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Stephen R. Hennigar  
*University of New Hampshire, Durham*

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EXPOSURE TO POLYBROMINATED DIPHENYL ETHERS (PBDEs) SUPPRESSES  
THE RELEASE OF PRO-INFLAMMATORY PRODUCTS BY ALVEOLAR  
MACROPHAGES *IN VITRO*

BY

STEPHEN R. HENNIGAR  
B.S., University of New Hampshire, 2008

THESIS

Submitted to the University of New Hampshire  
in Partial Fulfillment of  
the Requirements for the Degree of

Master of Science  
in  
Nutritional Sciences

May, 2010

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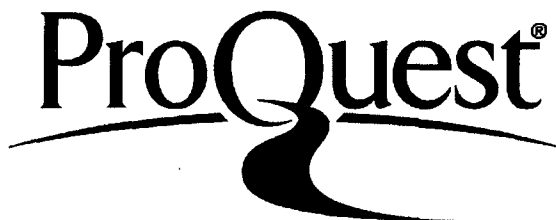
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Thesis Director, Anthony R. Tagliaferro, Ph.D.,  
Professor of Nutritional Sciences

---

Gale B. Carey, Ph.D.,  
Professor of Biology and Nutritional Sciences

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James P. McClung, Ph.D.,  
Nutritional Biochemist, U.S. Army Research  
Institute of Environmental Medicine

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Date

## DEDICATION

For my grandfather, Abraham Russel Taylor, who was instrumental in shaping me into the person I am today and who instilled in me the importance of always carrying *A Bag of Tools*.

### A BAG OF TOOLS

*Isn't it strange  
That princes and kings,  
And clowns that caper  
In sawdust rings,  
And common people  
Like you and me  
Are builders for eternity?*

*Each is given a bag of tools,  
A shapeless mass  
A book of rules;  
And each must make –  
Ere life is flown –  
A stumbling block  
Or a stepping stone.*

**R.L. SHARPE**

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## ABSTRACT

EXPOSURE TO POLYBROMINATED DIPHENYL ETHERS (PBDEs)  
SUPPRESSES THE RELEASE OF PRO-INFLAMMATORY PRODUCTS BY  
ALVEOLAR MACROPHAGES *IN VITRO*

by

Stephen R. Hennigar

University of New Hampshire, May, 2010

Endocrine disrupting chemicals have adverse effects on immune function that may result in respiratory conditions. Inhalation of dust is a major route of exposure to PBDEs; however, the impact of PBDEs on the immune response is unclear. The objective of this *in vitro* study was to determine the impact of PBDEs on the release of pro-inflammatory cytokines by activated alveolar macrophages. Porcine alveolar macrophages were grown in RPMI growth media supplemented with 10% porcine serum and incubated for 24-hours. After 24-hours, cells were activated by inoculation with PMA. In addition to PMA, different concentrations of the PBDE mixture DE-71 were introduced to the wells. After 6-hour incubation, conditioned media was removed and analyzed. Cells exposed to PMA and PBDEs released significantly less pro-inflammatory cytokines compared to controls. Suppression of pro-inflammatory cytokines-characteristic of a compromised immune system- suggests that persistent exposure to PBDEs may increase the susceptibility to respiratory conditions.

## CHAPTER I

### LITERATURE REVIEW

#### Preface

The purpose of this literature review is to examine the growing evidence that certain classes of synthetic chemicals are biologically active and may have subtle yet profound effects on the health of humans and wildlife. The literature review will begin with a brief discussion of environmental pollutants, specifically chemicals that have been shown to disrupt the endocrine system (i.e. endocrine disruptors). In doing so, I will focus on chemicals found to mimic the hormone estrogen (i.e. xenoestrogens). A brief history of some common xenoestrogens, their origins, and some of the plausible mechanisms of action related to health will be addressed. The review will then focus on one class of environmental pollutants and suspected xenoestrogen, polybrominated diphenyl ethers (PBDEs) – their properties, prevalence, routes of exposure, and health consequences. Lastly, a brief review of innate immunity as it relates to airway health will be presented. The possible interaction between PBDEs and innate immunity, specifically as it relates to alveolar macrophage function, will be the focus of the study reported in Chapter II.

#### Environmental Pollutants

Manufacturers have saturated the environment with synthetic chemicals since the Industrial Revolution. Some of the earliest and most well known chemicals manufactured included bisphenol A (BPA), originally used as a synthetic estrogen in the early 1920s

and now used in plastics; polychlorinated biphenyls (PCBs), introduced in 1929 and used in electrical equipment as well as many other products; and dichlorodiphenyltrichloroethane (DDT), introduced as a pesticide in 1938. Post World War II, synthetic chemical production increased exponentially, when companies previously manufacturing chemicals used for warfare, began the wide-scale manufacture of common domestic products (1). Currently, synthetic chemical production is at an all time high. Tens of thousands of chemicals are used in the manufacture of common products such as dyes, flame retardants, flavorings, medicines, perfumes, pesticides, pigments, plastics, plasticizers, resins, solvents, and countless others products (2). The U.S. Environmental Protection Agency (EPA) estimates that 87,000 chemicals are in use today, amounting to billions of pounds of chemicals produced annually (1). Although many of these chemicals were developed with good intentions and some are important for safety, they have recently been implicated with many unintended health risks.

In 1991, a group of scientists convened in Racine, Wisconsin to discuss the growing body of evidence that suggests many of these man-made chemicals have the potential to disrupt the endocrine system. The outcome of the conference, and what is now considered a monumental document, was the *Wingspread Consensus Statement*. Chaired by Dr. Theo Colborn, the group appropriately coined these synthetic chemicals “endocrine disruptors” (3). An endocrine disruptor is defined by the U.S. EPA as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process.” Diamanti-Kandarakis et al. (2009), extend this definition to say “from a physiological perspective,



an endocrine-disrupting substance is a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment” (4).

The endocrine system consists of glands (**Figure 1**), hormone-secreting cells, and target cells, all of which regulate important biological processes such as metabolism, growth, reproduction, and many other functions through the production and release of chemical messengers, called hormones. A hormone is a protein, steroid, amine, or eicosanoid, secreted by a cell that travels to a target cell to elicit a specific biological response via a receptor.

The glands of the endocrine system and the hormones they release influence the function of many biological processes. Other systems (i.e. nervous and immune systems) use different chemical messengers such as neurosecretions and cytokines respectively, to communicate. Hormones and chemical messengers allow for cross-talk to occur between these integrating systems (**Figure 2**). A wide range of hormonally-regulated processes such as those shown in **Figure 3** are therefore vulnerable to the subtle disruption of hormone mimicking chemicals (4).

Endocrine disruptors mimic or interfere with endogenous signaling pathways and can therefore disrupt receptor signaling. For example, certain chemicals act like the hormone estrogen (i.e. xenoestrogens). Xenoestrogens are capable of interfering with estrogen signaling in many different ways. Endocrine disruptors (including xenoestrogens) have the ability to bind directly to specific hormone receptors (i.e. estrogen, androgen, and thyroid hormone receptors) and affect hormone signaling; or

they can bind to orphan receptors, such as the aryl hydrocarbon receptor (AhR) or the steroid and xenobiotic receptor (SXR) and indirectly affect hormone signaling (5). Once bound, endocrine disruptors can act as agonists and mimic the action of a naturally occurring hormone (e.g. estrogens, androgens, and thyroid hormone) and its function; or act as antagonists and block the endogenous hormone from binding (e.g. anti-estrogens such as tamoxifen). To further complicate matters, most endocrine disruptors interfere with multiple hormone signaling pathways. For example, DDT has been shown to interact with estrogen and androgen receptors (5). Because of these capabilities, as well as the complex interactions of the endocrine system with many other systems of the body, endocrine disruptors have the ability to subversively affect health in a number of ways.

DDT was first made in 1873; however, it was not until 1938 that a Swiss chemist recognized its effectiveness as an insecticide. Incidentally, Dr. Paul Muller went on to win the Nobel Prize for the discovery in 1948. Due to its effectiveness, the production and use of DDT increased greatly in the 1940s. Shortly thereafter, it was discovered that DDT impacted the sexual development of birds; and later reports in humans indicated an increased risk of cancers, reproductive effects, and neurological toxicity (6). As a result, DDT was banned in the U.S. in 1972. It still, however, continues to be used in other parts of the world and continues to persist in the environment decades after the U.S. ban.

The wide-scale production of PCBs is another example of an endocrine disrupting chemical shown to have adverse effects on health. PCBs are flame retardant, chemically stable, have a high boiling point, and have electrical insulating properties. They are therefore found in a number of products including: electrical, heat transfer, and hydraulic equipment, plasticizers in paints, plastics, and rubber products, pigments, dyes, and

carbonless copy paper, as well as many other products (7). PCBs disrupt endocrine signaling by interacting with the AhR (8) as well as the thyroid hormone receptor (4). Studies have indicated that PCBs adversely affect the reproductive system (e.g. reduced birth rate, conception rates, and live birth rates in monkeys and other animals, and reduced sperm counts in rats), the nervous system (e.g. deficits in neurological development), the endocrine system (e.g. thyroid hormone disruption), and the immune system (e.g. reduced immune response, decreased resistance to infection, and an increased susceptibility to pneumonia and viral infections) (6, 7). They have also been shown to cause cancer in both animals and humans, although some suggest that cancer is a result of the PCB-induced suppression of the immune system (6). Due to their toxic effects on health, PCBs were banned from manufacture in the U.S. in 1979. Like most endocrine disruptors, however, PCBs were developed to have an exceptionally long half-life. So similar to DDT, PCBs persist in the environment decades after being banned.

Since the Wingspread Conference numerous other chemicals found in many of the products that we, as humans encounter throughout our everyday lives have emerged as endocrine disruptors. **Table 1** includes main groups of endocrine disrupting chemicals and some examples of each. Examples include but are not limited to synthetic industrial chemicals [dioxins, polybrominated diphenyl ethers (PBDEs), PCBs], plastics [bisphenol A (BPA)], plasticizers (phthalates), insecticides (carbaryl, DDT, dieldrin), fungicides (hexachlorobenzene), and pharmaceutical agents [diethylstilbestrol (DES)].

### **Polybrominated Diphenyl Ethers (PBDEs)**

Polybrominated diphenyl ethers (PBDEs) are a class of synthetic chemicals that are used extensively as flame retardants in numerous household and commercial products

including plastics in electrical appliances, televisions, and computers; and foams, carpets, and upholstery found in furniture, automobiles, and airplanes (9). PBDEs are added to these products to reduce their flammability and decrease the burning rate, so that in the event of a fire there is increased time to escape. Although designed to save lives, evidence is mounting that PBDEs may be doing more harm than good.

Unlike the long banned PCBs and dichlorodiphenyltrichloroethane (DDT), there is continued widespread production and use of PBDEs. Consequently, while the levels of PCBs and DDT have decreased since being banned, the levels of PBDEs found in human blood, milk, and tissue have increased exponentially (10) – with levels doubling every 3-5 years (11). Because PBDEs have a similar structure and many of the same properties of DDT and PCBs (**Figure 4**), there is reason to believe that exposure to PBDEs may result in similar health complications associated with these previously banned chemicals.

PBDEs consist of two phenyl rings joined by an ether linkage; differing in the number and position of the bromine atoms attached to the rings (**Figure 4A**). There are 209 possible configurations of PBDEs, and these congeners are grouped according to the number of bromine atoms in the molecule (i.e. 4-10). PBDEs are commercially developed as one of three formulations, named for the number of bromine atoms they contain: the penta- formulation, commercially known as DE-71 and Bromkal 70-5DE; the octa- formulation, commercially known as DE-79; and decaBDEs, commercially known as DE-83R (12). PentaBDEs are primarily composed of penta- congeners (50-62% by weight); octaBDEs are made up of hepta- (45%) and octa- (33%) congeners; and decaBDEs consists of BDE-209 (97-99%) (12). **Table 2** lists some of the major PBDE congeners and mixtures. It is important to note that five of the 209 different congeners

account for 90% of the PBDEs found in human tissues (BDE-47, -99, -100, -153, -154) (13) and that collectively these congeners make up the PBDE mixture, DE-71.

Furthermore, it is suspected that the pentaBDEs are the major contributor to the burden of PBDEs in both the environment and humans (12).

