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BIOLOGICAL EFFECTS OF THE SUDAN DYES

ROLE OF THE *Ah* CYTOSOLIC RECEPTOR

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Abstract—The hepatic induction of two cytochrome P_1-450-mediated activities [aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin *O*-deethylase (ETR)] was studied following the administration of the azo dyes Sudan I, II, III and IV. When using *Ah*-responsive C57BL/6J mice, Sudan dye II proved to be quite potent as an inducer causing almost maximal induction at doses as low as 40 mg/kg (1.4 μmoles/kg body weight); Sudan dyes I, III and IV caused one-half the maximal induction at four times that dose. In contrast, none of these compounds caused induction of AHH or ETR in the *Ah*-nonresponsive DBA/2J animals. When the dyes were given to B6D2F_1 × D2 backcross progeny, a strict correlation with the presence of the Ah^b allele and the inducibility of AHH and ETR was observed. When these compounds were examined as agonists for the *Ah* cytosolic receptor by their capacity to replace [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin binding, Sudan dye II was substantially more effective than Sudan dyes I, III and IV. When four repeated doses of Sudan dye II were administered intraperitoneally to *Ah*-responsive C57BL/6J mice and *Ah*-nonresponsive DBA/2J mice, Sudan dye II-induced immunotoxicity was markedly greater in C57BL/6J compared to DBA/2J mice.

The Sudan dyes§ are a group of azo compounds which have been employed as dyes for drugs, food, clothing, and histochemical analysis [1]. As recently as 1974, about 270,000 kg of Sudan dye I, 236,000 kg of Sudan dye II, 70,000 kg of Sudan dye III, and 1,075,000 kg of Sudan dye IV were produced in the United States [1]. More recently, however, their use has been prohibited by U.S. law [1].

Prima facie these azo dyes bear a slight structural resemblance to the classical P_1-450 inducers,‖ e.g. polyhalogenated aromatic compounds such as the polyhalogenated biphenyls and TCDD or polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and benzo[*a*]pyrene. A number of previous investigations have shown these Sudan dyes [2–4] and related azo compounds [5] to be inducers of P_1-450-associated enzymes in animals [2–4]. Since the induction of these activities is controlled by the *Ah* receptor, we have examined (a) the induction of P_1-450-associated enzymes by Sudan dyes I–IV *in vivo*, (b) the interaction of Sudan dyes I–IV with the cytosolic *Ah* receptor *in vitro*, and (c) the immunotoxic effects of Sudan dye II in *Ah*-responsive and *Ah*-nonresponsive mice.

MATERIALS AND METHODS

Chemicals. Sudan dye I, Sudan dye II, Sudan dye III, and Sudan dye IV were purchased from the Aldrich Chemical Co. (Milwaukee, WI). These compounds are 90–97% pure upon delivery; the contaminants are water and inorganic compounds (Aldrich Chemical Co., personal communication). Corn oil was obtained from the Fischer Scientific Co. (Pittsburgh, PA). [1,6-^{3}H]TCDD (sp. act. 55 Ci/mmole) was obtained from KOR Isotopes Inc. (Cambridge, MA).

Animals. B6 and D2 inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were on a 14-hr:10-hr day:night light cycle. Animals were permitted free access to water and food (Purina Chow Laboratory). Backcross (B6D2) × D2 were generated at the animal facilities at NICHD.

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§ Abbreviations: Sudan dye I, 1-phenylazo-2-naphthol; Sudan dye II, 1-(2,4-dimethylphenyl)azo-2-naphthol; Sudan dye III, 1-[4-(phenylazo)phenylazo]-2-naphthol; Sudan dye IV, 1-[4-(2-methylphenylazo)-2-methylphenylazo]-2-naphthol; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [^{3}H]TCDD, [1,6-^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B6, C57BL/6J; D2, DBA/2J; AHH, aryl hydrocarbon hydroxylase; ETR, ethoxyresorufin *O*-deethylase; PFC, plaque forming cells; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

‖ "P-450" denotes collectively all forms of membrane-bound cytochrome associated with NADPH-dependent monooxygenase activities and capable of binding CO when reduced. "P_1-450" is defined arbitrarily as that polycyclic aromatic-inducible form associated most closely with polycyclic aromatic-inducible aryl hydrocarbon (benzo[*a*]-pyrene) hydroxylase activity. "P-448" is defined arbitrarily as that form, or forms, having a Soret peak shifted maximally to the blue when reduced and combined with CO. 3-Methylcholanthrene-inducible P-448 and P_1-450 in mouse liver appear to be closely related to rabbit liver "LM_4" and "form 6" respectively.

