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Alteration in Basic Macrophage and Lymphocyte Cytokines from Benzene and Phenol in the Drinking Water of Male Institute of Cancer Research Mice

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ALTERATION IN BASIC MACROPHAGE AND LYMPHOCYTE CYTOKINES FROM BENZENE AND PHENOL IN THE DRINKING WATER OF MALE INSTITUTE OF CANCER RESEARCH MICE

by

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ABSTRACT

Alteration in Basic Macrophage and Lymphocyte Cytokines
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Major Professor: Dr. Howard M. Deer
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Groundwater contamination is a concern due to the large number of people that can become exposed to the contaminant. The chemicals benzene and phenol are known groundwater contaminants. The main health problem caused by benzene or phenol is bone marrow toxicity. Benzene and phenol are also immunotoxins reported to cause decreased thymic weights, altered lymphocyte mitogenic responses, and lower antibody production.

Cytokines are key signaling molecules produced by the cells of the immune system to activate other cells in the immune system, produce antibodies, and recruit other cells to sites of inflammation. The purpose of this study was to determine if exposure to benzene or phenol in drinking water for 30 days could lead to alterations in IL-1, IL-6,

and TNFα production in *in vitro* activated murine macrophages, or in IL-2, IL-3, and IFNγ production in *in vitro* activated murine lymphocytes. Cytokine mRNA and protein production were evaluated to determine if any alteration occurred.

Benzene and phenol exposure resulted in significantly decreased thymus weights. Interleukin-2 mRNA production was increased at the medium dose (200 mg/L) but the IL-2 protein secreted from the lymphocytes of benzene-treated mice was unchanged. The macrophages from benzene-treated mice showed a decrease at all dosage levels in both TNFα mRNA and protein production. These macrophages also produced increased IL-1α mRNA at the medium benzene concentration, although this increase did not mean an increase of IL-1α protein secreted. Mice given phenol at the medium (20 mg/L) and high (100 mg/L) dosages had decreased 30-day body weights. The production of IL-3 mRNA was decreased in the lymphocytes of mice receiving both low and high concentrations of phenol. Lowered TNFα mRNA values were observed in the macrophages from phenol-treated mice. Interleukin-1α mRNA production was increased in the macrophages of mice given the low (5 mg/L) dose of phenol. The TNFα cytokine protein was decreased at the low and medium doses, and the IL-1α protein level was decreased at the medium and high doses. The results indicate that benzene and phenol in groundwater should continue to be a concern for public and regulatory agencies.

(188 pages)

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Jay C Albretsen

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LIST OF ABBREVIATIONS

AAALAC	American Association for the Accreditation of Laboratory Animal Care
ACGIH	American Conference of Governmental Industrial Hygienists
APC	Antigen Presenting Cells
cAMP	Cyclic Adenosine Monophosphate
Con A	Concanavalin A
CPSC	Consumer Product Safety Commission
CSF	Colony Stimulating Factor
CTL	Cytotoxic Lymphocytes
DNA	Deoxyribonucleic Acid
Δ9-THC	Δ9-Tetrahydrocannabinol
ELISA	Enzyme-linked Immunosorbant Assay
GM-CSF	Granulocyte/Monocyte Colony Stimulating Factor
G-CSF	Granulocyte Colony Stimulating Factor
ICR	Institute of Cancer Research
IDO	Indole Amine 2,3-dioxygenase
IFNγ	Interferon Gamma
IL-1	Interleukin-1
IL-2	Interleukin-2

IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
I.P.	Intraperitoneal
LPS	Lipopolysaccharide
LTD4	Leukotriene D4
M7G	7-Methylguanylate
MHC I	Major Histocompatibility Complex Class I
MHC II	Major Histocompatibility Complex Class II
mRNA	Messenger Ribonucleic Acid
NFAT	Nuclear Factor of Activated T-cells
NF-KB	Nuclear Factor Kappa B
NK	Natural Killer Cells
PCR	Polymerized Chain Reaction
OBP	Oct-1 Binding Proteins

OD	Optical Density
PGE_2	Prostaglandin E_2
PHA	Phytohemagglutinin
PWM	Pokeweed Mitogen
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCE	Sister Chromatid Exchange
SRBC	Sheep Red Blood Cell
TFGβ	Transforming Growth Factor Beta
T_H1	T Helper 1 Cells
T_H2	T Helper 2 Cells
TLV	Threshold Limit Value
TNF	Tumor Necrosis Factor
TNFα	Tumor Necrosis Factor Alpha
UTR	Untranslated Region

CHAPTER 1

INTRODUCTION

The immune system is a remarkably complex combination of cells, regulatory proteins, and immunoglobulins. Our immune system has the direct benefit of protecting the body from disease and damage that can be caused by the millions of different antigens that exist in our environment. Because the immune system is constantly searching for antigens, it is designed to contact and remove these foreign substances quickly to prevent disease. Thus, the immune system is often activated by even small amounts of antigen or toxin exposed to the body.

Immunotoxicology is the science of determining how drugs, xenobiotics, and chemicals affect the immune system. An increasing number of toxins are being added to the list of immunotoxins every year. While there are many studies and much research in immunotoxicology, relatively little is known about the cellular mechanisms of a toxin's actions on the immune system. For example, benzene exposure is known to decrease total lymphocyte numbers as well as several subpopulations of lymphocytes (Luan, 1992). This decrease may be from depressed or altered cytokine production or because of another mechanism yet to be defined.

Cytokines are soluble, key signaling proteins produced by macrophages, lymphocytes, and several other cells in the body. They allow communications between macrophages and lymphocytes to occur, thus enabling the immune system to develop the necessary immunity against foreign antigens. Any alteration in cytokine production could

result in immunosuppression or immunostimulation, thereby decreasing or increasing macrophage and lymphocyte numbers. Benzene and phenol are known to decrease lymphocyte populations. A possible mechanism for the immunotoxicity of benzene and phenol could be because of their interference with normal cytokine production, thus preventing lymphocyte proliferation.

The Immune Response

The normal immune response can be divided into two categories: innate and adaptive immunity. Innate immunity refers to host defenses that mammals are all born with and this capability does not change in response to any particular antigen. Examples of innate immunity include phagocytosis of bacteria by macrophages and release of nonspecific acute phase proteins by the liver. In addition, complement proteins are always present in the plasma and will bind to any pathogen causing a cascade of events that eventually destroy the pathogen (Janeway and Travers, 1994). Innate immunity has the important role of providing immune protection for the host as soon as a pathogen enters the body. Principle cells in the innate immune response are macrophages and neutrophils (Gordon *et al.*, 1995; Tizard, 1992).

The adaptive immune response begins much later than the innate immune response, usually about 96 hours after a pathogen has entered the body. One of the most important properties of the adaptive immune response is the development of immunologic memory. Immunological memory gives the host the ability to respond more

rapidly to pathogens the second time. However, in order for the cells of adaptive immune response to become activated, the cells and stimulatory molecules of the innate immune response are needed. Lymphocytes are key cells in the adaptive immune response (Janeway and Travers, 1994).

Two signals are required by lymphocytes (T-cells and B-cells) before they become activated. The first signal is when an antigen binds to the antigen receptor on the cell surface of the lymphocyte. The second signal is known as a co-stimulatory signal and is produced only by certain cells, known as professional antigen presenting cells (APC). Macrophages, dendritic cells, and B-cells are the three types of APCs (Tizard, 1992). When an APC traps and presents an antigen to a T-cell, the APC also provides the necessary co-stimulatory signal for the T-cell to begin producing other regulatory proteins or cytokines. B-cells are activated to produce antibodies and macrophages are stimulated to become cytotoxic only after receiving the proper signal, or cytokine, from the T-cell (Germain, 1994; Janeway and Travers, 1994; Manthey *et al.,* 1994; McKean *et al.*, 1981). Any modulation in the production of these cytokines will ultimately lead to immunosuppression or immunostimulation (Batiuk *et al.,* 1995; Descotes and Vial, 1994; Cockfield *et al.,* 1993; Doherty *et al.,* 1993; Brabletz *et al.,* 1991; Emmel *et al.*, 1989). Thus, toxins, which are known immunomodulators, might be causing their immunotoxicity by interfering with normal cytokine production.

Cytokines are soluble regulatory proteins produced by many cells when they are given the proper stimulus (Zhu *et al.*, 1994b; Tizard, 1992). Cytokines play very important roles in promoting cell growth, differentiation, and activation of immune cells (Zhang *et al.*, 1994; Nussler and Thomson, 1992; Tizard, 1992). However, the immune system is not affected the same by all cell types that produce cytokines. For example, the major subtypes of T helper cells produce both similar and unique cytokines. The T_H1 cells produce interleukin-2 (IL-2) and interferon-γ (IFNγ). The T_H2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-10 (IL-10). Interleukin-3 and TNFα are produced by both T-cell subsets (Lawrence *et al.*, 1996; Nussler and Thomson, 1992; Street and Mosmann, 1991; Mosmann *et al*, 1991; Akira *et al.*, 1990; Mizel, 1989; Cher and Mosmann, 1987). These regulatory factors act on different cell types by autocrine or paracrine activity. Since distinct groups of cytokines are produced by these T-cell subsets, different cells are activated or suppressed by each T-cell subset. Macrophages are activated by T_H1 cells and T_H2 cells are suppressed. Conversely, T_H2 cells activate B-cells and suppress T_H1 cells (Janeway and Travers, 1994; Abbas *et al.*, 1991). Ultimately, the desired effect is to rid the body of foreign antigens.

Cytokines have also been shown to be multifunctional, and often cytokines have overlapping functions. For example, IL-1 and TNFα are potent inducers of IL-6. Interleukin-6 inversely regulates TNFα production and promotes the expression of IL-2 receptors on T-cells. Interleukin–2 induces IFNγ production, which in turn promotes the

expression of IL-2 receptors on T-cells. Interferon γ also suppresses the proliferation of T_H2 cells (Kishimoto, 1992). Some cytokines (like IL-2) can stimulate, or other cytokines (like IL-10) can suppress the immune system . In fact, some cytokines, like transforming growth factor-beta (TFG β), stimulate some cells and suppress others (Oppenheim and Neta, 1994; Nussler and Thomson, 1992). This pleiotropy of activity is not only exerted on the cells of the immune system, but also on many target cells and tissues, including the nervous system and hematopoietic system (Doherty *et al.,* 1993; Nussler and Thomson, 1992; Mizel, 1989). Modulation of the immune system requires several key cytokines and any imbalances in cytokine production can lead to problems such as immunodeficiency or graft rejection (Grimm *et al.*, 1995; Libertin *et al.*, 1994). Figure 1-1 lists key cytokines produced by macrophages and lymphocytes. These cytokines will be discussed individually in further detail.

Interleukin-1

Interleukin-1 is the term applied to two separate polypeptides (IL-1α and IL-1β). Both forms have a wide spectrum of inflammatory, metabolic, physiologic, hematopoietic, and immunologic functions. Interleukin-1 is produced by macrophages/monocytes, neutrophils, endothelial cells, epithelial cells, and fibroblasts (Nussler and Thomson, 1992; Akira *et al.,* 1990; Mizel, 1989). A key feature of IL-1 is its action on several inflammatory cell types to stimulate arachadonic acid metabolism and

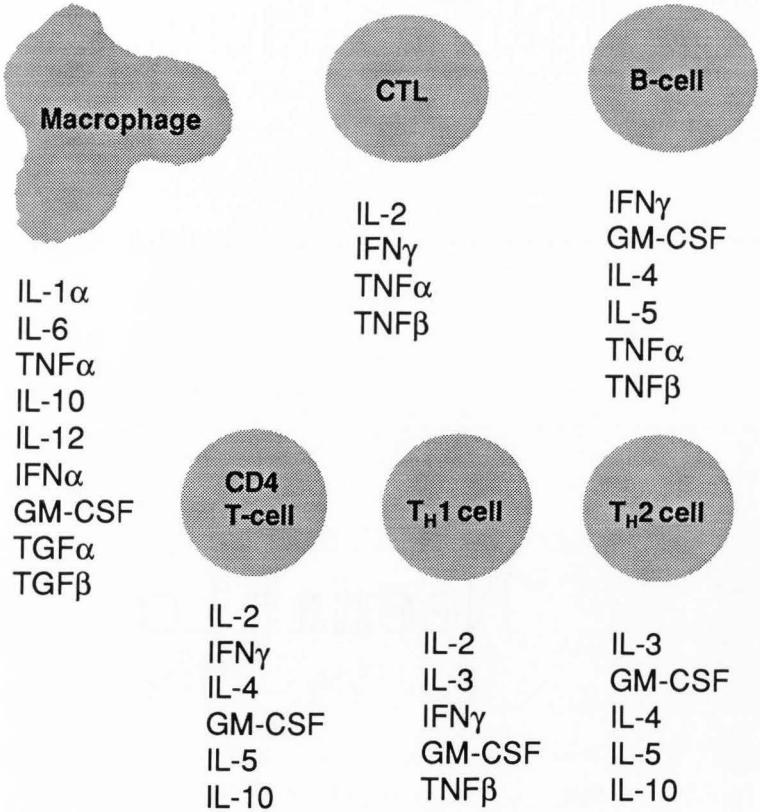

FIG. 1-1. Some cytokines produced by key immune system cells. Interleukin 1α, IL-6, TNFα, IL-2, IL-3, and IFNγ will be discussed in greater detail.

inflammatory protein production (Dinarello, 1992; Mizel, 1989). Interleukin-1 participates in T- and B-cell activation. Consequently, IL-1 receptors are expressed in resting lymphocytes. The T-cell activating property of IL-1 is measured by its ability to act in a co-stimulatory assay with suboptimal concentrations of antigens (Dinarello, 1992). Interleukin-1 amplifies T-cell activation by inducing the expression of IL-2 and IL-2 receptor genes particularly in conjunction with antigens, mitogens, calcium ionophores, or stimulators of protein kinase C (Houssiau *et al.*, 1989; Simic and Stobic, 1985). This cytokine also stimulates the production of IL-4 and assists in activating B-cells to produce antibodies (Mizel, 1989). The production of Granulocyte/Monocyte Colony Stimulating Factor (GM-CSF) and IL-3, as well as several other colony stimulating factors (CSF), is induced by IL-1 in the process of hematopoiesis (Bagby, 1989; Mizel, 1989). While IL-1 induces the production of many cytokines, it is the down-regulating properties of IL-1 on TNFα and the production of IL-1 receptor agonists that are believed to prolong survival in lethal infections of neutropenic mice (Vogels *et al.*, 1994).

Interleukin -6

Interleukin-6 (IL-6) is a glycoprotein with pleiotropic activities central to host defenses (Akira *et al.*, 1993). It is produced by a wide variety of cells, including both T and B-cells, macrophages/monocytes endothelial cells, fibroblasts, and several neoplastic cells (Akira *et al.*, 1990, 1993; Mizel, 1989). Among its varied activities, this cytokine

can act as a pyrogen, and stimulate mitogen-activated B-cells to produce IgM, IgG, and IgA, without causing B-cell proliferation (Kishimoto, 1992; Muraguchi *et al.*, 1988; Beagley *et al.*, 1989). This is consistent with the finding that IL-6 receptors are expressed on activated B-cells but not on resting B-cells (Taga *et al.*, 1987). While IL-1 is one of the most common stimulants of IL-6 production, other IL-6 stimulants include TNFα, platelet derived growth factor, virus infections, double stranded RNA, and cAMP (Nussler and Thomson, 1992; Tizard, 1992; Mizel, 1989). Interleukin-6 along with IL-3 will cause the induction of bone marrow stem cell differentiation and proliferation (Suzuki *et al.*, 1989). It also enhances phagocytosis and the expression of a number of macrophage differentiation antigens, such as Mac-1 and Mac-3 receptors, nonspecific esterase, lysozyme, and major histocompatibility complex class I (MHC I), thus assisting in the functional differentiation of the mature macrophage (Akira *et al.*, 1993).

Tumor necrosis factor alpha

Tumor necrosis factor alpha (TNFα) is an essential part of host defenses against viral, bacterial, parasitic, and autoimmune diseases (Mizel, 1989). It is produced by macrophages/monocytes, activated lymphocytes, natural killer (NK) cells, and keratinocytes. The production of TNFα is triggered by IL-2, interferon, bacterial lipopolysaccharides, viruses, bacterial toxins, fungal toxins, parasitic toxins, and some neoplastic cells (Cockfield *et al.*, 1993; Han *et al.*, 1990; Mizel, 1989; Hogan and Vogel, 1988). Conversely, many steroids inhibit TNFα production. Interleukin-2 and IFN

regulate TNF receptor expression (Le-Contel *et al.*, 1993; Nussler and Thomson, 1992). Tumor necrosis factor-α induces the synthesis of several cytokines, including IL-1, IL-2, IL-7, and IL-12. It has cytotoxic activity on malignant or infected cells, and activates cytotoxic lymphocytes (CTL) (Akira *et al.*, 1990; Fiers, 1991). Small doses of TNFα will cause both T- and B-cell proliferation, and will stimulate IL-2-induced immunoglobulin production and natural killer (NK) cell activity. Larger doses of TNFα will cause shock, cachexia, and graft rejection if given for extended periods of time (Smith *et al.*, 1995; Tizard, 1992). In addition, TNFα causes the release of acute phase proteins (proteins that play a necessary role in host defenses) by the liver (Nakagawatosa *et al.*, 1995).

Interleukin-2

Interleukin-2 (IL-2) is critical in initiating and potentiating the immune response, perhaps more than any other cytokine secreted by lymphocytes. This cytokine is produced mainly by activated T-cells, particularly T_H1 cells, and is also produced by cytotoxic lymphocytes (Janeway and Travers, 1994). The mechanism of IL-2 activation is the best understood of all known cytokines (Taniguchi and Minami, 1993). Briefly, IL-2 stimulates T-cell growth and differentiation and stimulates B-cell growth and immunoglobulin (particularly the J chain) synthesis (Zhu *et al.*, 1994a; Janeway and Travers, 1994). In addition, IL-5, TNFα, and IFN production is induced by IL-2.

Interleukin-2 receptor and TNFα receptor expression are regulated by IL-2 (Taniguchi and Minami, 1993; Janeway and Travers, 1994). Interleukin-2 also assists in the long-term growth of normal and antigen specific CTLs (Gillis *et al.*, 1978). In response to *Candida albicans*, IL-2 is one of the first cytokines to be produced and this production lasts for a significant period of time (Rosati *et al.*, 1995). Conversely, IL-2 production is inhibited by enteropathogenic *E. coli* (Klapproth *et al.*, 1995). By suppressing IL-2 production, pathogens are able to create life-threatening problems in the host.

Interleukin -3

Interleukin-3 (IL-3) is produced by antigen-stimulated T-cells, specifically T_H1 and T_H2 cells (Janeway and Travers, 1994). It is probably not necessary for hematopoiesis but serves to recruit additional cells (Tizard, 1992; Smith and Rennick, 1986). Interleukin-3 stimulates macrophage cytotoxicity and phagocytosis. It also induces Ia and LFA-1 adhesion molecule expression on macrophages, which increases the antigen presenting capacity of the macrophage to the T helper cells (Frendl and Beller, 1990). Interleukin-3 will also cause immunoglobulin secretion by B-cells and the proliferation of eosinophils, neutrophils, and monocytes (Tizard, 1992; Nussler and Thomson, 1992; Mizel, 1989). Interleukin-3 has been implicated as a contributing element in leukemogenesis, where the IL-3 gene is aberrantly activated and thus results in continuous cell growth (Mizel, 1989; Schrader, 1986). In the study by Frendl (1992), a dramatic increase in IL-1, IL-6, and TNF in peritoneal macrophages stimulated by IL-3

was noted. This suggests IL-3 to be a key cytokine causing the production of IL-6 and leading to macrophage activation (Ferran *et al.*, 1994).

Interferon gamma

Interferon gamma (IFNγ) is produced by T lymphocytes, principally T_H1 cells (Kasahara *et al.*, 1983; Janeway and Travers, 1994; Tizard, 1992). Interferon gamma is best known for its antiviral activities, although it also regulates the function of several cell types important in the immune response (Beigneux *et al.*, 1995; Fishman-Lobell *et al.*, 1994; Reynolds *et al.*, 1987; Friedman and Vogel, 1983). It serves to activate T-cells by stimulating the expression of IL-2 receptors. This expression of receptors allows the T-cells to proliferate and as a result these T-cells stimulated by IL-2 continue to produce IFNγ (Farrar *et al.*, 1986). In addition, IFN-γ regulates IL-3 production (Ferran *et al.*, 1994). This creates a network of interdependent T-cell responses and results in continued cytokine production. The resulting network increases tumor killing activity, the production of MHC class II, and phagocytosis in macrophage cells. It also enhances CTL and NK activation and growth (Trinchieri and Perussia, 1985). Interferon gamma induces oxygen-dependent or respiratory burst-dependent microbicidal activity in the macrophage. The macrophage is also stimulated by IFNγ to enhance indole amine 2,3-dioxygenase (IDO) action resulting in increased oxygen independent macrophage microbicidal activity (Pfefferkorn, 1984). Also, the synthesis of IgE is inhibited by IFNγ. In clinical trials, this down regulation of IgE resulted in the improvement of patients with

atopic dermatitis (Tan *et al.*, 1992). Finally, IFNγ has been reported to stimulate endothelial cells, causing a cascade of events and other cytokine production, which leads to graft rejection (Orosz, 1994). A summary of the cytokine communication network during an immune response as discussed previously is illustrated in Figure 1-2.

Regulation of Cytokine Gene Expression

Cytokines are essential signaling molecules in the development of the immune response. Each cytokine shows pleiotropism by acting on several target cells. Different cytokines show redundancy by acting on the same target cell as several other cytokines, each producing the same effect. Obviously, this is primarily the reason cytokines are regulated so tightly at transcriptional and translational levels. Without such regulation, any overproduction, or underproduction in a particular cytokine could result in very harmful consequences to the body. An example of regulation needed by cytokines is when TNF and IL-1 are overproduced in inflammatory autoimmune diseases, such as rheumatoid arthritis (Brennan and Feldman, 1992), or in the case of necrotizing fasciitis and myositis where exotoxin A produced by Strep A bacteria triggers an out-of-control production of TNF and several other cytokines (Nowak, 1994). Such immunosuppressive drugs as cyclosporin A and FK 506 downregulate IL-2 and other cytokines. This has been shown to be the main reason for the immunosuppression caused by these drugs (Hess *et al.*, 1988). Regulation of cytokine expression can occur during mRNA synthesis (transcriptional level), during mRNA processing

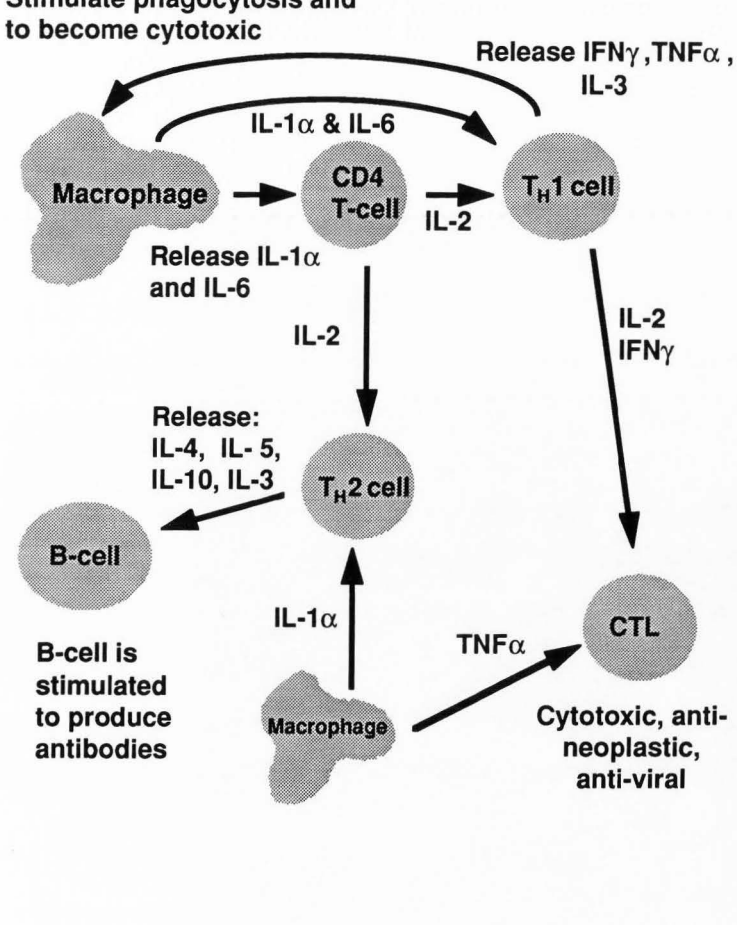

FIG. 1-2. The cytokine/cell communication network. Only IL- 1α, IL-6, TNFα, IL-2, IL-3, and IFNγ cytokines are mentioned.

(posttranscriptional level) , during protein synthesis (translational level), or during protein processing and packaging for export from the cell (posttranslational level).

Like other eukaryotic genes, cytokine genes contain regulatory elements such as promoters, enhancers, and silencers. These elements are composed of discrete DNA sequences called *cis*-acting elements, which are binding sites for sequence specific DNA binding proteins called *trans*-acting proteins (Darnell *et al.*, 1990). *Trans*-acting factors such as Nuclear Factor of Activated T-cells (NFAT), AP-1, AP-3, Nuclear Factor kappa B (NF-κB), NF-IL6, and Oct-1 Binding Proteins (OBP) bind to the upstream regions of the promoters on cytokine genes, thus regulating the start of transcription by RNA polymerase in a differential way (Riegel *et al.*, 1992; Chen-Kiang *et al.*, 1993; Rao, 1994). Drugs such as cyclosporin A and FK 506 have been shown to repress the induction of these transcriptional factors, thus preventing cytokine mRNA transcription (Woo *et al.*, 1995; Brabletz *et al.*, 1991; Emmel *et al.*, 1989).

