosol from C57BL/6J mice. Binding data were further analyzed by Scatchard plots (Fig. 6, *middle*) and by Woolf plots (Fig. 6, *bottom*). Scatchard and Woolf plots indicate that [^{3}H]TCDD binding is to a single class of sites in both DBA/2J and C57BL/6J cytosol. The kinetic parameters K_d and B_{max} were computed from Woolf plots (Table 2) rather than from Scatchard plots because the Woolf plot does not require experimental measurement of free radioligand and because the Woolf plot is less sensitive than the Scatchard plot to deviations of data points from perfect linearity (17). Full saturation studies with Woolf plot analyses were performed on hepatic cytosol from 21 DBA/2J mice in comparison with cytosol from 21 C57BL/6J mice. The concentration of specific [^{3}H]TCDD binding sites (B_{max}) in the population of C57BL/6J mice was significantly higher than in DBA/2J mice and the apparent affinity with which [^{3}H]TCDD bound Ah receptors was almost 1 order of magnitude higher in C57BL/6J than in DBA/2J cytosol (Table 2).

Discussion

Many previous attempts have been made to detect Ah receptors in cytosol from tissues of nonresponsive mice. Cytosolic Ah receptor in DBA/2 mice was not detectable in liver or thymus with charcoal adsorption assays (6, 20), in liver with hydroxylapatite adsorption assays (21), isoelectric focusing (22), gel permeation chromatography (23, 24), protein high performance liquid chromatography (25), photoaffinity labeling (26), or previous gradient centrifugation assays (4, 7, 24). In addition, cytosolic Ah receptor was not detectable by sucrose gradient assays in tissues from other nonresponsive strains including palate from AKR/J mice (27) and liver of congenic B6(Ah^d) mice (28).

Data reported here are the first direct demonstration that the cytosolic Ah receptor exists in genetically nonresponsive mice. The modified gradient centrifugation assay we used was derived empirically during studies of the cytosolic Ah receptor in human tissues (29) and human cells (30). The two most important technical changes are the use of molybdate in the buffer during homogenization and throughout all subsequent steps, along with elimination or reduction in the amount of

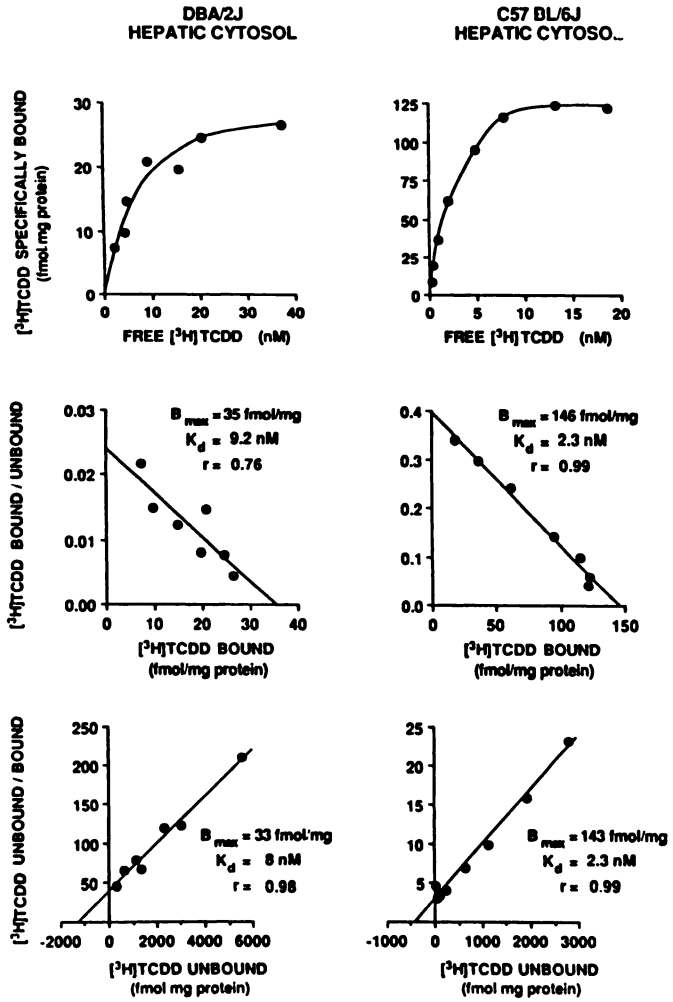

Fig. 6. Saturation analysis and determination of apparent affinity of binding of [^{3}H]TCDD in hepatic cytosol from a DBA/2J mouse versus a C57BL/6J mouse. Cytosol aliquots (5 mg of protein/ml, prepared in HEDGM buffer) were incubated at 4° for 2 hr with [^{3}H]TCDD at concentrations ranging from 2 to 40 nM (DBA/2J) or 0.25 to 20 nM (C57BL/6J). Specific binding in the 9 S peak was determined for each sample by analysis on sucrose gradients. Samples were not treated with charcoal/dextran. The Scatchard plots (*middle*) and Woolf plots (*bottom*) were derived from data shown in the saturation plots (*top*). Binding parameters shown in the Scatchard and Woolf plots were calculated by least squares linear regression. In the Woolf plot, the intercept on the abscissa represents $(-)K_d$, the intercept on the ordinate represents K_d/B_{max}, and the slope is equal to $1/B_{max}$. See Materials and Methods for further details.

