FUNCTIONAL ARYL HYDROCARBON RECEPTOR AND ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR IN MURINE SPLENOCYTES

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1. Introduction

The immune system has been identified as one of the most sensitive target organs for the toxic effects produced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This conclusion is based on extensive evaluations of immune status following exposure to TCDD in a variety of animal models, the most extensively studied being the mouse. In spite of a large data base identifying the alteration of a broad range of immunologic endpoints by TCDD, little is known about the actual mechanism responsible for immune perturbation by this compound. Previous studies which have capitalized on genetic differences in mouse strains and/or on differences in structure-activity relationships, have indirectly implicated the involvement of the AhR in the immunotoxicity by TCDD. Other laboratories have detected mRNA transcripts and protein for the AhR in mouse spleen but expression of the AhR and ARNT specifically in mouse splenocytes, devoid of connective tissue and red blood cells, has not been addressed, bringing to question the actual source of these two proteins.
Additionally, nuclear [^{3}H]-TCDD has been identified by sucrose density gradient centrifugation in human tonsilar lymphocytes thus suggesting the translocation of the AhR/ARNT-TCDD complex to the nucleus [1)]; however, no one has directly established the functionality of the AhR and ARNT (i.e., are they capable of binding to the dioxin-responsive enhancer following treatment with ligand) in purified lymphoid cell preparations. In the present investigation we have identified the Ah receptor and ARNT in splenocytes isolated from B6C3F1 mice by northern and western analysis. Additionally, we have quantitated the relative protein expression for both of these proteins in spleen cells as compared to that observed in liver within the same animal model. Lastly, we have established that both the AhR and ARNT are functional in splenocytes as demonstrated by their ability to bind to the dioxin-responsive enhancer (DRE) following TCDD treatment.

2. Methods

AhR mRNA expression in splenocytes, separated from the splenic capsule, was determined by Northern blot analysis. Total RNA was isolated using a modified method of Chomczynski and Sacci [2)]. Poly(A) RNA was isolated from the total RNA using the PolyATtract mRNA isolation system. Transcripts were resolved on an agarose gel, blotted onto a nylon membrane and hybridized with a ^{32}P labeled 1.87 kb fragment from the cloned mouse AhR.

Proteins isolated from whole cell lysates extracted from splenocytes and liver were analyzed for the AhR and ARNT by Western blot and slot blot analysis. For the Western analysis 100 ug of protein was loaded in each lane and resolved on 7.5% SDS-PAGE gels and transferred to nitrocellulose. For slot blot analysis increasing concentrations of protein were directly filtered onto a nitrocellulose membrane. Each blot was incubated with 2 ug/ml of anti-AhR (17-10B) or anti-ARNT (20-9B). Antibody binding was visualized by staining the blots with donkey anti-rabbit horseradish peroxidase linked immunoglobulins.

Binding of Hepa 1c1c7 or splenocyte nuclear proteins to a dioxin-responsive element was assessed by an electrophoretic mobility shift assay (EMSA), slightly modified from that previously described [3,4]. Six micrograms of nuclear protein from untreated or TCDD-treated Hepa 1c1c7 cells and splenocytes were incubated with a ^{32}P-labeled 26 base pair DRE oligonucleotide and protein-DNA complexes were resolved by a 4.0% nondenaturing PAGE gel, dried on 3MM filter paper and autoradiographed. Cold competitor DRE oligonucleotide was added at a 50-fold excess to show specific interaction of the proteins with the DRE oligonucleotide.

3. Results and Discussion

In the present studies, to avoid the possibility of confounding results, splenocytes devoid of capsule and red blood cells were evaluated by northern and western analysis for both AhR and ARNT. Northern analysis of poly(A) RNA isolated from splenocytes revealed a single band, approximately 6.6 kb, to which the AhR cDNA probe hybridized (Fig. 1). As previously shown, the B6C3F1 (C57BL/6 x C3H) mouse strain possesses two forms of the AhR which are codominately expressed. These two forms of the AhR correspond, to the Ahr^{b-1} (C57BL/6J) and Ahr^{b-2} (C3H) alleles [5]. In agreement with this, we identified two major AhR proteins of approximately 95 kDa and 104 kDa in B6C3F1 spleen cell lysates by western blot analysis (Fig. 2). The presence of ARNT in mouse splenocytes was confirmed by western blot analysis as an 87 kDa protein (Fig. 3). It is important to emphasize that we have used splenocyte suspensions in our analysis, which would include both lymphocytes and macrophages. Although we cannot rule out a contribution by macrophages, we believe our results are most consistent with a profile of activity in lymphocytes, primarily because macrophages constitute only about 5% of the splenocyte content from the B6C3F1 mouse. As such, we believe our results provide the most direct evidence to-date for the presence of the AhR and the ARNT protein in mouse-derived lymphocytes.

Not surprisingly because of the well-studied association of the AhR in the actions by TCDD in the liver, our quantitation of AhR by slot blot analysis revealed significantly greater amounts of the receptor in liver than spleen (approximately 2.5-fold more) (Table 1). However, it is quite intriguing that significantly higher amounts (approximately 2.3-fold more) of ARNT were found in splenocytes then liver (Table 1). This observation with respect to the relatively greater quantity of ARNT in splenocytes is in agreement with previous results by Carver and coworkers [6] in which they showed a trend towards greater expression of transcripts for ARNT as compared to AhR in rat spleen and thymus; however, the authors did not consider this difference in expression to be significant in lymphoid tissues [6]. It is tempting to speculate that the reason why there are greater amounts of ARNT in some tissues than others is that this skewed ratio of AhR to ARNT could increase the likelihood that the ligand-bound receptor would find its necessary binding partner (i.e., the ARNT protein) in tissues where there are low amounts of AhR. Carver and co-workers also speculated that the significance of the disparate levels of the AhR and ARNT may indicate other biological roles for ARNT [6].