A very active area of research is dealing with posttranslational mechanisms regulating mRNA stability and thereby regulating cytokine gene expression. Reiterated AUUUA pentamers within the 3'-untranslated regions (UTR) of IL-1α, IL-1β, IL-2, and IL-3, TNF, IFNγ, and GM-CSF appear to function as *cis* regulatory elements (Henics *et al.*, 1994; Han *et al.*, 1990). These sequences are common in mRNAs encoding proteins related to the inflammatory response (Janeway and Travers, 1994; Caput *et al.*, 1986) and they regulate both the turnover and translation of these cytokine mRNAs. The regulatory

elements control mRNA stability by binding directly to the AU-rich 3'-UTR and increase the stability of mRNA in neoplastic cells (Henics *et al.*, 1994). However, other research has shown that cyclohexamide-induced superinduction of IFNγ and TNF was due to the inhibition of the synthesis of RNase proteins responsible for the degradation of mRNA, targeting the AU rich region (Cockfield *et al.*, 1993). Further studies are in progress to characterize different AU-binding proteins and their role in the regulation of mRNA turnover and translation.

In eukaryotes, several mRNA structural characteristics influence translation. The mRNA 5' 7-methylguanylate (m7G) cap, the sequence surrounding the AUG start codon, the leader length, and the secondary structure of the mRNA both upstream and downstream of the AUG start codon all influence the initiation and progression of translation (Kozak, 1991; Darnell *et al.*, 1990). Furthermore, the secondary structural features of mRNA at the 3'-UTRs are also important for the translation of numerous cytokine mRNAs (Shaw and Kamen, 1986). Han *et al.* (1990) found that endotoxin-treated macrophages had repressed TNF biosynthesis because of the synthesis of an element binding to the 3' UTR of the TNF mRNAs.

Interleukin-1α and TNF are also regulated at the protein processing and secretion (posttranslational) levels. It is known that IL-1α and IL-β do not possess the typical NH_2 terminal hydrophobic secretary signal sequence necessary to allow IL-1 to translate directly into the endoplasmic reticulum via the classical exocytic secretory pathway

(Siders *et al.*, 1993; Hazuda *et al.*, 1991; Giri *et al.*, 1985). Newly synthesized IL-1α precursors are found in the cytoplasm. A protease enzyme, calpain, cleaves IL-1α to its mature form that has the necessary conformation and secretory signal (Siders *et al.*, 1993; Carruth *et al.*, 1991), allowing this mature IL-1α to be preferentially secreted by macrophages/monocytes (Siders *et al.*, 1993; Hazuda *et al.*, 1991). This preferential secretion of the mature IL-1α is shown by using Pentamidine, an aromatic diamine currently used in the treatment of *Pneumocystis carinii*. Pentamidine also specifically inhibits the cleavage of pro-IL-1α by inhibiting protease activity, thus inhibiting IL-1α secretion (Rosenthal *et al.*, 1991). A similar experiment with Δ^9-Tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of marijuana, and TNF inhibition has been researched. Fischer-Stenger *et al.* (1993) determined that Δ^9-THC blocks the intracellular conversion of pro-TNF to the mature secreted TNF, thus resulting in the suppression of macrophage tumoricidal activity.

Benzene

Chemical and physical properties

Benzene (C_6H_6) is an aromatic hydrocarbon with a molecular weight of 78.1. It is a volatile, colorless, and highly flammable liquid hydrocarbon. Pure benzene has a melting point of 5.5°C and a boiling point of 80.1°C. Compared to water, benzene has a density of 0.879 at 20°C (U.S. Environmental Protection Agency, 1980). Benzene is

soluble in water at concentrations that are toxic to aquatic organisms. Because of its lipophylic characteristic, benzene accumulates in tissues with a high lipid content. In addition, benzene also accumulates in areas of high metabolism such as the liver (Fishbein, 1984). The vapor pressure of benzene is 74.6 mm Hg at 20°C, and the solubility of benzene in water is 1780 mg/L at 25°C (U.S. Environmental Protection Agency, 1980). This indicates that benzene with its relatively high solubility could be easily washed out of the air and then easily evaporate back into the atmosphere because of its volatile nature. Benzene is miscible with acetone, alcohol, carbon disulfide, carbon tetrachloride, chloroform, ether, glacial acetic acid, and oils. The typical metabolic reactions of aromatic hydrocarbons (such as benzene) include chlorination, nitration oxidation, and sulfonation (Fishbein, 1984; U.S. Environmental Protection Agency, 1980). Benzene is also photooxidized in air and biodegraded in the environment (Korte and Klein, 1982). Some properties of benzene (and phenol) are listed in Table 1-1.

TABLE 1-1
Some Properties of Benzene and Phenol

Chemical	Structure	Mol. Weight	Melting Point	Boiling Point	Density (g/ml) at 20°C	Vapor Pressure (mmHg)	Solubility (g/L) at 25°C
Benzene	C_6H_6	78.1	5.5°C	80.1°C	0.879	74.6	1.8
Phenol	C_6H_6O	94.1	43°C	182°C	1.07	0.3513	66.7

Occurrence and usage

Benzene is manufactured primarily in the United States, Europe, and Japan. During 1995, benzene was the 16th most frequently produced chemical in the United States with 15.97 billion pounds made (Kirschner, 1996). One of the reasons for benzene's frequent production is because it is used in the manufacturing of many other products. Items such as pesticides, solvents, dyes, adhesives, rubber, medicines, and some food additives are all derived from benzene (Fishbein, 1984). In addition to already being a component of crude oil, benzene is added to unleaded gasoline to increase the octane rating. In the United States, the average amount of benzene in gasoline is approximately 0.8%. This amount is compared to European gasolines, which contain 5% benzene (Fishbein, 1984).

Most benzene losses to the environment occur into the air. A significant portion of this loss comes from oil refining, benzene production, and the production of other chemicals from benzene. Even more significant is the emission of benzene to the air from automobile exhaust. Benzene makes up 4% of the exhaust from a gasoline engine (U.S. Environmental Protection Agency, 1980). Losses of benzene to water in the U.S. are less but still significant. These losses are mainly from benzene production, other chemical production, and oil spills (Fishbein, 1984). Recently, the Environmental Protection Agency has been concerned with old and/or leaking underground gasoline storage tanks. This has been a source of groundwater contamination due to the direct link of the leaking storage tank directly into unconfined aquifers. Benzene is a nonaqueous phase liquid

(NAPL) and will follow water flow from groundwater or rainwater directly into the aquifer (Piver, 1992). Benzene concentrations in drinking water have been measured as high as 20,230 parts per billion (ppb) (range 0.6-20,230 ppb) or 20.23 mg/L (U.S. Office of Technology Assessment, 1984).

Populations at risk

Exposure levels and populations at risk to benzene exposure vary greatly. Obviously, workers at manufacturing plants using benzene are exposed to more benzene than other workers. The American Conference of Governmental Industrial Hygienists (ACGIH) has set the threshold limit value (TLV) for benzene at 10 ppb (ACGIH, 1995). Thus, workers are limited to their occupational exposure hazard from benzene. However, information of benzene concentrations in ambient air is limited. In metropolitan areas and by major roadways, benzene is found in higher concentrations than in other areas (Fishbein, 1984). Thus, motorists are a population at risk of being exposed to more benzene.

Benzene has been identified in cigarette smoke and smoke from other fires. It has been suggested that the average cigarette gives rise to 31 μg of benzene. Benzene in the smoke from other fires, while significant, is only a risk to people who are frequently around fires (Fishbein, 1984).

Benzene is also found naturally in trees, fruits, seeds, dairy products, eggs, and meat (U.S. Environmental Protection Agency, 1980). However, the U.S. Consumer

Product Safety Commission (CPSC) ruled in 1981 that benzene contaminant levels remaining in consumer products were unlikely to result in any significant consumer exposure to benzene (Fishbein, 1984).

Metabolism

Benzene metabolism occurs in the environment as well as in the body (Fishbein, 1984; Korte and Klein, 1982). In the body, most benzene metabolism occurs in the liver, although the bone marrow is also capable of benzene metabolism (Snyder and Kalf, 1994; Sawahata *et al.*, 1985). Benzene is converted to benzene oxide by cytochrome P-450's. The P-450 isoform 2E1 appears to exhibit the greatest affinity for benzene (Snyder and Kalf, 1994). Benzene oxide can undergo spontaneous rearrangement to phenol which may then be hydroxylated by P-450 to hydroquinone. Epoxide hydrolase may also take benzene oxide and convert it to benzene dihydrodiol. Benzene dihydrodiol with the help of a dehydrogenase is then converted to ortho-hydroquinone (catechol). The para-hydroquinone and ortho-hydroquinone intermediates can be further metabolized to reactive semiquinone or quinone products which are believed to be toxic (Snyder and Kalf, 1994; Dean, 1985; Sawahata *et al.*, 1985; Fishbein, 1984). Reactive intermediates of benzene bind to DNA and cause damage in the form of strand breaks and DNA adducts. Quinone reductase reduces quinones to hydroquinone via a 2-electron transfer. These quinone reductases are found mainly in the liver (Snyder and Kalf, 1994; Sawahata *et al.*, 1985). The bone marrow lacks quinone reductases and has peroxidases that enhance the

oxidation of phenol into reactive species (Sawahata *et al.*, 1985). In order to excrete benzene metabolites more readily and prevent reactive intermediate formation, the hydroquinone intermediates are further conjugated in the liver with glucuronides or sulfates (Sawahata *et al.*, 1985; Fishbein, 1984). It is well known that benzene causes bone marrow toxicities. However, even though the liver is the primary location for benzene metabolism, no toxicity to the liver is known. This liver protection is thought to be because of the additional enzymes in the liver, such as quinone-reductase and glutathione transferase, that keep these benzene metabolites in their reduced state and in easily excretable forms. The bone marrow enzymes just metabolize benzene to reactive intermediates (Snyder and Kalf, 1994; Sawahata *et al.*, 1985). These benzene intermediates are implicated in bone marrow toxicity (Manning *et al.*, 1994). A summary of benzene metabolism is given in Figure 1-3.

Benzene toxicity

Benzene is a known hematotoxin. It exerts its greatest effect on hematopoietic organs (Fishbein, 1984; Bolcsak and Nerland, 1983). Chronic benzene exposure results in pancytopenia--a condition with decreased numbers of circulating erythrocytes, leukocytes, and thrombocytes. This condition results in an aplastic anemia and fatty replacement of functional bone marrow (Green *et al.*, 1981). Continued exposure to benzene also leads to decreased numbers of lymphocytes in the blood, the bone marrow,

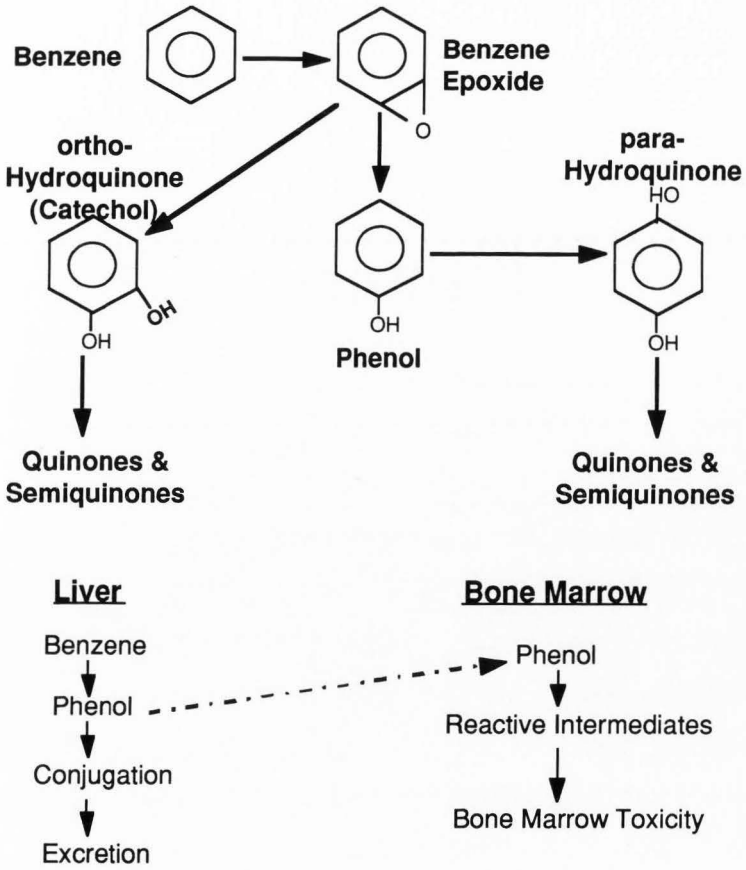

FIG. 1-3. The metabolism of benzene and phenol. The metabolism of benzene and phenol in the liver and in the bone marrow is also compared.

the thymus, and the spleen (Snyder and Kalf, 1994; McMurry *et al.*, 1991; Sawahata *et al.*, 1985; Dean, 1985). Studies have proven that oxidative benzene metabolites, rather than benzene itself, are required to cause these hematopoietic effects (Sawahata *et al.*, 1985; Irons, 1985; Bolcsak and Nerland, 1983).

Stem cell production decreases with benzene exposure as does the number of erythroid and myeloid progenitor cells (Snyder *et al.*, 1994; Dean, 1985; Tunek *et al.*, 1981, 1982) Benzene causes chromosomal loss and breakage in bone marrow cells (Chen *et al.*, 1994) and also inhibits the ability of bone marrow stromal cells to support hematopoiesis. Bone marrow stromal cells provide a supporting matrix for developing hematopoietic cells and they release cytokines regulating cell growth (Snyder and Kalf, 1994; Gaido and Wierda, 1985). They also produce prostaglandin E_2 (PGE_2). This prostaglandin acts as a negative regulator of hematopoiesis. Benzene administration resulted in increased levels of PGE_2 in the bone marrow (Gaido and Wierda, 1985) although benzene toxicity to stromal cells is not solely due to increased PGE_2. Interleukin-1α (IL 1α) is involved in lymphocyte development and the development of the pluripotent stem cell. It is considered a cytokine essential for hematopoiesis. Benzene inhibits the processing of the pre-IL-1α into the mature cytokine, which is capable of stimulating hematopoiesis (Renz and Kalf, 1991). Consequently, inhibiting IL-1α production is another way in which chronic benzene exposure leads to aplastic anemia.

Benzene's carcinogenic properties were first noted in epidemiologic studies done in the 1970's. Since that time benzene-induced leukemia has been reported both in man and animals from chronic benzene exposure (Dean, 1985; Fishbein, 1984). In rodents, lifetime exposure to benzene (300 ppm) resulted in some cases of myelogenous leukemia and an increased incidence of thymic lymphomas (Snyder and Kalf, 1994; Dean, 1985; Rozen and Snyder, 1985). Using the HL-60 promyelocytic leukemic cell line, it was found that benzene inhibits HL-60 differentiation into monocytes, but has no effect on granulocyte differentiation (Oliveira and Kalf, 1992). The HL-60 cell line mimicks the granulocyte/monocyte progenitor cell. Thus, benzene may alter the normal granulocyte/monocyte ratio, which may initiate or influence myeloid leukemogenesis (Synder and Kalf, 1994).

In another study, benzene upregulated leukotriene D4 (LTD4) production, which induces granulocytic differentiation. The production of LTD4 results in effects similar to those of granulocyte colony stimulating factor (G-CSF). However, while benzene does cause granulocyte differentiation, it requires the presence of IL-3 to provide the growth signal (Hazel *et al.*, 1995). Hydroquinone, a benzene metabolite, also induces granulocytic differentiation. Unlike benzene, it activates the LTD4 receptor rather than upregulating LTD4 production (Hazel *et al.*, 1995). Hydroquinone also caused more myeloid progenitor cells to be responsive to granulocyte/monocyte colony stimulating factor (GM-CSF), suggesting that alterations in normal myeloid progenitor cell

populations may be of relevance in the pathogenesis of myelogenous leukemia due to chronic benzene exposure (Irons *et al.*, 1992).

Benzene has also been implicated in causing menstrual disturbances and prenatal toxicities since benzene crosses the placenta. Kuna and Kapp (1981) showed that 50-500 ppm benzene vapor caused fetotoxicity in rats and the 500 ppm benzene vapor manifested a teratogenic potential. However, there has yet to be conclusive evidence to implicate benzene as a teratogen or mutagen (Dean, 1985; Fishbein, 1984). Prolonged benzene administration also causes elevated brain neurotransmitter release (Hsieh *et al.*, 1988a). In addition, nitric oxide production by bone marrow macrophages was increased when mice were exposed to benzene (Laskin *et al.*, 1995). Since nitric oxide suppresses cell growth, this suppression may also contribute to benzene's myelotoxicity (bone marrow toxicity).

Immunotoxicity of benzene

Although the evidence that many chemicals alter the immune system is not well established, benzene has been shown to be immunotoxic in several studies. The potential for immunosuppression is of concern because immunosuppression has been associated with cancer, increased infections, and other pathologic conditions (Luster and Rosenthal, 1993). Benzene caused leukopenia, lymphopenia, decreased splenic cellularity, decreased thymic weights, and decreased lymphocyte responsiveness to lipopolysaccharide (LPS), pokeweed mitogen (PWM), concanavalin A (Con A) and phytohemagglutinin (PHA)

mitogens (McMurry *et al.*, 1991; Hsieh *et al.*, 1988b). Luan (1992) showed that workers exposed to benzene had lymphopenia and a reduced number of both $CD2^+$ and $CD4^+$ lymphocytes. Both $CD8^+$ and $CD57^+$ lymphocyte cell subpopulations were also decreased, but their absolute percentages were unchanged. Thus, the cell-mediated immune response is altered after chronic benzene administration. Humeral immunity is also suppressed as can be seen by the decreased number of plaque forming cells at doses of 40 and 180 mg/kg/day. In addition, the response to sheep red blood cells (SRBC) was significantly depressed at a dose of 180 mg/kg/day (Hsieh *et al.*, 1988b). Benzene at a dose of 8 mg/kg/day resulted in increased responses to LPS, PWM, Con A, and PHA mitogens. At this dose, T lymphocytes were also more cytotoxic than controls, suggesting that at low doses benzene is immunostimulatory and at higher doses benzene is immunosuppressive (Hsieh *et al.*, 1988b). Toluene given with benzene reverses benzene's immunotoxicity (Hsieh *et al.*, 1990). Toluene competitively inhibits benzene biotransformation. Thus, less toxic metabolites of benzene are produced when toluene is given with benzene. The ultimate result is less toxicity due to fewer benzene metabolites being produced (Hsieh *et al.*, 1990).

As was mentioned previously, cytokines are key factors in the suppression or stimulation of all immune cells. Several reports have indicated that benzene results in decreased mature IL-1α production by bone marrow-derived macrophages (Niculescu and Kalf, 1995; Miller *et al.*, 1994). Benzene or its metabolites are known to inhibit calpain

enzyme activity, which processes pre-IL-1α into the mature IL-1α cytokine. Carbonnelle *et al.* (1995) found that both mRNA and protein synthesis were inhibited in human monocytes by hydroquinone a metabolite of benzene. However, a different study, using bone marrow derived leukocytes, found that benzene caused a small but significant increase in the production of IL-1. This same study found that TNFα production also increases, but benzene had no effect on IL-6 production (MacEachern *et al.*, 1992). Possible reasons for this discrepancy are that slightly different doses of benzene were used, different breeds of mice were used, and some dosage regimens were different. Benzene also suppressed the growth of an IL-2-dependent cell line, indicating that benzene suppressed IL-2 secretion by lymphocytes (Fan, 1992; Hsieh *et al.*, 1990).

Phenol

Chemical and physical properties

Phenol (C_6H_6O) is a monohydroxy derivative of benzene. It is a clear, colorless, hygroscopic, crystalline solid (at 25°C) with a molecular weight of 94.1, a melting temperature of 43°C, and a boiling point of 182°C. Phenol has a distinct tarry sweet odor, which is detectable at very low concentrations (0.05 ppm). The water solubility of phenol is 66.7g/L at 16°C. Although this solubility varies between 0-65°C, above 65°C phenol is miscible in all proportions. Phenol is soluble in most organic solvents (U.S. Environmental Protection Agency, 1986b, 1993; Bruce *et al.*, 1987). The density of

phenol is close to water at 1.07 g/ml, and its vapor pressure at 25°C is 0.3513 mmHg. Thus, phenol will not evaporate easily and it will be easily washed from the environment, ending up in water supplies (U.S. Environmental Protection Agency, 1993). Phenol and its metabolites are good electron donors for peroxidase enzyme oxidations. This oxidation reaction leaves electrophilic intermediates, which are thought to cause phenols toxic effects (Subrahmanyam *et al.*, 1990). Some properties of phenol (and benzene) are listed in Table 1-1.

In the environment, phenol is rapidly degraded in the presence of microbes. However, high concentrations of phenol (>3g/L) will destroy the microbes, thus inhibiting its degradation. In spite of phenol's rapid degradation potential, it has been found in aquifers 15-18 months after accidental spills had occurred (U.S. Environmental Protection Agency, 1993). The half life of phenol released to the atmosphere has been estimated at 0.7 to 14 hours. This value is dependent on the amount of other pollutants in the air (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987). In the body, phenol is rapidly absorbed and distributed to all tissues, the liver containing the highest concentrations of phenol (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987). Unlike benzene, phenol is not absorbed as rapidly by tissues high in lipid content such as fat or brain (Bruce *et al.*, 1987).

Occurrence and usage

Coal tar was first used as a source of phenol in 1934. Today, the majority of

phenol is produced synthetically from the sulfonation of benzene and its subsequent hydroxylation. In 1995, phenol was the 34th most commonly produced chemical in the United States with 4.16 billion pounds made (Kirschner, 1996). Phenol is also found naturally after the metabolism of benzene and is found in animal wastes and the decomposition of organic wastes (U.S. Environmental Protection Agency, 1986b). Exogenous sources of phenol include environmental chemicals (benzene, etc.) and medicines (i.e., Pepto-Bismol™ or others with phenylsalicilate).

Measurable levels of phenol have been detected in rivers, lakes, and underground water supplies. The U.S. Office of Technology Assessment (1984) found the levels of phenol ranged from 10 to 234,000 ppb (0.1-234 mg/L). Phenol is not considered to be a problem in urban/suburban air, although phenol can reach significant levels in industrial areas. The average concentration of phenol in automobile exhaust is 0.29 ppm (U.S. Environmental Protection Agency, 1993).

Populations at risk

Populations at risk to phenol exposure at high levels are limited to workers manufacturing or using phenol. The ACGIH has set the threshold limit value (TLV) for phenol at 5 ppm (ACGIH, 1995). They attached a "skin" notation to the TLV in 1961 (Bruce *et al.*, 1987). This notation indicates phenol as being a potential source of systemic poisoning if it comes in contact with the skin (ACGIH, 1995). Although phenol is found naturally, there is not concern of a widespread exposure risk probably due to

phenol's low volatility and water solubility (Bruce *et al.*, 1987). Phenol is also a known drinking water contaminant and becomes an exposure risk if a drinking water source becomes contaminated with phenol (U.S. Environmental Protection Agency, 1986a).

Metabolism

The metabolic fate of phenol is obviously very similar to benzene after it is converted to phenol by cytochrome P-450s. Figure 1-3 illustrates the metabolic fate of phenol. It is important to note that phenol is primarily conjugated with sulfate or glucuronide and eliminated in the urine (U.S. Environmental Protection Agency, 1993). Increased dietary protein will also increase urinary phenol output (U.S. Environmental Protection Agency, 1986b). As was mentioned earlier, the body normally excretes phenol, a by-product of food metabolism or aromatic amino acid metabolism. Therefore, the body is well prepared to metabolize and excrete phenol and its metabolites. However, phenol metabolism depends on the dose and species of animal. In rabbits given sublethal doses of phenol, 72 percent was excreted in the urine (Bruce *et al.*, 1987). In rabbits given lethal doses, only 3 percent of the phenol was excreted in the urine (Bruce *et al.*, 1987), suggesting the enzymes metabolizing phenol have reached their threshold limit and allowed phenol to reach toxic levels in the body. Pigs mainly excrete phenol as the phenol glucuronide conjugate, whereas cats cannot conjugate the phenol with glucuronide so they excrete it as the phenol sulfate or quinol sulfate (Bruce *et al.*, 1987). This variability in phenol metabolism is seen among all species, but the majority of animals including

humans conjugate phenol with sulfates and glucuronides. The mouse, like humans, conjugates phenol with mostly sulfates (Bruce *et al.*, 1987). This conjugation becomes the rate-limiting step in clearing the body of phenol. The metabolism of phenol is illustrated in Figure 1-3.

Phenol toxicity

Acute phenol exposure results in cardiac arrhythmias, blood pressure fluctuations, and respiratory distress. Phenol ingestion, even in small amounts, causes severe burns in the mouth and esophagus. Abdominal pain has also been reported after oral ingestion of phenol (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987). Rats given a single oral dose of 600 mg/kg died within 12-24 hr. Younger rats were found to be more susceptible to phenol at this dose causing them to die more quickly. Exposure to phenol can also cause ocular and nasal irritation, conjunctivitis, and corneal ulcerations (U.S. Environmental Protection Agency, 1993) which is consistent with phenol's irritant properties. Phenol, like other hepatic failure toxins (such as ammonia), induces coma and encephalopathy (Windus-Podehl *et al.*, 1983). When phenol levels get high enough to cause saturation of the conjugation processing enzymes in the body, more phenol is converted to hydroquinone. When phenol enters the bone marrow, hydroquinone levels rise and stimulate myelotoxicity (Legathe *et al.*, 1994; Subrahmanyam *et al.*, 1990). At this point, stromal cell function is also altered, presumably the same way benzene suppresses stromal cell activities (Gaido and Wierda, 1985).