The penta- and octaBDEs are more readily absorbed, more bioaccumulative, more bioactive, and are eliminated more slowly compared to the decaBDEs. The penta- and octaBDEs were first reported to be biologically active as neurotoxins and endocrine disruptors in laboratory animals (14). As a result, production of these congeners is banned in the European Union (EU) and several states in the U.S. The decaBDEs however, are still produced and used globally [according to Inventory Update Reports (IUR) between 50-100 million pounds were manufactured or imported in the U.S. in 2005 – the same as was reported in 2002 (15)]. Because PBDEs are not fixed in the polymer product by chemical bonding, bromine atoms are lost spontaneously over the lifetime of the product. Thus, over time, the more highly brominated decaBDEs can break down by photolytic or biological mechanisms and form the lower brominated, more bioactive and bioaccumulative PBDE congeners (12, 15, 16).

PBDEs are added to a wide variety of consumer products. PentaBDEs are most often found in polyurethane foam such as that used in couches, chairs, and automobile seats. OctaBDEs are used in televisions, computers, and other small appliances such as telephones and electronics; and decaBDEs are also used in plastics for consumer electronics, as well as plastic furniture and toys, wire insulation, and textiles such as upholstery. For reasons cited earlier, these products leak bromine atoms into ambient air with continued use and wear and tear over time.

Due to the ubiquity and persistence of PBDEs in nature, they are ingested regularly and accumulate readily in lipids of body tissues. They have been detected in indoor and outdoor air, dust, soil, streams and lakes and tissues of coldwater fish and domestic animals. They have also been found in the adipose, serum, and breast milk of humans (17). One source of exposure to PBDEs is food. Typically, animal-based foods contain higher concentrations of PBDEs than plant-based foods (18). Fish, meat, and dairy products contain the highest concentrations of PBDEs (19, 20). Due to its popularity in the Westernized diet, meat is suspected to be the major dietary source of PBDEs in the U.S. (20). Interestingly, a recent study showed that vegetarians have lower concentrations of PBDEs compared to omnivores and that serum PBDE concentrations increased in individuals who reported consuming a low, medium, and high amount of poultry and red-meat (21).

The levels of PBDEs found in human tissues in North Americans are of major concern, with levels one to two orders of magnitude higher than those detected in Europe and Japan (16). Lorber (2008), reports that similar levels of PBDEs are found in food while higher levels are found in soil, house dust, and air in the U.S. compared to abroad (12). In fact, recent evidence shows that inadvertent exposure to dust contributes more to the total body burden of PBDEs than diet (22). Furthermore, in a recent study it was estimated that 82% of total PBDE intake was obtained by inhalation of house dust (12, 23).

PBDEs are lipophilic and accumulate readily in adipose tissue and breast milk of nursing mothers (22). **Table 3** shows representative serum levels of PBDEs from a family from Northern California in 2004. Due to chronic PBDE exposure to the fetus via the

maternal circulation during pregnancy, to nursing infants via breast milk, and to children via house dust, infants and children have a particularly high body burden of PBDEs, reported as 3-4 times higher than adults (16). Moreover, exposure of fetuses, infants, and children to PBDEs during critical times of development raises increasing health concerns.

A growing body of literature suggests that PBDEs may be implicated in a number of potential health complications. Studies have linked PBDE exposure to altered function of thyroid, liver, and nervous systems, as well as impairment of behavioral development, disruption of reproductive hormones, and alterations in immune function. For example, PBDEs reported to lower thyroid hormone in animals and act as either agonists or antagonists at the androgen, progesterone, and estrogen receptors (17).

Studies documenting the effects of PBDEs on immune function are limited. In 2005, Reistad and Mariussen found that *in vitro* exposure of human neutrophil granulocytes to the DE-71 mixture induced respiratory burst (24). Fernie et al. (2005) exposed nestling American kestrels to pentaBDE congeners (-47, -99, -100, -153) and assessed the T-cell mediated-immune response via phytohemagglutinin (PHA) skin response test and humoral-immunity by measuring total plasma antibody (IgM + IgG) titers. PBDE-exposed birds showed an initial stimulation in the T-cell response that was suppressed as the concentrations of BDE-47 increased. Humoral immunity was also compromised, as kestrels exposed to PBDEs showed suppressed antibody titers. Researchers also saw fewer germinal centers in the spleen, reduced apoptosis in the bursa, and increased macrophages in the thymus. Collectively, these data suggested that exposure to PBDEs could result in an inadequate immune response to viruses and other potentially harmful pathogens (25). More recently, Lundgren et al. (2009) examined the

effects of PBDEs on cytokine responses in virus-infected mice. They found a suppression of cytokine [interleukin-13 (IL-13) and interferon- $\gamma$  (IFN- $\gamma$ )] and chemokine [macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ), regulated upon activation, normal T-cell expressed and presumably secreted (RANTES), and keratinocyte chemoattractant (KC)] levels in the serum of non-infected mice exposed to PBDEs, indicating that PBDEs may block immune signaling (26).

Because PBDEs have been implicated as xenoestrogens, their influence on immune function may be explained, in part, by the effects estrogen has on the immune system. Whether PBDEs are having similar effects on immune function and whether PBDEs are binding to the estrogen receptor to exert these effects remains to be determined. Due to limited studies examining the effects of PBDEs on the immune system and the conflicting results of the few studies conducted to date - more research in this area is warranted.

### **Immune Function**

The immune system is an intricate network of specialized cells dedicated to defending the body against foreign pathogens (**Figure 5**). A normally functioning immune system is crucial to survival. Infection occurs when the host encounters a pathogen that invades and infects the host's respiratory mucosal tissues.

**Figure 6** is an overview of the immune defense mechanisms against respiratory pathogens. Once the pathogen invades the cells, the innate immune response is initiated. Antigen presenting cells such as macrophages and dendritic cells are activated and produce an array of cytokines. For example, interferon- $\alpha/\beta$  (IFN- $\alpha/\beta$ ) is secreted and prevents viral replication. Also, classically-activated macrophages secrete the pro-



inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ), that induce the acute phase inflammatory (innate) response. The latter includes an increase in complement proteins, extravasation of leukocytes, and antigen presentation. Dendritic cells secrete interleukin-12 (IL-12), leading to the differentiation of naïve T<sub>Helper</sub> (T<sub>H</sub>) cells into T<sub>H1</sub> cells.

T<sub>H</sub> cells regulate the development of acquired immunity toward either a T<sub>H1</sub> or T<sub>H2</sub> pathway. T<sub>H1</sub> cells secrete interleukin-2 (IL-2) and IFN- $\gamma$ . IL-2 encourages the activation of cytotoxic T-cells, which travel to the infected area. Cytotoxic T-cells recognize antigen presented by major histocompatibility complex (MHC) class I molecules, which upon binding lead to cell death by apoptosis. Similarly, IFN- $\gamma$  activates natural killer (NK) cells to increase cell death by apoptosis. Macrophages then phagocytose and clean up the apoptotic cells and the intracellular antigen can then be cross-presented to cytotoxic T-cells.

Naïve T<sub>H</sub> cells also differentiate into T<sub>H2</sub> cells that secrete the anti-inflammatory cytokines interleukin-10 (IL-10) and interleukin-4 (IL-4). IL-10 suppresses the pro-inflammatory cytokines, while IL-4 induces the differentiation of B cells into plasma and memory B cells. Plasma cells secrete specific antibodies that bind infected cells and lead to apoptosis.

Recent evidence suggests that macrophages in the lung may be important modulators of immunity, directing T<sub>H</sub> cells toward either a T<sub>H1</sub> or T<sub>H2</sub> response (27). Macrophages are sentinel cells that respond to foreign antigens by secreting inflammatory cytokines and eicosanoids. Cytokines and eicosanoids are produced by the macrophage in response to external stimuli and influence other cells by binding to a

specific receptor on their surface. The major pro-inflammatory cytokines produced by macrophages include TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and the eicosanoid prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). In addition, macrophages secrete the anti-inflammatory cytokine, IL-10.

Pro-inflammatory cytokines are produced through the activation of the transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B). In the activated form, NF- $\kappa$ B is a heterodimer consisting of two proteins, a p65 subunit and a p50 subunit. In unstimulated cells, NF- $\kappa$ B is located in the cytoplasm bound to an inhibitory protein, I $\kappa$ -B that prevents it from entering the nucleus. Upon activation, I $\kappa$ -B kinase (IKK) phosphorylates I $\kappa$ -B, resulting in degradation of the inhibitory protein and allowing NF- $\kappa$ B to translocate to the nucleus to initiate the transcription of various cytokine genes. Many stimuli have been shown to activate NF- $\kappa$ B including cytokines such as TNF- $\alpha$  and protein kinase C activators such as PMA, a phorbol ester, and lipopolysaccharide (LPS), a bacterial endotoxin (28).

In healthy tissue, the macrophage is in a resting state. Once a pathogen is encountered, the macrophage initiates the inflammation process, activating and secreting pro-inflammatory cytokines. The release of cytokines results in vasodilation and increased vascular permeability that allows fluid, protein, and inflammatory cells to leave the blood stream and enter the tissue. As a result, the infected tissue becomes inflamed, causing the characteristic redness, heat, swelling, and pain associated with infection – a response that is necessary in order to resolve the infection.

Through the production of a multitude of different chemical signals and through the expression of a number of different receptors, macrophages have the potential to communicate via a number of different pathways. For example, macrophages express the

estrogen receptor and have been shown to be regulated by estrogen (29). Furthermore, estrogen has an inhibitory effect on TNF- $\alpha$  and other cytokines in many different cell types, such as human promonocytic cells, rat microglia, and mouse dendritic cells (reviewed by (29)). In this review Straub (2007), claims that these results are most likely due to inhibition of NF- $\kappa$ B (29). Ghisletti et al. (2005), provide evidence that the inhibitory effects of estrogen on NF- $\kappa$ B are mediated by blocking the binding and transcriptional activity of p65 and preventing nuclear translocation (29, 30). These latter findings suggest activation of macrophages and initiation of the initiation of inflammatory signals are modulated by estrogen. Whether similar effects on macrophage function occur in response to PBDEs, a suspected xenoestrogen, remains to be determined.

## CHAPTER II

### EXPOSURE TO POLYBROMINATED DIPHENYL ETHERS (PBDEs) SUPPRESSES THE RELEASE OF PRO-INFLAMMATORY PRODUCTS BY ALVEOLAR MACROPHAGES *IN VITRO*

#### Abstract

Endocrine disrupting chemicals have adverse effects on immune function that may result in respiratory conditions. Inhalation of dust is a major route of exposure to one endocrine disrupting chemical and xenoestrogen, polybrominated diphenyl ethers (PBDEs). The impact of PBDEs on the immune response, however, is unclear. The objective of this *in vitro* study was to determine the impact of PBDEs on the release of pro-inflammatory cytokines and eicosanoids by activated alveolar macrophages and to determine whether the effects are estrogen-receptor dependent. Porcine alveolar macrophages were grown in RPMI growth media supplemented with 10% porcine serum and incubated for 24-hours (5% CO<sub>2</sub>, 37°C). After 24-hours, cells were activated by inoculation with phorbol 12-myristate 13-acetate (PMA) (0.01 mg/ml) and ionomycin (0.05 mg/ml) in 1% DMSO. In addition to PMA, different concentrations of the pentaBDE mixture, DE-71, were introduced to the wells. To determine if the effects of PBDEs were similar to the effects of estrogen on cytokine release and whether PBDEs were working through the estrogen receptor, different concentrations of 17 $\beta$ -estradiol and the anti-estrogen, tamoxifen, were added to the wells. After 6-hour incubation in each of

the experiments, conditioned media was removed and stored at  $-80^{\circ}\text{C}$  until analysis of the following cytokines and  $\text{PGE}_2$  via ELISA:  $\text{TNF-}\alpha$ , IL-6, IL- $1\beta$ , and IL-10. Cells exposed to PMA and PBDEs released significantly less pro-inflammatory cytokines ( $\text{TNF-}\alpha$  and IL-6) and  $\text{PGE}_2$  compared to controls; IL- $1\beta$  and IL-10 were not detected in the culture medium. Cells exposed to PMA and estrogen released significantly less  $\text{TNF-}\alpha$  compared to controls and the addition of tamoxifen did not restore the inhibitory effect of PBDEs on  $\text{TNF-}\alpha$  release. Thus, the suppression of  $\text{TNF-}\alpha$  with DE-71 is similar to that of estrogen; however, the inhibitory effects of DE-71 are not mediated via the estrogen receptor. Suppression of pro-inflammatory cytokines- characteristic of a compromised immune system- suggests that persistent exposure to PBDEs may increase the susceptibility to respiratory disorders.

