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Activation of the Aryl Hydrocarbon Receptor: an Analysis of 2,3,7,8-Tetrachlorodibenzo-p-dioxin Dose-Response on Antigen-Specific Antibody

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A Thesis

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Abstract

The aryl hydrocarbon receptor is a ligand-activated transcription factor of the Per-Arnt-Sim (PAS) family of proteins that regulates cellular functions including immunity. It has been shown that 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), a high affinity ligand for the aryl hydrocarbon receptor (AhR), suppresses immune responses in mice. AhR activation prior to immunization with cholera toxin results in suppression of anti-cholera toxin antibody responses. This is observed as lower immunoglobulin (IgA) levels in both feces and serum 14 days after immunization. Thereafter, cholera toxin (CT)-specific IgA fecal responses recover, where serum CT-specific antibody levels do not; something unexplained by the current paradigm for AhR regulation of immune responses. Because the half-life of TCDD in mice is approximately 7 days, immunosuppressive TCDD levels can persist for multiple weeks. It is unknown if a high dose of TCDD is necessary for the suppression of the fecal IgA response or if lower doses can achieve the same outcome in both feces and serum. The question is: does activation of the aryl hydrocarbon receptor via weekly, low dose exposure to TCDD cause altered IgA/IgG responses equivalent to that observed following a single, high dose exposure? The pertinence of this experiment is to better explain how the aryl hydrocarbon receptor exerts a modifying influence in different body compartments and how the homeostatic pattern of the AhR is related; giving a better understanding of how it may be exploited clinically. This question will be addressed through lab-based experimentation model outlines provided, using female

C57B1/6 mice. Data were collected from immunologically relevant tissues (serum) and feces to ultimately determine levels of IgA and IgG.

This Thesis by: Isabella G. Aspromonte

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has been approved as meeting the requirement for the Degree of Bachelor of Science in the College of Natural and Health Sciences in the Department of Biology

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List of Abbreviations

AhR: Aryl hydrocarbon receptor

AOC: Area under the curve (data analytics)

TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin

CT: Cholera toxin

ELISA:

OD: Optical Density

IgA(G/M): Immunoglobulin A(G/M)

ASC: Antibody secreting cells

@TON: At time of necropsy

mcg(/kg): Micrograms/ micrograms per kilogram

2,3,7,8-Tetrachlorodibenzo-p-dioxin Dosing and Activating the Aryl Hydrocarbon Receptor: an Immunological Approach Assessing Cholera-Toxin Specific Antibody