In long-term exposure studies, phenol given up to 10,000 ppm (10,000 mg/L) in tapwater caused no effect in the liver, kidney, or any other organ. At 10,000 ppm, weight gains were decreased and water consumption was decreased, although no compound related microscopic pathology was noted (U.S. Environmental Protection Agency, 1993; Hsieh *et al.*, 1992). In an epidemiological study done in a community where an accidental spill of phenol into underground drinking water occurred, increases in human illnesses such as mouth sores, diarrhea, and dark urine were reported. In this instance, the authors estimated the people to have ingested 10-240 mg phenol/person/day (Baker *et al.*, 1978).

Phenol is fetotoxic and causes decreased birth weights in animals when given at doses of 7,000 ppm or greater (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987). Although phenol tests positive in *in vitro* teratogenesis studies, it has not been found to be teratogenic *in vivo* (Bruce *et al.*, 1987). Phenol is not mutagenic, however. In the laboratory when rat liver S9 microsomes were added to lymphocytes and then exposed to phenol, the incidence of sister chromatid exchange (SCE) increased (Bruce *et al.*, 1987; Dean, 1985). This increased SCE suggests that normally phenol is metabolized to DNA reactants at a low level. When S9 microsomes are added, this metabolism increases, causing phenol to become mutagenic (Dean, 1985). Phenol has been determined to be noncarcinogenic but it does have some tumor-promoting potential (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987).

Immunotoxicity of phenol

Phenol has previously been reported to be immunotoxic (Baker *et al.*, 1978). This is not surprising considering its close similarity to benzene. In fact, the concentration of phenol in the bone marrow increases following exposure to benzene (Sawahata *et al.*, 1985). As was mentioned previously, the peroxidases in the bone marrow convert phenol to reactive species (such as hydroquinones) resulting in myelotoxicity and immunotoxicity similar to benzene (Snyder and Kalf, 1994; Sawahata *et al.*, 1985). Phenol exposure is often through the skin. Keratinocytes within the skin are stimulated, when in contact with phenol, to produce TNFα, IL-1α, and interleukin-8 (IL-8). These factors may contribute to skin inflammation following phenol exposure (Wilmer *et al.*, 1994). Phenol also affects lymphocyte proliferation. When exposed to the mitogens PHA, PWM, and LPS, lymphocytes from mice treated with 95 mg/L phenol in the drinking water showed a decreased proliferative response. A decreased number of plaque-forming cells and antibody to sheep red blood cells (SRBC) was also noted in the mice exposed to this same dose of phenol (Hsieh *et al.*, 1992).

Summary

Benzene and phenol have been found to be immunotoxic. Humoral and cell-mediated portions of the immune system are altered, in addition to the other toxic effects caused by these chemicals. Immunomodulation is of growing concern due to the pathological effects on the body that a disfunctioning immune system can have. The

mechanisms of immunomodulation are variable between chemicals and many mechanisms are still unknown. The purpose of this study is to identify some of the molecular events that will lead to benzene or phenol immunotoxicity in mice. The likely cause for this immunotoxicity is from an alteration in the synthesis or release of cytokines by key immune cells such as the macrophage or T-cell. If these key cells are not stimulated appropriately, all other parts of the immune system can be adversely affected. In addition, there is no evidence, or conflicting evidence as to synthesis of cytokines at the transcriptional or translational levels by these chemicals.

In this study, benzene or phenol was added to the drinking water of the mice because previous research demonstrated that benzene or phenol in the drinking water caused some immunotoxic effects (Hsieh *et al.*, 1988b, 1992), and because these chemicals are known drinking water contaminants. Doses of benzene or phenol in the drinking water were equivalent to those used by Hsieh *et al.* (1988b, 1992) in order to duplicate similar immunotoxic effects. Murine macrophage functions were evaluated by their ability to produce IL-1α, IL-6, and TNFα. Murine lymphocyte functions were assessed by measuring their ability to produce IL-2, IL-3, and IFNγ. Cytokine production was measured at both the transcriptional (mRNA) and translational (protein) levels by using Northern blot, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), and sandwich Enzyme-Linked ImmunoSorbant Assay (ELISA) techniques. Northern blot and RT-PCR methods were used to assess cytokine mRNA levels, and the sandwich ELISA

method was used to determine cytokine protein levels after *in vitro* induction with mitogens in chemically treated and control animals.

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CHAPTER 2

ALTERATION OF KEY LYMPHOCYTE CYTOKINES AND CYTOKINE MESSENGER RNA BY BENZENE IN MALE INSTITUTE OF CANCER RESEARCH MICE

Abstract

Benzene is a known contaminant of groundwater. It has long been associated with myelotoxicity and recently has been found to have immunotoxic effects. Immunotoxicity has been linked to alterations in cytokines, which are key signaling molecules in the immune system. Modulation in the production of cytokines can cause increased susceptibility to disease or autoimmunity. This study was concerned with the potential effects that continuous 30-day exposure to benzene in the drinking water of male ICR mice would have on IL-2, IL-3, and IFNγ cytokine mRNA and protein production by lymphocytes. Benzene was added to drinking water at concentrations of 0, 40, 200, and 1000 mg/L and given to mice in control, low, medium, and high groups, respectively. As in previous studies, benzene at all dosage levels was associated with lower thymus weights. In Con A-stimulated lymphocytes from mice given the medium benzene dose, higher IL-2 mRNA levels were observed compared to the control group. In spite of increased IL-2 mRNA levels, the level of secreted IL-2 protein from the lymphocytes of benzene-treated mice remained similar to control levels. Benzene may be stimulating the transcription of IL-2 mRNA, but benzene may also be affecting IL-2 cytokine production

at the posttranscriptional, translational, or posttranslational level. Neither the mRNA nor the secreted protein levels of IL-3 and IFNγ were altered significantly in the lymphocytes from benzene-treated mice when compared to the lymphocytes from the control mice.

In conclusion, benzene can be associated with cytokine alterations in mice, although benzene does not appear to cause the same alterations in all cytokines. Therefore, benzene, even at low concentrations in the drinking water, should continue to be a concern of regulatory agencies.

Introduction

Benzene is widely distributed in our environment. It is found in petroleum and petroleum products, and it is present naturally in trees, seeds, dairy products, eggs, and meat in small amounts considered not to be hazardous. Benzene is considered a basic starting material in the manufacturing of several beneficial products including medicines (Fishbein, 1984; U.S. Environmental Protection Agency, 1980). Although benzene is known to have embryotoxic, carcinogenic, and teratogenic potential (Snyder and Kalf, 1994; Ungvary and Tatrai, 1985; Kuna and Kapp, 1981), its primary toxicity is causing dysfunction of the hematopoietic system (Snyder and Kalf, 1994; Sawahata *et al.*, 1985; Fishbein, 1984; Bolcsak and Nerland, 1983; U.S. Environmental Protection Agency , 1980). Repeated exposure to benzene caused pancytopenia, aplastic anemia, leukemia, and the development of chromosomal abnormalities (Dean, 1985; Green *et al.*, 1981). The mechanism for benzene's myelotoxicity is thought to be from the formation of oxidative

benzene metabolites, such as hydroquinone, rather than benzene itself (Snyder and Kalf, 1994; Legathe *et al.*, 1994; Sawahata *et al.*, 1985; Tunek *et al.*, 1981, 1982).

The majority of benzene pollution is in the air; however, because of benzene's solubility and known contamination in drinking water supplies, there is concern for the safety of our drinking water supplies. Benzene, a known additive in gasoline, has leaked from underground gasoline storage tanks into groundwater supplies, resulting in concentrations as high as 29,000 parts per billion (ppb) (U.S. Environmental Protection Agency, 1980). Since groundwater is the primary source of drinking water for about 50 percent of the nation, this could mean a large portion of the population is at risk of benzene exposure (U.S. General Accounting Office, 1988; U.S. Office of Technology Assessment, 1984). Although exposure to benzene may be minor, the toxic effects of benzene will accumulate over long periods of time.

Benzene exposure has been associated with immunotoxicity. In a study by Hsieh *et al.* (1988), oral ingestion of benzene at a low (8 mg/kg/day) dose in mice led to increased lymphocyte proliferation and antibody production. At higher doses (40 and 180 mg/kg/day), benzene-treated mice showed decreased lymphocyte proliferation and antibody production. The immunotoxicity of benzene was further demonstrated by an involution of the thymus, a depression in B- and T-cell mitogenesis, and a decrease in IL-2 secretion (Hsieh *et al.*, 1988, 1990).

Modulation of the immune response depends on cytokine production by key immune cells (Janeway and Travers, 1994; Tizard, 1992; Nussler and Thomson, 1992;

Mizel, 1989). Cytokines are soluble regulatory proteins produced by many cells including lymphocytes. Often, cytokines production in these cells is stimulated when the cells come in contact with an antigen (Janeway and Travers, 1994). These cytokines then stimulate cell proliferation, differentiation, and often the production of other cytokines. For example, IL-2 stimulates the proliferation of all lymphocytes, while IFNγ stimulates macrophages to become more cytotoxic. Depending on the cytokines they are exposed to, T-cells will differentiate into T_H1 or T_H2 cells (Kishimoto, 1992). Thus, cytokines play an important role in the immune system. Therefore, it is logical to theorize that the immunotoxicity caused by benzene could be due to the alteration of cytokine expression.

The purpose of this study was to determine both mRNA and secreted protein levels of cytokines produced by the lymphocytes of male ICR mice treated with benzene in their drinking water at levels already known to cause immunotoxicity. The cytokines IL-2, IL-3 and IFNγ were chosen for this study on the basis of their known importance in the regulation of the immune response (Janeway and Travers, 1994; Mizel, 1989).

Materials and Methods

Animals

Five-week-old male outbred Institute of Cancer Research (ICR) mice were obtained from Simonsen Laboratories (Gilroy, CA) and housed in an American

Association for the Accreditation of Laboratory Animal Care (AAALAC)-approved facility. The mice were maintained at a constant 22-24°C temperature and a 40-60 percent humidity with a 12-hr light/dark cycle. The mice were acclimated to this regimen for 1 week prior to the beginning of the study, and groups of five mice were randomly assigned to plastic cages containing ground corn cob bedding. Commercial rodent chow (Harlan Teklad, Madison, WI) and water were provided *ad libitum*.

Benzene exposure

Spectranalyzed benzene (99.9 percent purity, Fisher Scientific, Fair Lawn, NJ) was dissolved in tap water to make a stock solution of 1000 mg/L. From the stock solution, additional solutions of 40 mg/L and 200 mg/L were made by dilution. Benzene was administered to three treatment groups of mice (n=5 in each group) for 30 days at 40, 200, and 1,000 mg/L water for the low, medium, and high treatment groups, respectively. The control group received untreated tap water for 30 days. The benzene-treated water was put in glass water bottles, shaken daily, and changed every 3 days to minimize benzene decomposition and to maintain the desired benzene concentrations in the water. In order to duplicate results from past research, benzene was added to tap water and changed every 3 days just as performed by previous workers (Hsieh *et al.*, 1988). Water consumption and mouse weights were recorded every 3 days.

Gross observations

After 30 days, the mice were anesthetized with halothane gas to obtain blood

samples. The mice were then euthanized by cervical dislocation, and the spleen was removed aseptically, and placed into cold complete RPMI 1640 cell culture media [RPMI-1640 (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 mM 2-mercaptoethanol, 10 percent heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT)]. Gross pathological examination of other organs was performed and the liver, kidneys, and thymus were weighed.

Isolation and activation of splenic lymphocytes

A monocellular cell suspension from each spleen was prepared using a Stomacher lab blender (STOM 80, Tekman Co., Cincinnati, OH). Red blood cells were removed with ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). The remaining cells were plated on 100-mm tissue culture plates and incubated in a humidified 37°C, 5 percent CO_2 incubator for 1 hr to allow the adherence of macrophages. The nonadherent cells were collected after 1 hr and counted on a Coulter counter (Coulter Electronic Inc., Hialeah, FL). The concentration of nonadherent cells was adjusted to $2x10^6$ cells/ml, and 5 μg/ml Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) was added to activate the cells, then 15-20 ml of the activated cellular solution were replated on 100-mm cell culture dishes. These culture dishes were incubated for 8 hr to obtain cytokine mRNA, or 48 hr for isolation of cytokine protein in the supernatant.

Total RNA isolation and quantification

After 8 hr of activation with Con A, the lymphocytes were removed from the tissue culture plates and pelleted by centrifugation. Total RNA was extracted from the lymphocyte pellet with TRI REAGENT™ LS (Molecular Research Center, Cincinnati, OH) following the manufacturer's directions. Purified RNA was resuspended in ribonuclease (RNase) free DEPC-ddH_2O (diethylpyrocarbonate-distilled, deionized water), quantified using a spectrophotometer (Shimatzu Corp., Kyoto, Japan), and then stored at -70°C.

RNA analysis

First strand cDNA synthesis was performed using 1 µg total RNA and 0.5 µg oligo (dT_{15}) (Promega Corporation, Madison, WI) in a 20-µl total reaction volume containing 50 mM Tris-HCl (pH 8.3 at room temperature), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of dATP, dCTP, dGTP, and dTTP; 200 units of Superscript II RNase H^- Reverse Transcriptase (Life Technologies, Gaithersburg, MD) were then added and the samples incubated for 50 min at 42°C. The reverse transcriptase enzyme was heat inactivated at 70°C for 15 min and then the tubes were placed on ice. Two units of RNase H (United States Biochemical, Cleveland, OH) were added and the samples incubated at 37°C for 20 min. Twenty µl of ddH_2O was then added and the product was stored at -20°C until further analyses were performed.

Interleukin-2, IL-3, and IFNγ mRNA production were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Foley *et al.*, 1993). Primer sets for each of these cytokines were developed using Oligo 4.03 software (National Biosciences, Inc., Plymouth, MN). The primer sequences were as follows:

IL-2 : Sense = 5'-GCGCACCCACTTCAAGCTCC-3'

Antisense = 5'-AGCCCTTGGGGCTTACAAAA-3'

Product length - 611 bp

IL-3 : Sense = 5'-CCCTTGGAGGACCCAGAAC-3'

Antisense = 5'-GCCATGAGGAACATTCAGAC-3'

Product length - 567 bp

INFγ : Sense = 5'-GTTCTGGGCTTCTCCTCCTG-3'

Antisense = 5'-CGAATCAGCAGCGACTCCTT-3'

Product length - 510 bp

β–actin : Sense = 5'-AACACAGTGTTGTCTGGTGG-3'

Antisense = 5'ACGCAGCTCAGTAACAGTCC-3'

Product length - 265 bp

Two µl of the first strand reaction from above were used for each PCR reaction. The optimal primer and $MgCl_2$ concentrations were determined empirically for each cytokine. In a total reaction mixture volume of 50 ml, 10 mM Tris-HCL, pH 9.0, 50 mM KCl, and 0.1% Triton X-100 were added. The $MgCl_2$ concentrations were 5 mM, 4 mM,

2 mM, and 3 mM for IL-2, IL-3, IFNγ, and β-actin, respectively. The primer concentrations for IL-2, IL-3, IFNγ, and β-actin were 30 pmol, 30 pmol, 20 pmol, and 10 pmol, respectively. A TempCycler II thermocycler (Coy Corporation., Grass Lake, MI) was used for the PCR reaction. The temperatures and times used in the reactions were: 95°C for 5 min followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min.

The products from each PCR reaction were separated on 2% agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator (Fisher Scientific, Pittsburgh, PA). Negatives of each gel were taken using Polaroid Type 665 positive/negative film. The negatives were scanned, and the optical densities (OD) of each band were calculated using the ZERO-D scan software program (Stratagene Cloning Systems, LaJolla, CA). Total RNA in each band was adjusted using the β-actin standards. Average densities of RNA for control and treated groups were compared for statistical differences. The PCR products were confirmed to be the appropriate cytokine by sequencing PCR products from two mice.

Cytokine protein quantitation

Supernatants from 48-hr activated splenocyte cultures were obtained by removing the cells and media from the tissue culture dish, centrifuging to pellet the cells, and pouring off the supernatant. The supernatants were placed in separate vials and stored at -70°C until needed for IL-2, IL-3 and IFNγ quantification. All cytokines were quantitated

by sandwich ELISA techniques using kits obtained from Endogen, Inc. (Cambridge, MA) and following the manufacturer's instructions.

Statistical methods

Treatment effects for RT-PCR and cytokine ELISAs were analyzed using a one-way analysis of variance (ANOVA) followed by the Fisher least significant difference (LSD) statistical analysis. Statistical calculations were performed using Stat View™ SE and Graphics 1.03 software (Abacus Concepts, Inc., Berkeley, CA).

Results

The average concentrations of benzene in the drinking water of the mice were 0, 40, 200, and 1000 mg/L in control, low, medium, and high treatment groups, respectively (Table 2-1). No overt signs of toxicity were observed in any treatment group. However, both high and low dose groups had one mouse that became so aggressive after two weeks that they had to be placed in separate cages for the duration of the experiment. Food consumption did not change in any group throughout the experiment. However, the mice in the high dose group drank less water than the other groups.

Body and organ weights

There were no significant differences in the body weights of the mice at day 0 or at day 30 among control and treatment groups (Table 2-1). Each group of mice gained about the same amount of weight over the 30-day treatment period. Upon euthanasia, no

TABLE 2-1
Body Weights and Water Consumption in Mice Given Benzene in Their Drinking Water for 30 Days

Benzene Conc. in Water (mg/L)	Water Consumed[a] (ml/day)	Body Weights Day 0[b] (grams)	Body Weights Day 30[b] (grams)
0	8.1	22.0 ± 0.6	36.8 ± 1.2
40	9.0	21.0 ± 0.9	34.4 ± 1.3
200	7.6	21.2 ± 0.4	36 0± 1.4
1000	6.1	22.4 ± 0.5	36.4 ± 1.5

[a] Values are the mean from each group of five mice
[b] Values reported as the mean ± S.E. (n=5)

gross pathological abnormalities were found in control and treatment groups. Selected organ weights are given in Table 2-2. The spleen weights were greater in the treated groups than the control group. The high dose group had the greatest spleen, kidney, and liver weights although these differences were not statistically significant. The thymus weights were significantly ($p<0.05$) lower in all treatment groups when compared to the control group. The high dose group had the lowest average thymus weight.

Spleen cell numbers from homogenized spleens with red blood cells (RBC) and macrophages removed are reported in Table 2-2. While the control group had the lowest average number of splenocytes, this difference was not statistically significant.

TABLE 2-2
Selected Organ Weights and Spleen Cellularity of Mice Given Benzene in Their Drinking Water for 30 Days

Benzene Conc. in Water mg/L	Spleen Weight[a] (g/100g BW)	Kidney Weight[a] (g/100g BW)	Liver Weight[a] (g/100g BW)	Thymus Weight[a] (g/100g BW)	Spleen Cellularity[a] ($x10^6$ cells/ml)
0	0.26 ± 0.02	2.16 ± 0.04	5.55 ± 0.20	0.22 ± 0.03[b]	3.2 ± 0.3
40	0.31 ± 0.03	2.16 ± 0.08	6.02 ± 0.17	0.16 ± 0.02[c]	4.9 ± 0.4
200	0.27 ± 0.02	2.22 ± 0.05	5.48 ± 0.16	0.15 ± 0.01[c]	4.0 ± 0.8
1000	0.32 ± 0.07	2.40 ± 0.12	6.23 ± 0.31	0.13 ± 0.01[c]	4.2 ± 1.1

[a] Values are expressed as the mean ± S.E. (n=5)
[b,c] Different superscripts within a column are statistically different $p<0.05$

Cytokine mRNA production

The levels of IL-2, IL-3, and INFγ mRNA production in Con A-stimulated mouse lymphocytes are presented in Figures 2-1, 2-2, and 2-3. There was a greater IL-2 mRNA production at the low dose and significantly greater IL-2 mRNA production at the medium dose. At the high dose, the IL-2 mRNA production was lower such that it was not significantly different from the control group.

The same general trend is apparent for IL-3 mRNA production. Interleukin-3 mRNA levels were higher at the low dose and significantly higher at the medium dose when compared to IL-3 mRNA production of the control group. Again the high dose

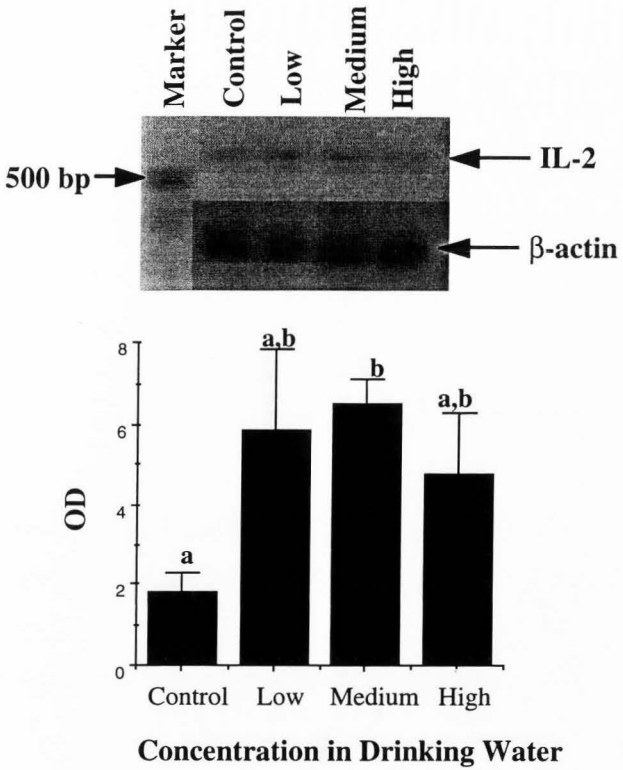

FIG. 2-1. Levels of IL-2 mRNA in Con A-activated splenocytes from benzene-treated mice. One μg total RNA from the Con A-activated (5 μg/2x10^6 cells for 10 hr) splenocytes was used to make cDNA and then amplified by PCR using murine IL-2 and β-actin primers. The densities from the band of each animal (n=4) were measured by ZERO-D scan. Values of IL-2 product were standardized for differences in total RNA using the β-actin product. Separate 2 percent agarose gels were used to run IL-2 and β-actin PCR products. Data are expressed as mean + S.E. Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups respectively. (^{a,b}) Different superscripts are different ($p<0.05$).

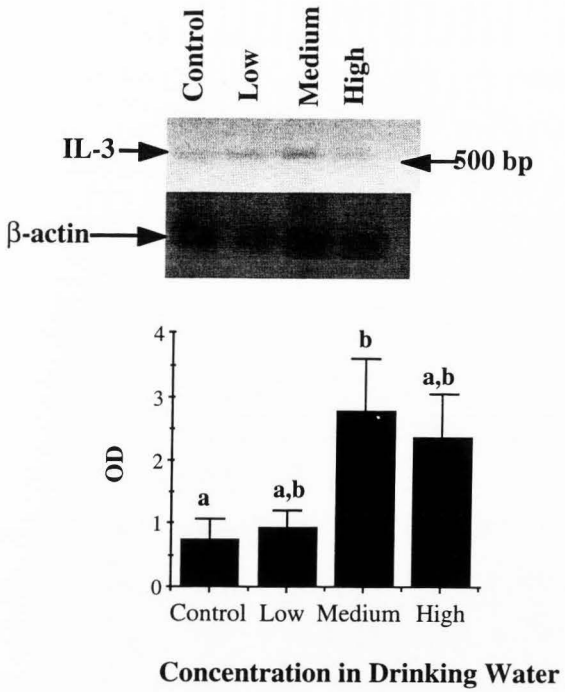

FIG. 2-2. Levels of IL-3 mRNA in Con A-activated splenocytes from benzene-treated mice. One μg total RNA from the Con A-activated (5 μg/2x10^6 cells for 10 hr) splenocytes was used to make cDNA and then amplified by PCR using murine IL-3 and β-actin primers. The densities from the band of each animal (n=4) were measured by ZERO-D scan. Values of IL-3 product were standardized for differences in total RNA using the β-actin product. Separate 2 percent agarose gels were used to run IL-3 and β-actin PCR products. Data are expressed as mean + S.E. Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups respectively. (^{a,b}) Different superscripts are different ($p<0.05$).

