**AN ABSTRACT OF THE THESIS OF**

Gregory K. DeKrey for the degree of Doctor of Philosophy in Toxicology presented on September 19, 1994. Title: Investigation of the Mechanism of 3,3',4,4',5,5'-Hexachlorobiphenyl-Induced Suppression of Cytotoxic T Lymphocyte Activity in C57Bl/6 Mice: Endocrine and Cytokine Dysregulation.

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Abstract approved: _____
Nancy I. Kerkvliet

Previous studies have reported that exposure to halogenated aromatic hydrocarbons (HAHs) [3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)] causes a dose-dependent suppression of the alloantigen-stimulated splenic cytotoxic T lymphocyte (CTL) response in mice. However, in vitro responses to alloantigen were unaffected by direct exposure to HxCB or TCDD. HAH exposure has been shown to alter the activity, production and/or circulating levels of numerous endocrine factors that are known to impact immune function. In these studies it was hypothesized that HAH-induced CTL suppression is mediated indirectly through an alteration of these immunomodulators. In HxCB-treated mice, a significant and dose-dependent elevation of plasma corticosterone (CS) (the major glucocorticoid in mice) was observed coincident with suppression of splenic CTL activity. However, treatment with RU 38486 (a glucocorticoid receptor antagonist) did not alter the degree of CTL suppression in HxCB-exposed mice. In TCDD-treated mice, dose-dependent suppression of CTL activity was observed in the absence of significant plasma CS elevation. These results suggest that CS elevation does not play a role in HAH-mediated CTL suppression. Direct administration of CS to alloantigen-challenged mice at dose rates sufficient to elevate plasma CS to HxCB-like levels did not alter splenic CTL activity. These results support the previous findings and we conclude that CS elevation alone does not play a role in HAH-mediated CTL suppression. In HxCB-treated mice, significant reduction of serum prolactin levels was observed following alloantigen challenge. However, comparable reduction of serum prolactin levels in bromocryptine-treated mice did not affect CTL activity. Production of $PGE_2$ by spleen cells was also shown to be reduced by HxCB treatment, but treatment with indomethacine alone, leading to markedly reduced $PGE_2$ levels, did not alter CTL activity. These results suggest that reduction of neither prolactin nor $PGE_2$ alone plays a role in

HxCB-mediated CTL suppression. Spleen cells from alloantigen-challenged, HxCB-treated mice produced significantly less interleukin-2 and interferon-γ, incorporated less $^{3}$H-thymidine (a measure of proliferation), and had a smaller population of $CD8^{+}$ cells (the cells responsible for CTL activity). Since interleukin-2 and interferon-γ are important for T cell proliferation and CTL maturation, these results suggest that HxCB may mediate CTL suppression through altered cytokine production.

# Investigation of the Mechanism of 3,3',4,4',5,5'-Hexachlorobiphenyl-Induced Suppression of Cytotoxic T Lymphocyte Activity in C57Bl/6 Mice: Endocrine and Cytokine Dysregulation

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Gregory K. DeKrey, Author

## ACKNOWLEDGEMENTS

This page provides me with the opportunity to mysspell and missyous the English language in a way that, hopefully, is not done elsewhere in this tome. It also provides me a chance to pass on thanks and other tidbits that have no other place in the chapters that follow.

I have learned many things while in grad school: where to get the most food in Corvallis for the lowest price, what a Huckleberry is, and how to "do" science, to name a few. One thing that I learned as a young lad and that has been reinforced as "words to live by" in grad school is this: you can pick your friends, you can pick your nose, but you can't pick your friends nose.

Many of the things that I will remember from the last seven years have nothing to do with what was accomplished at the lab bench regardless of how many hours were spent there. Science is good, sometimes even fun, and most of the time better than pumping gas. But science is a way to have a life, not a way of life. The parts of grad school that I will remember until my dying day are the walks in the forest, the long talks with friends, and the taste of a tomato grown in my own garden. It is to those simple pleasures of life that have helped me through this ordeal that I would like to make the first acknowledgement.

The next acknowledgement goes to the friends that I have known over the years; the friends that, in their own way, supported me with a meal, a kind word, or just being there. There is not room enough here to mention everyone who should be mentioned. To those people, you know who you are: thank you. A few other people must be mentioned. To Linda Hintzman, who put up with me in the beginning, thanks for the memories. To Kitty Gardiner, for support and counselling and for putting up with me now at the end, no amount of thanks and appreciation could ever be sufficient. To Cathy Neumann, from whom I received immeasurable personal and professional inspiration, and the milk of human kindness: thank you forever. To Peter Ruggiero, who can turn the gloomiest day into spring time: thanks and good luck. To John Hobbs, who provided me with the original inspiration to try doing something meaningful with my life: thanks for a purpose. To Spike, who never woke me up without a good reason (in her mind) and who lavished me with affection in her own bared-claw way, thanks for the pets. And, of course, to Jefferson Fowles who, aside from being just about the nicest guy I've ever known, has been there the whole time: thanks for listening, thanks for giving me feed back, thanks for helping out, thanks for being an example, thanks for the beer, and

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## CONTRIBUTION OF AUTHORS

The various coauthors of the chapters are listed in alphabetical order with their major contributions to the study.

| | |
|---|---|
| Baecher-Steppan, Linda B.: | Technical support |
| Deyo, James A.: | Technical support |
| Fowles, Jefferson R.: | Technical support |
| Hollingshead, Nancy C.: | Technical support |
| Kerkvliet, Nancy I.: | Technical support; critical review of the data, experimental design and manuscripts; patience |
| Oughton, Julie A.: | Technical support |
| Smith, Bradford B.: | Technical support; critical review of the data, experimental design and manuscripts |

"In this life," he said with a chuckle to the evening air, "in this World of Tears, you need a sense of humor, neh?"

-- James Clavell
*Gai-Jin*

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| | |
|---|---|
| 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin | TCDD |
| 3,3',4,4',5,5'-Hexachlorobiphenyl | HxCB |
| Adrenalectomized/adrenalectomy | ADX |
| Antigen presenting cell | APC |
| Area under the cytotoxicity curve | AUCC |
| Aromatic hydrocarbon | Ah |
| Castrated/castration | ODX |
| Corticosterone | CS |
| Cytotoxic T lymphocyte | CTL |
| Cytotoxicity | CTX |
| Effector cell:tumor cell | E:T |
| Glucocorticoid | GC |
| Halogenated aromatic hydrocarbons | HAH |
| Immunosuppressive dose | ID |
| Interferon | IFN |
| Interleukin | IL |
| Mixed lymphocyte-tumor cell | MLTC |
| Peritoneal cells | PC |
| Peritoneal fluid | PF |
| Polychlorinated biphenyl | PCB |
| Polychlorinated dibenzo-*p*-dioxin | PCDD |
| Polychlorinated dibenzofuran | PCDF |
| Prostaglandin | PG |
| Testosterone | TT |

# PREFACE

This thesis is comprised of eight chapters, six of which are manuscripts that have been submitted for publication. The status of these manuscripts ranges from submitted to published and is indicated at the beginning of each respective chapter. For manuscripts that are in press, copyright waivers from the publishers permitting their reproduction in this thesis have been filed with the Graduate School, Oregon State University.

# Investigation of the Mechanism of 3,3',4,4',5,5'-Hexachlorobiphenyl-Induced Suppression of Cytotoxic T Lymphocyte Activity in C57Bl/6 Mice: Endocrine and Cytokine Dysregulation

## CHAPTER I

## INTRODUCTION

The focus of the studies described here was to examine the mechanisms of immunotoxicity following exposure of mice to halogenated aromatic hydrocarbons. In this chapter, the intent is to 1) outline the organization and function of the immune system, 2) describe the uses and sources of HAHs, and 3) outline the toxicities associated with HAH exposure with an emphasis on immunotoxicity.

## THE IMMUNE SYSTEM

### Overview

The immune system, like other organ systems, is responsible for maintaining the homeostasis of an organism. The immune system is specialized to deal with threats to homeostasis such as viruses, bacteria, parasites and foreign bodies, and is called into play when other constitutive mechanisms of protection (e.g., skin, mucous membranes, lysozyme, stomach acid, commensal organisms, behavioral patterns) have failed. The immune system can also deal with some threats that arise from the internal environment such as altered or damaged "self" and tumors. Any substance that is capable of inducing an immune response, or reacting with products of an immune response, is called an immunogen.

### Innate and Acquired Immunity

A traditional view of the immune system recognizes a functional organization with two major arms: 1) the innate immune system, and 2) the acquired immune system [see reviews by Benjamini and Leskowitz (1988), Roitt et al. (1985), and Barrett (1988)]. In an animal, these two arms are interdependent and function simultaneously. Both the innate and acquired immune systems can respond to immunogens.

The innate immune system (also termed non-specific or natural) has the ability to respond rapidly to foreign bodies, bacteria, damaged cells, etc., without requiring

prior exposure. An innate immune response can involve preformed complement proteins as well as numerous effector cells (monocytes/macrophages, neutrophils, eosinophils and natural killer cells). The response of an innate effector cell against an immunogen may include phagocytosis, pinocytosis, and/or the release of degradative enzymes, toxic proteins and reactive molecules. The mechanisms, processes, specificity, and response time involved in an innate immune response do not change with repeated exposure to a specific disease agent.

The acquired immune system (also called adaptive), unlike the innate immune system, does not respond rapidly to immunogens, but, over time, acquires that ability to respond (Benjamini and Leskowitz, 1988; Roitt et al., 1985; Barrett, 1988). The onset of an acquired immune response may not be observed for several days. However, if a second contact is made with the immunogen, the onset of the subsequent (secondary) response will be more rapid and more intense.

The acquired immune system is composed of two arms, the humoral and cellular, the functions of which are mediated by B and T lymphocytes, respectively. B lymphocytes are so-called because of their developmental association with the Bursa of Fabricius in birds. Mammals do not have a Bursa of Fabricius, but an equivalent organ is believed to be the bone marrow. B cells are responsible for two major functions: 1) production of antibody, and 2) presentation of antigen (fragments of immunogens) to T cells (Barrett, 1988; Vitetta, 1989). Antibodies can bind to antigens with high specificity and inactivate them (in the case of viruses) or tag them for action by other components of the immune system (e.g., phagocytosis by reticuloendothelial cells, or cell lysis by complement or natural killer cells) (Barrett, 1988). In general, B cells can make antibodies either with or without the help of T cells. However, there are relatively few antigens that B cells can make antibody against without the help of T cells (T cell-independent antigens, i.e., bacterial endotoxins). The vast majority of antibody responses require T cell help (Benjamini and Leskowitz, 1988).

T lymphocytes are so called because of their developmental association with the thymus. T cells have two major functions: 1) they can act as regulatory cells for an immune response (e.g., helper cells and suppressor cells), and 2) they can directly lyse other cells (Benjamini and Leskowitz, 1988; Barrett, 1988).

## T Lymphocytes

Before they can become functional T cells, pro-T cells must migrate from the bone marrow to the thymus where they undergo cell division and a process of selection

[see reviews by Rothenberg (1992), Steele et al. (1993), and Nossal (1994)]. Selection is important because T cells have the potential to recognize both foreign and self-associated immunogens. Failure to ignore self-associated immunogens can lead to auto-immune diseases like arthritis and lupus erythematosis (Benjamini and Leskowitz, 1988). Thymic selection is a mechanism by which self-recognizing T cells are eliminated (programmed cell death) while the maturation of potential nonself-recognizing T cells is promoted. The selection process results in a very small percentage of T cells being released into the periphery as functional cells. However, the process cultivates the appropriate T cell repertoire for each animal. An example of an appropriate T cell repertoire can be demonstrated using inbred mouse strains. Genetically identical inbred mice can receive transplants of tissue from other mice of the same strain without detrimental effects. However, tissue transplants from a different strain of mouse will be rejected. Such a cross-strain graft is called an allograft.

T cells have receptors that can recognize antigens with high specificity. T cell receptors are heterodimeric cell surface proteins (Steele et al., 1993). Each monomer of the T cell receptor is the product of an intricate multigene rearrangement process that begins in the bone marrow and ends in the thymus (Robbins and Kumar, 1987). The gene rearrangement process provides each T cell with the potential to produce a receptor with a unique binding specificity. This property permits each T cell to recognize a different antigen. However, T cell receptors cannot recognize antigens without the assistance of other cells. These other cells are called antigen presenting cells (APCs). Within the cytoplasm of an APC, antigens are coupled to special proteins encoded within the major histocompatibility complex (MHC) of the genome. The MHC protein-antigen complex is then transported to the plasma membrane for presentation to T cells (Brodsky and Guagliardi, 1991; Germain and Margulies, 1993). In the case of allografts, the nonself MHC protein itself, in association with presented peptide, is thought to be antigenic (Sherman and Chattopadhyay, 1993).

T cell functions are performed by two major T cell subtypes: $CD4^+$ and $CD8^+$ cells (Benjamini and Leskowitz, 1988; Barrett, 1988). $CD4^+$ T cells express the CD4 glycoprotein and can act as regulatory cells [e.g., they can help B cells in the process of antibody production or coordinate delayed-type hypersensitivity responses (DTH)]. $CD8^+$ T cells express the CD8 glycoprotein and can act as regulatory cells (e.g., they can suppress immune responses) as well as directly lyse other cells. The major segregation in function of these two T cell types is the way that the CD4 and CD8 proteins restrict their interaction with other cells. Specifically, $CD8^+$ cells can only recognize antigen

when expressed in conjunction with a class I MHC protein, and $CD4^+$ cells can only recognize antigen when expressed in conjunction with a class II MHC protein. Class I protein is expressed on most cells, and therefore $CD8^+$ cells can recognize antigen on most cells. In contrast, class II protein is expressed by only a few cell types (called professional APCs: e.g., macrophages, dendritic cells, B cells), and therefore $CD4^+$ cells can only recognize antigen when presented on these cells. This segregation of antigen recognition plays an important role in regulation of immune responses.

Recognition of antigen can lead to T cell activation. T cell activation is a poorly understood process that leads to the generation of mature effector cells [see reviews by Gajewski et al. (1989); Bach et al. (1989); Abbas et al. (1991)]. Among the multitude of stages from precursor to mature effector cell are three major steps: 1) antigen binding, 2) costimulation by cytokines [e.g., interleukin (IL)-2, IL-4, interferon (IFN)-γ], and 3) cell division and differentiation into effector cells. In addition to the requirement of antigen for activation of T cells, the continued presence of antigen is required for maintenance of the activated state. Likewise, cytokines play a role throughout the T cell response. Cell division is important for a normal immune response as a mechanism of amplification. One T cell out of many thousands may have an appropriate T cell receptor sequence to recognize a particular antigen. However, an effective response to a disease agent may require millions of activated cells. This requirement is fulfilled by clonal expansion via proliferation.

## Cytotoxic T Lymphocytes (CTL)

CTL are important in protection against virus infection and are responsible, in part, for rejection of foreign tissue grafts [see reviews by Zinkernagel and Doherty (1979); Benjamini and Leskowitz (1988); Hutchinson (1991)]. In general, CTL are $CD8^+$ T cells. Following virus infection or grafting of nonself tissues, both $CD4^+$ and $CD8^+$ cells will become activated. $CD4^+$ cells help to stimulate the maturation of $CD8^+$ effector CTL by production of cytokines (Kitagawa et al., 1991). Once matured, CTL circulate through the body in search of virus-infected or foreign cells. CTL are so named because they can kill cells directly. Cell killing occurs by a "kiss of death" wherein a CTL, in close association with a target cell, releases the contents of cytoplasmic granules [containing cytotoxic enzymes (granzymes) and pore forming proteins (perforins)] as well as other toxic molecules (Podack et al., 1991; Vollenweider and

Groscurth, 1991). Target cells are killed by a poorly understood mechanism including osmotic lysis and programmed cell death. Both virus-infected cells and foreign cells are thought to be killed by similar mechanisms.

## HALOGENATED AROMATIC HYDROCARBONS

### Overview

Halogenated aromatic hydrocarbons (HAHs) include halogenated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, terphenyls, quaterphenyls, biphenylenes, naphthalenes, diphenyl ethers, azobenzenes, benzyltoluenes, phenols, anilines, and benzenes (figure I-1). Among these, polyhalogenated biphenyls, terphenyls, napthalenes, diphenyl ethers, benzyltoluenes, phenols and benzenes have been produced commercially (Safe, 1990; Lang, 1992). The others have not been intentionally produced in large quantities, but rather are generally biproducts of industrial (Rappe et al., 1979) or other chemical processes such as combustion or photolysis (Crosby and Moilamen, 1973; Hutzinger et al., 1973; De Voogt and Brinkman, 1989).

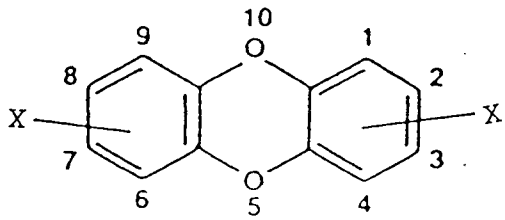
Those HAH produced commercially have been employed in a wide variety of industrial applications (Lang, 1992; Safe, 1990). Polychlorinated biphenyls (PCBs) have been utilized as heat transfer fluids, hydraulic fluids, dielectric fluids, lubricating and cutting oils, immersion oils, fire retardants, dedusting agents, laminating agents, as well as additives in paints, copy paper ink, pesticides, adhesives, sealants and plastics. Polychlorinated napthalenes, terphenyls, diphenyl ethers and benzyltoluenes have been used similarly to PCBs. Polybrominated biphenyls have been employed predominantly as fire retardants. Polyhalogenated phenols have been utilized as fungicides, and the monocyclic halogenated compounds have been used as intermediates in the synthesis of other products.

Due to their chemical and structural similarity, polycyclic HAHs (e.g., not benzenes, anilines or phenols) share many of the same broad physical and chemical properties. They are chemically and thermally stable, have low or no flammability, and a low vapor pressure at ambient temperatures. In addition, they are poor conductors of electrical current and are highly lipophilic. The stability and lipophilicity of these compounds correlates with an increasing halogen content (De Voogt and Brinkman, 1989).

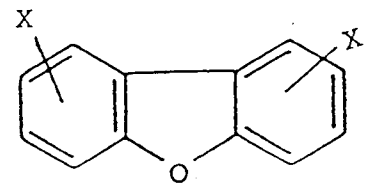
**Figure I-1. Halogenated Aromatic Hydrocarbons.**

The basic structures for some of the dicyclic HAHs are shown: polyhalogenated dibenzodioxins, dibenzofurans, biphenyls, naphthalenes, azobenzenes and benzyltoluenes. Terphenyls and quaterphenyls are similar in structure to biphenyls but consist of three and four aromatic rings, respectively, connected by carbon-carbon single bonds. Biphenylenes are similar in structure to biphenyls but with two carbon-carbon single bonds connecting the two aromatic rings. Diphenyl ethers are similar in structure to biphenyls but with a carbon-oxygen-carbon ether linkage connecting the aromatic rings rather than a carbon-carbon single bond.

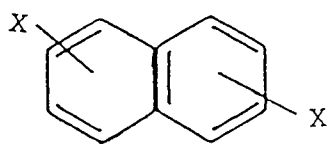
**Figure I-1.**



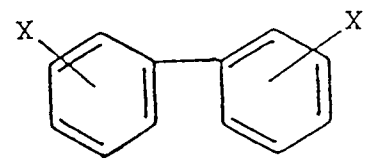

Dibenzodioxins



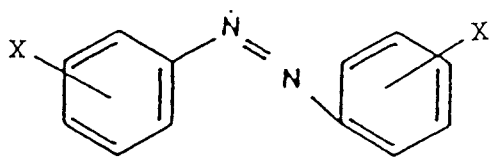

Dibenzofurans



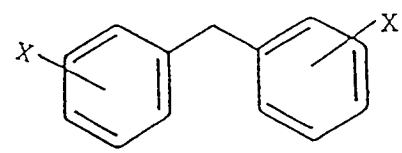

Naphthalenes




Biphenyls




Azobenzenes




Benzyltoluenes

## Sources

### *PCBs*

PCBs were first manufactured in 1881 but were not commercially produced in the United States until 1929 (Lang, 1992). PCBs have been produced internationally and are known commercially as Aroclor (Monsanto, United States), Clophen (Bayer, German Federal Republic), Phenclor and Pyralene (Caffaro, Italy), Kanèchlor (Kanegafuchi, Japan) and Fenchlor (Prodelec, France) (Erickson, 1986; De Voogt and Brinkman, 1989). Commercial PCBs were marketed as mixtures of various isomers based on the average percent of chlorination. For example, Aroclor 1254 contained a mixture of PCB isomers, as indicated by the 12 in 1254, and contained 54% chlorine by weight. Although 209 isomers of PCB are possible, only 132 have been identified in commercial PCB mixtures (Schulz et al., 1989). Because of concern over their toxicity, Monsanto Chemical Company, the major manufacture of PCBs in the United States, discontinued their commercial production in 1977. By 1984 an estimated 1.2 billion Kg of PCBs had been manufactured worldwide, more than 500 million Kg in the United States alone (Erickson, 1986; Tanabe, 1988).

PCBs were first identified in the environment by Jenson in 1966 and later shown to be ubiquitous (Lang, 1992). The widespread and varied uses of PCBs have permitted extensive disseminated sources of release. It has been estimated that 370 million Kg of PCB have found their way into the environment (Tanabe, 1988). Release of PCBs into the environment has occurred accidentally (e.g., leaking transformers), intentionally (e.g., as additives in plastics and dyes), and through negligence (e.g., improper disposal) (McFarland and Clarke, 1989; Lang, 1992).

### *Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)*

PCDDs and PCDFs can be produced through a multitude of pathways including combustion of municipal wastes and hazardous materials (including PCBs), chlorine bleaching processes, formation and reaction of chlorinated benzenes and phenols (as in the synthesis of the herbicide 2,4,5-T), and other industrial processes (Safe, 1990; Lang, 1992; Kimbrough, 1985; Tiernan et al., 1985). In general, the processes leading to the formation of PCDDs and PCDFs result in complex mixtures of congeners and isomers.

Release of PCDDs and PCDFs into the environment has occurred accidentally (e.g., following the rupture of an industrial chemical reaction vessel in Seveso, Italy),

through negligence (e.g., spreading of waste oil in Times Beach, Missouri, and improper disposal of hazardous waste) and incidentally (application of herbicides, and release of effluents and exhaust gases) (Dickson and Buzik, 1993; Menzer, 1991; Tiernan et al., 1985).

## Environmental Fate

The stability of HAHs, and slow degradation rates, can lead to their persistence in the environment once released. The three most probable routes of natural degradation are combustion, photolysis and biodegradation [see reviews by Tschirley (1986), Lang (1992) and Dickson and Buzik (1993)]. The degree to which each degradative mechanism can contribute to the degradation of HAHs may be dependent on the isomeric composition of the mixture. Because sufficiently high temperature natural combustion (~1500°C) is rare, and lower temperature combustion can lead to production of HAHs, combustion is not believed to play a major role in the degradation of HAHs (Drechsler, 1986). Photolysis requires extended exposure to sunlight, a requirement that limits this route of degradation because of the sorption of HAHs onto soils and sediments, and may restrict this degradative mechanism to air-born HAHs or HAHs in the surface layer of soils and water. It has been suggested that the only significant natural process for PCDD degradation is photolysis. In contrast, biodegradation (metabolism) has been suggested to be the major degradative route for some PCBs (Hooper et al., 1990). Both aerobic and anaerobic biodegradation has been reported. For aerobic biodegradation of PCBs by bacteria, the potential rate of metabolism decreases with increasing chlorine content (Furukawa et al., 1978). Thus, the rate of biodegradation of highly chlorinated HAHs in the environment may be slow. It has been estimated that the half-life of some HAHs in soil may exceed 10 years (Tschirley, 1986).

The ultimate fate of concern, with respect to man and wildlife, takes place upon exposure to HAHs (Safe, 1990; Lang, 1992; Oehme, 1991; Tiernan et al., 1985; Dickson and Buzik, 1993; Svensson et al., 1991; Schlatter, 1991). Because HAHs are lipophilic they tend to bioaccumulate with an estimated bioaccumulation factor (a ratio of concentrations: organism/environment), for PCBs, in the range of $10^4$-$10^6$. Exposure can occur by direct contact with the skin, by inhalation, or ingestion. For humans, food is the most common route of exposure and, once exposed, the whole body half-life is in the range of 7-11 years.

## TOXIC EFFECTS OF HAHS

### Overview

HAHs have become a major toxicologic concern for a variety of reasons: 1) HAHs are ubiquitous environmental contaminants (Lang, 1992; Tiernan et al., 1985), 2) some HAHs are highly potent toxicants in laboratory animals (Poland and Knutson, 1982; Kimbrough, 1985; Dickson and Buzik, 1993), and 3) HAHs have been involved in several tragic incidents of widespread human exposure with resulting toxicity (Cordle et al., 1978; Hsu et al., 1985; Yoshimura and Hayabuchi, 1985; Dickson and Buzik, 1993; Menzer, 1991; Tiernan et al., 1985). The effects of HAHs have been studied through epidemiology and controlled laboratory testing. The most intensively studied of the individual HAHs is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) because it has been reported to have the highest toxic potency (Poland and Knutson, 1982).

### Humans

The earliest reported cases of HAH-induced toxicity (1930s-1950s) occurred in humans following occupational exposure (Poland, 1991; James et al., 1993). Since then, a number of other accidental human exposures have occurred, both occupational and environmental, with some involving thousands of people. Among the most noteworthy include incidents in Yusho, Japan (1968), and in Yu-Cheng, Taiwan (1979), where people were exposed to PCBs and PCDFs through consumption of contaminated cooking oil. In 1971 in Times Beach, Missouri, TCDD contaminated oil was spread on roadways, and in 1976 in Seveso, Italy, people were exposed to TCDD following a reactor explosion at a pesticide plant.

The toxicologic effects of HAHs in humans have been reviewed (Dickson and Buzik, 1993; James et al., 1993; Safe, 1992; Kimbrough, 1987; Kimbrough, 1985; Yamashita and Hayashi, 1985). Among the reported effects of HAH exposure are chloracne, altered serum and liver enzyme levels, and disorders of the cardiovascular, nervous, immune, reproductive, endocrine, gastrointestinal, and urinary systems. Dermal toxicities, such as chloracne and hyperpigmentation, are known adverse health effects in humans exposed to PCBs. However, the ability of PCBs to induce dermal toxicity in some cases has been attributed to the presence of contaminating PCDFs. The ability of PCBs alone to induce some toxicities (e.g., respiratory) is controversial and again may have been due to the presence of contaminating PCDFs. Subjective symptoms (e.g., loss of appetite, tingling of hands) have been statistically correlated with blood PCB levels,

although no clinical correlations have been made. In children exposed to PCBs in utero, a fetal PCB syndrome has been described: brown skin and mucosal pigmentation (that disappeared after 2-5 months), early eruption of teeth, gingival hyperplasia, abnormal skull calcification, facial edema, and low birth weights. Symptoms were noted in babies even though the mothers displayed none. Cord blood PCB levels have been correlated with low growth rates and poor scores on behavioral and performance tests. However, it has been suggested that no significant acute or chronic health effects have been causally associated with exposure solely to PCBs (Kimbrough, 1987).

A number of studies have been conducted to examine the effects of PCDDs (most commonly TCDD) in humans [see reviews by Kimbrough (1985, 1987); Mocarelli et al. (1991); Dickson and Buzik (1993)]. Included are studies of people exposed to pure TCDD as well as complex mixtures. A wide variety of symptoms have been reported following TCDD exposure including sexual dysfunction, weight loss, porphyria, hyperkeratosis, eye irritation, and headaches. However, to date, the only toxic signs that have been clearly correlated with exposure to TCDD are chloracne and hyperkera•tosis. The highest estimated human exposure levels to TCDD occurred at Seveso with levels in serum lipids exceeding 20,000 ppt in a few individuals (Needham et al., 1991). A number of suspected pathologies were examined in people from Seveso including reproductive, neurologic, and hepatic toxicities as well as teratogenesis, but no significant trends have been observed (Mocarelli et al., 1991). A study of Vietnam veteran's conducted by the Centers for Disease Control examined the potential effects of TCDD via exposure to the pesticide Agent Orange. Their findings suggested that exposure to Agent Orange did not increase a veterans risk of developing a variety of suspected cancers or of fathering a child with birth defects (Houk, 1991). A more recent study on cancer mortality in chemical workers has suggested a link between TCDD exposure for $\geq$1 year and excess cancer deaths after a latency of $\geq$20 years (Fingerhut et al., 1991).

## Laboratory Animals

### *A spectrum of toxic effects*

Results from studies with laboratory animals have indicated that HAHs are toxic. Among the reported effects of HAHs in animals are a wasting syndrome, thymic atrophy, splenic atrophy, immune suppression, teratogenicity, reproductive toxicity, hepatotoxicity, porphyria, chloracne, tissue specific hyperplasia, squamous metaplasia,

suppression of some enzyme activities, enzyme induction, altered hormone and hormone receptor levels, and carcinogenesis [see reviews by James et al. (1993); Lucier et al. (1993); Dickson and Buzik (1993); Safe (1990); Poland and Knutson (1982)]. Importantly, the physiological effects of HAHs on animals varies between species. The spectrum of toxic effects observed in a particular species does not encompass all of the effects listed above. Even within a species the effects of HAHs can vary with strain, sex and age. However, exposure alone does not automatically lead to toxicity. The most influential factors determining the effects of HAHs on animals are the dose and composition (congeneric and isomeric) of the HAH mixture. Structure-activity relationship studies have shown that the most toxic of the HAHs are the polyhalogenated dibenzodioxins, dibenzofurans and biphenyls. Generally, the more halogenated HAHs are the most toxic. However, the arrangement of halogen atoms on the aromatic rings also plays an important role in determining toxic potency. The most toxic HAHs are the tetra-, penta-, or hexahalogenated compounds with full halogen substitution at the most lateral positions. Toxic potency tends to decrease with increasing substitution at the nonlateral positions. The most toxic HAHs have been shown to be approximate isostereomers of TCDD, the penultimate toxic HAH.

### *An aromatic hydrocarbon receptor model*

In their studies of benzo[a]pyrene metabolism, Nebert (1989) found that hepatic aryl hydrocarbon hydroxylase (AHH) activity could be highly induced in some inbred strains of mice (e.g., C57Bl/6) but not in others (e.g., DBA/2). Continued study of this phenomenon (Nebert et al., 1993) has shown that AHH [now called cytochrome P450 1A1 (CYP1A1)] activity is induced by many aromatic hydrocarbons such as benzo[a]pyrene, 3-methylcholanthrene, PCBs, and PCDDs. CYP1A1 inducibility is an autosomal dominant trait in inbred mouse strains and is linked to the expression of the *b* allele of the aromatic hydrocarbon (*Ah*) locus. The protein product of the *Ah* locus [first described by Poland et al. (1976)] is a receptor, called the Ah receptor, that binds aromatic hydrocarbon ligands (Whitlock, 1987; 1990). Mice of the C57Bl/6 strain are homozygous for the $Ah^b$ allele and show CYP1A1 inducibility when exposed to Ah receptor ligands. In contrast, mice of the DBA/2 strain are homozygous for the $Ah^d$ allele and show CYP1A1 inducibility only when exposed to much higher doses of aromatic hydrocarbon ligands. The difference in CYP1A1 inducibility between these two mouse strains has been attributed to amino acid sequence differences in the $Ah^d$ allelic

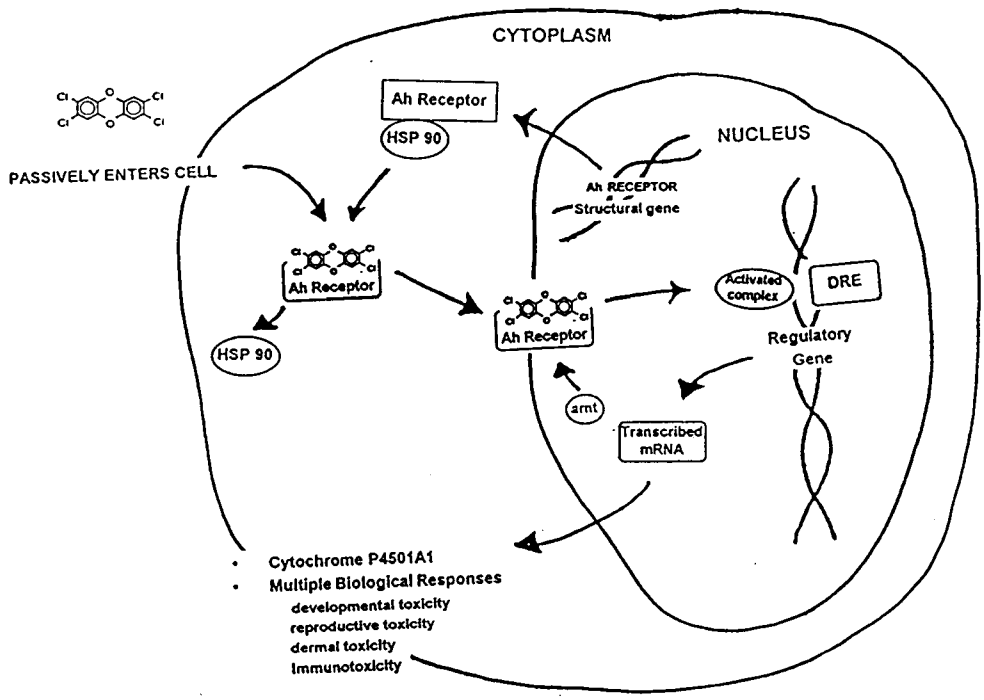
gene product resulting in lower binding affinity of aromatic hydrocarbon ligands (Chang et al., 1993).

