2007

Modulation of the 3'IgH Regulatory Region (3'IgH RR), a prospective in vitro screening tool for identifying potential immunotoxicants

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MODULATION OF THE 3'IgH REGULATORY REGION (3'IgH RR), A PROSPECTIVE IN VITRO SCREENING TOOL FOR IDENTIFYING POTENTIAL IMMUNOTOXICANTS

A thesis submitted in partial fulfillment
for the requirements of the degree of
Master of Science

By

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B.S., Wright State University, 2003

2007
Wright State University

WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

June 7, 2007

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Rebecca A. Henseler ENTITLED Modulation of the 3'IgH Regulatory Region (3'IgH RR), a prospective in vitro screening tool for identifying potential immunotoxicants BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Henseler, Rebecca A. M.S., Department of Biological Sciences, 2007.
Modulation of the 3'IgH Regulatory Region (3'IgH RR), a prospective in vitro screening tool for identifying potential immunotoxicants.

The immune system is critical to human survival. However, assessing alterations of immune function by potential immunotoxicants is complicated by the diffuse nature of the immune system, which is composed of various effector cells each with differing effector functions. Current immunotoxicity testing is limited to animal studies. We have developed a model, which may provide an in vitro alternative to animal studies in identifying immunotoxicants that specifically target B cell function (i.e., alteration of immunoglobulin (Ig) or expression and antibody secretion). This model consists of a well-characterized B cell line, CH12.LX, which appears to appropriately model primary B cell function. We have stably transfected the CH12.LX cell line with a transgene regulated by an enhancer, the 3'Ig heavy chain regulatory region (3'IgH RR), purported to control Ig heavy chain gene expression. Our previous work has identified the 3'IgH RR as a sensitive target of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) possibly mediated by activation of the aryl hydrocarbon receptor (AhR) signaling pathway. We have tested our model with several structurally diverse chemicals shown to activate the AhR signaling pathway and found an association between AhR activation and inhibition

of both 3'IgH RR activation and Ig protein expression. We have also tested two chemicals previously shown to activate humoral immunity through non-AhR receptors. Results indicate that our model appropriately identifies immunomodulators of two receptor-signaling pathways, each leading to altered immunoglobulin expression. (Supported by the Colgate-Palmolive Grants for Alternative Research and the Boonshoft School of Medicine, WSU)

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I. 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

Dioxin is a term used to refer to a class of chemicals that have similar structures and a common route of action. This class includes polychlorinated dibenzodioxins, dibenzofurans, and biphenyls. These chemicals have been ubiquitous environmental contaminants since the beginning of the 20th century but have not been viewed as a concern until the past thirty years. Human exposure usually occurs through consumption of contaminated food, air, or through inhalation of dust and smoke (Mandal 2005). Prior to the enforcement of specific environmental regulations the major sources of dioxins were through the production of the pesticides, 2,4,5-tricholorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid, and paper bleaching. The knowledge of these types of contamination has led to banning of certain pesticides and development of new ways to bleach paper. Dioxins are still a concern today, but now the primary source of dioxins has shifted to combustion processes where dioxins are a secondary product. Dioxins are a concern to our environment, because of the implications that they have on animal and human welfare, which include reproductive toxicity, neurotoxicity, potential carcinogenicity, and immunotoxicity (Mandal 2005). The hallmark of this family of contaminants is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This chemical is believed to be one of the most potent and dangerous of chemicals in this family, and in turn has gained the most concern and research devoted to it. Some of the documented toxicities that occur with exposure to TCDD are loss of body weight, teratogenicity, porphyria,

chloracne, tumor promotion, disruption in endocrine homeostasis, and immunotoxicity (Poland and Knutson 1982; Mandal 2005). The effects of TCDD and other dioxins are believed to be mediated through binding to the aryl hydrocarbon receptor (AhR) and influencing gene expression (Mandal 2005).

II. ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AhR) is an intracellular protein that is part of the helix-loop-helix PER-ARNT-SIM (PAS) family. The PAS family of proteins play an essential role in the metabolism of xenobiotic compounds, adaptation to hypoxia, and the regulation of circadian rhythm (Gu, Hogenesch et al. 2000). The AhR is a ubiquitous protein that is present in the cytoplasm of most cells and is complexed with other proteins including HSP 90 until activated by ligand. TCDD is one of the most studied ligands of the AhR and is frequently used to study the AhR signaling pathway. Once bound by a ligand, the AhR dissociates from the cytosolic complex and translocates to the nucleus where it heterodimerizes with the AhR nuclear translocator (ARNT) protein. The AhR/ARNT complex then functions as a regulator of gene transcription by binding to dioxin responsive enhancers (DRE) within sensitive genes (Denison, Fisher et al. 1989). The most thoroughly studied gene regulated by TCDD-induced activation of the AhR signaling pathway is cytochrome P4501A1 (CYP1A1). However, increasing evidence supports a role of the AhR in many of the toxic effects induced by TCDD, including immunotoxicity. (Figure 1)

III. BASIC IMMUNOLOGY

Immunology is the study of how the body reacts to foreign or non-self substances referred to as antigens which include viral, bacterial, fungal, parasitic and any nonself proteins or substances. The body has two combating forces against these infectious agents that help to prevent infection: the innate and adaptive immune responses. Innate immunity is the body's first line of defense, and is characterized as non-specific, rapid (i.e., reacts within minutes of invasion) and short-term protection. Its main physiological function is to destroy intruding microorganisms through a non-specific process carried out mainly by macrophages and neutrophils that clear pathogens by phagocytosis and proteolysis. Tissue macrophages are the first to encounter pathogens that have broken through the protective epithelial layer. Pathogen-activated macrophages begin to release proteins known as cytokines that initiate an inflammatory response, resulting in the recruitment of neutrophils to the site of infection. If the infection is unsuccessfully cleared by the innate immune response, the adaptive immune response becomes activated to clear the infection. Adaptive immunity can be defined as a specific and diversified response to individual antigens, which offers long-term protection through the generation of immunologic memory, a process that elicits a quicker response to future encounters by the same antigen.

Adaptive immunity is primarily mediated by T cells and B cells that originate in the bone marrow. B cells will remain in the bone marrow for maturation until they are

distributed to peripheral lymphoid organs. T cells, on the other hand, originate in the bone marrow than migrate to the thymus to complete maturation, followed by recruitment to peripheral lymphoid organs. Both cells are capable of specific antigen recognition, resulting in each individual B or T cell recognizing only one antigen determinant. Both B and T cells have effector molecules that aid in the fight against the intruding antigens. T cells release cytokines in response to foreign pathogens, while B cells are capable of secreting antibodies in response to an antigen. T cells and B cells work together at times in the clearance of antigen. Both B cells and T cells have surface receptors that aid in the recognition of an antigen. On a B cell there is the B cell receptor, which is responsible for recognition of the specific antigen. This receptor is composed of a membrane bound immunoglobulin (referred to as an antibody in its secreted form) which is associated in a noncovalent complex with Igα and Igβ chains. The B cell receptor sends signals to the B cell once it has bound an antigen, and either directly activates the B cell to proliferate and differentiate into antibody secreting cells or leads to presentation of the antigen via MHC class II surface molecules to helper T cells, which secrete proteins (cytokines) that cause the B cell to proliferate and differentiate into antibody secreting cells.

IV. B CELLS AND ANTIBODY PRODUCTION

B cells, major effector cells in adaptive immunity, have the primary function of producing and secreting high concentrations of antibodies that can specifically bind and clear invading antigens from the body. Each B cell makes antibodies with only one antigen specificity and will only produce antibodies when activated by that specific antigen. The basic immunoglobulin (Ig) is composed of two heavy chains and two light chains. Each chain can be further broken down into variable regions and constant regions. The variable region determines the antigen specificity and also forms the antigen binding site. Binding sites for the antigen are formed from a composite of one light and one heavy chain. Since Ig is composed of two heavy chains and two light chains, there are two antigen binding sites. There are five major isotypes of Ig (IgG, IgM, IgA, IgD, and IgE), each defined by its heavy chain constant region (C_H), which is represented by lower-case Greek letters as follows: IgG=Cγ, IgM=Cμ, IgA=Cα, IgD=Cδ, and IgE=Cε. IgG can be further broken down into four subclasses (IgG1, IgG2, IgG3, and IgG4 for humans; IgG1, IgG2a, IgG2b, and IgG3 for mice), and in humans there are two subclasses of IgA (IgA1 and IgA2), which is not true for mice (one IgA class). All naive B cells express IgM and IgD on their cell surface, but the early stages of an antibody response are dominated by IgM production and secretion. Later on in the response or upon a second encounter with the same antigen, production of IgG or IgA will dominate the antibody response. Isotype switching from IgM to IgG or IgA occurs

through a DNA recombination process called class switch recombination in which the heavy chain variable (V) region becomes associated with a different C_H region resulting in the deletion of DNA between these regions and somatic recombination. Isotype switching does not alter antibody specificity but instead has an effect on the functions that an antibody can engage, resulting in a quicker and stronger antibody response.