Chemical treatment. For the induction experiments, B6 and D2 mice (four per group) were injected intraperitoneally with the Sudan dyes (without further purification) or 3-methylcholanthrene in 0.1 ml of corn oil. Three control animals from each strain were treated with 0.1 ml of corn oil. For the immunotoxicity studies, three animals were inoculated daily for 4 days with Sudan dye II in 0.05 ml corn oil.

For the backcross analysis, progeny of B6D2F_1 × D2 cross were injected intraperitoneally with β-naphthoflavone (80 mg/kg) 36 hr prior to receiving a dose of zoxazolamine (300 mg/kg). Those animals which were paralyzed for less than 1 hr were regarded as *Ah*-responsive while those which were paralyzed for more than 1 hr were of the *Ah*-nonresponsive phenotype [6]. After a 3-week waiting period, all backcross progeny received Sudan dye II (80 mg/kg) 48 hr prior to being killed.

AHH and ETR determination. Forty-eight hours after the treatment with the azo dyes or 3-methylcholanthrene, the animals were killed and their livers were homogenized in 0.25 M sucrose–0.05 M Tris buffer, pH 7.5. The homogenate was centrifuged at 600 *g* for 5 min. The resulting supernatant fraction was centrifuged successively for 20 min at 10,000 *g* and for 60 min at 105,000 *g*. The 105,000 *g* microsomal pellet was resuspended in the initial homogenization buffer and stored at −70° until used. AHH and ETR activities were determined by previously published procedures [7, 8].

Sucrose density gradient analysis of the Ah *receptor.* The mice were killed by cervical dislocation. From ten untreated B6 mice the livers were combined, minced, and washed repeatedly with potassium phosphate-buffered saline (0.85% NaCl) until visibly clear of hemoglobin. All further operations were carried out at 4°. The material was homogenized with a Teflon–glass tissue grinder in 3 vol. of HEDG buffer [25 mM Hepes, 1.5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 10% glycerol (v/v), pH 7.6] containing 0.1 M NaCl. The homogenate was centrifuged at 10,000 *g* for 15 min, and the supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The supernatant (cytosolic) fractions were quickly frozen in 1 ml aliquots and stored in liquid nitrogen. It is known that no losses of *Ah* receptor occur during storage of these fractions for at least 6 months [9]. The objective of the receptor competition assay is to measure the capacity of a given chemical to displace [^{3}H]TCDD binding from the cytosolic receptor. Cytosol (1 ml) was incubated for 1 hr at 4° with 1 nM [^{3}H]TCDD in the absence or presence of a 100 nM concentration of the test compound; the cytosol was exposed simultaneously to the test compound (when present) and the radioligand. Dextran-coated charcoal was used to remove unbound [^{3}H]TCDD, following which centrifugation was performed at 235,000 *g* for 16 hr at 2° in a linear 5–20% sucrose density gradient. Twenty-five fractions (0.2 ml) from each gradient were each counted by scintillation spectrometry. Percent displacement of [^{3}H]TCDD from the *Ah* receptor by each test compound was calculated by dividing the test peak values (addition of the Sudan dye and [^{3}H]TCDD alone) and then multiplying by 100. Each compound was tested three times for competition. Further details of this assay have been described [9, 10].

Immunotoxicology. This assay is based on the capacity of radially diffusing antibody from spleen cells to bind to sheep red blood cells. In the presence of complement, the antibody will lyse sheep red blood cells in a monolayer mixture, thereby producing cleared areas, or plaques. The experimental procedure was similar to previously published methodology [11, 12]. Animals were injected intraperitoneally with 0.1 ml of a 10% sheep red blood cell (MA Bioproducts, Walkersville, MD) suspension. Ninety-six hours later, the spleens were removed and homogenized in a Potter–Elvehjem homogenizer, and nucleated spleen cells were counted. Spleen cells from pooled animals (10^6 cells in 0.10 ml) were mixed with 0.3 ml of RPMI 1640 (GIBCO, Grand Island, NY), 0.15 ml of a 10% sheep red blood cell solution, and 0.05 ml of guinea pig serum (MA Bioproducts) at 4°. One hundred microliters of this mixture was placed in a "Cunningham" slide chamber [13] and incubated for 75 min at 37°. PFC were determined by macroscopic examination.