It has been suggested that most, if not all, toxicity induced by TCDD is mediated through the Ah receptor (Lucier et al., 1993). Although this is less clear for all HAHs, numerous studies have indicated similar relationships for many PCDDs, PCDFs and PCBs (Safe, 1990). Figure I-2 schematically summarizes a proposed mechanism for Ah receptor-mediated effects. The Ah receptor is a ~90,000 MW protein that has been found in numerous cell types derived from animals and humans (Safe, 1988). In the absence of ligand, the Ah receptor exists in the cytosol as a heterotrimer in association with heat shock proteins. The Ah receptor-ligand complex translocates to the nucleus where it is found in association with the ARNT protein. Binding of the ARNT protein to the Ah receptor-ligand complex permits binding to specific sequences in the genome known as dioxin response elements (DREs), also known as xenobiotic response elements (XREs). Ah receptor-ligand-ARNT complex binding to DREs has been shown to alter the transcription of down-stream sequences. The most extensively studied example of Ah-mediated enhanced gene transcription with subsequent increased protein production is the induction of CYP1A family of enzymes following aromatic hydrocarbon exposure (Nebert et al., 1993). The transcription of other genes may be regulated by the same mechanism. In addition to induction of both phase I (CYP1A1, CYP1A2) and phase II (menadione oxidoreductase, aldehyde dehydrogenase, UDP glucuronosyltransferase, glutathione transferase) hepatic enzymes, HAH-induced thymic involution, teratogenicity and immunotoxicity have been correlated with an Ah-mediated mechanism (Poland and Knutson, 1982).

The most toxic HAH, TCDD, has been shown to bind with very high affinity to the Ah receptor ($K_d$ ~1 x $10^{-10}$M) (Lucier et al, 1993). Interestingly, the affinity of 3-MC for the Ah receptor is ~4-fold lower than that for TCDD, while the ability of 3-MC to induce CYP1A1 activity is ~1000-fold lower. A recent study has suggested that part of the difference in toxicity between 3-MC and TCDD is linked to the different metabolic transformation rates of the two compounds (Riddick et al., 1994). Therefore, the rate of metabolic transformation, as well as the Ah receptor binding affinity, may play a role in determining the toxicity of HAHs.

**Figure I-2. The Ah receptor model.**

A general schematic of the Ah receptor model is shown. A ligand, shown here as TCDD, enters the cell via passive diffusion and binds to the Ah receptor. Prior to ligand binding, the Ah receptor is complexed with heat shock protein (HSP). After ligand binding, the ligand-receptor complex can translocate to the nucleus where it is found in association with the aromatic receptor nuclear transport (ARNT) protein. The ligand-receptor-ARNT protein complex can bind to dioxin response elements (DREs) influencing transcriptional events. Numerous biological effects (e.g., expression of P4501A1 protein, reproductive toxicity, developmental toxicity, dermatotoxicity, and immunotoxicity) of HAH have been correlated with an Ah-mediated mechanism.



## Immunotoxicity

### *Humans*

Reports of humans accidentally exposed to HAHs have suggested that HAHs are immunosuppressive in humans [see reviews by Vos and Luster (1989), Holsapple et al. (1991b), and Vos et al. (1991)]. In people exposed to PCBs in cooking oil, the reported immunological findings included decreased immunoglobulin titers (IgA and IgM), a decreased percentage of total circulating T lymphocytes, and suppressed DTH skin responses, but enhanced in vitro T cell mitogen-induced proliferation of blood lymphocytes (Lü and Wu, 1985). The incidence and severity of respiratory symptoms (e.g., respiratory distress, respiratory infection) correlated with PCB concentrations in blood and sputum samples (Shigematsu et al., 1978). Children born to exposed women had a higher incidence of bronchitis. Interestingly, the results of studies of humans occupationally exposed to PCBs have not indicated any significant immunological effects. Subsequent analysis of cooking oil samples indicated the presence of PCDFs in addition to PCBs. Much of the toxicity observed in Yusho and Yu-Cheng has since been attributed to the presence of PCDFs in the cooking oil (James et al., 1993).

The results of immunological studies on cohorts of humans accidentally exposed to TCDD are more ambiguous than those of PCB/PCDF exposed people. Immunological findings in people exposed to TCDD at Seveso, Italy, or Times Beach, Missouri, have been inconsistent over several studies. For example, a cohort from Times Beach was shown to have suppressed DTH responses in one study, whereas the results of a follow-up study indicated no alteration of the DTH response. Further, no apparent immunological abnormalities were found in children exposed to TCDD in Seveso, although some of them presented with chloracne, whereas a second study involving a different set of subjects found significant elevation of blood complement levels that correlated with the incidence of chloracne. The ability of TCDD to alter immune function in humans is inconclusive.

### *Laboratory animals*

Exposure of animals to HAHs has been shown to significantly increase their susceptibility to infectious diseases (Vos et al., 1991; Vos and Luster, 1989). The resistance of mice to infection with viruses (Clark et al., 1983; Imanishi et al., 1980), bacteria (Hinsdill et al., 1980; Thigpen et al., 1975; Luster et al., 1980) and parasites (Loose et al., 1978) is decreased by HAH exposure. These findings are important

because they suggest that HAHs can pose real health risks to animals and support the suggestion that HAHs are immunotoxic in humans.

HAH-induced immune suppression has been demonstrated in numerous animal species (e.g., ducks, mice, rats, guinea pigs, rabbits and monkeys), but the mouse has been used to the greatest extent for studying the underlying mechanisms. Although the effects of both complex mixtures and purified isomers have been examined, TCDD has been by far the most intensively studied of the HAHs. The effects of HAHs on immune function have been extensively reviewed (Kerkvliet, 1984; Thomas and Faith, 1985; Vos and Luster, 1989; Holsapple et al., 1991b; Vos et al., 1991; Kerkvliet and Burleson, 1994).

Among the earliest reported effects of HAHs in laboratory animals was atrophy of lymphoid organs. Flick et al. (1965) was the first to demonstrate significant atrophy of lymphoid tissues in chickens fed PCBs. Subsequent studies have shown that lymphoid atrophy, particularly of the thymus, is a common response following exposure to HAHs (Vos and Luster, 1989). Although thymic involution by itself is not indicative of immune suppression, it is a hallmark of exposure to HAHs in nearly all animal species studied (Holsapple et al., 1991b). Approximate stereoisomers of TCDD have the greatest potency for causing thymic involution. Thus, in mice 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) was more potent then 2,2',4,4',6,6'-, 2,2',3,3',6,6'- and 2,2',4,4',5,5'-hexachlorobiphenyl at causing thymic involution (Biocca et al., 1981). HAH-induced thymic involution has also been correlated with expression of the high-affinity Ah receptor (Silkworth and Grabstein, 1982; Kerkvliet et al., 1990b). Mice that express the high-affinity Ah receptor (e.g., C57Bl/6) are approximately 10-fold more sensitive to HAH-induced thymic involution than DBA/2 mice (Vos and Luster, 1989). A number of mechanisms have been proposed to explain HAH-induced thymic involution including inhibition of terminal deoxynucleotidyl transferase (TdT) activity in bone marrow and thymus cells leading to an impaired capacity of prothymocytes to populate the thymus (Fine et al., 1990), $Ca^{++}$-dependent endonuclease activation in thymocytes (McConkey and Orrenius, 1989), and suppression of the ability of thymic epithelial cells to support thymocyte proliferation (Greenlee et al., 1985). Although the thymus is clearly affected by HAHs, the influence of thymic toxicity may only be relevant to immature animals with developing immune systems since thymectomy has been shown to have no effect on immune responses in either vehicle or HAH-treated adult animals (Kerkvliet and Brauner, 1987).

The effect of HAHs on immune function in immature rodents is potent, and sufficient exposure to cause immune suppression can occur in utero and/or postnatally (via nursing of exposed mothers) (Vos and Luster, 1989; Kerkvliet and Burleson, 1994). Thomas and Hinsdill (1979) reported that offspring from mice exposed to TCDD in the diet (5 ppb) had suppressed humoral [antibody responses to sheep red blood cells (SRBC)] and cellular (DTH) immune responses while the mothers were able to respond normally to bacterial challenge. These results suggest that young animals are more sensitive than adults to the immunosuppressive effects of HAHs. This is supported by the observation that mice exposed perinatally to TCDD (20 μg/Kg to the mother over four weeks) led to suppressed bone marrow colony formation (granulocyte-macrophage); prolonged growth of transplanted tumors was observed at lower doses (Luster et al., 1980). In rats, exposure to TCDD perinatally (20 μg/Kg total dose to the dams) led to significant suppression of DTH responses for up to 108 days after weaning; normal responses were observed by 245 days after weaning suggesting that the immunosuppressive effects of TCDD dissipate over time (Faith and Luster, 1979).

In adult animals also, HAHs have been shown to cause significant suppression of cellular immune function. The effects of HAHs on cellular immunity were first examined because of the reported effects of HAHs on the thymus. In vivo exposure of adult animals to HAHs has been shown to cause suppression of DTH responses, graft-versus-host responses, allospecific CTL activity, host defense responses to virus challenge and T cell mitogen responses (see reviews by Kerkvliet, 1984; Vos and Luster, 1989). In contrast, no suppressive effect on T cell antigen-driven responses has been observed following direct exposure of lymphocytes to HAHs in vitro (Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988b). Suppression of antigen-driven T cell responses has been demonstrated in some in vitro studies but only if lymphocytes were first exposed to HAHs in vivo (*ex vivo* studies) (Clark et al., 1983; Kerkvliet and Baecher-Steppan, 1988b). The results of these studies suggest that HAH-induced effects on T cells in adult animals is indirect. HAH-induced suppression of cellular immunity, like thymic toxicity, is mediated through the Ah receptor (Clark et al., 1983; Kerkvliet et al., 1990b). The effect of HAHs on CTL function in adult animals is discussed below.

Suppression of humoral immunity by HAHs has been reported in numerous animal models including rabbits, guinea pigs, monkeys, rats and mice (Vos and Luster, 1989; Holsapple et al., 1991b; Kerkvliet and Burleson, 1994). In the mouse, HAH-induced humoral immune suppression has been linked to an Ah-mediated mechanism (Kerkvliet et al., 1990a; House et al., 1990). Acute exposure of adult Ah respon-

sive mice to as little as 1 μg TCDD per Kg body weight has been shown to significantly suppress the antibody response to SRBC (Vecchi et al., 1980). Suppression of both primary and secondary antibody responses (measured as serum antibody titers) by HAHs has been reported (Loose et al., 1977; Hinsdill et al., 1980). In addition, HAHs can suppress both T cell-independent (TI) and T cell-dependent (TD) antibody responses in mice (Kerkvliet and Brauner, 1987). Interestingly, the TD anti-SRBC response in mice was more sensitive to HAH-induced suppression (by 10-fold) than the TI response to trinitrophenyl toluene-conjugated lipopolysaccharide (TNP-LPS) (Kerkvliet and Brauner, 1987). These results suggest that HAH-induced suppression of T cells plays a role in the suppression of TD antibody responses in vivo.

In vitro generated antibody responses are also sensitive to suppression by HAHs suggesting that HAHs can affect B cells by a direct mechanism (Holsapple et al., 1991b). Indeed, Luster et al. (1988) have shown that, while B cell viability and antigen-driven proliferation are unaltered, B cell differentiation (measured by expression of plasma cell markers) and antibody production is suppressed by HAH exposure in vitro. However, in contrast to in vivo antibody responses, in vitro TI and TD antibody responses are nearly equally sensitive to HAH-induced suppression (Holsapple et al., 1986). These results suggest that B cell function is more sensitive to the direct suppressive effect of HAH than is T helper cell function. Interestingly, Holsapple et al. (1986) and Davis and Safe (1991) have reported that the Ah-dependence of HAH-induced immune suppression observed in vivo is lost in vitro. An explanation for this lack of Ah dependence in vitro is unclear.

Some components of the innate immune system can also be affected by exposure to HAHs. Following acute TCDD exposure (5 μg/Kg) in mice, suppression of neutrophil-mediated tumor cytolysis was shown, although reactive oxygen production and degranulation were unaffected (Ackermann et al., 1989). Suppression of complement activity in mice has been demonstrated following acute or subchronic exposure to a total dose of 14 μg/Kg (White et al., 1986). Other functions (e.g., NK-mediated tumor cytolysis and macrophage activity) are unaffected by HAH exposure (Vos and Luster, 1989; Kerkvliet and Burleson, 1994).

Rosenthal et al. (1989) has shown that hepatic clearance of endotoxin was significantly reduced by exposure to TCDD. Reduced endotoxin clearance in TCDD-treated animals may partially explain the enhanced sensitivity of TCDD-treated animals to endotoxin-induced shock (Vos et al., 1978), as well as the greater susceptibility of mice to infection by gram negative bacteria (Hinsdill et al., 1980). However, hyperin-

flammation following injection with SRBC in the absence of specific endotoxin challenge has also been reported [Moos et al., 1994; Kerkvliet and Oughton, 1993) and suggests an enhanced response to inflammatory stimuli. In support of this, increased levels of TNF, a major inflammatory mediator, have been shown in TCDD-treated mice following injection with SRBC (Moos et al., 1994) and endotoxin (Clark et al., 1991b). Further, pretreatment of TCDD-exposed mice with anti-TNF antibodies or TNF-soluble binding protein reduced hyperinflammatory responses (Moos et al., 1994; Clark et al., 1991a; Taylor et al., 1992). Altered regulation of another imflammatory mediator, IL-1ß, by TCDD has also been suggested (Sutter et al., 1991). It is unclear if increases in these inflammatory mediators play a role in HAH-mediated immune suppression.

***Suppression of CTL activity***

Numerous investigators have examined the effects of HAHs on CTL activity in mice (Clark et al., 1981; Clark et al., 1983; Nagarkatti et al., 1984; Hanson and Smialowicz, 1994; Kerkvliet and Baecher-Steppan, 1988a,b; Kerkvliet et al., 1990b). The effects of a variety of HAHs have been examined, including purified isomers and complex mixtures. But the two most intensively studied HAHs, with respect to the CTL response, are HxCB and TCDD. Both HxCB and TCDD have been shown to dose-dependently suppress alloantigen-specific CTL activity in vivo (Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b). In addition, in host resistance models thought to be reflective of CTL responses, HAH treatment significantly decreased the survival rate of mice challenged with viruses (Imanishi et al., 1980; Clark et al., 1983).

Like other toxic responses, suppression of CTL activity is mediated through the Ah receptor. Using mice congenic for the *Ah* locus, Kerkvliet et al. (1990b) has shown that the sensitivity of mice to HAH-induced CTL suppression correlated with expression of the b allele. In addition, CTL suppression has been shown to be Ah receptor mediated in structure activity studies using polychlorinated biphenyl isomers with varying affinities for the Ah receptor (Clark et al., 1983; Kerkvliet et al., 1990b).

The ultimate mechanism of HAH-induced CTL suppression has not been determined, but two major hypotheses have been proposed. The first involved HAH-enhanced T cell suppressor function. This was proposed by Clark et al. (1981) who reported that thymus cells from TCDD-treated mice suppressed the in vitro generation of CTL activity by peripheral lymph node cells. This hypothesis has not been supported by the results of other investigators (Hanson and Smialowicz, 1994). To the contrary,

Kerkvliet and Baecher-Steppan (1988b) have reported that, rather than enhancing suppressor cell activity, HxCB treatment led to suppression of splenic suppressor cell activity. Further study may be required to explain these differing results.

A more recent hypothesis (Kerkvliet and Baecher-Steppan, 1988b) suggested that HAHs cause suppression of CTL activity through an indirect method. This was proposed because of the observation that HxCB, when added directly to mixed lymphocyte cultures, did not suppress the lymphoproliferative response (Kerkvliet and Baecher-Steppan, 1988b). Similar results have been reported by Clark et al. (1981) for TCDD added directly to mixed lymphocyte cultures.

One such indirect mechanism may involve dysregulation of the production of, or an altered sensitivity to, endogenous immunomodulators. The circulating levels of numerous endogenous immunomodulators have been shown to be altered by exposure to HAH, e.g., lowered testosterone (TT) levels (Moore et al., 1985), elevated glucocorticoid (GC) levels (Gorski et al., 1988; Kerkvliet et al., 1990b), lowered thyroid hormone levels (Pazdernik and Rozman, 1985), increased prostaglandin (PG)-$E_2$ production (Quilley and Rifkind, 1986) and lowered prolactin levels (Moore et al., 1989; Russell et al., 1988; Jones et al., 1987). Since GCs and $PGE_2$ have specifically been shown to suppress CTL function (Leung and Mihich, 1982; Wolf and Droege, 1982; Henney et al., 1972; Gillis et al., 1979; Schleimer et al., 1984), and reduction of endogenous prolactin levels leads to suppression of immune function (Gala, 1991; Berczi et al., 1983), the potential exists for HAHs to mediate the suppression of CTL activity through these indirect immunomodulators.

## HYPOTHESIS

In the studies described here the underlying hypothesis was: HAH-induced suppression of CTL activity in C57Bl/6 mice is mediated indirectly through alteration of endogenous immunomodulators. HxCB and TCDD were used as model immunotoxic HAHs.

Two major approaches were used to test the hypothesis: 1) examination of the effects of HAHs on circulating immunomodulator levels or production of immunomodulators by spleen cells; and 2) manipulation of circulating immunomodulator levels to antagonize or mimic the effects of HAH exposure with subsequent evaluation of CTL function. In the first approach, the effects of HAHs on eight endogenous immunomodu-

lators were examined (CS, prolactin, $PGE_2$, TT, IL-2, IL-4, IL-6 and IFN-$\gamma$). In the second approach, manipulation of three immunomodulators was attempted (CS, $PGE_2$, prolactin).

# CHAPTER II

## PCB-INDUCED IMMUNE SUPPRESSION: CASTRATION, BUT NOT ADRENALECTOMY OR RU 38486 TREATMENT, PARTIALLY RESTORES THE SUPPRESSED CTL RESPONSE TO ALLOANTIGEN

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## ABSTRACT

The cytotoxic T lymphocyte (CTL) response to allogeneic P815 tumor in C57Bl/6 mice is dose-dependently suppressed following treatment with 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB). Elevation of plasma corticosterone (CS) is also observed coincident with CTL suppression. Since immune suppression is inducible by glucocorticoid (GC) administration, the role of elevated CS was investigated as an indirect mechanism of HxCB-induced immunotoxicity. In multiple experiments, HxCB treatment (10 mg/Kg body weight) consistently reduced CTL activity by 70%-85% in male mice. Adrenalectomy (ADX) failed to alter the suppression of CTL activity by HxCB. However, the mortality rate was high (≥70%) in these experiments and plasma CS elevation persisted in HxCB-treated ADX survivors. Therefore, the use of ADX mice was inadequate to determine if CS elevation leads to CTL suppression following HxCB treatment. Daily administration of the GC receptor antagonist RU 38486 (150 mg/Kg body weight, po.) also failed to alter the suppression of CTL activity in HxCB-treated mice; however, spleen cellularity was significantly increased, suggesting functional GC receptor antagonism. Male mice were more sensitive to HxCB-induced CTL suppression than female mice, and HxCB-induced plasma CS elevation was greater in male mice. Castration (ODX) failed to reduce the elevation of plasma CS in HxCB-treated male mice. However, ODX partially alleviated CTL suppression in HxCB-treated male mice. Taken together, these data suggest that (1) GC receptor antagonism does not alleviate HxCB-induced CTL suppression, (2) suppression of CTL may be enhanced in male mice by HxCB effects in the testes or by an HxCB-induced altered sensitivity of the immune system to testes-specific factors.


## INTRODUCTION

Polychlorinated biphenyls (PCBs) are a member of a large group of structurally related halogenated aromatic hydrocarbons (HAH) that are ubiquitous environmental contaminants with wide ranging toxicologic potential for both man and wildlife (McFarland and Clarke, 1989). Concern for these compounds revolves around their toxic potency which is dependent on isomer, exposure route and the species studied (Vickers et al., 1985). Toxic potency has also been shown to segregate with binding affinity for the cytosolic aromatic hydrocarbon receptor (AhR) (Silkworth and Grabstein, 1982; Silkworth and Antrim, 1985; Kerkvliet et al., 1990a,b), the affinity for which is determined by the chlorine substitution pattern of each isomer (Bandiera et al., 1982). The prototype Ah receptor ligand, and the most potent HAH is

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) is among the most potent of the PCBs.

Immune suppression is a hallmark of HAH toxicity in many animal species (Poland and Knutson, 1982). Although the effects of HAH on immune function have been widely studied (reviewed by Vos and Luster, 1989), the mechanism(s) for the immunotoxic effects have yet to be resolved. Past studies have shown that HAH suppress CTL responses to tumor allograft (Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988a). The potency of suppression in vivo correlated directly with the binding affinity of HAH congeners for the AhR (Clark et al, 1983; Kerkvliet et al., 1990b). However, neither TCDD (Clark et al., 1981) nor HxCB (Kerkvliet and Baecher-Steppan, 1988b), which are both highly immunosuppressive in vivo, directly altered T cell responses when added to mixed lymphocyte cultures. These results suggest that an indirect mechanism may be responsible for the suppression of CTL function observed in vivo.

Coincident with suppression of the CTL response by PCBs in vivo is the elevation of plasma corticosterone (CS) levels (Kerkvliet et al., 1990b). The degree of CS elevation correlates with the dose and binding affinity of PCB congeners for the Ah receptor. Elevations of circulating glucocorticoid (GC) levels after treatment with other HAH have also been reported (Sanders et al., 1977 and 1974; Gorski et al., 1988a; Jones et al., 1987; DiBartolomeis et al., 1987). Deviations from normal levels of GC by PCBs may have significance to their immunotoxicity since GC affect macrophages, as well as B and T cells (del Rey et al., 1984; Bradley and Mishell, 1982; Schechter and Feldman, 1977). Importantly, development of CTL activity has been shown to be sensitive to suppression by GC (Gillis et al., 1979; Schleimer et al., 1984).

In the studies reported here, several methods to regulate GC were used to examine the relationship between elevated plasma CS levels and suppression of the CTL response in HxCB-treated mice. ADX and ODX were used in attempts to reduce CS production. RU 38486 treatment was used in an attempt to block the effects of elevated CS at the receptor level.

## METHODS

### ANIMALS

Male or female C57Bl/6 mice, 7-11 weeks of age, were used in all experiments. Mice were obtained from Jackson Laboratories (Bar Harbor, ME), Bantin and Kingman

(Freemont, CA), or Taconic Farms (Germantown, NY). Animals were housed in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experimentation. Animal rooms were maintained with a 12 hr light/dark cycle (fluorescent, 7:30 AM lights on) and constant temperature (79 ± 1 for ADX, 72 ± 1 for others [°F]) and 50% humidity. Animals were housed with a maximum population of four per cage in polycarbonate shoe-box cages which were randomly assigned to positions in a cage rack. Animals were provided with Bed-O-Cob bedding (The Andersons, Maumee, OH), Wayne Rodent Blox (Harlan Sprague Dawley Co., Bartonville, IL) and drinking water *ad libitum* (0.9% saline for ADX animals, tap water for all others). Unless otherwise indicated, mice were surgically modified in our laboratory while under ketamine and xylazine anesthesia. Following ADX, animals were rested a minimum of 3 days prior to HxCB treatment. Following ODX, animals were rested a minimum of 7 days prior to HxCB treatment.

## HXCB TREATMENT

Environmental standard grade (99% purity) 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) was obtained from Ultrascientific, Hope, RI. HxCB was dissolved in acetone and mixed with peanut oil (Nabisco Brands Inc., East Hanover, NJ); the acetone was evaporated under a stream of nitrogen for a final concentration of 1.0 mg/mL. Animals were given 0 or 10 mg/kg HxCB (0.1 mL per 10 g body weight) by gavage one day prior to P815 injection. Ten mg/Kg HxCB was a dose previously shown to cause reproducible and statistically significant suppression of CTL activity (Kerkvliet and Baecher-Steppan, 1988a).

## P815 INJECTION

The P815 mastocytoma cell line was propagated in ascites form by weekly passage in DBA mice, the strain of origin. C57Bl/6 mice ($H\text{-}2^b$) were inoculated with $1.0 \times 10^7$ viable P815 cells ($H\text{-}2^d$) by i.p. injection in a 0.5 mL volume of Hanks Balanced Salt Solution (HBSS).

## EXPERIMENTAL DESIGN

Unless otherwise indicated, the time course for experimentation is given relative to HxCB treatment as follows: HxCB treatment on day 0; P815 injection on day 1; termination on day 11. Experiments were terminated by euthanizing all animals in the

morning (8:30-11:00 AM), the low period in the murine diurnal CS rhythm (Shimizu et al., 1983). In some experiments animals were killed by cervical dislocation followed by decapitation, and drained blood was collected. In most experiments, animals were killed by an overdose of $CO_2$ and blood was collected by heart puncture. Death due to an overdose of $CO_2$ was rapid (approximately 20 seconds) and did not cause a detectable elevation of CS. Serum or EDTA-treated plasma was stored at -20°C until analyzed. Male mice were used in all experiments except where indicated.

## RU 38486 TREATMENT

RU 38486 (17-ß-hydroxy-11-ß-(4-dimethylaminophenyl)-17-α-(propanyl)-estra-4,9-dien-3-one), was received as a generous gift from Roussel-Uclaf, Romainville, France. RU 38486 was suspended in 0.25% carboxymethyl cellulose and 0.2% Tween 80 and given orally twice daily (0.1 mL per 10 g body weight) for a total dose of 150 mg/Kg/day. Two separate treatment regimens were conducted in different experiments: (1) administration for 10 days beginning on the day of HxCB treatment; (2) administration for three days beginning on day 3, 5 or 7 post P815 injection. Controls received placebo (carboxymethyl cellulose/Tween 80) by the same treatment regimen.

## CTL ACTIVITY

Splenic CTL activity was measured in a chromium-51 release assay as described previously (Kerkvliet and Baecher-Steppan, 1988a). The percent cytotoxicity (CTX) at each effector:tumor cell (E:T) ratio was calculated by the equation:

$$\%CTX = \frac{cpm_i - cpm_{ni}}{cpm_{mr} - cpm_{ni}} \times 100,$$

where $CPM_i$ = cpm using spleen cells from P815-injected animals, $CPM_{ni}$ = cpm using spleen cells from nonP815-injected animals and $CPM_{mr}$ = the maximum cpm released from cultures incubated with sodium dodecyl sulfate. E:T ratios of 3.7:1, 11:1, 33:1 and 100:1 were used. In order to compare the overall cytotoxic potential, the area under the cytotoxicity curve (AUCC) was calculated for each animal using the trapezoidal rule and log(E:T) ratios from 3.7:1 to 100:1. AUCC is given as units % CTX • log(E:T).

## CORTICOSTERONE AND TESTOSTERONE

Serum or plasma CS or TT levels were determined using [$^{125}$I]-coupled double antibody radioimmunoassay (RIA) kits (ICN Biochemicals, Carson, CA, for CS; Diagnostic Products Corp., Los Angeles, CA, for TT). Protocols optimized by the manufacturers were used. Standard curves were generated with each assay. The lower limits of detection for each assay were 25 ng CS/mL and 0.05 ng TT/mL. The maximum interassay coefficients of variance for internal controls were 15.7% (CS) and 12.9% (TT) as determined by the manufacturers. The maximum intraassay variations were 8.8% (CS) and 17.7% (TT). For statistical purposes, samples with analyte levels below the lower limit of detection were assigned the limit value.

## STATISTICAL ANALYSIS

Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Significant treatment effects were determined by analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of SAS. Comparisons between two means were performed using *t* tests (TTEST of SAS). Comparisons between more than two means were performed using LSD multiple comparison *t* tests (GLM of SAS). Regression analysis was performed using the REG procedure of SAS. Values of $p \leq 0.05$ were considered statistically significant.

# RESULTS

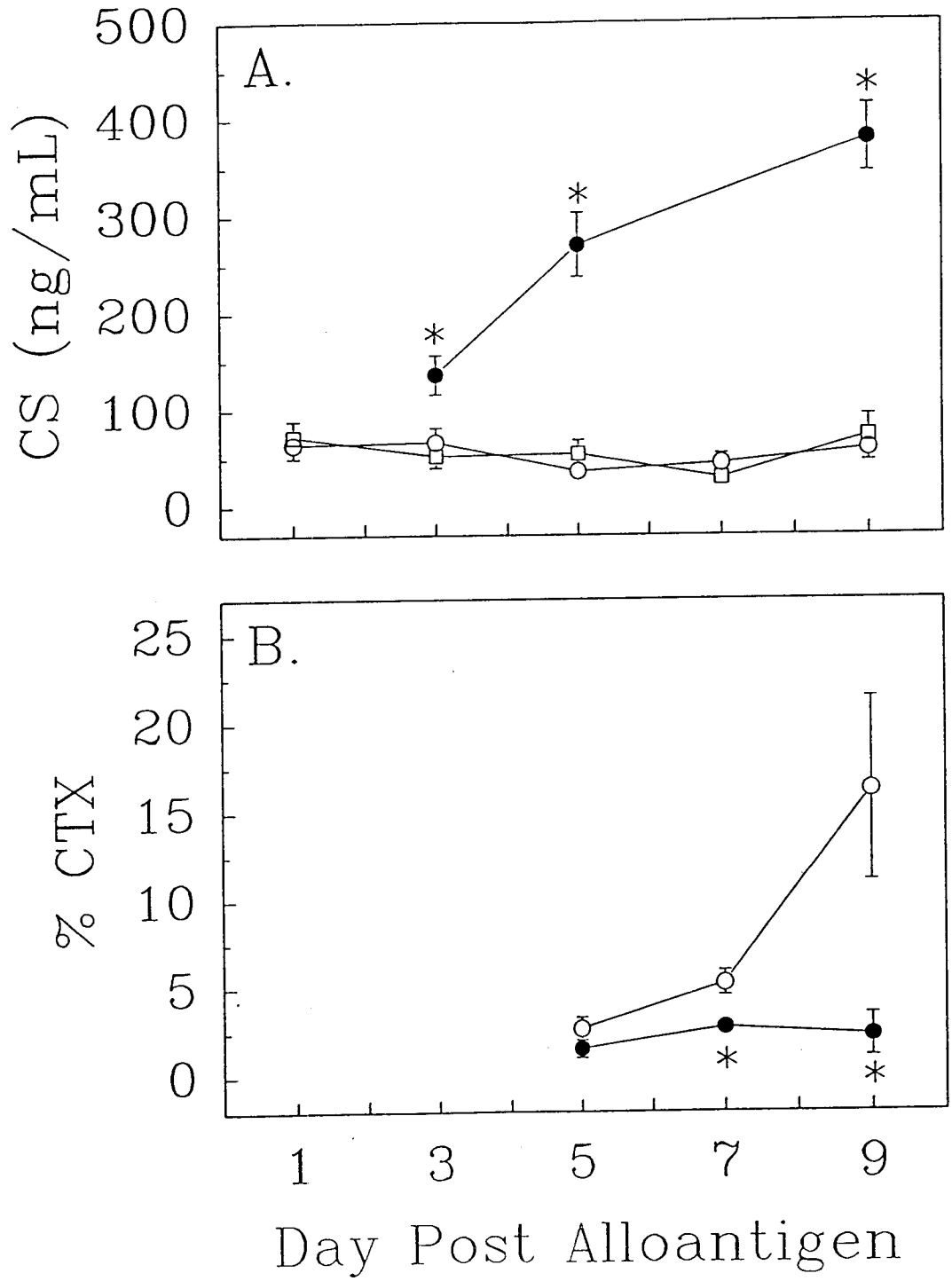
## COINCIDENT EFFECTS OF HxCB OVER TIME FOLLOWING P815 INJECTION

The effects of HxCB on CS levels and CTL activity are shown in figures II-1A and II-1B, respectively. Significant CS elevation was evident in HxCB-treated mice 3 days after P815 injection; the degree of CS elevation increased through day 9. P815 injection alone did not alter CS levels on any day measured. In other experiments, mice treated with HxCB alone (10 mg/Kg) had significantly elevated CS levels only on day 4 (data not shown). CTL activity in HxCB-treated mice was significantly suppressed compared with vehicle treated mice at all time points after day five.