V. IMMUNE EFFECTS INDUCED BY TCDD

TCDD is one of the most studied of the dioxins, and effects on the immune system appear to be one of the most sensitive endpoints of TCDD toxicity (Kerkvliet 2002). Studies of altered immune function by TCDD date all the way back to the 1970's when innate, humoral and cell-mediated immunity of rodents was shown to be directly impacted by exposure to TCDD. Past studies also showed that rodents who encountered doses of TCDD were more susceptible to disease, unable to reject transplanted tumors, and tumor growth and metastasis increased (Holsapple, Morris et al. 1991; Kerkvliet 2002) . TCDD has been shown to have a negative influence on various cells of the immune system including T and B cells (Kerkvliet 2002). The alterations of T cell immunity include decreased production of cytotoxic T lymphocytes, thymic atrophy, and suppressed hypersensitivity responses (Kerkvliet 2002) . The B cell has also been identified as a direct target of TCDD exposure. In the sheep red blood cell (SRBC) assay, in which SRBC's activate B cells through the required help of T cells and macrophages, TCDD has been shown to inhibit this response (Dooley and Holsapple 1988). However, TCDD has also been shown to directly inhibit B cell differentiation into plasma cells when activated by lipopolysaccharide (LPS) (Dooley and Holsapple 1988). LPS is a complex lipid structure found in the outer membrane of most gram negative bacteria which can activate and differentiate B cells in the absence of T cells and

macrophages. Therefore, B cells are a direct target of TCDD exposure (Dooley and Holsapple 1988)

VI. AHR AND B CELLS

Previous studies have shown that TCDD is capable of binding to the AhR in murine B cells and inhibiting B cell differentiation and antibody secretion, and this inhibition is proposed to be mediated by the AhR (Marcus, Holsapple et al. 1998; Sulentic, Holsapple et al. 1998; Sulentic, Holsapple et al. 2000). This hypothesis has been tested in a number of different studies including one study that tested two different B cell lines, one that expresses high levels of AhR (CH12.LX) and one that lacks AhR expression (BCL-1). Only the CH12.LX cell line showed TCDD-induced inhibition of Ig heavy chain expression and secretion, which appeared to be mediated through binding of the AhR to DRE motifs (Sulentic, Holsapple et al. 1998; Sulentic, Holsapple et al. 2000). Further investigation of the role of the AhR was shown in an AhR knockout mouse study. This study showed that AhR-deficient and wildtype mice (both from the same strain of mouse) were capable of normal cell-mediated and humoral immune responses when presented with an antigen, but when dosed with TCDD, the antibody immune response in the AhR-deficient mice was refractory to suppression (Vorderstrasse, Steppan et al. 2001). This outcome suggests a key role of the AhR in the dysregulation of B cell function by TCDD. Furthermore, studies have identified DRE binding sites within a 3' region of the Ig heavy chain gene (only expressed in B cells), known as the 3'Ig heavy chain regulatory region, which may mediate TCDD-induced inhibition of Ig heavy chain expression and Ig secretion (Sulentic, Holsapple et al. 2000).

VII. 3'IMMUNOGLOBULIN HEAVY CHAIN REGULATORY REGION

One important gene within B cells is the Ig heavy chain gene. Regulation of this gene is accomplished by several elements that include a V_H promoter that lies upstream of a variable region and is responsible for regulation of Ig heavy chain (IgH) gene expression; a E_μ region that lies in between the variable region and the μ constant region (C_μ) and is responsible for regulating VDJ joining (Calame and Eaton 1988; Serwe and Sablitzky 1993); and most recently discovered a 40-kb region 3' of the α constant region (C_α) known as the 3'Ig heavy chain regulatory region (3'IgH RR). The 3'IgH RR is believed to play an important role in Ig heavy chain expression and class switch recombination (Lieberson, Giannini et al. 1991). (Figure 2)

The 3'IgH RR is composed of four separate hypersensitive regulatory domains (hs3, hs1,2, hs3B, hs4), with the first three domains being active mainly in mature B-cells and the hs4 enhancer active throughout the lifespan of a B cell (Chauveau, Pinaud et al. 1998). There are several binding sites within the 3'IgH RR for transcription factors that appear to regulate its activity. These transcription factors include B cell specific activator protein (BSAP), NF-κB, octamer (OCT), OCA- B, and AP-1. As mentioned above, DRE motifs have also been identified within the 3'IgH RR (Sulentic, Holsapple et al. 2000). TCDD was shown to induce AhR-DRE binding within the 3'IgH RR as well as inhibition of 3'IgH RR activity, which correlates with TCDD-induced inhibition of μ heavy chain expression and IgM secretion (Sulentic, Holsapple et al. 2000; Sulentic,

Zhang et al. 2004). Since structurally diverse chemicals from dietary, pharmaceutical and industrial origin have been demonstrated to activate the AhR signaling pathway, a potential AhR-mediated modulation of 3'IgH RR activity may have broad toxicological implications. The primary focus of the research reported here was to test this premise by utilizing an in vitro B cell line model and assessing the effects of several chemicals, including a diverse array of AhR and non-AhR ligands on transcriptional activity mediated by the 3'IgH RR.

VIII. AhR AGONISTS

The chemicals were chosen based on previous studies that have demonstrated activation of the AhR, whether indirect or direct. Historically, the ligands that bind to the AhR have had the characteristics of being planar, aromatic, and hydrophobic as in the case of TCDD, but recently other chemicals that associate with the AhR deviate from this set of characteristics (Denison and Nagy 2003). Chemicals were also chosen in a manner that would provide a selection of structurally diverse chemicals ranging from dietary, pharmaceutical, and environmental origin. These may have a higher potential of human exposure than the historic ligands that have been shown to activate the AhR signaling pathway.

A. Indolo(3,2,b)carbazole

Indolo(3,2,b)carbazole (ICZ) is an important chemical when considering immune regulation because of its similarity to TCDD, which include structural and functional similarities. Two important deviations from TCDD include the lack of halogens and low lipophilicity, which results in lower accumulation in cells than TCDD. ICZ is a dietary breakdown product of indole-3-carbinol that is present in cruciferous vegetables such as brussel sprouts and broccoli. ICZ is catalyzed from indole-3-carbinol in the acid environment of the stomach, or it can also be produced as a metabolic product of intestinal bacteria from trytophan (Chen, Riby et al. 1995). A previous study showed that people who consume 100 grams of cruciferous vegetables a day will in turn be producing

1.3μg of ICZ (Bjeldanes, Kim et al. 1991). In comparison with TCDD, ICZ is capable of inducing CYP1A1 at a rate of 10^3-10^4 less than TCDD (Chen, Riby et al. 1995). Furthermore, ICZ has an affinity for the AhR that is approximately $3.7x10^2$ lower than TCDD and induces nuclear translocation of the AhR 100 times lower than TCDD (Pohjanvirta, Korkalainen et al. 2002)

B. Omeprazole

Acid reflux related diseases are an increasing problem for the human population with a total of 2.3% of the US population being affected. The choice of therapy had been histamine-2-receptor agonists, but has recently shifted to proton pump inhibitors (Robinson 2005). Omeprazole is one of the main proton pump inhibitors used, and mechanistically it works by inhibiting the H^+/K^+-ATPase in gastric parietal cells (Quattrochi and Tukey 1993). The use of this medication is given in tablet form of up to 40mg, which can result in a plasma concentration of more that 5.21μM (PDR 2006). Structurally, Omeprazole is planar with a sulfoxide-containing chain connecting a benzimidazole ring and a substituted pyridine ring. The planarity matches one of the hallmark characteristics of being a ligand for the AhR (Dzeletovic, McGuire et al. 1997). Previous studies have shown Omeprazole to be capable of inducing CYP1A1, but they have failed to show direct binding to the AhR based on competitive binding studies. Contrary to a lack of AhR binding, within this study Omeprazole was shown to induced nuclear translocation of the AhR and DRE binding in rat hepatocytes (Lemaire, Delescluse et al. 2004)

C. Carbaryl

Carbaryl is a common insecticide that humans come into contact with through the consumption of water, plants or fish that have absorbed some of the chemical. Most people are not in direct danger of exposure to Carbaryl, but instead it is mainly a threat to the workers who produce it and are in daily contact with it. This is because of the steady deterioration of the chemical and its short half life. It has been shown that possible Carbaryl exposure of formulators and applicators can range from 59-74mg/hr through the dermal route and up to 1mg/hr through respiration (Comer, Staiff et al. 1975). Carbaryl has been shown to induce CYP1A1 through an AhR-dependent mechanism, and has been shown to directly bind the AhR. Even though this chemical is neither planar, nor polycyclic aromatic, it is considered a weak agonist that is about $3x10^5$ fold less potent than TCDD (Denison, Phelan et al. 1998).