### **Introduction**

Respiratory infections (e.g. rhinovirus and influenza) are the most prevalent and pathogenic form of infectious disease (31, 32). Infection occurs when the host encounters a pathogen, which invades and infects the host's respiratory mucosal tissues. Once the pathogen invades the cells, the innate immune response is initiated (see Chapter I: Immune Response for a detailed explanation). The immune response is orchestrated by a number of finely tuned immune cells. When all is functioning normally, the process of resolving an infection takes approximately 7-14 days and is characterized by coughing, sore throat, fever, nasal/ear congestion, fatigue, and body aches (31). When the immune system is compromised, individuals may have an impaired ability to clear invading pathogens. Recent evidence suggests that exposure to PBDEs may compromise the immune response thereby increasing susceptibility to infectious disease.

The respiratory tract is continuously exposed to foreign antigens from the environment. Effective defense mechanisms are therefore essential in the clearance of potentially infectious antigens. Due to chronic PBDE exposure via inhalation, PBDEs may compromise the activation of innate immunity, which is essential for lung homeostasis and the prevention of chronic respiratory conditions.

The macrophage is the sentinel cell of immunity. It acts both directly on foreign antigens, or pathogens, and indirectly as an antigen-presenting cell. Macrophages reside in several tissues including the liver, bone, spleen, connective tissue, and lung. In the absence of a pathogen or in healthy tissue, the macrophage is in a resting state. Upon activation by a pathogen, the macrophage initiates a series of defense mechanisms such as phagocytosis and the release of pro-inflammatory cytokines and eicosanoids. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ) are pro-inflammatory products phenotypic of classically-activated activated macrophages. Macrophages have also been shown to produce large quantities of the eicosanoid, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) that has both pro- and anti-inflammatory effects (33). These chemical signals not only activate physiological processes that support the development of inflammation, but also communicate with other effector cells such as T-helper lymphocytes that play a role in the development of acquired immunity.

Alveolar macrophages account for approximately 93% of the macrophages in the lung (32). Due to their position in the interstitial spaces of the bronchi and alveoli, in the vascular compartment, and in the alveolus, alveolar macrophages come into contact with the majority of the inhaled environmental antigens ubiquitous in ambient air (34, 35). It is therefore thought that activated macrophages that reside in the lung and pleural cavities

are the main regulators of lung homeostasis (36). Furthermore, through the production of a multitude of different chemical signals mentioned previously and through the expression of a number of different receptors, alveolar macrophages have the capability to communicate with a number of different pathways. For example, alveolar macrophages express the estrogen receptor and have been shown to be regulated by estrogen (29).

The immune response to PBDEs is not well characterized. To date, no study has examined the effects of PBDEs on alveolar macrophage function. Therefore, the objective of this *in vitro* study was to determine the impact of PBDEs on the release of pro-inflammatory products by activated alveolar macrophages and to determine if the effects on inflammatory products are estrogen-receptor dependent. For the *in vitro* work presented in this paper, I used the 3D4/31 commercial porcine alveolar macrophage cell line.

## **Materials and Methods**

### **Alveolar macrophages**

The 3D4/31 cell line, established from primary porcine alveolar macrophages (37), was obtained from ATCC; Manassas, VA. Cells were plated out and cryopreserved in liquid nitrogen at passage #22.

### **Materials**

All materials were purchased from Sigma-Aldrich; St. Louis, MO, unless indicated otherwise. The DE-71 formulation (a mixture of BDE-99, -47, -100, and -153) of pentabrominated diphenyl ether (pentaBDE) was purchased from Cambridge Isotope

Laboratories, Inc.; Andover, MA. The  $17\beta$ -estradiol was a gift from Dr. Paul Tsang (Department of Molecular, Cellular, and Biomedical Sciences; University of New Hampshire). Enzyme-linked immunosorbant assays (ELISAs) were acquired from R&D Systems, Inc., Minneapolis, MN to measure the following pro-inflammatory products: TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and PGE<sub>2</sub>; and the anti-inflammatory cytokine, IL-10. Cell viability was confirmed with a lactate dehydrogenase (LDH) assay (Cayman Chemical Company; Ann Arbor, MI). The RPMI-1640 and non-essential amino acids were purchased from ATCC; fetal bovine serum (FBS) and porcine serum were purchased from MP Biomedicals, Solon, OH.

#### Cell culture protocol

For each experiment, a cryovial of passage #22 3D4/31 porcine alveolar macrophages were removed from storage, seeded into T<sub>75</sub> flask(s) in RPMI growth media supplemented with 10% FBS and 1% non-essential amino acids, and incubated at physiological conditions (5% CO<sub>2</sub>, 37°C). This protocol was adapted, in part, in collaboration with doctoral student Jay Myers. At 60-70% confluency, cells were passaged (#23) and seeded into 24-well plate(s) (300,000 cells/well) in RPMI growth media supplemented with 10% porcine serum and 1% non-essential amino acids and incubated (5% CO<sub>2</sub>, 37°C) for 24-hours. After 24-hours, cells were activated by inoculation with phorbol 12-myristate 13-acetate (PMA). The activating stimulus was prepared by solubilizing PMA (0.01 mg/mL) and ionomycin (I<sub>o</sub>) (0.05 mg/mL) in 1% dimethyl sulfoxide (DMSO). In addition to the activating stimulus, different concentrations of the experimental treatments and respective vehicle controls were introduced to the wells and the plate(s) were incubated (5% CO<sub>2</sub>, 37°C). After a 6-hour



period, conditioned media was removed, centrifuged, and stored at -80°C until further analysis.

### Experiment 1: PBDE exposure & cytokine and eicosanoid release

The purpose of the first experiment was to determine the effects of PBDEs on cytokine and eicosanoid release. Stock solutions were prepared by solubilizing the DE-71 mixture in DMSO at seven different concentrations (0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, 1000 ng/mL, 1500 ng/mL, and 2000 ng/mL). The final DMSO concentration in the solutions was less than 1%. The following cytokines and PGE<sub>2</sub> were measured via ELISA: TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-10. For TNF- $\alpha$  measurements, cells were exposed to PMA and each of the seven different concentrations of DE-71 [n = 18 (0.1 – 2000 ng/mL) and n = 27 (0 ng/mL)]; for all other cytokine and PGE<sub>2</sub> measurements, cells were exposed to PMA and 0.1, 1, 10, 100, and 1000 ng/mL DE-71 (n = 15-18/treatment).

### Experiment 2: Cell Viability

LDH is a cytosolic enzyme released into the culture medium when the cell is damaged due to either apoptosis or necrosis and can therefore, be used as an indicator of cytotoxicity. To assess cell viability, cells were seeded in a 96-well plate at a density of 100,000 cells/well in 120  $\mu$ L RPMI growth media supplemented with 1% FBS and 1% non-essential amino acids. After a 24-hour incubation period (5% CO<sub>2</sub>, 37°C), experimental treatments were added to the wells and the plate was incubated (5% CO<sub>2</sub>, 37°C) for 6-hours. Experimental treatments included 1) culture medium, 2) DMSO (1%), 3) PMA (0.01 mg/mL) and I<sub>0</sub> (0.05 mg/mL) in 1% DMSO, and 4-10) PMA plus seven different concentrations of DE-71 (0.1, 1, 10, 100, 1000, 1500, 2000 ng/mL). After 6-

hours, the plate was centrifuged at 400 x g for five minutes. In the corresponding wells of a new 96-well plate, standards were added in triplicate (n = 3) and 100  $\mu$ L of supernatant from each well of the centrifuged plate was added (n = 8). In accordance with the manufacturer's protocol, 100  $\mu$ L of reaction solution (9.6 mL of assay buffer + 100  $\mu$ L of the following: NAD<sup>+</sup>, lactic acid, a tetrazolium salt (INT), and reconstituted diaphorase) was added to each well and the plate was placed on an orbital shaker and incubated at room temperature with gentle shaking for 30 minutes. The absorbance at 490 nm was then read with a plate reader. According to the protocol, LDH measures cell death due to chemical compounds or environmental factors using a coupled two-step reaction. In the first step, LDH catalyzes the reduction of NAD<sup>+</sup> to NADH and H<sup>+</sup> by oxidation of lactate to pyruvate. Diaphorase then uses NADH and H<sup>+</sup> to catalyze the reduction of INT to the intensely colored formazan, which absorbs at 490-520 nm. The amount of formazan produced is proportional to the amount of LDH released into the culture medium as a result of cytotoxicity.

### Experiment 3: Estrogen exposure and TNF- $\alpha$ release

The purpose of the third experiment was to determine the effects of estrogen on TNF- $\alpha$  release. Five different physiological and supraphysiological concentrations of 17 $\beta$ -estradiol were prepared by diluting the 640  $\mu$ g/mL stock solution in ethanol (EtOH) (12.5, 25, 50, 250, and 1000 pg/mL). The final EtOH concentration in the solutions was less than 1%. Cells were exposed to PMA and each of the five different concentrations of 17 $\beta$ -estradiol (n = 5/treatment). The release of TNF- $\alpha$  was measured via ELISA.

#### Experiment 4: Tamoxifen exposure and TNF- $\alpha$ release

Experiment four assessed whether the effects of PBDEs on cytokine release was mediated via the estrogen receptor. A stock solution was prepared by solubilizing the tamoxifen in DMSO, so that the final concentration was 10  $\mu$ M. Cells were exposed to PMA and 0.1 and 1 ng/mL of DE-71 and 12.5 pg/mL of 17 $\beta$ -estradiol with or without the addition of tamoxifen (n = 9/treatment). The release of TNF- $\alpha$  was measured via ELISA.

#### Statistical Analysis

Statistical analysis was performed using commercially available statistical software (SPSS 18; SPSS Inc., Chicago, IL). For all experiments, treatment effects were determined by analysis of variance (ANOVA) using the general linear model (GLM) with a Bonferroni adjustment. Differences with a *p*-value less than 0.05 were considered statistically significant.

### **Results**

For all experiments, neither cytokines nor eicosanoids were detected in any of the control conditions when in the absence of PMA (i.e. cells exposed to vehicle alone). Therefore, only conditions in which measurable levels of product were detected are reported.

#### Experiment 1: PBDE exposure & cytokine and eicosanoid release

Alveolar macrophages treated with PMA alone released 340  $\pm$  23 pg/mL of TNF- $\alpha$ . Cells exposed to each of the seven different concentrations of DE-71 (0.1, 1, 10, 100, 1000, 1500, and 2000 ng/mL) and PMA (n = 18/treatment) released significantly less

TNF- $\alpha$  compared to cells exposed to the activating stimulus alone (n = 27) (**Figure 7**). In addition, cells exposed to two of the lower concentrations of DE-71 (1 and 10 ng/L) in addition to PMA, released significantly less TNF- $\alpha$  compared to cells exposed to 1000 ng/mL DE-71 and PMA (**Figure 7**). Furthermore, the addition of higher concentrations of DE-71 (1500 and 2000 ng/mL) plus PMA resulted in a significant decrease in TNF- $\alpha$  compared to cells exposed to 0.1 and 1000 ng/mL DE-71 plus PMA (**Figure 7**).

A significant suppression in IL-6 was also evident in cells exposed to 0.1, 1, 10, 100, 1000 ng/mL DE-71 compared to PMA alone (n=15/treatment) (**Figure 8**). All concentrations of DE-71 (0.1, 1, 10, 100 ng/mL) plus PMA released significantly less IL-6 compared to cells exposed to 1000 ng/mL DE-71 plus PMA (**Figure 8**).

Cells released  $1010 \pm 101$  pg/mL PGE<sub>2</sub> when exposed to PMA alone; at 0.1, 1, 10, and 1000 ng/mL DE-71, PGE<sub>2</sub> was significantly suppressed (n=18/treatment) compared to PMA alone (**Figure 9**).

Cytokines IL-1 $\beta$  and IL-10 were not detected in any treatment or control conditions (data not shown).

### Experiment 2: Cell Viability

**Figure 10** shows the LDH cytotoxicity results. Alveolar macrophages in culture medium released  $5.94 \pm 0.3$  mU/mL LDH. Cells exposed to 1, 100, and 1000 ng/mL DE-71 plus PMA released significantly less LDH ( $3.96 \pm 0.1$ ,  $3.24 \pm 0.2$ , and  $3.38 \pm 0.4$  mU/mL, respectively) compared to cells in culture medium alone but the same as cells exposed to PMA alone. When exposed to the highest concentration of DE-71 (2000 ng/mL) plus PMA, cells released significantly more LDH ( $8.83 \pm 0.7$  mU/mL) compared to all other treatments. With the addition of 1500 ng/mL DE-71 plus PMA, cells released

significantly more LDH ( $5.95 \pm 0.6$  mU/mL) compared to cells exposed to PMA plus 1, 100, and 1000 ng/mL DE-71, and significantly less LDH compared to cells exposed to PMA plus 2000 ng/mL DE-71. DE-71 at 2000 ng/mL also was higher than all control conditions. The experiment was repeated with the same reagents one week later and those results are reported in the Appendix. No differences in LDH activity were observed between treatments (see Appendix for interpretation).