TABLE 2

Affinity of [^{3}H]TCDD binding and concentration of binding sites in DBA/2J mice versus C57BL/6J mice

Data were derived from saturation assays and Woolf plot analyses, as described in Materials and Methods and in the legend to Fig. 6. Values represent the mean ± standard error for 21 animals in each group.

	DBA/2J	C57BL/6J
K_d (nM)	16 ± 2.5	1.8 ± 0.2
B_{max} (fmol/mg of protein)	55 ± 6.4	133 ± 6.8

charcoal used to adsorb excess radioligand before gradient analysis. For maximum detection of the cytosolic Ah receptor in nonresponsive, strains it also is necessary to increase the concentration of [^{3}H]TCDD that is incubated with the cytosol. Detection of the cytosolic receptor in nonresponsive strains appears to depend upon a combination of these simple (but critical) technical factors rather than any single factor. For example, our laboratory previously had tested molybdate several times to determine whether its presence would permit the Ah receptor to be detected in DBA/2 cytosol; these earlier attempts were not successful.[1] Tukey *et al.* (5) also tested molybdate to determine whether it would stabilize the Ah receptor in DBA/2N hepatic cytosol and reported that the receptor still was not detectable. In both cases, however, cytosol was incubated with relatively low concentrations of [^{3}H]TCDD (1 to 10 nM) and samples were treated with a high concentration of charcoal (1 mg of charcoal/mg of cytosol protein). Thus, the small amount of [^{3}H]TCDD that might have become specifically bound would be lost at the charcoal treatment stage. This earlier work is cited not to point out "failures," but to emphasize that the delicate specific binding of [^{3}H]TCDD to the Ah receptor in nonresponsive mice can easily be lost during assay.

A technique that has been invaluable in molecular characterization and partial purification of the Ah receptor is photoaffinity labeling with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin (26, 31). This compound exhibits high affinity reversible binding to the cytosolic Ah receptor and can be photoactivated to covalently bind to the receptor (26). Because the ^{125}I label provides very high specific activity, it would seem that 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin should be an excellent candidate for photoaffinity labeling of the low affinity receptor in DBA/2 mice, yet no labeled peptides corresponding to the Ah receptor were detected in hepatic cytosol from congenic nonresponsive C57BL/6J(Ah^d) mice (26). It would be of great interest, in light of our findings, to determine whether slight modifications of this procedure (e.g., elimination of charcoal treatment) might permit photoaffinity labeling of the cytosolic Ah receptor from nonresponsive mice and whether subsequent electrophoretic characterization would reveal any significant difference in relative molecular mass between the low affinity product of the Ah^d allele and the two known high affinity variants that have been characterized for the Ah^b allele (32).

The loss of specific binding in charcoal-treated cytosol from DBA/2J mice, compared with C57BL/6J mice, generally seems to be in proportion to the difference in affinity of [^{3}H]TCDD binding between the two strains. Charcoal may easily strip ligand from the receptors of nonresponsive mice simply because the binding is of relatively low affinity.

It is perhaps more difficult to interpret the nature of the molybdate stabilization phenomenon in nonresponsive versus responsive mice. The precise mode of molybdate action still is not resolved in any system, but the most recent evidence indicates that molybdate interacts directly with steroid receptor proteins (perhaps at sulfhydryl groups) and maintains the association of unliganded receptor with the abundant heat-shock protein, hsp 90 (33). Molybdate-stabilized steroid receptors exist as an 8 to 10 S complex with hsp 90 on sucrose gradients at low ionic strength; molybdate greatly retards disaggregation of this complex when cytosol is exposed to conditions of high ionic strength (33). Thus, one potential function of molybdate in DBA/2J cytosol might be to maintain the Ah receptor in the 9 S state as a complex with hsp 90. Very recently, hsp 90 has been shown to form complexes with the Ah receptor in rat hepatic cytosol (34, 35) and in responsive Hepa-1 mouse

[1] Unpublished data.

