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Looking Inside the Cell for Mechanisms of Immunotoxicity: Experimental Design and Approaches Aimed Toward Elucidation of 2,3,7,8-Tetrachlordibenzo-p-dioxin-mediated B Cell Dysfunction

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ABSTRACT *: One of the major focuses and perhaps the greatest challenges during the past decade in the discipline of immunotoxicology has been the elucidation of the molecular mechanisms responsible for immunotoxicity by specific agents. Much is currently understood about the basic underlying intracellular processes that control leukocyte effector function. This fundamental information in cell biology can now be applied toward developing systematic approaches, through the application of cell and molecular biology techniques, to identify the intracellular targets and processes disrupted by immunotoxicants. The objective of this paper is two fold. First to discuss fundamental principles of experimental design aimed at elucidation of cellular mechanisms in immunotoxicology; and second to discuss the application of molecular biology techniques in characterizing the mechanism of TCDD-induced B cell dysfunction as a working example.*

Key Words *: Immunotoxicology, TCDD, B cell, Ah receptor*

I. INTRODUCTION

During the past decade, one of the major focus areas in the discipline of immunotoxicology, and perhaps the greatest challenge thus far, has been to elucidate the effects xenobiotics induce at the intracellular level. The importance of this stems from the fact that lymphocytes in general are quiescent cells which are critically dependent on undergoing the highly ordered process of activation, proliferation and ultimately differentiation in response to antigenic stimulation prior to performing effector functions. Progression through the aforementioned series of events is tightly regulated by distinct signal transduction cascades induced through external stimuli initiated at the cell surface not only at the level of the T cell and B cell antigen receptors but also through secondary receptor ligand interactions such as CD40/CD40 ligand interactions for B-cell activation, B7/CD28 interactions for T-cell activation, lymphokine/lymphokine receptor interactions, etc. The signaling cascades activated by external stimuli represent the intracellular circuitry that activates as well as represses the transcription of genes whose products control the movement of resting lymphocytes through cell cycle (proliferation) and then through the cellular differentiation process. It has become readily apparent that many immunotoxicants, originally identified using immune function assays, mediate their biological activity by disrupting the signaling pathways in lymphocytes that control the orderly process of activation, proliferation and differentiation. The recent development of new experimental techniques in the areas of biochemistry, cell and molecular biology has made it possible to begin investigating how immune altering agents actually mod-

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Abbreviations: TCDD, 2,3,7,8-Tetrachlordibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; AFC, antibody forming cell response; sRBC, sheep erythrocytes; IgM, immunoglobulin M; LPS, lipopolysaccharide; DRE, dioxin response element; EMSA, electrophoretic mobility shift assay; CYP1A1, cytochrome P450 1A1; EROD, ethoxyresorufin-O-deethylase; RT-PCR, reverse transcription-polymerase chain reaction, PCDD, polyclorinated dibenzo-p-dioxin; SAR, structure activity relationship; IC50 and EC50, 50% of inhibitory or effective concentration, respectively; MCDD, 1-monochlorodibenzo-p-dioxin; TriCDD, 2,3,7-trichlorodibenzo-p-dioxin; HxCDD; 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin; NF-κB, nuclear factor of κ light chain in B cells

ify the cellular responses of leukocytes at the level of signal transduction and gene expression. Thus the integration of molecular biologic approaches has significantly extended our basic understanding of the mechanisms by which xenobiotics modulate the immune system. Very briefly, recent studies aimed at further elucidating the mechanism responsible for immune modulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) will be utilized to illustrate this point as well as to discuss in more general terms experimental design in immunotoxicology.