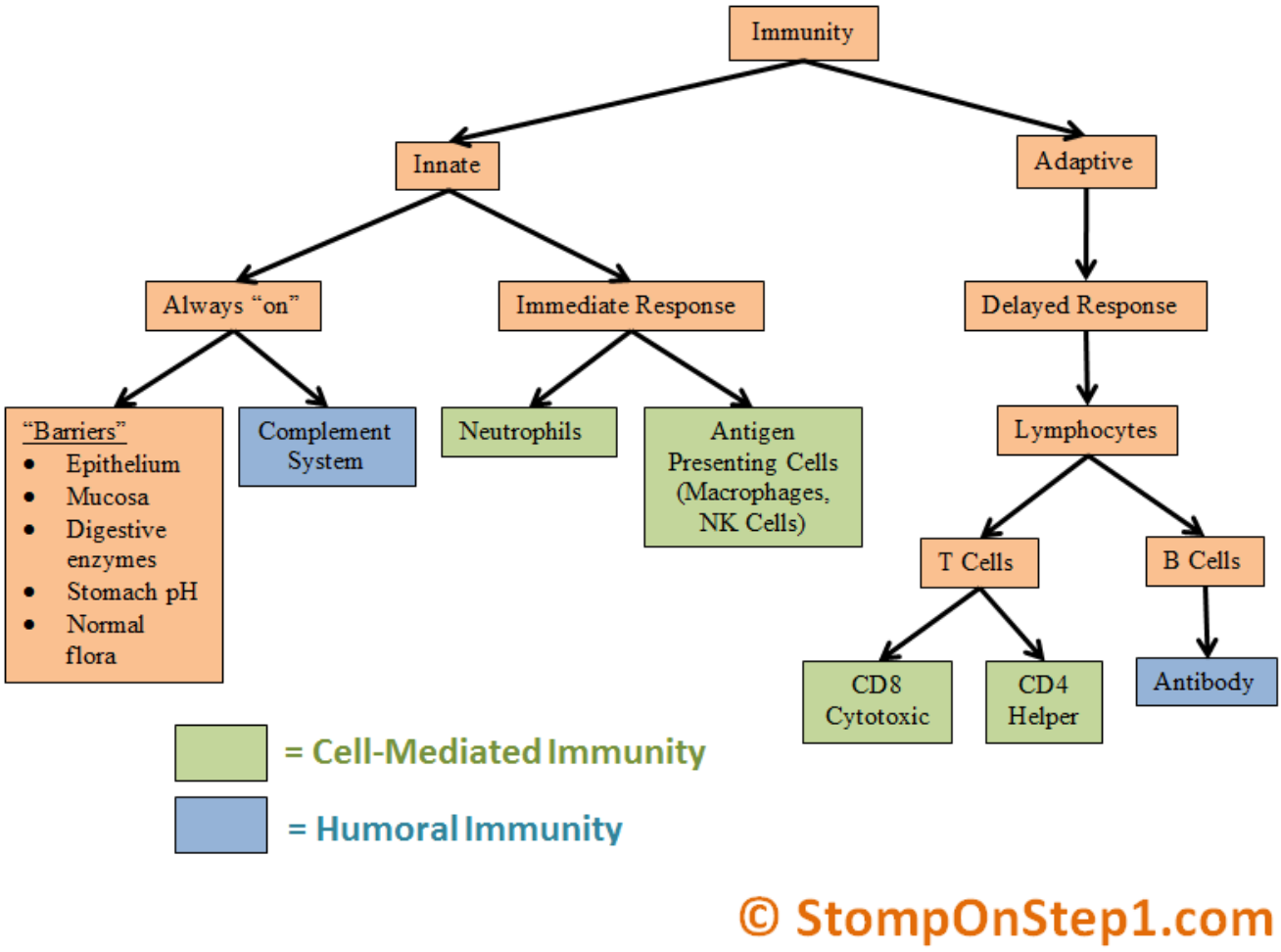
Review of Related Literature

Organization of Immune System

The innate and adaptive immune systems are the two largest parts in the organization of the immune system. The innate immune response is known as the "rapid response" and is the first line of defense against any invader. There are three major components to the innate response, being: barriers, patrolling cells, and soluble defense factors. If the innate immune system is not able to target the invader, the adaptive immune system is activated in addition. The adaptive immune response is the delayed response that has cells to secrete antibodies (plasma cells), coordinate other cells for better defense, and attack self (CTL). There are two major components in adaptive immunity: being humoral and cell-mediated immunity; as exhibited in Figure A. Humoral immunity is antibody mediated and is expressed through B lymphocytes. Its mode of action is antibody secretion and its primary defense is against extracellular pathogens. Cell-mediated immunity is expressed by T-lymphocytes and its mode of action is direct cell-to-cell contact or secreted cytokines. Cell-mediated immunity has a primary defense against intracellular pathogens; something that antibodies are not capable of doing (Alam, 1998). In summary, "mechanisms by which pathogen-specific innate immune recognition activates antigen-specific adaptive immune responses and the roles of different types of innate immune

recognition in host defense from infection and injury" are crucial in the prospect of morbidity and mortality (Iwasaki, 2010).

Figure A



Antibody Secretion via Humoral Immunity

Immunoglobulins/antibodies are glycoproteins secreted as a product of the humoral immune response by B-cells as a way for the body to neutralize and target antigens (Hsu, 2018). Antigens are molecules that initiate an immune response in the body: this can include attachment to threatening pathogens such as viruses and bacteria, transplanted organs or tissues, or even self cells (autoimmunity). During B cell development in the bone marrow, each immature B cell produces an antigen receptor that is unique in its specificity. Attachment of antigen to a naive B cell receptor initiates B cell activation (often with helper T cell). Activated by the binding of an antigen to a specific matching receptor on its surface, the B cell proliferates into a clone via colonal selection of a B cell (Burnet 1957). Following, clonal cells differentiate producing plasma cells and memory B cells. Memory B cells are used for immunologic memory or secondary response. Plasma cells secrete antibodies/immunoglobulin that will bind to the antigen for eventual destruction.

In relation to this project directly, it has been shown that exposure to AhR agonists has disrupted the adaptive immune response. Additionally, for B cells, AhR activation reduces the probability of naive B cell commitment to IgM secretion and suppresses class switch recombination to IgG and IgA isotypes, whereas antibody production

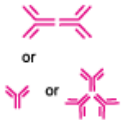
per activated B cell is unchanged, and clonal expansion is only mildly impacted (Yang, 2014). This is supported in **Figure C.** Additionally, the AhR alters expression of factors that control antibody secreting cell (ASC) migration.