**Figure II-1. Coincident effects of HxCB treatment on plasma CS levels and splenic CTL activity.**

Data are presented as mean ± S.E. for 6-10 male mice per group. Animals were injected with HBSS or P815 cells one day following treatment with 0 or 10 mg/kg HxCB. Animals were killed on days indicated. For statistical purposes (panel A), plasma samples with CS levels below the lower limit of detection (25 ng/mL) were given the value 25 ng/mL. Percent CTX data (panel B) is given for an E:T ratio of 33:1. Data for double-vehicle-treated animals are provided as normal reference points (open squares). Open circles indicate vehicle-treated, P815-injected animals and closed circles indicate HxCB-treated, P815-injected animals. * indicates a significant difference between treatment groups for P815-injected animals at $p < 0.05$.

**Figure II-1.**


A.
B.
CS (ng/mL)
% CTX
500
400
300
200
100
0
25
20
15
10
5
0
1
3
5
7
9
Day Post Alloantigen

## INFLUENCE OF ADRENALECTOMY ON HxCB EFFECTS

Experiments utilizing ADX mice were complicated by a high rate of mortality among HxCB-treated ADX mice. Results shown in table II-1 are representative of several experiments. A mean time to death of 7 days following P815 injection was consistently observed for HxCB-treated ADX mice; the time to death correlated with the time of maximum tumor burden (data not shown). As observed in surviving mice, ADX had no effect on HxCB-induced suppression of CTL activity or elevation of CS levels (table II-1).

## EFFECTS IN RU 38486-TREATED MICE

Treatment with RU 38486 twice daily during the 10 days following HxCB treatment did not alter body weight gain or spleen cellularity (table II-2), plasma CS levels (figure II-2), or CTL activity (figure II-3) in vehicle-treated/P815-injected mice. In contrast, RU 38486 treatment significantly attenuated some of the effects of HxCB treatment: body weight loss was reduced 50%, and spleen cellularity was increased 144% when compared to HxCB-treated mice given placebo twice daily (table II-2). Interestingly, the elevation of plasma CS levels by HxCB was significantly enhanced (2.6 fold) by RU 38486 treatment (figure II-2). The degree of HxCB-induced CTL suppression was significantly enhanced by RU 38486 treatment when measured as AUCC (from 72% suppressed to 84% suppressed), although % CTX was significantly different only at the 11:1 E:T ratio (figure II-3).

Administration of RU 38486 to vehicle-treated mice for three-day periods from day 3 through day 9 post P815 injection did not alter body weight gain or spleen cellularity (table II-3). However, RU 38486 treatment of vehicle-treated mice did lead to enhancement of splenic CTL activity when RU 38486 was given on days 7-9 (table II-3 and figure II-4). Administration of RU 38486 to HxCB-treated mice on days 3-5 or days 7-9 had no effect on body weight gain, splenic CTL activity (table II-3 and figure II-4) or the HxCB-induced elevation of plasma CS (data not shown). Administration of RU 38486 to HxCB-treated mice on days 7-9 appeared to increase spleen cellularity (not significant, table II-3) and did significantly increase spleen weight when measured as a percent of body weight (data not shown). Effects of RU 38486 treatment on days 5-7 post P815 injection could not be assessed in HxCB-treated mice because of a 100% mortality rate (table II-3). In addition, two out of six mice that were given RU 38486 on days 3-5 post P815 injection died.

**Table II-1. Effects of HxCB treatment and ADX in P815-injected mice[a]**

| ADX | HxCB (mg/Kg) | % Mort.[b] | Body Weight Change (g)[c] | AUCC[d] | Corticosterone (ng/mL) |
|---|---|---|---|---|---|
| - | 0 | 0 | $3.1 \pm 0.3^{e}$ | $73.7 \pm 1.6^{e}$ | $71 \pm 16^{e}$ |
| + | 0 | 10 | $1.2 \pm 0.5^{f}$ | $86.8 \pm 6.1^{e}$ | $77 \pm 12^{e}$ |
| - | 10 | 0 | $-1.0 \pm 0.8^{g}$ | $15.7 \pm 3.8^{f}$ | $551 \pm 111^{f}$ |
| + | 10 | 77 | $-1.5 \pm 0.6^{g}$ | $25.4 \pm 4.8^{f}$ | $369.3 \pm 61^{f}$ |

[a]Values represent mean ± S.E. for 5-7 male mice. Animals were ADX or sham operated 3 days prior to HxCB treatment. All animals were injected with P815 one day following HxCB treatment. Survivors were killed 10 days after HxCB treatment.

[b]Mortality given as the percent of initial group size of 5-30 animals.

[c]Body weight change was computed as the difference in weights on the day of surgery and the day of necropsy. The mean initial body weight (± S.D.) was 21.9 ± 1.6 g and there was no difference between groups.

[d]Area under the cytotoxicity curve (AUCC) is given for E:T ratios of 4:1, 11:1, 33:1, 100:1.

[e,f,g]Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Table II-2. Effects of HxCB and 10 days of RU 38486 treatment in P815-injected mice[a]**

| RU 38486 | HxCB (mg/Kg) | Body Weight Change (g)[b] | Spleen Cells (x $10^6$) |
|---|---|---|---|
| - | 0 | $1.8 \pm 0.2^c$ | $167.4 \pm 7.9^c$ |
| + | 0 | $1.9 \pm 0.4^c$ | $174.1 \pm 6.2^c$ |
| - | 10 | $-2.4 \pm 0.2^d$ | $87.4 \pm 2.3^d$ |
| + | 10 | $-1.2 \pm 0.6^e$ | $125.6 \pm 6.0^e$ |

[a]Values represent mean ± S.E. for 6-7 male mice given HxCB one day prior to injection with P815 cells; RU 38486 was given twice daily from the day of HxCB treatment to the day of necropsy (day 10 post HxCB treatment).

[b]Body weight change was computed as the difference in weights on the day of HxCB treatment and the day of necropsy. The mean initial body weight (± S.D.) was 22.9 ± 1.6 g and there was no difference between groups.

[c,d,e]Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Table II-3. Effects of HxCB and three days of RU 38486 treatment in P815-injected mice[a]**

| RU 38486 (mg/Kg/d) | HxCB (mg/Kg) | Body Weight Change (g)[b] | Spleen Cells (x $10^6$) | AUCC[c] |
|---|---|---|---|---|
| *A. RU 38486 given on days 3-5 post antigen injection* | | | | |
| 0 | 0 | $2.6 \pm 0.2^e$ | $149.2 \pm 7.5^e$ | $49.2 \pm 2.9^e$ |
| 150 | 0 | $2.6 \pm 0.3^e$ | $142.7 \pm 5.6^e$ | $50.1 \pm 1.9^e$ |
| 0 | 10 | $-2.0 \pm 0.8^f$ | $77.7 \pm 4.0^f$ | $19.0 \pm 2.9^f$ |
| 150 | 10 | $-0.4 \pm 0.8^f$ | $98.2 \pm 6.3^f$ | $18.2 \pm 2.9^f$ |
| *B. RU 38486 given on days 5-7 post antigen injection* | | | | |
| 0 | 0 | $1.6 \pm 0.2^e$ | $132.7 \pm 4.3^e$ | $55.5 \pm 1.7^e$ |
| 150 | 0 | $1.7 \pm 0.4^e$ | $154.6 \pm 6.8^e$ | $51.9 \pm 1.5^e$ |
| 0 | 10 | $-1.7 \pm 0.6^f$ | $81.8 \pm 10.8^f$ | $20.1 \pm 2.8^f$ |
| 150 | 10 | ND[d] | ND | ND |
| *C. RU 38486 given on days 7-9 post antigen injection* | | | | |
| 0 | 0 | $2.3 \pm 0.2^e$ | $158.6 \pm 5.9^e$ | $47.0 \pm 1.2^e$ |
| 150 | 0 | $2.2 \pm 0.2^e$ | $159.4 \pm 9.5^e$ | $52.5 \pm 1.4^f$ |
| 0 | 10 | $-1.2 \pm 0.4^f$ | $86.1 \pm 6.3^f$ | $18.8 \pm 3.2^g$ |
| 150 | 10 | $-0.8 \pm 0.3^f$ | $102.2 \pm 4.9^f$ | $18.2 \pm 3.4^g$ |

[a]Values represent mean ± S.E. for 4-7 male mice given HxCB one day prior to injection with P815 cells; RU 38486 was given twice daily for 3 days as indicated. All animals were killed on day 11 post HxCB treatment.

[b]Body weight change was computed as the difference in weights on the day of HxCB treatment and the day of necropsy. The mean initial body weight (± S.D.) was 21.2 ± 1.4 g and there was no difference between days or groups.

[c]Area under the cytotoxicity curve (AUCC) is given for E:T ratios of 4:1, 11:1, 33:1, 100:1.

[d]ND indicates Not Determined because all animals died prior to necropsy. The mean time to death was 7 days post P815 injection.

[e,f,g]Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Figure II-2. The effects of RU 38486 administration for 10 days on plasma CS levels in HxCB-treated mice.**

Mice were treated as described in table II-2. Data for plasma CS in male mice are presented as geometric means (bars) with individual animal data indicated by closed or open circles if above or below the lower limit of detection (20 ng/mL, dotted line), respectively. For statistical purposes, samples with levels below the lower limit of detection were given the value 25 ng/mL. † Indicates a significant HxCB effect between placebo-treated mice at $p < 0.05$. * indicates a significant RU 38486 effect between HxCB-treated mice at $p < 0.05$.

**Figure II-3. The effects of RU 38486 administration for 10 days on CTL activity in HxCB-treated mice.**

Animals were treated as described in table II-2. Symbols indicate double-vehicle-treated animals (open circles), HxCB-treated/vehicle-treated animals (closed circles), vehicle-treated/RU 38486-treated animals (open triangles) and HxCB-treated/RU 38486-treated animals (closed triangles). The % CTX, at each E:T ratio, and AUCC for HxCB-treated mice were significantly less than in vehicle-treated mice ($p < 0.05$). * indicates a significant RU 38486 effect at the indicated E:T ratio between HxCB-treated mice at $p < 0.05$. Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Figure II-4. The effects of RU 38486 administration for three days on CTL activity in HxCB-treated mice.**

Animals were treated as described in table II-3. Symbols indicate vehicle-treated/placebo-treated animals (open circles), HxCB-treated/placebo-treated animals (closed circles), vehicle-treated/RU 38486-treated animals (open triangles) and HxCB-treated/RU 38486-treated animals (closed triangles). At each E:T ratio, the % CTX for HxCB-treated mice was significantly less than in vehicle-treated mice ($p < 0.05$). * indicates a significant RU 38486 effect at an individual E:T ratio between vehicle-treated mice at $p < 0.05$. RU 38486 did not significantly alter CTL activity in HxCB-treated mice.

**Figure II-2.**

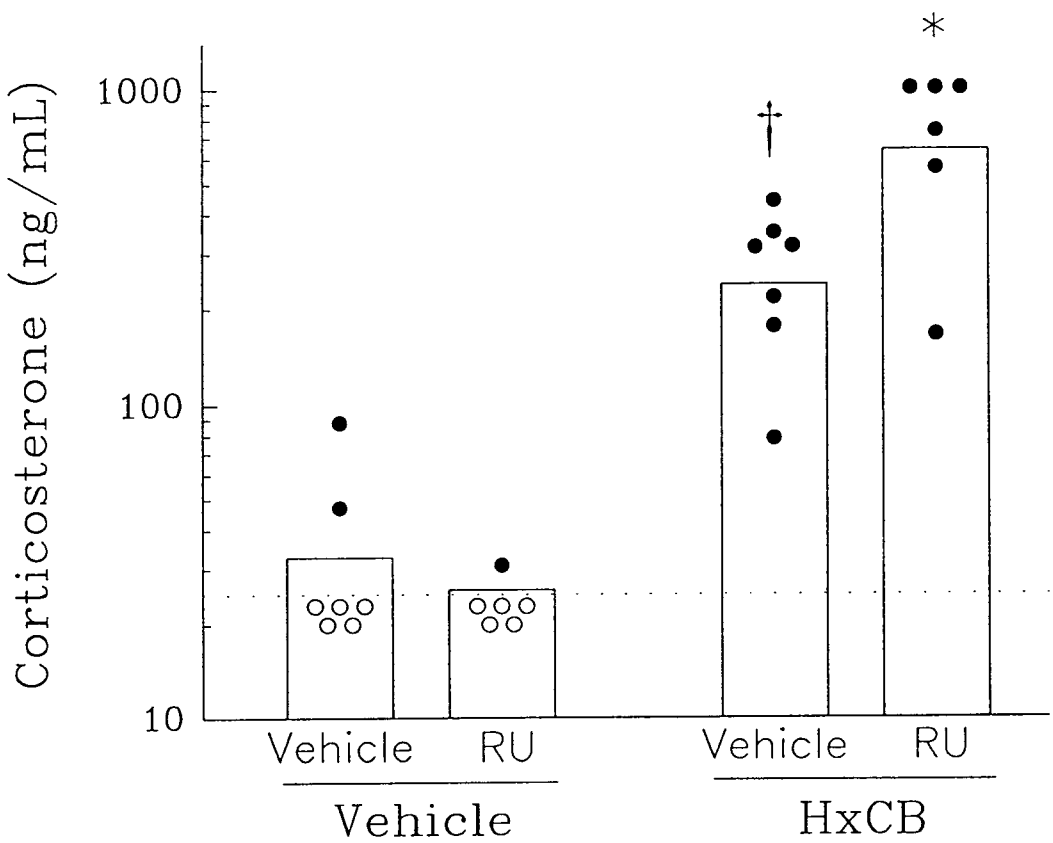

Corticosterone (ng/mL)
1000
100
10
*
†
Vehicle
RU
Vehicle
RU
Vehicle
HxCB

**Figure II-3.**

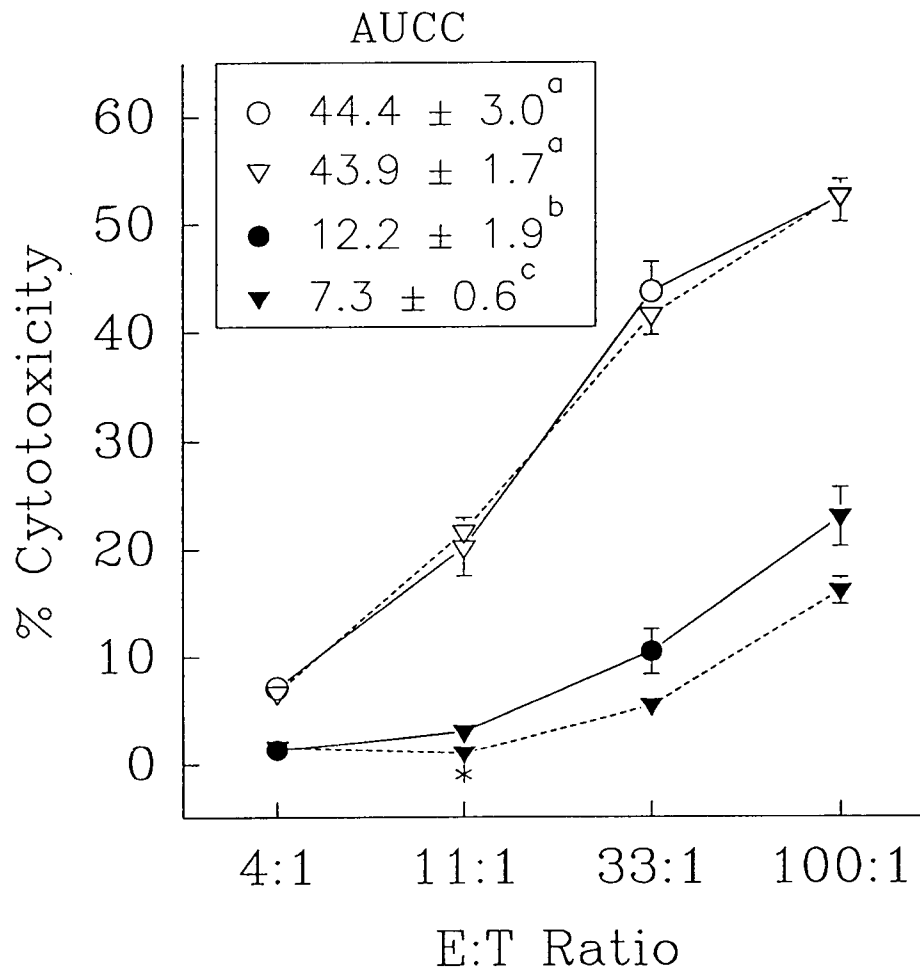
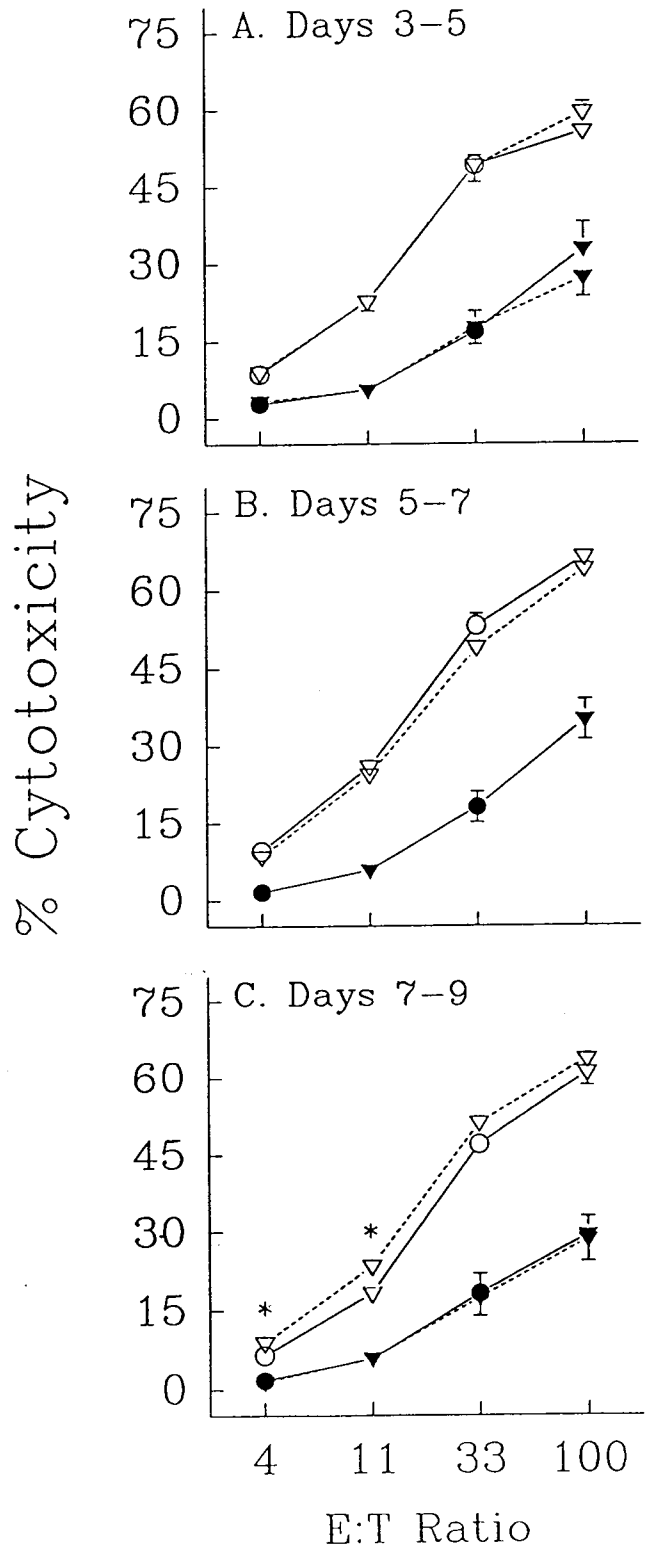

AUCC
44.4 ± 3.0 a
43.9 ± 1.7 a
12.2 ± 1.9 b
7.3 ± 0.6 c
% Cytotoxicity
60
50
40
30
20
10
0
4:1
11:1
33:1
100:1
E:T Ratio

**Figure II-4.**


A. Days 3-5
B. Days 5-7
C. Days 7-9
% Cytotoxicity
75
60
45
30
15
0
*
*
4
11
33
100
E:T Ratio

## EFFECTS OF HxCB IN MALE AND FEMALE MICE

Compared with male mice, female mice gained less body weight, and had lower spleen cellularity following P815 injection (table II-4). CTL activity in female mice was significantly lower than in male mice (figure II-5). In HxCB-treated mice, males were more sensitive to immune suppression than females: CTL activity (AUCC) was reduced 68% in males and 48% in females (figure II-5). HxCB treatment reduced spleen cellularity 50% in males and 17% in females (table II-3). HxCB treatment led to a loss of body weight in male mice, whereas the gain in body weight was reduced by HxCB treatment in female mice (table II-3). Plasma CS levels were significantly increased by HxCB treatment in both male and female mice, but the degree of elevation in male mice was greater than that observed in female mice (figure II-6).

## EFFECTS OF HxCB IN CASTRATED MALE MICE

As shown in table II-5, ODX of P815-injected mice significantly increased spleen cellularity (114%), but had no effect on CTL activity (figure II-7). In contrast, ODX significantly enhanced CTL activity by 2.4 fold, as measured by AUCC, in HxCB-treated mice (figure II-7). However, CTL suppression by HxCB was not fully eliminated by ODX. In addition, ODX reduced the loss of body weight in HxCB-treated mice (table II-5). HxCB treatment significantly lowered plasma TT levels by 80%, whereas ODX lowered plasma TT levels to nearly undetectable levels in both vehicle- and HxCB-treated mice (figure II-8A). ODX did not alter the HxCB-induced elevation of plasma CS (figure II-8B).

## REGRESSION ANALYSIS OF CTL ACTIVITY VERSUS CS LEVELS

Data were compiled from individual experiments and subjected to regression analysis to determine if CTL suppression was correlated with increased CS levels. Only data from HxCB-treated mice were analyzed since CS levels in many control samples were measured at or below the lower limit of detection. As shown in figure II-9, no significant correlation was found between CTL activity (AUCC) and CS level in HxCB-treated mice.

**Table II-4. Effects of HxCB treatment in male and female P815-injected mice[a]**

| SEX | HxCB (mg/Kg) | Body Weight Change (g)[b] | Spleen Cells (x $10^6$) |
|---|---|---|---|
| Male | 0 | $3.6 \pm 0.3^c$ | $122.2 \pm 5.1^c$ |
| Male | 10 | $-0.5 \pm 0.4^d$ | $62.1 \pm 1.9^d$ |
| Female | 0 | $2.1 \pm 0.2^e$ | $106.6 \pm 7.3^e$ |
| Female | 10 | $1.6 \pm 0.3^e$ | $88.1 \pm 2.7^f$ |

[a]Values represent mean ± S.E. for 5-8 animals. All animals were injected with P815 cells one day following HxCB treatment. All animals were killed 11 days following HxCB treatment.

[b]Body weight change was computed as the difference in weights on the day of HxCB treatment and the day of necropsy. The mean initial body weight (± S.D.) for male and female mice was 18.8 ± 1.4 g and 16.2 ± 0.7 g, respectively.

[c,d,e,f]Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Table II-5. Effects of ODX and HxCB treatment in P815-injected mice[a]**

| ODX | HxCB (mg/Kg) | Body Weight Change (g)[b] | Spleen Cells (x $10^6$) |
|---|---|---|---|
| - | 0 | $1.8 \pm 0.2^{cd}$ | $135.0 \pm 6.7^{c}$ |
| + | 0 | $2.9 \pm 0.2^{c}$ | $154.1 \pm 7.5^{d}$ |
| - | 10 | $-1.8 \pm 0.7^{d}$ | $73.6 \pm 4.2^{e}$ |
| + | 10 | $-0.7 \pm 0.3^{c}$ | $78.9 \pm 5.8^{e}$ |

[a]Values represent mean ± S.E. for 5-8 male mice castrated or sham operated 7 days prior to treatment with HxCB. All animals were injected with P815 cells one day following HxCB treatment. All animals were killed 11 days following HxCB treatment.

[b]Body weight change was computed as the difference in weights on the day of HxCB treatment and the day of necropsy. The mean initial body weight (± S.D.) for sham and ODX mice was 24.7 ± 1.2 g and 23.0 ± 1.5 g, respectively.

[c,d,e]Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Figure II-5. The effects of HxCB treatment on CTL activity in male and female mice.**

Animals were treated as described in table II-4. Symbols indicate vehicle-treated male animals (open circles), HxCB-treated male animals (closed circles), vehicle-treated female animals (open triangles) and HxCB-treated female animals (closed triangles). At each E:T ratio, the % CTX for HxCB-treated mice was significantly less than in vehicle-treated mice ($p < 0.05$). * indicates a significant sex effect at individual E:T ratios between vehicle-treated mice ($p < 0.05$). Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Figure II-6. Differential effects of HxCB on plasma CS levels in male and female mice.**

Animals were treated as described in table II-4. Bars indicate the geometric mean with individual animal data indicated by closed or open circles if above or below the lower limit of detection (25 ng/mL, dotted line), respectively. For statistical purposes, samples with levels below the lower limit of detection were given the value 25 ng/mL. † Indicates a significant sex effect between HxCB-treated mice at $p < 0.05$. * indicates a significant HxCB effect between male or female mice at $p < 0.05$.

**Figure II-7. The effects of ODX on CTL activity in HxCB-treated mice.**

Animals were treated as described in table II-5. Symbols indicate vehicle-treated/sham-operated animals (open circles), HxCB-treated/sham-operated animals (closed circles), vehicle-treated/ODX animals (open triangles) and HxCB-treated/ODX animals (closed triangles). At each E:T ratio, the % CTX for HxCB-treated mice was significantly less than in vehicle-treated mice ($p < 0.05$). * indicates a significant ODX effect at individual E:T ratios between HxCB-treated animals ($p < 0.06$). Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests.

**Figure II-8. The effects of ODX on plasma CS and TT levels in HxCB-treated mice.**

Animals were treated as described in table II-5. Bars indicate the geometric mean with individual animal data indicated by closed or open circles if above or below the lower limit of detection (dotted lines), respectively. For statistical purposes, samples with levels below the lower limit of detection were given the value 0.05 ng/mL (TT) or 25 ng/mL (CS). † indicates a significant HxCB effect between sham-operated mice at $p < 0.05$. * indicates a significant ODX effect between vehicle- or HxCB-treated mice at $p < 0.05$.

**Figure II-9. The lack of correlation between plasma CS levels and CTL suppression.**

CTL activity (AUCC) is presented versus plasma CS data compiled from 5 separate experiments. Animals were injected with P815 cells one day following treatment with HxCB at 0 or 10 mg/kg body weight. All animals were killed 11 days following HxCB treatment. The equation of the regression line for data from HxCB-treated animals is $Y = -0.0107 \times X + 16.7865$ ($R^2 = 0.0791$). The slope is not significantly different from 0.

**Figure II-5.**

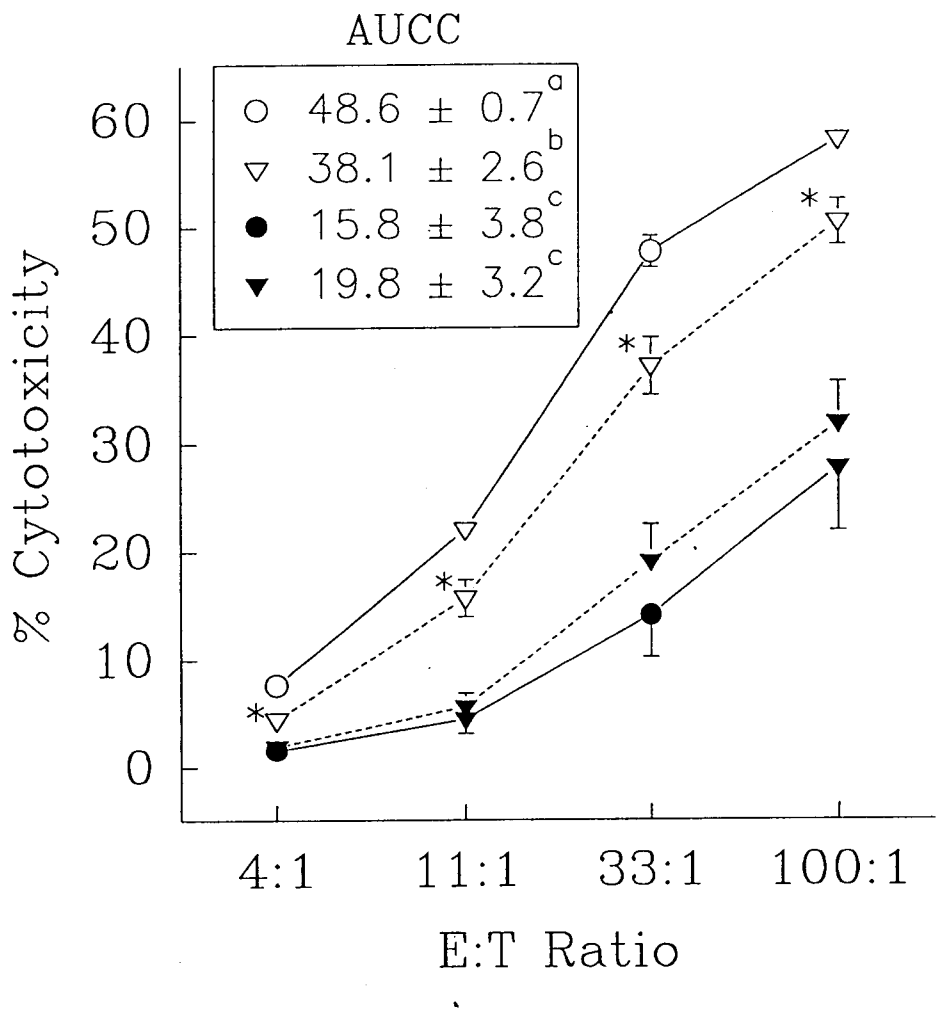

AUCC
○ 48.6 ± 0.7 a
▽ 38.1 ± 2.6 b
● 15.8 ± 3.8 c
▼ 19.8 ± 3.2 c
% Cytotoxicity
60
50
40
30
20
10
0
4:1
11:1
33:1
100:1
E:T Ratio

**Figure II-6.**

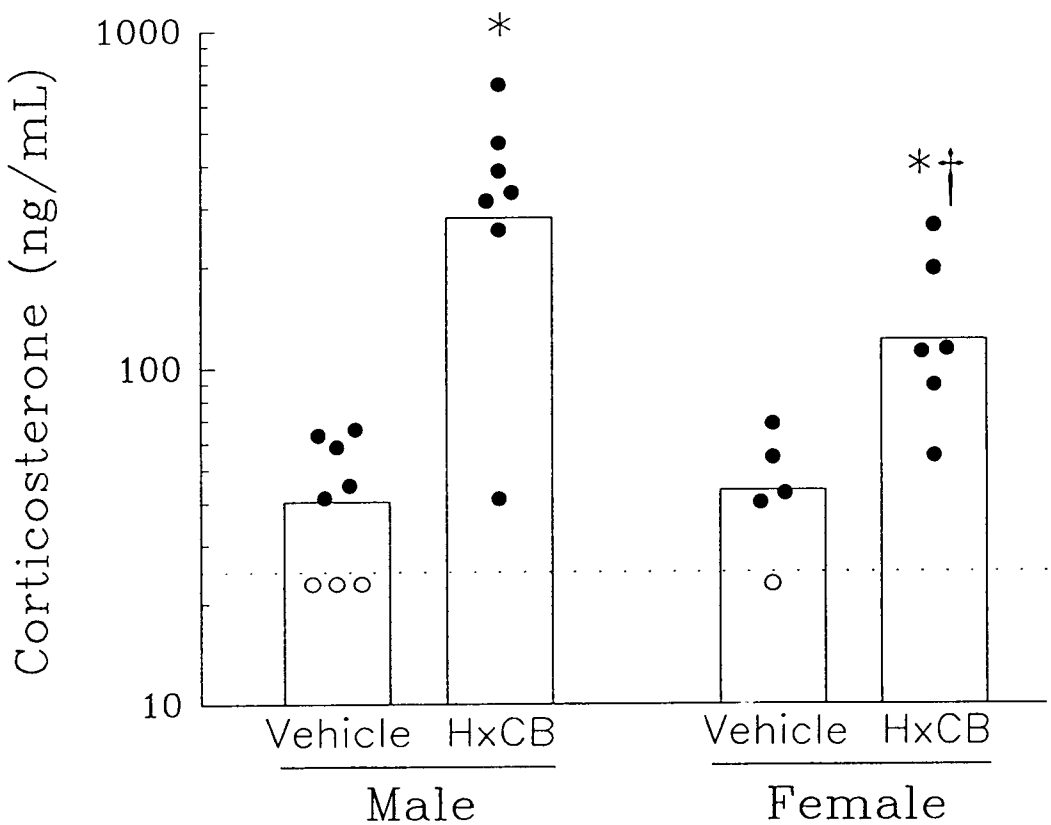

1000
100
10
Corticosterone (ng/mL)
*
*†
Vehicle
HxCB
Vehicle
HxCB
Male
Female

**Figure II-7.**

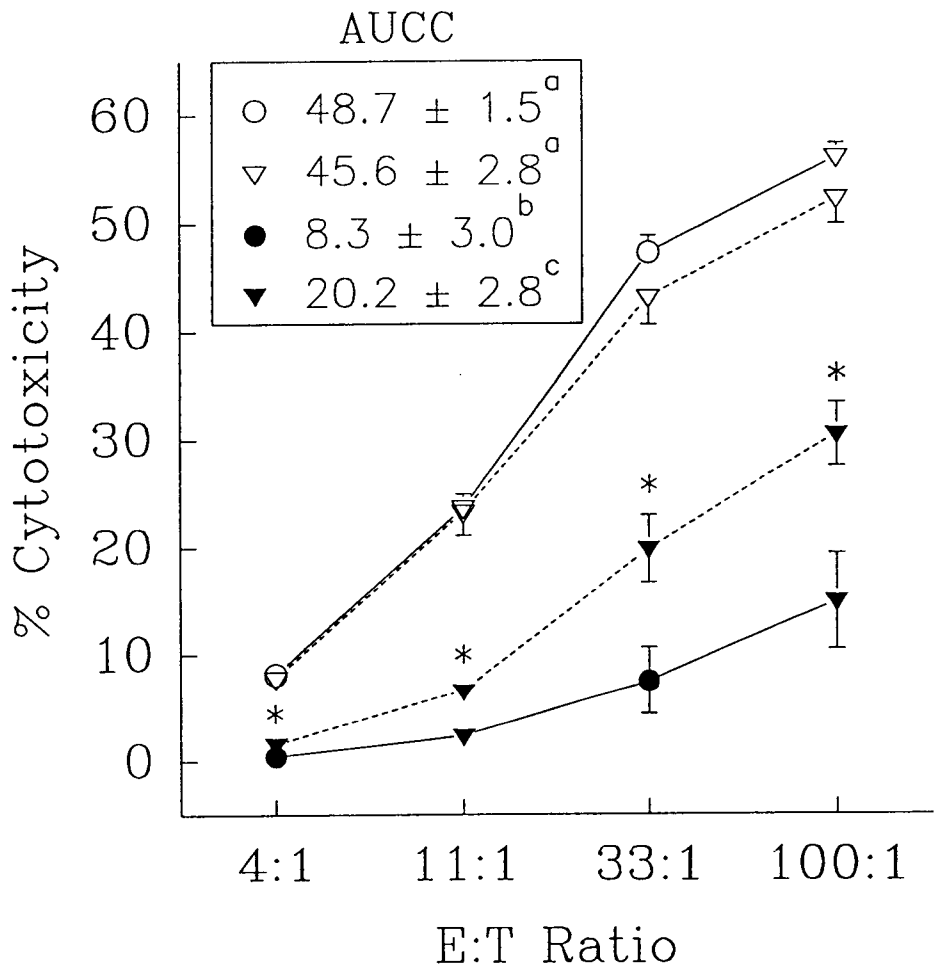

AUCC
48.7 ± 1.5 a
45.6 ± 2.8 a
8.3 ± 3.0 b
20.2 ± 2.8 c
% Cytotoxicity
60
50
40
30
20
10
0
4:1
11:1
33:1
100:1
E:T Ratio