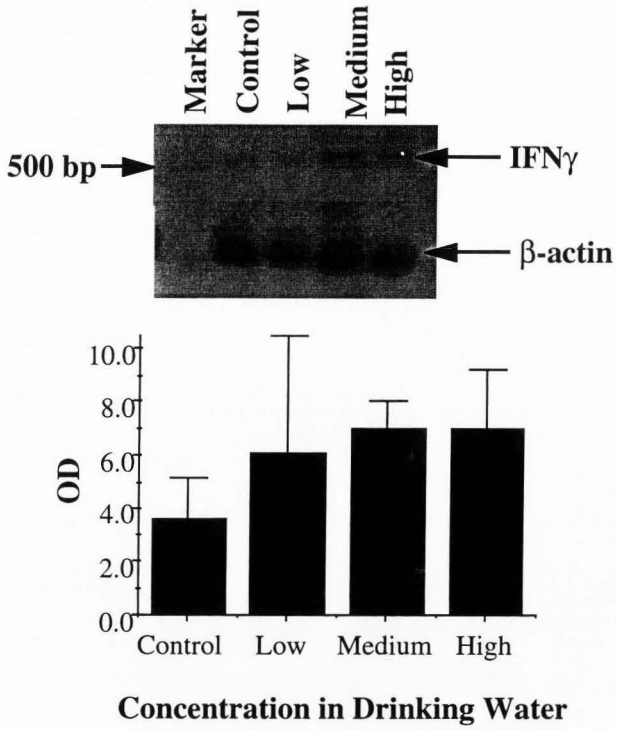

FIG. 2-3. Levels of IFNγ mRNA in Con A-activated splenocytes from benzene-treated mice. One μg total RNA from the Con A-activated (5 μg/2x10^6 cells for 10 hr) splenocytes was used to make cDNA and then amplified by PCR using murine IFNγ and β-actin primers. The densities from the band of each animal (n=4) were measured by ZERO-D scan. Values of IFNγ product were standardized for differences in total RNA using the β-actin product. Separate 2 percent agarose gels were used to run IL-2 and β-actin PCR products. Data are expressed as mean + S.E. Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups respectively.

group mRNA levels were lower and not statistically different from the IL-3 mRNA production of the control group.

The IFNγ mRNA production, determined by RT-PCR, was slightly different from IL-2 and IL-3 mRNA production. The mRNA production at the low dose (40 mg/L) and the medium dose (200 mg/L) was highest, but this difference was not statistically significant. At the high dose (1000 mg/L), the mRNA production was similar to the medium dose group, but again, was not significantly different from the control group.

Cytokine protein production

The levels of IL-2, IL-3 and IFNγ in Con A-stimulated mouse splenocytes are presented in Figures 2-4, 2-5, and 2-6, respectively. The production of IL-2, IL-3, and IFNγ of the control group was not significantly different from the treatment groups. Interleukin-2 protein production was highest at the low dose, less at the medium dose, and slightly higher in the high dose group (Figure 2-4). All treatment groups had greater IL-2 production than the control group, similar to the pattern of IL-2 mRNA, although the medium dose group had the highest level of mRNA.

The pattern of IL-3 protein secretion was very similar to the IL-3 mRNA levels (Figures 2-2 and 2-5). However, even though IL-3 protein for the low and medium dose groups was higher than the control group, these levels were not significantly different. Lymphocytes from mice given the high dose of benzene appeared to be secreting less IL-3 protein than the medium group.

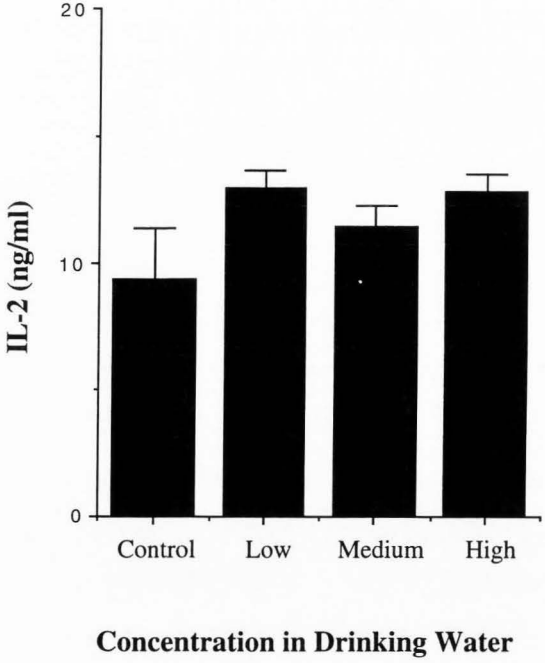

FIG. 2-4. Levels of IL-2 secreted protein from activated splenocytes of benzene-treated mice. The splenocytes ($2x10^6$/ml) from control and benzene-treated mice were activated with 5 μg/ml Con A. Supernatants were taken after 48 hours, and IL-2 was detected in the serum by ELISA. Data are expressed as mean + S.E. (n=4). Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively.

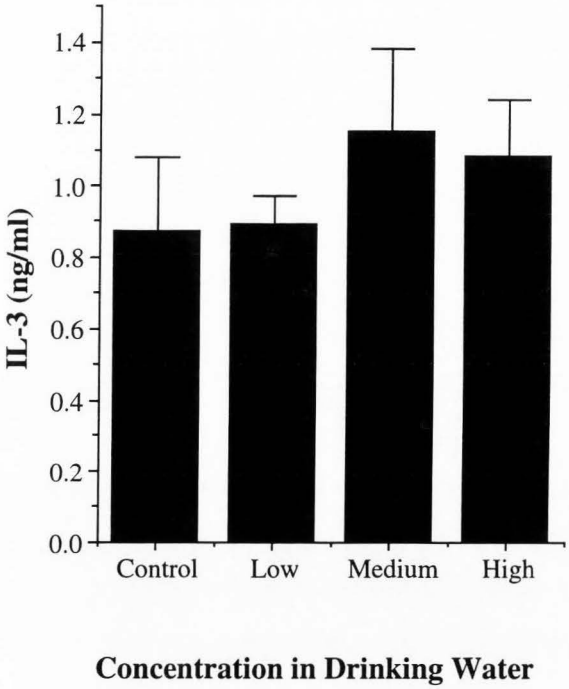

FIG. 2-5. Levels of IL-3 secreted protein from activated splenocytes of benzene-treated mice. The splenocytes ($2x10^6$/ml) from control and benzene-treated mice were activated with 5 μg/ml Con A. Supernatants were taken after 48 hours, and IL-3 was detected in the serum by ELISA. Data are expressed as mean + S.E. (n=4). Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively.

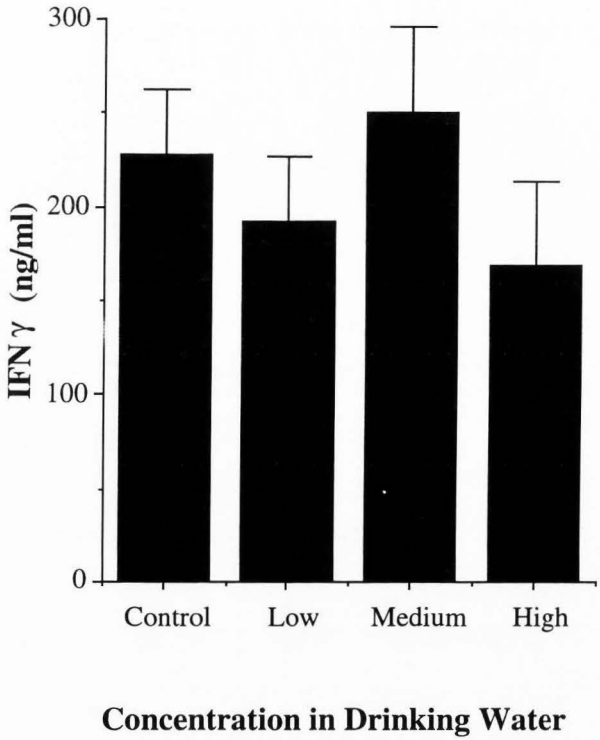

FIG. 2-6. Levels of IFNγ secreted protein from activated splenocytes of benzene-treated mice. The splenocytes ($2x10^6$/ml) from control and benzene-treated mice were activated with 5 μg/ml Con A. Supernatants were taken after 48 hours, and IFNγ was detected in the serum by ELISA. Data are expressed as mean + S.E. (n=4). Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively.

The medium dosage group had the greatest INFγ protein level (Figure 2-6), similar to INFγ mRNA levels. However, the high and low dose groups had less IFNγ production than the control group, unlike the trend of IFNγ mRNA production, for which all treatment groups were higher than the control group.

Discussion

Alteration in cytokine production can be linked to autoimmune problems and disease (Janeway and Travers, 1994; Tizard, 1992; Nussler and Thomson, 1992; Mizel, 1989). Immunosuppressive drugs, such as cyclosporin A, modulate cytokine production leading to immunotoxicity (Brabletz *et al.*, 1991; Emmel *et al.*, 1989). Benzene is immunotoxic in mice (Hsieh *et al.*, 1988), but the mechanism of this immunotoxicity is unknown. Determining the nature and extent of cytokine alterations in benzene-treated mice will help to shed light on the molecular mechanisms involved in benzene immunotoxicity. Therefore, in this research, splenic lymphocytes from benzene-treated male mice were used to study the *in vitro* expression of cytokine mRNA and the secretion of cytokine proteins.

No overt signs of toxicity resulted from the doses of benzene given (40, 200, 1000 mg/L) although the high dose group drank less water than the control group. At the concentration given to the high dose mice (1000 mg/L), benzene could be detected by smell in the water and it is likely that benzene could be tasted in the water as well.

Benzene-treated mice had significantly lower thymus weights than the control mice (Table 2-1). These results support previous studies that used similar doses of benzene and showed thymic atrophy (Hsieh *et al.*, 1988, 1990).

Benzene is known to affect lymphocytes more readily than other cells in the bone marrow or peripheral blood (Hsieh *et al.*, 1988; Rozen and Snyder, 1985; Green *et al.*, 1981). The permeability of the lymphocyte liposomal membrane increases during benzene exposure, causing a release of lysosomal enzymes into the cytoplasm, which ultimately destroys the lymphocyte (Moszczynsky and Lisiewicz, 1984). The reduction in B and T lymphocyte numbers leads to decreased thymic weights due to cortical depletion (Rozen and Snyder, 1985). The fact that at all doses of benzene used in this study resulted in thymic atrophy supports these findings.

No significant differences were noted in the weights of other organs or in the number of splenocytes obtained from control or benzene-treated mouse spleens. In a previous study where mice were given benzene at 180 mg/kg/day in their drinking water, benzene-treated mice had lower spleen weights, decreased spleen cellularity, and decreased thymic weights (Hsieh *et al.*, 1988). As was noted earlier, benzene is particularly toxic to lymphocytes (Rozen and Snyder, 1985; Moszczynsky and Lisiewicz, 1984). Because lymphocytes make up a large portion of the cellularity in the spleen, it is expected that splenocytes will decrease in number after benzene treatment. However, in this study, no statistically significant differences were found for spleen weights, probably due to the large variability within the groups. This large variability can

be partly explained by the variation in amount of drinking water consumed by each mouse; thus the dose of benzene may have varied between each mouse and the extent of toxicity would differ, resulting in large variation in measured traits. Future studies may want to dose the mice by oral gavage so all mice receive equal doses.

The IL-2 protein production by Con A-stimulated splenocytes from benzene-treated mice was not significantly different from the control mice. However, IL-2 mRNA production was greatest at the low (40 mg/L) and medium (200 mg/L) doses. Differences in transcriptional and translational events indicate that benzene may have a multifaceted effect on cytokines. Like all eukaryotic genes, the IL-2 gene is under the control of several DNA binding proteins (Riegel *et al.*, 1992). DNA binding proteins such as NFAT-1, Oct-1, and NF-KB bind directly upstream of the promoter to initiate transcription of the IL-2 gene. Cyclosporin A and FK 506 stop IL-2 mRNA production by inhibiting the production of NFAT-1, thus preventing transcription of the IL-2 gene (Brabletz *et al.*, 1991). While cyclosporin inhibits the production of NFAT-1, it appears to have no effect on the production of AP-1. Furthermore, cyclosporin first increases the production of NF-KB and at higher doses inhibits it production (Emmel *et al.*, 1989). This research suggests that benzene is activating transcription of the IL-2 gene at low and medium doses, but at the high dosage, benzene may be inhibiting IL-2 transcription. One possible explanation for this effect could be the interaction of benzene with nuclear

binding proteins. At low benzene doses, nuclear binding proteins are activated, but at high benzene doses they are inhibited.

However, the higher levels of IL-2 mRNA are not associated with higher IL-2 protein production (Figure 2-4). It is possible that benzene is causing some effect at the posttranscriptional, translational, or posttranslational level. The IL-2 mRNA has reiterated sequences at the 3' untranslated region (UTR) of the mRNA (Henics *et al.*, 1994; Janeway and Travers, 1994), making the IL-2 mRNA labile with a half life of only 8 hr. These regions not only modulate mRNA turnover, they are also important in regulating mRNA translation (Henics *et al.*, 1994). If benzene increased production of cytoplasmic proteins responsible for mRNA degradation, translational modification of the IL-2 protein would occur. This alteration may explain why no significant increases in IL-2 protein were seen even when the IL-2 mRNA levels were increased. Interleukin-2, more than any other cytokine secreted by T-cells, plays a key role in activating the immune response. Therefore, failure to stimulate IL-2 properly can lead to immune dysfunction (Riegel *et al.*, 1992).

Interleukin-3 supports the viability and differentiation of hematopoietic progenitor cells (Kishimoto, 1992; Mizel, 1989). The function of IL-3 in an immune response may be to recruit additional immune cells (macrophages and lymphocytes) to sustain or amplify the responses (Mizel, 1989). The results of this research indicated that IL-3 mRNA production was greater in both the low and medium dose groups (Figure 2-2). While the trend was similar in IL-3 cytokine protein levels, these differences were

not significant. Benzene may be enhancing the production of one or more nuclear activating proteins responsible for IL-3 gene transcription. However, the IL-3 gene binds different transcription factors than the IL-2 gene. CK-1, CLE-1, and NF-GMa are some of the transcription factors used by the IL-3 gene (Kishimoto, 1992). Nevertheless, benzene's effects on IL-3 production appear to be occurring at the transcriptional level.

The significance of IFNγ in the immune response has been studied extensively (Janeway and Travers, 1994; Cockfield *et al.*, 1993). Its main functions are to activate macrophages, natural killer (NK) cells, and to stimulate antiviral properties in cells. Interferon-γ also causes the differentiation of B-cells resulting in IgG synthesis and is a key cytokine in immune cell activation and viral elimination (Janeway and Travers, 1994). The results of this study indicate that benzene stimulates IFNγ mRNA production only slightly. However, even though IFNγ mRNA production was stimulated, the IFNγ protein production was not significantly different from the control. In the study by Cockfield *et al.* (1993), cyclosporin A appeared to have transcriptional control of the IFNγ gene, but the authors suggested that posttranscriptional stability could also have been modified. Interleukin-2 has also been reported to regulate IFNγ gene transcription. The reported mechanism for IL-2 induction of the IFNγ gene is by activation of protein kinase C and the coordinate increase in intracellular calcium (Cockfield *et al.*, 1993, Farrar *et al.*, 1986).

In conclusion, benzene appears to cause a generalized cytokine transcriptional activation at lower doses (40 or 200 mg/L). At higher doses this activation is not as pronounced. Protein synthesis did not always correlate with cytokine mRNA levels, suggesting a direct effect of benzene on posttranscriptional, translational, or posttranslational events. Thus, even though cytokine mRNA is induced, the protein levels are not. A lack of cytokine could become significant when the animal is challenged by some pathogen and the immune system does not respond properly because the lymphocytes do not secrete the cytokine even though increased levels of mRNA are present. Further research in the area of benzene's posttranslational, translational, or posttranscriptional actions on cytokine mRNA is warranted. Thus, regulatory agencies should be concerned by low levels of benzene in underground water supplies.

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CHAPTER 3

ALTERED CYTOKINE PRODUCTION IN PERITONEAL MACROPHAGES DUE TO BENZENE IN THE DRINKING WATER OF MALE INSTITUTE OF CANCER RESEARCH MICE

Abstract

The presence of pollutants in groundwater is attracting attention because a large proportion of the population use groundwater as the primary source of drinking water. Benzene is a known contaminant of drinking water, and when ingested it causes myelotoxicity and damages the immune system. In this study, benzene was given to mice via their drinking water for 30 days at dosages of 0, 40, 200, and 1000 mg/L in control, low, medium, and high treatment groups, respectively.

Fewer peritoneal macrophages were obtained from the benzene treated mice than from control mice. Immunotoxicity of benzene was suggested because lower tumor necrosis factor alpha (TNFα) mRNA and protein secretion occurred at all treatment levels compared to the controls. In contrast, interleukin-1 (IL-1α) mRNA was higher in the low and medium dose groups than in control groups. At the high dose, IL-1α mRNA production was lowest. The level of IL-1α protein secretion was less in the low group suggesting a post-translational event that prevented IL-1α release. Interleukin-6 protein secretion was not altered by benzene. Thus, production of certain cytokines was altered in the macrophages from mice treated with benzene.

Introduction

Benzene, the simplest aromatic hydrocarbon, is found everywhere. This chemical is found naturally in petroleum products, and benzene is considered a basic starting material in the manufacturing of several beneficial products including medicines (Fishbein, 1984; U.S. Environmental Protection Agency, 1980). Benzene is also one of the most widely distributed pollutants. Due to the lipophilic nature of benzene and the production of several biologically reactive intermediates after metabolic biotransformation, benzene poses a significant health hazard (Irons *et al.*, 1992; Fishbein, 1984; Bolcsak and Nerland., 1983). Prolonged benzene exposure has caused pancytopenia, aplastic anemia, leukemia, the development of chromosomal abnormalities (Dean, 1985; Green *et al.*, 1981), and is known to cause dysfunction of the hematopoietic system (Snyder and Kalf, 1994; Ungvary and Tatrai, 1985; Sawahata *et al.*, 1985; Fishbein, 1984; Bolcsak and Nerland, 1983; Kuna and Kapp, 1981; U.S. Environmental Protection Agency, 1980). The formation of oxidative benzene metabolites is thought to be the mechanism for the toxic effects associated with benzene exposure (Snyder and Kalf, 1994; Legathe *et al.*, 1994; Sawahata *et al.*, 1985; Tunek *et al.*, 1981, 1982).

Benzene has also been associated with immunotoxicity. Oral ingestion of benzene in mice can cause altered lymphocyte proliferation and antibody production, in addition to decreased IL-2 production, depressed B-and T-cell mitogenesis, and an involution in thymic mass (Hsieh *et al.*, 1988,1990). Benzene-induced depressions in lymphocyte

numbers and functions may impair immunocompetence because lymphocytes play a key role in the immune system (Janeway and Travers, 1994).

The normal immune response not only depends on cells such as the lymphocyte and macrophage, but also on cytokine production by these cells (Janeway and Travers, 1994; Tizard, 1992; Nussler and Thomson, 1992; Mizel, 1989). Cytokines are soluble regulatory proteins produced by many cells. Often, cytokines are stimulated to be produced in cells of the immune system when they come in contact with an antigen (Janeway and Travers, 1994). Cytokine production stimulates cell proliferation, differentiation, and the production of other cytokines. For example, IL-1 and TNFα induce IL-6 production. Interleukin-6 inversely regulates TNFα production and stimulates the expression of IL-2 receptors on T-cells. T-cells will differentiate into T_H1 or T_H2 cells depending on which cytokines are produced (Janeway and Travers, 1994; Kishimoto, 1992). Thus, cytokines play an important role in the immune system and it is reasonable to theorize that benzene could alter the expression of one or more cytokines, leading to immunotoxicity.

Recent studies have found that benzene (600 mg/kg for 2 days) prevented pre-IL-1α from being converted to the active mature IL-1α that is excreted from the cell (Renz and Kalf, 1991). In another study using benzene (660 mg/kg for 3 days), IL-1 and TNFα production were increased (MacEachern and Laskin, 1992). In the same study, benzene appeared to have no effect on IL-6 production. From these reports, it is clear that

benzene is altering cytokine production, and more studies need to be conducted to clarify its role in cytokine production.

The purpose of this study was to determine both the cytokine mRNA levels and secreted protein levels in the macrophages of mice treated with benzene in their drinking water at levels already known to cause immunotoxicity. The cytokines IL-1α, IL-6, and TNFα were chosen on the basis of their importance in the initial regulation of the immune response (MacEachern and Laskin, 1992; Renz and Kalf, 1991; Akira *et al.*, 1990). Because of benzene's solubility and known contamination into drinking water supplies from leaky gasoline storage tanks (U.S. Environmental Protection Agency, 1980), there is concern for the safety of our drinking water supplies, especially since groundwater is the primary source of drinking water for about 50 percent of the nation (U.S. General Accounting Office, 1988; U.S. Office of Technology Assessment, 1984). Therefore, in this study benzene was added to the drinking water of mice for 30 days.

Materials and Methods

Animals

Five-week-old Institute of Cancer Research (ICR) outbred mice were obtained from Simenson Laboratories in Gilroy, California. The mice were acclimatized for 7 days in an AAALAC accredited animal care facility. They were housed in a room with a 12-hr light/dark cycle, an ambient temperature of 22°C ± 1°C, and a 50 percent (± 10 percent) relative humidity. Animals were randomly assigned to control and treatment groups and

housed five mice per group in plastic cages with ground corn cob bedding. They were maintained on commercial rodent chow (Harlan Teklad, Madison, WI) and tap water *ad libitum*.

Benzene exposure

Spectranalyzed benzene (99.9 percent purity, Fisher Scientific, Fair Lawn, NJ) was dissolved in tap water to make a stock solution of 1000 mg/L. From the stock solution, additional solutions of 40 mg/L and 200 mg/L were made. Benzene was administered to the three treatment groups for 30 days at 40, 200, and 1,000 mg/L water for the low, medium, and high treatment groups, respectively. The control group received untreated tap water. The benzene-treated water was put in glass water bottles, shaken daily, and changed every 3 days to minimize benzene decomposition and to maintain the desired benzene concentrations in the water. In order to duplicate results from past research, benzene was added to tap water and changed every 3 days just as performed by previous workers (Hsieh *et al.*, 1988). Water consumption and mouse weights were recorded every 3 days.

Isolation of peritoneal macrophages

Mice were injected intraperitoneally with 3 ml of brewer thioglycollate broth (Difco Laboratories, Detroit, MI) on day 26 to increased peritoneal macrophage numbers and allow the stimulated macrophages time to return to normal before the macrophages were removed. Four days later the mice were euthanized with carbon dioxide. Peritoneal

lavage was performed aseptically with 10 ml of ice-cold complete RPMI cell culture medium [RPMI-1640 and 10 percent heat inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 mM 2-mercaptoethanol], and 10 μg/ml heparin. Peritoneal lavage medium was withdrawn from the peritoneum of each mouse and macrophages were counted in the media using a hemocytometer. The macrophages were adjusted to 10^6 cells/ml of complete RPMI medium, and then 5 ml of the cell suspension was plated in 60 mm cell culture dishes. The macrophages were activated using 10 μg/ml lipopolysaccharide (LPS) (Sigma Chemical, St. Louis, MO). Cell culture dishes with activated macrophages were then incubated in a cell culture chamber at 37°C with 5 percent CO_2 for 10 hr to obtain macrophage RNA, or 48 hr to collect supernatants for cytokine protein quantification.

Total RNA isolation and quantification

Cell culture dishes to be used for RNA isolation were taken from the incubator after 10 hr. The medium was removed from the plates and TRI REAGENT™ LS (Molecular Research Center, Cincinnati, OH) was added to the adherent cells following the manufacturer's directions. Total RNA was isolated and purified from the cells following the manufacturer's directions and then resuspended in FORMAzol™ (Molecular Research Center). The RNA concentration was determined spectrophotometrically and the RNA was stored in FORMAzol™ at -20°C.

Northern blot analyses of IL-1α, IL-6, TNFα, and control β-actin were performed using antisense riboprobes labeled with α^{32}P-CTP to a specific activity of 2 x 10^7 cpm (Ausubel *et al.*, 1991). Antisense transcripts were made using bacteriophage T3 or T7 RNA polymerase (Promega, Madison, WI) and cloned cDNA for β-actin, IL-1α, IL-6, and TNFα following manufacturer's directions. The IL-1α cDNA clone (ATCC #63106) was in pBluescript SK+. To prepare the probe, the clone was cut with Hind III and transcribed with T3 RNA Polymerase. Tumor necrosis factor alpha, a 1230 bp cDNA clone with Eco R1 ends in pBluescript II KS+ (Dr. A.D. Weinberg, University of California, San Diego, CA), was cut with Hind III and transcribed with T7 RNA polymerase. The IL-6 cDNA clone, 800 bp with Pst I ends in pBluescript II KS+ (Dr. A.D. Weinberg, University of California, San Diego, CA), was cut with Hind III and transcribed with T7 RNA polymerase. The human β-actin cDNA clone (ATCC #78554) was cut with Hind III and transcribed with T3 RNA polymerase. All cDNA clones were sequenced from both ends. These sequences conformed to their respective cDNA sequences in Genbank (Genetics Computer Group, Madison, WI).

Ten micrograms of total RNA were separated on a 1.2 percent agarose gel containing 1.1 percent formaldehyde, and transferred to MAGNA NT nylon membranes (Micron Separations Inc., Westboro, MA). The membranes were stained with 0.02 percent methylene blue and photographed with Polaroid type 667 film so the positions of the 18S and 28S ribosomal RNA bands, as well as the RNA ladder size markers, could be

marked on the membrane. These marks were later used to estimate the size of the RNA bands in each lane. The membranes were prehybridized for 1 hr followed by hybridization for 16-24 hr in buffers containing 50 percent formamide, and then washed under high stringency conditions (5X SSPE/0.5 percent SDS for 15 min at room temperature, 1X SSPE/1.0 percent SDS for 15 min at 37°C, 0.1X SSPE/1 percent SDS at 65°C for 15 min). The blots were then exposed for 24-168 hr to Kodak X-AR radiographic film at -70°C using Dupont Lightning Plus screens. The bands on the film were quantitated by scanning their densities using the ZERO-D scan software program (Stratagene Cloning Systems, LaJolla, CA). Total RNA in each lane was adjusted using the β-actin standards and the densities of control and treatment groups were compared.

Cytokine quantification

The supernatants from activated macrophage cell cultures incubated for 48 hr were collected and used to quantitate IL-1α, IL-6 and TNFα cytokine production. All supernatants were frozen at -70°C until assayed. These cytokines were quantitated using sandwich ELISA kits for each cytokine (Endogen, Inc., Cambridge, MA) according to manufacturer's directions.