Table 1. Relative Band Intensities Determined by Densitometry Analysis of the Slot Blots

	Protein Lysate (ug)								
	0	5	10	20	40	60	80	100	200
AhR									
Splenocyte	ND	ND	ND	0.37	0.65	0.99	1.18	1.73	3.41
Liver	ND	ND	0.49	0.99	1.42	1.54	3.46	5.73	9.07
ARNT									
Splenocyte	ND	0.81	2.70	5.50	11.63	15.57	19.15	19.80	27.46
Liver	ND	ND	1.00	3.84	5.42	5.95	7.01	7.69	11.45

The previous demonstration that protein bound-[^{3}H]-TCDD translocates from cytosol to the nucleus in human tonsilar lymphocytes [1] is especially significant to our present studies since this was the first evidence suggesting that lymphoid cells may possess "functional" AhRs. However, the report by Lorenzen and Okey was inconsistent with results from studies by Denison and coworkers in which they were unable to show binding of the AhR/ARNT heterodimer to the DRE by electrophoretic mobility shift using TCDD-treated splenic cytosol from a variety of animal species including rat, guinea pig and hamster [7]. Taken together, the available results indicated that it was not enough to demonstrate specific binding by radiolabeled TCDD, or even the presence of the AhR in lymphoid cells, without questioning whether the AhR is "functional" in lymphocytes. In the present studies, we tested the functionality of the AhR/ARNT heterodimer by its ability to bind to the DRE following treatment of splenocytes with TCDD (Fig. 4, lane 6). Furthermore, this binding was demonstrated to be specific for the DRE motif as indicated by the ability of unlabeled DRE to compete for the binding of the heterodimer with ^{32}P-labeled DRE (Fig. 4, lane 7) . These results confirm that the AhR and ARNT are in fact functional in B6C3F1-derived lymphocytes. We suspect that one reason this same result was not observed by Denison and coworkers may be due to the fact that the optimum conditions for AhR/ARNT binding to the DRE are modestly different when using liver cytosolic preparations than that found in splenic preparations.

4. Conclusions

Although our present results indicate that lymphocytes possess the necessary components for immune suppression to be mediated by the AhR, several previously reported observations have challenged the exclusivity of this model and still need to be resolved. Nevertheless, we believe that the present studies clearly establish B6C3F1 splenocytes as a suitable model to begin to elucidate the involvement of the AhR in mediating immunotoxicity by TCDD and structurally-related compounds.

5. Acknowledgments

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6. References

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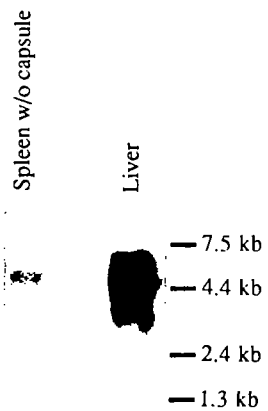

Figure 1

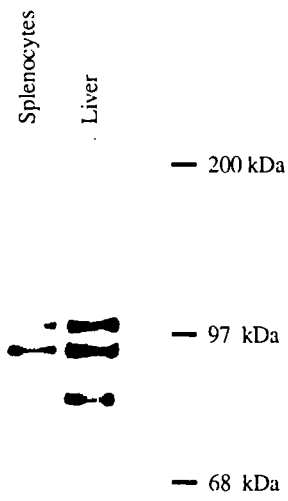

Figure 2

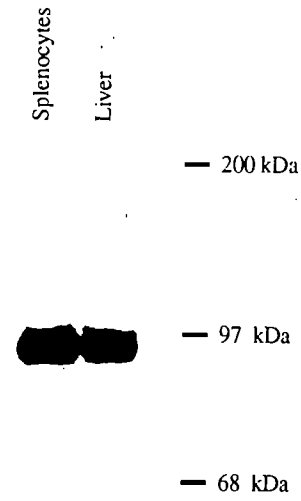

Figure 3

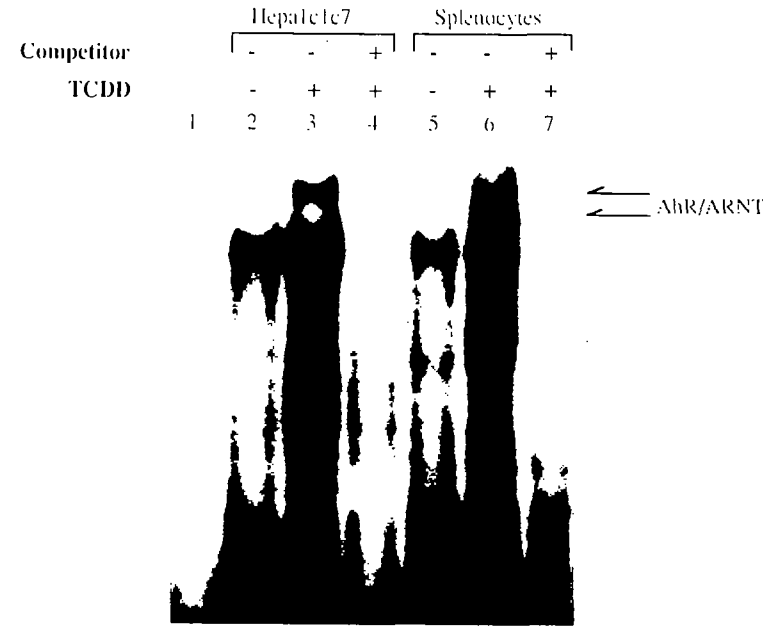

Figure 4