RESULTS

The chemical structures of Sudan dyes I through IV, 3-methylcholanthrene and TCDD are illustrated in Fig. 1. The Sudan dyes were tested for their capacity to induce AHH and ETR activity in B6 and D2 animals (Tables 1 and 2). When Sudan dye II was examined, maximal AHH induction occurred at a concentration of 80 mg/kg (2.7 μmoles/kg) with an ED_{50} of 20–40 mg/kg dose range. Sudan dye II, twice recrystallized in methanol, displayed a similar level of AHH and ETR induction (data not shown). In contrast, doses as high as 160 mg/kg of Sudan dyes I and II produced about 60% of maximal AHH induction. Thus, a dose of 40 mg/kg Sudan dye I caused less than a 2-fold increase in AHH and a 4-fold increase in ETR. At the maximal dose tested (320 mg/kg), Sudan dyes I and III caused maximal AHH induction. Sudan dye IV may not produce maximal induction even at 320 mg/kg (Table 1). Sudan dye II (at 320 mg/kg) was not tested due to its ability to maximally induce AHH and ETR in B6 mice at 160 mg/kg. None of the Sudan dyes significantly induced AHH and ETR activity in the D2 mice (Table 1).

To further substantiate the involvement of the Ah^b allele, enzyme induction was studied in backcross animals. All progeny of a B6D2 × D2 cross were injected with Sudan dye II (80 mg/kg). The backcross analysis (Table 3) show that those mice phenotyped as *Ah*-responsive (Ah^b) possessed induced AHH and ETR enzyme activities, while mice of the Ah^d phenotype (*Ah*-nonresponsive) exhibited no induced enzyme activities.

The capacity of the Sudan dyes to compete for binding of 1 nM [^{3}H]TCDD to the cytosolic *Ah* receptor was examined (Table 1 and Fig. 2). At a final concentration of 100 nM, Sudan dye II was a potent competitor (78% displacement) for the *Ah* receptor, whereas Sudan dye I was moderately potent (48% displacement). Sudan dyes III and

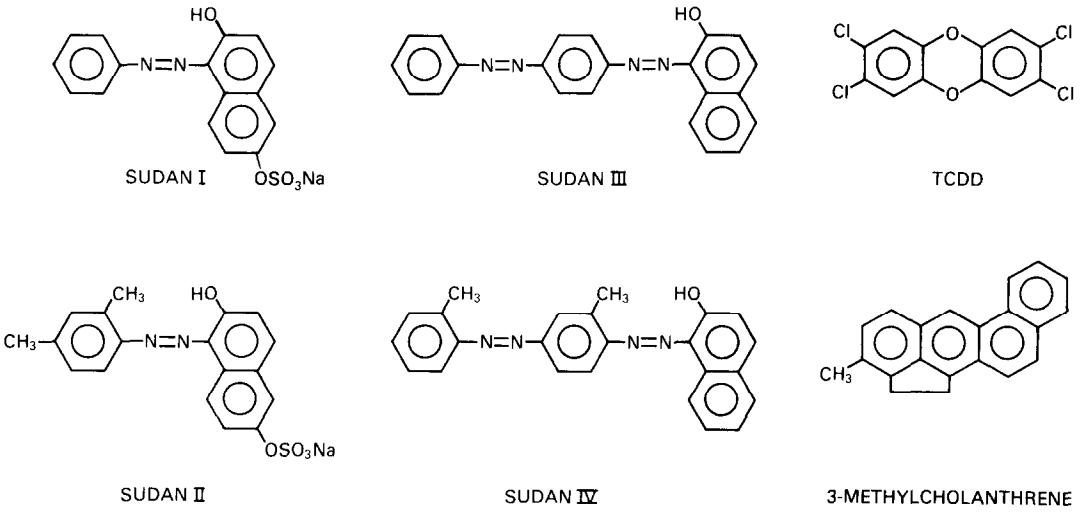

Fig. 1. Chemical structures of the four Sudan dyes along with the structures of 3-methylcholanthrene and TCDD for comparison.

IV marginally displaced [^{3}H]TCDD at these concentrations.