D. Primaquine

Malaria is a very serious disease. According to the Centers for Disease Control and Prevention (CDC), 350-500 million cases of malaria occur worldwide with over two million people dying each year. Primaquine has been the drug treatment of choice and cure for relapse of *Plasmodium vivax* and *ovale* blood stage infections. Primaquine has been used as a treatment for malaria since 1952 and has remained the hallmark treatment for malaria when compared to any other recently discovered treatments(Hill, Baird et al. 2006) . This drug has been shown to have adverse effects at high dosages, which include methaemoblobinaemia and haemolytic anaemia (Fontaine, Delescluse et al. 1999). Previous studies in hepatoma cells have demonstrated AhR activation and CYP1A1 induction following Primaquine treatment. Primaquine has an affinity for the AhR at approximately $5x10^5$ times less than TCDD but is able to displace TCDD from the AhR

(Backlund and Ingelman-Sundberg 2004). Primaquine is usually given in 15 or 30 mg tablets for 14 days and studies have shown that after an acute dosage of 15mg there was a peak concentration of 65.0 ng/ml (0.25μM) (Ward, Mihaly et al. 1985).

IX. NON-AhR AGONISTS

The chemicals listed above all work through the AhR receptor; however, regulation of the 3'IgH RR and Ig expression as reviewed above may involve multiple transcription factors. We chose to look at two chemicals that work through different receptors that are expressed in B cells.

A. Carbachol

Carbachol is a cholinergic agonist for the muscarinic and nicotinic receptors. It is commonly used in the treatment of glaucoma. Glaucoma is a serious eye disease in which the intraocular pressure in the eye is elevated. Carbachol is prescribed as drops that produce constriction of the iris and ciliary body resulting in the reduction of ocular pressure associated with glaucoma (Duncan and Collison 2003). In a previous study Carbachol was shown to increase the T-cell dependent IgM response to sheep red blood cells (Pruett, Han et al. 1992). Both T and B cells express cholinergic receptors (Sato, Fujii et al. 1999; Skok, Grailhe et al. 2005) .

B. Terbutaline

Terbutaline is an agonist for the β_2-adrenergic receptor, which is present on B-cells. It is a commonly prescribed drug for the treatment of many lung diseases, such as asthma or emphysema, to help eliminate shortness of breath by opening up airway passages (Sears and Lotvall 2005). In previous studies Terbutaline has been shown to

enhance Ig expression in B cells through the induction of OCA-B binding to octomer binding sites within the 3'IgH (Podojil, Kin et al. 2004; Podojil and Sanders 2005).

X. SIGNIFICANCE AND OBJECTIVES

The purpose of this research was to determine the effect on 3'IgH RR activity of structurally diverse chemicals from dietary, environmental, or pharmaceutical origin which induce the AhR signaling pathway. Previous results from this lab have identified the 3'IgH RR as a sensitive target of TCDD. This study determined if inhibition of 3'IgH RR is unique to TCDD or if other chemicals (ICZ, Omeprazole, Carbaryl, and Primaquine) that activate the AhR signaling pathway also target 3'IgH RR. Since TCDD represents a broad class of chemicals and increasing evidence suggests that the spectrum of potential AhR ligands is much broader than typical halogenated hydrocarbons, the potential for AhR-mediated inhibition of 3'IgH RR activity is of great toxicological significance to human health and maintaining immunocompetence mediated by antibodies.

XI. MATERIALS AND METHODS

A. Chemicals

Omeprazole, Primaquine, Carbaryl, Carbachol, and Terbutaline were all purchase through Sigma Aldrich (Milwaukee, WI). Chemical purities were as followed: Primaquine (98.9%), Carbaryl (99.8%), Omeprazole (100%), Carbachol (>99%), and Terbutaline (>99%) Indolo(3,2,b)carbazole was generously donated by Dr. Leonard F. Bjeldanes, (University of California, Berkely, CA). LPS (Sigma) was used as a B cell stimulant in all experiments.

B. Cell line model

The CH12.LX murine B cell line was utilized in the transient transfection experiments with a luciferase reporter regulated by the 3'IgH RR. This cell line, previously characterized by Bishop and Haughton (Bishop and Haughton 1986), was derived from the murine CH12 B cell lymphoma that arose in B10.H-2^{a}H-4^{b}p/Wts mice (B10.A x B10.129) and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC). To more closely resemble the endogenous IgH chromosome, a modified version of the CH12.LX cell line (CH12.γ2b-3'IgH) that stably expresses a γ2b transgene regulated by the 3'IgH RR was also utilized. The γ2b mini-locus (Shi and Eckhardt 2001) that our lab used in making the CH12.γ2b-3'IgH cell line was generously donated by Dr. Laurel Eckhardt (Hunter College of the City University of New York, New York, NY).

C. Transient transfection

Transient transfections were performed as followed: CH12.LX cells ($2.2x10^7$) were resuspended into 440µl of culture media with 22µg 3'IgH RR or V_H reporter plasmid. 200uL of the solution was then transferred to two 2-mm gap electroporation cuvettes. Cells were electroporated using an electro cell manipulator (ECM 630) with the voltage at 250 volts, the capacitance at 250µF, and the resistance at 300 ohms. The total 400µL electroporated solution was then resuspended into 3.6ml of culture media ($5.0x10^6$ cells). 280µL of the resuspended solution was then added to 7 tubes containing 6.72mL of culture media each, resulting in $2x10^5$ cells/ml. CH12.LX cells were immediately treated with or without LPS and ICZ, Carbaryl, Omeprazole, Primaquine, Carbachol, Terbutaline, TCDD, or DMSO. 2ml from each tube were aliquoted into 12-well plates for triplicate samples at a concentration of $2.0x10^5$ cells/ml. The 12-well plates were incubated at 37°C, 5% CO_2 for 48hr. After the 48hr incubation period, cells were lysed using 1x reporter lysis buffer. The samples were immediately frozen at -80°C. To measure luciferase enzyme activity, samples were thawed at room temperature then centrifuged at 14,000 RPM at 4°C for 5 min. The lysate was transferred to new tubes, and 20µl of lysate was analyzed with a luminometer (Berthold detection systems, Sirius). The luciferase activity was represented as relative light units.

D. Analysis of γ2b gene and Ig protein by Enzyme-Linked Immunosorbent Assay (ELISA)

For both the γ2b transgene and Ig protein analysis, stably transfected CH12.LX cells were treated with LPS and ICZ, Omeprazole, Carbaryl, Primaquine, Carbachol, Terbutaline, TCDD, or DMSO and plated in 12 well plates. For γ2b analysis cells were

plated at a concentration of $2.5x10^4$ cells/ml, followed by a 48hr incubation period, while cells used for Ig protein analysis were plated at $2.0x10^5$ cells/ml, followed by a 24hr incubation period. Cells were then centrifuged at 3000 rpm at 4°C for 5 min, and then lysed with a mild lysis buffer with added protease inhibitors and frozen at -80˚C until analysis.

At the time of analysis the cell lysates were thawed at room temperature and the protein concentration determined by Bradford assay in order to standardize samples to protein concentration. Standards for the Bradford were prepared with varying amounts of Elga water, BSA, and 2µl of the mild lysis buffer plus protease inhibitor solution, allowing for final standard concentrations ranging between 1-14µg of BSA and final volumes of 800ul. 2µl of supernatant for each chemical sample were dispensed into appropriately marked glass tubes with 798µl Elga water and 200µl Bradford reagent. 200µl of the standards and samples were added to 96-well plates followed by absorbance readings on a plate reader (Molecular Devices, Spectramax plus 384).

The values calculated for the amount of protein in each sample were then used to determine amounts of each sample needed to obtain 2µg of total protein for the ELISA analysis of γ2b levels and Ig levels. The analysis was done by a standard sandwich ELISA, in which the coating antibody and the detection antibody are specific for the same antigen. The detection antibody is conjugated to HRP (horesradish peroxidase) enzyme which cleaves a substrate producing a color change. Absorbance of the reaction product will be determined by the plate reader previously mentioned above, using a kinetic reading and standard curve to determine concentrations of γ2b and Ig.

E. Statistics

The mean ± S.E. was determined for each treatment group (n=3). To determine statistical significance between treatment groups and vehicle controls, the data were analyzed by a one-way ANOVA followed by a Dunnett´s two-tailed *t*-test. For IC_{50} generation, a complete concentration-response curve for each chemical was obtained. Concentration-response curves were fit by a four-parameter logistic concentration-response equation given as Y = Bottom + (Top-Bottom)/(1+10^((LogIC_{50}-X)*Hill Slope)). X is the logarithm of concentration. Y is the response. The derived parameter, IC_{50} was the nanomolar (nM) to micromolar (μM) concentration generating half-maximal response. The IC_{50} value for each AhR activator was expressed as the average plus the 95% confidence interval which was generated from the best-fit value of the IC_{50} parameter between data sets (n=3 to 4).