**Figure II-8.**

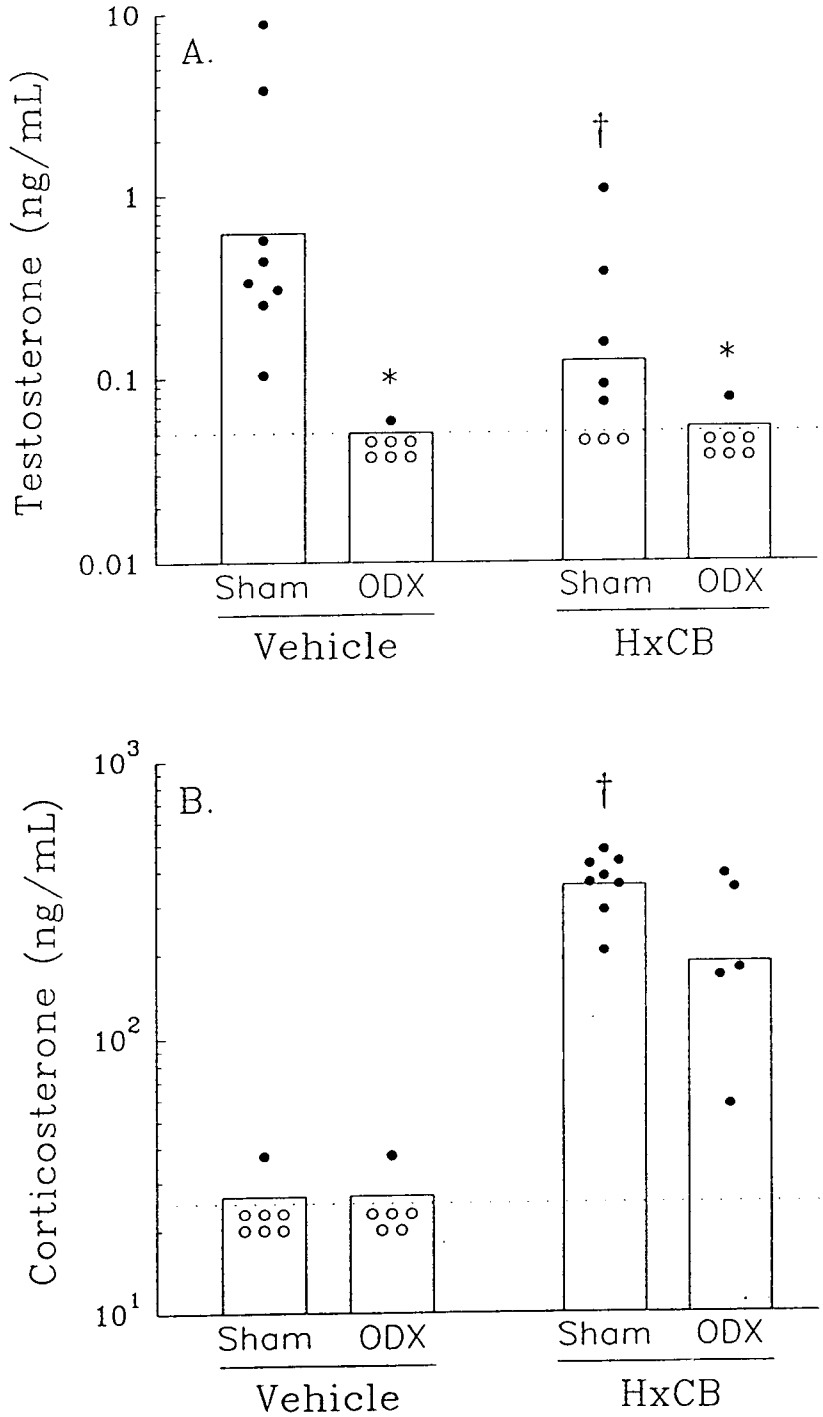
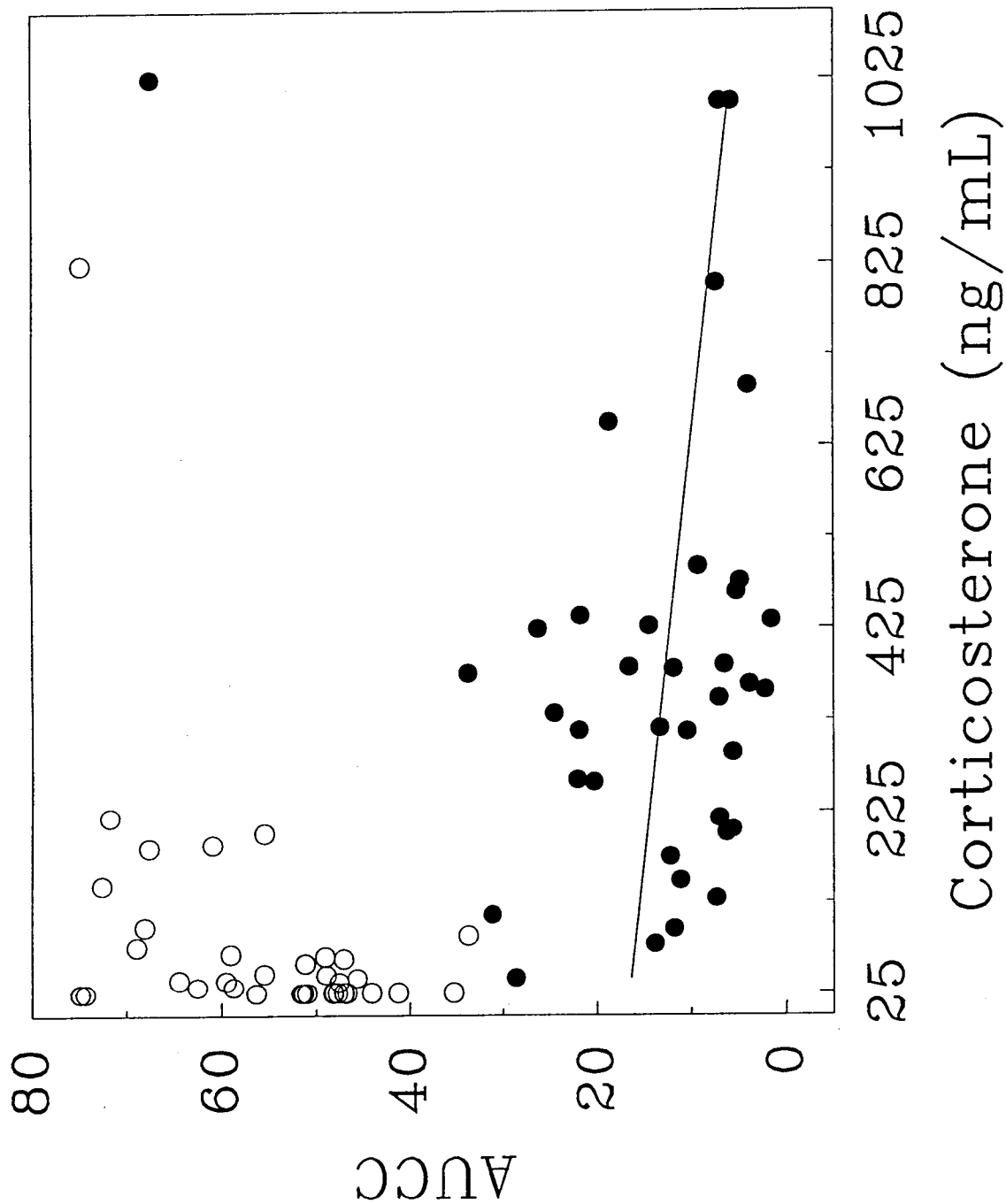

A.
Testosterone (ng/mL)
10
1
0.1
0.01
*
†
*
Sham
ODX
Sham
ODX
Vehicle
HxCB
B.
Corticosterone (ng/mL)
$10^3$
$10^2$
$10^1$
†
Sham
ODX
Sham
ODX
Vehicle
HxCB

**Figure II-9.**



## DISCUSSION

Reduced CTL activity and elevation of plasma CS occurred coincidentally in HxCB-treated mice over the time-course of CTL development following injection with P815 cells. Kerkvliet and Baecher-Steppan (1988a) showed that the kinetics of CTL generation was not altered by HxCB treatment in P815-injected mice, with the peak day of splenic CTL activity occurring on day 10 post P815 injection. They also showed that the growth of peritoneal P815 cells was not altered by HxCB treatment, suggesting that CTL suppression is not due to a reduction of the antigen challenge in HxCB-treated mice. Recently, we have also shown that the expression of class I antigens ($H\text{-}2D^d$) on peritoneal P815 cells, taken from C57Bl/6 mice, was not altered by HxCB treatment (unpublished data). Thus, the coincident nature of CS elevation and immune suppression suggested a possible cause-effect relationship between them. In the studies reported here, attempts were made to reduce plasma CS levels in HxCB-treated mice to determine the impact on the CTL response.

The use of ADX mice was inadequate to determine if CS elevation led to CTL suppression following HxCB treatment. Studies using ADX animals were complicated by a high rate of mortality ($\geq$70%) among HxCB-treated animals, potentially caused by hypoglycemia. Although glucose levels of moribund HxCB-treated ADX animals were not routinely measured, the few animals that were sampled had extremely low plasma levels ($\leq$40 mg/dL). In addition, hypoglycemia has been reported in TCDD-treated rats (Potter et al., 1983; Gorski and Rozman, 1987). The likely mechanism responsible for hypoglycemia is reduced gluconeogenesis. GC stimulate gluconeogenesis and reduce glucose utilization in the periphery, two compensatory mechanisms that would be reduced in ADX animals. Further, HAH have been shown to reduce the activities of gluconeogenesis enzymes in rats (Messner et al., 1976; Hsia and Kreamer, 1985; Weber et al., 1991). Gorski et al. (1988b) showed that ADX increased the mortality rate and reduced the mean time to death in TCDD-treated rats. The dose of HxCB used here (10 mg/Kg), when given alone, was nonlethal for P815-naive male C57Bl/6 mice; the survival time exceeded 84 days for unaltered mice and 21 days for ADX mice (data not shown). Therefore, it seems likely that the energy demands of rapidly proliferating tumor cells placed a lethal burden on the energy production capacity of HxCB-treated ADX mice. Interestingly, HxCB-treated ADX survivors had unexpectedly elevated plasma CS levels. This was potentially due to accessory-adrenal production of CS (Dunn, 1970; Hummel, 1958) since no adrenal glands were found in these animals at necropsy. Survival appeared to depend on the bioavailability of CS since supplementa-

tion with exogenous CS (2 mg/Kg/d) reduced the mortality rate of HxCB-treated ADX mice (data not shown). The survival requirement for CS is supported by the observation that HxCB-treated mice had a 30% mortality rate if given RU 38486 on days 3-5 post P815 injection, and a 100% mortality rate if given RU 38486 on days 5-7. In the latter group, the mean time to death was 7 days post P815 injection which corresponds with the day of maximal tumor burden (Kerkvliet and Baecher-Steppan, 1988a). The lower mortality rate in HxCB-treated animals that received RU 38486 on days 3-5 may be due to a lower tumor burden. Rapid clearance of tumor from the peritoneal cavity occurs following day seven post P815 injection (Kerkvliet and Baecher-Steppan, 1988a), an event that may account for the lack of death observed in animals given RU 38486 on days 7-9.

Because of the inability to eliminate CS elevation in HxCB-treated mice via ADX, attempts were made to antagonize the physiologic effects of CS elevation by administering RU 38486, a competitive receptor antagonist of GCs and progesteroids (Philibert, 1984). Preliminary experiments showed that thymic involution induced by 50 mg/Kg of corticosterone-21-acetate ip. was blocked by 24 hour pretreatment with RU 38486 at 150 mg/kg, po. (data not shown). Therefore, this dose of RU 38486 was used in subsequent studies with HxCB. It was hypothesized that HxCB-induced CTL suppression was due to elevated CS and would be attenuated by daily treatment with RU 38486 following HxCB exposure. To the contrary, when CTL activity was analyzed as AUCC, a small but significant enhancement of the HxCB-induced CTL suppression was observed. The mechanism for this increased suppression by RU 38486 is unknown. It is unlikely that the observed hyperelevation of plasma CS would contribute to CTL suppression if RU 38486 were functioning as an effective GC antagonist. The efficacy of RU 38486 in HxCB-treated mice was suggested by its effects on body weight gain and spleen cellularity, effects that were not evident in RU 38486-treated control mice having normal CS levels. However, it is possible that the efficacy of RU 38486 is diminished after prolonged treatment in mice (Dr. S. Pruett, personal communication). Therefore, an additional study was conducted in which RU 38486 treatments were limited to durations of three days during the period in which CS elevation is known to occur in HxCB-treated/P815-injected mice. Administration of RU 38486 did not alter the HxCB-induced suppression of CTL activity when given on days 3-5 or days 7-9 post P815 injection. These results argue against the hypothesis that CS elevation is responsible for CTL suppression in HxCB-treated mice. The 100% lethality of RU 38486, when given to HxCB-treated mice on days 5-7 post P815 injection, further suggests its

efficacy as a GC antagonist in this model. When compared to the lack of lethality in HxCB-treated mice given RU 38486 for 10 days, these data suggest that the efficacy of RU 38486 as a GC antagonist is reduced with prolonged treatment.

Previous studies in our laboratory have shown that male mice are more sensitive to HxCB-induced suppression of the CTL response when compared to female mice (Kerkvliet and Baecher-Steppan, 1988a). In addition, as shown here, HxCB treatment led to a greater elevation of plasma CS in male mice compared to female mice. It was hypothesized that the higher CS levels of male mice might explain the greater CTL suppression observed. Further, studies by Mebus et al. (1987) have shown that treatment with TCDD can affect the metabolism of steroids in the testes of rats. Abnormal CS production in the testes of HxCB-treated mice might result in the significantly higher CS levels observed in male mice. Therefore, ODX male mice were tested for their sensitivity to HxCB. ODX did not eliminate the HxCB-induced elevation of plasma CS; therefore, the testes are not of significant concern as a site of CS production in this model. However, unlike ADX or RU 38486 treatment, ODX significantly enhanced CTL activity in HxCB-treated mice. This may be attributable to the elimination of testes-specific factors. TT has been shown to suppress immune function (Hirasawa and Enosawa, 1990), and ODX has been shown to increase both lymphoid organ weights and antigen-specific immune responses (Castro, 1974a and 1974b). However, because ODX in vehicle-treated mice did not enhance the CTL response, the data suggest that HxCB may alter the sensitivity of the immune system to testes-derived factor(s), and/or that HxCB treatment alters the production of testes-specific immunomodulatory factor(s). In the latter case, TT alone is not a likely candidate because circulating levels were decreased in HxCB-treated mice. HxCB effects on the testes could explain the higher sensitivity of male mice to HxCB-induced immune suppression. However, the basis of the remaining HxCB-induced CTL suppression in female and ODX male mice remains unknown.

Following treatment with various HAH, both rats and mice have been shown to have elevated circulating GC levels (Sanders et al., 1974 and 1977; Gorski et al., 1988a). DiBartolomeis et al. (1987) and Jones et al. (1987) have suggested that morning CS elevation in TCDD-treated rats is caused by a dysregulation of the normal diurnal CS rhythm. In contrast, Dunn et al. (1983) showed that Aroclor 1254 did not alter the diurnal rhythm of CS in mice. Results in our laboratory have indicated that, while the normal evening CS elevation was significantly enhanced by HxCB treatment, the diurnal CS cycle of mice was unaffected 6 days following HxCB treatment. This was observed

for both P815-injected and -naive mice (unpublished results). Therefore, morning CS elevation in HxCB-treated, P815-injected mice is most likely due either to augmented production or reduced clearance of CS. Evidence to support the latter cause has been observed in this laboratory: CS supplementation (2 mg/Kg/day) of P815-injected ADX mice led to higher plasma CS levels following HxCB treatment versus vehicle treatment.

Recently, it has been reported that treatment of rats with HAH leads to a reduction in the binding capacity of GC receptor in both liver (Lin et al., 1991; Ryan et al., 1989; Sunahara et al., 1989) and skeletal muscle (Max and Silbergeld, 1987). A desensitization of the hypothalamus to the negative feedback of CS, via a reduced GC receptor binding capacity, could conceptually explain higher plasma CS levels following HxCB exposure. Such an effect might be enhanced by receptor antagonists leading to even higher CS levels, as was shown here following RU 38486 treatment. Further, if a reduction in the binding capacity of GC receptor is a generalized effect of HxCB treatment in all tissues, the degree of CS elevation observed here for HxCB-treated mice may have less of an impact on immune function, or other physiological processes, than an elevation of equal magnitude in untreated mice.

# CHAPTER III

## SUPPRESSION OF CYTOTOXIC T LYMPHOCYTE ACTIVITY BY 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN OCCURS IN VIVO, BUT NOT IN VITRO, AND IS INDEPENDENT OF CORTICOSTERONE ELEVATION

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## ABSTRACT

Previous studies have shown that 2,3,7,8-tetrachlorodibenz*o*-p-dioxin (TCDD) is a potent immunosuppressive compound. In our laboratory, TCDD and structurally related polychlorinated biphenyls (PCBs) have been shown to suppress alloantigen-specific cytotoxic T lymphocyte (CTL) activity in C57Bl/6 mice. PCB-induced CTL suppression occurs coincident with significant elevation of plasma glucocorticoid (GC) levels (>500 ng/mL). Since GC elevation can cause immune suppression, this study was conducted to determine if TCDD-induced CTL suppression is correlated with elevation of plasma corticosterone (CS), the major GC in mice. Single oral doses of TCDD (2.5-40 μg/Kg) induced a dose-dependent suppression of CTL activity with a calculated 50% immunosuppressive dose ($ID_{50}$) occurring at 7.2 μg/Kg. When total lytic units (LU)/spleen were calculated, the $ID_{50}$ was 2.8 μg/Kg. In contrast, plasma CS levels were not significantly altered at doses below 40 μg/Kg. These data suggest that TCDD-induced CTL suppression is not dependent on CS elevation. The direct effect of TCDD on CTL generation was tested by adding TCDD at $10^{-13}$-$10^{-9}$M to in vitro mixed lymphocyte-tumor cell (MLTC) cultures. No alteration of CTL activity was observed after five days of culture at any TCDD concentration. In contrast, CS alone significantly suppressed CTL activity in vitro. CS-induced CTL suppression in vitro was neither enhanced nor inhibited by the presence of TCDD. These results suggest that TCDD causes CTL suppression in vivo by a mechanism that does not involve CS.


## INTRODUCTION

2,3,7,8-Tetrachlorodibenz*o*-p-dioxin (TCDD) is a highly toxic and unintended by-product of numerous chemical processes (Safe, 1990). In experimental animals, the immune system is among the most sensitive targets of TCDD toxicity (Holsapple et al., 1991a; Vos and Luster, 1989; Kerkvliet and Burleson, 1994). In mice, TCDD-induced suppression of both humoral and cellular immune responses has been shown to be dependent on binding to the aromatic hydrocarbon (Ah) receptor (Kerkvliet et al., 1990a,b; Clark et al., 1983; Harper et al., 1993). The structure and toxicity of TCDD is similar to that of other halogenated aromatic hydrocarbons (HAH) (e.g., halogenated biphenyls, dibenzofurans and dibenzodioxins), many of which can also bind to the Ah receptor (Safe, 1990). However, of all the HAH, TCDD has the highest affinity for the Ah receptor and the highest toxic potency (Safe, 1990).

In this laboratory, 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB), a model Ah receptor binding and immunosuppressive polychlorinated biphenyl, has been used to

study the mechanism of HAH induced suppression of alloantigen-specific cytotoxic T lymphocyte (CTL) activity in C57Bl/6 mice (DeKrey et al., 1993a, 1994a,b; Kerkvliet et al., 1990b; Kerkvliet and Baecher-Steppan, 1988a,b). HxCB exposure leads to a dose dependent suppression of CTL activity coincident with a marked elevation of plasma corticosterone (CS) levels (Kerkvliet et al., 1990a; DeKrey et al., 1993a). CS is the major glucocorticoid (GC) in mice. Since GC have been shown to cause suppression of immune function in numerous models (Boumpas et al., 1991; Cupps and Fauci, 1982; Roudebush and Bryant, 1993; Billingham et al., 1951), CS elevation was suspected as an indirect mechanism of CTL suppression by HxCB. An indirect mechanism was supported by the observation that HxCB does not induce suppression of T cell responses in vitro (Kerkvliet and Baecher-Steppan, 1988b). However, subsequent studies showed that neither adrenalectomy nor treatment with RU 38486 (a GC receptor antagonist) altered the degree of CTL suppression in HxCB-exposed mice. These results suggested that CS elevation does not play a significant role in HxCB-mediated CTL suppression (DeKrey et al., 1993a).

The involvement of CS elevation in TCDD-mediated CTL suppression has been suggested by a previous study in this laboratory (Kerkvliet et al., 1990a). In the present study, the potential role of CS in TCDD-mediated CTL suppression was further examined. Mice were exposed to TCDD orally with single doses of 2.5-40 μg/Kg, and the dose responses for CTL suppression and CS elevation were compared. In addition, the direct effects of TCDD on CTL generation were examined in vitro in mixed lymphocyte-tumor cell (MLTC) cultures. Potential interactive effects of TCDD and CS on CTL activity in vitro were also examined.

## METHODS

### Animals

Male C57Bl/6 mice, 6 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed individually in polycarbonate shoe-box cages in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experimentation. Cages were randomly assigned to positions in a cage rack, and animals were provided with Bed-O-Cob bedding (The Andersons, Maumee, OH). Food (Wayne Rodent Blox, Harlan Sprague Dawley Co., Bartonville, IL) and tap water were available *ad libitum*. Animal rooms were maintained with a 12 hr light/dark cycle (fluorescent,

7:30 AM lights on) and constant temperature (72 ± 1 [°F]) and 50% humidity. The mice remained free of all common murine pathogens as assessed by serum titers (Charles River Professional Services, Wilmington, MA).

## Chemicals

Environmental standard grade (99% purity) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Ultrascientific, Hope, RI. For in vivo use, TCDD was dissolved in acetone and mixed with peanut oil (Nabisco Brands Inc., East Hanover, NJ). The acetone was evaporated under a stream of nitrogen. Serial dilutions of TCDD were made in peanut oil. Animals were given peanut oil or TCDD (0.1 mL per 10 g body weight) by gavage. For in vitro use, TCDD was dissolved in DMSO and diluted in medium. Corticosterone (CS) (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and diluted in medium. Control medium contained ethanol or DMSO.

## Animal Treatment

The P815 mastocytoma cell line was propagated in ascites form by weekly passage in DBA mice (H-$2^d$), the strain of origin. P815 cells were harvested from DBA mice after 6-8 days of in vivo growth. C57Bl/6 mice (H-$2^b$) were injected ip. with 1.0 x $10^7$ viable P815 cells in 0.5 mL of Hanks Balanced Salt Solution (HBSS).

## In Vivo CTL Induction

C57Bl/6 mice were injected with P815 cells one day after TCDD treatment. All animals were killed 10 days later between the hours of 8:30 and 11:00 AM. Plasma was collected and stored at -20°C until analyzed for CS content. Spleens were removed and single-cell suspensions were prepared by teasing with forceps in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 5% FBS (Rehatuin, Intergen, Purchase, NY), 20 mM HEPES buffer and 5 x $10^{-5}$M 2-mercaptoethanol. Using this method, spleen cell viability was generally >90% (Kerkvliet and Baecher-Steppan, 1988a). Erythrocytes were lysed by incubating spleen cells in water for 10 seconds. CTL activity was determined using a 4-hour $^{51}$Cr release assay as described previously (DeKrey et al., 1993a; Kerkvliet and Baecher-Steppan, 1988a) and is presented as percent cytotoxicity. LU/spleen were calculated using a method adapted from Bryant et al. (1992) by the equation:

$$\text{LU/Spleen} = \frac{\text{Total Spleen Cells}}{10^6 \cdot e^{\{(\bar{Y}^* - C \cdot \overline{\ln(x)} - \ln(P/(A-P)))/C\}/T}},$$

where $Y^*$ = logit transformed percent lysis, $C$ = mean slope of the logit transformed percent lysis curve for each group, $x$ = effector:target (E:T) ratio, $A$ = maximum limiting percent lysis (75%), $P$ = reference lysis (10%), and $T$ = number of target cells.

## In Vitro CTL Generation

CTL activity was generated in vitro in 5-day MLTC cultures. To prevent cell division, P815 cells ($P815_m$) were incubated with mitomycin C (Sigma) at 50 µg/mL for 30 minutes in MEM (Gibco, Grand Island, New York) supplemented with 10% FBS, 20 mM HEPES buffer, 50 µg/mL gentamicin, 5 x $10^{-5}$M 2-mercaptoethanol and 2mM glutamine. $P815_m$ were washed 4X prior to use. Single cell suspensions of spleen cells from antigen-naive C57 mice were prepared as described above without lysis of erythrocytes. Spleen cells were cultured at a 100:1 ratio with $P815_m$ in wells of a round bottom 96 well tissue culture plate (Corning, Corning, NY). Each well contained 6 x $10^5$ spleen cells and 6 x $10^3$ $P815_m$ in a final volume of 200 µL with ethanol and DMSO at concentrations of 0.01% and 0.02%, respectively. Some cultures contained spleen cells only and served as naive controls. Cultures were incubated in 5% $CO_2$ at 37°C for five days.

To determine CTL activity, 1 x $10^4$ $^{51}$Cr-labelled P815 cells were added to each well in 50 µL medium and cultures were again incubated. After four hours, 125µL of culture medium was harvested from each well and counted for gamma emission. The percent cytotoxicity (CTX) of each culture was calculated by the equation:

$$\%Cytotoxicity = \frac{sample - naive}{mr - naive} \times 100,$$

where *sample* = cpm using $P815_m$-stimulated spleen cells, *naive* = cpm using P815-naive spleen cells and *mr* = the maximum cpm released from cultures incubated with sodium dodecyl sulfate.

## CS RIA

Plasma CS levels were determined using [$^{125}$I]-coupled double antibody radioimmunoassay (RIA) kits (ICN Biochemicals, Carson, CA). A protocol optimized by the manufacturer was used. The lower limit of detection was 25 ng CS/mL. The maximum

inter- and intra-assay coefficients of variance for internal controls were 15.7% and 8.8%, respectively, as determined by the manufacturer. Samples with analyte levels below the lower limit of detection were assigned the limit value.

### Statistics

Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Significant treatment effects were determined by one-way and two-way analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of SAS. Comparisons between two means were performed using *t* tests (TTEST of SAS). Comparison of more than two treatment means to a control mean was performed using Dunnett's multiple comparison *t* test (GLM of SAS). All-ways comparisons of more than two means were performed using LSD multiple comparison *t* tests (GLM of SAS). Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### Effects of TCDD on Splenic CTL Activity and Plasma CS Levels

Alloantigen-specific CTL activity was generated in vivo and measured in spleen cells on day 10. As shown in figure III-1, TCDD exposure caused a dose-dependent suppression of splenic CTL activity. Regression analysis of the dose response curve (2.5-20 μg/Kg) using data for an effector:target ratio of 100:1 indicated a 50% immunosuppressive dose ($ID_{50}$) of 7.2 μg/Kg. A significant and dose-dependent decrease in spleen cells was also observed (figure III-2A). Thus, when the total splenic CTL potential was calculated as lytic units/spleen, slightly suppression was observed at a dose of 2.5 μg/Kg (not significant) and significant suppression was observed at doses of 5 μg/Kg and higher (figure III-2B). Regression analysis of the LU/spleen dose response curve (2.5-40 μg/Kg) indicated an $ID_{50}$ of 2.8 μg/Kg. In contrast to CTL suppression, a significant elevation of plasma CS levels was observed only at the 40 μg/Kg dose, the highest dose of TCDD tested (figure III-3). Plasma levels of CS obtained from mice treated with TCDD at 40 μg/Kg were approximately 230 ng/mL.

**Figure III-1. TCDD exposure causes a dose-dependent suppression of splenic CTL activity.**

The mean percent cytotoxicity is shown for six animals per group. Animals were given TCDD one day prior to ip. injection of P815 cells. All animals were killed 10 days later. CTL activity was determined as described in Methods. Regression analysis was performed on data from TCDD-treated animals (2.5-20 μg/Kg) at an effector:target ratio of 100:1 for calculation of an $ID_{50}$ dose: $y = (-43.34) \log(x) + (64.18)$; $R = 0.99$; $ID_{50} = 7.2$ μg/Kg.

**Figure III-2. TCDD exposure causes a dose-dependent suppression of LU/spleen and total spleen cells.**

Total spleen cells (A) and LU/spleen (B) are shown as mean ± SEM for six animals per group. Animals were given TCDD one day prior to ip. injection of P815 cells. All animals were killed 10 days later. LU were calculated as described in Methods. * Indicates the mean is significantly different from vehicle-treated control ($p < 0.05$ by ANOVA and Dunnett's t test). Regression analysis was performed on data from TCDD-treated animals (indicated by open circles) for calculation of an $ID_{50}$ dose: $\log(y) = (-1.50) \log(x) + (3.20)$; $R = 0.99$; $ID_{50} = 2.8$ μg/Kg.

**Figure III-3. TCDD exposure causes significant elevation of CS levels but only at a dose of 40 μg/Kg.**

Plasma CS levels are shown as mean ± SEM for six animals per group. Animals were given TCDD one day prior to ip. injection with P815 cells. All animals were killed 10 days later. Plasma CS levels were determined as described in Methods. * Indicates the mean CS level is significantly different from vehicle-treated control ($p < 0.05$ by ANOVA and Dunnett's t test).