hepatoma cells (35). The fact that Ah receptor from DBA/2J mice sediments at ≈9 S in our assays suggests (but does not prove) that hsp 90 also is associated with the ligand-binding subunit (or subunits) of the Ah receptor in nonresponsive strains. What is unusual in regard to the molybdate effect is that the Ah receptor in *responsive* strains of mice such as C57BL/6J is highly stable in the 9 S form under high-salt conditions, even in the absence of molybdate (19); molybdate, therefore, has very little effect on the cytosolic receptor from responsive mice. The inference from the effectiveness of molybdate at stabilizing the Ah receptor from nonresponsive mice is that the receptor from nonresponsive strains differs in at least two aspects from the receptor in responsive mice, as follows: 1) the affinity of the receptor for ligands is lower in nonresponsive mice than in responsive mice and 2) the ability of the ligand-binding subunit(s) from nonresponsive mice to form a 9 S complex with hsp 90 (or other soluble proteins) is weaker in nonresponsive mice than in responsive mice. This latter point is evidenced by the fact that molybdate is essential for detecting 9 S cytosolic receptor in nonresponsive strains (this report) but does not increase the amount of 9 S receptor detected in responsive strains (Ref. 18 and this report). It is possible that responsive strains of mice possess an endogenous "molybdate-like" factor [such as has been reported to stabilize the glucocorticoid receptor in rat liver (36)] and that this factor is deficient in nonresponsive strains of mice. Some heritable variation in inducibility of P_1-450 might be related to receptor-stabilizing factors rather than being limited to a gene or genes encoding ligand-binding subunit(s) of the receptor per se. It also is possible that a single mutation might simultaneously reduce affinity of the receptor for its ligands and also interfere with the ability of the receptor to interact with hsp 90.

Our measurements of the relative affinity of Ah receptors in nonresponsive versus responsive mice appear to confirm the hypothesis set forth several years age (9–11) that the insensitivity of nonresponsive strains to MC-like inducers was due to the presence of a low affinity variant form of Ah receptor in nonresponsive animals. Measurement of the affinity of binding in nonresponsive mice was complicated somewhat by the low amount of receptor in cytosol from these animals. However, the sucrose gradient procedure is an effective means of detecting and quantitating the amount of specifically bound ligand. Moreover, the receptor from DBA/2J hepatic cytosol was stable for up to 24 hr in the presence of ligand and molybdate. The K_d values that we report for the interaction of [^{3}H]TCDD with the Ah receptor do not necessarily represent the true dissociation constants. Several previous studies have shown that the apparent K_d for the interaction of [^{3}H]TCDD or tritiated dibenzofurans with the Ah receptor varies with the protein concentration employed in hydroxylapatite adsorption assays or in isoelectric focusing (37–39); the apparent affinity increases as the protein concentration is decreased. Recently Bradfield *et al.* (18) reported that the apparent affinity of a very high specific activity radioligand, [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin, varied as much as 2 orders of magnitude, depending upon the concentration of cytosolic protein being studied. In our experiments the protein concentration was fixed at 5 mg/ml for all samples. Thus, although the apparent K_d values we derived may not reflect the true affinity of [^{3}H]TCDD for receptor, the relative affinities and the comparisons between DBA/2J and C57BL/6J mice remain valid. These affinity estimates indicate that the decrease in affinity of Ah receptor in DBA/2J mice is in proportion to the shift in the dose-response curve for AHH induction by TCDD between these two strains (2, 3).

It has been known for some time that "nonresponsive" mice are not truly nonresponsive. As cited above, TCDD clearly induces AHH activity in DBA/2 mice to nearly the same level as in C57BL/6 mice. It also has been repeatedly demonstrated that nonresponsive mice do exhibit Ah-receptor-mediated toxicity from TCDD and related halogenated aromatic compounds if given higher doses than responsive strains; this is consistent with the presence of a receptor with reduced affinity (2, 40–42). Among certain recombinant inbred lines of mice it also has been reported that MC can induce AHH activity (43). Finally, embryonic cells from so-called nonresponsive strains do exhibit AHH induction when treated in culture with nonhalogenated compounds such as MC, BP, or BA (44, 45). Using the same assay techniques reported in this paper, we have detected substantial amounts of cytosolic receptor in embryonic tissues and embryonic cells from nonresponsive strains of mice (45).

The results reported here should resolve a long-standing paradox, i.e., how did TCDD-treated nonresponsive mice manage to mount a P_1-450 induction response when no cytosolic receptor for TCDD appeared to exist in these animals? Previous experiments (4, 5) showed that the nuclear form of Ah receptor could be detected in nonresponsive mice but did not reveal any cytosolic receptor. This left open the possibility that TCDD bound directly to a nuclear protein in nonresponsive strains, rather than acting via a cytosolic intermediate. Our present experiments directly demonstrate that several nonresponsive strains do, in fact, have a low affinity form of Ah receptor in cytosol. Thus, the initial event in the induction response in both responsive and nonresponsive strains involves binding to cytosolic receptor. These data further strengthen the evidence that the Ah receptor mediates induction P_1-450 and toxic responses to specific halogenated and nonhalogenated aromatic hydrocarbons.

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