XII. RESULTS

A. Inhibition of LPS-induced 3'IgH RR activity by AhR ligands in transiently transfected CH12.LX cells.

AhR binding within the 3'IgH RR has been previously identified, along with inhibition of the 3'IgH RR by the AhR ligand TCDD. However, the role of the AhR in this effect has not been determined. TCDD represents only a single chemical in a large group of chemicals that can modulate AhR activity. Therefore, if the effect of TCDD on 3'IgH RR activity is AhR-dependent, than the 3'IgH RR may be targeted by a broad group of chemicals. To determine if the modulation of the 3'IgH RR produced by TCDD is a common effect of all AhR ligands, we examined four non-dioxin AhR ligands ICZ, Primaquine, Carbaryl, and Omeprazole for their effect on luciferase activity in CH12.LX B-cells transiently transfected with a luciferase reporter regulated by the 3'IgH RR. These assays showed that similar to TCDD all four chemicals were capable of suppressing LPS-induced 3'IgH RR activity in a concentration-dependent manner. Inhibition of the 3'IgH RR by each chemical was concentration-dependent, with maximal suppression (without deterioration of viability) occurring at the following concentrations: ICZ-500nM, Primaquine-50μM, Carbaryl-100μM, and Omeprazole-62.5μM (Figure 3 A-D). This assay was taken a step further to investigate if it is only the 3'IgH RR region that is modulated or if the activity of the V_H promoter is also affected. The V_H promoter lies upstream of the variable region and is responsible for initiation of transcription at

theIg heavy chain (IgH) gene. The V_H promoter transiently transfected into the same B-cell line was activated with LPS stimulation, but to a lesser degree than the 3'IgH RR reporter. Also, the concentrations of the chemicals that inhibited 3'IgH RR activation did not modulate the V_H promoter (Figure 4 B). Consistent with previous studies identifying the sensitivity of activated versus resting B cells to TCDD (Marcus, Holsapple et al. 1998; Sulentic, Holsapple et al. 1998; Crawford, Sulentic et al. 2003), these AhR activators had no effect on background activity of 3'IgH RR (Figure 4 A).

B. Inhibition of 3'IgH RR activity by structurally diverse AhR ligands follows a structure activity relationship in CH12.LX cells stably expressing a transgene regulated by the 3'IgH RR.

The transient transfection results supported further investigating the phenomenon of 3'IgH RR inhibition by AhR agonists other that TCDD. While transient transfections provide a quick way to obtain results, there can be considerable variation from experiment to experiment. When transiently transfecting a plasmid into a cell you are not guaranteed a certain amount of copies per cell or even that every cell will receive a copy. Additionally, in transient transfections the 3'IgH RR reporter is not wrapped around chromatin as is the case for endogenous genes. In order to more closely approximate the situation on the IgH chromosome and to avoid the many limitations of transient transfection experiments, an experimental model (CH12.γ2b-3'IgH) developed in CH12.LX cells that stably expresses a γ2b heavy chain transgene under the regulation of the 3'IgH RR was used (Shi and Eckhardt 2001). The CH12.γ2b-3'IgH cell line expresses the 3'IgH RR in the context of chromatin just like endogenous genes, and each cell contains one copy of the γ2b transgene as determined by PCR analysis (Sulentic,

CEW, unpublished results). These cells have also been characterized as IgA expressing B cells and do not express endogenous γ2b or IgG2b as determined by flow cytometry and ELISA. All of the AhR agonists suppressed the LPS-inducible transcription of the γ2b transgene in the CH12.γ2b-3'IgH cells (Figure 5 A-D). In addition to the results of this assay following the same tendency of down-regulation of the 3'IgH RR shown in the transient transfection assay, the down-regulation of the γ2b gene between the different chemicals followed a structure activity relationship to their AhR binding affinity (Figure 6). The rank order potency established by the IC_{50} showed that TCDD, as expected, has the greatest potency in inhibiting γ2b transgene expression, followed by ICZ, Carbaryl, Omeprazole, and Primaquine. This is relatively consistent with their affinities for the AhR, based on past research. TCDD has been shown to have the greatest affinity followed next by ICZ. Carbaryl, Primaquine, and Omeprazole, based on past research, have similar affinities for the AhR.

C. Endogenous Ig protein expression was suppressed by the AhR agonists.

While the modulation of the 3'IgH RR may provide an important tool in identifying AhR activators and other chemicals that may alter B cell function, it may not correlate with an alteration of endogenous Ig expression. An important function of B cells is to produce and secrete antibodies following an encounter with a foreign agent, as in the case of LPS. As demonstrated above a diverse set of AhR agonists are capable of suppressing the 3'IgH RR which is thought to regulate Ig expression. Therefore, it was important to establish a relationship between the suppression of our 3'IgH RR models, i.e. transient and stable reporters, and the effect on endogenous Ig protein expression. All

of the AhR agonists suppressed the LPS-induced expression of endogenous IgA (Figure 7). Instead of secreted IgA, intracellular IgA was measured and standardized to total protein concentration so as to avoid any confounding influences due to an effect of the chemicals on cellular proliferation.

D. Modulation of the 3'IgH RR and Ig protein expression by non-AhR Agonists.

Up to this point the focus of this study was directed at AhR agonists, but it was also important to examine the effects of non-AhR agonists since the 3'IgH RR is regulated by several transcription factors which are likely activated through different signaling pathways. We chose to evaluate the effects of Terbutaline and Carbachol in our γ2b model since each activates a distinct signaling receptor and have been shown to modulate B cell activation. Terbutaline is a β_2-adrenergic receptor agonist and has been shown to enhance Ig expression in activated B cells through induction of OCA-B binding to the 3'IgH RR (Podojil, Kin et al. 2004; Podojil and Sanders 2005). Carbachol is an agonist for muscarinic and nicotinic receptors and has been shown to increase the T-cell dependent antibody forming cell response (Pruett, Han et al. 1992). Our studies demonstrated that Terbutaline up-regulated the expression of the γ2b transgene in LPS-stimulated cells, while Carbachol had no effect (Figure 8 A-B). The up-regulation of the transgene by terbutaline was concentration-dependent, with the greatest effect at a concentration of 100μM. Terbulatine and Carbachol did not modulate the V_H promoter in LPS-stimulated cells (Figure 9 B). Terbutaline and Carbachol also had no affect on background activity when evaluating the 3'IgH RR with the luciferase reporter (Figure 9 A) or the γ2b transgene reiterating the need for prior stimulation of the B cell (Figure 10

A-B). We once again showed that the effects on the γ2b transgene expression in LPS-stimulated cells mimicked the effects on Ig protein expression with Terbutaline increasing endogenous IgA protein expression in LPS-stimulated CH12.γ2b-3'IgH cells and carbachol having no effect (Figure 11). These findings validate our model in its ability to identify chemical-induced modulation of the 3'IgH RR, and the correlation between 3'IgH RR regulation and endogenous IgA protein expression.

XIII. DISCUSSION

This study focused on chemical-induced modulation of the 3'IgH RR which appears to correlate with the effects of these chemicals on Ig expression. The 3'IgH RR is composed of four separate hypersensitive regulatory domains (hs3, hs1,2, hs3B, hs4), each one being active at different times through the lifespan of a B cell (Chauveau, Pinaud et al. 1998). As mention previously the 3'IgH RR contains numerous regulatory binding sites, including at least two functional DRE motifs (Sulentic, Holsapple et al. 2000). Previous studies within this lab have demonstrated TCDD-induced AhR-DRE binding within the 3'IgH RR as well as inhibition of 3'IgH RR activity that correlated with TCDD-induced inhibition of μ heavy chain expression and IgM secretion(Sulentic, Holsapple et al. 2000; Sulentic, Zhang et al. 2004).

TCDD is the prototypical AhR ligand and represents a large class of aromatic hydrocarbons. However, a major focus of the present study was to determine if inhibition of 3'IgH RR activation applied to a diverse range of non-dioxin AhR activators. For these studies we utilized the CH12.LX B-cell line that has been well-characterized in regards to TCDD-induced inhibition of B cell function and to the function of the AhR signaling pathway (Sulentic, Holsapple et al. 1998). We chose to evaluate the following non-dioxin AhR activators: the dietary metabolite ICZ; the antimalarial drug primaquine; the pesticide Carbaryl; and the proton pump inhibitor Omeprazole. ICZ, Primaquine, Carbaryl, and Omeprazole have all been shown to be AhR activators (Denison, Phelan et

al. 1998; Pohjanvirta, Korkalainen et al. 2002; Backlund and Ingelman-Sundberg 2004; Lemaire, Delescluse et al. 2004). ICZ, which is a breakdown product of cruciferous vegetables, has the greatest potential exposure to humans based on a daily consumption of cruciferous vegetables. Primaquine is a common choice for the treatment of malaria which is widespread in tropical and subtropical regions. Carbaryl exposure is not as broad as some of the other chemicals, but it is specific to certain lines of work where insecticides are needed. Finally, Omeprazole is a common pharmaceutical drug used in the treatment of stomach acid related illnesses. Additionally, Omeprazole is unique compared to the other chemicals in that it , as yet, not been shown to directly bind to the AhR, but it does induce nuclear translocation of the AhR, DRE binding, and CYP1A1 induction in rat hepatocytes (Lemaire, Delescluse et al. 2004).