**Figure III-1.**

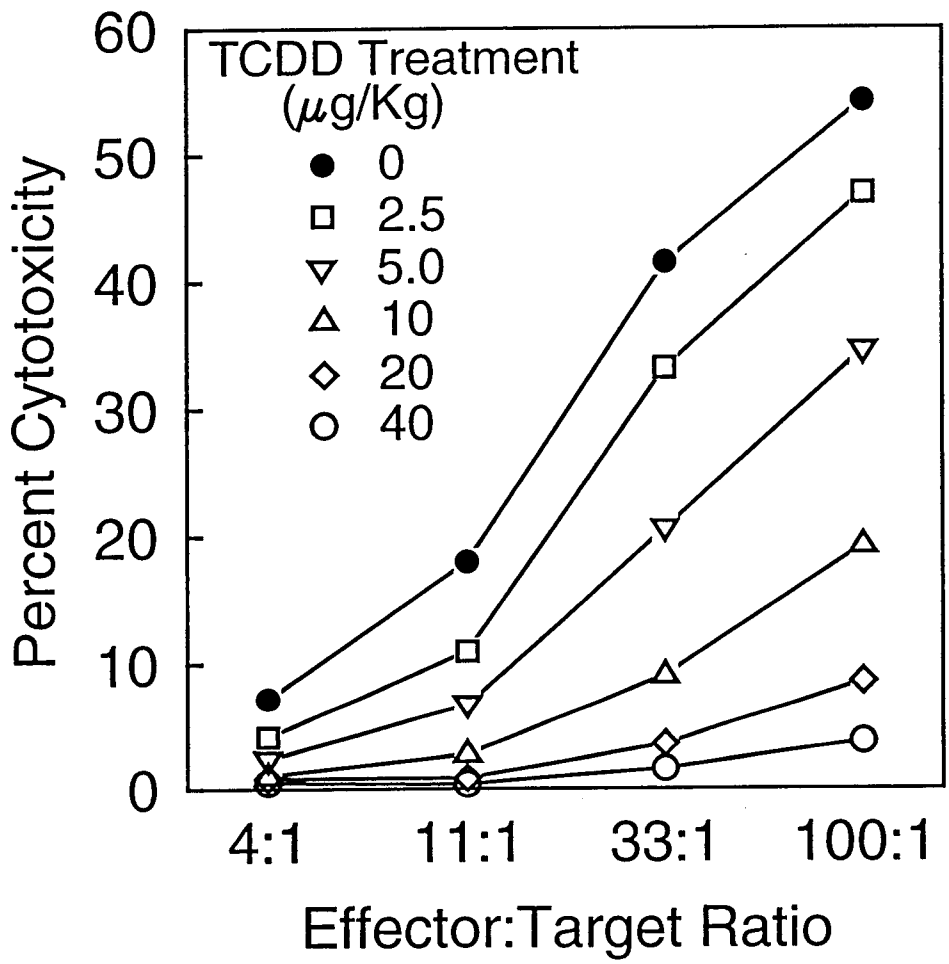

TCDD Treatment
(μg/Kg)
0
2.5
5.0
10
20
40
Percent Cytotoxicity
60
50
40
30
20
10
0
4:1
11:1
33:1
100:1
Effector:Target Ratio

**Figure III-2.**

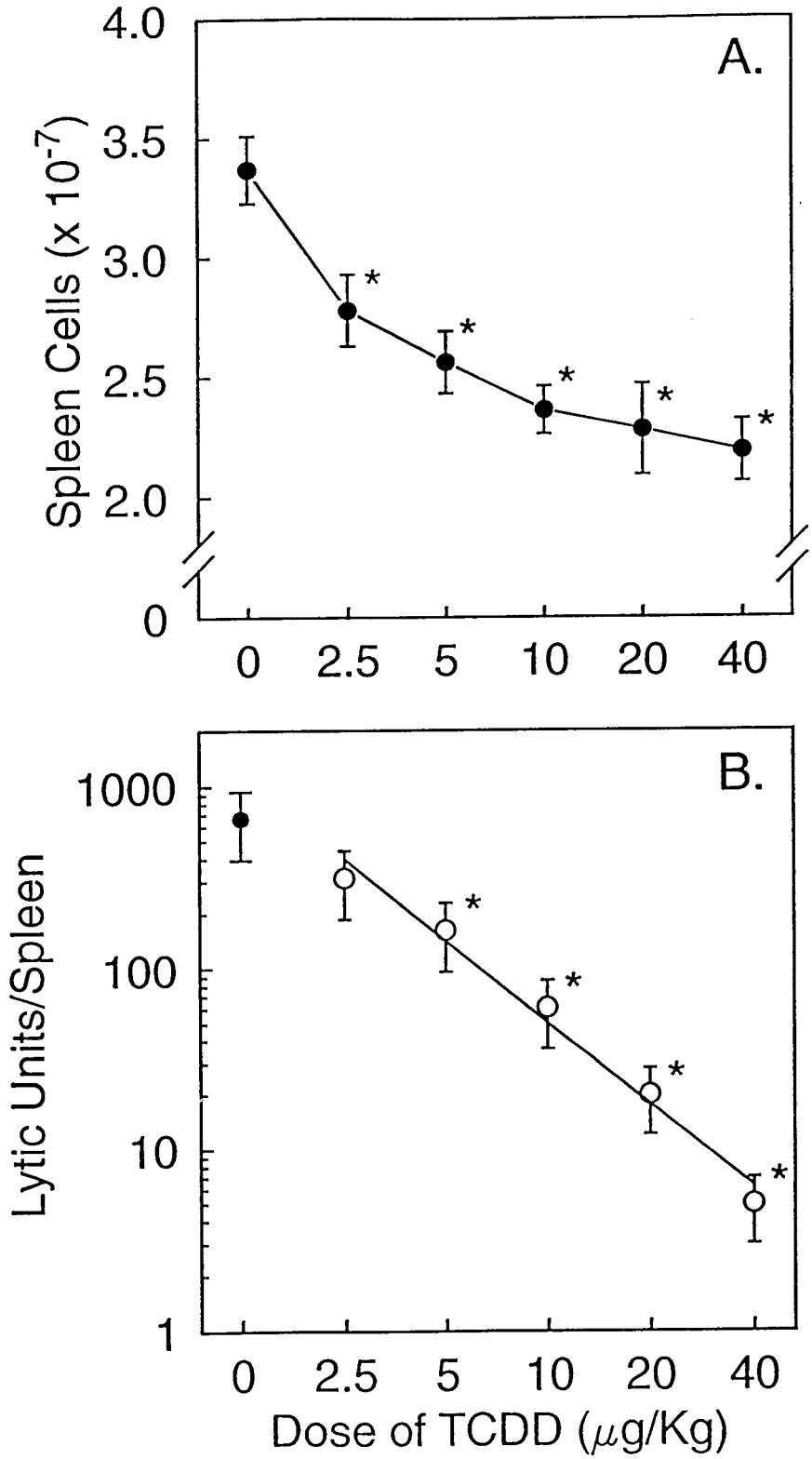
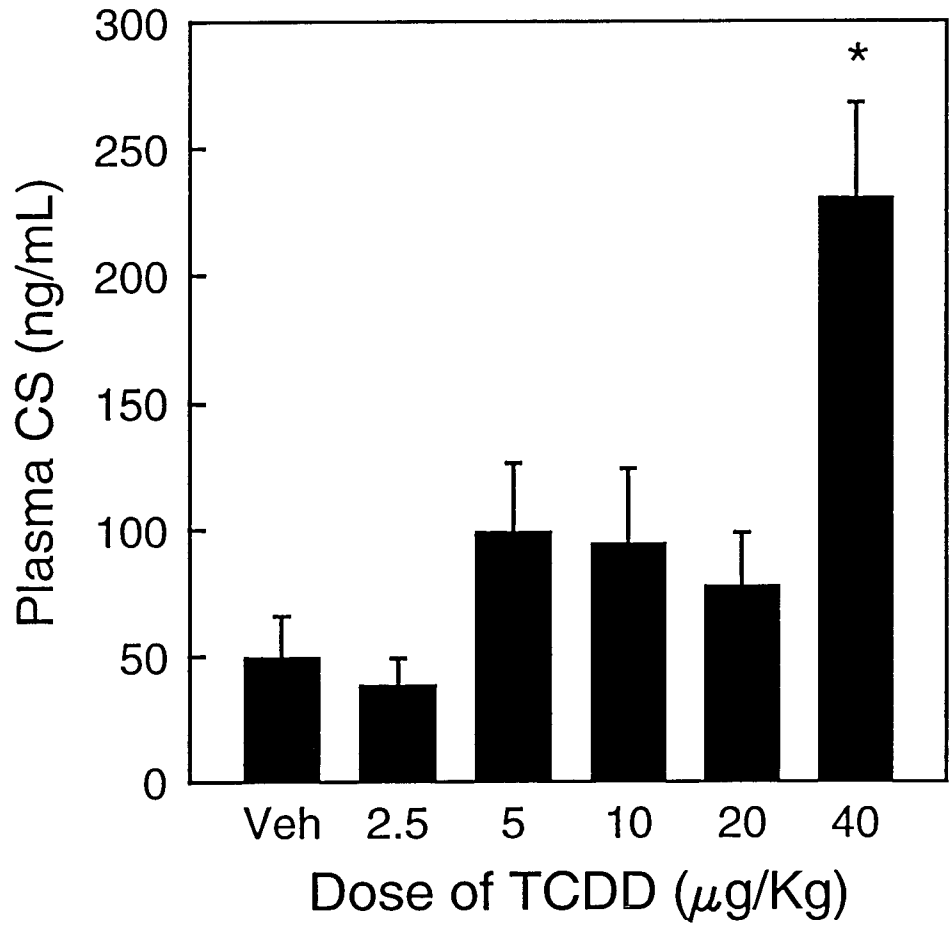

A.
Spleen Cells (x $10^{-7}$)
4.0
3.5
3.0
2.5
2.0
0
0
2.5
5
10
20
40
*
B.
Lytic Units/Spleen
1000
100
10
1
Dose of TCDD ($\mu$g/Kg)

**Figure III-3.**


300
250
200
150
100
50
0
Plasma CS (ng/mL)
*
Veh
2.5
5
10
20
40
Dose of TCDD (μg/Kg)

**Effects of TCDD and CS on the Generation of CTL Activity in Vitro**

Alloantigen-specific CTL activity was generated in spleen cells in vitro and measured on day five of culture (figure III-4). Spleen cells cultured in the absence of alloantigen ($P815_m$) did not develop CTL activity (data not shown). Addition of TCDD to MLTC cultures at concentrations of $10^{-13}$-$10^{-9}$M did not alter CTL activity. In contrast, addition of CS to MLTC cultures caused significant and dose-dependent suppression of CTL activity (figure III-4). Addition of TCDD to MLTC cultures in the presence of CS neither enhanced nor inhibited CS-mediated CTL suppression (figure III-4) indicating a lack of interaction between TCDD and CS on CTL activity.

## DISCUSSION

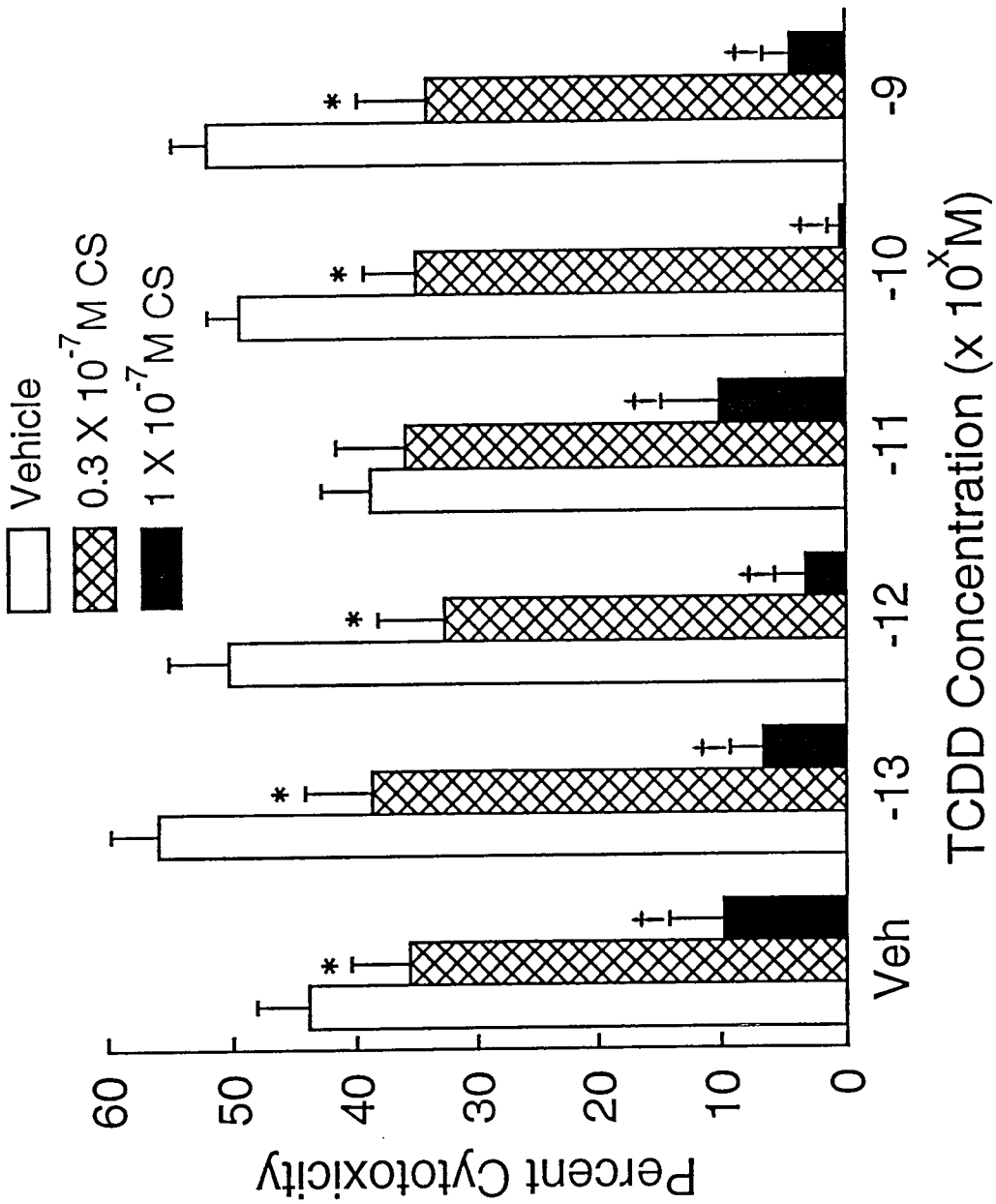
The goal of these studies was to examine the role of CS elevation in HAH-induced CTL suppression. Previous studies have shown a dose-dependent elevation of plasma CS levels that paralleled CTL suppression in alloantigen-challenged, HxCB-treated mice (Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990a; DeKrey et al., 1993a). However, neither adrenalectomy nor RU 38486 treatment altered the degree of HxCB-induced CTL suppression (DeKrey et al., 1993a) suggesting that CS elevation alone was not responsible for CTL suppression. In this study, following TCDD treatment, a dose dependent suppression of alloantigen-specific CTL activity was observed in the absence of a coordinate elevation of plasma CS levels. These results support the conclusions of the previous study (DeKrey et al., 1993a) by suggesting that HAH-induced suppression of CTL activity is not caused by elevation of plasma CS levels.

In this study, the lowest dose of TCDD that caused significant suppression of CTL activity was 5 μg/Kg. However, when based on LU/spleen, the calculated $ID_{50}$ dose of TCDD was slightly lower at 2.8 μg/Kg. The results of this study are in contrast to those of Clark et al. (1981) who showed significant suppression of splenic CTL activity at a cumulative TCDD dose of 0.4 μg/Kg. Further, these results are also in contrast to those of a more recent study by Hanson and Smialowicz (1994) who used the methods of Clark et al. (1981) and showed no suppression of splenic CTL activity in mice treated with either a single dose of TCDD (up to 7.2 μg/Kg) or multiple doses of TCDD (up to 12 μg/Kg cumulative total). A discussion of the disparities between these two previous studies has been published elsewhere (Hanson and Smialowicz, 1994).

**Figure III-4. TCDD does not suppress in vitro generated CTL activity nor alter CS-induced CTL suppression.**

The mean percent cytotoxicity is shown for 9-10 replicate cultures per treatment. Similar results were obtained in four other trials. Cultures were established and CTL activity was determined as described in Methods. * Indicates the mean CTL activity is significantly lower than in vehicle-treated cultures with the same TCDD concentration; † indicates the mean CTL activity is significantly lower than both vehicle-treated cultures and cultures containing CS at 3 x $10^{-8}$M with the same TCDD concentration ($p < 0.05$ by ANOVA and LSD t test).

**Figure III-4.**


Vehicle
0.3 X 10$^{-7}$M CS
1 X 10$^{-7}$M CS
Percent Cytotoxicity
0
10
20
30
40
50
60
Veh
-13
-12
-11
-10
-9
TCDD Concentration (x 10$^{x}$M)
*

An explanation for the difference in the results between this study and that of Hanson and Smialowicz (1994) may lie in the different methodologies used. For example, this study used a single oral dose regimen for TCDD exposure one day prior to challenge with P815 cells. In contrast, the study by Hanson and Smialowicz (1994) used ip. injection of TCDD with exposure preceding P815 challenge by one week. The total excretion half-life of TCDD has been shown to be approximately nine days by Birnbaum (1986) and 12-13 days by Gasiewicz et al. (1983). Thus, the highest dose of TCDD used by Hanson and Smialowicz (1994) approaches the lowest dose at which CTL suppression was observed in this study. Therefore, normal experimental variation at a similar dose of TCDD could account for the different results obtained. Further, the study by Hanson and Smialowicz (1994) used female C57Bl/6 mice. In this laboratory, female C57Bl/6 mice have been shown to be less sensitive than male C57Bl/6 mice to CTL suppression by HxCB (DeKrey et al., 1993a). Although it is not clear if a similar sex difference is observed following TCDD exposure, a sex difference may also contribute to an explanation for the different results obtained by these two studies.

TCDD-induced elevation of circulating CS levels has been previously shown in both rats (Gorski et al., 1988b; DiBartolomeis et al., 1987; Jones et al., 1987; Bestervelt et al., 1993) and mice (Kerkvliet et al., 1990a). CS elevation has also been shown in mice exposed to other HAH (Sanders et al., 1974, 1977; Kerkvliet et al., 1990a; DeKrey et al., 1993a). The apparent potency of TCDD to elevate plasma CS levels in mice, relative to its immunosuppressive potency, is less than that of HxCB. A dose of TCDD that causes ~85% suppression of CTL activity (20 μg/Kg) leads to a 1.6-fold increase in plasma CS levels (not significant). In contrast, a dose of HxCB that causes ~85% suppression of CTL activity (10 mg/Kg) also causes nearly 8-fold elevation of plasma CS levels (Kerkvliet et al., 1990a). An explanation for the apparent differences in relative potencies is unclear since CS elevation and CTL suppression have been associated with Ah receptor binding by both TCDD and HxCB (Kerkvliet et al., 1990a).

Alloantigen-specific CTL generation from spleen cells in vitro has been shown to be unaffected by preexposure to TCDD in vivo (Hanson and Smialowicz, 1994). However, examination of the direct effects of TCDD on in vitro generated CTL have not been previously reported. In this study, addition of TCDD at concentrations up to $10^{-9}$M to MLTC cultures did not alter the degree of alloantigen-specific CTL activity generated. These results suggest that TCDD acts via an indirect mechanism to suppress CTL activity in vivo. Kerkvliet and Baecher-Steppan (1988a) have shown that alloantigen-driven proliferation in mixed lymphocyte cultures was unaltered by the addition of HxCB at

concentrations up to $10^{-6}$M. Combined, these results suggest that an indirect mechanism for suppression of alloantigen responses may be common to HAH. It should be noted that the lack of correlation between in vitro results and in vivo observations following HAH exposure is not unique to CTL responses and has been reported for antibody responses as well (Davis and Safe, 1991).

In contrast to TCDD, addition of CS to MLTC cultures directly inhibited CTL generation. CS-mediated CTL suppression in vitro was dose-dependent with a 50% immunosuppressive concentration of $<1 \times 10^{-7}$M. Suppression of in vitro generated CTL activity by CS was expected since others have shown that development of allogeneic responses can be suppressed by GCs both in vivo (Billingham et al., 1951; Cupps and Fauci, 1982; Conlon et al., 1985; Freise et al., 1991) and in vitro (Borel, 1976; Cupps and Fauci, 1982; Schleimer et al., 1984). In cultures containing both CS and TCDD, no alteration of CS-induced CTL suppression, either increase or decrease, was observed at any concentration of TCDD tested. The lack of an interaction between TCDD and CS was interesting given that Abbott et al. (1992) have shown an enhanced teratogenic effect of GC when coadministered with TCDD, and numerous others have reported alteration of GC receptor binding affinity in rats treated with TCDD (Csaba et al., 1991; Lin et al., 1991; Ryan et al., 1989; Sunahara et al., 1989; Max and Silbergeld, 1987).

In summary, our results indicate that TCDD-induced suppression of CTL activity and elevation of plasma CS levels are not coincident effects. In addition, no interaction between TCDD and CS was observed in vitro. These results suggest that plasma CS elevation is not involved in suppression of CTL activity by HAH.

# CHAPTER IV

## EFFECTS OF EXOGENOUS CORTICOSTERONE TREATMENT ON ALLOANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTE ACTIVITY IN MICE

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## ABSTRACT

The intent of this study was to examine the effects of stress-like plasma corticosterone (CS) elevation on the generation of alloantigen-specific cytotoxic T lymphocyte (CTL) activity in mice. Elevation of plasma CS was achieved by infusion of exogenous CS via osmotic pumps. CS infusion at 16 mg/Kg/day on days -4 through 10 relative to alloantigen challenge led to slight, but significant, suppression of CTL activity on day 10 but no elevation of plasma CS levels. Serial sampling of mice infused with CS at 0.09, 0.9 or 9 mg/Kg/day over a 14 day period indicated that only the 9 mg/Kg/day infusion rate caused significant plasma CS elevation. Peak CS levels (~500 ng/mL) were observed one day after the start of CS infusion, but CS levels fell to below 200 ng/mL by day seven and were ~50 ng/mL on day 12 indicating that elevated plasma CS levels could not be maintained for extended periods by CS infusion. An attempt to define the windows of CS sensitivity during CTL development was made by infusing mice with CS at doses of 10-16 mg/Kg/day on days 0-3, 3-6, 4-7, 5-8, and 6-9, relative to alloantigen challenge; however, CS infusion had no effect on CTL activity. In contrast, dexamethasone infusion (9.4 mg/Kg/day) on days 0-3 suppressed CTL activity by ~90% indicating that the generation of CTL activity is sensitive to high dose GC treatment, but is refractory to stress-like CS elevation. In mixed lymphocyte-tumor cell (MLTC) cultures, CTL activity was suppressed by CS ($2.5 \times 10^{-8}$M) if added on the first day of culture but not if added on subsequent days. These results suggest that CTL are most sensitive to CS-induced suppression if exposed near to the time of alloantigen challenge.


## INTRODUCTION

Glucocorticoids (GCs) are potent immunosuppressive and antiinflammatory compounds [see reviews by Cupps and Fauci (1982), and Boumpas et al., (1991; 1993)]. GCs have been used in both human medicine and laboratory animal research. Synthetic, rather than natural GCs, are used most often because of their longer half-lives and greater potency. The immunosuppressive potency of synthetic GCs (e.g., dexamethasone) has led to their use as benchmark immunosuppressive compounds in immunotoxicity testing (Exon et al., 1990).

Because of the immunosuppressive potency of exogenously administered GCs, enhanced production of endogenous GCs and elevation of circulating GC levels has been suspected to induce immune suppression. This is supported by numerous studies that have correlated stress-induced elevation of endogenous GCs, such as that induced by

shock (Keller et al., 1983), rotation (Riley, 1981) and noise (Monjan and Collector, 1977), with altered immune function (reviewed by Pruett et al., 1993). More recently, immune suppression has been correlated with elevation of circulating GC levels in laboratory animals exposed to immunotoxic xenobiotic compounds [e.g., ethanol (Jerrells et al., 1990), benzene and toluene (Hsieh et al., 1991), morphine (Bryant et al., 1991; Pruett et al., 1992; Sei et al., 1991), gallium arsenide (Burns et al., 1994), and phenytoin (Hirai and Ichikawa, 1991)].

In this laboratory, a correlation between endogenous GC elevation and suppression of cytotoxic T lymphocyte (CTL) activity has been investigated in mice exposed to 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB), a toxic polychlorinated biphenyl isomer. Treatment of mice with HxCB leads to a dose-dependent suppression of splenic CTL activity coincident with a dose-dependent elevation of plasma CS levels (Kerkvliet et al., 1990b). In mice treated with HxCB at 10 mg/Kg body weight, suppression of CTL activity by ~80% has been correlated with peak plasma CS levels reaching 200-550 ng/mL (DeKrey et al., 1993a; Kerkvliet et al., 1990b). Since exogenous GC treatments have been reported to suppress in vivo generated CTL activity in mice (Eishi et al., 1983; Freise et al., 1991; Conlon et al., 1985; Kajiwara, 1988; Borel, 1976), elevation of plasma CS levels was suspected as a mechanism of HxCB-induced CTL suppression (Kerkvliet et al., 1990b). However, treatment with RU 38486 (a GC receptor antagonist) did not alter the degree of CTL suppression in HxCB-exposed mice (Dekrey et al., 1993) suggesting that CS elevation does not play a significant role in HxCB-mediated CTL suppression.

In the study reported here, the ability of exogenous CS to suppress in vivo-generated CTL activity was examined as a way to examine the immunosuppressive potency of elevated endogenous CS. Exogenous CS was administered by constant sc. infusion using osmotic pumps. Osmotic pumps were implanted at various times relative to alloantigen challenge to examine the time-dependent sensitivity of CTL to suppression by CS. In addition, for purposes of comparison, the time-dependent sensitivity of in vitro generated CTL to CS exposure was examined using mixed lymphocyte-tumor cell (MLTC) cultures.

## METHODS

### Animals.

Male C57Bl/6 mice, 6 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in polycarbonate shoe-box cages in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experimentation. A maximum of four animals were housed per cage. Cages were randomly assigned to positions in a cage rack, and animals were provided with Bed-O-Cob bedding (The Andersons, Maumee, OH). Food (Wayne Rodent Blox, Harlan Sprague Dawley Co., Bartonville, IL) and tap water were available *ad libitum*. Animal rooms were maintained on a 12 hr light/dark cycle (fluorescent, 7:30 AM lights on) and constant temperature (72 ± 1 [°F]) and 50% humidity. The mice remained free of all common murine pathogens as assessed by Charles River Professional Services, Wilmington, MA.

### Chemicals

Dexamethasone sodium phosphate was obtained from Anthony Products (Arcadia, CA) as an aqueous solution at 4 mg/mL and was used without further dilution. For in vivo use, corticosterone (CS) (Sigma Chemical Co., St. Louis, MO) was dissolved in polyethylene glycol (PEG) 400 (Sigma). Osmotic pumps (Alza Corp., Palo Alto, CA) were filled with dexamethasone, CS or polyethylene glycol 400 vehicle. For in vitro use, CS was dissolved in ethanol and diluted in culture media.

### Animal treatment.

While mice were under anesthesia (ketamine and xylazine), pockets were formed to the left of the dorsal midline and osmotic pumps were placed into them. Some mice were also adrenalectomized (DeKrey et al., 1993a) at the time of pump implantation. Some mice were bled by clipping the end of the tail, and blood was collected into EDTA treated capillary tubes. Plasma was isolated and stored at -20°C until analyzed.

The P815 mastocytoma cell line was propagated in ascites form by weekly passage in syngeneic DBA mice (H-$2^d$). P815 cells were harvested from DBA mice after 6-8 days of in vivo growth. C57Bl/6 mice (H-$2^b$) were injected ip. with 1.0 x $10^7$ viable P815 cells in 0.5 mL of Hanks Balanced Salt Solution (HBSS). Mice were killed 10 days later at the time of peak CTL activity (Kerkvliet and Baecher-Steppan, 1988a).

Spleens were removed and single-cell suspensions were prepared as described previously (Kerkvliet and Baecher-Steppan, 1988a) in RPMI 1640 (BioWhittaker) containing 5% FBS (Rehatuin, Intergen, Purchase, NY), 20 mM HEPES buffer and 5 x $10^{-5}$M 2-mercaptoethanol.

**MLTC (Mixed Lymphocyte-Tumor Cell) Cultures.**

The MLTC culture conditions used in these studies were based on the methods of House et al. (1989). P815 cells ($P815_m$) were incubated with mitomycin C (Sigma) at 50 µg/mL for 30 minutes in MEM (Gibco, Life Technologies Inc., Grand Island, NY) containing 10% FBS (Rehatuin), 20 mM HEPES buffer, 50 µg/mL gentamicin, 5 x $10^{-5}$M 2-mercaptoethanol and 2mM glutamine. $P815_m$ were washed 4X prior to use. Single cell suspensions of spleen cells from C57Bl/6 mice were prepared as described. Spleen cells (1.5 x $10^7$) were cocultured with 3 x $10^5$ $P815_m$ in 25 cm tissue culture flasks or 6 well tissue culture plates (Corning, Corning, NY) and 10 mL medium. CS- or ethanol-containing medium was added on various days after culture initiation. The final concentration of ethanol in all cultures was 0.02%. Cultures were incubated in 10% $CO_2$ at 37°C. After five days, the CTL activity of viable cells from each culture was measured. The concentration of viable cells in each culture was determined as follows: 50 µL culture samples were incubated with equal volumes of pronase (5 mg/mL) for 10 minutes at 37°C. Each sample was then diluted in Isoton (Coulter Electronics, Hialeah, FL) and Zapoglobin (Coulter) and incubated at room temperature for 1-10 minutes. The concentration of cells in each diluted sample was determined using a Coulter Counter (Coulter).

**CTL Assay**

CTL activity was measured in a 4-hour $^{51}Cr$ release assay as described previously (Kerkvliet and Baecher-Steppan, 1988a; DeKrey et al., 1993a). The percent cytotoxicity (CTX) at each effector:tumor cell (E:T) ratio was calculated by the equation:

$$\%CTX = \frac{test - naive}{mr - naive} \times 100,$$

where *test* = cpm using P815-stimulated spleen cells, *naive* = cpm using P815-naive spleen cells and *mr* = the maximum cpm released from cultures incubated with either sodium dodecyl sulfate or tween 80. Since preliminary studies indicated that freshly isolated spleen cells were ≥90% viable (data not shown), the viability of spleen cells was not determined prior to assay for in vivo generated CTL. The viability of cultured cells was determined in each experiment and E:T ratios were calculated based on viable cells. In order to compare the overall cytotoxic potential of animals or MLTC cultures, lytic units (LU)/spleen were calculated using a method adapted from Bryant et al. (1992) by the equation:

$$\text{LU/Spleen} = \frac{\text{Total Spleen Cells}}{10^6 \cdot e^{\{(\overline{Y}^* - C \cdot \overline{\ln(x)} - \ln(P/(A-P)))/C\}/T}},$$

where $Y^*$ = logit transformed percent lysis, $C$ = mean slope of the logit transformed percent lysis curve for each group, $x$ = effector:target (E:T) ratio, $A$ = maximum limiting percent lysis (75%), $P$ = reference lysis (30%), and $T$ = number of target cells. LU are presented as per spleen for in vivo data and per culture for in vitro data.

## CS RIA

Plasma CS levels were determined using [$^{125}$I]-coupled double antibody radioimmunoassay (RIA) kits (ICN Biochemicals, Carson, CA). A protocol optimized by the manufacturer was used. The lower limit of detection was 25 ng CS/mL. The maximum inter- and intra-assay coefficients of variance for internal controls were 15.7% and 8.8%, respectively, as determined by the manufacturer. Samples with analyte levels below the lower limit of detection were assigned the limit value of 25 ng/mL.

## Statistics

Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Comparisons of two sample means were performed using *t* tests (TTEST of SAS). Comparisons of more than two sample means were performed using general linear models (GLM of SAS). Unless otherwise indicated, values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### Study 1: The effects of 14 day continuous CS infusion on CTL activity

The intent of this study was to determine if infusion of exogenous CS via osmotic pump would suppress alloantigen-specific CTL activity in mice. Based on CS solubility tests in this laboratory, a CS infusion rate of 16 mg/Kg/day was the highest infusion rate that could be achieved using 14 day osmotic pumps (model 2002). This infusion rate is approximately 10-fold higher than the rate sufficient to elevate CS to basal levels (~50 ng/mL) in adrenalectomized mice (DeKrey, unpublished results). Therefore, 16 mg/Kg/day was chosen as the highest infusion rate for this study. Four days prior to alloantigen challenge, mice were implanted with osmotic pumps designed to deliver CS at 1, 2, 4, 8 or 16 mg/Kg/day for 14 days. All mice were killed 10 days after alloantigen challenge.

As shown in figure IV-1, infusion of CS at 16 mg/Kg/day led to a slight, but significant, suppression of CTL activity. In contrast, no significant suppression of CTL activity was observed in mice that received CS at dose rates of 8 mg/Kg/day (figure IV-1) or lower. The total splenic CTL potential, calculated as LU/spleen, was reduced by 41% and 64% in mice infused with CS at 8 and 16 mg/Kg/day, respectively, but no statistically significant reduction of LU/spleen was observed at any dose rate (table II-1). Interestingly, organ weights were significantly affected by CS at lower dose rates than that required to alter CTL activity. As shown in table II-1, the minimal dose rate that led to a significant reduction in thymus weight was 2 mg/Kg/day, whereas a dose rate of 4 mg/Kg/day led to significant loss of spleen weight and cells. Higher CS dose rates led to more pronounced effects in a dose dependent manner.