#### Experiment 3: Estrogen exposure and TNF- $\alpha$ release

Alveolar macrophages treated with PMA secreted  $204 \pm 3.6$  pg/mL TNF- $\alpha$ . Cells exposed to the activating stimulus plus each of the five different concentrations of 17 $\beta$ -estradiol (12.5, 25, 50, 250, and 1000 pg/mL) released significantly less TNF- $\alpha$  compared to cells exposed to PMA alone ( $169 \pm 3.5$ ,  $162 \pm 2.5$ ,  $159 \pm 1.4$ ,  $164 \pm 3.7$ , and  $169 \pm 3.1$  pg/mL, respectively) (**Figure 11**).

#### Experiment 4: Tamoxifen exposure and TNF- $\alpha$ release

**Figure 12** shows the effects of tamoxifen on the release of TNF- $\alpha$  in alveolar macrophages exposed to varying treatments. Cells treated with tamoxifen in addition to PMA released significantly less TNF- $\alpha$  compared to PMA alone ( $102 \pm 3.8$  vs.  $120 \pm 2.5$  pg/mL, respectively). PMA plus 0.1 and 1 ng/mL DE-71 resulted in a significant suppression in TNF- $\alpha$  compared to PMA alone ( $98 \pm 3.5$  and  $97 \pm 2.0$  pg/mL, respectively); the suppression remained with the addition of tamoxifen; and there was no difference compared to PMA plus DE-71 alone. When PMA and 17 $\beta$ -estradiol were added to the culture medium, there was a significant reduction in TNF- $\alpha$  compared to PMA alone ( $97 \pm 2.9$  pg/mL); a reduction similar to that seen with DE-71 treatment and

tamoxifen. The reduction in TNF- $\alpha$  was restored when tamoxifen in combination with PMA and 17 $\beta$ -estradiol was added to the culture medium ( $112 \pm 3.6$  pg/mL).

### **Discussion**

For the first time, we provide evidence that exposure of activated porcine alveolar macrophages to the PBDE mixture DE-71 suppresses the release of pro-inflammatory products *in vitro* and that the suppression at lower concentrations is independent of cell toxicity. In our model, PBDEs suppress the release of the pro-inflammatory products TNF- $\alpha$ , IL-6, and PGE<sub>2</sub>. IL-1 $\beta$  and IL-10 were not detected in the culture medium. Furthermore, we show that there is a similar dampening of TNF- $\alpha$  when activated porcine alveolar macrophages are in the presence of estrogen; however, the effects seen with PBDEs are not mediated via the estrogen receptor.

The suppression of TNF- $\alpha$  and IL-6 in the present study may be explained in part by disrupted signaling of nuclear factor- $\kappa$ B (NF- $\kappa$ B). Due to their position in the alveolar lumen of the lung, alveolar macrophages are chronically exposed to inhaled environmental antigens ubiquitous in ambient air (34). Upon encountering an antigen, macrophages become activated and produce pro-inflammatory cytokines to rid the body of the pathogen. Pro-inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  are regulated through the activation of the transcription factor, NF- $\kappa$ B. In the activated form, NF- $\kappa$ B is a heterodimer consisting of two proteins, a p65 subunit and a p50 subunit. In unstimulated cells, NF- $\kappa$ B is located in the cytoplasm bound to an inhibitory protein, I $\kappa$ -B that prevents it from entering the nucleus. Upon activation, I $\kappa$ -B kinase (IKK) phosphorylates I $\kappa$ -B, resulting in degradation of the inhibitory protein and allowing NF- $\kappa$ B to translocate to the nucleus to initiate the transcription of various cytokine genes.

Many stimuli have been shown to activate NF- $\kappa$ B including TNF- $\alpha$  and protein kinase C activators such as PMA, a phorbol ester, and lipopolysaccharide (LPS), a bacterial endotoxin (28).

TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , all have powerful physiological effects. For example, TNF- $\alpha$  induces a local inflammatory response (i.e. vasodilation and increased vascular permeability) that allows fluid, protein, and inflammatory cells to leave the blood stream and enter the tissue. As a result, the infected tissue becomes inflamed, causing the characteristic redness, heat, swelling and pain associated with infection.

The eicosanoid PGE<sub>2</sub> is a potent inflammatory mediator derived from arachidonic acid through the cyclooxygenase (COX) enzymes (33). Depending on the inflammatory stimulus and the receptor to which PGE<sub>2</sub> binds, PGE<sub>2</sub> has the ability to act in either a pro- or anti-inflammatory manner (38). The pro-inflammatory immune effects of PGE<sub>2</sub> are similar to those of the pro-inflammatory cytokines (i.e. vasodilation, increased vascular permeability, fever, and pain), whereas the anti-inflammatory effects include suppressing lymphocyte proliferation and inhibiting the production of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1. PGE<sub>2</sub> is not a product of the NF- $\kappa$ B pathway and thus, the concomitant reduction in PGE<sub>2</sub> seen in the present study suggests that other mechanisms may be involved.

The failure to detect IL-1 $\beta$  and IL-10 in the culture medium is most likely due to the inherent heterogeneity of macrophages. Macrophages are a dynamic cell, with the ability to change their phenotype based on different environmental signals. Based on both their location (i.e. lung, interstitial, peritoneal, liver, etc.) and their spectrum of activation (i.e. classically-activated, alternatively activated), macrophages have been

shown to produce different products (39, 40). In some instances this corresponds to enhanced cytokine output and phagocytic activity – resulting in a heightened immune response. This is not, however, always the case. Different environmental signals can give rise to macrophages that are less equipped to produce cytokines and result in an increased susceptibility to infection (40). Others in our lab are currently investigating the further characterization of the 3D4/31 cell line (41).

In the present study, the suppression of TNF- $\alpha$ , IL-6, and PGE<sub>2</sub> in cells exposed to lower concentrations of DE-71 (0.1 – 1500 ng/mL) appears to be independent of cytotoxicity. Cells exposed to PMA and the highest concentration of DE-71 (2000 ng/mL) released significantly more LDH compared to cells exposed to control conditions and cells exposed to PMA and lower concentrations of DE-71 (0.1 – 1500 ng/mL). As the concentration of DE-71 increased, there was a linear decrease in the amount of LDH released, until 1000 ng/mL DE-71. At that concentration there was an observed linear increase in LDH that was significant at 2000 ng/mL DE-71. These data suggested that DE-71 was toxic to alveolar macrophages at a concentration between 1000 and 1500 ng/mL and that the suppression of TNF- $\alpha$  in cells exposed to PMA plus 1500 and 2000 ng/mL DE-71 may be in part, due to cell death. On the other hand, these findings indicated that at nontoxic levels, there was a linear decrease in LDH that suggested the effects of PBDEs on production of cytokines and eicosanoids was related to a down regulation in metabolism of macrophages. For example, PMA has been shown to increase glucose oxidation in alveolar macrophages (42). Furthermore, Hoppe and Carey (2007) provide evidence of a significant increase in lipolysis and a significant decrease in glucose oxidation in adipocytes isolated from Sprague-Dawley rats treated with



pentaBDEs (43).

The endocrine disrupting potential of PBDEs has largely been studied in regard to their ability to disrupt thyroid hormone homeostasis. Many environmental pollutants, however, have proven to be xenoestrogens (44-47). Due to the structural similarity between PBDEs and other environmental pollutants, such as DDT and PCBs, there is high likelihood that PBDEs may be estrogenic as well (48-50). Indeed, Meerts et al. (2001), provide evidence that several PBDE congeners are agonists of the ER $\alpha$  and ER $\beta$  receptors (49) and in the time since, further evidence of the estrogenic activity of PBDEs has followed (51, 52). In one study Mercado-Feliciano and Bigsby (2008), showed that DE-71 behaves as a weak estrogen in both MCF-7 breast cancer cells and ovariectomized mice (52). In a different study, the same researchers showed that the weak estrogenic effects of DE-71 are due to the activation of individual congeners, however, the exact mechanism remains to be elucidated (51).

To determine if PBDEs are acting as xenoestrogens we compared the effects of PBDEs and estrogen on TNF- $\alpha$  release. Cells exposed to PBDEs showed a dose-dependent U-shaped curve. At low concentrations (0.1 and 1 ng/mL DE-71) there was a steady decline in TNF- $\alpha$  and as the concentration of DE-71 increased (10, 100, 1000 ng/mL DE-71), the production of TNF- $\alpha$  increased – although it remained suppressed compared to PMA alone throughout.

A similar relationship was described by Tomaszewska et al. (2003), who exposed mouse leukemic macrophages to estrogen (53). Researchers exposed cells to an activating stimulus (LPS) alone, and the activating stimulus in addition to five different concentrations of estrogen (17 $\beta$ -estradiol) (12.5, 25, 50, 250, 1000 pg/mL). Stimulated

cells exposed to 12.5, 25, and 50 pg/mL 17 $\beta$ -estradiol released significantly less TNF- $\alpha$  compared to stimulated cells alone ( $p < 0.05$ ); no differences were seen with 250 and 1000 pg/mL 17 $\beta$ -estradiol. In a recent review the response was described as a dose-dependent U-shaped curve, where at physiological concentrations a suppression in TNF- $\alpha$  was seen but disappeared at supraphysiological concentrations (29). In the present study, we exposed porcine alveolar macrophages to an activating stimulus (PMA) alone and the activating stimulus in addition to the same concentrations of 17 $\beta$ -estradiol (12.5, 25, 50, 250, 1000 pg/mL) and found a similar relationship. Unlike Tomaszewska et al. (2003), we found a significant suppression of TNF- $\alpha$  with all concentrations of 17 $\beta$ -estradiol.

Estrogen has an inhibitory effect on TNF- $\alpha$  and other cytokines in many different cells types (reviewed by (29)). For example, IL-6 is inhibited by estrogen in human promonocytic cells (54); Vegeto (2001), describes a dose-dependent LPS-induced suppression of PGE<sub>2</sub> in rat primary microglia cultures exposed to varying levels of 17 $\beta$ -estradiol (55); and IL-12 and TNF- $\alpha$  are reduced in mouse dendritic cells with a shift toward IL-4 and IL-10 (56). Straub (2007), claims that these results are most likely due to inhibition of NF- $\kappa$ B (29). Ghisletti et al. (2005), provide evidence that the inhibitory effects of estrogen on NF- $\kappa$ B are mediated by blocking the binding and transcriptional activity of p65 and preventing nuclear translocation (30). For these experiments, mice peritoneal macrophages were exposed to LPS alone; 1 and 100 nM of 17 $\beta$ -estradiol or tamoxifen (an antagonist of the estrogen receptor) and subsequently LPS; and competition experiments where tamoxifen was added before the addition of 17 $\beta$ -estradiol (100 nM). Immunocytochemical analysis revealed a significant increase in cytoplasmic

p65 in cells exposed to 17 $\beta$ -estradiol, indicating the reduced capacity of NF- $\kappa$ B to translocate to the nucleus and produce pro-inflammatory products. Conversely, significantly less cytoplasmic p65 was seen in cells exposed to tamoxifen and in the competition experiments, indicating the translocation of NF- $\kappa$ B to the nucleus and the production of pro-inflammatory products.

Despite the reported estrogenic activity of PBDEs, it does not appear that they are acting via the estrogen receptor in the current model. In the present study, tamoxifen, an antagonist of the estrogen receptors- $\alpha$  and - $\beta$ , did not restore the inhibitory effect of PBDEs in porcine alveolar macrophages; tamoxifen did, however, restore the suppression seen with 17 $\beta$ -estradiol. These findings suggest that the PBDEs are acting in an estrogen-independent manner. Mercado-Feliciano and Bigsby (2008), provide evidence that DE-71 can enhance the estrogenic effects beyond those produced by a saturating dose of estrogen. The researchers suggest that PBDEs are behaving like estrogen through nonclassical pathways (52).

Endocrine disruptors have the ability to disrupt receptor signaling in a 1) direct manner by binding to specific hormone receptors or 2) in an indirect manner by binding to alternative receptors and affecting hormone signaling through various mechanisms such as competing for common cofactors (5). Once bound to a receptor, endocrine disruptors act as either: agonists and bind to a specific hormone receptor and mimic that naturally occurring hormone (e.g. in this case estrogen) and its function, or as an antagonist and bind to a specific hormone receptor and block the endogenous hormone from binding (e.g. anti-estrogens such as tamoxifen). When inactive, nuclear receptors are located in the cytoplasm or in the nucleus bound to chaperone proteins and co-

repressors. When a ligand binds to the receptor, there is a conformational change allowing for the dissociation of the chaperone and co-repressor complex and the receptors to dimerize. If the ligand is an agonist of the receptor, coactivators are recruited. This facilitates the activated complex to bind to response elements and regulate (activation or inhibition) transcription of target genes. If the ligand is an antagonist of the receptor, coactivators are prevented from binding and the complex is not activated.

