

Dioxin and Host Susceptibility to Infection

Introduction

Dioxin, an environmental pollutant, increases host susceptibility to infections. A long-term objective of our project is to identify and characterize the cellular and molecular mechanisms by which dioxin increases susceptibility to infections. Effective host defense against pathogenic microorganisms depends on timely recognition of the microbe, activation of immediate containment measures and ultimately the generation of a specific and definitive adaptive immune response. Recent research has identified a family of molecules known as the Toll like receptors (TLRs) that play a key role in pathogen recognition and initiation of inflammatory and immune response. Upon recognition of their cognate ligands, TLRs induce the expression of cytokines, chemokines, costimulatory molecules that support the development of an appropriate immune response that arms the host against the invading organisms. Our central hypothesis is that dioxin increases host susceptibility to infection by modulating the TLRs expression and TLR signaling. The short term specific aims of this study are:

AIM #1: To examine the effect of acute exposure of pro-monocytic cell lines to dioxin on the expression and function of TLR 2 and TLR 4

AIM #2: To examine the effect of prolonged exposure of pro-monocytic cell lines to environmentally relevant concentrations on expression and function of TLR2 and TLR4

AIM #3: To examine the effect of prolonged exposure of pro-monocytic cell lines on expression of TLR signaling components.

These studies should provide us preliminary data to support our hypothesis that exposure to environmental pollutants may increase the susceptibility to infections by modulating TLR expression and function.

Background

2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), most commonly known as dioxin, is a member of a large family of halogenated aromatic hydrocarbons (HAH) that include polychlorinated and polybrominated biphenyls (PCB and PBB), polychlorinated dibenzofurans and dioxins (CDF and PCDD) respectively. TCDD is composed of two benzene rings, each containing two chlorine atoms, joined by two oxygen atoms (Figure 1). Many people harbor traces of dioxins and related compounds as a result of exposure to pesticides in their diet or airborne dioxins released by certain type waste incineration. These environmental chemicals have raised much public concern because of their toxicity to the immune system and resulting alterations in host susceptibility to infections. Dioxin has been studied in greater detail than other environmental pollutants because it has been found to be highly active at extremely low levels as described by Whitlock and Clark et al. The environmental protection agency (EPA) regulates dioxin in drinking water at 13 parts per quintillion. In a recent report the EPA estimates total exposure to dioxin and related compounds at 40-60 pg/gram of body fat and the report further states that non-cancer effects of dioxin may be a more urgent threat to humans (Stone).

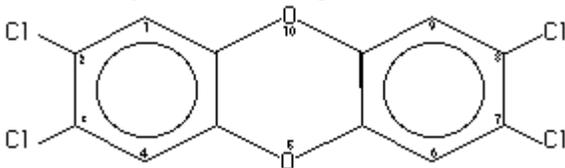


Figure 1.

Although dioxin has been reported to increase the susceptibility of the host to infections (see below), little is known about the mechanisms by which it decreases the host resistance to infections. This project seeks to examine the impact of dioxin on the level of expression of TLRs and their signaling components as well as TLR function. TLR 2 and TLR 4 are selected as a model to study the effect of dioxin on

susceptibility to bacterial infections because these two receptors are not only intimately involved in initiating innate immunity against bacterial infections but are also involved in initiating the adaptive immune response and in shaping the course of these responses. Diverse aspects related to this proposal are briefly reviewed in the following sections.

Effect of dioxin on host resistance

Increased susceptibility of the host to infections is one of the end points in the assessment of health risk of immunotoxic substances. The impact of dioxin on host resistance to certain infections has been studied in animal models of human diseases. Various studies have shown that exposure to TCDD decreases functional activity in the immune system and results in an increased susceptibility to viral, bacterial and parasitic diseases. Vos et al., Thigpen et al., and Hindsill et al, all have shown that rats and mice exposed to TCDD were increasing susceptible to the gram-negative bacterium *Salmonella*. Vos et al. and Thomas and Hindsill have established that rats and mice demonstrated decreased resistance to the bacterium *Escherichia coli* by after TCDD exposure. Further, White et al. has shown that increased mortality resulted when TCDD treated mice were challenged with *Streptococcus pneumoniae*, a gram-positive bacterium. Enhanced susceptibility to viral infections has been reported by Clark et al. Their study illustrated that mice treated with TCDD had enhanced mortality to *herpes simplex tube II strain 33 virus*. Finally, Tucker et al. have established parasitic disease vulnerability in their study which showed only a single dosage of TCDD resulted in a decreased resistance to the parasite *Plasmodium yoelii 17 XNL* in female mice.