Consistently, our studies demonstrated that all four AhR activators were capable of inhibiting LPS-induced 3'IgH RR activity and suppressing the production Ig protein. This suggests that modulation of the 3'IgH RR is not specific to TCDD, but more likely is a target of all AhR activators. Furthermore, the inhibition of γ2b transgene expression in the CH12.γ2b-3'IgH cells followed a structure activity relationship for AhR binding or activation affinity, further supporting a role of the AhR in the inhibition of 3'IgH RR activity. These effects were dependent on activation of the B cell. This study focused on LPS-induced B cell activation. There is a possibility that the inhibitory effects of the AhR activators are specific to LPS stimulation; however, this is unlikely since several groups have previously identified an inhibition of Ig secretion or the antibody forming cell response by TCDD following B cell activation with other stimuli including T-cell dependent stimuli (Holsapple, Morris et al. 1991) . Since B cells are most sensitive to

TCDD during activation, the potential impairment of Ig expression by AhR activators in humans will depend on the timing of chemical exposure in relation to an immune response. Timing is also an important consideration when it comes to the inhibitory properties of the AhR activators, both in humans and in vitro. In other words, the AhR activators will likely need to be at an effective concentration prior to the initiation of an antibody response. Indeed, previous times of addition studies with TCDD have identified the critical window of exposure to be within the first 24 hr of stimulation (Tucker, Vore et al. 1986) .

These results may have important implications to human health, particularly considering that AhR ligands include a diverse group of chemicals and that humans may be exposed to several AhR ligands simultaneously and perhaps under chronic conditions that may contribute to reaching a threshold level of AhR activation perhaps leading to inhibition of 3'IgH RR activation and Ig expression. However, it is also a possibility that multiple AhR agonists may compete for AhR binding and potentially decrease the overall effect particularly if a lower affinity AhR ligand is in excess of a higher affinity ligand. An additional consideration is the half-life of the AhR ligands. If they have short half-lives they may well be cleared from the body before they could induce immunosuppressive properties, so for many agonists continuous exposure at high levels may be necessary to induce immunosuppresive effects. Whether multiple AhR ligands in vivo will induce additive, synergistic, or antagonistic effects has yet to be determined. Furthermore, the fact that some AhR ligands are antagonists and have been identified in fruit and vegetable extracts (Denison and Nagy 2003) further complicates predictions regarding the overall effect on 3'IgH RR activity and Ig expression in humans potentially

exposed to multiple AhR modulators. Regardless, the 3'IgH RR has at least two functional DRE sites and is a sensitive target of TCDD and other AhR modulating chemicals. An additional caveat is that this study utilized the mouse 3'IgH RR which has differences in structure and transcriptional regulation compared to the human 3'IgH RR. However, both the mouse and human 3'IgH RR contain two DRE-like binding sites.

As stated previously ICZ is a breakdown product of cruciferous vegetables. ICZ is catalyzed from indole-3-carbinol in the acid environment of the stomach, or produced as a metabolic product of intestinal bacteria from trytophan (Chen, Riby et al. 1995). A previous study showed that people who consume 100 grams of cruciferous vegetables a day will in turn be producing 1.3μg of ICZ (Bjeldanes, Kim et al. 1991). Interestingly, indole-3-carbinol is widely sold in health food stores as a dietary supplement which increases the possibility of certain population groups, particularly vegetarians, reaching blood concentrations of ICZ that could impact 3'IgH RR activity, even though there is still debate over the potential toxicological relevance in humans due to the short half-life.

Omeprazole is the common choice of treatment for stomach acid related illnesses. It is generally given in tablet form of up to 40mg, which can result in a plasma concentration of more than 5.21μM (PDR 2006) which is approximately 10-fold lower than the concentration inducing maximal inhibition of 3'IgH RR activation in vitro. However, the accumulated plasma concentration following a multi-day administration has not been defined. Furthermore, with pharmaceutical drugs there is no guarantee that patients will not take a higher dose than is recommended or the possibility of errors in doctor or pharmacy prescriptions. Additionally, as stated above there may be co-

exposure to other AhR activators that may contribute to reaching an effective concentration to mediate an effect on 3'IgH RR activity and Ig expression.

Primaquine is a widely used treatment for malaria, while Carbaryl is used as an insecticide. Malaria causes disease in approximately 400 million people every year and kills between 1 and 3 million every year in Sub-Saharan Africa. Primaquine is usually given in 15 or 30mg tablets for 14 days and studies have shown that after acute dosage of 15mg there was a peak concentration of 65.0 μM (Ward, Mihaly et al. 1985). This peak concentration, combined with the possibility of bioaccumulation over the course of the therapy may result in an effective concentration to suppress 3'IgH RR activity and Ig expression. Furthermore, individuals living in third world countries, where malaria is most prevalent, may already have decreased immune function due to malnutrition and/or other factors; suppression of 3'IgH RR activation may further compromise an already weakened immune system. Carbaryl on the other hand is more likely to affect a smaller population, specifically individuals that directly apply the insecticide. Studies have shown that possible Carbaryl exposure of formulators and applicators can range from 59-74mg/hr through the dermal route and up to 1mg/hr through respiration (Comer, Staiff et al. 1975); however, the plasma concentration was not determined. This leaves the question of how much this chemical will have suppressive properties on the immune system, but it stands to reason that if chronic exposure occurs, especially without proper protective clothing, plasma concentrations could reach levels that might lead to an effect on 3'IgH RR activation and immunocompetence.

Our model also detected chemical-induced enhancement of 3'IgH RR activation. Terbutaline, which is a β_2-adrenergic agonist, was able to potentiate LPS-induced 3'IgH

RR activation and IgA protein expression. This demonstrates that our model is not limited to detecting inhibition of 3'IgH RR activity by AhR ligands, but can also detect chemicals using distinct cellular pathways that lead to the modulation of transcription factors other than the AhR which regulate 3'IgH RR activity. Additionally, these results show that the 3'IgH RR can be regulated in both directions. These results are consistent with a previous study in which Terbutaline was shown to up regulate the 3'IgH RR in primary B cells and provides further significance to the present study in that a different stimulus other than LPS was used and that it was done in primary B cells (Podojil, Kin et al. 2004; Podojil and Sanders 2005) This shows that 3'IgH RR regulation is not limited to LPS activation, and that our B cell line model is representative of primary B cells. In contrast to Terbutaline, the muscarinic and nicotinic agonist Carbachol did not alter 3'IgH RR activation suggesting many possibilities, including the following. 1) Activation of cholinergic receptors does not directly activate the appropriate signaling pathway to lead to 3'IgH RR modulation. 2) The appropriate co-stimuli were not present. For example, in a previous study Carbachol was shown to increase the T-cell dependent IgM response to sheep red blood cells. So Carbachol might be T-cell dependent in its regulation of immune responses. 3) Cholinergic receptors may not be functional in CH12.LX cells.

In summary, modulation of the 3'IgH RR is not limited to TCDD and appears to be AhR-dependent. It is becoming increasingly evident that AhR ligands (agonist and antagonists) are not restricted to industrial or environmental sources but include therapeutic drugs and dietary components present in dietary supplements, fruits and vegetables (Denison and Nagy 2003). Multiple sources of potential exposure to AhR

ligands and accumulation may produce additive or synergistic activation of the AhR signaling pathway therefore posing a potential risk to humans, particularly to toxic responses such as B cell dysfunction, which may not occur when exposed to only one source. Additionally, regulation of the 3'IgH RR is not limited to AhR agonists, but includes modulators of other signaling and transcriptional pathways therefore increasing the potential array of chemicals that may modulate 3'IgH RR activity and affect immunocompetence. We believe that our B cell model may provide a valuable screening and mechanistic tool in identifying chemicals that can directly alter 3'IgH RR activity and Ig expression which is of significance to human health and to the detection of possible immunotoxicants.