Antibodies

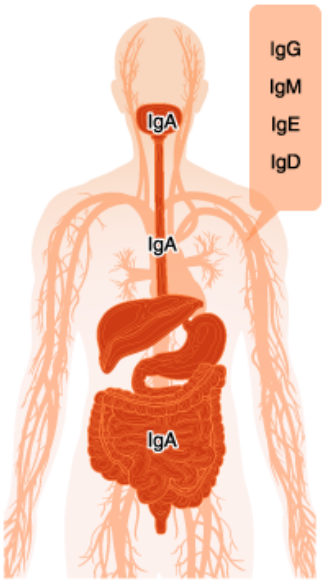
Self and environmental factors can influence how the body's antibody response is initiated and, in turn, how much, and of what antibody isotype is produced more or less in response. Immunoglobulin A (IgA) is the predominant class of antibody in secretions and is found in mucous, saliva, tears, and blood (Parham, 2000). IgA is translocated to mucosal surfaces and is one of the first isotypes in defense against foreign invaders (pathogens) on the outside of the body. Each immunoglobulin isotype (IgA, IgG, IgM, etc.) has defining structures and traits that differ from the next making them most prominent in different areas of the body; which are exhibited in **Figure B**. There are two different isotypes of IgA in humans, being IgA1 and IgA2; IgA1 being found most often in serum, and IgA2 being found most often in secretions (i.e. lumen of GI tract, and eventually, feces). Each antibody is eventually attached to a singular antigen to serve as a chemical signal for the antibody itself, phagocytosis by macrophages, or T-lymphocytes to destroy. Dysregulation of antibody responses is linked to altered disease resistance and insufficient antibody can lead to morbidity and mortality. On the other hand, production of antibody against self can also cause morbidity and mortality. Activation of the aryl hydrocarbon receptor (AhR) alters antibody responses, most often by suppression.

Figure B

Types and characteristics of antibodies

IgG		• Highest opsonization and neutralization activities. • Classified into four subclasses (IgG1, IgG2, IgG3, and IgG4).
IgM		• Produced first upon antigen invasion. Increases transiently.
IgA		• Expressed in mucosal tissues. Forms dimers after secretion.
IgD		• Unknown function.
IgE		• Involved in allergy.

Distribution in the body



The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor is a ligand-activated transcription factor (Wagage, 2014) that is found in most nucleated cells of vertebrates, including mammals (Okey, 2007). It has been suggested that the AhR is essential in multiple bodily processes such as cell growth and proliferation, differentiation, regulation of autoimmunity, inflammation, apoptosis and cancer progression (Moghe, 2013). As well, the AhR has been shown to have a roll in pluripotency and stemness (Roman et all., 2017). Though the AhR is shown to serve a role in immunity, how and why has been a subject to raise questions in the scientific community since its discovery. There have been numerous suggestions of the

AhR's purpose and roll, most of which having to do with mediating toxicity. Relevant literature suggests that the AhR suppresses cholera toxin specific immune response when activated as in the experimental model used here.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

TCDD is a polychlorinated dibenzo-p-dioxin that will be used to activate the aryl hydrocarbon receptor. A number of endogenous and exogenous ligands can activate the AhR; TCDD being the most common exogenous chemical used to activate the AhR. TCDD has a 7-10 day half-life in mice and it has the highest affinity of all AhR activating compounds (Stejskalova et al, 2011). Previously, it has been shown that one large dose of TCDD suppresses a cholera toxin specific IgA response in feces and serum. Figure C exhibits results from previous studies which exposed mice to a single large dose of TCDD and the recovery of the antibody response over time.