Given the current findings, it is likely PBDEs are acting indirectly and perhaps through a different receptor all together, however, no clear evidence exists as to which receptor is involved. One plausible candidate is the aryl hydrocarbon receptor (AhR). AhR is a ligand-activated transcription factor that has been shown to be involved with the cellular responses of many xenobiotics including PCBs (8). The activation of the AhR is similar to that of other nuclear receptors. AhR resides in the cytoplasm bound to chaperone proteins. When a ligand binds, AhR is translocated to the nucleus, the chaperone proteins are dissociated, and AhR dimerizes with the AhR nuclear translocator (ARNT). The ligand-AhR-ARNT complex can then bind to response elements, which leads to gene transcription (57). The ligand-AhR-ARNT has also been reported to downregulate the transcription of some genes (58). In the same study, Tijet et al. (2006), goes on to say that estrogen and AhR are reciprocally regulated because of the overlap and antagonism of the two pathways. Nonetheless, a recent review examining the effects of PBDEs on different receptors, Luthe et al. (2008), speculate that due to their structural conformation, PBDEs do not possess AhR activity (59). Further research is needed to address these issues.

Another possibility is a recently discovered orphan nuclear receptor. In 1998, three independent research groups discovered and named the receptor the steroid and xenobiotic receptor (SXR) (the name I will use throughout), the pregnane X receptor (PXR), and the pregnane activated receptor (PAR), respectively (60-62). SXR is a nuclear receptor that has been described as a xenobiotic sensor that regulates the clearance of xenobiotics in the liver and intestine (63). Interestingly, it has also been shown to be highly expressed in alveolar macrophages (64). Many endogenous compounds (e.g. steroids such as estrogen) and exogenous compounds (e.g. pharmaceuticals such as tamoxifen and endocrine disruptors such as PBDEs) are known activators of SXR (63). Due to this capability, cross-talk between SXR and other nuclear receptors exists. For example, SXR has been shown to interact with NF- $\kappa$ B, thereby providing a potential link between SXR ligands and inflammation (63). Many studies have shown that activation of SXR suppresses the effects of NF- $\kappa$ B, although the exact mechanism remains to be elucidated. Our data from the tamoxifen experiments suggest the suppressive effects of PBDEs may be acting via SXR. Furthermore, tamoxifen has been shown to be a potent activator of the SXR and may therefore explain the apparent decrease in TNF- $\alpha$  with tamoxifen alone, as well as the redundant effects seen with PBDEs and tamoxifen (65). Conversely, in the tamoxifen study mentioned earlier, Ghisletti et al. (2005) saw a negligible amount of cytoplasmic p65 with LPS and tamoxifen – an indication of increased pro-inflammatory cytokines. These apparently disparate results could be explained by the inherent cell- or species-specific differences (i.e. mouse peritoneal vs. porcine alveolar macrophages). Another possible explanation could be the dose of tamoxifen (1 and 100 nM vs. 10  $\mu$ M).

In summary, proper functioning of the macrophage through chemical signals such as cytokines and eicoanoids is essential for the innate immune response and host defense. Thus, the observed suppression in pro-inflammatory cytokine and eicosanoid production in PMA-activated porcine alveolar macrophages exposed to DE-71 is a novel finding. Furthermore, we show that the suppression seen with DE-71 is similar to that of estrogen; however, our results demonstrate that the inhibitory effects of DE-71 are not mediated via the estrogen receptor. Given the present findings, there is concern that PBDEs may compromise innate immune function, thereby increasing the susceptibility and the time required to resolve an infection. These data suggest that subacute chronic inflammation may be explained in part by persistent exposure to airborne PBDEs. Whether these effects are seen *in vivo*, remains to be determined.

## CHAPTER III

### IMPLICATIONS

The concept of flame retardants is a good one. Reducing the flammability and decreasing the burn rate of products so that there is an increased time to escape in the event of a fire is critical. There is a growing body of evidence that chronic exposure to flame retardants may have important (yet not well understood) health risks. Research conducted in our laboratory suggests that exposure to lower concentrations of PBDEs may increase an individual's susceptibility to and the time needed to resolve an infection. These findings could have important public health implications.

Respiratory infections (e.g. rhinovirus and influenza) are the most prevalent and pathogenic form of infectious disease (31, 32). Infection occurs when the host encounters a pathogen, which invades and infects the host's respiratory mucosal tissues. Once the pathogen invades the cells, the innate immune response is initiated (see Chapter I: Immune Response for a detailed explanation). The process of resolving an infection takes approximately 7-14 days and is characterized by coughing, sore throat, fever, nasal/ear congestion, fatigue, and body aches (31). We provide evidence that exposure to PBDEs may compromise this response.

Porcine alveolar macrophage exposed to PBDEs, show a marked reduction in the pro-inflammatory response, leading to a decrease in effector cell (i.e. macrophage) function and an inappropriate development of the adaptive immune response. The suppression of the immune system seen with exposure to PBDEs, especially at lower

concentrations, may provide an ‘open window’ of susceptibility to infection. During this ‘open window’ of susceptibility, viruses and bacteria may gain entry, thereby increasing the risk and/or duration of infection.

Furthermore, the suppression in pro-inflammatory cytokines of the innate immune response may compromise the role of the alveolar macrophage in eliciting adaptive immunity. It is therefore plausible that exposure to PBDEs could play a role in the development of atopic disease and chronic inflammatory disorders. The decreased ability of alveolar macrophages to generate pro-inflammatory cytokines could shift acquired immunity away from cell mediated and more toward humoral immunity. A shift toward the anti-inflammatory  $T_H2$  phenotype and away from the pro-inflammatory  $T_H1$  phenotype has been shown in a number of diseased and inflammatory conditions. For example, a shift toward a  $T_H2$  response is seen in allergic diseases such as asthma, allergic rhinitis, food hypersensitivity, atopic dermatitis; and chronic respiratory disorders (31, 66). The same trend may occur with chronic exposure to PBDEs – that is, the  $T_H1$  response may be suppressed too much – allowing pathogens to gain entry and leading to an increased susceptibility to infection.

Current data suggests there is a relationship between exposure to PBDEs and the susceptibility to infection. In light of these suspected health conditions, research and legislation should be directed toward further investigation of how PBDEs may be affecting human and animal health, and at the same time, developing alternative flame retardant products. Due to the persistence of PBDEs in the environment, proper disposal of products containing PBDEs is essential. Furthermore, it is important that individuals recognize the risks associated with chemical pollutants and employ ways to reduce



exposure. Most importantly, populations that are most susceptible to infection – pregnant women, infants, and children – may be more at risk.

## REFERENCES

1. Colborn T. Neurodevelopment and endocrine disruption. *Environ Health Perspect* 2004;112:944-9.
2. Baillie-Hamilton PF. Chemical toxins: a hypothesis to explain the global obesity epidemic. *J Altern Complement Med* 2002;8:185-92.
3. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 1993;101:378-84.
4. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev* 2009;30:293-342.
5. Ruegg J, Penttinen-Damdimopoulou P, Makela S, Pongratz I, Gustafsson JA. Receptors mediating toxicity and their involvement in endocrine disruption. *EXS* 2009;99:289-323.
6. Longnecker MP, Rogan WJ, Lucier G. The human health effects of DDT (dichlorodiphenyltrichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health. *Annu Rev Public Health* 1997;18:211-44.
7. U.S. EPA. Polychlorinated Biphenyls (PCBs). Washington, DC:U.S. Environmental Protection Agency, 2010.
8. Swedenborg E, Ruegg J, Makela S, Pongratz I. Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. *J Mol Endocrinol* 2009;43:1-10.
9. McDonald TA. A perspective on the potential health risks of PBDEs. *Chemosphere* 2002;46:745-55.
10. Darnerud PO, Eriksen GS, Johannesson T, Larsen PB, Viluksela M. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ Health Perspect* 2001;109 Suppl 1:49-68.
11. Zhu LY, Hites RA. Temporal trends and spatial distributions of brominated flame retardants in archived fishes from the Great Lakes. *Environ Sci Technol* 2004;38:2779-84.
12. Lorber M. Exposure of Americans to polybrominated diphenyl ethers. *J Expo Sci Environ Epidemiol* 2008;18:2-19.

13. McDonald TA. Polybrominated diphenylether levels among United States residents: daily intake and risk of harm to the developing brain and reproductive organs. *Integr Environ Assess Manag* 2005;1:343-54.
14. Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? *Environ Health Perspect* 2004;112:9-17.
15. U.S. EPA. Polybrominated Diphenyl Ethers (PBDEs) Action Plan. Washington, DC:U.S. Environmental Protection Agency, 2009:1-16.
16. Costa LG, Giordano G. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology* 2007;28:1047-67.
17. Costa LG, Giordano G, Tagliaferri S, Cagliari A, Mutti A. Polybrominated diphenyl ether (PBDE) flame retardants: environmental contamination, human body burden and potential adverse health effects. *Acta Biomed* 2008;79:172-83.
18. Bocio A, Llobet JM, Domingo JL, Corbella J, Teixido A, Casas C. Polybrominated diphenyl ethers (PBDEs) in foodstuffs: human exposure through the diet. *J Agric Food Chem* 2003;51:3191-5.
19. Schecter A, Colacino J, Patel K, Kannan K, Yun SH, Haffner D, Harris TR, Birnbaum L. Polybrominated diphenyl ether levels in foodstuffs collected from three locations from the United States. *Toxicol Appl Pharmacol* 2009;243:217-24.
20. Schecter A, Papke O, Harris TR, Tung KC, Musumba A, Olson J, Birnbaum L. Polybrominated diphenyl ether (PBDE) levels in an expanded market basket survey of U.S. food and estimated PBDE dietary intake by age and sex. *Environ Health Perspect* 2006;114:1515-20.
21. Fraser AJ, Webster T, McClean MD. Diet contributes significantly to the body burden of PBDEs in the general U.S. population. *Environ Health Perspect* 2009;117:1520-5.
22. Wu N, Herrmann T, Paepke O, Tickner J, Hale R, Harvey LE, La Guardia M, McClean MD, Webster TF. Human exposure to PBDEs: associations of PBDE body burdens with food consumption and house dust concentrations. *Environ Sci Technol* 2007;41:1584-9.
23. Stapleton HM, Kelly SM, Allen JG, McClean MD, Webster TF. Measurement of polybrominated diphenyl ethers on hand wipes: estimating exposure from hand-to-mouth contact. *Environ Sci Technol* 2008;42:3329-34.
24. Reistad T, Mariussen E. A commercial mixture of the brominated flame retardant pentabrominated diphenyl ether (DE-71) induces respiratory burst in human neutrophil granulocytes in vitro. *Toxicol Sci* 2005;87:57-65.

25. Fernie KJ, Mayne G, Shutt JL, Pekarik C, Grasman KA, Letcher RJ, Drouillard K. Evidence of immunomodulation in nestling American kestrels (*Falco sparverius*) exposed to environmentally relevant PBDEs. *Environ Pollut* 2005;138:485-93.
26. Lundgren M, Darnerud PO, Blomberg J, Friman G, Ilback NG. Polybrominated diphenyl ether exposure suppresses cytokines important in the defence to coxsackievirus B3 infection in mice. *Toxicol Lett* 2009;184:107-13.
27. Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada Calvo F, Henry E, Closset R, Dewals B, Thielen C, Gustin P, de Leval L, Van Rooijen N, Le Moine A, Vanderplasschen A, Cataldo D, Drion PV, Moser M, Lekeux P, Bureau F. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest* 2009;119:3723-38.
28. Epstein FH. Nuclear Factor- $\kappa$ B - A pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997;336:1066-1071.
29. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* 2007;28:521-74.
30. Ghisletti S, Meda C, Maggi A, Vegeto E. 17 $\beta$ -estradiol inhibits inflammatory gene expression by controlling NF- $\kappa$ B intracellular localization. *Mol Cell Biol* 2005;25:2957-68.
31. Martin SA, Pence BD, Woods JA. Exercise and respiratory tract viral infections. *Exerc Sport Sci Rev* 2009;37:157-64.
32. Marriott HM, Dockrell DH. The role of the macrophage in lung disease mediated by bacteria. *Exp Lung Res* 2007;33:493-505.
33. Vancheri C, Mastruzzo C, Sortino MA, Crimi N. The lung as a privileged site for the beneficial actions of PGE<sub>2</sub>. *Trends Immunol* 2004;25:40-6.
34. Lambrecht BN. Alveolar macrophage in the driver's seat. *Immunity* 2006;24:366-8.
35. Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* 2008;8:142-52.
36. Cailhier JF, Sawatzky DA, Kipari T, Houlberg K, Walbaum D, Watson S, Lang RA, Clay S, Kluth D, Savill J, Houghs J. Resident pleural macrophages are key orchestrators of neutrophil recruitment in pleural inflammation. *Am J Respir Crit Care Med* 2006;173:540-7.