Effects of dioxin on cells of the immune system

One of the most sensitive and earliest indicators of dioxin toxicity is changes in the function of immune system. Frazier et al has shown that animals exposed to dioxin show thymic involution quantitative changes in percentage of lymphocytes decreased antibody responses to both thymic dependent and independent antigens reduced cytotoxic T cell functions. Studies in human subjects exposed to dioxin have shown both an increase and decrease and no effect in cell numbers and their proliferative responses to T cell mitogens have been reported. *In vitro* studies with dioxin on human peripheral blood lymphocytes showed alterations in surface marker expression and inhibition of immunoglobulin synthesis by human tonsillar lymphocytes. Currently there is little information on the effect of dioxin on the production of a number of cytokines important in immune regulation, by human monocyte/macrophages and lymphocytes. Furthermore, the biochemical and molecular mechanism by which dioxin exerts its effects on lymphocyte and macrophage functions remains unclear. Studies in other cell types (transformed keratinocytes) showed that dioxin alters the expression of growth factor (IL-1 β , TGF α) genes by both transcription and post transcriptional mechanisms

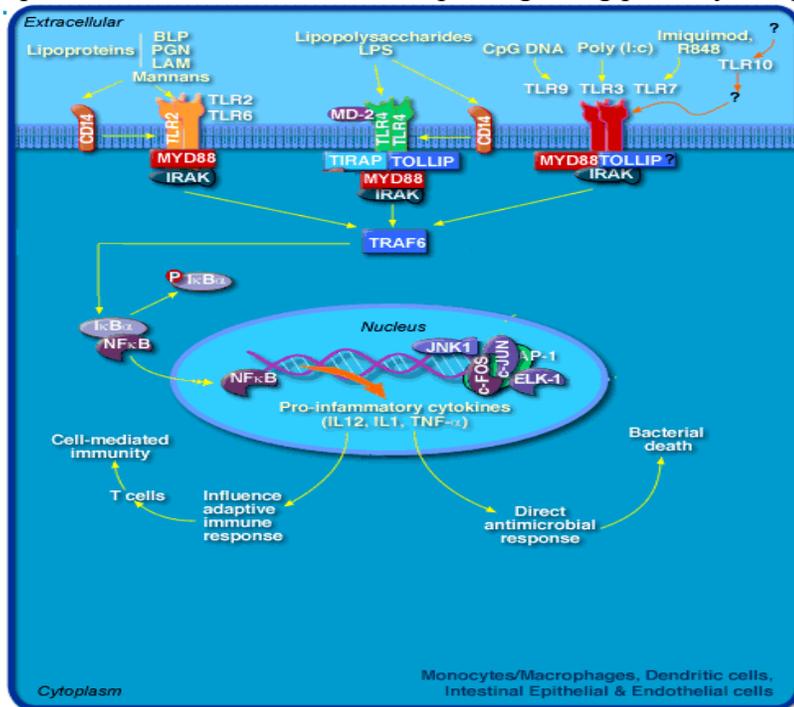
TOLL Like Receptors (TLRs)

Toll like receptors are pattern recognition receptors that recognize conserved molecular patterns on microbes (Pathogen Associated Molecular Patterns, PAMPS) and link innate and adaptive immune system. At present, 10 TLR proteins have been identified in humans. TLR family members are transmembrane proteins. TLR2 recognizes peptidoglycans of Gram positive bacteria, where as TLR 4 detects LPS of Gram negative bacteria. Although PAMPS have also been identified for TLR 3(dsRNA) the, TLR5 (flagellin) and TLR 9(hypomethylated CpG motifs) have been identified, none have identified for TLR7, TLR8 and TLR 10.

TLR Signaling

Common to all TLRs is a complex signaling pathway. The family of TLRs utilizes an adaptor protein, MyD88 in the initial signaling step. MyD88 then recruits members of the IL-1 receptor-associated kinase (IRAK) family, specifically IRAK 1 and IRAK 4 (Barton and Medzhitov). Both IRAK 1 and IRAK4 are responsible for phosphorylation and activation of the Tumor necrosis factor receptor-associated factor 6

(TRAF6). TRAF6 and its associated complex's then activate the kinase complex I κ B α (Barton and Medzhitov). Phosphorylation and consequently degradation of I κ B α releases nuclear factor kappa B (NF- κ B), which can then activate NF- κ B genes triggering the production of the pro-inflammatory cytokines interleukin(IL)-12, IL-1 and tumor necrosis factor α (TNF α) (Barton and Medzhitov). Schematic representation of the Toll-like receptor signaling pathway is represented below (figure 2)