Statistical methods

Data from the Northern blots and cytokine ELISA's were analyzed by using a one-way analysis of variance (ANOVA) followed by the Fisher least significant

difference (LSD) statistical analysis. Statistical calculations were done using Stat View™ SE and Graphics 1.03 software (Abacus Concepts, Inc., Berkeley, CA).

Results

The daily intake of benzene by each group of mice was 0, 40, 200, and 1000 mg/L in the control, low, medium, and high dose groups, respectively (Table 3-1). The high dose group drank less water than the other groups.

Continuous exposure to various amounts of benzene in the drinking water of male ICR mice did not cause any clinical signs of toxicity. No gross abnormalities were noted in the mice upon euthanasia. In addition, there was no noticeable change in food consumption between control and treatment groups. The average weight of the groups at the beginning of the study or at day 30 did not differ significantly.

TABLE 3-1
Body Weights and Peritoneal Macrophage Numbers in Mice Given Benzene in Their Drinking Water for 30 Days

Benzene Conc. in Water (mg/L)	Water Consumed[a] (ml/day)	Body Weights Day 0[b] (grams)	Body Weights Day 30[b] (grams)	Peritoneal Macrophages[b] ($x10^6$ cells/ml)
0	8.8	25.0 ± 0.5	37.5 ± 0.9	2.32 ± 0.14
40	8.6	25.6 ± 0.9	35.3 ± 1.1	1.49 ± 0.31
200	8.9	25.2 ± 0.6	38.3 ± 1.7	1.84 ± 0.27
1000	7.2	25.8 ± 0.7	36.8 ± 1.7	1.91 ± 0.23

[a] Values are the mean from each group of 5 mice
[b] Values are the mean ± S.E. (n=5)

It should be noted (see Chapter II) that there were no significant differences in spleen, liver, and kidney weights among similar groups of mice treated with identical concentrations of benzene during the same period of time. However, thymus weights were significantly lower ($p<0.05$) in the benzene treatment groups (Table 2-2). Overall, benzene-treated groups of mice produced fewer peritoneal macrophages when stimulated by the I.P. injection of brewers thioglycollate medium. The low dose group produced the fewest macrophages and the control group produced the greatest number of macrophages.

Interleukin-1α, IL-6, and TNFα gene expression was determined by Northern blot analysis. While not significant statistically, a prominent trend in both IL-1α and IL-6 showed that mRNA production steadily increased at the low and medium dosage levels, then dropped at the high dose (Figure 3-1 and 3-2). In the case of IL-1α, mRNA production at the high dose was less than the control mRNA production. The results also indicate that benzene-treated mice had significantly lower amounts of TNFα mRNA produced at all tested dosage levels when compared to the control group (Figure 3-3). No apparent differences in the levels of β-actin mRNA production were seen among any of the groups.

Macrophages from the low dosage group had lower IL-1α cytokine protein secretion than the control group (Figure 3-4). Although the level of IL-1α for the medium dose was significantly higher ($p<0.05$) than the low dose, both medium and high dosage groups secreted about the same amounts of IL-1α as the control group. Interleukin-6

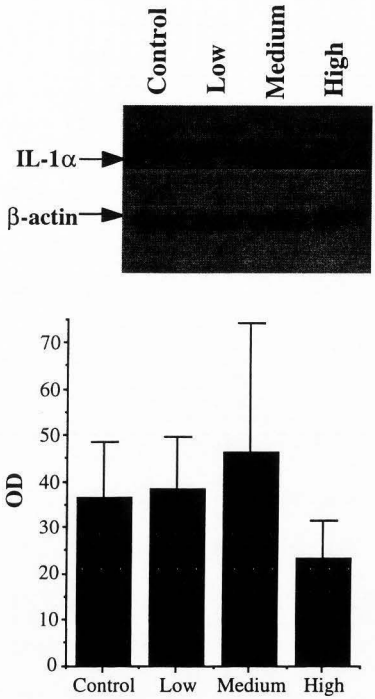

FIG. 3-1. The effects of benzene on IL-1α mRNA production from LPS-activated macrophages. Total RNA was isolated from LPS-activated peritoneal macrophages (10 μg/10^6 cells for 10 hr) obtained from control and benzene-treated mice. The RNA (10 μg) was analyzed by Northern blot using murine IL-1α and was standardized using β-actin ^{32}P labeled riboprobes. Band densities were measured by ZERO-D scan and the values of the total RNA per lane were equalized using the β-actin standards. Data are expressed as the mean ± S.E. of the animals within each group (n=3). Hybridizations of IL-1α and β-actin were performed separately. Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively.

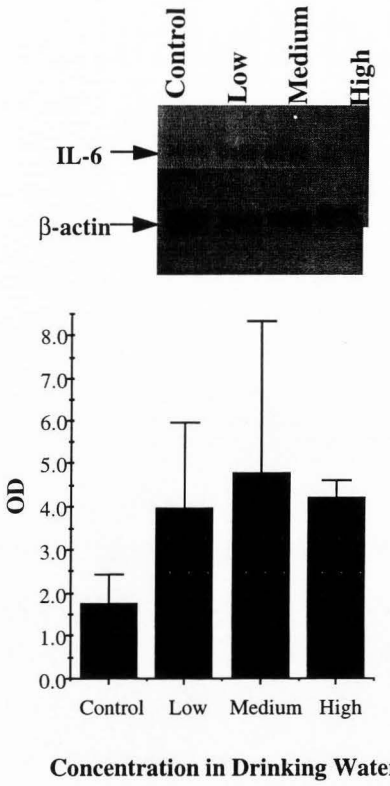

FIG. 3-2. The effects of benzene on IL-6 mRNA production from LPS-activated macrophages. Total RNA was isolated from LPS-activated peritoneal macrophages (10 μg/10^6 cells for 10 hr) obtained from control and benzene-treated mice. The RNA (10 μg) was analyzed by Northern blot using murine IL-6 and was standardized using β-actin ^{32}P labeled riboprobes. Band densities were measured by ZERO-D scan and the values of the total RNA per lane were equalized using the β-actin standards. Data are expressed as the mean ± S.E. of the animals within each group (n=3). Hybridizations of IL-6 and β-actin were performed separately. Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively.

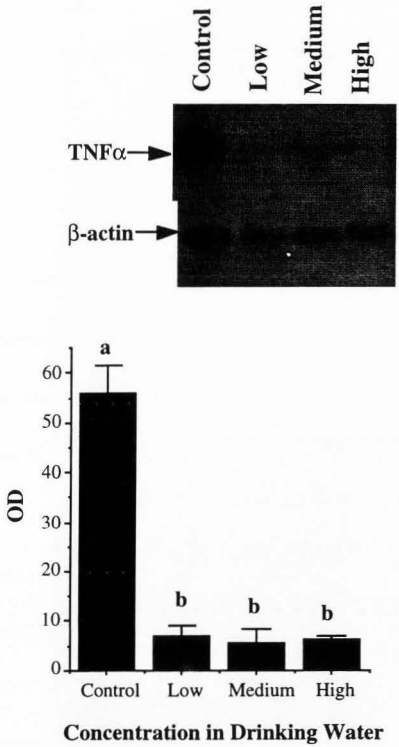

FIG. 3-3. The effects of benzene on TNFα mRNA production from LPS-activated macrophages. Total RNA was isolated from LPS-activated peritoneal macrophages (10 μg/10^6 cells for 10 hr) obtained from control and benzene-treated mice. The RNA (10 μg) was analyzed by Northern blot using murine TNFα and was standardized using β-actin ^{32}P labeled riboprobes. Band densities were measured by ZERO-D scan and the values of the total RNA per lane were equalized using the β-actin standards. Data are expressed as the mean ± S.E. of the animals within each group (n=3). Hybridizations of TNFα and β-actin were performed separately. Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$).

cytokine protein production was not changed by benzene treatment at any dosage level (Figure 3-5). However, IL-6 protein production, as determined by sandwich ELISA, was similar to IL-6 cytokine mRNA production (i.e., low and medium dosages increased over the control value and high dosage group was about the same as the medium group). Benzene significantly depressed TNFα secretion ($p<0.05$) at all dosage levels (Figure 3-6). In fact, benzene-treated mice produced very little TNFα cytokine. The macrophages from benzene-treated mice produced between 0.05 and 0.1 ng/ml TNFα while the control group produced an average of 32.4 ng/ml TNFα. Levels of IL-6 and TNFα mRNA followed IL-6 and TNFα protein secretion; however, there was no similarity between IL-1α mRNA and protein levels. While IL-1α mRNA for the low and medium groups tended to increase, IL-1α protein production was lower or similar to control values for low and medium dose groups, respectively. The high dose group had a lowered level of IL-1α cytokine mRNA compared to the control group, but the level of IL-1α protein secretion was greater than the control value.

Discussion

The basis of this study rests on the theory that an analysis of toxin-induced changes in cytokine gene expression and cytokine protein production will help to determine the molecular mechanisms that lead to the immunotoxicity caused by the toxin. The results of this research demonstrate that macrophages from mice given benzene in

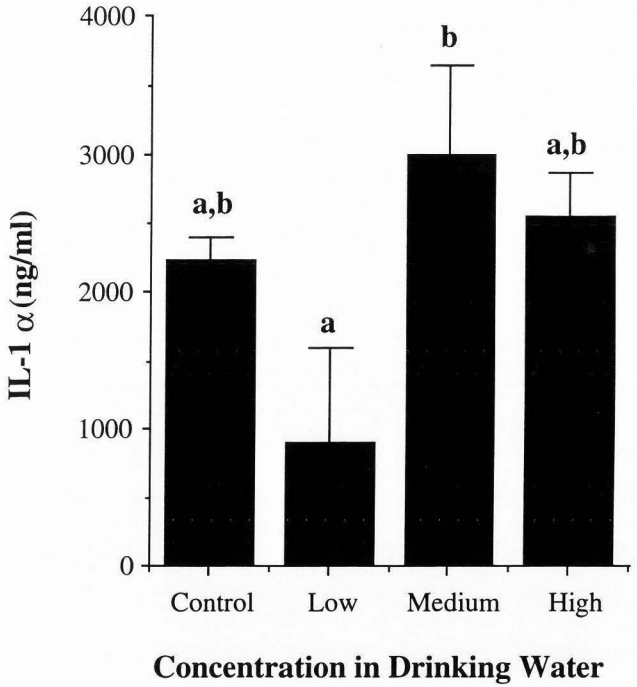

FIG. 3-4. Effects of benzene on IL-1α production from activated macrophages of mice ingesting benzene through drinking water. The macrophages ($1x10^6$/ml) from control and benzene-treated mice were activated with 10 μg/ml LPS. Supernatants were taken after 48 hours and IL-1α was detected in the serum by ELISA. Data are reported as the mean ± S.E. for animals within each treatment group (n=4). Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$).

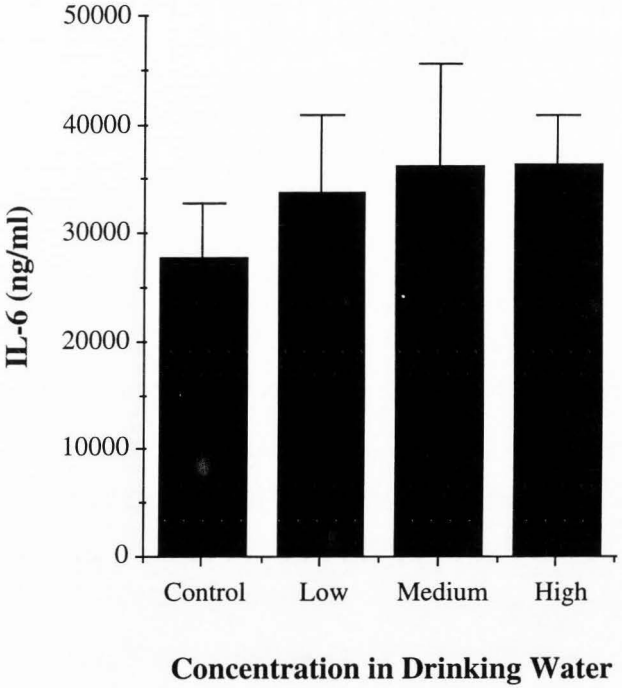

FIG. 3-5. Effects of benzene on IL-6 production from activated macrophages of mice ingesting benzene through drinking water. The macrophages ($1x10^6$/ml) from control and benzene-treated mice were activated with 10 μg/ml LPS. Supernatants were taken after 48 hours and IL-6 was detected in the serum by ELISA. Data are reported as the mean ± S.E. for animals within each treatment group (n=4). Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively.

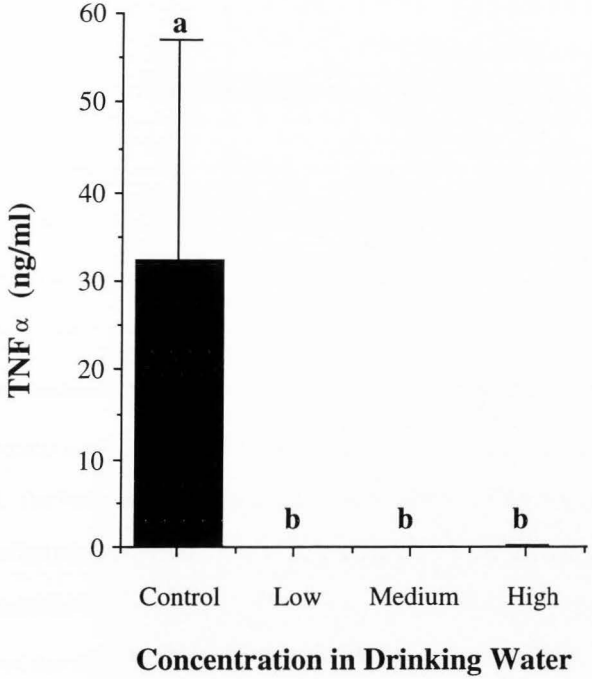

FIG. 3-6. Effects of benzene on TNFα production from activated macrophages of mice ingesting benzene through drinking water. The macrophages ($1x10^6$/ml) from control and benzene-treated mice were activated with 10 μg/ml LPS. Supernatants were taken after 48 hours and TNFα was detected in the serum by ELISA. Data are reported as the mean ± S.E. for animals within each treatment group (n=4). Drinking water concentrations of benzene were 0, 40, 200, and 1000 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$).

their drinking water had altered cytokine mRNA and protein production. This effect was particularly evident for TNFα as levels were severely inhibited in macrophages from mice given all concentrations of benzene.

Although the dosages of benzene were much lower in this study than in some experiments studying the effects of benzene on cytokine production (MacEachern and Laskin, 1992; Renz and Kalf, 1991), the sensitivity of the immune system to environmental toxins can be suggested by the fact that even 40 mg/L of benzene in the drinking water caused a significant reduction in TNFα mRNA and protein levels and appeared to stimulate both IL-1α and IL-6 cytokine mRNA transcription.

Normally an antigen is phagocytized by a macrophage and then presented to a T-helper cell. The T-helper cell becomes activated and releases cytokines to activate the macrophage and stimulate more macrophage migration to that area (Janeway and Travers, 1994). Interleukin-1α, IL-6, and TNFα are essential in the proper activation of lymphocytes, thus activating the rest of the immune system (Dinarello, 1992; Akira *et al.*, 1990). In this study, fewer numbers of peritoneal macrophages were obtained from the treatment groups when compared to the control group. Benzene is not known to be directly toxic to the macrophage, as it is to lymphocytes (Moszczynsky and Lisiewicz, 1984). Fewer macrophages numbers could be from less responsive macrophages leading to a suppressed immune response following the intraperitoneal injection of brewers thioglycollate. Indeed, macrophages obtained from benzene-treated mice were less

responsive as indicated by altered IL-1α and TNFα mRNA production and cytokine secretion.

Another reason for fewer peritoneal macrophages from benzene treated mice may be related to benzene and its metabolites in the bone marrow. It has been previously shown that benzene administration (660 mg/kg daily for 3 days) caused an increased number of mature active macrophages in the bone marrow of mice (MacEachern and Laskin, 1992). This activation of macrophages in the bone marrow may have prevented the I.P. injection of brewer thioglycollate broth from attracting as many macrophages into the peritoneum, and therefore, fewer macrophages were collected in the lavage.

Both TNFα mRNA and protein levels were depressed with all doses of benzene (Figures 3-3 and 3-6). One possible explanation for this depression is that the transcriptional activation of TNFα is being blocked. Like IL-1α and IL-6, the TNF gene has several activating factor binding sites upstream of the promoter (Akira *et al.*, 1990). In the case of cyclosporin A and FK 506, which are known immunosuppressants, their immunosuppression is due to inhibition of protein factor production, which binds to the IL-2 enhancer (Brabletz *et al.*, 1991). Likewise, if benzene prevented the formation of NF-KB, AP-1, or Sp1 nuclear binding proteins known to have binding sights on the TNFα gene (Akira *et al.*, 1990), the production of TNFα mRNA would decrease. If less TNFα mRNA is produced, it is only logical to assume less translation of mRNA would occur and thus less cytokine would be produced. Another reason for lower TNFα

protein production could be that posttranslational events might be inhibited. In the normal macrophage, TNFα mRNA is translated into a 26 KD pre-TNFα. This pre-TNFα is then cleaved to a 17 KD secreted form of TNFα. This intracellular conversion of pre-TNFα to the secreted TNFα is inhibited by Δ9-Tetrahydrocannabinol (Fischer-Stenger *et al.*, 1993). The fact that there was almost no TNFα production in the benzene-treated groups, while these same groups had some TNFα mRNA production, although it was less than control TNFα mRNA production, suggests that both transcription and posttranslational mechanisms may be affected by benzene.

There was a significant increase in the production of IL-1α protein at the medium dose, but at the high dose, protein production began to decrease. This pattern of IL-1α protein secretion is similar to IL-1α mRNA production (Figures 3-1 and 3-3). The report of a biphasic response to benzene is not novel. Hsieh *et al.* (1988) found that at a benzene dose of 8 mg/kg/day, splenocyte responses to mitogens such as LPS and Con A were increased, but as the dose of benzene increased, splenocyte responses to mitogens lessened. As mentioned, cyclosporin A inhibits nuclear activating proteins causing immunosuppression (Emmel *et al.*, 1989). Still, cyclosporin A does not inhibit all nuclear binding proteins. In fact, some proteins such as AP-1 appear to not be affected by cyclosporin up to doses of 1000 ng/ml. Other proteins, such NF-KB, are stimulated at low doses of cyclosporin A and then inhibited as the dose increases (Emmel *et al.*, 1989). A possible explanation of benzene's biphasic reaction could be that one or more

nuclear activating factors are stimulated at lower doses of benzene and as the benzene increases, the same nuclear activating factors are depressed. This biphasic response is also indicative of benzene being toxic to the macrophage at the high dose.

Interleukin-1α mRNA is translated into a pre-IL-1 α protein in a similar fashion as TNF α. This pre-IL-1 α requires the enzyme calpain to cleave it into the mature form, which is then secreted (Miller *et al.*, 1994; Dinarello, 1992). The benzene metabolite hydroquinone is known to inhibit pre-IL-1α to the mature IL-1α by decreasing calpain activity (Miller *et al.*, 1994). In the present study, IL-1 α protein levels did not appear to be depressed, except at the low dose. Perhaps the benzene concentrations used in this study never reached a level that would inhibit calpain activity in the macrophages. While hydroquinone has been implicated in calpain inhibition, other metabolites of benzene have not. In fact, the benzene metabolite catechol (see Figure 1-3) does not produce toxicity when administered alone (Fishbein, 1984; Irons *et al.*, 1992).

Another possible explanation for the levels of IL-1α from benzene-treated macrophages has to do with the tight regulatory control exerted by the body on all cytokine production. It is known that all cytokines have mRNA with AU-rich 3' UTR regions. This region on the mRNA is recognized by certain cytosolic proteins that destroy the mRNA. As the mRNA half life decreases, its ability to be translated into protein is also decreased (Janeway and Travers, 1994; Shaw and Kamen, 1986). As benzene and benzene metabolite levels increase, the activity of these cytosolic enzymes

may also be reduced, thus increasing the half life of IL-1α mRNA and its ability to be translated into protein.

In conclusion, benzene given in doses of 40, 200, and 1000 mg/L likely caused several events that may ultimately lead to immunotoxicity. Interleukin-1α, IL-6, and TNFα are essential factors in stimulating lymphocytes, and activating the immune system. The depression in TNFα alone is enough to prevent activation of T_H1 cells and macrophages. When variations in normal IL-1α production are combined with the depressed TNFα production, it is possible that immune system functions will be altered.

The small number of animals used in this study and the large variation within groups led to few statistical significances. One possible reason for the variation may be because of the differences in water consumption by each mouse, altering the effect of benzene because a similar dose was not given to each mouse. Future studies may want to alter the method of benzene administration for more consistent dosages so more statistically significant results may be achieved. This research lends support for the concerns of benzene-contaminated drinking water supplies and its possible immunotoxic consequences.

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CHAPTER 4

VARIATION IN ESSENTIAL CYTOKINE PROTEIN AND CYTOKINE MESSENGER RNA PRODUCTION IN THE LYMPHOCYTES OF MALE INSTITUTE OF CANCER RESEARCH MICE GIVEN PHENOL IN THEIR DRINKING WATER

Abstract

The major metabolite of benzene is phenol. Industry produces and uses large quantities of phenol in manufacturing processes. Phenol has been shown to pollute underground water supplies. The effects of long-term, low-dose exposure to phenol include suppressed lymphocyte responsiveness to antigens and decreased antibody production. Cytokines are key signaling molecules used by immune cells to induce cell proliferation and antibody production, and to recruit additional cells. In this study, the immunotoxic effects of phenol on lymphocyte cytokine production were evaluated using phenol in the drinking water of mice for 30 days. The mice received doses of 0, 5, 20, and 100 mg/L in the control, low, medium, and high dose groups, respectively. Phenol ingestion resulted in decreased thymus weights at all dose levels. Splenocytes were removed from the mice and activated *in vitro* with Concanavalin A (Con A). The levels of IL-2, IL-3, and IFNγ mRNA were measured using RT-PCR, and the IL-2, IL-3, and IFNγ secreted protein levels were measured by sandwich ELISA. A dose-related increase in IL-2 and IFNγ mRNA in lymphocytes from the low and medium phenol treatment

groups was observed. The IL-3 mRNA production was lower in lymphocytes from low and high groups of phenol-treated mice when compared to the control group. Unlike the pattern of mRNA production, IL-3 cytokine secretion did not differ between the control and phenol groups, suggesting some kind of posttranscriptional regulation of the IL-3 mRNA. Interleukin-2 and IFNγ secreted protein levels were not statistically different from the control. Thus, phenol may be altering cytokine production in lymphocytes, and possibly regulates these cytokines at both the transcriptional and posttranscriptional levels.

Introduction

Phenol is a monohydroxy derivative of benzene , and is the primary metabolite of benzene *in vivo* (Sawahata *et al.*, 1985; Fishbein, 1984). Many chemical, oil, and steel industries produce phenol in large quantities. Phenol is used as a reagent in the process manufacturing resins, dyes, disinfectants, antiseptics, and antimicrobial agents, and is a solvent in the petroleum refining process. Phenol is produced after the decomposition of animal wastes and from the normal metabolism in the body of some ingested medicines (such as Pepto-Bismol™) and aromatic amino acid metabolism (U.S. Environmental Protection Agency, 1986, 1993; Bruce *et al.*, 1987).

In experimental animals, phenol is fetotoxic, genotoxic, and myelotoxic (Subrahmanyam *et al.*, 1990; Gaido and Wierda 1985). Other determined health effects that have been associated with phenol include cardiac arrhythmias and depression,

respiratory depression, neuromuscular hyperexcitability, coma, encephalopathy, severe irritation, corrosion of the skin or other tissues, and spleen, liver, and kidney damage (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987; Windus-Podehl *et al.*, 1983). In addition, phenol in drinking water has been reported to cause increased disease susceptibility in humans (Baker *et al.*, 1978). Phenol is also immunotoxic. Suppression of B- and T-cell lymphoproliferation after lipopolysaccharide (LPS), phytohemagglutinin (PHA), and pokeweed mitogen (PWM) exposure following phenol addition to the drinking water of mice has been reported (Hsieh *et al.*, 1992). Decreased antibody production by the mice was also reported in that same study.

Cytokines are proteins that render signals to cells, providing a communication network between many cells in the body. These cytokines have been found to be key factors in promoting growth, differentiation, and activation of immune cells (Janeway and Travers, 1994; Tizard, 1992). Thus, it is possible that the mechanism of phenol's toxicity to immune cells is from an alteration of key cytokine production by immune cells such as the lymphocyte or macrophage. Very little research has been done to determine if phenol is altering cytokine production.

The purpose of this study was to examine both the cytokine mRNA and protein levels produced by lymphocytes of mice given phenol in their drinking water at levels already known to cause immunotoxicity (Hsieh *et al.*, 1992). Cytokines IL-2, IL-3, and IFNγ were chosen on the basis of their importance in immune response regulation

(Janeway and Travers, 1994; Taniguchi and Minami, 1993; Mizel, 1989; Smith and Rennick, 1986; Friedman and Vogel, 1983; Kasahara *et al.*, 1983). Because of the concern for the hazards of pollutants (such as phenol) present in groundwater used by a large proportion of the population for drinking water (U.S. General Accounting Office, 1988; U.S. Office of Technology Assessment, 1984), phenol was added to the drinking water of the mice used in this study.