37. Weingartl HM, Sabara M, Pasick J, van Moorlehem E, Babiuk L. Continuous porcine cell lines developed from alveolar macrophages: partial characterization and virus susceptibility. *J Virol Methods* 2002;104:203-16.
38. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest* 2001;108:15-23.
39. Hubbard AK. Effects of xenobiotics on macrophage function: evaluation in vitro. *Methods* 1999;19:8-16.
40. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958-69.
41. Myers JL, Tagliaferro AR. Classic activation of 3D4/31 porcine alveolar macrophages using phorbol 12-myristate 13-acetate and ionomycin. *FASEB J* 2009;23:908.4.
42. Hoidal JR, Repine JE, Beall GD, Rasp FL, Jr., White JG. The effect of phorbol myristate acetate on the metabolism and ultrastructure of human alveolar macrophages. *Am J Pathol* 1978;91:469-82.
43. Hoppe AA, Carey GB. Polybrominated diphenyl ethers as endocrine disruptors of adipocyte metabolism. *Obesity (Silver Spring)* 2007;15:2942-50.
44. Sonnenschein C, Soto AM. An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* 1998;65:143-50.
45. Zava DT, Blen M, Duwe G. Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. *Environ Health Perspect* 1997;105 Suppl 3:637-45.
46. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 1995;103:582-7.
47. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* 1995;103 Suppl 7:113-22.
48. Meerts IA, Letcher RJ, Hoving S, Marsh G, Bergman A, Lemmen JG, van der Burg B, Brouwer A. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. *Environ Health Perspect* 2001;109:399-407.

49. Hooper K, McDonald TA. The PBDEs: an emerging environmental challenge and another reason for breast-milk monitoring programs. *Environ Health Perspect* 2000;108:387-92.
50. Pijnenburg AM, Everts JW, de Boer J, Boon JP. Polybrominated biphenyl and diphenylether flame retardants: analysis, toxicity, and environmental occurrence. *Rev Environ Contam Toxicol* 1995;141:1-26.
51. Mercado-Feliciano M, Bigsby RM. Hydroxylated metabolites of the polybrominated diphenyl ether mixture DE-71 are weak estrogen receptor-alpha ligands. *Environ Health Perspect* 2008;116:1315-21.
52. Mercado-Feliciano M, Bigsby RM. The polybrominated diphenyl ether mixture DE-71 is mildly estrogenic. *Environ Health Perspect* 2008;116:605-11.
53. Tomaszewska A, Guevara I, Wilczok T, Dembinska-Kiec A. 17beta-estradiol- and lipopolysaccharide-induced changes in nitric oxide, tumor necrosis factor-alpha and vascular endothelial growth factor release from RAW 264.7 macrophages. *Gynecol Obstet Invest* 2003;56:152-9.
54. Jain SK, Rogier K, Prouty L. Protective effects of 17beta-estradiol and trivalent chromium on interleukin-6 secretion, oxidative stress, and adhesion of monocytes: relevance to heart disease in postmenopausal women. *Free Radic Biol Med* 2004;37:1730-5.
55. Vegeto E, Bonincontro C, Pollio G, Sala A, Viappiani S, Nardi F, Brusadelli A, Viviani B, Clana P, Maggi A. Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J Neurosci* 2001;21:1809-18.
56. Pettersson A, Ciumas C, Chirsky V, Link H, Huang YM, Xiao BG. Dendritic cells exposed to estrogen in vitro exhibit therapeutic effects in ongoing experimental allergic encephalomyelitis. *J Neuroimmunol* 2004;156:58-65.
57. Okey AB. An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI. *Toxicol Sci* 2007;98:5-38.
58. Tijet N, Boutros PC, Moffat ID, Okey AB, Tuomisto J, Pohjanvirta R. Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. *Mol Pharmacol* 2006;69:140-53.
59. Luthe G, Jacobus JA, Robertson LW. Receptor interactions by polybrominated diphenyl ethers versus polychlorinated biphenyls: a theoretical Structure-activity assessment. *Environ Toxicol Pharmacol* 2008;25:202-210.

60. Blumberg B, Evans RM. Orphan nuclear receptors--new ligands and new possibilities. *Genes Dev* 1998;12:3149-55.
61. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Wilson TM, Zetterstrom RH, Perlmann T, Lehmann JM. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 1998;92:73-82.
62. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA* 1998;95:12208-13.
63. Zhou C, Verma S, Blumberg B. The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism. *Nucl Recept Signal* 2009;7:e001.
64. Raunio H, Hakkola J, Pelkonen O. Regulation of CYP3A genes in the human respiratory tract. *Chem Biol Interact* 2005;151:53-62.
65. Nagaoka R, Iwasaki T, Rokutanda N, Takeshita A, Koibuchi Y, Horiguchi J, Shimokawa N, Iino Y, Morishita Y, Koibuchi N. Tamoxifen activates CYP3A4 and MDR1 genes through steroid and xenobiotic receptor in breast cancer cells. *Endocrine* 2006;30:261-8.
66. Dietert RR, Zelikoff JT. Early-life environment, developmental immunotoxicology, and the risk of pediatric allergic disease including asthma. *Birth Defects Res B Dev Reprod Toxicol* 2008;83:547-60.
67. Chalubinski M, Kowalski ML. Endocrine disrupters - potential modulators of the immune system and allergic response. *Allergy* 2006;61:1326-35.
68. Colborn T, Dumanoski D, Myers JP. *Our stolen future : are we threatening our fertility, intelligence, and survival? : a scientific detective story : with a new epilogue by the authors.* New York: Penguin Group, 1997.



## **TABLES**

**Table 1.**

Group	Examples
Herbicides	Alachlor, Atrazine, Nitrofen, Trifluralin
Fungicides	Hexachlorobenzene, Maneb, Zineb
Insecticides	Carbaryl, DDT, Dieldrin, Organophosphates, Toxaphene
Industrial chemicals	Bisphenol A, Dioxins, PBDEs, PCBs, Phthalates, Styrenes
Heavy metals	Cadmium, Lead, Mercury

Main groups and examples of endocrine-disrupting chemicals [adapted from (3, 67)].  
DDT, dichlorodiphenyltrichloroethane; PBDEs, polybrominated diphenyl ethers; PCBs, polychlorinated biphenyls.

**Table 2.**

Major PBDE congeners or mixtures	
BDE-47	2,2', 4,4'-tetrabromodiphenyl ether
BDE-99	2,2', 4,4',5-pentabromodiphenyl ether
BDE-100	2,2', 4,4',6-pentabromodiphenyl ether
BDE-153	2,2', 4,4',5,5'-hexabromodiphenyl ether
BDE-154	2,2', 4,4',5,6'-hexabromodiphenyl ether
BDE-209	Decabromodiphenyl ether
DE-71	PentaBDE technical mixture (BDE-99, 44%; BDE-47, 32%; BDE-100, 9%; BDE-153, 4%)
DE-79	OctaBDE technical mixture (BDE-183, 35%; BDE-197, 22%; BDE-207, 14%; BDE-196, 9%)

Adapted from (16).

**Table 3.**

Serum levels of PBDE's in a family from Northern California in 2004

Family member	Age	$\Sigma(5)$ PBDEs <sup>a</sup>
Father	35	64
Mother	36	106
Female child	5	247
Male toddler	1.5	418

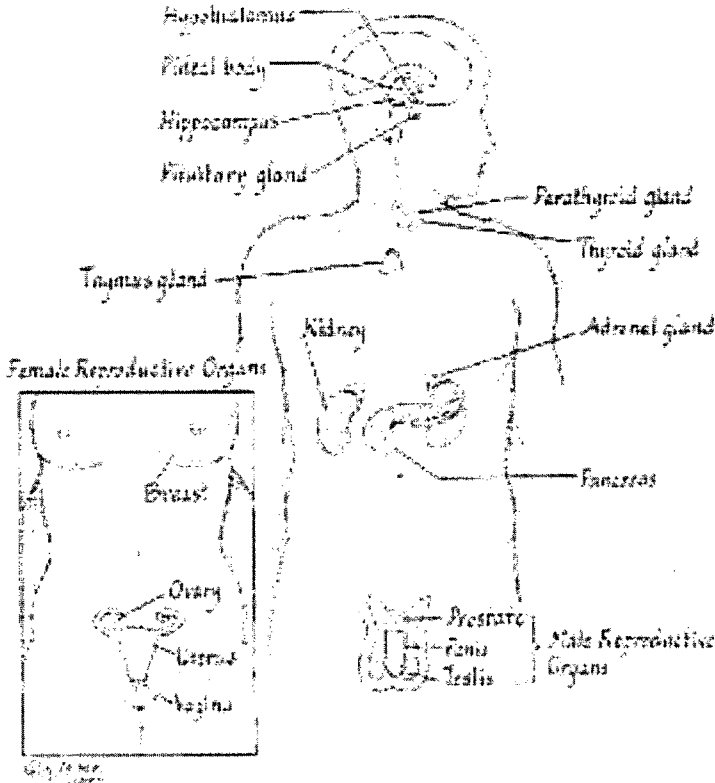
PBDE levels are expressed as ng/g lipid weight.

<sup>a</sup> Sum of five BDE's (BDE-47, -99, -100, -153, -154).

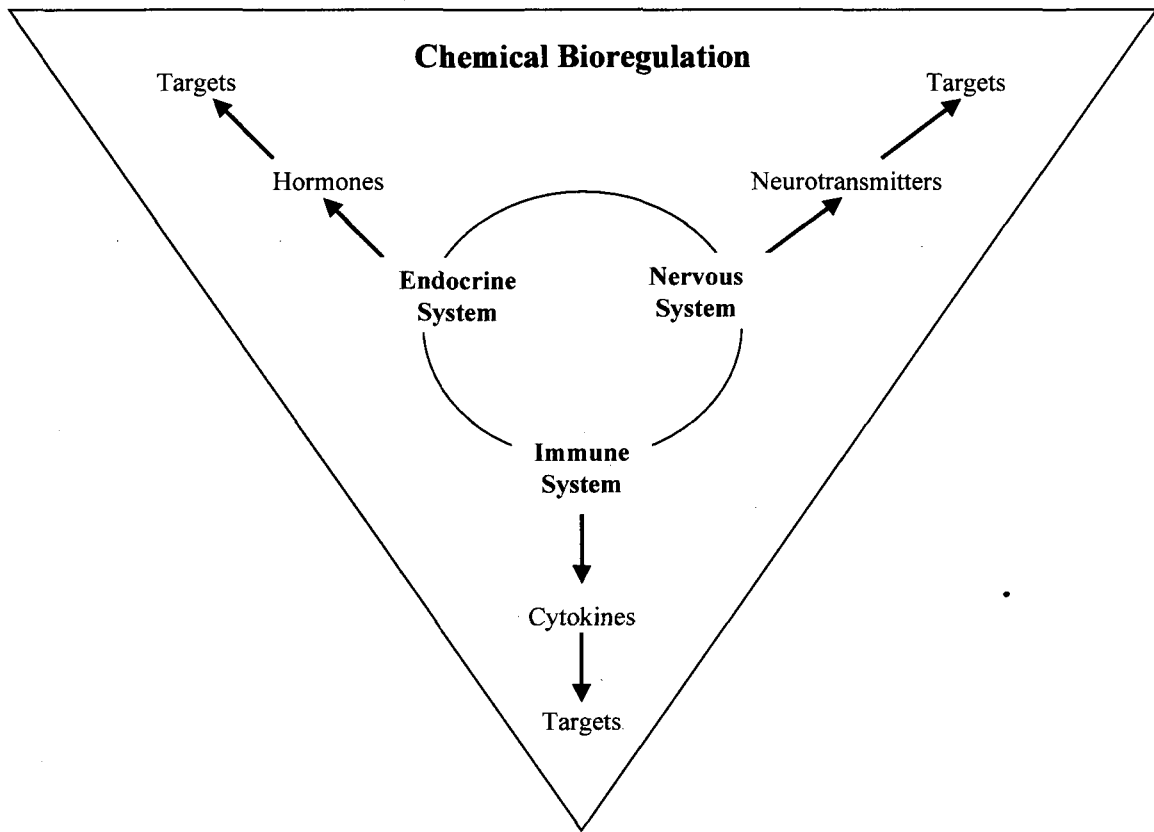
Adapted from (16).