Experimental design and Methods

AIM #1: To examine the effect of acute exposure of pro-monocytic cell lines to dioxin on the expression and function of TLR 2 and TLR 4

TLR 2 detects Gram positive organisms where as TLR4 senses Gram negative organisms. Because dioxin exposure is associated with increased infections with both Gram positive and gram negative organisms, in this investigation, we will study the expression and function of TLR 2 and TLR 4 U937 cells will be incubated with different concentrations of dioxin (1ng/ml, 10ng/ml, 50ng/ml, 100ng/ml). At different time intervals (3, 6, 9, 12, 15, 18, 21, 24 27 and 30 days post exposure) the cells will be stained with phycoerythrin (PE) labeled monoclonal antibodies against TLR2, TLR4 and isotype control antibody. The stained cell will be analyzed by FACScan flow cytometer and receptor density and percent positive cell will be determined. The Fluorescence signals from labeled cells were analyzed either using FACScan (Becton-Dickenson, San Jose, California) for excitation at 488 nm forward and side scatters will be used to gate and exclude cellular debris and results will be recorded for 10,000 cells. Statistical analysis will be performed by the Kolmogorov-Smirnov test using Cell Quest Software System (Becton-Dickinson, Menlo Park, CA). A "D" value of >0.2 is considered statistically significant.

Cells exposed to dioxin and cells not exposed dioxin (control cells) will be incubated with peptidoglycan, a ligand for TLR 2 or with LPS, a ligand for TLR 4. Cell culture supernatants will be collected at 24 and 48 hr post incubation and IL-1 and TNF- α production will be quantified using ELISA kits. ELISA will be done as suggested by the recommendations of the manufacturer of the kit.

AIM #2: To examine the effect of prolonged exposure of pro-monocytic cell lines to environmentally relevant concentrations on expression and function of TLR2 and TLR4

U937 cells will be incubated with different concentrations of dioxin (range from 0.1ng/ml, 0.01ng/ml, 0.001ng/ml, 0.0001ng/ml). At different time intervals (3, 6, 9, 12, 15, 18, 21, 24 27 and 30 days post exposure) the cells will be stained with PE- labeled anti-TLR2, TLR4 or isotype control antibody. The stained cell will be analyzed by FACScan flow cytometer and receptor density and percent positive cell will be determined.

Cells pre-exposed to dioxin and cells not exposed to dioxin will be incubated with peptidoglycan derived from Gram positive bacteria or Lipopolysaccharide (LPS) derived from Gram negative bacteria and the production of cytokines such as IL-1, TNF- α will be quantified by ELISA

AIM #3: To examine the effect of prolonged exposure of pro-monocytic cell lines to on expression of TLR signaling components.

TLRs transduce signals through adapter protein which in turn recruits down stream targets such as IRAK, TRAF6. Recruitment of TRAF6 to the signaling complex leads to activation and translocation of nuclear factor κ B (NF κ B) to nucleus resulting in turning on transcription of target genes (e.g. IL-1 and TNF) genes. To gain insights into mechanisms by which dioxin modulates TLR signaling, we will study the level of expression MyD88, IRAK, TRAF 6, and NF κ B by western blotting and correlate the level of expression with the function of TLRs.

Western blotting

Cells (5×10^6) treated with or without dioxin will be harvested and whole cell extracts will be prepared by lysing the cell pellet in 50 μ l cold TGNT buffer with protease and phosphatase inhibitors (100 mM Tris-Cl pH 7.4, 20% glycerol, 100 mM NaCl, 2% Triton X-100, 20 mM EGTA, 100 mM NaF, 4 mM PMSF, 4 mM Na₃VO₄, and 2 mM PNPP). Aliquots of cell lysates containing 25 μ g of total protein will be resolved by electrophoresis on 10% SDS-Polyacrylamide gels and transferred onto PVDF membranes by electroblotting. The membranes will be blocked with 5% nonfat dried milk in TBST buffer, and sequentially probed with appropriate dilutions of the following antibodies: anti- MyD88, anti-IRAK, anti-TRAF6 anti-NF- κ B. The blots will be washed with TBST buffer and then incubated with HRP-conjugated anti-mouse or anti-rabbit antibody Blots will be developed with ECL Plus detection system (Amersham Pharmacia Biotech Inc, Piscataway, New Jersey). To verify normalized protein loading and transfer efficiency, the blots will be probed with anti-actin antibody (Chemicon, Temecula, California).