Materials and Methods

Animals

Five-week-old male outbred Institute of Cancer Research (ICR) mice were obtained from Simonsen Laboratories (Gilroy, CA), and were housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-approved facility. The mice were maintained at a constant 22-24°C temperature and a 40-60 percent humidity with a 12-hr light/dark cycle. The mice were acclimated to this regimen for 1 week prior to the beginning of this study. Groups of five mice were randomly assigned to plastic cages containing ground corn cob bedding. Commercial rodent chow (Harlan Teklad, Madison, WI) and water were provided *ad libitum*.

Phenol exposure

Phenol (USP Phenol 90% w/w. Fisher Scientific, Fair Lawn, NJ) was dissolved in tap water to make a stock solution of 100 mg/L. From the stock solution, additional

solutions of 5 mg/L and 20 mg/L were made. Phenol was administered to the three treatment groups of mice (n=5 in each group) for 30 days at 5, 20, and 100 mg/L water for the low, medium, and high treatment groups, respectively. The control group received untreated tap water for 30 days. The phenol treated water was put in glass water bottles with stainless steel sipper tubes, shaken daily, and changed every 3 days to minimize phenol decomposition and to maintain the desired phenol concentrations in the water. In order to duplicate results from past research, phenol was added to tap water and changed every 3 days just as performed by previous workers (Hsieh *et al.*, 1992). Water consumption and mouse weights were recorded every 3 days.

Gross observations

After 30 days, the mice were anesthetized with halothane gas to obtain blood samples. The mice were then euthanized by cervical dislocation and the spleen was removed aseptically and placed into cold complete RPMI 1640 cell culture media [RPMI-1640 (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 mM 2-mercaptoethanol, 10 percent heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT)]. Gross pathological examination of other organs was performed and the liver, kidneys, and thymus were weighed.

Isolation and activation of splenic lymphocytes

A monocellular cell suspension from each spleen was prepared using a Stomacher lab blender (STOM 80, Tekman Co., Cincinnati, OH). Red blood cells were removed with ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). The remaining cells were plated on 100-mm tissue culture plates and incubated in a humidified 37°C, 5 percent CO_2 incubator for 1 hr to allow the adherence of macrophages. The nonadherent cells were collected after 1 hr and counted on a Coulter counter (Coulter Electronic Inc., Hialeah, FL). The concentration of nonadherent cells was adjusted to $2x10^6$ cells/ml, 5 μg/ml Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) was added to activate the cells, and 15-20 ml of the activated cellular solution were replated on 100-mm cell culture dishes. These culture dishes were incubated for 8 hr to obtain cytokine mRNA or 48 hr to find cytokine protein in the supernatant.

Total RNA isolation and quantification

After 8 hr of activation with Con A, the lymphocytes were removed from the tissue culture plates and pelleted by centrifugation. Total RNA was extracted from the lymphocyte pellet with TRI REAGENT™ LS (Molecular Research Center, Cincinnati, OH) following the manufacturer's directions. Purified RNA was resuspended in ribonuclease (RNase) free DEPC-ddH_2O (diethylpyrocarbonate-distilled, deionized water), quantified using a spectrophotometer (Shimatzu Corp., Kyoto, Japan), and then stored at -70°C.

RNA analysis

First strand cDNA synthesis was performed using 1 µg total RNA and 0.5 µg oligo (dT_{15}) (Promega Corporation, Madison, WI), in a 20-µl total reaction volume containing 50 mM tris-HCl (pH 8.3 at room temperature), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of dATP, dCTP, dGTP, and dTTP; 200 units of Superscript II RNase H^- Reverse Transcriptase (Life Technologies, Gaithersburg, MD) were then added and the sample was incubated for 50 min at 42°C. The reverse transcriptase enzyme was heat inactivated at 70°C for 15 min then the tubes were placed on ice. Two units of RNase H (United States Biochemical, Cleveland, OH) were added and the samples incubated at 37°C for 20 min. Twenty µl of ddH_2O was then added and the product was stored at -20°C until further analyses were performed.

Interleukin-2, IL-3, and IFNγ mRNA production were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Foley *et al.,* 1993). Primer sets for each of these cytokines were developed using Oligo 4.03 software (National Biosciences, Inc., Plymouth, MN). The primer sequences were as follows:

IL-2 : Sense = 5'-GCGCACCCACTTCAAGCTCC-3'

Antisense = 5'-AGCCCTTGGGGCTTACAAAA-3'

Product length - 611 bp

IL-3 : Sense = 5'-CCCTTGGAGGACCCAGAAC-3'

Antisense = 5'-GCCATGAGGAACATTCAGAC-3'

Product length - 567 bp

INFγ: Sense = 5'-GTTCTGGGCTTCTCCTCCTG-3'

Antisense = 5'-CGAATCAGCAGCGACTCCTT-3'

Product length - 510 bp

β–actin : Sense = 5'-AACACAGTGTTGTCTGGTGG-3'

Antisense = 5'ACGCAGCTCAGTAACAGTCC-3'

Product length - 265 bp

Two µl of the first strand reaction from above were used for each PCR reaction. The optimal primer and $MgCl_2$ concentrations were determined empirically for each cytokine. In a total reaction mixture volume of 50 ml, 10 mM Tris-HCL, pH 9.0, 50 mM KCl, and 0.1 percent Triton X-100 were added. The $MgCl_2$ concentrations were 5 mM, 4 mM, 2 mM, and 3 mM for IL-2, IL-3, IFNγ, and β-actin, respectively. The primer concentrations for IL-2, IL-3, IFNγ, and β-actin were 30 pmol, 30 pmol, 20 pmol, and 10 pmol, respectively. A TempCycler II thermocycler (Coy Corporation, Grass Lake, MI) was used for the PCR reaction. The temperatures and times used in the reactions were 95°C for 5 min followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min.

The products from each PCR reaction were separated on 2 percent agarose gels, stained with ethidium bromide and visualized with a UV transilluminator (Fisher Scientific, Pittsburgh, PA). Negatives of each gel were taken using Polaroid Type 665

positive/negative film. The negatives were scanned and the optical densities of each band were calculated using the ZERO-D scan software program (Stratagene Cloning Systems, LaJolla, CA). Total RNA in each band was adjusted using the β-actin standards and the average densities of RNA for control and treated groups were compared for statistical differences. The PCR products were confirmed to be the appropriate cytokine by sequencing PCR products from three mice.

Cytokine protein quantitation

Supernatants from 48-hr activated splenocyte cultures were obtained by removing the cells and media from the tissue culture dish, centrifuging to pellet the cells, and pouring off the supernatant. The supernatants were placed in separate vials and stored at -70°C until needed for IL-2, IL-3, and IFNγ quantification. All cytokines were quantitated by sandwich ELISA techniques using kits obtained from Endogen, Inc. (Cambridge, MA) and following the manufacturer's instructions.

Statistical methods

Treatment effects for RT-PCR and cytokine ELISAs were analyzed using a one-way analysis of variance (ANOVA) followed by the Fisher least significant difference (LSD) statistical analysis. Statistical calculations were done using Stat View™ SE and Graphics 1.03 software (Abacus Concepts, Inc., Berkeley, CA).

Results

The doses of phenol were 0, 5, 20, and 100 mg/L for the control group, and the low, medium and high treatment groups, respectively (Table 4-1). Animals exposed to phenol did not elicit any overt signs of toxicity and there were no obvious differences in food consumption. In addition, there was very little difference in water consumption between control and treatment groups. There were no gross pathological differences between control and treatment groups.

Weights and spleen cellularity

The results of continuous ingestion to phenol for 30 days on the body and organ

Table 4-1
Body Weights and Water Consumption in Mice Given Phenol in Their Drinking Water for 30 Days

Phenol Conc. in Water (mg/L)	Water Consumed[a] (ml/day)	Body Weights Day 0[b] (grams)	Body Weights Day 30[b] (grams)
0	8.3	27.5 ± 0.8	37.2 ± 1.3
5	8.4	28.1 ± 0.6	35.5 ± 2.0
20	8.2	28.4 ± 1.2	37.4 ± 2.0
100	8.4	28.0 ± 0.6	36.7 ± 1.3

[a] Values are the mean from each group of 5 mice
[b] Values are the mean ± S.E. (n=4)

TABLE 4-2
Selected Organ Weights and Spleen Cellularity in Mice Ingesting Phenol in Their Drinking Water for 30 Days

Phenol Dose (mg/L)	Spleen weight[a] (g/100g BW)	Kidney weight[a] (g/100g BW)	Liver weight[a] (g/100g BW)	Thymus weight[a] (g/100g BW)	Spleen cellularity[a] ($x10^6$ cells/ml)
0	0.25 ± 0.02^b	2.06 ± 0.10	6.50 ± 0.21	0.19 ± 0.03^b	2.15 ± 0.16
5	0.21 ± 0.01^b	2.21 ± 0.13	5.81 ± 0.23	0.09 ± 0.01^c	$3.17 \pm 0..26$
20	0.19 ± 0.02^c	2.28 ± 0.11	6.23 ± 0.14	0.10 ± 0.01^c	3.04 ± 0.55
100	0.24 ± 0.02^b	2.49 ± 0.20	6.70 ± 0.27	0.12 ± 0.02^c	2.76 ± 0.17

[a] Values are the mean ± S.E. (n=4)
[b,c] Different superscripts within a column are different $p<0.05$

weights are listed in Tables 4-1 and 4-2. There were no significant differences among the starting weights and the ending weights for each group. The treatment groups had lower spleen weights, but this was not a significant difference. Thymus weights were significantly lower ($p<0.05$) in the treated groups when compared to the controls. However, the thymus weights among treatment groups were not significantly different. Neither liver nor kidney weights were significantly different between control and treatment groups, but as the dose of phenol increased, average kidney weight tended to increase.

After removing red blood cells and macrophages from the spleen homogenate, the

remaining cells were counted. No significant difference in the number of splenocytes between the control and treated groups was found although the average cellularity of the spleen tended to be higher in the treatment groups.

Cytokine mRNA production

The effect of phenol on IL-2, IL-3, and INFγ mRNA production was measured by RT-PCR (Figures 4-1, 4-2, and 4-3, respectively). There were no significant alterations in IL-2 and IFNγ mRNA levels between lymphocytes from the control and the phenol treated mice from the low and medium phenol dosages (Figures 4-1 and 4-3). The IL-3 mRNA production in lymphocytes from mice given low and high doses was significantly depressed (Figure 4-2). At the medium dosage level, lymphocytes produced significantly more IL-3 mRNA than the did lymphocytes from the high phenol group, although this production was not significantly different from the control group. No difference in the production of β-actin mRNA was observed among the groups.

Cytokine ELISA's

Supernatants from splenocytes that were cultured for 48 hr in the presence of Con A were used to quantitate IL-2, IL-3, and IFNγ production. The results are illustrated in Figures 4-4, 4-5, and 4-6. No significant differences were noted in the cytokine protein production from sandwich ELISA. It should be noted that the pattern of protein production does not follow that of cytokine mRNA production for IL-3 at the high and

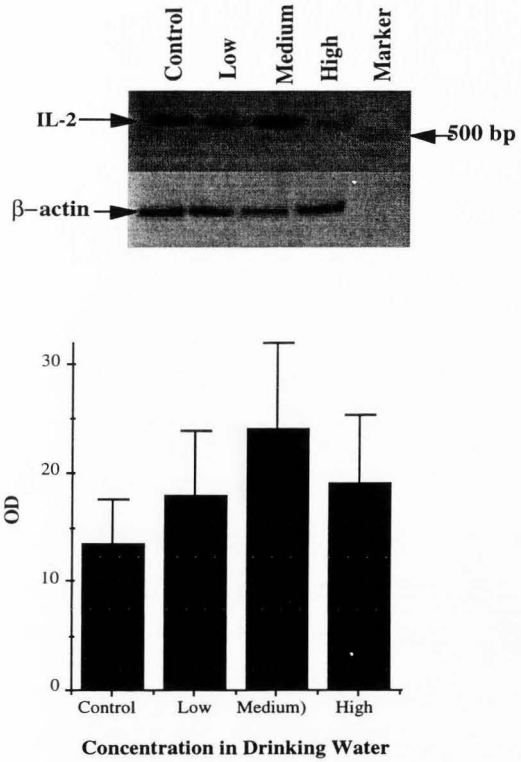

FIG. 4-1. Levels of IL-2 mRNA in Con A activated splenocytes from phenol-treated mice. One μg total RNA from the Con A-activated (5 μg/2x10^6 cells for 10 hr) splenocytes was used to make cDNA and then amplified by PCR using murine IL-2 and β-actin primers. The densities from the band of each animal (n=4) were measured by ZERO-D scan. Values of IL-2 product were standardized for differences in total RNA using the β-actin product. Separate 2 percent agarose gels were used to run IL-2 and β-actin PCR products. Data are expressed as mean + S.E. Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively.

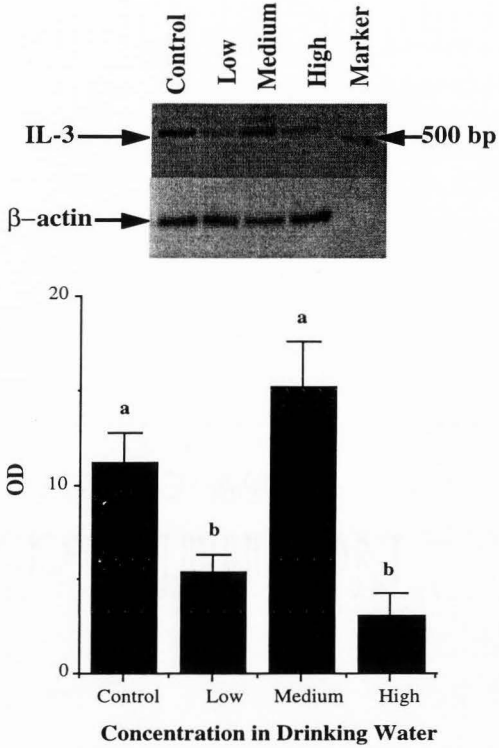

FIG. 4-2. Levels of IL-3 mRNA in Con A activated splenocytes from phenol-treated mice. One μg total RNA from the Con A-activated (5 μg/2x10^6 cells for 10 hr) splenocytes was used to make cDNA and then amplified by PCR using murine IL-3 and β-actin primers. The densities from the band of each animal (n=4) were measured by ZERO-D scan. Values of IL-3 product were standardized for differences in total RNA using the β-actin product. Separate 2 percent agarose gels were used to run IL-3 and β-actin PCR products. Data are expressed as mean + S.E. Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$).

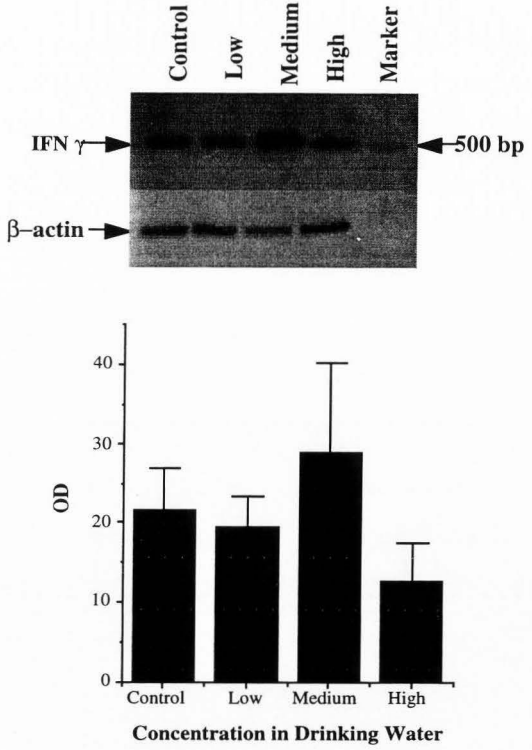

FIG. 4-3. Levels of IFNγ mRNA in Con A activated splenocytes from phenol-treated mice. One μg total RNA from the Con A-activated (5 μg/2x10^6 cells for 10 hr) splenocytes was used to make cDNA and then amplified by PCR using murine IFNγ and β-actin primers. The densities from the band of each animal (n=4) were measured by ZERO-D scan. Values of IFNγ product were standardized for differences in total RNA using the β-actin product. Separate 2 percent agarose gels were used to run IFNγ and β-actin PCR products. Data are expressed as mean + S.E. Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively.

low doses. All of the lymphocytes secreted nearly equal amounts of the IL-3 cytokine despite the lowered IL-3 mRNA levels in phenol-treated mice. The INFγ cytokine mRNA level at the highest dose decreased slightly from the medium dose group and the cytokine protein level echoes this decreased mRNA production. Interleukin-2 showed higher cytokine protein levels at the low dose compared to the control group. This increase is similar to the cytokine IL-2 mRNA produced, with higher levels at the low and medium doses, and less at the high dose. However, IL-2 protein secretion was lower in lymphocytes from mice given the medium dose of phenol, and decreased further in mice given the high dose of phenol.

Discussion

Phenol, or monohydroxy benzene, is considered a myelotoxicant and immunotoxicant (Legathe *et al.*, 1994; Hsieh *et al.*, 1992). Phenol's toxicity depends on its concentration in the bone marrow or lymphoid organs and the conjugation of phenol in the liver (Bruce *et al.*, 1987; Subrahmanyam *et al.*, 1990). The bone marrow uses peroxidases to metabolize phenol to hydroquinone and catechol. These peroxidases are capable of converting phenol to reactive species. Phenol can also be activated through co-oxidation with prostaglandin, forming reactive semiquinone and quinone macromolecules (Eastmond *et al.*, 1987; Tunek *et al.*, 1982). Therefore, even though benzene and phenol are closely related chemically, phenol may be metabolized into different amounts of the

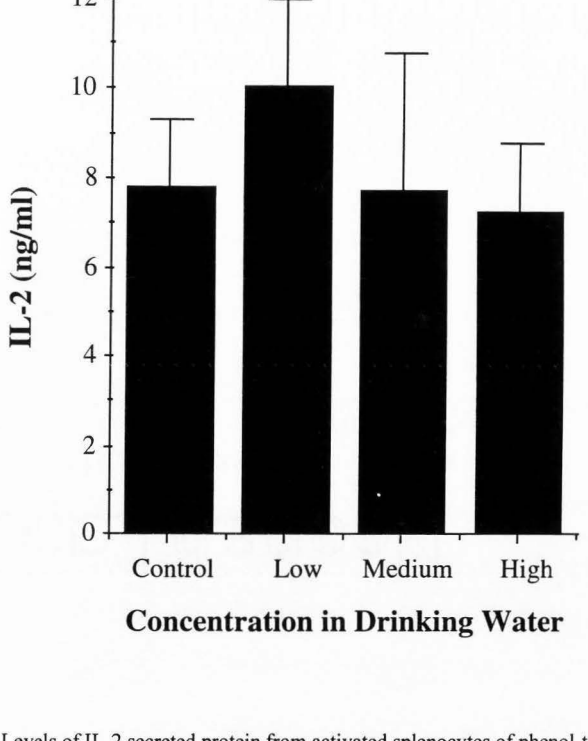

FIG. 4-4. Levels of IL-2 secreted protein from activated splenocytes of phenol-treated mice. The splenocytes ($2x10^6$/ml) from control and phenol-treated mice were activated with 5 μg/ml Con A. Supernatants were taken after 48 hours, and IL-2 was detected in the serum by ELISA. Data are expressed as mean + S.E. (n=4). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively.

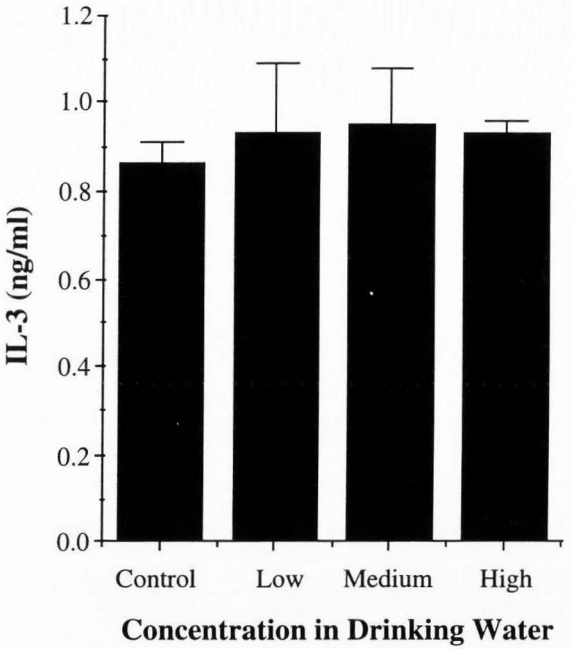

FIG. 4-5. Levels of IL-3 secreted protein from activated splenocytes of phenol-treated mice. The splenocytes ($2x10^6$/ml) from control and phenol-treated mice were activated with 5 μg/ml Con A. Supernatants were taken after 48 hours, and IL-3 was detected in the serum by ELISA. Data are expressed as mean + S.E. (n=4). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively.

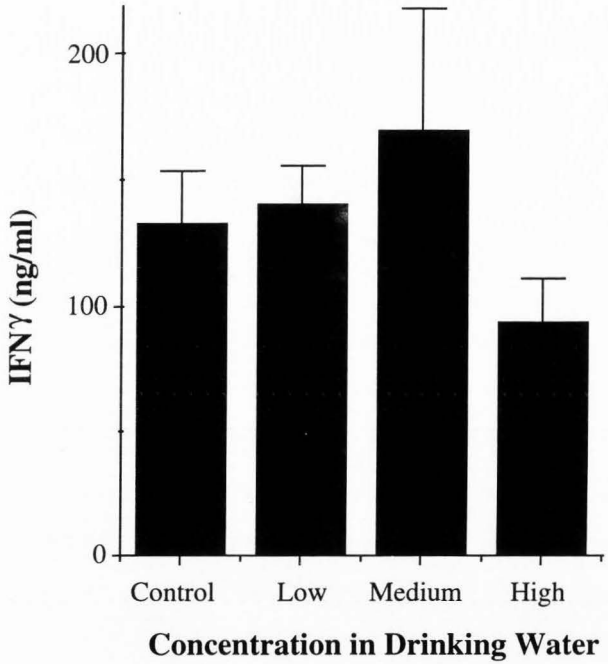

FIG. 4-6. Levels of IFNγ secreted protein from activated splenocytes of phenol-treated mice. The splenocytes ($2x10^6$/ml) from control and phenol-treated mice were activated with 5 μg/ml Con A. Supernatants were taken after 48 hours, and IFNγ was detected in the serum by ELISA. Data are expressed as mean + S.E. (n=4). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively.

reactive intermediates than benzene depending on species of animal, health, and liver status (Bruce *et al.*, 1987).

Phenol caused lowered thymus weights, but this effect did not appear to be dose related as all treatment groups had similar thymus weights. Reduced numbers of lymphocytes causing lower thymic mass may explain the decreased thymus weights. The metabolites of phenol, particularly polyhydroxy metabolites such as hydroquinone, are quite destructive to lymphocytes (Rozen and Snyder, 1985; Green *et al.*, 1981). Thus, the thymus atrophies after phenol injestion due to cortical cellular depletion. In previous research using higher doses of phenol (75 mg/kg), hydroquinone administered with phenol resulted in enhanced binding of reactive intermediates to blood and bone marrow molecules (Subrahmanyam *et al.*, 1990), suggesting that phenol stimulates the peroxidase mediated hydroquinone metabolism to reactive intermediates, and it is these intermediates that cause myelotoxicity and the destruction of lymphocytes or lymphocyte precursors.

The lymphocytes from phenol-treated mice show higher levels of IL-2 at the medium dose than the control group (Figure 4-1). At the high dose, IL-2 mRNA levels were lower, but the IL-2 cytokine protein production peaks at the low dose and then decreases in the medium and high doses (Figure 4-4). This suggests phenol may have some effect on the IL-2 cytokine both at transcriptional and posttranscriptional levels. Like all cytokines, the IL-2 mRNA has an AU rich 3'-UTR (Henics *et al.*, 1994; Janeway and Travers, 1994). This region is recognized by the ribosome as well as by cytoplasmic enzymes responsible for RNA degradation. Perhaps phenol is altering the cytoplasmic

proteins responsible for mRNA degradation or affecting the binding of essential nuclear binding proteins like NFAT-1 (Riegel *et al.*, 1992) to cause the changes in IL-2 production seen in this study. This is the mode of action in cyclosporin A immunosuppression (Brabletz *et al.*, 1991; Emmel *et al.*, 1989).

The low and high doses of phenol given to mice produced lymphocytes that when activated by Con A transcribed significantly less IL-3 mRNA (Figure 4-2), suggesting a correlation between phenol and the transcription of IL-3 mRNA at the doses used in this study. On the IL-3 gene, several nuclear activating proteins have binding sites upstream of the promoter. Proteins such as CK-1, CLE-1, and NF-GMa bind to the sites initiating transcription by RNA polymerase (Kishimoto, 1992). Phenol possibly inhibited the production of one or more of these nuclear activating proteins, thereby causing less IL-3 mRNA production.

Interleukin-3 primarily supports the viability and differentiation of hematopoietic progenitor cells (Kishimoto, 1992; Mizel, 1989). It recruits additional immune cells necessary to sustain or amplify the immune response (Mizel, 1989). The normal to slightly increased amounts of IL-3 produced may explain why the phenol-treated mice had slightly increased cellularity in the spleen at the low and medium doses (Table 4-2). The IL-3 protein secreted by lymphocytes of phenol-treated mice was nearly the same as the control group in spite of less IL-3 mRNA being produced (Figure 4-5). Some kind of posttranscriptional, translational, or posttranslational modification of the IL-3 mRNA is suggested by nearly equal amounts of IL-3 being secreted by control and treatment groups

in this study. A possible explanation for this would relate to the AU rich 3'-UTR on the IL-3 mRNA (Henics *et al.*, 1994; Janeway and Travers, 1994). Phenol may prevent the normal cytosolic enzymes from breaking down the IL-3 mRNA allowing it to be translated to more protein, thus making the nearly equal amounts of IL-3 protein from less mRNA when compared to the control group.