XIV. LITERATURE CITED

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APPENDICES

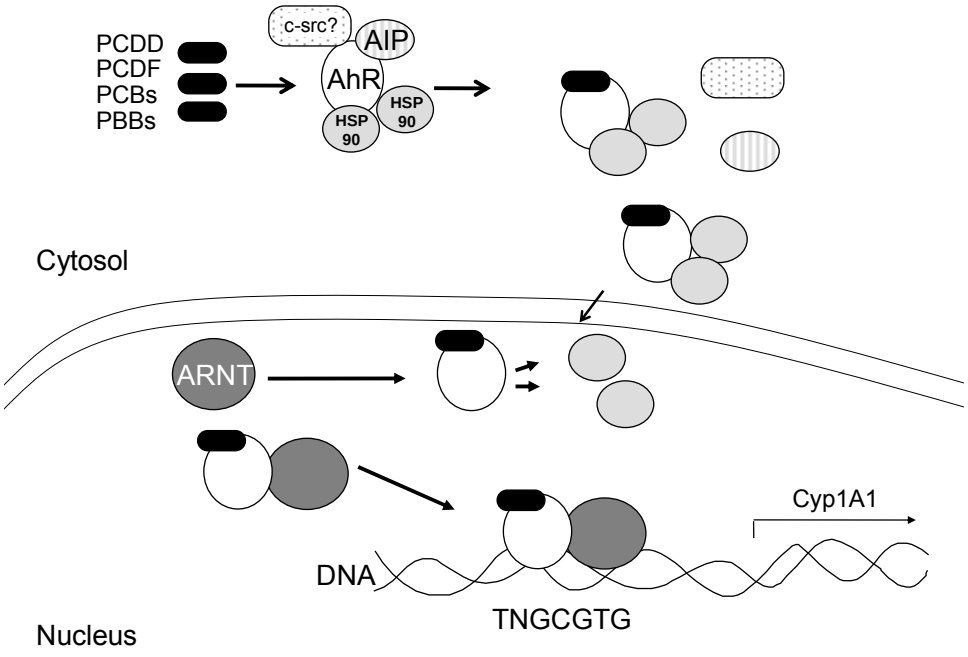


Figure 1. Aryl Hydrocarbon Receptor (AhR) signaling pathway. Regulation of cytochrome P4501A1 (CYP1A1) through ligand-induced activation of the AhR signaling pathway.

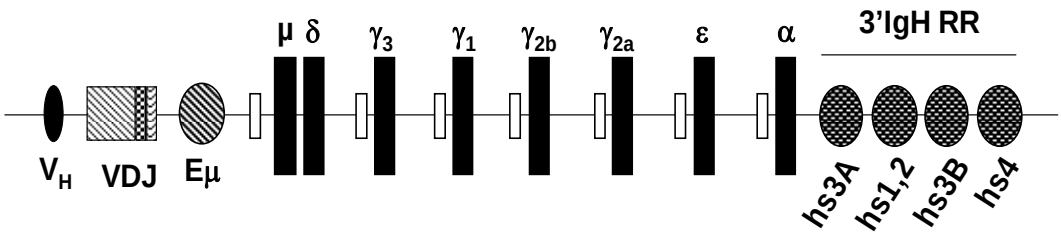


Figure 2. Murine Immunoglobulin heavy chain (IgH) locus. Schematic of the IgH locus and its various regulatory elements.

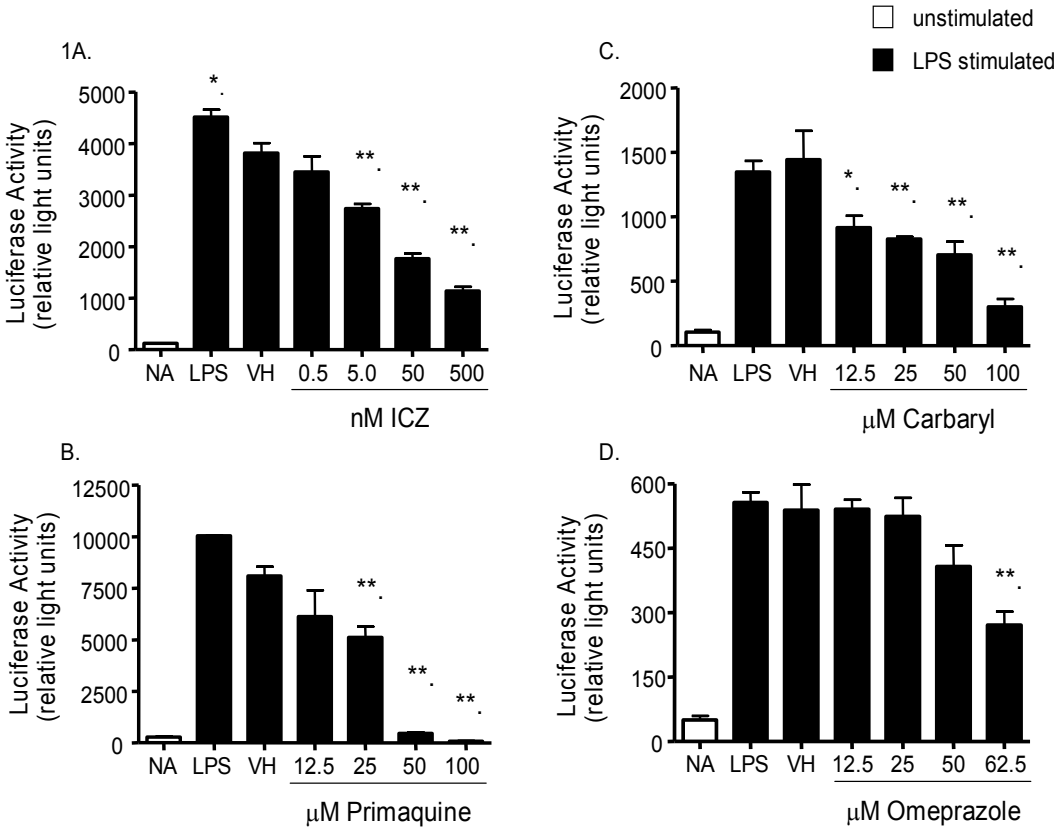


Figure 3. Inhibition of the 3'IgH RR luciferase reporter by AhR activators. CH12.LX cells were transiently transfected with the 3'IgH RR reporter plasmid then treated with vehicle (VH, 0.01% DMSO for ICZ and Primaquine or 0.1% DMSO for Carbaryl and Omeprazole) or varying concentrations of indolo(3,2,b)carbazole (ICZ) (A), Primaquine (B), Carbaryl (C), or Omeprazole (D), in the presence of LPS (3 μg/ml) for 48hr. Luciferase enzyme activity is shown on the y-axis as relative light units, n=3. NA denotes naïve control. Significance from VH control at $p<0.05$ (*) or $p<0.01$ (**) was determined by a 1-way ANOVA and Dunnett's post-hoc test.

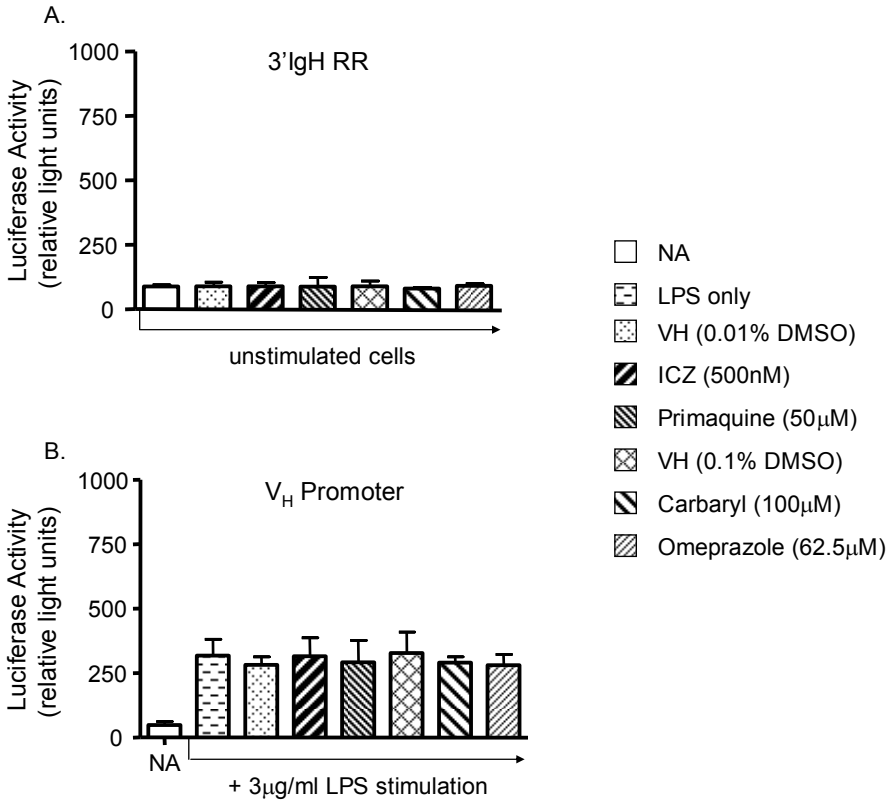


Figure 4. Regulation of the V_H Promoter and the 3'IgH RR. CH12.LX cells were transiently transfected with the 3'IgH RR plasmid (A) or the V_H promoter plasmid (B) then treated with vehicle (VH, 0.01% DMSO) or specific concentrations of ICZ, Primaquine,Carbaryl, or Omeprazole, in the presence or absence of LPS (3 μg/ml) for 48hr. Luciferase enzyme activity is shown on the y-axis as relative light units, n=3. NA denotes naïve control. Significance from VH control at $p<0.05$ (*) or $p<0.01$ (**) was determined by a 1-way ANOVA and Dunnett's post-hoc test.