II. FUNDAMENTAL APPROACHES IN MECHANISM-BASED IMMUNOTOXICOLOGIC EXPERIMENTAL DESIGN

In spite of the rapid advancements in cell and molecular biology, fundamental information about the actions of a given xenobiotic on the immune system is essential prior to employing many of the approaches discussed in this paper. The first and most critical criteria prior to the rational application of a wide range of *in vitro* assays and approaches aimed at the elucidation of the molecular mechanism of a xenobiotic is the demonstration that the agent in fact alters immune competence after *in vivo* administration. Historically, the application of an immunotoxicologic tier testing approach consisting of a battery of immune function assays which systematically evaluate the profile of immunotoxicity of an agent, after *in vivo* administration, has been widely and successfully employed ADDIN ENRfu (Luster *et al.*, 1988). Although a discussion of the immunotoxicologic tier approach is beyond the scope of this paper, mention needs to be made of the anti-sheep erythrocyte (sRBC) IgM antibody forming cells (AFC) response. This assay represents the cornerstone of the tier approach as it has proven to be the single most sensitive and consistent assay employed in the identification of immunotoxic agents. In the case of TCDD, this halogenated aromatic hydrocarbon is a widely established inhibitor of humoral immune responses in rodents including the *in vivo* anti-sRBC IgM AFC response ADDIN ENRfu (Holsapple, 1995; Holsapple *et al.*, 1991). A second critical factor is the identification of the cell type(s) that is targeted by the immunotoxicant. Although numerous strategies have been utilized by various laboratories to determine the specific cell types target by immunotoxicants, two general approaches have been most extensively employed toward this end. One is to evaluate humoral immune responses to defined antigens requiring different cellular cooperativity (e.g., sRBC as a T-cell dependent antigen, versus DNP-Ficoll, as a T-cell independent antigen, versus LPS, as a polyclonal B cell activator). Although antibody production is a B cell effector function, humoral responses directed against sRBC require macrophages and T cells as accessory cells; against DNP-Ficoll macrophages are required as accessory cells, and against LPS no accessory help is required. Comparisons of the immunomodulatory activity of a given agent to modify responses against each of these antigens allows the identification of sensitive target cell populations. A second commonly used approach is the employment of cell fraction-reconstitution in conjunction with the *in vitro* anti-sRBC IgM AFC response. The latter involves the isolation of leukocytes, normally spleen cells, from vehicle and xenobiotic treated animals, their fraction into macrophage, B cell and T cell pools and then cross reconstitution of the leukocyte populations in various combinations between vehicle and treated cells to assess the sensitivity of specific cell types. Both approaches when employed to characterize TCDD have demonstrated the most profound effect being the inhibition of B cell function. Conversely, only a modest inhibition of helper T cell function and no affect on macrophage function was observed by cell fractionation experiments associated with TCDD-treatment ADDIN ENRfu (Dooley and Holsapple, 1988). Collectively, these studies have identified the B cell as a sensitive target for inhibition by TCDD. The third critical issue concerning the preliminary characterization of immunotoxic xenobiotics is to determine whether the agent acts directly on leukocytes. The significance of this point cannot be overemphasized since it will dictate whether or not direct addition studies of the agent to *in vitro* leukocyte cultures can be employed as a biologically relevant approach. Clearly, if the mechanism of action for the immunotoxicant were dependent on the induction of immunomodulatory factors from nonlymphoid organs, direct addition of such an agent to leukocyte cultures would not be a relevant approach. Similarly, because leukocytes have very limited drug metabolizing capa-

bility, agents requiring metabolic bioactivation are likewise not easily studied *in vitro* in the absence of metabolic activation systems. In the case of TCDD, its *in vivo* profile of immunotoxic action is comparable to that induced *in vitro* by direct addition of TCDD to naËÊe immunocompetent cells in culture.