Phenol only slightly modified IFNγ mRNA production in this study. At low and medium doses of phenol, the IFNγ production in activated lymphocytes increased. However, at the high dose mRNA production decreased (Figure 4-3). This general trend followed the IFNγ protein secretion level (Figure 4-6), suggesting that phenol may transcriptionally control IFNγ production. Cyclosporin A is also thought to transcriptionally control IFNγ production possibly by inhibiting calcium dependent activation genes (Cockfield *et al.*, 1993). Interleukin-2 has been shown to regulate IFNγ gene transcription by activating protein kinase C, increasing intracellular calcium (Farrar *et al.*, 1986). Phenol may also be controlling protein kinase C, or one of the many nuclear activating proteins for the IFNγ gene.

In summary, since the normal metabolism of phenol allows the body to rid itself of phenol quickly (Bruce *et al.*, 1987; Fishbein, 1984), the doses of phenol given in this experiment may not have been enough to overload the normal excretory mechanisms and allow a high enough production of the reactive intermediates to result in more pronounced immunotoxic effects. However, IL-2, IL-3, and IFNγ are key cytokines in activating the

immune response (Riegel *et al.*, 1992). Altering their production even slightly may result in consequences when the animal comes in contact with other pathogens and requires these cytokines for activating other immune cells. Further research using higher doses of phenol is indicated to characterize the mechanisms behind the immunotoxicity of phenol.

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CHAPTER 5

VARIATION IN ESSENTIAL MACROPHAGE CYTOKINE MESSENGER RNA AND CYTOKINE PROTEIN OF MALE INSTITUTE OF CANCER RESEARCH MICE DUE TO PHENOL IN THE DRINKING WATER

Abstract

Recently, trace amounts of phenol have been found in groundwater. Phenol, a major metabolite of benzene, is known to have immunotoxic effects, even in small dosages. The immunotoxic effects of phenol include decreased antibody production and decreased lymphocyte responsiveness to mitogen. Cytokines are soluble regulatory proteins that provide a communication network between cells in the immune system. Alterations in normal cytokine production would cause decreased antibody production and lower lymphocyte responsiveness to mitogens. Phenol may be causing immunotoxicity by varying normal cytokine production.

In this study, male ICR mice were given 0, 5, 20, and 100 mg/L of phenol in the drinking water of the control, low, medium, and high groups, respectively, for 30 days. The levels of IL-1α, IL-6, and TNFα mRNA production were determined in all groups by Northern blot analyses of the LPS-activated peritoneal macrophages from each mouse. Secreted amounts of IL-1α, IL-6, and TNFα from activated peritoneal macrophages were determined by ELISA. Phenol caused less IL-1α protein to be secreted, particularly in the medium and high dose groups despite the increased levels of IL-1α mRNA produced

by the phenol-treated peritoneal macrophages. Phenol is inhibiting IL-1α translation or the proper processing of the translated protein possibly by inhibiting calpain protease, which cleaves pre-IL-1α to the mature secreted IL-1α. The levels of TNFα mRNA were lower in all phenol dosage groups compared to the controls. Phenol only caused less secreted TNFα protein at the low and medium doses in the activated peritoneal macrophages. In fact, at the high dose of phenol, TNFα secreted protein was similar to the control group, in spite of less TNFα mRNA produced. This may be because phenol affected cytosolic enzymes responsible for breaking down cytokine mRNA. Although less mRNA was present, mRNA longevity increased so more protein was translated. Phenol did not seem to affect IL-6 mRNA or protein secretion at any dose of phenol used in this experiment. However, as described above, phenol altered IL-1α and TNFα cytokine levels in normal macrophages. This modulation of cytokines is likely part of the cellular mechanism inducing immunotoxicity from phenol exposure.

Introduction

Phenol, a monohydroxy derivative of benzene, is the primary metabolite of benzene *in vivo* (Sawahata *et al.*, 1985; Fishbein, 1984). Industry and other natural sources produce phenol in large quantities. In fact, 4.16 billion pounds of phenol were produced in 1995, which makes it the 34th most commonly produced chemical in the USA (Kirschner, 1996). Phenol is used as a disinfectant, an antiseptic, an antimicrobial agent, a solvent in the petroleum refining process, and is used to manufacture resins and dyes

(U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987). Phenol is produced after the decomposition of animal wastes and from the normal metabolism of medicines, such as Pepto-Bismol™ and from aromatic amino acids (U.S. Environmental Protection Agency, 1986).

The detrimental health effects that have been associated with phenol include cardiac arrhythmias and depression, respiratory depression, neuromuscular hyperexcitability, coma, encephalopathy, severe irritation, and corrosion on contact of the skin or other tissues, and spleen, liver, and kidney damage (U.S. Environmental Protection Agency, 1993; Bruce *et al.*, 1987; Windus-Podehl *et al.*, 1983). In addition, phenol in the drinking water has been reported to have caused increased disease susceptibility (Baker *et al.*, 1978). In experimental animals, phenol is fetotoxic, genotoxic, and myelotoxic (Subrahmanyam *et al.*, 1990; Gaido and Wierda, 1985). In the study by Hsieh *et al.* (1992), lymphocytes of mice that had been given phenol in their drinking water showed suppression of B- and T-cell lymphoproliferation after lipopolysaccharide (LPS), phytohemagglutinin (PHA), and pokeweed mitogen (PWM) exposure. Antibody production was also decreased in the lymphocytes of these same mice.

While it is thought that the toxic products of phenol metabolism (such as hydroquinone) are involved in the mechanism of phenol toxicity, the exact mechanisms have not been well characterized (Subrahmanyam *et al.*, 1990; Tunek *et al.*, 1981, 1982). For example, the reason phenol suppresses B- and T-cell lymphoproliferation has not been determined. The immune system is a very complex system of cells and regulatory

proteins. Research has shown that regulatory proteins, or cytokines, are produced when antigens are presented to immune cells like macrophages and lymphocytes. These cytokines have been found to be key factors in promoting cell growth, differentiation, and activation of immune cells (Janeway and Travers, 1994; Tizard, 1992). Thus, a probable mechanism for the immunotoxicity of phenol is through the alteration of key cytokine production in immune cells such as the lymphocyte or macrophage. The objective of this study was to determine if the macrophages of mice given phenol had any modulation of IL-1, IL-6, and TNFα mRNA or protein production. These cytokines were selected because of their importance in the initial regulation of the macrophage, and the importance of these cytokines in the stimulation of further immune responses (Akira *et al.*, 1990). Because phenol is a known contaminant of drinking water supplies (U.S. General Accounting Office, 1988; U.S. Office of Technology Assessment, 1984), phenol was added to the drinking water of male ICR mice for this research.

Materials and Methods

Animals

Five-week-old Institute of Cancer Research (ICR) outbred mice were obtained from Simenson Laboratories (Gilroy, CA). The mice were acclimatized for 7 days in an AAALAC accredited animal care facility. They were housed in a room with a 12-hr light/dark cycle, an ambient temperature of 22°C ± 1°C, and a 50% (± 10%) relative humidity. Animals were randomly assigned to control and treatment groups and housed

five mice per group in plastic cages with ground corn cob bedding. They were maintained on commercial rodent chow (Harlan Teklad, Madison, WI) and tap water *ad libitum*.

Phenol exposure

Phenol (USP Phenol 90% w/w. Fisher Scientific, Fair Lawn, NJ) was dissolved in tap water to make a stock solution of 100 mg/L. From the stock solution, additional solutions of 5 mg/L and 20 mg/L were made. Phenol was administered to the three treatment groups of mice (n=5 in each group) for 30 days at 5, 20, and 100 mg/L water for the low, medium, and high treatment groups, respectively. The control group received untreated tap water for 30 days. The phenol-treated water was put in glass water bottles with stainless steel sipper tubes, shaken daily, and changed every 3 days to minimize phenol decomposition and to maintain the desired phenol concentrations in the water. In order to duplicate results from past research, phenol was added to tap water and changed every 3 days just as performed by previous workers (Hsieh *et al.*, 1992). Water consumption and mouse weights were recorded every 3 days.

Isolation of peritoneal macrophages

Mice were injected intraperitoneally with 3 ml of brewer thioglycollate broth (Difco Laboratories, Detroit, MI) on day 26 to increased peritoneal macrophage numbers and allow the stimulated macrophages time to return to normal before the macrophages were removed. Four days later the mice were euthanized with carbon dioxide. Peritoneal lavage was performed aseptically with 10 ml of ice-cold complete RPMI cell culture

medium [RPMI-1640 and 10 percent heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 mM 2-mercaptoethanol], and 10 µg/ml heparin. Peritoneal lavage medium was withdrawn from the peritoneum of the mouse and macrophages were counted in the media using a hemocytometer. The macrophages were adjusted to 10^6 cells/ml of complete RPMI medium, and then 5 ml of the cell suspension was plated in 60-mm cell culture dishes. The macrophages were activated using 10 µg/ml lipopolysaccharide (LPS) (Sigma Chemical, St. Louis, MO). Cell culture dishes with activated macrophages were then incubated in a cell culture chamber at 37°C with 5 percent CO_2 for 10 hr to obtain macrophage RNA, or 48 hr to collect supernatants for cytokine protein quantification.

Total RNA isolation and quantification

Cell culture dishes to be used for RNA isolation were taken from the incubator after 10 hr. The medium was removed from the plates and TRI REAGENT™ LS (Molecular Research Center, Cincinnati, OH) was added to the adherent cells following the manufacturer's directions. Total RNA was isolated and purified from the cells following the manufacturer's directions and then resuspended in FORMAzol™ (Molecular Research Center). The RNA concentration was determined spectrophotometrically and the RNA in FORMAzol™ was stored at -20°C.

Northern blot analyses of IL-1α, IL-6, TNFα, and control β-actin were performed using antisense riboprobes labeled with α^{32}P-CTP to a specific activity of 2 x 10^7 cpm (Ausubel *et al.*, 1991). Antisense transcripts were made using bacteriophage T3 or T7 RNA polymerase (Promega, Madison, WI) and cloned cDNA for β-actin, IL-1α, IL-6, and TNFα following manufacturer's directions. The IL-1α cDNA clone (ATCC #63106) was in pBluescript SK+. To prepare the probe, the clone was cut with Hind III and transcribed with T3 RNA Polymerase. Tumor necrosis factor alpha, a 1230 bp cDNA clone with Eco RI ends in pBluescript II KS+ (Dr. A.D. Weinberg, University of California, San Diego, CA), was cut with Hind III and transcribed with T7 RNA polymerase. The IL-6 cDNA clone, 800 bp with Pst I ends in pBluescript II KS+ (Dr. A.D. Weinberg, University of California, San Diego, CA), was cut with Hind III and transcribed with T7 RNA polymerase. The human β-actin cDNA clone (ATCC #78554) was cut with Hind III and transcribed with T3 RNA polymerase. All cDNA clones were sequenced from both ends. These sequences conformed to their respective cDNA sequences in Genbank (Genetics Computer Group, Madison, WI).

Ten micrograms of total RNA were separated on a 1.2 percent agarose gel containing 1.1 percent formaldehyde and transferred to MAGNA NT nylon membranes (Micron Separations Inc., Westboro, MA). The membranes were stained with 0.02 percent methylene blue and photographed so the positions of the 18S and 28S ribosomal RNA bands, as well as the RNA ladder size markers, could be marked on the membrane.

These marks were later used to estimate the size of the RNA bands in each lane. The membranes were prehybridized for 1 hr followed by hybridization for 16-24 hr in buffers containing 50 percent formamide and then washed under high stringency conditions (5X SSPE/0.5 percent SDS for 15 min at room temperature, 1X SSPE/1.0 percent SDS for 15 min at 37°C, 0.1X SSPE/1 percent SDS at 65°C for 15 min). The blots were then exposed for 24-168 hr to Kodak X-AR radiographic film at -70°C using Dupont Lightning Plus screens. The bands on the film were quantitated by scanning their densities using the ZERO-D scan software program (Stratagene Cloning Systems, LaJolla, CA). Total RNA in each lane was adjusted using the β-actin standards and the densities of control and treatment groups were compared.

Cytokine quantification

The supernatants from activated macrophage cell cultures incubated for 48 hr were collected and used to quantitate IL-1α, IL-6, and TNFα cytokine production. All supernatants were frozen at -70°C until assayed. These cytokines were quantitated using sandwich ELISA kits for each cytokine (Endogen, Inc., Cambridge, MA) according to manufacturer's directions.

Statistical methods

Data from the Northern blots and cytokine ELISA's were analyzed by using a one-way analysis of variance (ANOVA) followed by the Fisher least significant

difference (LSD) statistical analysis. Statistical calculations were done using Stat View™ SE and Graphics 1.03 software (Abacus Concepts, Inc., Berkeley, CA).

Results

The mice were given phenol in their drinking water at concentrations of 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. No clinical signs of toxicity were evidenced in any of the treated groups. However, the low dose group had two mice that did show signs of aggression such that one mouse had to be in a separate cage for the entire experiment (30 days). No differences in water or food consumption were observed between control and treatment groups.

There was not a significant difference in body weights between groups of mice at the beginning of this study (day 0). By day 30, the medium and high dose groups weighed significantly less than the control and the low groups (Table 5-1). It is interesting to note that the mouse in the separate cage for the entire study weighed the least at the end of the 30-day treatment period (data not shown).

Peritoneal macrophage numbers and gross pathology

Upon euthanasia, no observable gross pathological differences were seen between the control or treatment groups. There were no significant differences in the number of macrophages between control and treatment groups, although the high dose group had the most peritoneal macrophages overall (Table 5-1). In Chapter 4, it was reported that mice given similar phenol treatments showed significantly decreased thymus weights in all

TABLE 5-1
Body Weights and Peritoneal Macrophage Numbers in Mice Given Phenol in Their Drinking Water for 30 Days

Phenol Conc. in Water (mg/L)	Water Consumed[a] (ml/day)	Body Weights Day 0[b] (grams)	Body Weights Day 30[b] (grams)	Peritoneal Macrophages[b] ($x10^6$ cells/ml)
0	8.4	30.3 ± 0.6	38.3 ± 0.7[c]	1.84 ± 0.19
5	10.6	28.9 ± 0.7	39.6 ± 0.9[c]	1.58 ± 0.33
20	8.6	30.4 ± 0.5	36.3 ± 0.8[d]	1.16 ± 0.29
100	8.3	28.9 ± 0.7	35.6 ± 0.5[d]	2.24 ± 0.15

[a] Values are the mean from each group of 5 mice
[b] Values are the mean ± S.E. (n=4)
[c,d] Different superscripts within a column are significantly different $p<0.05$

dosage groups and the spleen weights were significantly lower in the medium dosage group when compared to the control group (Table 4-2).

Cytokine mRNA levels

No significant differences were observed in the production of IL-6 and TNFα cytokine mRNA between control and treatment group macrophages; however, the trend seen in the levels is still important to note (Figures 5-1, 5-2, and 5-3). Both IL-1α and IL-6 mRNA levels were higher at the low dose level of phenol than the control group. At the medium dose level the cytokine mRNA began to decrease, and in the high dose group, mRNA production from activated macrophages was similar to the control group. The

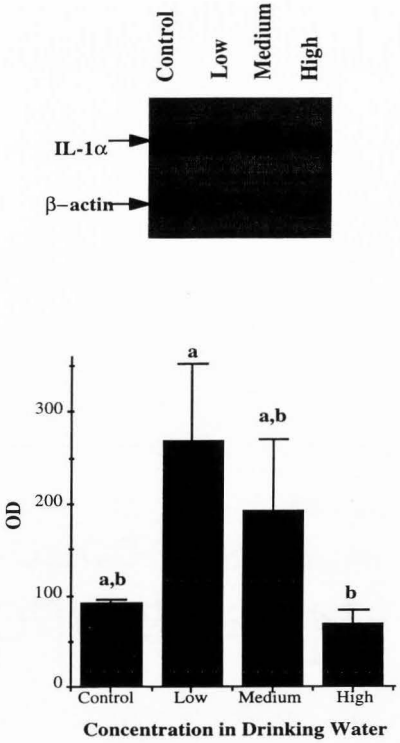

FIG. 5-1. The effects of phenol on IL-1α mRNA production from LPS-activated macrophages. Total RNA was isolated from LPS-activated peritoneal macrophages (10 μg/10^6 cells for 10 hr) obtained from control and phenol-treated mice. The RNA (10 μg) was analyzed by Northern blot using murine IL-1α and was standardized using β-actin ^{32}P labeled riboprobes. Band densities were measured by ZERO-D scan, and the values of the total RNA per lane were equalized using the β-actin standards. Data are expressed as the mean ± S.E. of the animals within each group (n=3). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$). Hybridizations of IL-1α and β-actin were performed separately.

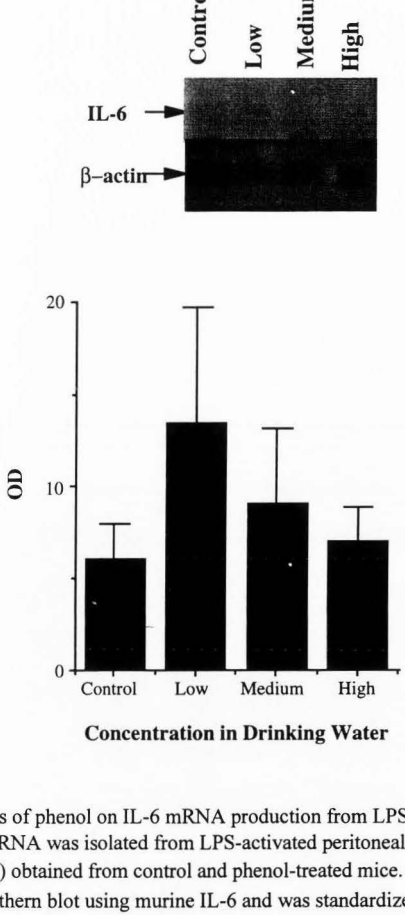

FIG. 5-2. The effects of phenol on IL-6 mRNA production from LPS-activated macrophages. Total RNA was isolated from LPS-activated peritoneal macrophages (10 μg/10^6 cells for 10 hr) obtained from control and phenol-treated mice. The RNA (10 μg) was analyzed by Northern blot using murine IL-6 and was standardized using β-actin ^{32}P labeled riboprobes. Band densities were measured by ZERO-D scan, and the values of the total RNA per lane were equalized using the β-actin standards. Data are expressed as the mean ± S.E. of the animals within each group (n=3). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. Hybridizations of IL-6 and β-actin were performed separately.

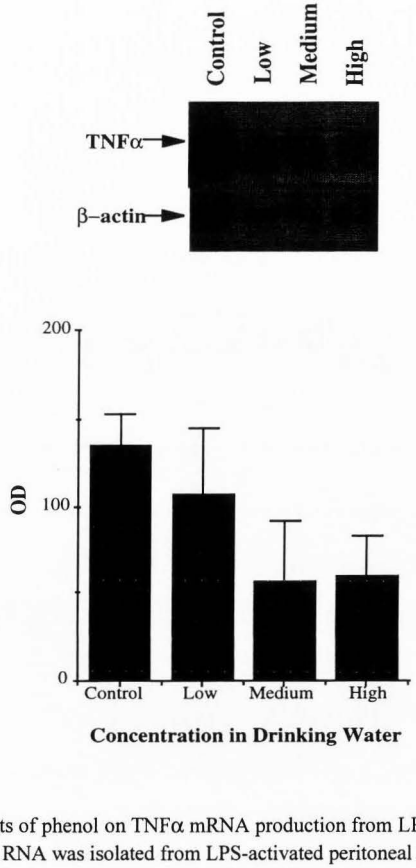

FIG. 5-3. The effects of phenol on TNFα mRNA production from LPS-activated macrophages. Total RNA was isolated from LPS-activated peritoneal macrophages (10 μg/10^6 cells for 10 hr) obtained from control and phenol-treated mice. The RNA (10 μg) was analyzed by Northern blot using murine TNFα and was standardized using β-actin ^{32}P labeled riboprobes. Band densities were measured by ZERO-D scan, and the values of the total RNA per lane were equalized using the β-actin standards. Data are expressed as the mean ± S.E. of the animals within each group (n=3). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. Hybridizations of TNFα and β-actin were performed separately.

reduction in IL-1α mRNA production in macrophages from mice given the highest dose of phenol was significant when compared to IL-1α production from macrophages of mice given the lowest dose of phenol. The levels of TNFα were lower when compared to the control in all treatment levels. These reduced TNFα mRNA levels were lowest at the medium and high treatment groups. The medium and high dose groups produced less IL-6 mRNA than the low dose group.

Cytokine protein production

The amounts of IL-1α cytokine protein secreted, as determined by sandwich ELISA using 48-hr LPS-stimulated macrophage supernatants, were significantly less in the medium phenol dosage group when compared to the control group (Figure 5-4). The low dose of phenol was nearly the same as the control IL-1α production and the high dose group was nearly the equal to the low dose group in IL-1α protein secretion. Interleukin-6 protein secreted from peritoneal macrophages of phenol-treated mice was about equal at all dosage levels and was just slightly less than the control group (Figure 5-5). Finally, TNFα cytokine protein production in macrophages of phenol-treated mice was less than the controls, but this reduction was significant only in the macrophages of mice given the low and medium doses of phenol (Figure 5-6). The macrophages from mice given the high dose of phenol secreted nearly the same amount of TNFα protein compared to the control group.

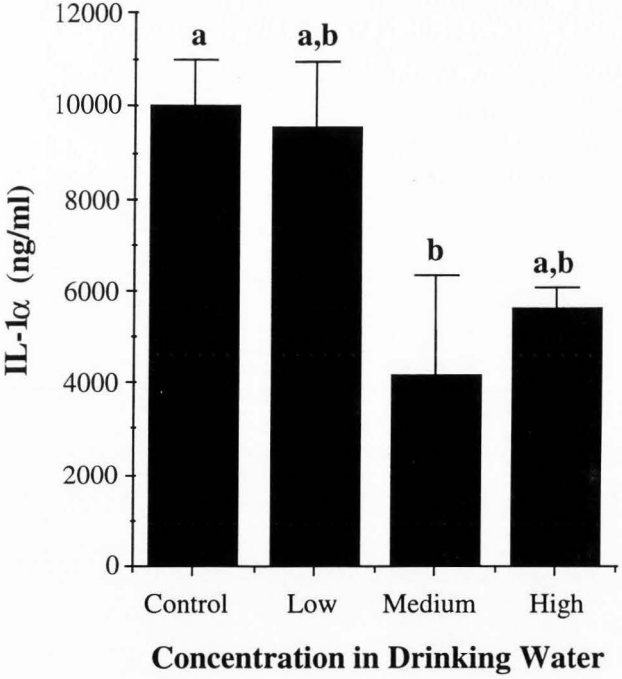

FIG. 5-4. Effects of phenol on IL-1α production from activated macrophages of mice ingesting phenol through drinking water. The macrophages ($1x10^6$/ml) from control and phenol-treated mice were activated with 10 μg/ml LPS. Supernatants were taken after 48 hours and IL-1α was detected in the serum by ELISA. Data are reported as the mean ± S.E. for animals within each treatment group (n=4). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$).

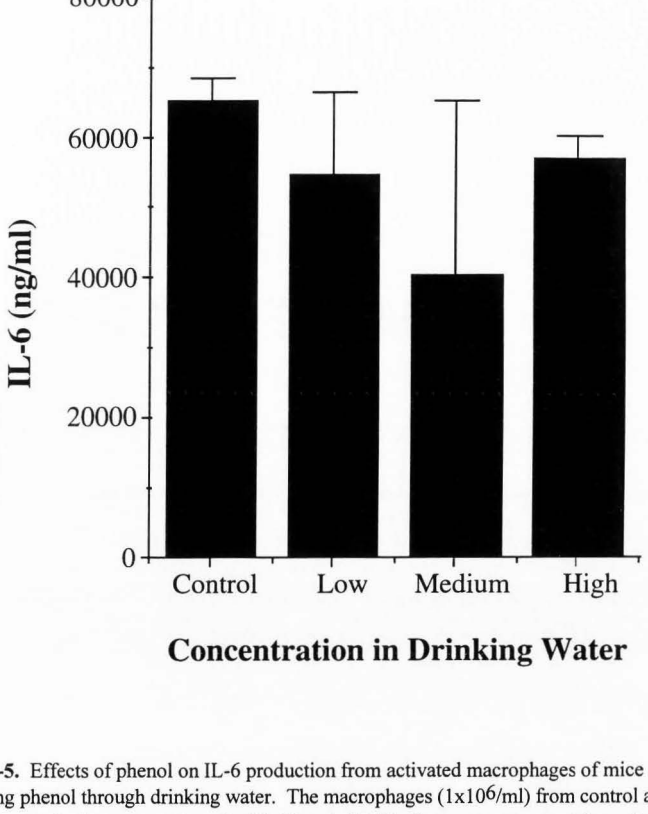

FIG. 5-5. Effects of phenol on IL-6 production from activated macrophages of mice ingesting phenol through drinking water. The macrophages ($1x10^6$/ml) from control and phenol-treated mice were activated with 10 μg/ml LPS. Supernatants were taken after 48 hours and IL-6 was detected in the serum by ELISA. Data are reported as the mean ± S.E. for animals within each treatment group (n=4). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively.