At the time of euthanasia, plasma was collected to verify elevated CS levels in CS treated animals. Interestingly, unlike the dose-dependent effects on organ weights, no dose-dependent elevation of plasma CS was observed (table II-1). Significant plasma CS elevation was only observed in mice that received CS at a rate of 4 mg/Kg/day.

### Study 2: Time dependent elevation of plasma CS levels in mice infused with exogenous CS

The intent of this study was to examine the time-dependent effects of CS infusion on plasma CS levels in mice. In the previous study, a dose-dependent elevation of plasma CS levels was expected in mice receiving continuous CS infusion, but none was observed. However, since only one time point was examined (14 days after the start of

CS infusion) and it was unclear if the CS levels observed were representative of the actual CS levels that occurred on the previous days. Therefore, in this study, plasma CS levels were examined in CS treated mice on various days after the start of CS infusion up to day 12. ADX mice were used to permit serial blood sampling (approximately every three days by tail vein) without causing elevation of plasma CS levels due to stress-induced endogenous production. CS was administered at dose rates of 0.09, 0.9 or 9.0 mg/Kg/day via osmotic pumps which were fitted with a length of tubing that delayed the onset of CS infusion until the day after surgery. In this way, the adrenalectomized status of each mouse (lack of detectable plasma CS levels) could be verified prior to the start of CS infusion.

As shown in figure IV-2, infusion of CS at a rate of 0.09 mg/Kg/day did not elevate plasma CS levels above the preinfusion baseline level. Infusion of CS at a rate of 0.9 mg/Kg/day led to a plasma CS level of approximately 60 ng/mL which was maintained over the duration of sampling. Infusion of CS at a rate of 9.0 mg/Kg/day significantly elevated plasma CS levels to ~490 ng/mL on day one after beginning CS infusion. However, at this infusion rate, the level of CS declined on each successive day of sampling thereafter. By day 12 after the start of infusion, plasma CS had declined to ~50 ng/mL, a level that was equivalent to that observed in morning sampled nonADX mice (Shimizu et al., 1983; DeKrey et al., 1993a). These results indicated that elevation of plasma CS to ~500 ng/mL could be achieved at a CS dose of 9 mg/Kg/day (and probably higher doses), but elevated steady state levels could not be maintained, and significantly elevated CS levels could not be maintained for longer than 3-4 days.

**Study 3: The effects of CS infusion during three-day time windows after alloantigen challenge**

The intent of this study was to examine the time-dependent effects of HxCB-like plasma CS elevation on the generation of alloantigen-specific CTL activity in vivo. Osmotic pumps designed to infuse CS at a rate of 0 or 10 mg/Kg/day for three day periods were implanted into mice on days 3-6 post alloantigen challenge. In this way, mice were exposed to CS on days 3-6, 4-7, 5-8, or 6-9. This schedule was based on the fact that HxCB did not induce elevated CS levels prior to day three (figure IV-3).

**Table IV-1. Dose-dependent effects of CS infused for 14 days on LU/spleen, organ weights and plasma CS levels of alloantigen-challenged[a].**

[a]Osmotic pumps were implanted into all CS-treated mice four days prior to alloantigen challenge as described in Methods. All animals were killed on day 10 post alloantigen challenge.

[b]Indicates body weight on the day of necropsy. No significant difference in body weight change was observed over the course of the study.

[c]Values are given as mean ± SEM for 6-8 animals per group. Organ weights are given as ratios of body weight.

*Indicates the mean is significantly different from untreated control by analysis of variance and post hoc Bonferroni t-tests ($p < 0.05$).

Table IV-1.

| Corticosterone (mg/Kg/day) | LU/Spleen | Body Weight[b] (g) | Spleen Weight[c] (mg/g BW) | Spleen Cells (x $10^7$) | Thymus Weight (mg/g BW) | Plasma CS (ng/mL) |
|---|---|---|---|---|---|---|
| 0 | 481 ± 79 | 26.9 ± 0.6 | 6.0 ± 0.2 | 11.1 ± 0.7 | 0.91 ± 0.08 | 32 ± 2 |
| 1 | 414 ± 77 | 28.7 ± 0.5 | 5.7 ± 0.4 | 10.8 ± 0.5 | 0.79 ± 0.16 | 29 ± 3 |
| 2 | 547 ± 110 | 28.6 ± 0.7 | 5.6 ± 0.3 | 10.8 ± 0.6 | 0.53 ± 0.06* | 52 ± 4 |
| 4 | 432 ± 138 | 25.2 ± 0.6 | 4.4 ± 0.2* | 7.6 ± 0.5* | 0.31 ± 0.03* | 90 ± 10* |
| 8 | 283 ± 127 | 25.7 ± 1.0 | 4.2 ± 0.5* | 6.9 ± 0.7* | 0.36 ± 0.13* | 47 ± 8 |
| 16 | 175 ± 30 | 27.7 ± 0.3 | 4.6 ± 0.4* | 8.0 ± 1.0* | 0.39 ± 0.07* | 42 ± 8 |

**Figure IV-1. Effect of CS infusion from day -4 through day 10 relative to alloantigen challenge on CTL activity.**

CTL activity is presented as percent lysis at various effector:tumor (E:T) cell ratios (mean ± SEM) for 6-8 mice per group. CS-treated animals were implanted with osmotic pumps four days prior to alloantigen challenge. The pumps were designed to release CS at a constant rate (16 mg/Kg/day) for 14 days. Animals were killed on day 10 post alloantigen challenge and splenic CTL activity was measured as described in Methods. *Indicates the mean is significantly different from untreated control at the E:T ratio specified ($p < 0.05$).

**Figure IV-2. Lack of prolonged plasma CS elevation following infusion of exogenous CS.**

Animals were adrenalectomized and implanted with osmotic pumps containing CS as described in Methods. The pumps were designed to release CS at a constant rate for 14 days. The pumps were fitted with a length of tubing that delayed infusion of CS until one day after implantation. Serial blood samples were collected from the tails of mice on various days after ADX, and plasma was analyzed for CS levels by RIA. Data are presented as mean ± SEM for 5-6 mice per group.

**Figure IV-3. HxCB treatment causes elevation of plasma CS levels beginning on day three post alloantigen challenge.**

Plasma CS levels in alloantigen-challenged mice are shown as adapted from DeKrey et al., 1993a. Data represent mean ± SEM for 6-10 animals per group. Mice were treated with HxCB at 0 or 10 mg/Kg one day prior to ip. injection with 1 x $10^7$ allogeneic P815 cells. Animals were killed on the days indicated. For statistical purposes, plasma samples with CS levels below the lower limit of detection (25 ng/mL) were given the value 25 ng/mL. *Indicates a significant difference between treatment groups on the day specified ($p < 0.05$).

**Figure IV-1.**

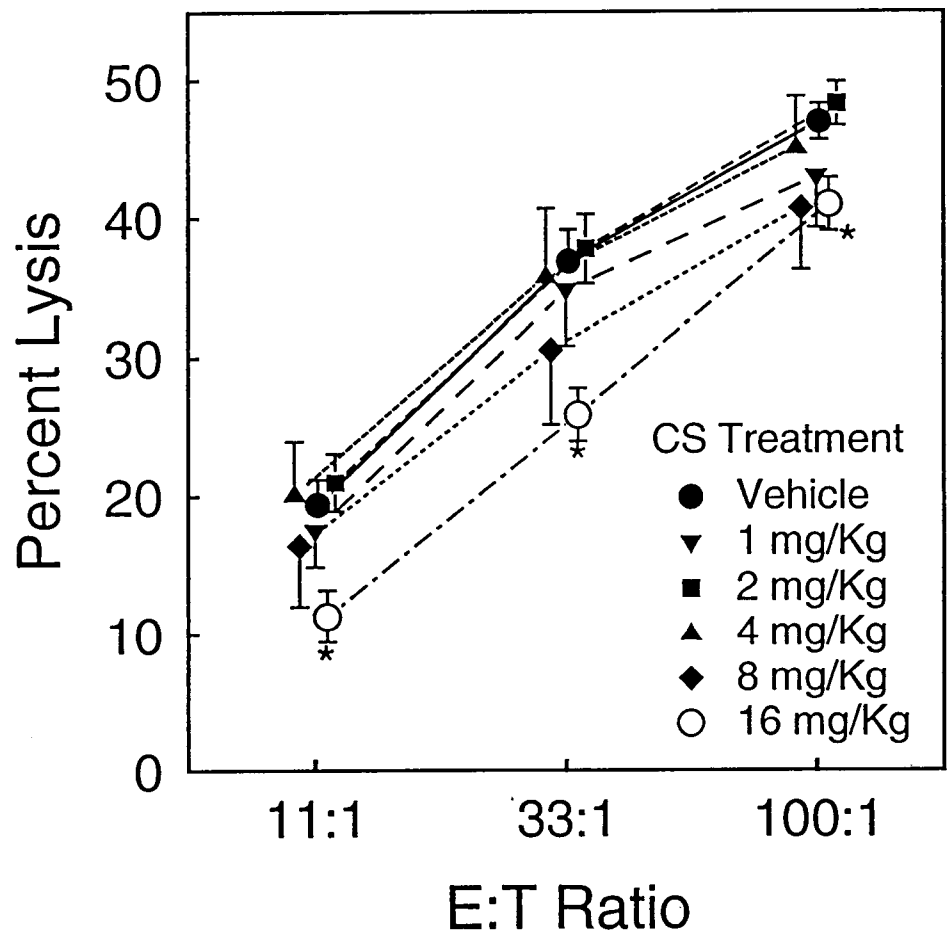

Percent Lysis
50
40
30
20
10
0
11:1
33:1
100:1
E:T Ratio
CS Treatment
Vehicle
1 mg/Kg
2 mg/Kg
4 mg/Kg
8 mg/Kg
16 mg/Kg
*
*
*

**Figure IV-2.**

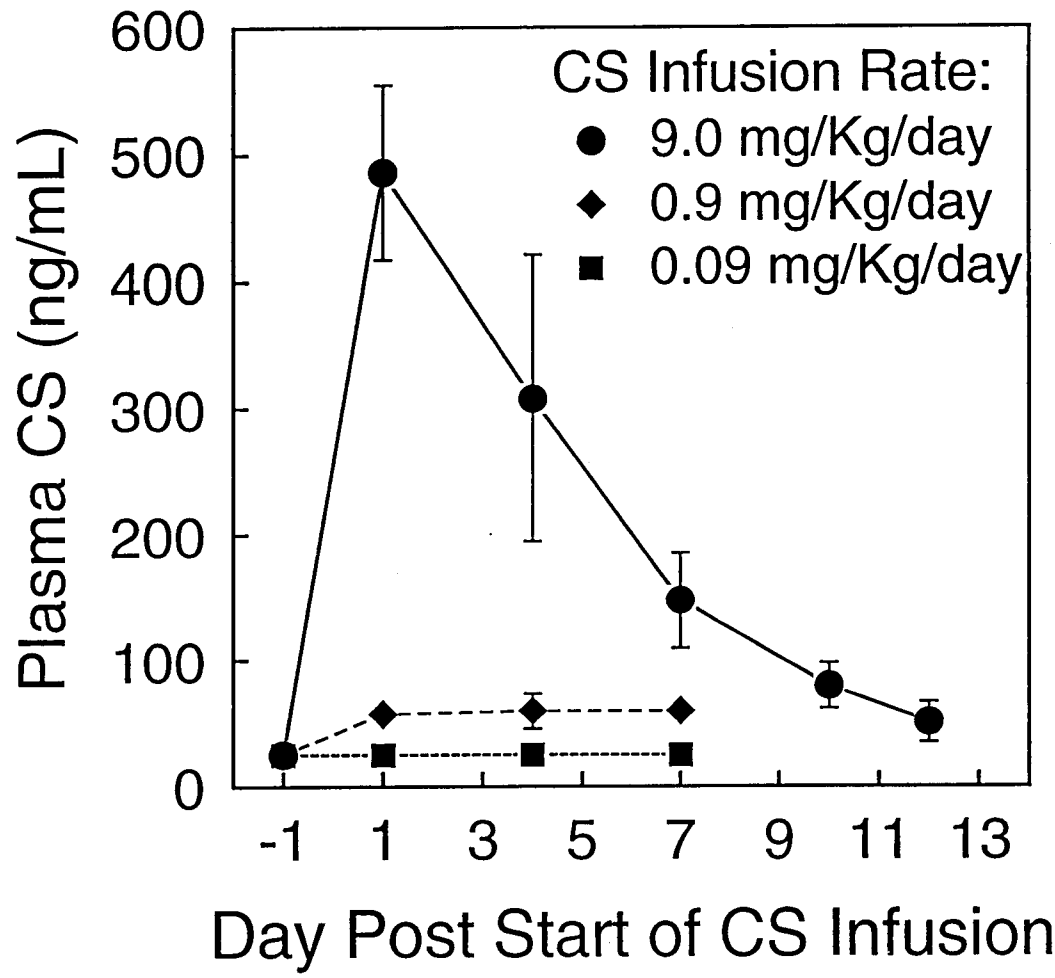
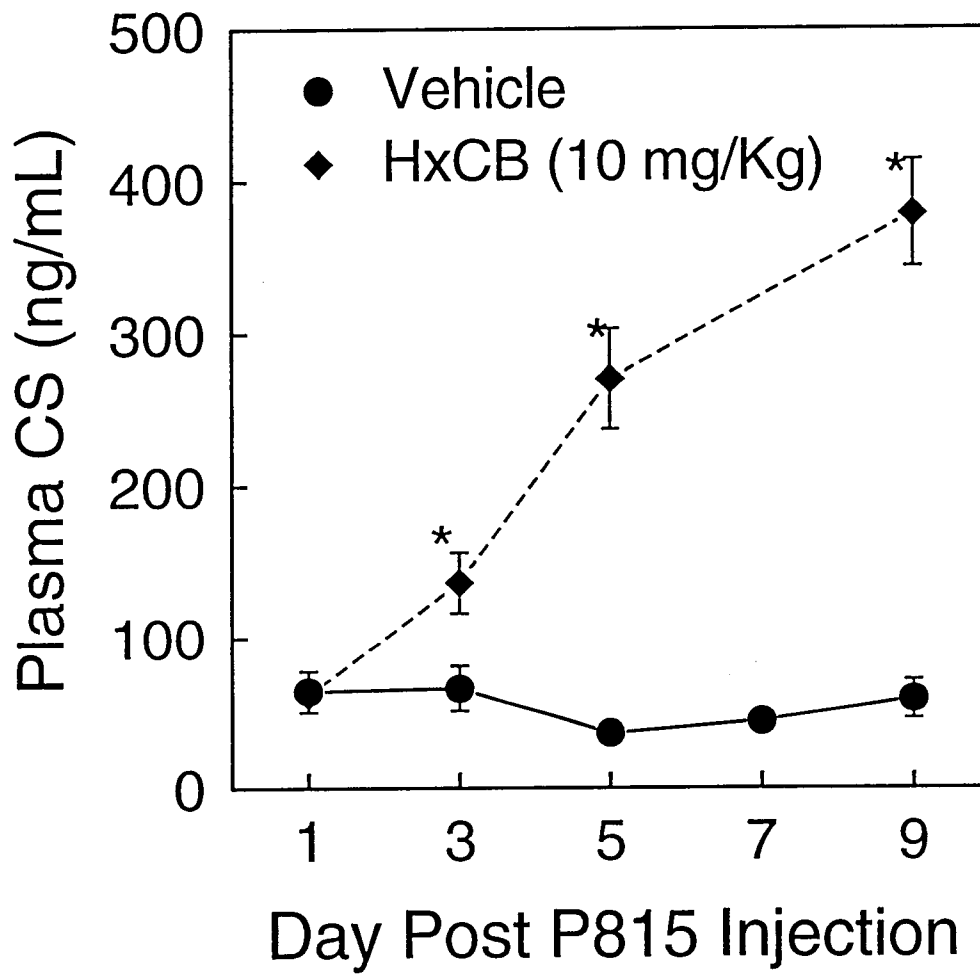

600
500
400
300
200
100
0
Plasma CS (ng/mL)
CS Infusion Rate:
9.0 mg/Kg/day
0.9 mg/Kg/day
0.09 mg/Kg/day
-1
1
3
5
7
9
11
13
Day Post Start of CS Infusion

**Figure IV-3.**


500
400
300
200
100
0
Plasma CS (ng/mL)
Vehicle
HxCB (10 mg/Kg)
*
*
*
1
3
5
7
9
Day Post P815 Injection

Infusion of CS over days 3-9 had no significant effect on the generation of CTL activity measured either as percent lysis (figure IV-4) or as LU/spleen (table IV-2). In contrast, spleen weights were significantly decreased in all CS-treated animals, and thymus weights were reduced in all CS-treated animals except those that were implanted with pumps on day six (table IV-2). Spleen cellularity was not significantly affected by CS exposure.

**Study 4: Effects of CS or dexamethasone infusion on days 0-3 post alloantigen challenge**

Since no effective CTL suppression was observed in mice treated with CS after day three post alloantigen challenge, in this study we examined the effects of CS treatment prior to day three. A dose rate of CS at 16 mg/Kg/day was sufficient to cause suppression of CTL activity in study one. Therefore, the same dose rate was used in this study. The effects of a high dose rate of dexamethasone (9.4 mg/Kg/day), a known immunosuppressive GC (Exon et al., 1990), were examined in a separate trial. Osmotic pumps designed to infuse vehicle, CS or dexamethasone for three day periods were implanted into mice approximately six hours prior to alloantigen challenge. CTL activity was measured 10 days later.

As shown, CS infusion at 16 mg/Kg/day on days 0-3 had no suppressive effect on the generation of CTL activity measured either as percent lysis (figure IV-5A) or as LU/spleen (table IV-3). In contrast, dexamethasone treatment (9.4 mg/Kg/day) over the same time period markedly suppressed CTL activity measured as both percent lysis (figure IV-5B) and LU/spleen (table IV-3). Interestingly, both CS and dexamethasone caused significant reduction of thymus weight and total spleen cells, with the effects of dexamethasone being more pronounced (table IV-3). CS, but not dexamethasone, caused significant reduction of spleen weight (table IV-3).

**Table IV-2. Dose-dependent effects of CS infused for 14 days on LU/spleen, organ weights and plasma CS levels of alloantigen-challenged[a].**

[a]Osmotic pumps were implanted on the day indicated relative to alloantigen challenge as described in Methods. All animals were killed on day 10 post alloantigen challenge. Values indicate mean ± SEM for 5-7 animals per group. Organ weights are given as ratios of body weight.
[b]Indicates the body weight on the day of necropsy. No significant difference in body weight change was observed between treatment groups over the course of the study.
*Indicates the mean is significantly different from vehicle-treated control ($p < 0.06$).

**Table IV-3. LU/spleen and organ weights of mice treated with CS or dexamethasone on days 0-3 post alloantigen challenge[a]**

[a]Osmotic pumps were implanted approximately six hours prior to alloantigen challenge as described in Methods. All animals were killed on day 10 after alloantigen challenge.
[b]Indicates the body weight on the day of necropsy. A significant difference in body weight was reflective of body weight change over the course of the study. Values indicate mean ± SEM for 5-7 animals per group. Organ weights are given as ratios of body weight.
[c]The total dose of dexamethasone is indicated. Animals that received dexamethasone were implanted with two osmotic pumps each.
*Indicates the mean is significantly different from vehicle-treated control ($p < 0.05$).
†Indicates the mean is significantly different from vehicle-treated control ($p < 0.05$).

Table IV-2.

| CS Infusion Period (Days) | Corticosterone (mg/Kg/day) | LU/Spleen | Body Weight (g)[b] | Spleen Weight (mg/g BW) | Spleen Cells (x $10^7$) | Thymus Weight (mg/g BW) |
|---|---|---|---|---|---|---|
| 3-6 | 0 | 925 ± 60 | 23.1 ± 0.5 | 5.5 ± 0.2 | 10.4 ± 0.7 | 0.42 ± 0.03 |
| | 10 | 856 ± 79 | 22.5 ± 0.3 | 4.7 ± 0.3* | 9.4 ± 0.5 | 0.27 ± 0.02* |
| 4-7 | 0 | 621 ± 62 | 23.5 ± 0.6 | 4.9 ± 0.5 | 9.2 ± 0.7 | 0.54 ± 0.07 |
| | 10 | 773 ± 118 | 23.9 ± 0.2 | 3.9 ± 0.2* | 8.8 ± 0.4 | 0.25 ± 0.01* |
| 5-8 | 0 | 705 ± 54 | 23.2 ± 0.4 | 5.1 ± 0.2 | 9.4 ± 0.3 | 0.44 ± 0.05 |
| | 10 | 656 ± 63 | 22.3 ± 0.5 | 4.1 ± 0.2* | 8.5 ± 0.4 | 0.25 ± 0.02* |
| 6-9 | 0 | 790 ± 83 | 22.8 ± 0.7 | 5.6 ± 0.2 | 9.1 ± 0.8 | 0.39 ± 0.04 |
| | 10 | 756 ± 100 | 22.0 ± 0.6 | 4.3 ± 0.2* | 8.0 ± 0.4 | 0.31 ± 0.04 |

Table IV-3.

| Day of Pump Implantation | Glucocorticoid (mg/Kg/day) | LU/Spleen | Body Weight[b] (g) | Spleen Weight (mg/g BW) | Spleen Cells (x $10^7$) | Thymus Weight (mg/g BW) |
|---|---|---|---|---|---|---|
| 0 | 0 | 892 ± 109 | 23.9 ± 0.6 | 5.8 ± 0.3 | 14.0 ± 0.9 | 0.58 ± 0.07 |
| | 16 (CS) | 726 ± 110 | 23.1 ± 0.7 | 4.2 ± 0.2* | 9.4 ± 0.8* | 0.37 ± 0.04* |
| 0 | 0 | 278 ± 157 | 23.9 ± 0.6 | 4.4 ± 0.6 | 9.8 ± 1.4 | 1.46 ± 0.21 |
| | 9.4 (Dex)[c] | 1.3 ± 0.3* | 25.9 ± 0.4* | 3.2 ± 0.4 | 2.1 ± 0.6* | 0.22 ± 0.02* |

**Figure IV-4. CTL activity is not altered by infusion of exogenous CS on days 3-9 post alloantigen challenge.**

CTL activity is presented as percent lysis at various effector:tumor cell ratios (mean ± SEM) for 5-7 mice per group. Animals were implanted with osmotic pumps containing CS or vehicle on days 3-6 post alloantigen challenge as indicated. The pumps were designed to release CS at a constant rate (10 mg/Kg/day) over the course of three days. Animals were killed on day 10 post alloantigen challenge and splenic CTL activity was measured as described in Methods. Pumps alone did not alter the CTL activity of mice during any time period (data not shown).

**Figure IV-5. Effects of CS or dexamethasone on CTL activity when infused on days 0-3 post alloantigen challenge.**

CTL activity is presented as percent lysis at various effector:tumor cell ratios (mean ± SEM) for 5-7 mice per group. Animals were implanted with osmotic pumps containing CS, dexamethasone (Dex) or vehicle six hours prior to alloantigen challenge. The pumps were designed to release CS at a constant rate for three days. Animals were killed on day 10 post alloantigen challenge and splenic CTL activity was measured as described in Methods. *Indicates the mean is significantly different from vehicle-treated control at the E:T ratio specified ($p < 0.05$).

**Figure IV-4.**

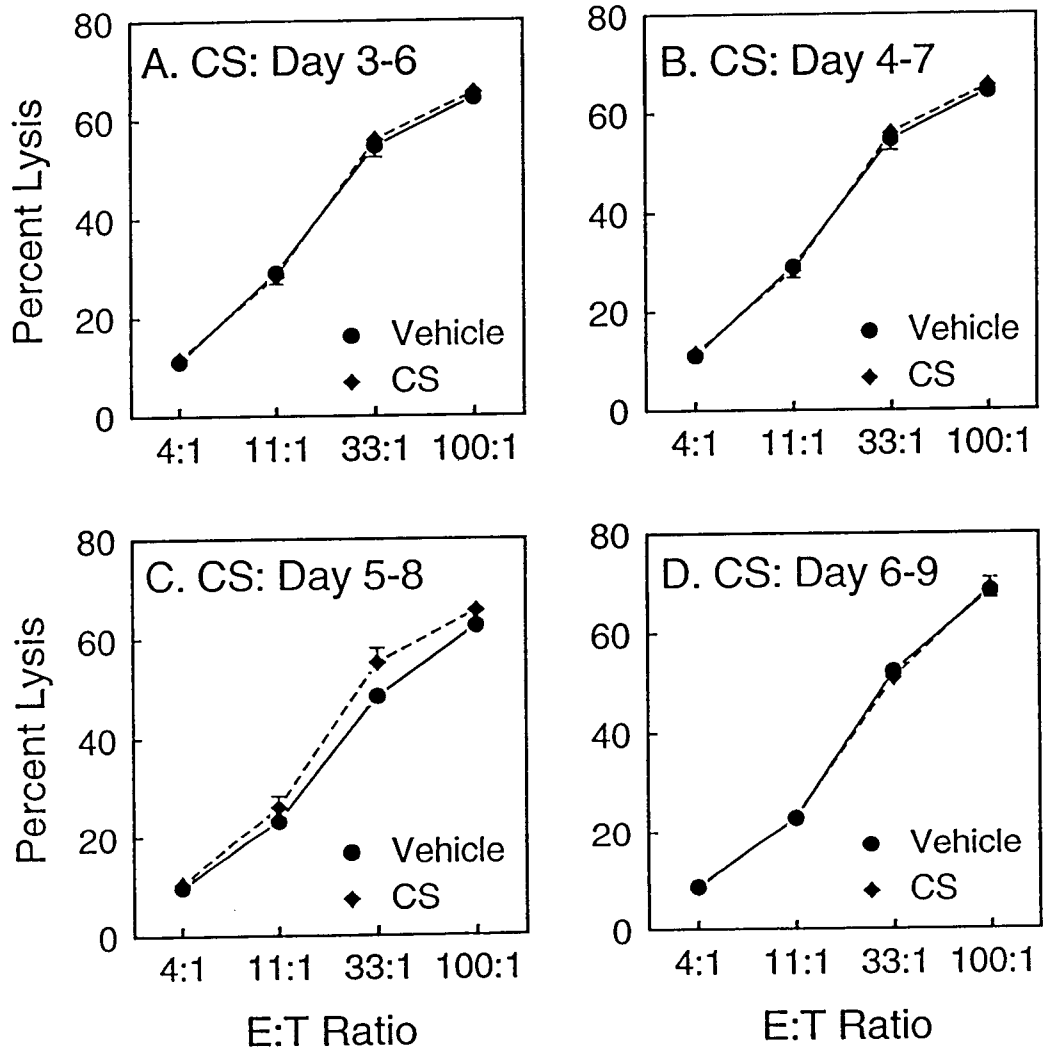
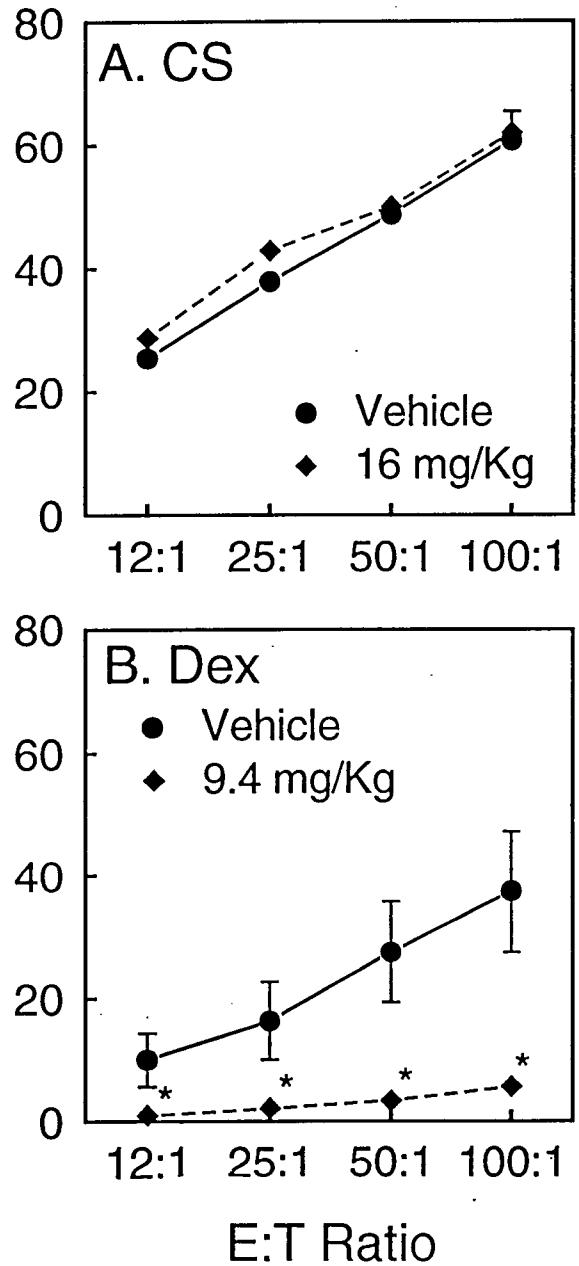

A. CS: Day 3-6
B. CS: Day 4-7
C. CS: Day 5-8
D. CS: Day 6-9
Percent Lysis
E:T Ratio
Vehicle
CS
4:1
11:1
33:1
100:1
0
20
40
60
80

**Figure IV-5.**


80
60
40
20
0
A. CS
Vehicle
16 mg/Kg
12:1
25:1
50:1
100:1
80
60
40
20
0
B. Dex
Vehicle
9.4 mg/Kg
*
*
*
*
12:1
25:1
50:1
100:1
E:T Ratio

**Study 5: Effects of CS on in vitro generated CTL**

MLTC cultures were used to examine the direct effects of CS on the generation of CTL activity in vitro. As shown in figure IV-6A, addition of CS to MLTC cultures resulted in a concentration-dependent suppressive effect on viable cell recovery. CS concentrations less than $10^{-8}$M did not alter cell viability. However, the viability of cultured cells decreased markedly over the concentration range $10^{-8}$-$10^{-7}$M CS, with cell recovery decreasing to <10% of controls at higher concentrations. Viable cell recovery from cultures with 2.5 x $10^{-8}$M and 5.0 x $10^{-8}$M CS were 40% and 24% of vehicle-treated cultures, respectively.

Due to poor cell recovery, CTL activity could not be assessed in cultures with CS concentrations >$10^{-8}$M. Therefore, the effects of CS between $10^{-8}$ and $10^{-7}$M on the generation of CTL activity were examined. As shown in figure IV-6B, CTL activity (percent lysis) was significantly suppressed in a concentration-dependent manner by exposure to CS. CS at a concentration of 2.5 x $10^{-8}$M suppressed CTL activity by 74% (calculated as LU/culture). Because higher CS concentration caused significantly lower cell recovery (figure IV-6B), CS at 2.5 x $10^{-8}$M was used in subsequent experiments.

The time-dependent sensitivity of in vitro generated CTL to CS exposure was examined by addition of CS to cultures on various days post culture initiation. In vitro generation of CTL activity was sensitive to CS exposure at 2.5 x $10^{-8}$M when present from the day of culture initiation (percent lysis, figure IV-7; LU/culture, table IV-4). In contrast, addition of CS on any later day did not alter CTL activity (figure IV-7 and table IV-4). The level of CTL activity generated in vehicle-treated cultures was consistent across days of vehicle addition and was the same as in non-vehicle-treated cultures (data not shown).

## DISCUSSION

Elevation of endogenous CS has been linked to suppression of immune function following stress or exposure to xenobiotic compounds (Pruett et al., 1993). However, no previous studies have directly examined the immunological effects of CS elevation to the approximate levels observed in stressed or xenobiotic-exposed mice. The intent of the studies reported here was 1) to determine if CS infusion could significantly elevate plasma CS to stress- or xenobiotic-like levels, and 2) to examine the effects of such CS elevation on CTL activity.

**Table IV-4. Time-dependent effects of CS on LU/MLTC culture[a]**

| Day of CS Addition | Corticosterone (x $10^{-8}$M) | LU/Culture |
|---|---|---|
| 0 | 0 | 11.2 ± 2.9 |
| | 2.5 | 1.7 ± 0.8* |
| 1 | 0 | 11.5 ± 4.0 |
| | 2.5 | 6.7 ± 3.7 |
| 2 | 0 | 12.6 ± 2.4 |
| | 2.5 | 13.8 ± 5.6 |
| 3 | 0 | 13.2 ± 2.3 |
| | 2.5 | 11.2 ± 4.3 |
| 4 | 0 | 13.0 ± 3.6 |
| | 2.5 | 7.2 ± 1.4 |

[a]LU/MLTC culture were determined as described in Methods for three cultures (separate spleen cell pools) per treatment group. Spleen cell pools were shared across treatment groups. The data are representative of three separate experiments.

*Indicates the mean is significantly different from vehicle-treated control ($p < 0.05$).