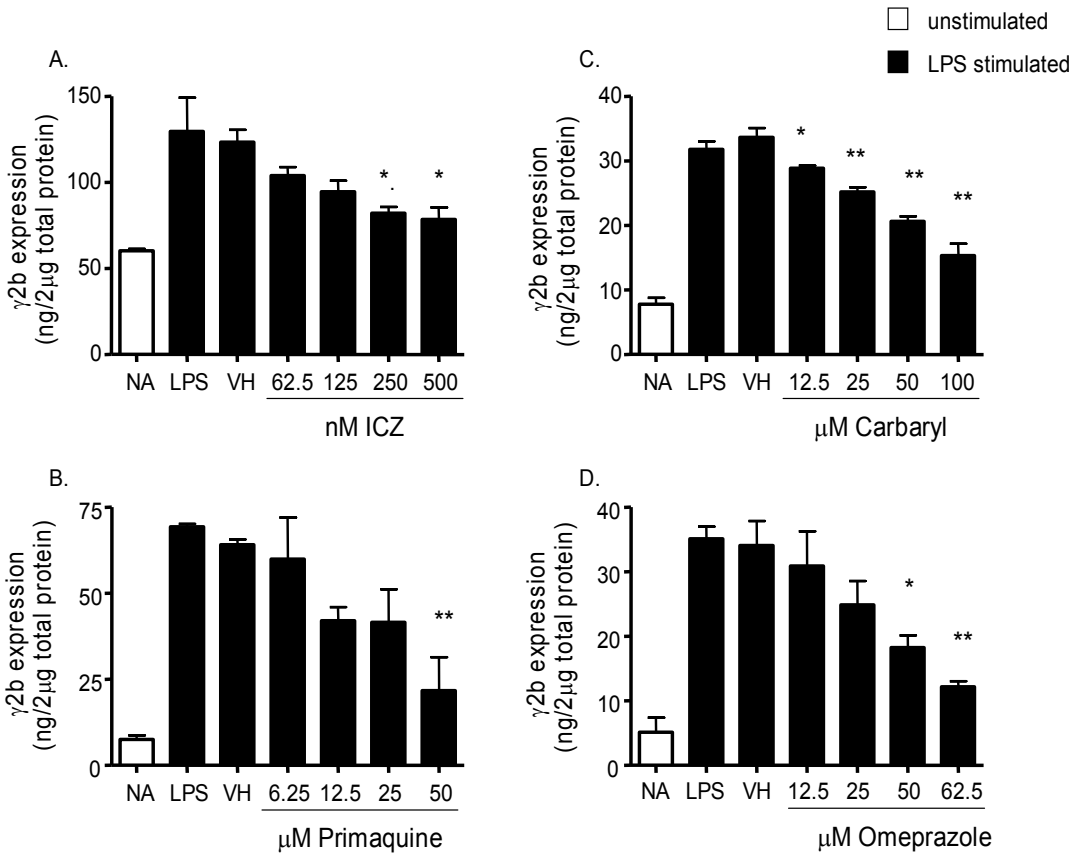
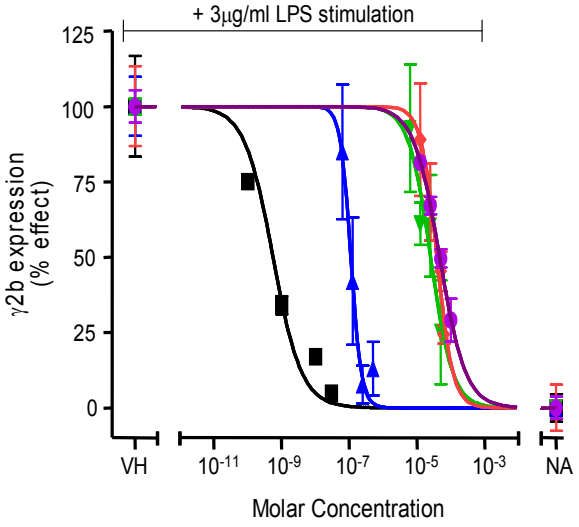


Figure 5. Inhibition of the γ2b transgene by AhR activators. Stably transfected CH12.γ2b-3'IgH cells were treated with vehicle (VH, 0.01% DMSO for ICZ and Primaquine and 0.1% DMSO for Carbaryl and Omeprazole) or varying concentrations of indolo(3,2,b)carbazole (ICZ) (A), Primaquine (B), Carbaryl (C), or Omeprazole (D), in the presence of LPS (3μg/ml) for 48hr. γ2b protein expression in the cell lysate was determined by standard ELISA and standardized to 2μg of total protein. n=3 Significance from VH control at $p<0.05$ (*) or $p<0.01$ (**) was determined by a 1-way ANOVA and Dunnett's post-hoc test.



AhR activator	IC_{50}	95% CI
TCDD	$5.0x10^{-10}$	$1.7x10^{-10}$ to $1.5x10^{-9}$
ICZ	$1.2x10^{-7}$	$9.6x10^{-8}$ to $1.4x10^{-7}$
Carbaryl	$3.8x10^{-5}$	$3.1x10^{-5}$ to $4.6x10^{-5}$
Primaquine	$2.2x10^{-5}$	$1.8x10^{-5}$ to $2.7x10^{-5}$
Omeprazole	$3.8x10^{-5}$	$3.3x10^{-5}$ to $4.5x10^{-5}$

Figure 6. IC_{50} of AhR activators. Percent inhibition was calculated from 3 to 4 separate concentration-response curves for each chemical (one representative curve shown) and fit to a four-parameter logistic concentration-response equation to generate the average IC_{50} for each chemical. NA denotes naïve control.

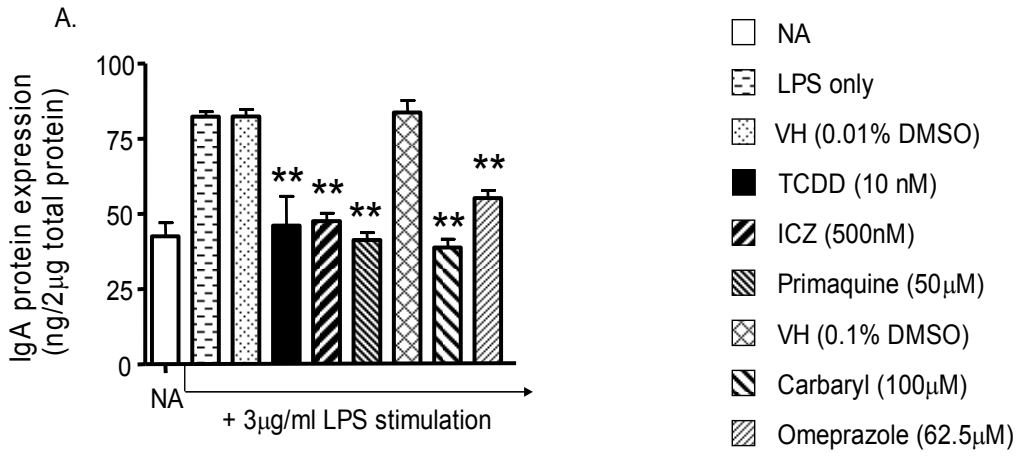


Figure 7. Altered IgA secretion correlated with effects on 3'IgH RR activity. CH12.γ2b-3'IgH cells were treated with vehicle (VH, 0.01% DMSO for ICZ and primaquine or 0.1% DMSO for carbaryl and omeprazole) or specific concentrations of indolo(3,2,b)carbazole (ICZ), primaquine, carbaryl, or omeprazole, in the presence of LPS (3 μg/ml) for 24hr. IgA protein expression in the cell lysate was determined by standard ELISA and standardized to 2μg of total protein. n=3. NA denotes naïve control. Significance from VH control at $p<0.05$ (*) or $p<0.01$ (**).