III. APPROACHES FOR ELUCIDATION OF THE MOLECULAR MECHANISM FOR B CELL DYSFUNCTION BY TCDD

Predicated on the above basic observations in conjunction with those established in nonlymphoid cell models, a rational mechanism-based approach will be discussed that has been applied to the study the mechanisms of TCDD-mediated B cell dysfunction. The putative mechanism of action by TCDD is believed to be mediated through the aryl hydrocarbon (Ah) receptor which upon ligand binding translocates to the nucleus to function as a transcription factor regulating the expression of a wide variety of genes. It is important to emphasize that the aforementioned putative mechanism of action for TCDD has been elucidated primarily from studies performed in liver-derived cellular preparation. Interestingly, earlier studies aimed at elucidating the mechanism responsible for immunotoxicity by TCDD led to findings, which challenged the exclusivity of the Ah receptor model. First, neither detection nor induction by TCDD of ethoxyresorufin-O-deethylase (EROD) activity, a hallmark biological response to AhR ligands, had been convincingly demonstrated in leukocytes. Second, employment of the electrophoretic mobility shift assay (EMSA) using rat and guinea pig spleen extracts failed to show binding of the AhR-ARNT heterodimer to the DRE following TCDD-treatment ADDIN ENRfu (Denison 1991) Third, the low affinity AhR ligand, 2,7-dichlorodibenzo-p-dioxin and TCDD produced comparable inhibition of the anti-sRBC IgM antibody forming cell response following subchronic treatment of mice *in vivo* and following direct addition to naive splenocytes *in vitro* ADDIN ENRfu (Holsapple *et al.*, 1986). Lastly, subchronic TCDD treatment produced a marked immune suppression in both AhR low responding DBA/2 and AhR high responding B6C3F1 mice ADDIN ENRfu (Morris *et al.*, 1992). In light of the above, we directed studies to delineate the AhR-mediated signal transduction events and gene regulation in B-cells to address two fundamental questions pertaining to the mechanism of immunotoxicity by TCDD. First, is the AhR obligatory for the inhibition of IgM secretion by B-cells? Second, is CYP1A1 induction by TCDD mechanistically related to the inhibition by TCDD of LPS-induced IgM expression? Answering these fundamental questions is an integral part of the following underlying hypothesis currently being test in our laboratory: "Inhibition of B cell function by TCDD is mediated through the AhR which acts as a DNA binding protein to adversely regulate immunologically relevant genes possessing DREs in their regulatory domains".

Our initial efforts were focused on determining whether expression of AhR receptor and it dimerization partner, ARNT, could be detected within the immune system. Employing mouse splenocytes, a heterogeneous leukocyte preparation consisting of approximately 45% B cells, 45% T-cells and 5~8% macrophages, Northern analysis and Western blotting, clearly demonstrated that both AhR and ARNT were expressed ADDIN ENRfu (Williams *et al.*, 1996). Moreover, direct addition of TCDD to cultured splenocytes induced nuclear translocation and DNA binding of the AhR-ARNT complex to dioxin responsive elements (DRE), as demonstrated by electrophoretic mobility shift assay (EMSA). We believe that the inability of previous investigators to identify the transformed AhR by EMSA analysis is most likely due to technical aspects pertaining to assay conditions. Specifically the EMSA conditions previously utilized had been optimized for hepatic and not splenic nuclear protein preparations.

To more directly characterize the role of the AhR in B-cells, two complementary models were employed, purified primary splenic B-cells and two murine B-cell lines, one of which expresses AhR and ARNT (CH12.LX), and one which is deficient in AhR but expresses ARNT (BCL-1). The BCL-1 cell line was an especially useful model since it provided the opportunity to examine the direct effects of TCDD on B cells in the absence of AhR. Primary splenic B-cells, which were approximately 92% pure, as determined by fluorescent activated cell sorting, were found to express both AhR and ARNT at the mRNA and protein level, as confirmed by quantitative RT-PCT and Western blot ADDIN ENRfu (Marcus *et al.*, 1998). Equally impor-