The immunotoxic effects of Sudan dye II were tested in B6 and D2 animals (Table 4). Animals were treated with 80 or 160 mg/kg Sudan dye II in corn oil for 4 days, followed by injection of sheep red blood cells on day 5. Four days following the injection of the sheep red blood cells, the spleens were removed and PFC were determined. The effects of Sudan dye II appear to be associated with the *Ah* locus. There was a significant ($P < 0.05$) decrease in PFC (measured as PFC/10^6 cells or as PFC/spleen) in the *Ah* responsive B6 mice. In contrast, Sudan dye II had minimal effects on PFC induction in D2 mice: less than a 25% decrease occurred when measured either as PFC/10^6 cells or as PFC/spleen. The first variable, PFC/10^6 spleen cells, shows that there was a 55% decrease in "immunologically reactive cells" on a per-cell basis in B6 mice. The other variable, PFC per whole spleen, reflects both the previous variable and the decrease in recoverable spleen cells. B6 mice exhibited an overall 75% decrease in PFC/spleen.

DISCUSSION

The Sudan dyes, much like polycyclic hydrocarbons, induced AHH and ETR activity in B6 mice. The differential inducibility of these animals is determined by the fact that the B6 mouse strain possesses a cytosolic receptor which avidly binds with the various inducing agents, whereas the D2 strain possesses an altered receptor that is incapable of binding most inducers. Sudan II maximally induced

Table 1. Induction of *Ah* locus-associated hepatic enzyme activities and percent displacement of [^{3}H]TCDD from the *Ah* receptor by Sudan dyes

Chemical	Dose (mg/kg)	Inbred strain	AHH*	ETR*	% D†
Control		B6	378 ± 45	113 ± 6	
		D2	397 ± 31	128 ± 5	
Sudan dye I	160	B6	1600 ± 192	1450 ± 59	48 ± 2
	320	B6	2590 ± 310	2340 ± 95	
	320	D2	286 ± 10	98 ± 6	
Sudan dye II	160	B6	2760 ± 195	2560 ± 107	78 ± 1
	160	D2	270 ± 28	136 ± 73	
Sudan dye III	160	B6	2042 ± 496	2245 ± 76	24 ± 5
	320	B6	2810 ± 106	2440 ± 104	
	320	D2	299 ± 27	138 ± 10	
Sudan dye IV	160	B6	1269 ± 152	1570 ± 204	30 ± 8
	320	B6	1821 ± 102	1810 ± 128	
	320	D2	293 ± 32	141 ± 90	

* AHH and ETR activities are expressed in moles per min per mg microsomal protein.
† Displacement of 1 nM [^{3}H]TCDD by 100 nM unlabeled Sudan dyes. Refer to Materials and Methods for details (values are means ± S.D.).

Table 2. Induction of *Ah* locus-associated enzymatic activities by various doses of Sudan dyes I and II or 3-methylcholanthrene in the liver of B6 mice

Chemical	Dose (mg/kg)	AHH*	ETR*
Control		297 ± 38	108 ± 14
Sudan dye I	40	454 ± 54	470 ± 23
	80	773 ± 31	1410 ± 17
	160	1720 ± 154	2030 ± 142
	320	2590 ± 233	2340 ± 163
Sudan dye II	10	1030 ± 218	730 ± 130
	20	1720 ± 205	1170 ± 146
	40	2700 ± 405	2480 ± 570
	80	3280 ± 177	2920 ± 262
	160	3300 ± 554	2660 ± 345
3-Methylcholanthrene	20	459 ± 55	813 ± 40
	40	1130 ± 45	2820 ± 338
	80	2180 ± 196	2940 ± 205
	120	2680 ± 402	3350 ± 301

* AHH and ETR activities are expressed in pmoles per min per mg microsomal protein.

Table 3. Enzyme activities from backcross analysis of progeny from (B6D2) F_1 and D2 parents injected with Sudan dye II (80 mg/kg)

Ah phenotype*	AHH†	ETR†
d	185	8.0
d	215	13.7
d	165	9.3
d	255	8.6
d	190	15.7
d	206	9.2
d	158	15.0
b	3010	2302
b	2650	2201
b	1710	975
b	1930	1448
b	2100	1695
b	2160	1716

* Determined by zoxazolamine paralysis test [6]. Ah^b = *Ah*-responsive; Ah^d = *Ah*-nonresponsive.