## FIGURES

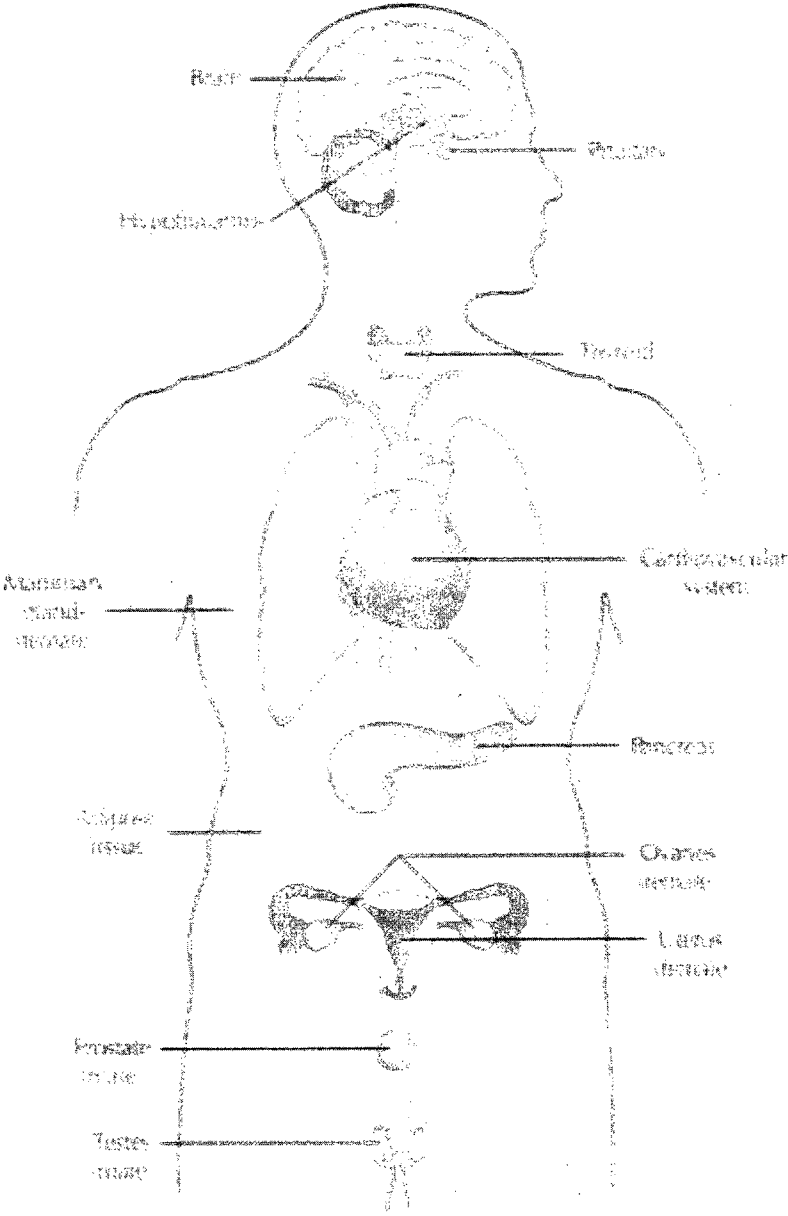
Figure 1. The glands of the endocrine system [Illustration by K. Born (1995) (68)].



**Figure 2.** The body's integrating systems and the cross-talk that occurs between the different systems via hormones and other chemical messengers (adapted from figure created by William Condon, Ph.D.).



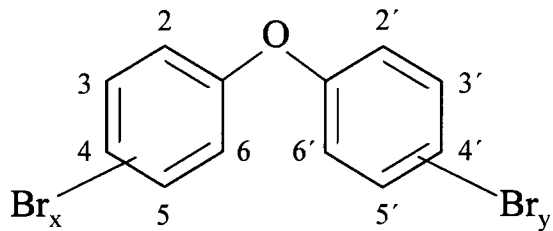
**Figure 3.** Systems and organs targeted by endocrine disrupting chemicals (4).



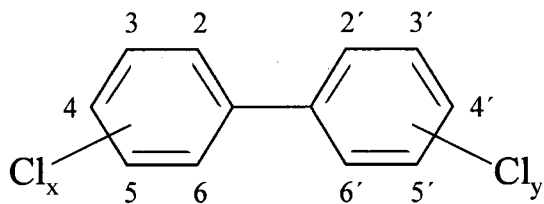


**Figure 4.** Structure of: A. polybrominated diphenyl ethers (PBDEs), B. polychlorinated biphenyls (PCBs), and C. dichlorodiphenyltrichloroethane (DDT).

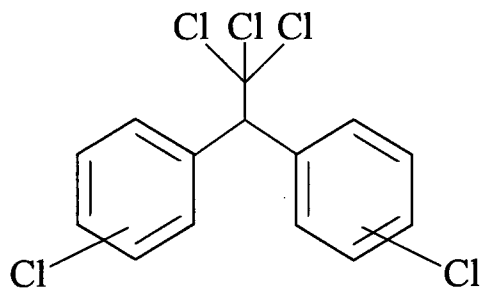
A.



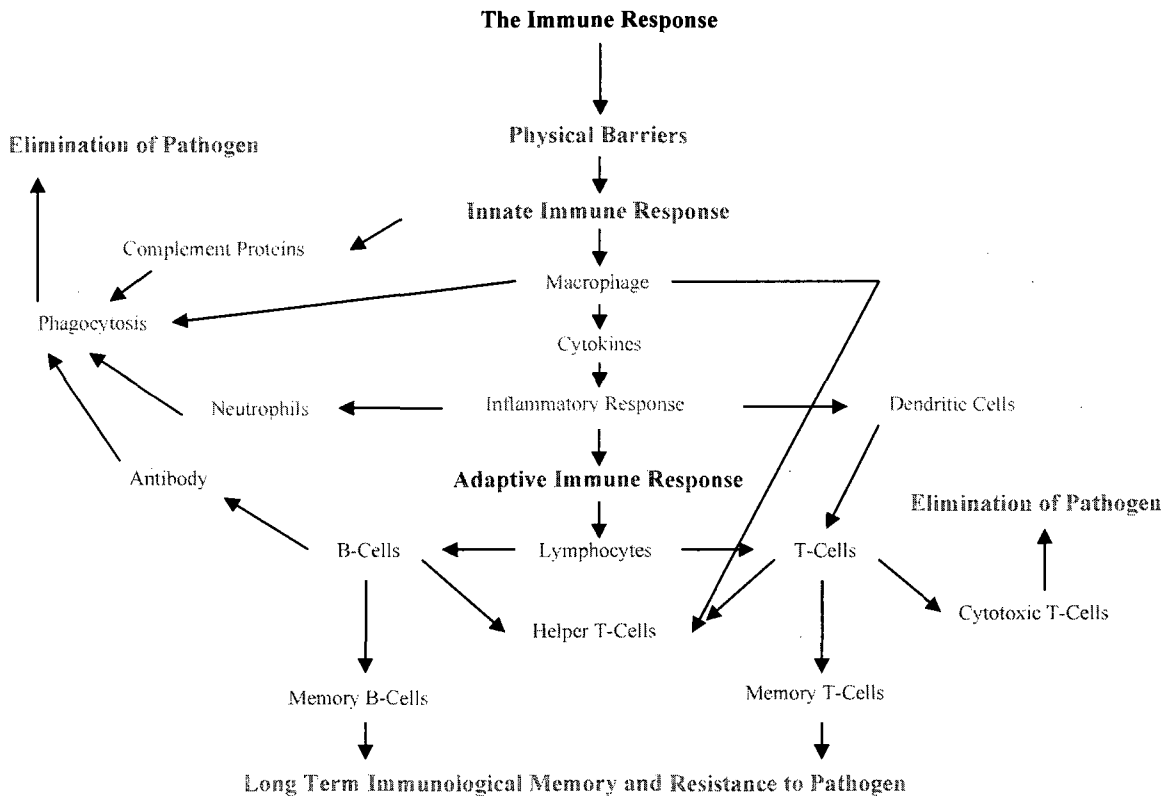
B.



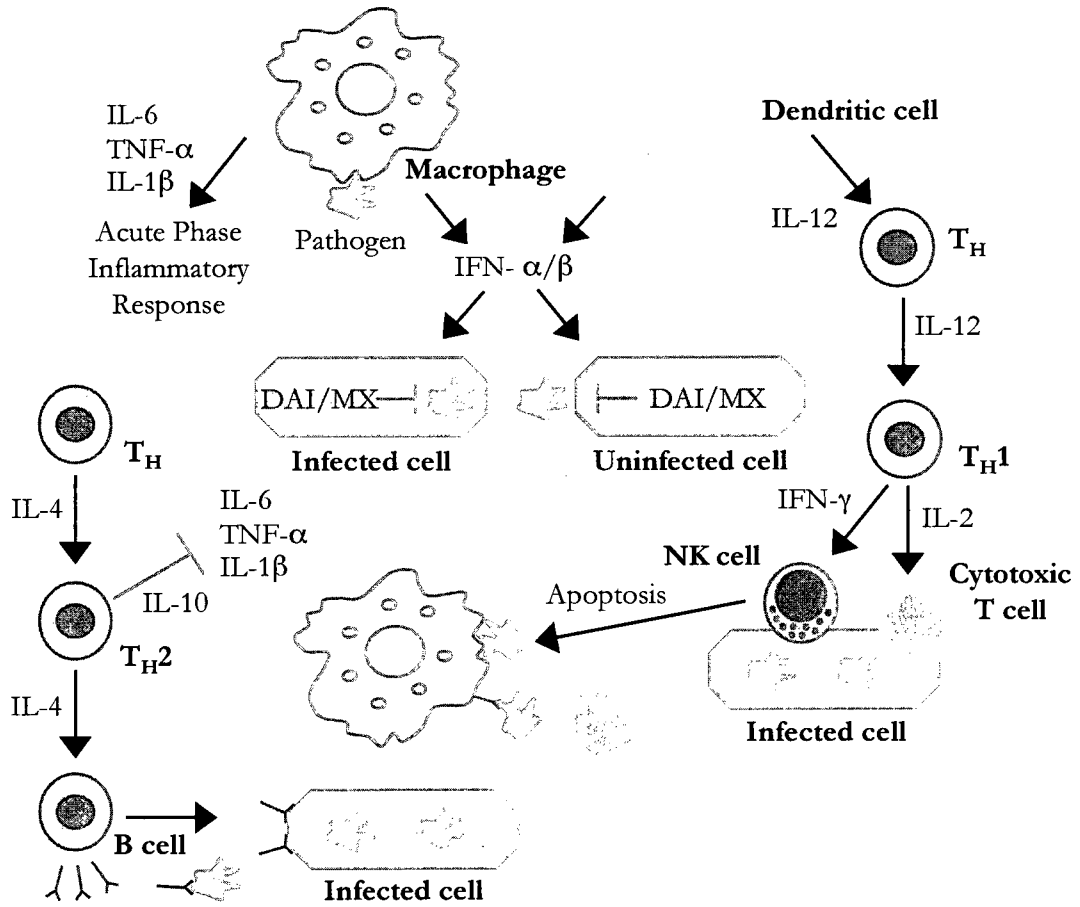
C.



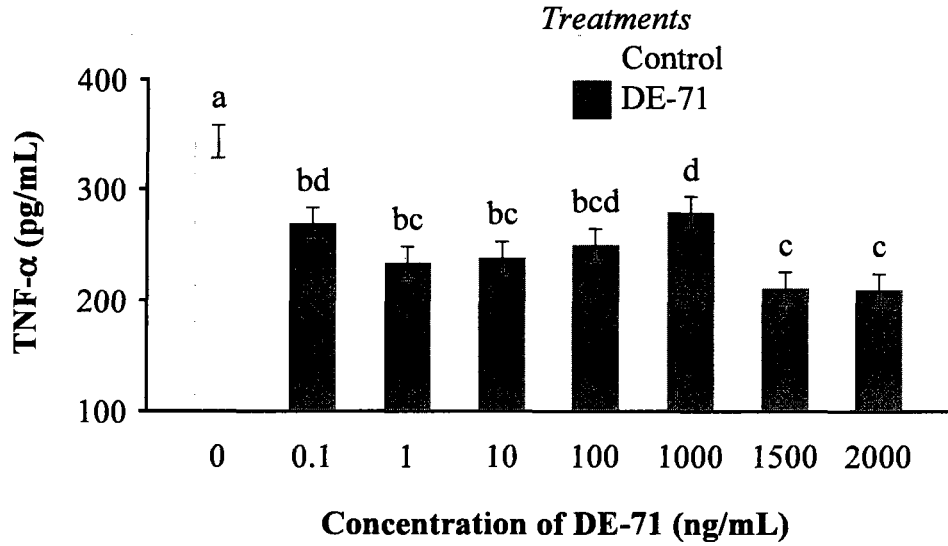
**Figure 5.** The intricate network of the immune system (adapted from figure created by Amy Moore, Ph.D.).