U937 Cell Culture

A pro-monocytic cell line, U937, (ATCC) will be cultured in various concentrations of TCDD. An acute study will contain 2×10^6 cells in five different concentrations of TCDD: Control (no TCDD), 1ng/ml, 10 ng/ml, 50ng/ml and 100ng/ml. A non-acute study will have 1×10^6 cells in the following concentrations: Control (no TCDD), 0.0001ng/ml, 0.001ng/ml, 0.01ng/ml and 0.1ng/ml. All cells will be in a medium containing RPMI-1640, 10% Fetal Bovine Serum, 10% Penicillin, Streptomycin and L-Glutamine supplement. Stock TCDD (50 μ g/ml) (Cambridge Isotope Laboratories) will be diluted in RPMI to obtain desired concentrations.

Specific Responsibilities

- A. Maintain all cell lines (5 acute, 5 Non-acute).
 - a. Count cell concentration
 - b. Centrifuge cells in old medium and replace with fresh medium
 - c. Examine cells for contamination of yeast or mycoplasma.

- B. Freeze excess cells when cell concentration becomes too great.
- C. Analyze expressional down-regulation by TLR antibody staining and flow cytometry acquisition.
- D. Maintain record of expressional down-regulation.
- E. Assay MyD88, IRAK, NF- κ B, and TRAF6 through Western Blotting
- F. Analyze TNF- α , and IL-1 cytokines by ELISA
- G. Record and Analyze Data
- H. Journal article write-up
- I. UROP Symposium Poster assembly

Time Line

	No v.	Dec	Jan	Feb	Mar h	Apr il	Ma y
Stock Cell Expansion and Begin Study							
Maintain Cell Lines							
Allow Cells to Culture in Various Dioxin Concentrations							
Stain Cells to Determine Expressional Decline							
Begin Functional Study Once Expressional Decline is Established							
Analyze Data							
Prepare Publication and Poster							

Budget

	<u>Item</u>	<u>Quantity</u>	<u>Price</u>	<u>Company</u>
1.	ELISA Kit (TNF- α)	1 Plate	\$475	Biosource
2.	ELISA KIT (IL-1)	1 Plate	\$475	Biosource
2.	TCDD	4 x 1.2 ml	\$275	Cambridge
3.	Mouse IgG	2 ml	\$350	EBiosci.
5.	TLR 2	2 ml	\$250	EBiosci.
6.	TLR 4	2 ml	\$250	EBiosci.
			\$2075	

Total Project Budget- \$2075

Total Funds Requested- \$1000

References

Barton G, Medzhitov R (2003) Toll-Like Receptor Signaling Pathways. Sci. STKE (Connections Map, as seen April 2003), http://stke.sciencemag.org/cgi/cm/stkecm;CMP_8643.

Clark DA, Sweeney G, Safe S, Hancock E, Kilburn DG, Gaudie J. Cellular and genetic suppressional of cytotoxic T cell generation by haloaromatic hydrocarbons. *Immunopharmacology* 1983;6:143-153. basis for

Clark G, Tritscher A, Bell D, Lucier G. 1992. Integrated approach for evaluating species interindividual differences in responsiveness to dioxins and structural analogs. *Environmental Perspectives*. 98:125-132. and

- Hindsdill RD, Couch DL, Speirs RS. Immunosuppression in mice induced by dioxin (TCDD) in feed. *J Environ Pathol Toxicol* 1980;4(2-3):401-425
- Stone R. 1994. Dioxin report faces scientific gauntlet. *Science*. 265:1650.
- Thigpen JE, Faith RE, McConnell EE, Moore JA. Increased susceptibility to bacterial infection as a sequel of exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. *Infect Immun* 1975;12:1319-1324.
- Thomas PT, Hindsdill Rd. The effect of perinatal exposure to tetrachlorodibenzo-p-dioxin on the immune response of young mice. *Drug Chem Toxicol* 1979;2:77-98.
- Tucker AN, Vore SJ, Luster MI. Suppression of B cell differentiation by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. *Mol Pharmacol* 1986;29:372-377.
- Vos JG, Kreeftenberg JG, Engel HWB, Minderhoud A, Van Noorle Jansen LM. Studies on 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-induced immune suppression and decreased resistance to infection: endotoxin hypersensitivity, serum zinc concentrations and effect of thymosin treatment. *Toxicology* 1978;9:75-86.
- White KL, Lysy HH, McCay JA, Anderson Act. Modulation of serum complement levels following exposure to polychlorinated dibenzo-p-dioxins. *Toxicol Appl Pharmacol*. 1986;84:209-219.
- Whitlock JP. 1990. Genetic and molecular aspects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin action. *Ann. Rev. Pharmacol.* 30:251-277