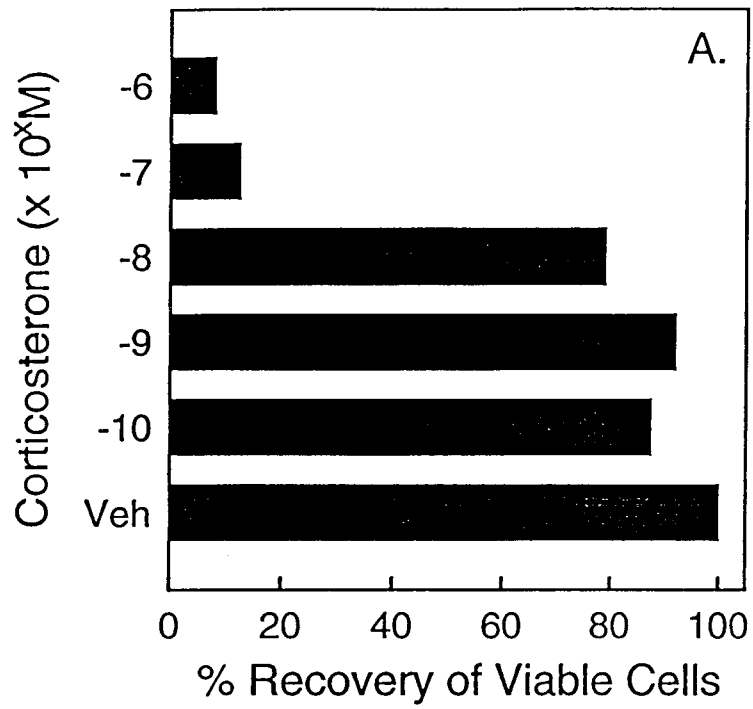
**Figure IV-6. Effects of CS on viability and CTL activity in MLTC cultures.**

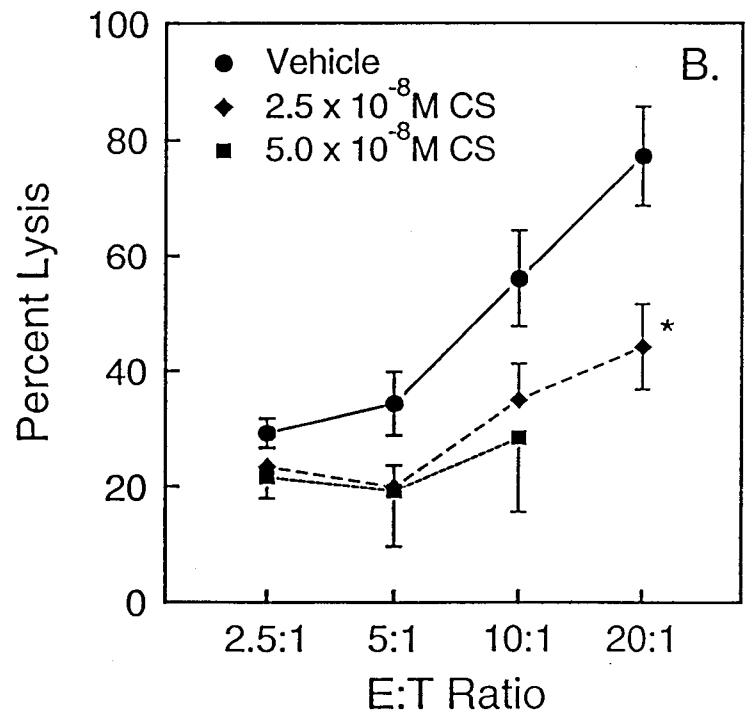
Spleen cells ($1.5 \times 10^7$) were cultured with $3 \times 10^5$ mitomicin C-treated P815 cells in the presence of CS from the time of culture initiation. After five days, the CTL activity of each culture was determined with E:T ratios based on viable cell numbers. The viability of cells (panel A) is given as percent of control (0.02% ethanol) for duplicate cultures. The CTL activity against $^{51}Cr$-labels P815 cells (panel B) is given as mean ± SEM of six replicate cultures. CTL activity at an E:T ratio of 20:1 could not be determined for cultures containing $5.0 \times 10^{-8}$M CS due to low cell viability (panel B). *Indicates the mean is significantly different from vehicle-treated control at the E:T ratio specified ($p < 0.05$). LU/culture for vehicle and CS ($2.5 \times 10^{-8}$M) exposed cultures were 76.7 ± 15.5 and 19.9 ± 5.0 [mean ± SEM], respectively and were significantly different ($p < 0.05$).

**Figure IV-7. Effects of CS on CTL activity when added on various days after the start of MLTC cultures.**

CTL activity is presented as percent lysis at various effector:tumor (E:T) cell ratios (mean ± SEM) for three cultures (separate spleen cell pools) per treatment group. The data are representative of three separate experiments. Spleen cells ($1.5 \times 10^7$) were cocultured with $3 \times 10^5$ mitomicin C-treated P815 cells for five days. CS was added on days 0-4 to a final concentration of 0 or $2.5 \times 10^{-8}$M (vehicle-treated cultures contained 0.02% EtOH). Spleen cell pools were shared across treatment groups. E:T ratios were based on viable cell numbers. *Indicates the mean is significantly different from vehicle-treated control at the E:T ratio specified ($p < 0.05$).

**Figure IV-6.**


A.
Corticosterone (x 10^X M)
-6
-7
-8
-9
-10
Veh
0
20
40
60
80
100
% Recovery of Viable Cells


B.
Vehicle
2.5 x 10^-8 M CS
5.0 x 10^-8 M CS
Percent Lysis
100
80
60
40
20
0
*
2.5:1
5:1
10:1
20:1
E:T Ratio

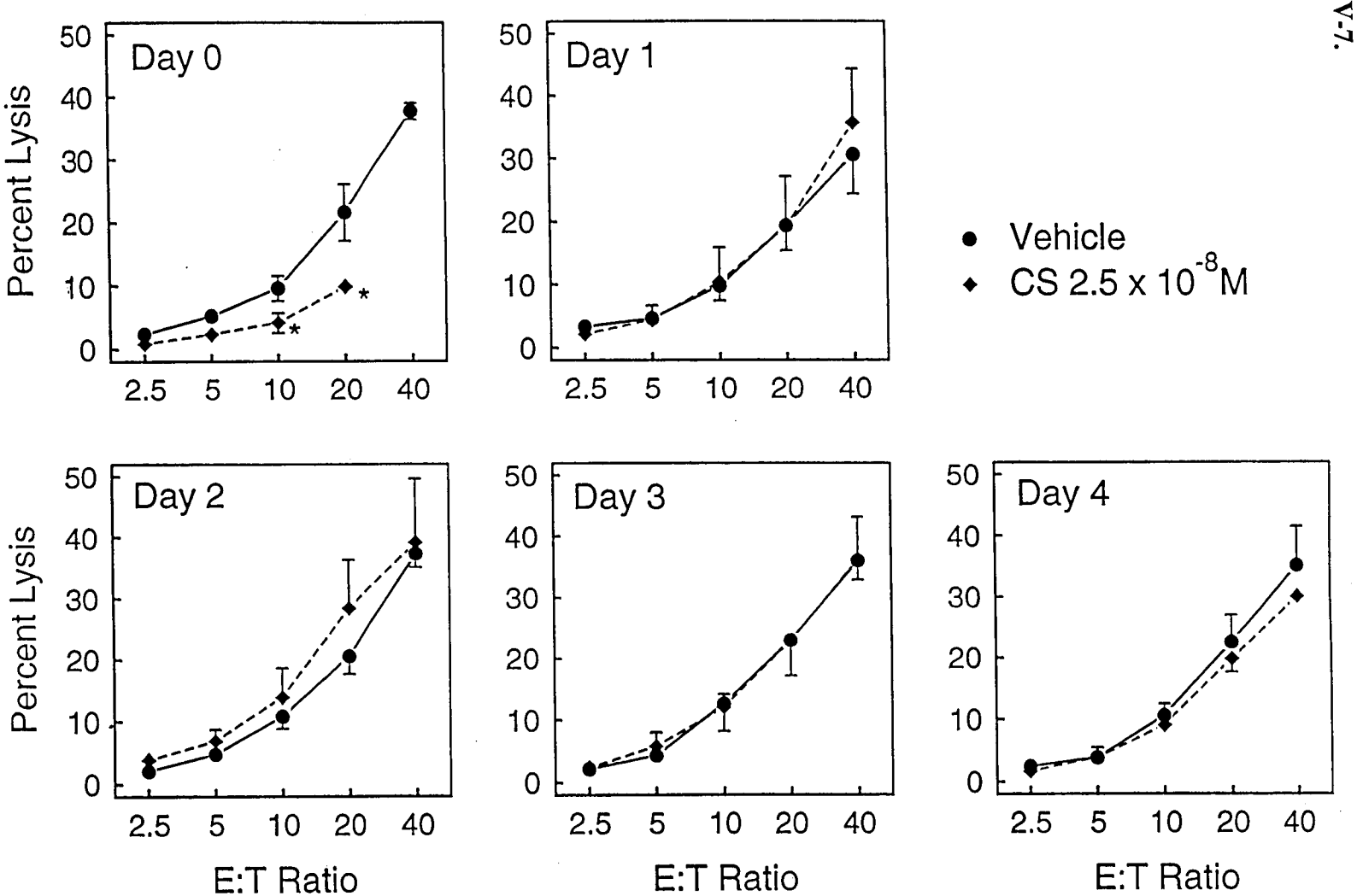

Day 0
Day 1
Day 2
Day 3
Day 4
Percent Lysis
E:T Ratio
0
10
20
30
40
50
2.5
5
10
20
40
Vehicle
CS 2.5 x 10-8M

Figure IV-7.

Infusion of exogenous CS significantly elevated plasma CS to stress-like levels; however, elevated steady state levels could not be maintained, and significant elevation of CS levels could not be maintained for longer than 3-4 days. Infusion of CS at a dose rate of 9 mg/Kg/day was sufficient to elevate plasma CS levels to ~500 ng/mL on the day after the start of CS infusion. However, CS levels steadily declined over the following 11 days to normal plasma levels of ~50 ng/mL. The mechanism behind the decrease in plasma CS levels is not known. Induction of drug metabolism is a classic response following treatment with many drugs including glucocorticoids (Gibson and Skett, 1986; Juchau, 1990), and calculations based on cortisol infusion have suggested that removal of cortisol from blood is increased when the capacity of cortisol-binding globulin is exceeded (Tait and Burstein, 1964). Therefore, it is likely that the decrease of plasma CS levels over time was caused by an increase in the clearance rate of CS. This would explain the normal plasma CS levels in mice after 14 days of CS infusion at 16 mg/Kg/day. The fact that normal plasma CS levels were maintained in mice infused with CS at 0.9 mg/Kg/day suggests that high dose CS infusion may be required for an increase in the clearance rate of CS. Interestingly, the highest plasma CS level observed after 14 days of CS infusion, significantly higher than observed in any other dose group (90 ng/mL), occurred at an infusion rate of 4 mg/Kg/day. These results suggest that a CS infusion rate >4 mg/Kg/day may be required for an increase in the clearance rate of CS.

Because plasma CS levels are not significantly elevated in HxCB-treated mice until three days after alloantigen challenge, we examined the direct effects of exogenous CS treatment on CTL activity by beginning CS infusion three days after alloantigen challenge. Infusion of CS at 10 mg/Kg/day was expected to elevate plasma CS to peak levels of ~500 ng/mL, similar to the peak CS levels observed in HxCB-treated mice [200-550 ng/mL (DeKrey et al., 1993a; Kerkvliet et al., 1990b)]. However, since significantly elevated plasma CS levels could not be expected for much longer than three days, we examined the effects of CS infusion during three day windows of time. Interestingly, CS infusion had no effect on CTL activity. These results suggest that CS elevation in HxCB-treated animals may not play a role in suppression of CTL activity.

The only significant, albeit slight, effect of CS on CTL activity in the in vivo studies described here was observed when CS infusion was begun four days prior to alloantigen challenge (16 mg CS/Kg/day for 14 days). However, due to the time-dependent decrease of plasma CS levels, it is unlikely that significant plasma CS elevation persisted for more than 5 days from the time of pump implantation. Since infusion

of CS at the same dose rate on days 0-3 relative to alloantigen challenge had no effect on CTL activity, these results suggest that the days prior to antigen challenge are the most sensitive for CS-induced CTL suppression. These results are supported by the findings of Conlon et al. (1985) who reported that the sensitivity of mice to GC-induced CTL suppression decreased after alloantigen challenge. Injection of hydrocortisone (2.5 mg per animal, ip.) into mice two days prior to alloantigen challenge significantly suppressed the CTL response; however, hydrocortisone injection on days two or seven after alloantigen challenge had no effect on CTL activity (Conlon et al., 1985).

In contrast to infusion of CS, infusion of dexamethasone at 9.4 mg/Kg/day on days 0-3 relative to alloantigen challenge caused a marked suppression of CTL activity. These data indicate that the CTL response is not insensitive to GC-induced suppression during that time. Indeed, the CTL response to alloantigen in mice has been shown to be sensitive to dexamethasone-induced suppression as late as 9-11 days following antigen challenge (Borel, 1976). Dexamethasone is >50-fold more potent as an immunosuppressive GC than corticosterone (Schleimer et al, 1984). Therefore, these data suggest that a higher dose of CS given after alloantigen challenge might also suppress CTL activity.

The generation of CTL activity in vitro was suppressed in the presence of CS. When added at the initiation of MLTC cultures, 2.5 x $10^{-8}$M CS was sufficient to suppress CTL activity by 43%. However, when CS was added to MLTC cultures on any subsequent day, no suppressive effect on CTL activity was observed. Similar results were reported by Suehiro (1987) who showed that CTL activity in mixed lymphocyte cultures could be suppressed by dexamethasone (1 x $10^{-8}$M) if added on the first day of culture, but no suppression of CTL activity was observed when dexamethasone was added 48 hours later at a 100-fold higher concentration. Taken together, these in vitro results support the in vivo results of these studies and the findings of Conlon et al. (1985), suggesting that the CTL response is most sensitive to GC-induced suppression when exposure occurs near to the time of antigen challenge.

In their review of stress responses and immune suppression, Pruett et al. (1993) suggested that "suppression of one or more immunological parameters is reasonably consistent [with plasma CS elevation] when peak CS levels exceed ~200 ng/mL and when assessment of immunological parameters is done at least 6-12 hours after exposure of the animals to the stressor." In the studies described here, there is reasonable confidence that the dose rate of CS administered to mice was sufficient to elevate plasma CS to stress-like levels, well in excess of 200 ng/mL, in all in vivo experiments. However, only a slight in vivo effect was observed and in only one study. Interestingly, the timing

of CS exposure relative to the time of alloantigen challenge was more important for suppression of CTL activity than the timing of CS exposure relative to the time at which CTL activity was measured. It is not known if a similar relationship exists for virus-induced CTL activity. The degree of CTL suppression observed in CS exposed mice was minor compared to that observed in stress-induced immune suppression models (e.g., antibody or delayed-type hypersensitivity responses) (Riley, 1981; Landy et al., 1982) although the plasma CS levels were reasonably similar. This suggests that the CTL response may be less sensitive to plasma CS elevation than other immune responses. However, since CS is not the only mediator of stress-induced immune suppression (Pruett et al., 1993), further comparative study will be required to determine the differential sensitivities of immune functions to CS.

# CHAPTER V

## SUPPRESSION OF PROLACTIN AND CYTOTOXIC T LYMPHOCYTE ACTIVITY IN PCB-TREATED MICE

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## ABSTRACT

Halogenated aromatic hydrocarbons (HAH) are ubiquitous environmental contaminants. Studies in rats have shown that HAH treatment can lead to dysregulation of circulating hormone levels, including prolactin. Reduction of prolactin levels in both rats and mice is inhibitory to immune function. Previous studies have reported suppression of alloantigen-specific cytotoxic T lymphocyte (CTL) activity in mice treated with 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB). Here we report that treatment of mice with HxCB (10 mg/Kg body weight) leads to a significant reduction of serum prolactin levels (by 89% to 3.7 ng/mL) on day 10 post alloantigen-injection (P815 mastocytoma), the day of peak alloantigen-specific CTL activity. Prolactin levels were not altered on day 3 post alloantigen injection. Treatment with bromocryptine (5 mg/Kg/day) reduced serum prolactin levels slightly on day 3 and significantly (94% to 2.1 ng/mL) on day 10 post alloantigen injection. Splenic CTL activity was not altered by treatment with bromocryptine. The data presented here suggest that reduction of prolactin levels alone, to the extent observed in HxCB-treated mice, is not causative for CTL suppression.

## INTRODUCTION

Polychlorinated biphenyls (PCBs) are a member of a large group of structurally related HAH including halogenated dibenzo-*p*-dioxins and dibenzofurans. HAH are ubiquitous environmental contaminants with wide ranging toxicologic potential for both man and wildlife (McFarland and Clarke, 1989). Immune suppression is a hallmark of HAH toxicity in many animal species (Poland and Knutson, 1982). The toxic potency of HAH is dependent on the isomer, exposure route, species studied (Vickers et al., 1985), and binding affinity for the cytosolic aromatic hydrocarbon (Ah) receptor (Silkworth and Grabstein, 1982; Silkworth and Antrim, 1985; Kerkvliet et al., 1990a,b). The prototype Ah receptor ligand, and the most potent HAH is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); HxCB is among the most potent of the PCBs. The suppressive effects of HAH on immune function include both humoral and cellular responses (reviewed by Vos and Luster, 1989). Although the immunosuppressive effects of HAH have been widely studied, the mechanism(s) for the immunotoxic effects have yet to be resolved.

Previous studies have utilized the CTL response to tumor allografts as a model to study HAH-induced suppression of T cell function (Clark et al., 1981, 1983; Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b). However, neither TCDD (Clark et al., 1981) nor HxCB (Kerkvliet and Baecher-Steppan, 1988b), which are both highly immunosuppressive in vivo, altered T cell responses in vitro when added to mixed lym-

phocyte cultures. These findings suggest that HAH induce CTL suppression via an indirect mechanism.

One possible indirect mechanism for HAH-induced CTL suppression is an alteration of the production of, or sensitivity to, endogenous immunomodulators. The circulating levels of numerous endogenous immunomodulators have been shown to be altered by exposure to HAH, e.g., testosterone (Moore et al., 1985), glucocorticoid (GC) (Gorski et al., 1988a) and thyroid hormones (Pazdernik and Rozman, 1985). Studies have also shown reduced levels of circulating prolactin in rats treated with TCDD (Moore et al., 1989; Russell et al., 1988; Jones et al., 1987). Kerkvliet et al. (1990b) reported that HxCB treatment of alloantigen-injected mice led to elevation of plasma corticosterone, the major GC in mice. GCs have been reported to inhibit the release of prolactin in rats (Leung et al., 1980). Reduction of prolactin levels in both rats and mice by hypophysectomy or bromocryptine treatment is inhibitory to immune function (reviewed by Gala [1991] and Berczi et al. [1983]). Taken together, the potential for prolactin dysregulation in HxCB-treated mice suggested a possible indirect mechanism for CTL suppression following alloantigen injection. Therefore, prolactin was investigated as a potential mediator in HxCB-induced CTL suppression.

## METHODS

### MATERIALS

C57Bl/6 and DBA/2 mice were obtained from Jackson Laboratories (Bar Harbor, ME). The P815 mastocytoma cell line was propagated in ascites form by weekly passage in DBA mice (H-$2^d$), the strain of origin. HxCB (3,3',4,4',5,5'-hexachlorobiphenyl, Ultrascientific, Hope, RI) was dissolved in HPLC grade acetone (Aldrich Chemical Co., Milwaukee, WI) and mixed with peanut oil (Nabisco Brands Inc., East Hanover, NJ). The acetone was evaporated under a stream of nitrogen for a final concentration of 1 mg/mL. Solutions of a dopamine type 2 agonist, bromocryptine (a generous gift from Sandoz Pharmaceuticals Corp., East Hanover, NJ), were prepared fresh for each experiment. Bromocryptine and an equal mass of tartaric acid (Sigma Chemical Co., St Louis, MO) were dissolved in 70% ethanol and then diluted 10 fold in distilled water. Solutions of bromocryptine, from 0.3-30 mg/mL, were filter sterilized and loaded into osmotic mini-pumps (model 2002, Alza Corp., Palo Alto, CA). The pumps were designed to release drug at a constant rate of 1 μL/hour for 14 days.

## ANIMALS

Male or female C57Bl/6 mice (H-$2^b$), 6-8 weeks of age, were used in all experiments. Animals were housed individually in polycarbonate shoe-box cages with Bed-O-Cobs (The Andersons, Maumee, OH) and were randomly assigned to positions in a cage rack. Animals were provided with Wayne Rodent Blox (Harlan Sprague Dawley Co., Bartonville, IL) and tap water *ad libitum*. Animal rooms were maintained with a 12 hr light/dark cycle (fluorescent, 7:30 AM lights on) and constant temperature (72°F ± 1°F) and 50% humidity. Animals were housed in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experimentation.

## TREATMENTS

In all studies, mice were injected ip. with 0.5 mL HBSS containing 0 or 1 x $10^7$ viable allogeneic P815 cells. In study 1, animals were given 0 or 10 mg/kg HxCB (0.1 mL per 10 g body weight) by gavage 1 day prior to alloantigen injection. In studies 2 and 3, animals received bromocryptine via osmotic mini-pump: two days prior to alloantigen injection, animals were anesthetized with ketamine and xylazine and pumps were implanted sc. along the dorsal midline. In all studies, experiments were terminated by euthanizing all animals in the morning (8:30-11:00 AM) with an overdose of $CO_2$. Death due to an overdose of $CO_2$ was rapid, approximately 30 seconds. Blood was collected by heart puncture. Serum was stored at -20°C.

## ASSESSMENT OF CTL ACTIVITY

Splenic CTL activity was measured in a chromium-51 release assay as described previously (Kerkvliet and Baecher-Steppan, 1988a). The percent cytotoxicity (CTX) at each effector:tumor (E:T) ratio was calculated by the equation:

$$\%CTX = \frac{cpm_i - cpm_{ni}}{cpm_{mr} - cpm_{ni}} \times 100,$$

where $CPM_i$ = cpm using spleen cells from P815-injected animals, $CPM_{ni}$ = cpm using spleen cells from nonP815-injected animals and $CPM_{mr}$ = the maximum cpm released from cultures incubated with sodium dodecyl sulfate.

## ANALYSIS OF SERUM PROLACTIN LEVELS

Serum prolactin levels were determined using a double antibody radioimmunoassay. Mouse prolactin and rabbit anti-mouse prolactin were obtained from Dr. J.F. Parlow (UCLA Medical Center, Torrence, CA). Sheep anti-rabbit antisera was obtained as a second antibody reagent from sheep of the Oregon State University herd that were injected with purified rabbit IgG and complete Freund's adjuvant. Standard curves were generated with each assay, and the lower limit of detection for each assay was 1 ng/mL. The maximum interassay coefficient of variance for internal controls was 7.9% as determined in this laboratory; the maximum intraassay variation was 5.9%.

## STATISTICS

Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Significant treatment effects were determined by *t* test analysis using the TTEST procedure of SAS. Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### STUDY 1: EFFECTS OF HxCB TREATMENT OVER TIME FOLLOWING P815 INJECTION

The immunosuppressive effects of HxCB treatment on CTL activity were confirmed in this study. As shown in figure 1, splenic CTL activity in HxCB-treated male mice, measured as percent cytotoxicity (CTX), was significantly suppressed on day 10 post alloantigen injection at all effector:tumor (E:T) ratios examined.

As shown in figure 2A, serum prolactin levels were not altered by HxCB treatment in alloantigen-naive male mice on day 3 or day 10 post ip. injection with 0.5 mL HBSS. In alloantigen-injected male mice, HxCB treatment significantly reduced serum prolactin levels (to 11% of vehicle control) on day 10 but not day 3 post alloantigen injection (figure 2B). Injection with alloantigen alone did not alter serum prolactin levels when measured on either day 3 or day 10 post injection (compare figure 2A vehicle to figure 2B vehicle).

Significant reductions of serum prolactin levels following HxCB treatment were confirmed for male mice on day 10 post alloantigen injection (figure 3). In contrast, pro-

lactin levels in HxCB-treated female mice were not altered (figure 3). Because male mice were more sensitive to the prolactin-reducing effects of HxCB, male mice were used in all subsequent experiments.

## STUDY 2: DOSE-RESPONSE EFFECTS OF BROMOCRYPTINE ON PROLACTIN LEVELS

A dose-dependent reduction of serum prolactin levels was observed in bromocryptine-treated male mice when measured 10 days post alloantigen injection (figure 4). Dose rates of bromocryptine of 10 mg/Kg/day or greater lowered serum prolactin levels to below the lower limit of analytical detection (1 ng/mL). A dose rate of bromocryptine at 3 mg/Kg/day reduced serum prolactin levels to 2.97 ng/mL. By extrapolation, a dose rate of 5 mg/Kg/day was considered sufficient to reduce prolactin levels to those observed in HxCB-treated male mice, and was, therefore, used in the subsequent study.

## STUDY 3: EFFECTS OF BROMOCRYPTINE ON PROLACTIN LEVELS AND CTL ACTIVITY

Treatment of male mice with bromocryptine at 5 mg/Kg/day reduced serum prolactin levels moderately on day 3 ($p = 0.052$) and significantly (to 8% of vehicle control) on day 10 post alloantigen injection (figure 5). The levels of prolactin observed in bromocryptine-treated male mice were similar to those observed in HxCB treated male mice (see figure 2). In addition, the combination of alloantigen injection and pump implantation did not significantly alter the levels of prolactin observed in vehicle-treated mice on either day 3 or day 10 when compared to mice that received neither pumps nor alloantigen (day 0, figure 5). Importantly, however, as shown in figure 6, despite the greatly reduced serum prolactin level, splenic CTL activity on day 10 post alloantigen injection was not altered by bromocryptine treatment.

**Figure V-1. The effects of HxCB treatment on CTL activity.**

Data for splenic CTL activity (percent CTX) are given for 7-8 animals per group with circles indicating the arithmetic mean ± S.E. Male mice were treated as described for study 1 in Methods. * indicates a significant difference between treatment groups for the specified E:T ratio at $p < 0.05$.

**Figure V-2. The effect of HxCB treatment and alloantigen injection on serum prolactin levels in male mice.**

Data for serum prolactin levels are given for 7-8 animals per group with bars indicating the geometric mean and with individual data indicated by closed circles. Male mice were treated as described for study 1 in Methods. Animals were injected with HBSS vehicle (panel A) or allogeneic P815 cells (panel B). For statistical purposes, serum samples with prolactin levels below the lower limit of analytical detection (1 ng/mL, represented by a dotted line) were given the value 1 ng/mL. * indicates a significant difference between treatment groups for the specified day at $p < 0.05$.

**Figure V-3. A comparison of the effect of HxCB treatment on serum prolactin levels in male and female mice.**

Data for serum prolactin levels are given for 7-8 animals per group with bars indicating the geometric mean and with individual data indicated by closed circles. Animals were treated as described for study 1 in Methods. For statistical purposes, serum samples with prolactin levels below the lower limit of analytical detection (1 ng/mL, represented by a dotted line) were given the value 1 ng/mL. * indicates a significant difference between treatment groups for the specified sex at $p < 0.05$.

**Figure V-4. The dose-response effects of bromocryptine treatment on serum prolactin levels.**

Data for serum prolactin are given as the geometric mean ± S.E. for 3 animals per group. Animals were treated as described for study 2 in Methods. The lower limit of analytical detection (1 ng/mL) is represented by a dotted line. A normal range (95% confidence interval), indicated by dashed lines, is given for animals killed 10 days post alloantigen injection.

**Figure V-5. The effects of bromocryptine treatment on prolactin levels over time.**

Data for serum prolactin are given for 4-6 animals per group with bars indicating the geometric mean and with individual data indicated by closed circles. Animals were treated as described for study 3 in Methods. For statistical purposes, serum samples with prolactin levels below the lower limit of analytical detection (1 ng/mL, represented by a dotted line) were given the value 1 ng/mL. * indicates a significant difference between treatment groups for the specified day at $p < 0.05$.

**Figure V-6. The effects of bromocryptine treatment on CTL activity.**

Data for splenic CTL activity (percent CTX) are given for 5-6 animals per group circles indicating the arithmetic mean ± S.E. Animals were treated as described for study 3 in Methods.