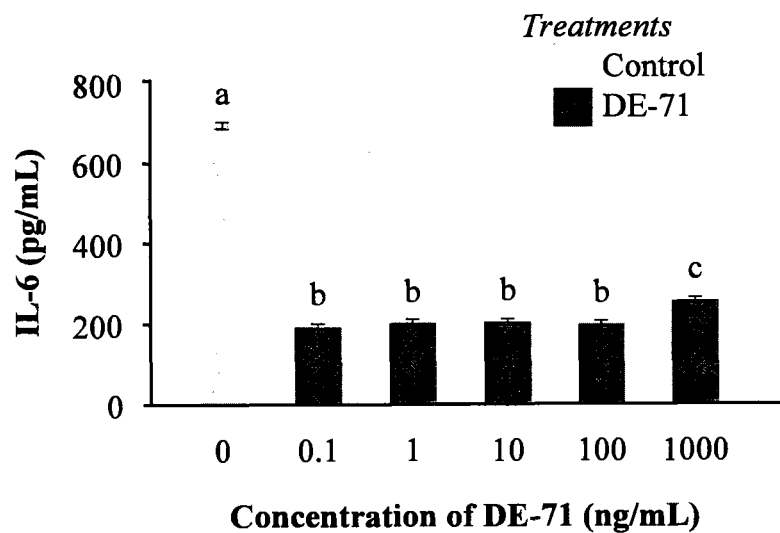
**Figure 6.** Overview of the immune defense mechanisms against respiratory pathogens [adapted from (31)].



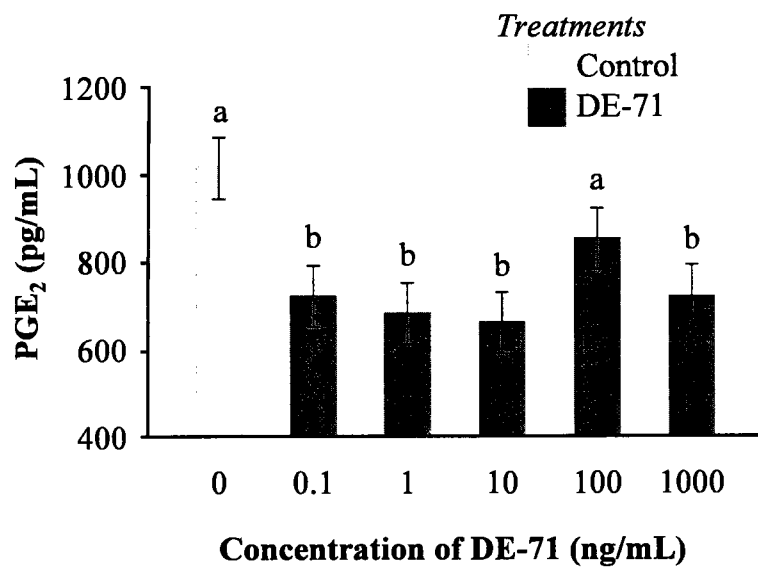
**Figure 7.** Effects of PBDEs on TNF- $\alpha$  release by activated porcine alveolar macrophages. All treatments included PMA (10  $\mu$ L/mL). Values are means  $\pm$  SE; n = 18 (0.1-2000 ng/mL) and n = 27 (0 ng/mL). Means with different letters are significantly different ( $p < 0.05$ ).



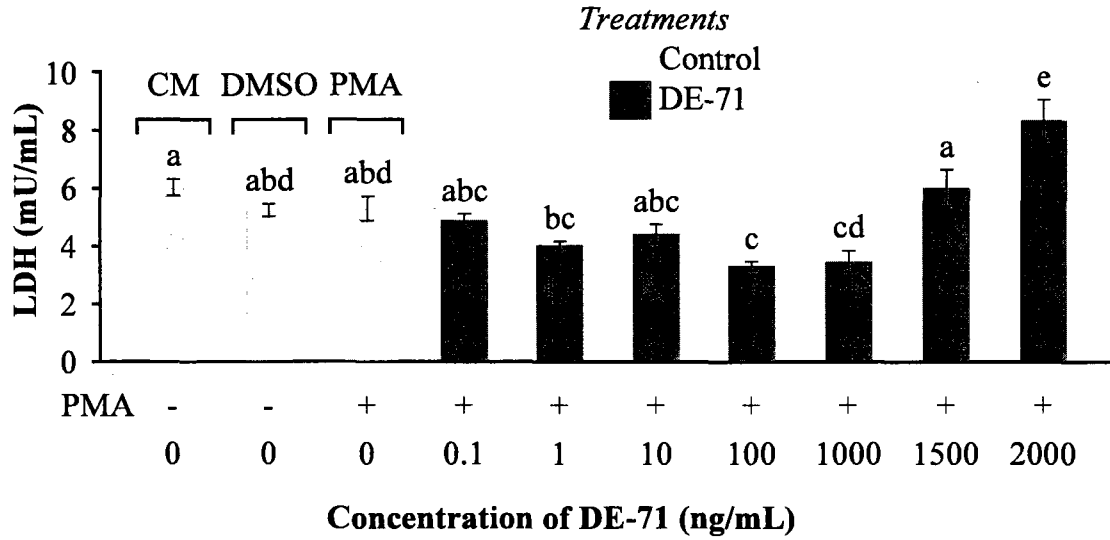
**Figure 8.** Effects of PBDEs on IL-6 release by activated porcine alveolar macrophages. All treatments included PMA (10  $\mu$ L/mL). Values are means  $\pm$  SE; n = 15/treatment. Means with different letters are significantly different ( $p < 0.05$ ).



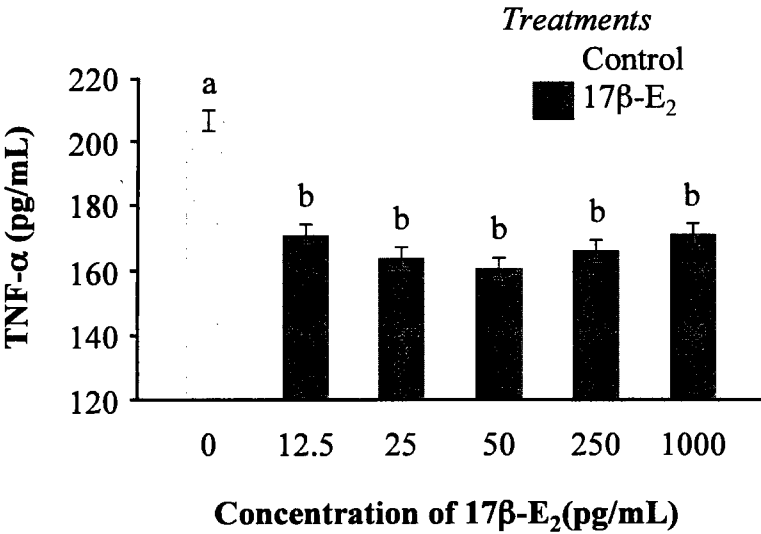
**Figure 9.** Effects of PBDEs on PGE<sub>2</sub> release by activated porcine alveolar macrophages. All treatments included PMA (10 μL/mL). Values are means ± SE; n = 18/treatment. Means with different letters are significantly different (*p* < 0.05).



**Figure 10.** Lactate dehydrogenase (LDH) cytotoxicity. Cells were exposed to culture medium (CM); 1% dimethyl sulfoxide (DMSO); phorbol 12-myristate 13-acetate (PMA) (0.01 mg/mL) and ionomycin (Io) (0.05 mg/mL) in 1% DMSO; and PMA plus seven different concentrations of DE-71. Values are means  $\pm$  SE; n=8/treatment. Means with different letters are significantly different ( $p < 0.05$ ).

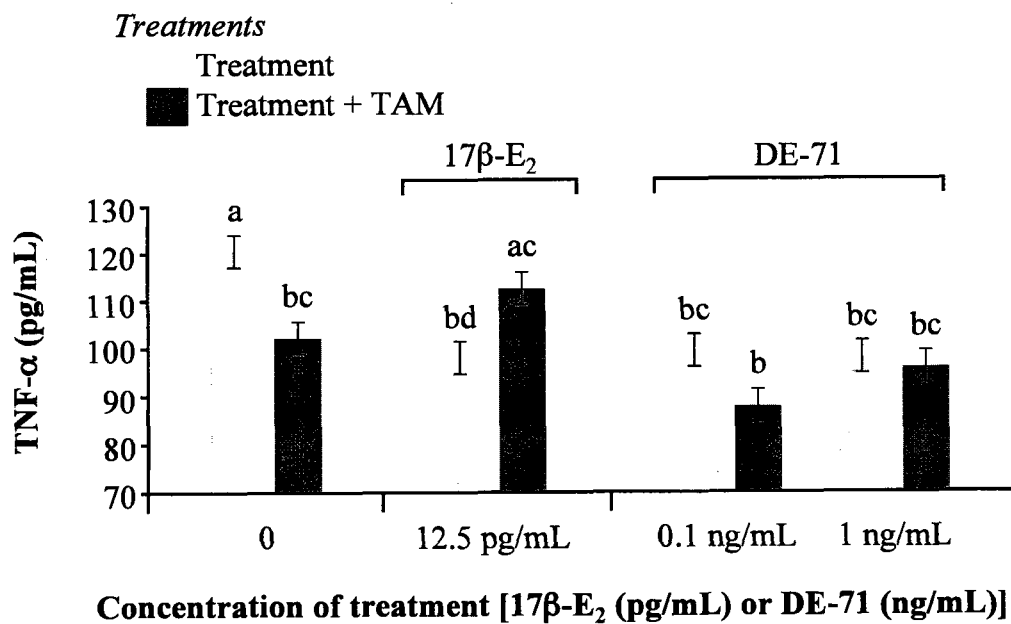


**Figure 11.** Effects of  $17\beta$ -estradiol ( $17\beta$ -E<sub>2</sub>) on TNF- $\alpha$  release by activated porcine alveolar macrophages. All treatments included PMA (10  $\mu$ L/mL). Values are means  $\pm$  SE; n = 5/treatment. Means with different letters are significantly different ( $p < 0.05$ ).





**Figure 12.** Effects of tamoxifen (TAM) on TNF- $\alpha$  release by activated porcine alveolar macrophages. All treatments included PMA (10  $\mu$ L/mL). Values are means  $\pm$  SE; n = 9/treatment. Means with different letters are significantly different ( $p < 0.05$ ).



## **APPENDIX**

### Lactate Dehydrogenase (LDH) Cytotoxicity

		LDH activity (mU/mL)
Controls	Culture medium	0.659 ± 0.133
	DMSO	0.511 ± 0.133
	PMA	0.366 ± 0.133
DE-71	0.1 ng/mL	0.554 ± 0.133
	1 ng/mL	0.490 ± 0.133
	10 ng/mL	0.260 ± 0.133
	100 ng/mL	0.197 ± 0.133
	1000 ng/mL	0.261 ± 0.133
	1500 ng/mL	0.156 ± 0.133
	2000 ng/mL	0.156 ± 0.133

Cells were exposed to culture medium; dimethyl sulfoxide (DMSO)(1%); phorbol 12-myristate 13-acetate (PMA) (0.01 mg/mL) and ionomycin (Io) (0.05 mg/mL) in 1% DMSO; and PMA plus seven different concentrations of DE-71. Values are means ± SE; n=8/treatment.

To confirm the results obtained in the first experiment, a second LDH cytotoxicity assay was performed one week later. In the second experiment, no difference in LDH activity was observed between treatments. Despite the conflicting results at the higher concentrations (1500 and 2000 ng/mL) and the magnitude of difference in the levels of LDH in the two LDH cytotoxicity experiments, the trend for the lower concentrations (0.1 – 1000 ng/mL DE-71) remains the same (i.e. a linear decrease in LDH). The disparity of the magnitude of difference in the levels of LDH in the two experiments is most likely due to the time of the assay. In the first LDH experiment, all reagents were prepared, the assay was performed, and the leftover reagents were stored for later use. In the second LDH experiment, the leftover reagents were used – which could explain the decreased sensitivity of the assay.