tant, CYP1A1 expression was induced by TCDD at both the mRNA and protein level confirming that AhR and ARNT were functional in the primary B-cells. However, these studies also demonstrated that the expression of CYP1A1 was modest and explains why past measurements of TCDD-mediated EROD substrate activity in leukocyte preparations were inconclusive. Further mechanistic studies using CH12.LX and BCL-1 cells were employed to explore whether the AhR is, in fact, essential in TCDD-mediated inhibition of immunoglobulin secretion. One of the critical features of both the CH12.LX and BCL-1 cell lines which facilitated their application for these studies is that both exhibit only a modest basal level of IgM secretion and that they are readily induced to secrete IgM by treatment with lipopolysaccharide (LPS). This responsiveness of both the CH12.LX and BCL-1 cells to LPS suggests that both possess the critical signaling cascades that regulate immunoglobulin gene expression that exist in primary B-cells. Interestingly, the CH12.LX cell line exhibited a marked and concentration dependent inhibition of LPS-induced IgM secretion at concentrations as low as 0.03 nM TCDD. Conversely, the BCL-1 cells were found to be completely refractory to inhibition by TCDD (3.0 nM) on LPS-induced IgM expression ADDIN ENRfu (Sulentic *et al.*, 1998). Concordantly, TCDD induced CYP1A1 mRNA expression in a time and concentration dependent manner in CH12.LX cells; whereas, CYP1A1 mRNA was not only not induced by TCDD but was also undetectable in BCL-1 cells as assessed by RT-PCR.

A limited structure activity relationship study was conducted with a variety of dioxin congeners exhibiting different binding affinities for the AhR in CH12.LX cells to compare CYP1A1 mRNA induction, inhibition of LPS-induced IgM secretion and inhibition of mRNA expression ADDIN ENRfu (Sulentic *et al.*, (in press)). The rationale for this approach was based on the belief that if the IC50 concentrations for inhibition of μ expression and IgM secretion for the selected congeners were similar to their respective EC50 concentrations for induction of CYP1A1 mRNA expression, these findings would suggest that TCDD regulated CYP1A1 and IgM expression through a common mechanism of action (i.e., DRE-mediated). Conversely, if there was no or a poor correlation between inhibition of LPS-induce μ expression and IgM secretion versus induction of CYP1A1 mRNA expression for each of the respective congeners, then the results would suggest distinct mechanisms of action. The following specific polyclorinated dibenzo-p-dioxin (PCDD) congeners were selected based on previously reported rank order potency for AhR binding affinity and AhR-dependent induction of CYP1A1, as TCDD > HxCDD > TriCDD >> MCDD with MCDD exhibiting no affinity for the AhR and incapable of inducing EROD activity ADDIN ENRfu (Poland and Glover 1976; Poland *et al.*, 1979). The binding affinities (kd) for TCDD, HxCDD and TriCDD are 0.27, 0.77 and 1.92 nM, respectively ADDIN ENRfu (Poland and Glover 1976). The results from the SAR study demonstrated a strong correlation between ED50 values for induction of CYP1A1 mRNA expression and IC50 values for the inhibition of LPS induced μ mRNA expression and IgM secretion by PCDD congeners in the CH12.LX B-cells.

Results from the SAR studies coupled with the strong inhibition by TCDD to inhibit LPS-induced mRNA expression prompted the examination of the promoter region of the mouse immunoglobulin heavy chain gene for DRE. Although no DRE where located in the immunoglobulin heavy chain promoter, two DRE-like sites were identified in the 3'α enhancer which is composed of four functional enhancer domains [Cα3'E, 3'αE(hs1,2), hs3 and 3'α-hs4]. One DRE-like site was identified in the 3'αE(hs1,2) and a second in the 3'α-hs4 enhancer domains. Although not fully understood, it has been suggested that these enhancers form a locus control region since they can act synergistically to regulate the expression of the immunoglobulin heavy chain genes, μ, δ, γ, ε, and α, which encode heavy chain proteins for IgM, IgD, IgG, IgE and IgA, respectively ADDIN ENRfu (Pettersson *et al.*, 1997). Analysis by EMSA of nuclear extracts isolated from TCDD-treated CH12LX cells revealed TCDD-inducible binding to a 41 bp oligonucleotide derived from the 3'αE(hs1,2) and a rather broad binding complex to a 41 bp oligonucleotide derived from the 3'α-hs4 ADDIN ENRfu (Sulentic *et al.*, (in press)). In each case, the oligonucleotide sequences derived from the respective regulatory domains contained the DRE-like motifs. To serve as a negative control, nuclear extracts were also isolated from TCDD-treated BCL-1 cells and analyzed by EMSA utilizing the 3'α-hs4