† AHH and ETR activities are expressed in pmoles per min per mg microsomal protein.

AHH at 80 mg/kg (2.7 μmoles/kg) and had an ED_{50} between 20 and 40 mg/kg. This makes Sudan dye II a more potent inducer than 3-methylcholanthrene (Table 2) and almost all of the polycyclic aromatic hydrocarbons. Thus, with 3-methylcholanthrene a dose of ~80 mg/kg (2.98 μmoles/kg) was required for maximal induction. In contrast, Sudan dyes I, III, and IV were much weaker inducers and required doses of up to 320 mg/kg to maximally induce activity (Table 1).

The backcross analysis provided information that the pharmacological response to Sudan dye II exposure segregates with the Ah^b allele, and thereby, associates with the *Ah* locus. The data in Table 3 shows, by the zoxazolamine paralysis test, that the animals which possess the Ah^b phenotype are also induced by Sudan dye II. This 100% association in the backcross analysis indicates that this response is specific for the Ah^b allele and is, most likely, not due to the numerous other genetic differences between B6 and D2 mice.

To investigate the mechanistic basis for these results, we studied the capacity of the Sudan dyes to interact with the *Ah* receptor. This receptor appears to be the primary determinant of AHH induction [9, 14–16]. The receptor binds to an inducing chemical (e.g. 3-methylcholanthrene), the resulting inducer–receptor complex undergoes a temperature-dependent translocation [17] into the nucleus, and the result is the transcription of the numerous enzymes involved in the *Ah* locus-mediated response [9, 14–16]. It has been shown that potent inducers of AHH activity *in vivo* bind avidly to the receptor, but weaker inducers display lower affinity [9, 10]. The data in Table 1 and Fig. 2 show measurements of the capacity of the Sudan dyes to compete with [^{3}H]TCDD for binding to the cytosolic receptor. Sudan dye II was a potent agonist (78% displacement) for the *Ah* receptor, whereas Sudan dye I was moderately potent (48% displacement). It is of interest that Sudan dye II has two methyl groups where Sudan dye I lacks these structural moieties (Fig. 1). In contrast, Sudan dyes III and IV caused more limited competition, 24% and 30% respectively. The ability of Sudan dyes I and II to act as potent competitors is in good agreement with the finding that they are potent inducers of AHH activity *in vivo*. The rather limited competition of Sudan dyes III and IV is in contrast to their significant potency in inducing AHH activity *in vivo* (Table 1).

Any direct extrapolation of potency as an agonist or competitor for the receptor *in vitro* (Table 1) to potency as an inducer *in vivo* (Tables 1 and 2) may be influenced by at least four considerations. First,

Table 4. Effect of Sudan dye II on the PFC response of B6 and D2 mice

Dose/day	Inbred strain	Number of spleen cells ($\times 10^{-6}$)	PFC / 10^6 spleen cells	PFC / spleen
Control	B6	83.2	541	45,000
80 mg/kg	B6	78.6	322	24,000
160 mg/kg	B6	42.0	271	11,400
Control	D2	120	935	112,000
80 mg/kg	D2	122	1,070	131,000
160 mg/kg	D2	125	927	116,000