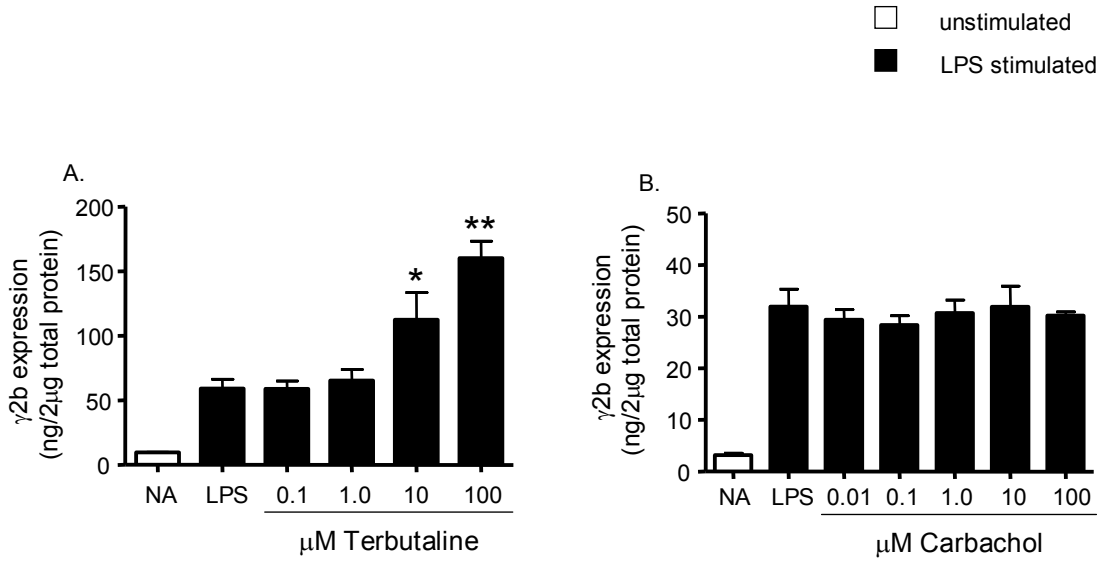


Figure 8. Inhibition of the γ2b transgene by non-AhR ligands. Stably transfected CH12. γ2b-3'IgH cells were treated with varying concentrations of terbutaline (A), carbachol (B) in the presence of LPS (3μg/ml) for 48hr. γ2b protein expression in the cell lysate was determined by standard ELISA and standardized to 2μg of total protein. n=3. Significance from LPS control at $p<0.05$ (*) or $p<0.01$ (**) was determined by a 1-way ANOVA and Dunnett's post-hoc test.

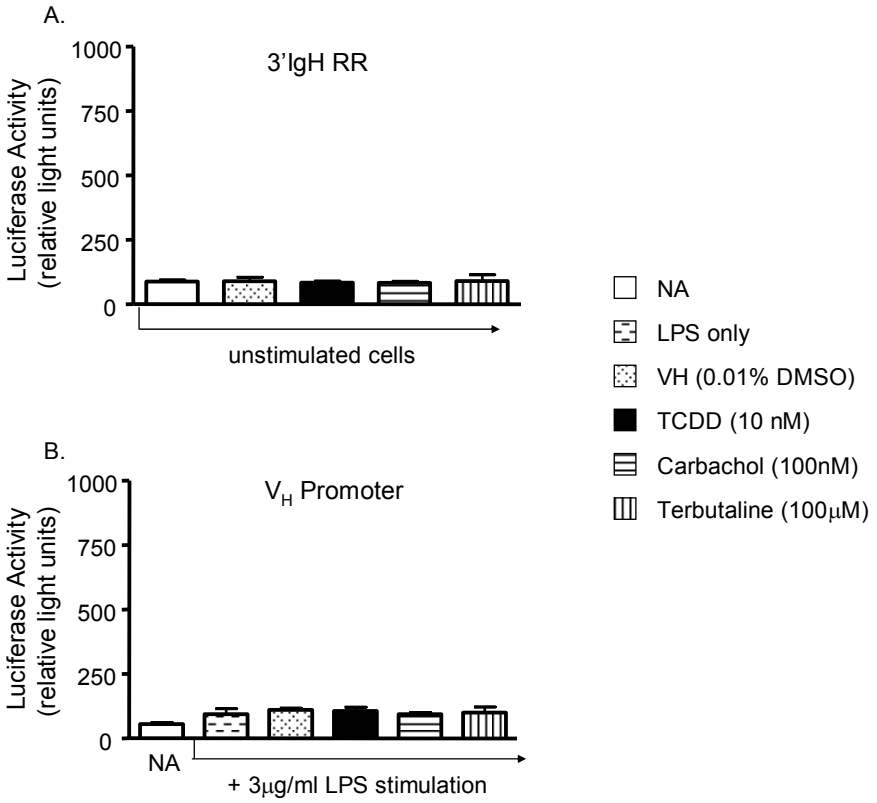


Figure 9. Regulation of the V_H Promoter and the 3'IgH RR. CH12.LX cells were transiently transfected with the 3'IgH RR (A) or the V_H Promoter plasmid (B) then treated with vehicle (VH, 0.01% for TCDD) or specific concentrations of TCDD, terbutaline, or carbachol in the presence or absence of LPS (3 μg/ml) for 48hr. Luciferase enzyme activity is shown on the y-axis as relative light units, n=3. NA denotes naïve control. Significance from NA (VH control for TCDD) (A) or from LPS (LPS+VH control for TCDD) (B) at $p<0.05$ (*) or $p<0.01$ (**) was determined by a 1-way ANOVA and Dunnett's post-hoc test.

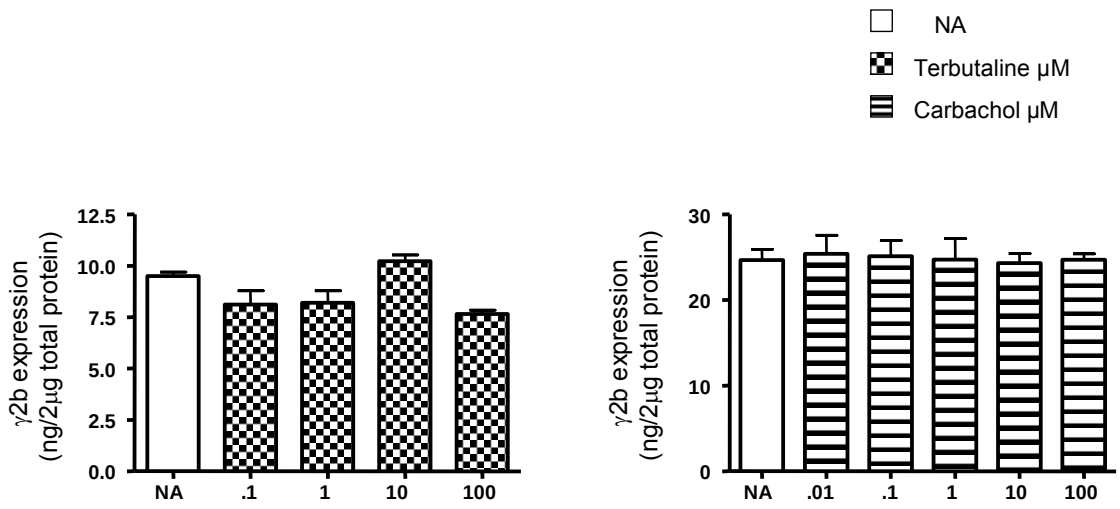


Figure 10. Regulation of the γ2b transgene by Non-AhR ligands in unstimulated CH12.γ2b-3'IgH cells. Stably transfected CH12.γ2b-3'IgH cells were treated with varying concentrations of terbutaline (A), or carbachol (B) in the absence of LPS-stimulation for 48hr. γ2b protein expression in the cell lysate was determined by standard ELISA and standardized to 2µg of total protein. n=3. Significance from NA control at $p<0.05$ (*) or $p<0.01$ (**) was determined by a 1-way ANOVA and Dunnett's post-hoc test.

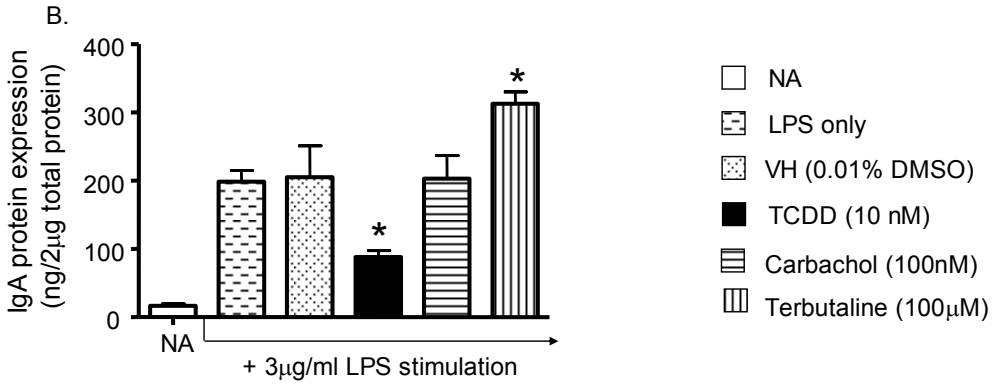


Figure 11. Altered IgA secretion correlated with effects on 3'IgH RR activity. CH12.γ2b-3'IgH cells were treated with vehicle (VH, 0.01% DMSO for TCDD) or specific concentrations TCDD, terbutaline, or carbachol in the presence of LPS (3 μg/ml) for 24hr. IgA protein expression in the cell lysate was determined by standard ELISA and standardized to 2μg of total protein. n=3. NA denotes naïve control. Significance from NA or VH control at $p<0.05$ (*) or $p<0.01$ (**).