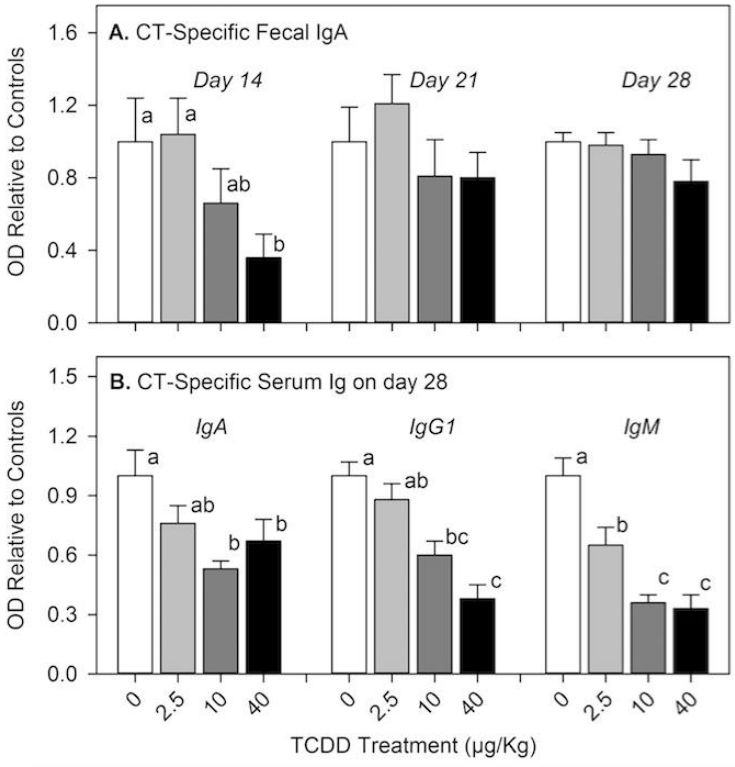


Figure C

Cholera Toxin

Cholera toxin is a complex of two units produced by the pathogen *Vibrio cholerae* and has been found to serve usefully in biology experimentation. It has been found that either alone or as an adjuvant for use with other antigens, cholera toxin exhibits a wide variety of application across many experimental fields (Wessling, 2017). Cholera toxin is comprised of varying units in its complex structure, the toxic "A" subunit causing mobility and mortality, where "B" protein subunits have been found to bind to the surface of mammalian target cells. Cholera toxin is unique in that it serves as both an antigen and

adjuvant. An adjuvant non-specifically enhances immune response, which serves usefully in experimental immunology. It has been shown that cholera toxin immunization "strongly potentiates the immunogenicity of most antigens" (Bharati, 2011).

Dosage

Dose is often determined based off of the half-life of a drug (or the time that concentration of a given substance decreases by half). It is this determination of dose that affects the behavior of a drug and the response to a drug. If dose is changed, toxicity, activity, and morbidity and mortality rates will also change. If it is assumed that dosage changes the toxicity and action of a drug, it can be predicted that varying doses will change the reaction seen, such as IgA and IgG levels (which will help determine the effects from activation of the AhR).. It has been shown in previous experimentation that one, large dose (40 mcg/Kg concentration) of TCDD followed one day later with the first of four oral cholera toxin immunizations results in suppressed levels of CT-specific IgA. conjunction with an oral cholera toxin immunization model results in suppressed levels of CT-specific IgA. It then comes in to question if smaller doses, more frequently, using the same cholera toxin immunization regimen will produce a similar response.

Research Question

Does activation of the aryl receptor via weekly, low dose exposure to TCDD cause altered cholera-toxin specific IgA/IgG responses equivalent to that observed after a single, high dose exposure?

Hypothesis

- Weekly low dose exposure to TCDD causes altered cholera-toxin specific IgA/IgG responses equivalent to that observed after a single high dose exposure.
- Prediction 1: Weekly low dose exposure to TCDD will initially cause suppression of serum and fecal CT-specific antibody levels equivalent to that seen after a single high dose exposure to TCDD.
- Prediction 2: Recovery of CT-specific fecal antibody levels, but not serum antibody levels, will occur by four weeks after weekly low dose exposure to TCDD and single high dose exposure.

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Northern Colorado. All efforts were made to minimize suffering.

Animals, immunizations, and treatments

The animals used were selected from the University of Northern Colorado animal facility. Female C57 mice between 6 and 8 weeks of age at the time of initial treatment

were used. 10 mice in total were used for each of the two trials of the experiment (3-4 animals per treatment group). Each mouse was assigned an independent cage with standard food and water schedules maintained. Cholera Toxin immunization and TCDD treatments were administered by Dr. Gregory DeKrey via gavage according to the time line attached. Animals were given peanut oil (Group 1) or TCDD as either three treatments (Group 2, 20 mcg/Kg total dose) or a single treatment (Group 3, 40 mcg/Kg total dose).

Weights were taken and recorded twelve times through the experiment as shown. Through both trials, there were no notable changes in weights in any of the mice (that would represent extreme stress and TCDD toxicity).

Sample processing and analysis

Fecal pellets were collected fresh after production by a mouse and lyophilized. All animals were euthanized via CO2 overdose and serum was collected directly from the heart. Serum was separated from whole blood via centrifugation. Lyophilized fecal pellets were extracted with saline. Both serum and fecal extracts were analyzed for levels of antibodies using an ELISA method as described by Lycke et al. (1999). Data at each dilution were collected as optical density (OD) and used to compute areas under the curve (AUC) values for each animal. Data are shown for samples collected three weeks after the initial treatment.