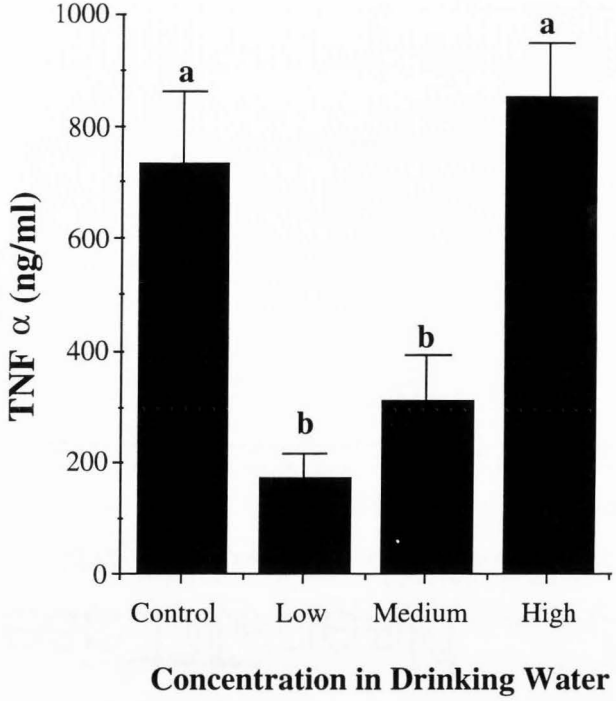

FIG. 5-6. Effects of phenol on TNFα production from activated macrophages of mice ingesting phenol through drinking water. The macrophages ($1x10^6$/ml) from control and phenol-treated mice were activated with 10 μg/ml LPS. Supernatants were taken after 48 hours and TNFα was detected in the serum by ELISA. Data are reported as the mean ± S.E. for animals within each treatment group (n=4). Drinking water concentrations of phenol were 0, 5, 20, and 100 mg/L in the control, low, medium, and high groups, respectively. (^{a,b}) Different superscripts are significantly different ($p<0.05$).

Discussion

Cytokines are important signaling molecules in the immune system. This importance is exemplified in T-cells, which if not activated, fail to produce cytokines and the whole immune system is not properly activated. When T-cells are activated, the production of specific cytokines tells the immune system to produce more antibodies or enables macrophages to become cytotoxic and able to destroy phagocytized bacteria (Janeway and Travers, 1994).

In Chapter 3, benzene was shown to alter TNFα and IL-1α production. These cytokines may have been altered at transcriptional, post transcriptional, or post translational levels by benzene. Of greatest importance was benzene's suppression of TNFα mRNA and cytokine production. In the present study, phenol was given in place of benzene. Although phenol is quite similar to benzene, there were differences in cytokine production. Analysis of phenol-induced changes in cytokine gene expression or protein production will help determine the molecular mechanisms behind its immunotoxic potential.

Phenol (monohydroxy benzene) is a hydrocarbon of significant importance in drinking water supplies (U.S. Environmental Protection Agency, 1986; Baker *et al.*, 1978). Phenol is metabolized by the liver into hydroquinone or catechol. Often it is these products administered with phenol that result in myelotoxicity (Subrahmanyam *et al.*, 1990; Eastmond *et al.*, 1987). It is reasonable to theorize that the metabolic products of phenol are also responsible for the affects of phenol seen in this study.

At the medium and high doses, the mice weighed less at 30 days when compared to the control group. This weight reduction was not noted in a previous study using similar doses of phenol (Hsieh *et al.*, 1992). Although the difference was not statistically significant, mice given similar doses of phenol weighed less than the control group in Chapter 4. Since weight loss has not previously been associated with phenol treatments, perhaps the cause of this weight loss is from reasons besides phenol in the drinking water. For example, the weight loss could be due to the normal aggressiveness of the male mice. Five male mice were placed in one cage for 30 days. Some mice seemed to spend most of their awake time fighting amongst themselves and not letting the other mice in the cage eat as much as if they had not been bothered. The result is mice that weigh less, although no gross pathological abnormalities would be expected from these mice. The low group did have one mouse that was so aggressive that it was placed in a separate cage.

Another reason for the weight loss in the medium and high doses of phenol-treated mice could be due to the encephalopathic effects of phenol. Windus-Podehl *et al.* (1983) gave rats intraperitoneal doses of phenol ranging from 300 to 600 µmoles. In their study, rats given phenol developed coma as the doses of phenol increased. To illustrate the importance of the liver in causing phenol-induced coma, additional chemicals such as ammonium acetate or pentobarbital were given with the phenol. These additional chemicals lowered the dose of phenol necessary to cause coma. In the present study, no coma was observed at any phenol dosage level. However, the phenol-treated mice were observed to sleep more than the other mice. If the treated mice did sleep more than the

control mice, obviously those treated would have spent less time eating. Consequently, lower 30-day weights would be expected, but again, no other gross pathological abnormalities would have been found.

The levels of cytokine produced by macrophages from phenol-treated mice were also altered. Phenol-treated mice had macrophages with higher IL-1α mRNA production at the low dose, but at the medium and high doses mRNA production lowered in a dose response fashion. However, the production of secreted IL-1α protein did not follow the mRNA production, indicating that phenol may be affecting more than just the transcription of the IL-1α gene.

Like all eukaryotic genes, nuclear activating proteins play important roles in binding upstream of the IL-1α promoter and initiating transcription (Dinarello, 1992). At the low dose of phenol, transcription of the IL-1α gene is probably increased by stimulating one or more of these transcription factors, but at higher doses, IL-1α could repress these nuclear activating proteins. This biphasic response on nuclear activating factor production is the mechanism of cyclosporin A immunotoxicity (Emmel *et al.*, 1989). The end results are more IL-1α mRNAs at the low dose of phenol and less IL-1α mRNAs at the medium and high doses of phenol.

A very significant part of IL-1α control comes at the posttranslational level. The IL-1α mRNA is translated into 34 kilodalton (KD) pre-IL-1α, which is then cleaved by an enzyme into the mature 17 KD protein that is able to be secreted (Siders *et al.*, 1993;

Hazuda *et al.*, 1991; Giri *et al.*, 1985). The important enzyme in this cleavage process was further characterized to be a member of the calpain protease family (Carruth *et al.*, 1991). Hydroquinone, a metabolite of benzene and phenol, is known to block this calpain protease activity, causing less IL-1α to be secreted (Miller *et al.*, 1994). Because phenol has the potential to block calpain, it was not surprising to find that secreted IL-1α protein levels did not correspond to IL-1α mRNA levels. Therefore, even with increased amounts of IL-1α mRNA present, the calpain protease cleavage of translated pre-IL-1α into mature secreted IL-1α is inhibited and less IL-1α is secreted from the macrophage.

Phenol treatment does not appear to alter IL-6 mRNA or protein production significantly in this study. While the IL-6 mRNA trend shows more IL-6 mRNA in the macrophages of mice given the low dose of phenol when compared to the controls, macrophages from phenol-treated mice had slightly less IL-6 protein production. Since normal cells do not produce IL-6 unless stimulated appropriately and since IL-1 is one of the most important stimulants of IL-6 (Mizel, 1989), it is not surprising that the phenol-treated groups were not producing more IL-6 than the control group.

Tumor necrosis factor alpha mRNA production is lower in all treatment groups when compared to the control group in this study. The transcriptional control of TNFα mRNA is likely from the binding of nuclear activating factors that bind and initiate the transcription of TNFα mRNA (Akira *et al.*, 1992). Perhaps phenol is preventing nuclear activating factors from binding, causing less TNFα mRNA production.

Like IL-1α, TNFα mRNA is translated into pre-TNFα proteins. The 26 KD pre-TNFα is then cleaved into the 17 KD secreted TNFα. The posttranslational event that cleaves pre-TNFα to the mature TNFα is prohibited by Δ9-Tetrahydrocannabinol (Fischer-Stenger *et al.*, 1993). The results from this research indicate macrophages from phenol-treated mice produce less TNFα protein at the low dose. The medium dose group produced slightly more TNFα than the low group. The high dose produced even more TNFα and was about equal to the TNFα protein produced by the control group (Figure 5-6). The results of TNFα mRNA and protein production are not consistent with phenol having any posttranslational control of TNFα protein production. Rather, TNFα protein production may be controlled by the posttranscriptional regulation of proteins that degrade mRNA. Just like other cytokines, TNFα has AU rich 3'-UTR. This region makes the mRNA more susceptible to the mRNA degrading proteins in the cytoplasm. This region and resulting short half life of the mRNA are all part of the tight control that is necessary to regulate these cytokines in our body (Janeway and Travers, 1994; Han *et al.*, 1990).

Phenol caused lower TNFα mRNA levels, which translates into less TNFα protein as is seen at the low dose level. However, as phenol increases, it may have inhibited the mRNA degrading proteins in the cytosol. Thus, even though less TNFα

mRNA is produced, the mRNA has a longer half life and is able to be translated into more TNFα protein (Figures 5-3 and 5-6).

In conclusion, phenol, even at the low levels used in these studies, is recognized by the immune system and results in altered cytokine production. Giving phenol in the drinking water resulted in a wide variation in cytokine production among mice because it is expected that no mouse drank the same amount. Future studies may want to give phenol orally to the mice so that equal doses can be given. A reduced variation among mice may result in greater statistical significance.

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CHAPTER 6

SUMMARY

Some of the molecular events involved in benzene- and phenol-induced immunotoxicity were evaluated in male ICR mice. The mice received doses of benzene or phenol in their drinking water over a 30-day period. The mRNA and secreted cytokine protein levels of IL-1α, IL-6, and TNFα in LPS-activated peritoneal macrophages, and IL-2, IL-3, and IFNγ in Con A-activated lymphocytes were measured. The *in vitro* mRNA production for specific cytokines was identified and evaluated using Northern blot or RT-PCR procedures. Secreted protein levels of specific cytokine were quantitated by sandwich ELISA.

The results of this research indicate both benzene and phenol alter normal cytokine production in lymphocytes and macrophages. The doses of benzene and phenol used in this study also resulted in lower thymus weights, in support of previous research. Although not manifested in the benzene treated groups, phenol treated mice had lower day-30 body weights. As demonstrated in other studies, when levels of phenol in the body reach high concentrations, the liver is unable to metabolize phenol properly, allowing phenol to enter the brain. In this study, the level of phenol never reached a level in the brain to induce coma, but the phenol-treated mice appeared to sleep more than the control group, which could explain their decreased body weights. Besides the body

weight loss and lower thymic weights, no other gross abnormalities in the mice could be attributed to benzene or phenol at any dose used in this study.

In the peritoneal macrophages from benzene-treated mice, the levels of TNFα mRNA and secreted protein were less at all levels. As the concentration of benzene increased, the production of TNFα mRNA was lower in peritoneal macrophages, suggesting a dose-related response.

The peritoneal macrophages from phenol-treated mice also showed a dose-related decline in the TNFα mRNA production. The measurement of secreted TNFα protein indicated that macrophages from phenol-treated mice also depressed TNFα production at the low dose, with a lesser effect at the medium dose, and no change in TNFα protein secretion at the high dose when compared to the control. Thus, phenol may be influencing more than just transcriptional control of the TNFα gene. A possible reason for the pattern of TNFα protein production in macrophages from phenol-treated mice is that phenol may be influencing mRNA degrading proteins, especially those that recognize the 3' AU rich region of cytokines. Consequently, even when TNFα mRNA production is lower, TNFα protein is still made because the TNFα mRNA is retained. Additional research is warranted in this area to further elucidate phenol's mechanism of action on TNFα mRNA production and the translation of the TNFα mRNA.

Benzene also affected IL-1α. Because the IL-1α mRNA production and secreted protein levels do not match at each dosage of benzene used in this experiment, benzene

may affect a posttranslational mechanism. In fact, Miller *et al.* (1994), have shown that metabolites of benzene do inhibit the action of calpain protease necessary to cleave pre-IL-1α to the mature secreted IL-1α. This inhibition of the calpain protease by benzene could explain why lower amounts of IL-1α were found in the macrophages from benzene-treated mice at all dosages even though higher levels of IL-1α mRNA's were detected in the macrophages of mice given the low and medium dosages of benzene.

Phenol-treated mice also had macrophages that showed a similar pattern of IL-1α mRNA production and protein secretion. The same metabolites of benzene that inhibit the calpain protease also are metabolites of phenol. Therefore, it appears phenol may be inhibiting IL-1α protein secretion by inhibiting calpain protease cleavage of pre-IL-1α into the mature secreted IL-1α.

Neither benzene nor phenol had much effect on IL-6 production. This result is similar to other research reports showing that benzene treatment had no effect on IL-6 production. The variation in IL-6 mRNA and protein secretion between individuals in a group may account for some of this nonsignificance. Dosing animals with benzene or phenol by oral gavage may eliminate some of this variation. Additional research may need to be done to confirm if benzene or phenol does have an effect on IL-6 production.

The Con A-activated lymphocytes from benzene-treated mice were evaluated for their production of IL-2, IL-3, and IFNγ mRNA and protein production. Little significance was found between control and treatment groups in both benzene and phenol-

treated mice. Generally the pattern of protein secretion mirrored the pattern of mRNA production, indicating that there was coupling of mRNA and protein synthesis. In benzene-treated mice, IL-2 mRNA production was greater in the medium dose of benzene. However, IL-2 secretion by the lymphocytes of benzene treated mice did not differ from the control, suggesting benzene may be influencing the translation of IL-2 mRNA, or the release of the IL-2 protein from the lymphocyte. In the lymphocytes of mice treated with the low and high doses of phenol, IL-3 mRNA production was lower, although IL-3 protein secretion did not differ from the control. Phenol may be influencing cytoplasmic RNA degrading proteins, allowing more IL-3 protein to be produced from less IL-3 mRNA.

Generally, patterns of mRNA production showed that at high doses of benzene or phenol, mRNA production seemed to be declining. This reduced mRNA production indicates that the high doses of benzene and phenol are toxic to the macrophage and lymphocyte. Future research may want to study the effects of higher concentrations of benzene and phenol on the macrophage and the lymphocyte to see if this alters not only cytokine production, but also other cellular activities. In addition, this study did not differentiate between T_H1 and T_H2 lymphocytes and the different cytokines that each cell line produces. Additional research is needed to determine if one of these T-helper cell lines is being activated or suppressed by benzene or phenol.

In conclusion, benzene and phenol appear to alter normal cytokine production by macrophages and lymphocytes. When normal cytokine production is altered, the immune

system cannot be activated properly and becomes more susceptible to disease. Figures 6-1 and 6-2 illustrate how benzene and phenol may influence macrophage and lymphocyte activation.

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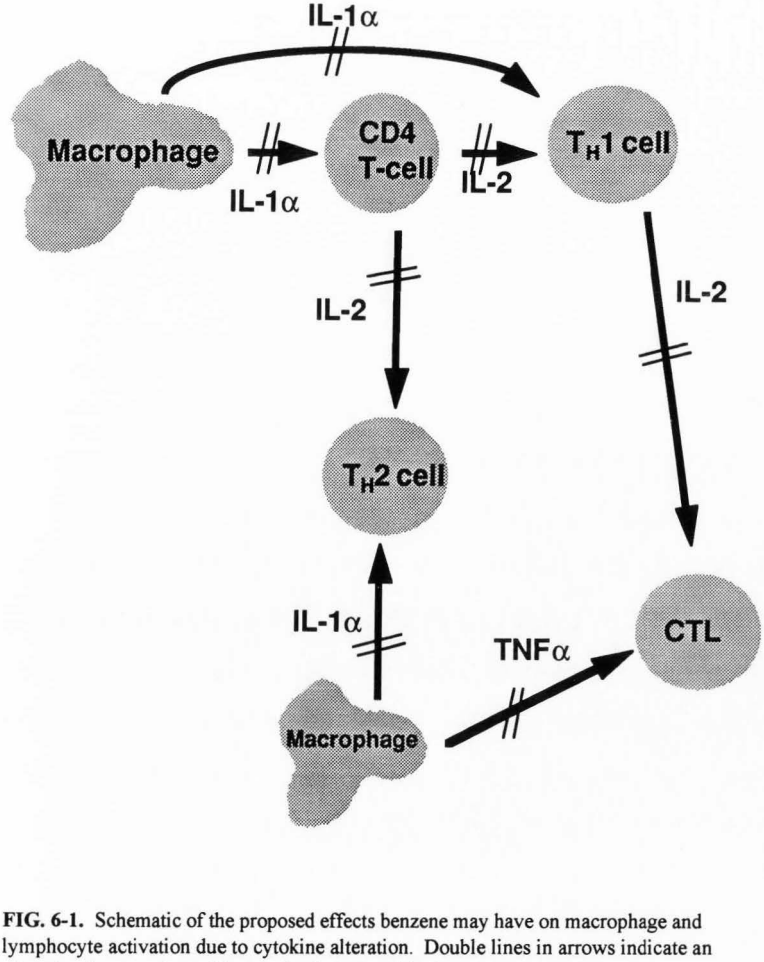

FIG. 6-1. Schematic of the proposed effects benzene may have on macrophage and lymphocyte activation due to cytokine alteration. Double lines in arrows indicate an alteration in normal cytokine production caused by benzene exposure.

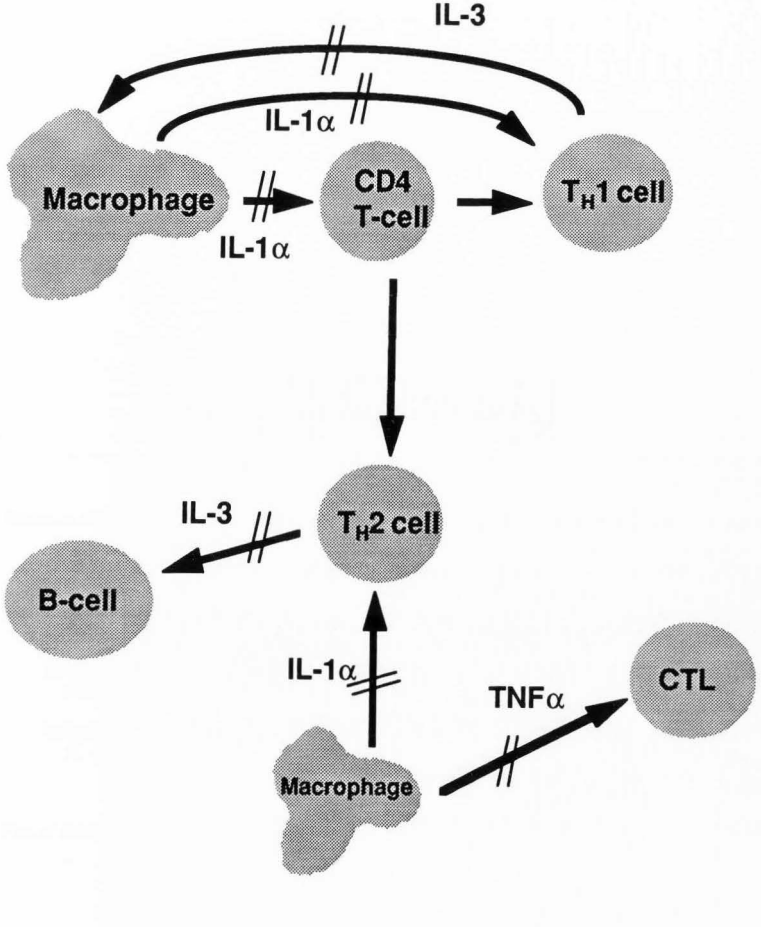

FIG. 6-2. Schematic of the proposed effects phenol may have on macrophage and lymphocyte activation due to cytokine alteration. Double lines in arrows indicate an alteration in normal cytokine production caused by phenol exposure.

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EDUCATION

Utah State University, Logan, Utah (October 1992 - June 1996)
Ph.D. Toxicology. August 1996 (anticipated). Dissertation title: Alteration in basic macrophage and lymphocyte cytokines from benzene and phenol in the drinking water of male ICR mice.

Purdue University, West Lafayette, Indiana (August 1984 - May 1988)
Doctor of Veterinary Medicine. May 1988.

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PROFESSIONAL POSITIONS

- Attending Veterinarian, Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah (1995- present).
- Part-time Instructor, Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah (1993- present)
- Research Assistant, Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah (1993-present).
- Clinical Resident Veterinarian, Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah (1992-1995)

- Associate Veterinarian, Northwood Veterinary Hospital, Anderson, Indiana (1988 - 1992).
- Research Assistant, Swine Department, Purdue University, West Lafayette, Indiana (1986-1988).
- Research Assistant, Parasitology Laboratory, Purdue University, West Lafayette, Indiana (1984-1986).

RESEARCH SKILLS

Proficient in the following laboratory procedures:

- Genetic engineering, isolation, and characterization of nucleic acids.
- Reverse Transcription-Polymerase Chain Reaction (RT-PCR).
- cDNA cloning, electrophoresis, and sandwich ELISA.
- Cell culture growth, treatment, and analysis.
- *In vivo* and *in vitro* evaluation of general toxicity and immunotoxicity.
- Operation of a Coulter Counter.
- Use of X-ray machines and X-ray developing machines.
- Laboratory handling of toxic and radioactive materials.
- Kodak Echtachem Blood Chemistry Analyzer processes.

Veterinary Skills

Proficient in all aspects of both small and large animal general veterinary practice. In addition, also skilled in the following specialties:

- Handling of laboratory animals, experience in oral gavage and intraperitoneal injection of toxic chemicals/drugs.
- Palpating cows to determine reproductive performance.
- Small animal dermatology and orthopedics.
- Embryo transfer in sheep and goats.
- Interpreting radiographs.

Computer Skills

- Word Processing Software - Both Macintosh and IBM versions of Microsoft Word and WordPerfect.
- Numerical Analysis Software - Quattro Pro, Microsoft Excel, Cricket Graph, StatView SE.
- Presentation Software - Harvard Graphics and Microsoft PowerPoint.
- Molecular Biology Related Software - Genetics Computer Group (GCG), Oligo 4.0, Zero D Scan.

RESEARCH EXPERIENCE

- Principle Investigator studying immunotoxicology of xenobiotics (1993 - 1996).

  The main goal of these studies was to find out the immunomodulatory effects and possible molecular mechanisms of the immunotoxicity of mycotoxins (Aflatoxin B_1 and T-2 toxin), benzene, and phenol in male CD-1 mice. Effects on cytokines at transcriptional (mRNA) and translational (protein) levels were studied, assuming the hypothesis that these toxins may exert their immunotoxic effects via interference with the synthesis of different cytokines in T lymphocytes (IL-2, IL-3 and IFNγ) and macrophages (IL-1α, IL-6 and TNFα).

- Research Assistant examining retinoic acid and angiogenic growth factors on the growth of solid tumors (1994 - 1996).

  The effect of retinoic acid (Vitamin A derivative) induced reduction of solid tumors was studied. This involved studying the effect of retinoic acid on the production of vascular endothelial growth factor (VEGF) and VEGF mRNA in various cell cultures.

- Research Assistant investigating hereditary chondrodysplasia in lambs (1993-1996).

  The goal of these studies was to determine the gene associated hereditary chondrodysplasia in sheep (spider lamb syndrome). Required radiographing the proximal ulna of lambs to determine if the lamb did have hereditary chondrodysplasia, then checking for genetic marker similarities.

- Research Assistant evaluating the effects of sub-clinical hypocalcium on post-parturient Holstein cows (1992-1994).

  Blood was taken from cows and heifers pre-parturition, at paturition, and post parturition to determine blood calcium and magnesium levels. The cows were evaluated for post parturient problems, such as ketosis, displaced abomasum, and metritis.

- Research Assistant testing raccoons for *Baylisascaris procyonis* (1984 - 1985).

  Identified raccoons infected with the roundworm *Baylisascaris procyonis*.

- Research Assistant isolating and identifying *Sarcocystis* oocysts (1984 - 1985).

  Isolated *Sarcocystis* oocysts from infected dogs and identified *Sarcocystis* cysts from infected cattle.

TEACHING EXPERIENCE

- Instructor for graduate course, General Pharmacology (1996).

  A general course teaching basic pharmacokinetics, drug applications, and toxicology principles. Emphasis was given on current research.

- Instructor for undergraduate Herd Health (1993-1995).

A course on general animal health and preventative medicine, with emphasis given to dairy animals.

AWARDS AND HONORS

- Graduate Research Assistantship, Utah State University (1995 - 1996).
- Hills Veterinary Nutrition Award, Purdue University (1988).
- College of Agriculture Scholarship, Utah State University (1983 - 1984).

PROFESSIONAL ORGANIZATIONS

- Society of Toxicology, (1996).
- Utah Veterinary Medical Association, (1992 - present).
- American Veterinary Medical Association, (1988 - present).

GRANTS

Utah Agricultural Experiment Station New Faculty Research Funds, $12,000, 1994 - 1995. Subclinical hypocalcemia and its relationship to periparturient disorders in dairy cows.

SCHOLARLY WORKS IN PROGRESS

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Albretsen J., Holyoak R., Cockett N., 1996. Efficacy of radiography in detecting hereditary chondrodysplasia (spider lamb syndrome) in the proximal ulna of lambs.

PRESENTATIONS AT SCIENTIFIC MEETINGS

Jenson E.P., Cockett N.E., Shay T.L., Nielsen D., **Albretsen J.,** Holyoak R., and Bunch T.D., 1994. No evidence of linkage between nine genetic markers and the Spider Lamb gene in sheep. Proc. West. Sect. Meeting, Amer. Soc. Anim. Sci.

Shay T.L., Cockett N.E., Nielsen D., **Albretsen J.,** and Bunch T.D., 1996. No evidence of linkage between 54 genetic markers and the Spider Lamb gene in sheep. XXV International Conference on Animal Genetics.