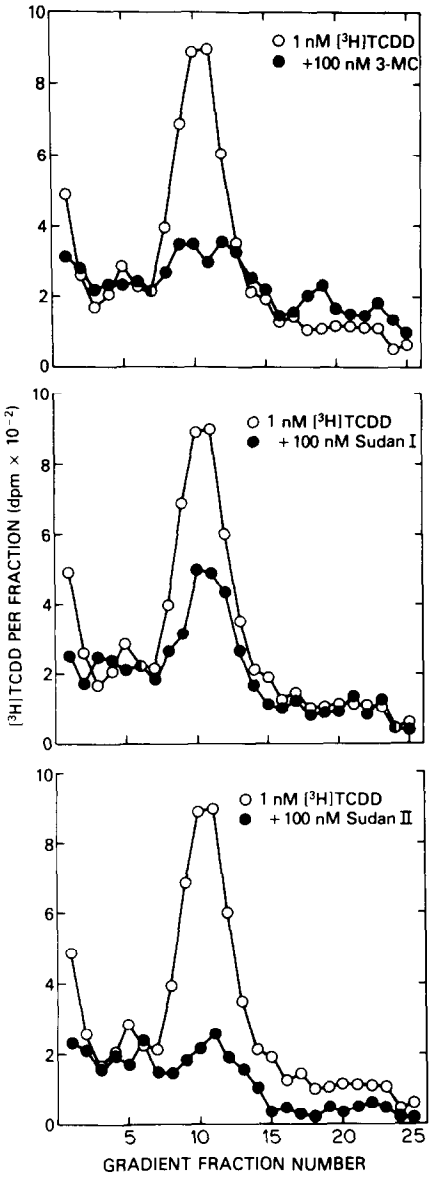

Fig. 2. Displacement of [³H]TCDD from the *Ah* receptor by 3-MC, Sudan I or Sudan II. In 1 ml of B6 liver cytosol, 1 nM [³H]TCDD was added in the presence or absence or 100-fold molar excess of 3-methylcholanthrene (top panel), Sudan dye I (center panel) or Sudan dye II (bottom panel). Each competitor was tested three times.

if an inducing compound is rapidly metabolized *in vivo* it may appear less potent. Second, if the pharmacokinetic distribution of a compound results in the limited accumulation in the tissue being assayed for biological activity, it will appear less potent. Third, there are compounds which bind to the receptor but do not translocate to the nucleus [18]. Fourth, if the actual inducing agent is not the parent compound itself but one of its metabolites [19], the parent compound may not appear to be a competitor for the receptor.

One of the interesting and well documented biological effects of Sudan dyes I and III is their capacity to inhibit the toxic, carcinogenic, and clastogenic effects of 7,12-dimethylbenz[*a*]anthracene [20, 21]. Thus, in female rats when intragastric Sudan dyes I, III and IV are administered repeatedly prior to a single intravenous 7,12-dimethylbenz[*a*]anthracene dose, they significantly inhibit the adrenal necrosis, mammary tumors, and chromosomal aberrations caused by 7,12-dimethylbenz[*a*]anthracene [20, 21]. This capacity has been linked [20] indirectly with the induction of the quinone oxidoreductase (EC 1.6.99.2). Hence, compounds which maximally induce quinone oxidoreductase are potent inhibitors of the toxic and carcinogenic effects of the 7,12-dimethylbenz[*a*]anthracene. However, since the induction of this enzyme is linked to the *Ah* locus [22], it may be difficult mechanistically to differentiate the effects of Sudan dye pretreatment. Thus, the effects of Sudan dye could be due to P_1-450 induction, which thereby alters 7,12-dimethylbenz[*a*]anthracene metabolism, or they could be due to the induction of certain other *Ah* receptor-mediated induced enzymes (UDP glucuronosyltransferase, quinone oxidoreductase, etc.).

In view of the fact that the Sudan dyes induce AHH activity and are known, albeit weak, carcinogens [1] and mutagens [23, 24], we postulated that the pharmacological and immunotoxic effects of exposure to these compounds are associated with the *Ah* locus. From the results of testing Sudan dye II immunotoxicity in B6 or D2 animals and the backcross analysis it is likely that the effects of Sudan dye II are associated with the *Ah* locus. Thus, there was a significant ($P < 0.05$) decrease in PFC in the *Ah*-responsive B6 mice but minimal effects on the PFC induction in the *Ah*-nonresponsive D2 mice (Table 4). The observed decrease of recoverable spleen cells in B6 animals is typical of many chemicals which interact with the cytosolic *Ah* receptor [16].

There are at least two possible mechanisms for the immunotoxic response to Sudan dye II. First, an *Ah* receptor-mediated induction of Sudan dye metabolism, analogous to the metabolism of polycyclic hydrocarbons, may result in metabolism of Sudan dye II to a more active product. Repeated doses of Sudan dye II may, therefore, induce forms of cytochrome P_1-450 which preferentially metabolize and activate Sudan dye II. A second mechanism would entail direct *Ah* receptor-mediated toxicity independent of metabolism. In the case of many of the polyhalogenated hydrocarbons, they apparently are not readily metabolized to reactive metabolites, and the observed toxicity is directly associated with occupation of the *Ah* receptor [16]. Hence, the toxicity we observe may be independent of further metabolism of the Sudan dyes. In summary, we found that the Sudan dyes interacted with the *Ah* cytosolic receptor, induced AHH *in vivo*, and showed *Ah* receptor-dependent immunotoxicity in two strains of mice.

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