Experimental Schedule

Day	Group	Treatment	Obtained
-1	All groups assigned treatment groups. And individual numbers. Heaviest and lightest mouse placed in control group.	Individual cages chosen for each mouse, placed in one area in animal facility to prevent bias. Cages labelled with mouse # and treatment group.	Weights obtained, recorded in grams.
0	Control	Peanut oil administration based off weight.	Weights obtained.
0	Group 1	TCDD in concentration 1mcg/mL given based off of weight	Weights obtained.
0	Group 2	TCDD in concentration 4mcg/mL given based off of weight	Weights obtained.
1	All groups	Cholera Toxin given based off weights	Weights obtained
7	Control	Peanut oil administration based off weight.	Weights obtained
7	Group 1	TCDD in concentration 1mcg/mL given based off of weight	Weights obtained
7	Group 2	Peanut oil administration based off weight.	Weights obtained
8	All groups	Cholera Toxin given based off weights	Feces collected, frozen for future analysis.
14	Control	Peanut oil administration based off weight.	Weights obtained

Day	Group	Treatment	Obtained
14	Group 1	TCDD in concentration 1mcg/mL given based off of weight	Weights obtained
14	Group 2	Peanut oil administration based off weight.	Weights obtained
15	All groups	Cholera Toxin given based off weights	Feces collected, frozen for future analysis.
21	All groups	Necropsy	Weights obtained, Feces collected & frozen. Serum collected and frozen.

Preliminary Data:

Experiment 1 (averages only)

Group	Control P.O.	Group 1 (1mcg/kg)	Group 2 (4mcg/kg)
AUC average serum	7.9495	8.0138	8.0173
AUC average feces	1.8675	0.4046	0.8076

Experiment 2 (averages only)

Group	Control P.O.	Group 1 (1mcg/kg)	Group 2 (4mcg/kg)
AUC average serum	6.3406	0.9141	1.1180
AUC average feces	1.8852	0.1314	0.2643

Discussion

The AhR exerts a modifying force on immune function when activated, suppressing antibody. Suppression of antibody is useful in clinical application in instances such as: autoimmunity, graft, and organ rejection. For this reason, hypotheses included principles of toxicology and pharmacology. Data from experiment 1 and 2 show similarities in terms of fecal antibody, as predicted from previous literature. It is shown that fecal antibody levels are consistently suppressed in both experiments, and there does not seem to be an obvious difference between TCDD groups. These data support the hypothesis that weekly low dose exposure to TCDD causes altered cholera-toxin specific IgA/IgG responses equivalent to that observed after a single high dose exposure. As the current pharmaceutical therapy model encourages small, frequent dosage times calculated based off of half life, this model will directly translate in to research on AhR activation. Though

neither TCDD groups exhibit toxicity in the 21 day experimental period, long term toxicity of a large dose model is possible, something to consider with the activation of the AhR. It is unknown whether a small dose model lowers the probability for toxicity; however, given the trend of medicating, it is predicted that a small dose approach will be used. It was shown through these data that weekly low dose exposure to TCDD will initially case suppression of serum and fecal CT-specific antibody levels equivalent to that seen after a single high dose exposure to TCDD.

In previous research, it has been shown that fecal antibody levels can be suppressed via activation of the AhR; however, serum antibody levels do not respond in the same way as fecal antibody levels, regardless of their similar isotype population. This suggests that the activation of the AhR has differing affects throughout the body. In previous experimentation, it has been shown that we can suppress serum antibody levels similarly to fecal antibody levels, however, these levels to do not recover in the 21 day experimental window, as fecal antibody levels do. It was hypothesized and observed successfully that recovery of CT-specific fecal antibody levels, but not serum antibody levels, occurred by four weeks after weekly low dose exposure to TCDD and single high dose exposure. The serum antibody levels differ from experiment 1 to 2, unlike fecal values. The data from experiment 1 shows no suppression of serum levels, but more obvious suppression in the second experiment. It is consistent that in experiment 1 and 2

that serum antibody levels do not recover in the 4 week period, validating the second prediction. Serum, across experiments, shows a more unpredictable suppression of antibody levels than feces.

Through this experiment it has been shown that the activation of the AhR via TCDD in varying doses exudes a similar response, and recovers predictably in feces. Further, it can be predicted that this same suppression is happening in serum, but, without recovery in the 21 day period, and varying between trails.

Future Studies

While the hypotheses were correct, consistent serum data will need to be produced and compared to further analyze clinical application. Additionally, a post antigen challenge model must be presented. These two ideas will give a better understanding of AhR activation throughout the body, and how those recover. A post antigen challenge model will also then be able to address if the AhR activation is similar in both sick (post antigen) and healthy (pre antigen) animals/ patients.

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