derived probe. Interestingly, a TCDD-inducible 3'α-hs4 binding complex was identified by EMSA using BCL-1 cell nuclear extracts; however, this binding complex exhibited a different migration profile as observed with nuclear extracts derived from TCDD treated CH12.LX cells. Closer examination of the 3'α-hs4-derived oligomer revealed the presence of a NF-κB element that overlaps the DRE-like site. EMSA-Western analysis of CH12.LX and BCL-1 cell nuclear extracts was employed to identify the composition of the TCDD-inducible 3'α-hs4 DNA binding complexes. EMSA-Western immunoblots identified AhR -ARNT as one of the 3'α-hs4 binding complexes in nuclear extracts from CH12.LX cells but not BCL-1 cells. Based on the presence of a NF-κB element in the 3'α-hs4 oligomer, EMSA-Western immunoblots were also performed using antibodies specific for NF-κB/cRel family member proteins. The studies identified TCDD-induction of p65, RelB, p50 and cRel using nuclear extracts from CH12.LX cells ADDIN ENRfu (Sulentic *et al.*, (in press)). These NF-κB proteins formed a series of 3'α-hs4 DNA binding complexes that were distinct from AhR-ARNT. Similarly, TCDD-induced the NF-κB/cRel family members, p65, RelB and c-Rel but not p50 in BCL-1 cells. The latter findings are especially interesting since they clearly demonstrate that the induction of NF-κB proteins in B cells are mediated through a mechanism of action that is independent of the AhR.

To assess the transcriptional relevance of TCDD-induced AhR/ARNT binding to DRE-like sites and concomitant induction of NF-κB binding on 3'α-hs4 enhancer activity a luciferase reporter gene construct was prepared which is under the control of the 3'α-hs4 enhancer. Specifically, we amplified the immunoglobulin heavy chain variable region promoter and 3'α-hs4 enhancer element from B6C3F1 genomic DNA which was subcloned into an upstream and downstream multiple cloning site of a luciferase structural gene. Transient transfection of 3'α-hs4 to CH12.LX cells CH12LX cells showed relative high basal level activity, which was enhanced by both TCDD and LPS treatment. Furthermore, the enhancement of 3'α-hs4 activity by TCDD was partially blocked by the AhR antagonist, α-naphthoflavone ADDIN ENRfu (Kang *et al.*, 2000). These results suggest that TCDD treatment of B cells results in an increase in 3'α-hs4 transcriptional activity; however, presently unclear is the relative contribution of the NF-κB and AhR in mediating this effect. Studies are presently underway to more fully characterize the relative contribution both AhR and NF-κB family member proteins exert on the transcriptional activity via the 3'α-hs4 enhancer following TCDD treatment and the functional consequences of this modulation on immunoglobulin regulation. Significance of these studies is that they putatively represent the first direct mechanistic link between TCDD treatment and inhibition of immunoglobulin expression.

IV. SUMMARY

The advent of molecular approaches makes this a very exciting time for the discipline of immunotoxicologic. The tools are now available which make it possible to genuinely look inside the cell and to identify the specific components of the intracellular machinery that are targeted by immunotoxicants as illustrated above with TCDD. Drugs and chemicals that adversely impact immune competence can be critically investigated not only at the level of which cell-types they target and what respective effector functions they disrupted (e.g., secrete proteins such as immunoglobulin or lymphokines, phagocytize microorganisms; etc.) but also as to which critical signal transduction cascades and genes they disrupt to produce the loss of effector function. Inherent in the knowledge that comes with a firm understanding of the cellular and molecular mechanisms responsible for immunotoxicity will come more effective risk assessment, the ability to predict which agents have a strong potential for being immune modulators and the identification of novel intracellular targets for more effective immune therapy.

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