**Figure V-1.**

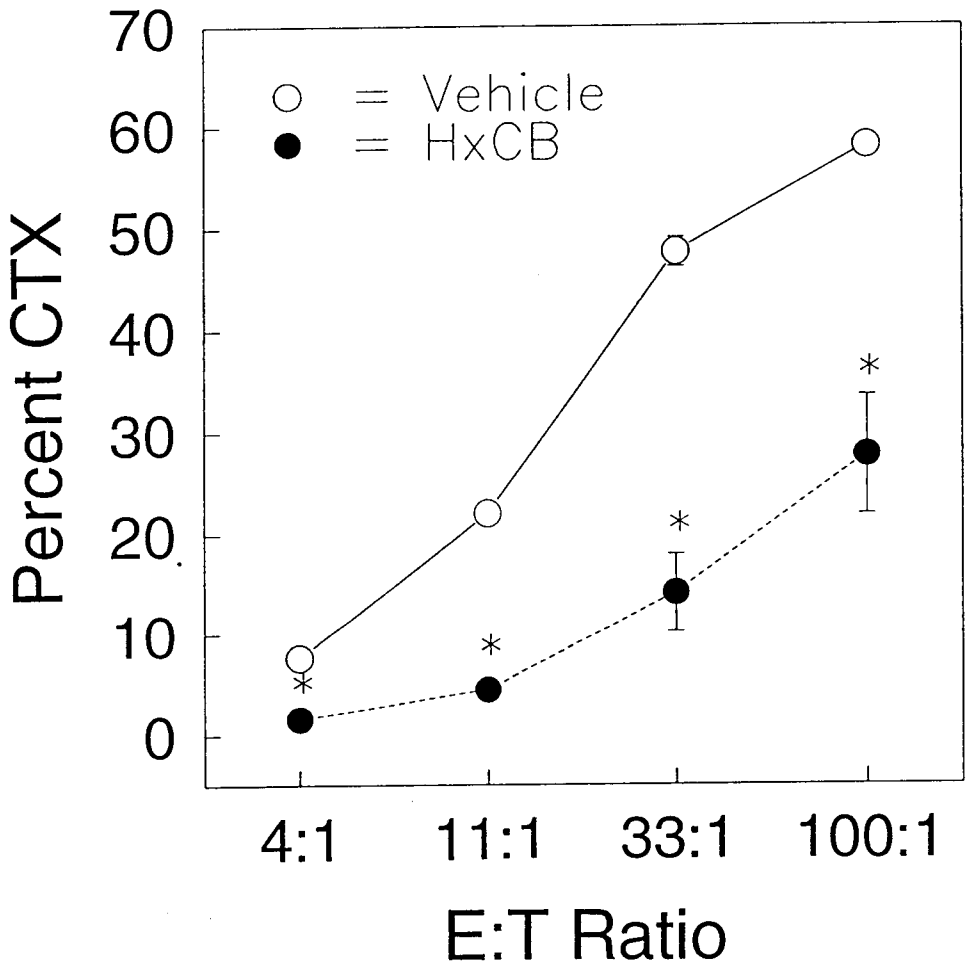

70
60
50
40
30
20
10
0
Percent CTX
○ = Vehicle
● = HxCB
*
*
*
*
4:1
11:1
33:1
100:1
E:T Ratio

**Figure V-2.**

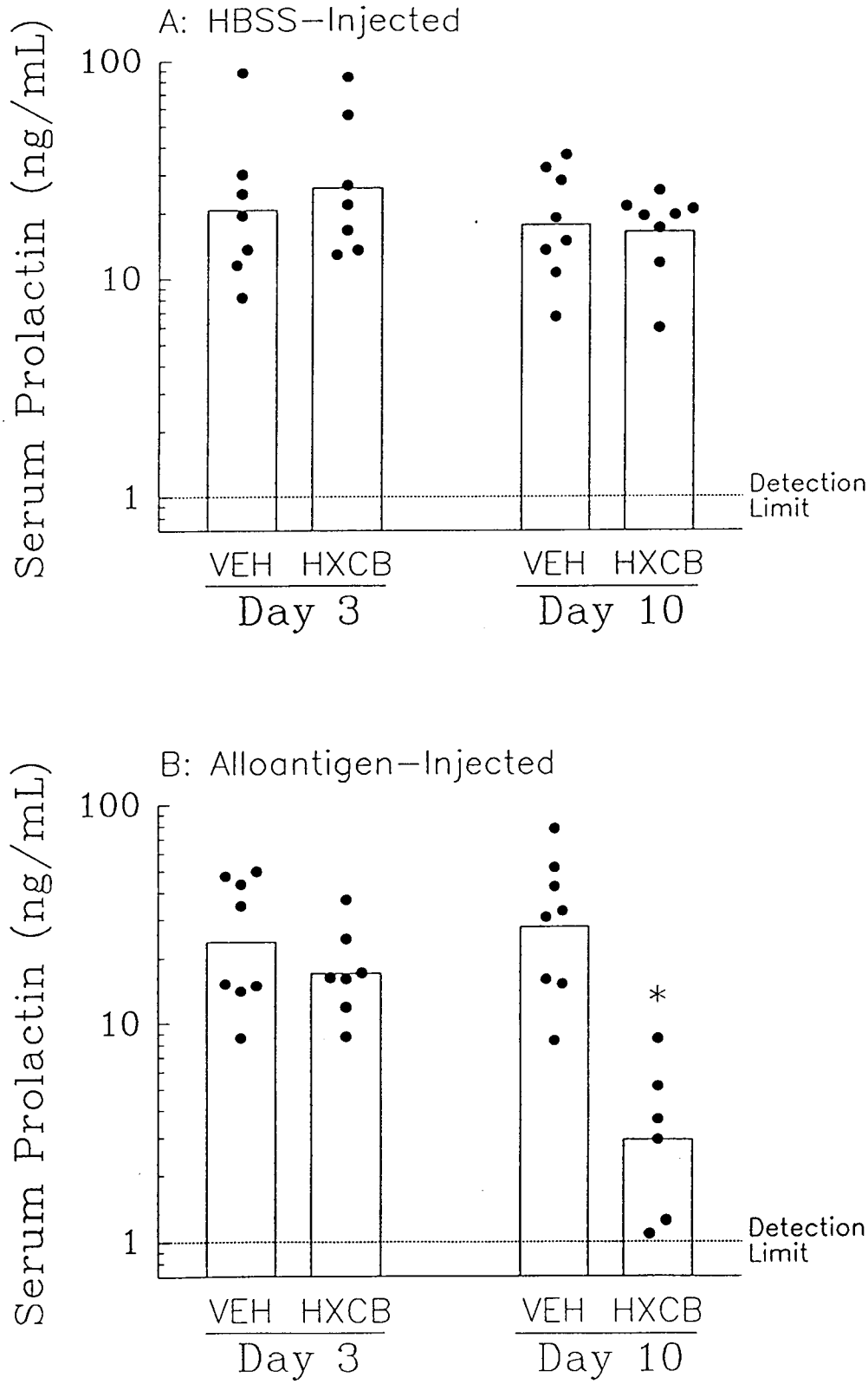

A: HBSS–Injected
Serum Prolactin (ng/mL)
100
10
1
Detection Limit
VEH HXCB
Day 3
VEH HXCB
Day 10
B: Alloantigen–Injected
Serum Prolactin (ng/mL)
100
10
1
*
Detection Limit
VEH HXCB
Day 3
VEH HXCB
Day 10

**Figure V-3.**

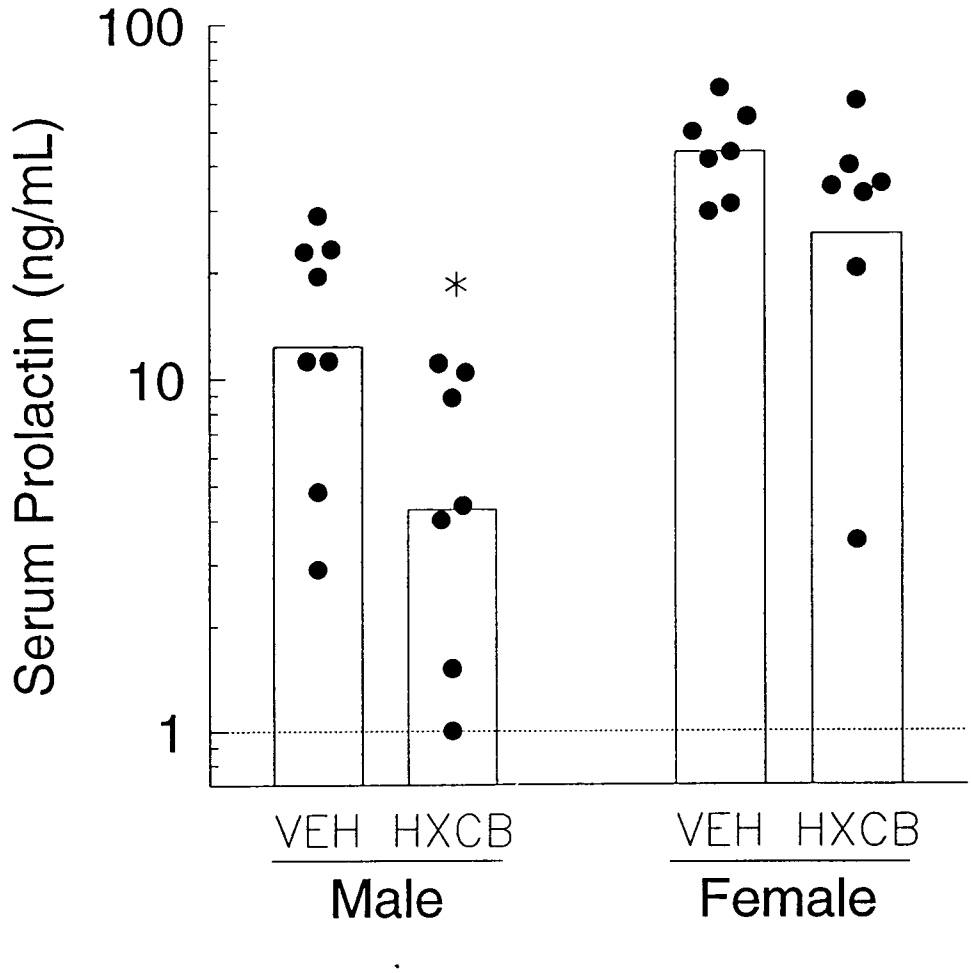


**Figure V-4.**

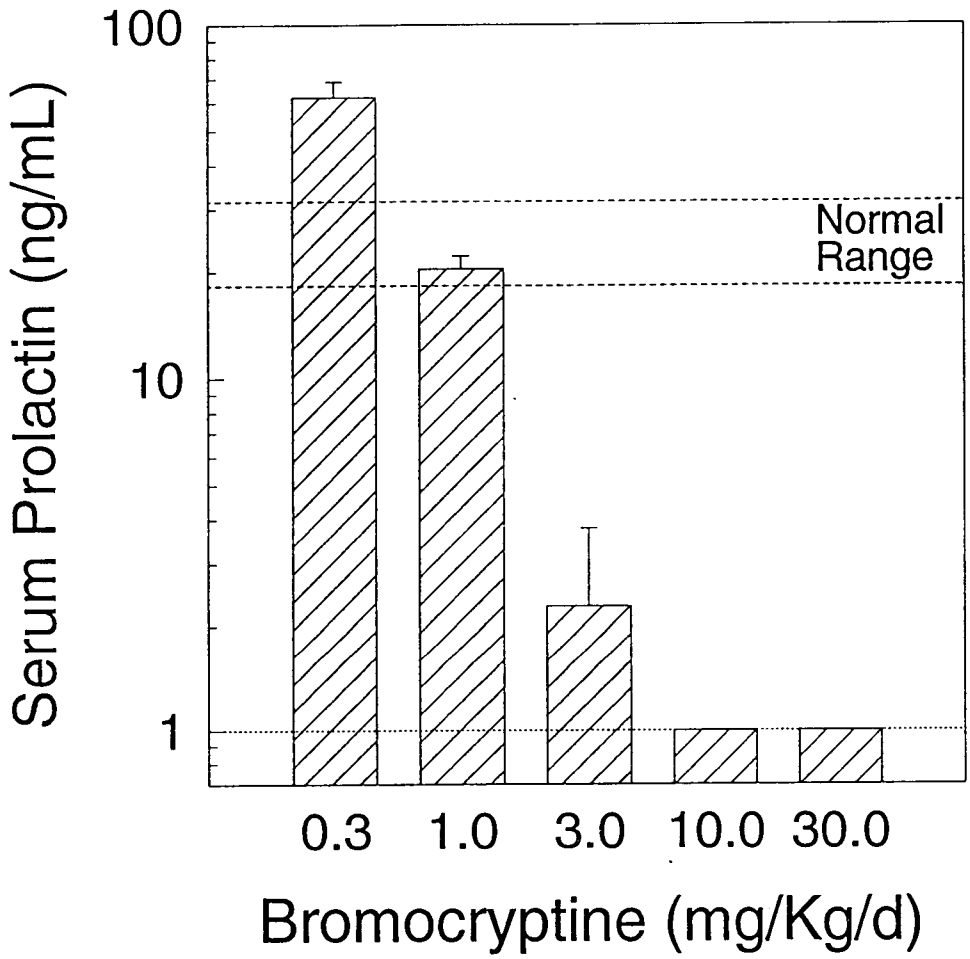

100
10
1
Serum Prolactin (ng/mL)
Normal
Range
0.3
1.0
3.0
10.0
30.0
Bromocryptine (mg/Kg/d)

**Figure V-5.**

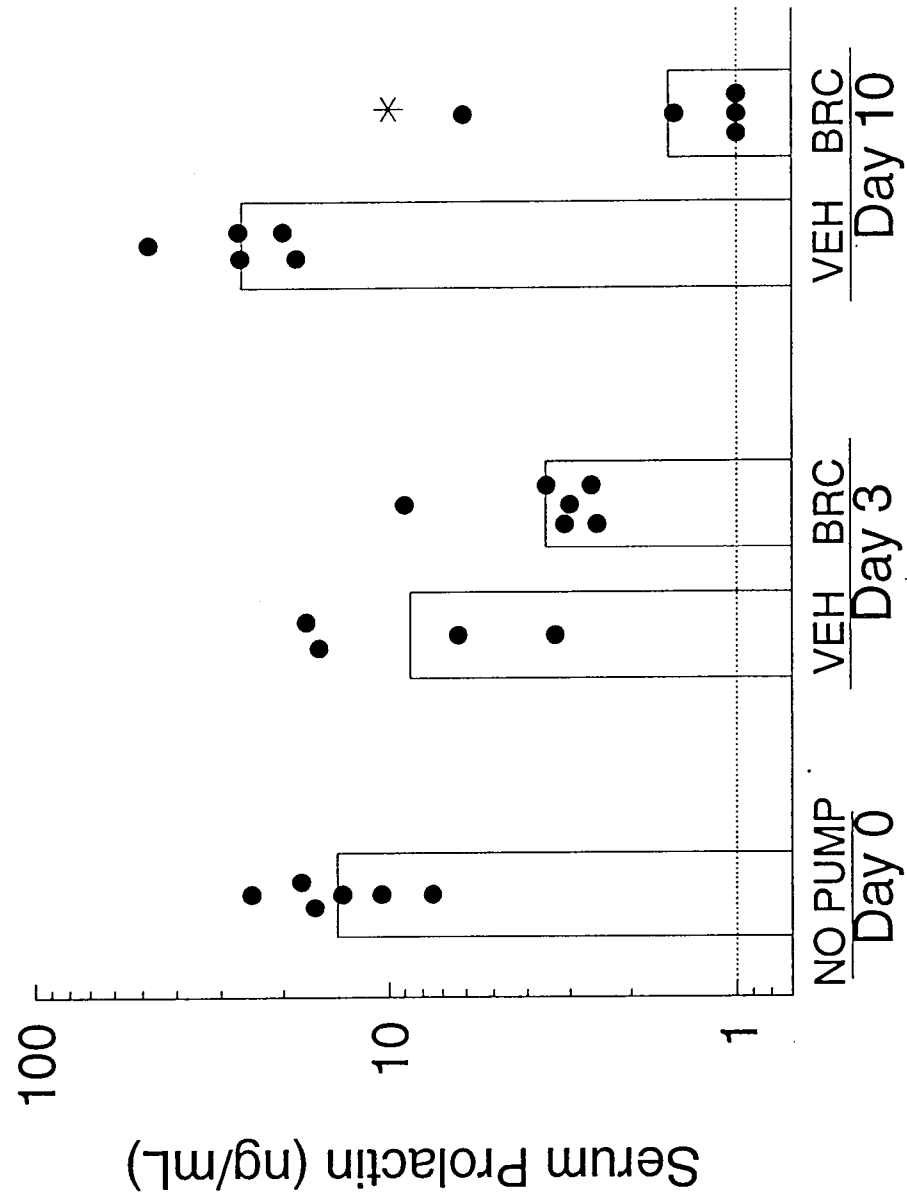
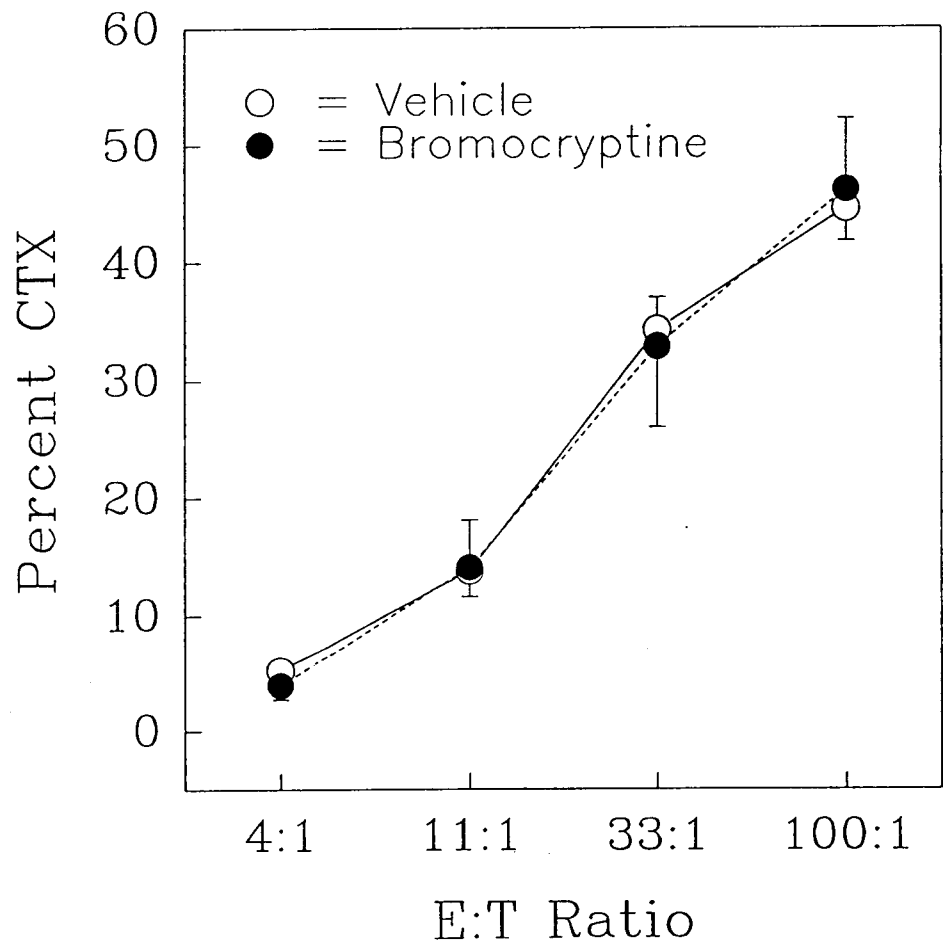

Serum Prolactin (ng/mL)
100
10
1
NO PUMP
Day 0
VEH
BRC
Day 3
VEH
BRC
Day 10

**Figure V-6.**


60
50
40
30
20
10
0
Percent CTX
○ = Vehicle
● = Bromocryptine
4:1
11:1
33:1
100:1
E:T Ratio

## DISCUSSION

The first objective of the studies reported here was to measure serum prolactin concentrations in mice after exposure to an immunosuppressive dose of HxCB. It was hypothesized that HxCB exposure in mice could lead to dysregulation of circulating prolactin levels for two reasons: 1) exposure to TCDD, an HAH structurally related to HxCB, had been previously reported to cause prolactin dysregulation in male rats (Moore et al., 1989; Russell et al., 1988; Jones et al., 1987); and 2) GCs, which are elevated in the plasma of HxCB-treated mice (Kerkvliet et al., 1990b; DeKrey et al., 1993a), have been reported to inhibit the release of prolactin in rats (Leung et al., 1980). Since, corticosterone was significantly elevated by day 3 post alloantigen injection in HxCB-treated mice, with peak levels observed on day 10 (DeKrey et al., 1993a), we measured prolactin levels on day 3 and day 10 in the present studies. Therefore, as shown here, prolactin levels were examined on both days three and 10. Results showed that prolactin levels were significantly reduced on day 10, but not on day three, in mice that received both HxCB treatment and alloantigen injection. Interestingly, prolactin levels were not altered on either day in mice that received either HxCB treatment alone or alloantigen injection alone. These results suggest an interactive effect of HxCB treatment and alloantigen injection on prolactin levels. The nature of this interaction is unclear.

A second objective of our studies was to examine the potential of prolactin as an indirect immunomodulator in HxCB-treated mice. Prolactin has been shown to be an important cofactor for lymphocyte proliferation (Berczi et al., 1991; Clevenger et al., 1991). Hypophysectomy or treatment with bromocryptine have been shown to reduce immune responses in rats, and supplementation with exogenous prolactin has been shown to restore immune function (Berczi et al., 1984, 1983, 1981; Nagy and Berczi, 1981, 1978; Bernton et al., 1988; Nagy et al., 1983). Although others have used bromocryptine to reduce prolactin levels at the time of antigen challenge (Nagy et al., 1983), in our study, bromocryptine treatment was used to reduce prolactin levels after antigen challenge. In this way, both the degree and time-course of hypoprolactinemia induced by bromocryptine mimicked that observed in HxCB-treated mice. Importantly, bromocryptine treatment did not effect CTL activity even though prolactin levels were significantly reduced on day 10. Thus, these results suggest that hypoprolactinemia, by itself, is not the cause of CTL suppression in HxCB-treated mice.

In our studies, male mice were more sensitive than female mice to the effects of HxCB with respect to reduction of serum prolactin levels. It has been previously reported that male mice are also more sensitive than female mice with respect to HxCB-induced elevation of plasma corticosterone and suppression of alloantigen-specific CTL activity (Kerkvliet et al., 1990b; DeKrey et al., 1993a). Importantly, CTL activity was significantly suppressed in female mice (Kerkvliet et al., 1990b; DeKrey et al., 1993a) at a dose of HxCB (10 mg/Kg body weight) that caused no alteration of prolactin levels. These results support the conclusion that hypoprolactinemia is not the cause of CTL suppression in HxCB-treated mice.

# CHAPTER VI

# POLYCHLORINATED BIPHENYL-INDUCED SUPPRESSION OF CYTOTOXIC T LYMPHOCYTE ACTIVITY: ROLE OF PROSTAGLANDIN-$E_2$

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## ABSTRACT

Prostaglandin (PG)$E_2$ was investigated for its role in suppression of splenic cytotoxic T lymphocyte (CTL) activity following exposure to 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) in mice. Following ip. alloantigen injection, $PGE_2$ levels significantly increased in peritoneal fluid and in spleen cell culture supernatants. HxCB exposure 1) significantly elevated $PGE_2$ levels above control in peritoneal fluid, 2) significantly reduced production of $PGE_2$ by spleen cells, and 3) did not alter $PGE_2$ production by peritoneal cells. The levels of $PGE_2$ observed were below (>100 fold) those shown by others to cause immune suppression, and splenic CTL activity was unaltered by indomethacine treatment sufficient to reduce peritoneal $PGE_2$ to undetectable levels. We conclude that altered $PGE_2$ production is not involved in suppression of CTL activity by HxCB.

## INTRODUCTION

Halogenated aromatic hydrocarbons (HAH) are widespread environmental contaminants that pose potential health risks for both man and wildlife (McFarland and Clarke, 1989). Included among the HAH are the structurally related halogenated biphenyls, dibenzo-*p*-dioxins and dibenzofurans. HAH have been studied as individual congeners and as commercial mixtures of congeners. The toxic potency of an individual congener correlates with its binding affinity to the intracellular aromatic hydrocarbon (Ah) receptor (Silkworth and Antrim, 1985; Silkworth and Grabstein, 1989; Kerkvliet et al., 1990a,b). Binding affinity is dependent on the position(s) and degree of halogen substitution (Silkworth and Antrim, 1985; Silkworth and Grabstein, 1989). The prototype Ah receptor ligand, and the most potent HAH is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) is among the most potent of the halogenated biphenyls.

Immune suppression, a hallmark of HAH toxicity in many species, has been widely studied [reviewed by Vos and Luster, 1989), but the cellular mechanism(s) underlying the immunotoxic effects have yet to be resolved. Numerous studies have described the in vivo suppression of alloantigen-specific cytotoxic T lymphocyte (CTL) activity following exposure to TCDD (Clark et al., 1981; Clark et al., 1983) and HxCB (Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b; DeKrey et al., 1993a). In addition to suppressing CTL activity, HxCB also markedly reduces the production of both IL-2 and interferon-$\gamma$ in alloantigen challenged mice (DeKrey et al., 1993b; Steppan et al., 1993) as well as the in vivo clonal expansion of $CD8^+$ spleen cells (Brauner and

Kerkvliet, 1989). Like other HAH-induced toxicities, suppression of CTL activity in vivo is dependent on binding to the Ah receptor (Kerkvliet et al., 1990b; Clark et al., 1983).

In contrast to in vivo responses, in vitro alloantigen-specific T cell responses are not suppressed by TCDD (Clark et al., 1981) or HxCB (Kerkvliet and Baecher-Steppan. 1988b) suggesting that an indirect mechanism is responsible for HAH-induced CTL suppression in vivo. One possibility is an alteration of the production of, or sensitivity to, the endogenous immunomodulator prostaglandin (PG)$E_2$. $PGE_2$ is a potent immunomodulator (reviewed by Goodwin and Ceuppens, 1983, and Phipps et al., 1991) that is produced by a variety of cell types including macrophages (Humes et al., 1982; Bachwich et al., 1986), fibroblasts (Lin et al., 1992; Candela et al., 1993), follicular dendritic cells (Heinen et al., 1986), and endothelial cells (Schrör, 1985). $PGE_2$ suppresses immune function in ways that are similar to that observed in mice exposed to HAH. T lymphocyte activation and proliferation is inhibited by $PGE_2$ (Goodwin et al., 1977, 1978; Shapiro et al., 1993) as is CTL function (Henney et al., 1972; Leung and Mihich; 1982; Wolf and Droege, 1982). $PGE_2$ has been shown to inhibit the production of several lymphokines including IL-2 (Rappaport and Dodge, 1982; Betz and Fox, 1991) and interferon-$\gamma$ (Hasler et al., 1983; Betz and Fox, 1991. Furthermore, Quilley and Rifkind (1986) have shown that exposure to HxCB increases the production of prostaglandin $PGE_2$ in chick embryo hearts. Thus, the potential for enhanced production of $PGE_2$ in HxCB-exposed mice exists.

In the present study, we hypothesized that 1) exposure of mice to HxCB followed by alloantigen challenge would result in enhanced $PGE_2$ production, and 2) inhibition of prostaglandin production by treatment with indomethacine would alleviate HxCB-induced CTL suppression.

## METHODS

### ANIMALS

Male C57Bl/6 mice, 6 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed individually in polycarbonate shoe-box cages in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experimentation. Cages were randomly assigned to positions in a cage rack, and animals were provided with Bed-O-Cob bedding (The Andersons, Maumee, OH). Food (Wayne

Rodent Blox, Harlan Sprague Dawley Co., Bartonville, IL) and tap water were available *ad libitum*. Animal rooms were maintained with a 12 hr light/dark cycle (fluorescent, 7:30 AM lights on) and constant temperature (72 ± 1 [°F]) and 50% humidity.

## ANIMAL TREATMENT

The P815 mastocytoma cell line was propagated in ascites form by weekly passage of 6 x $10^5$ cells in DBA mice (H-$2^d$), the strain of origin. P815 cells were harvested from DBA mice after 6-8 days of in vivo growth. C57Bl/6 mice (H-$2^b$) were injected ip. with 1.0 x $10^7$ viable P815 cells in 0.5 mL of Hanks Balanced Salt Solution (HBSS). Environmental standard grade (99% purity) 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) was obtained from Ultrascientific, Hope, RI. HxCB was dissolved in acetone and mixed with peanut oil (Nabisco Brands Inc., East Hanover, NJ); the acetone was evaporated under a stream of nitrogen for a final concentration of 1.0 mg/mL. Animals were given 0 (vehicle only) •or 10 mg/kg HxCB (0.1 mL per 10 g body weight) by gavage. A dose of HxCB at 10 mg/Kg body weight has been previously shown to cause reproducible and statistically significant suppression of CTL activity (Kerkvliet and Baecher-Steppan, 1988a; DeKrey et al., 1993a). Indomethacine (Sigma Chemicals, St. Louis, MO) was dissolved in ethanol to 17 mg/mL and then diluted in phosphate buffered saline (PBS) to 170 µg/mL for injection. Animals were injected ip. with indomethacine (1.7 mg/Kg/day) or PBS of equal volume.

## EXPERIMENTAL DESIGN AND SAMPLE COLLECTION

Animals were exposed to HxCB or vehicle one day prior to injection with P815 (day 0). Some animals were injected with indomethacine on days 4-8 post P815 injection. Animals were transported from the animal facility to the laboratory during the evening of the day prior to killing. Animals were killed with an overdose of $CO_2$ between 8:30 AM and 11:00 AM on day 9 post P815 injection.

At necropsy, peritoneal fluid (PF) and cells (PC) were collected by lavage with 5 mL cold RPMI-1640 containing 1% FBS, 20 mM HEPES buffer, 50 µg/mL gentamicin and 1% sodium citrate. Cell free PF was isolated by centrifugation and stored at -70°C until analyzed. PC were cultured (2.5-4.4 x $10^7$ cells/mL) for six hours at 37°C with 5% $CO_2$ in serum free Ultraculture (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine, 50 µg/mL gentamicin, 20 mM HEPES buffer and 5 x $10^{-5}$M 2-mercaptoethanol. Spleens were removed and single cell suspensions were prepared; erythrocytes were lysed by incubating with water for 10 seconds. Spleen cells

($1.0 \times 10^7$) were cultured in 2 mL volumes in 24-well tissue culture plates (Corning, Corning, NY) for six hours at 37°C with 5% $CO_2$ in the presence of $1 \times 10^6$ freshly harvested P815 cells. Supernatants from PC and spleen cell cultures were harvested and stored at -70°C until analyzed.

## ANALYSIS OF $PGE_2$

$PGE_2$ was purified from 1 mL samples of PF or culture supernatant by solid-phase extraction using C18 minicolumns (Amersham Corp., Arlington Heights, IL) according to the method of (Kelly et al., 199x). $PGE_2$ was derivatized with methyl oxime overnight and $PGE_2$ methyl oximate was quantified by [$^{125}$I]-coupled double antibody radioimmunoassay (Amersham) using a method optimized by the manufacturer. Standard curves were generated with each assay. The lower limit of detection for each assay was 1.25 pg $PGE_2$. The maximum interassay coefficient of variance for internal controls was 10% and the maximum intraassay variation was 6.8% as determined by the manufacturer. For statistical purposes, samples with analyte levels below the lower limit of detection were assigned the value 1.2 pg/mL.

## ANALYSIS OF CTL ACTIVITY

Splenic CTL activity was measured in a 4-hour chromium-51 release assay as described previously (Kerkvliet and Baecher-Steppan, 1988a). The percent cytotoxicity (CTX) at each effector:target cell (E:T) ratio was calculated by the equation:

$$\%CTX = \frac{cpm_i - cpm_{ni}}{cpm_{mr} - cpm_{ni}} \times 100,$$

where $CPM_i$ = cpm using spleen cells from P815-injected animals, $CPM_{ni}$ = cpm using spleen cells from nonP815-injected animals and $CPM_{mr}$ = the maximum cpm released from cultures incubated with sodium dodecyl sulfate. E:T ratios of 3.7:1, 11:1, 33:1 and 100:1 were used.

## STATISTICAL ANALYSIS

Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Significant treatment effects were determined by analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of SAS. Comparisons between two means were per-

formed using *t* tests (TTEST of SAS). Comparisons between more than two means were performed using LSD multiple comparison *t* tests (GLM of SAS). Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### STUDY 1: $PGE_2$ LEVELS IN PF AND SPLEEN CELL SUPERNATANTS

$PGE_2$ levels in PF were significantly increased on days 6-9 in response to P815 injection in both vehicle- and HxCB-exposed mice (figure 1A) with a maximum (3.3 pg/mL) on day seven in both treatment groups. On day nine, although peritoneal $PGE_2$ levels had declined in both treatment groups, levels in HxCB-exposed mice were significantly higher (146%) than in vehicle-exposed mice. The volumes of PF recovered from animals on each day were not different between treatment groups (data not shown). As shown in figure 1B, the production of $PGE_2$ by cultured PC obtained on days 5-9 was similar for both treatment groups and there was no difference in amount produced between days.

As shown in figure 2, the production of $PGE_2$ by spleen cells following P815 injection increased dramatically from day three to day six in both vehicle- and HxCB-exposed mice. $PGE_2$ production continued to increase in vehicle-exposed mice on days 7-9, while $PGE_2$ production declined in HxCB-exposed mice during this time period.

### STUDY 2: EFFECTS OF HXCB AND INDOMETHACINE TREATMENT

Because PGE2 levels were measurable in the first study beginning on day five post P815 injection, daily indomethacine treatments in this study were begun on day four. As shown in figure 3, HxCB exposure caused significant suppression of splenic CTL activity. Indomethacine treatment, by itself, had no effect on CTL activity in vehicle-exposed mice, and indomethacine treatment did not alter the degree of HxCB-induced CTL suppression despite the fact that indomethacine reduced $PGE_2$ levels in PF to below the limit of detection in both vehicle- and HxCB-exposed mice (table VI-1).

On the other hand, indomethacine treatment alone significantly increased the number of spleen cells (table VI-1) while HxCB reduced spleen cell numbers. Indomethacine did not clearly influence this effect of HxCB since the percent decrease induced by HxCB was similar in both controls and indomethacine-treated mice.

**Table VI-1. Effects of HxCB and indomethacine treatment on spleen cell numbers and PF $PGE_2$ levels[a]**

| HxCB | Indomethacine | Spleen Cells ($\times 10^6$) | $PGE_2$ (pg/mL) |
|---|---|---|---|
| - | - | $15.3 \pm 1.0^c$ | $4.2 \pm 1.3$ |
| + | - | $9.8 \pm 0.4^d$ | $5.7 \pm 0.5$ |
| - | + | $19.3 \pm 0.8^e$ | $< 1.25^b$ |
| + | + | $13.1 \pm 0.6^f$ | $< 1.25^b$ |

[a]Values represent the mean ± S.E. for 4-5 animals. All animals were injected with P815 cells one day after exposure to HxCB. Animals were injected with indomethacine on days 4-8 post P815 injection and killed on day nine. One animal in the double-vehicle treatment group died prior to day nine. PF was collected and processed as indicated in Materials and Methods.

[b]$PGE_2$ levels in all samples for this group were below the limit of detection.

[c,d,e,f]Values with different superscripts are significantly different ($p < 0.05$) as determined by ANOVA followed by multiple comparison *t* tests. No statistical interaction was found between HxCB and indomethacine treatment.

**Figure VI-1. $PGE_2$ levels in PF and PC culture supernatant over time post P815 injection.**

Data are presented as mean ± S.E. for 4-8 mice (panel A) or 4-5 mice (panel B) per group. Animals were injected ip. with 1 x $10^7$ P815 cells one day following exposure to HxCB at 0 or 10 mg/kg. Animals were killed on the days indicated. For statistical purposes, samples with $PGE_2$ levels below the lower limit of detection (1.25 pg/mL, dotted line) were given the value 1.2 pg/mL. For panel A, the number of samples below the limit of detection for vehicle- and HxCB-exposed groups, respectively, are given as follows (as a ratio of the total sample size): day 1, 5/7 & 4/6; day 3, 5/5 & 4/5; day 5, 3/4 & 5/5; day 6, 0/7 & 0/7; day 7, 0/7 & 0/8; day 9, 2/8 & 0/8. * indicates a significant difference between treatment groups on a single day ($p < 0.05$).

**Figure VI-2. $PGE_2$ levels in spleen cell culture supernatant over time post P815 injection.**

Data are presented as mean ± S.E. for 4-8 mice per group. Animals were treated as described in figure 1. For statistical purposes, samples with $PGE_2$ levels below the lower limit of detection (1.25 pg/mL, dotted line) were given the value 1.2 pg/mL. * indicates a significant difference between treatment groups on a single day ($p < 0.05$).

**Figure VI-3. Indomethacine treatment does not alter HxCB-induced splenic CTL suppression.**

Data are presented as mean ± S.E. for 4-5 animals per group. Animals were injected ip. with 1 x $10^7$ P815 cells one day following exposure to HxCB at 0 or 10 mg/kg. Animals were injected ip. with indomethacine at 0 or 1.7 mg/Kg/day on days 4-8 as indicated. All animals were killed on day nine. The % CTX for HxCB-exposed mice was significantly less than vehicle-exposed mice at each E:T ratio ($p < 0.05$).

Figure VI-1.

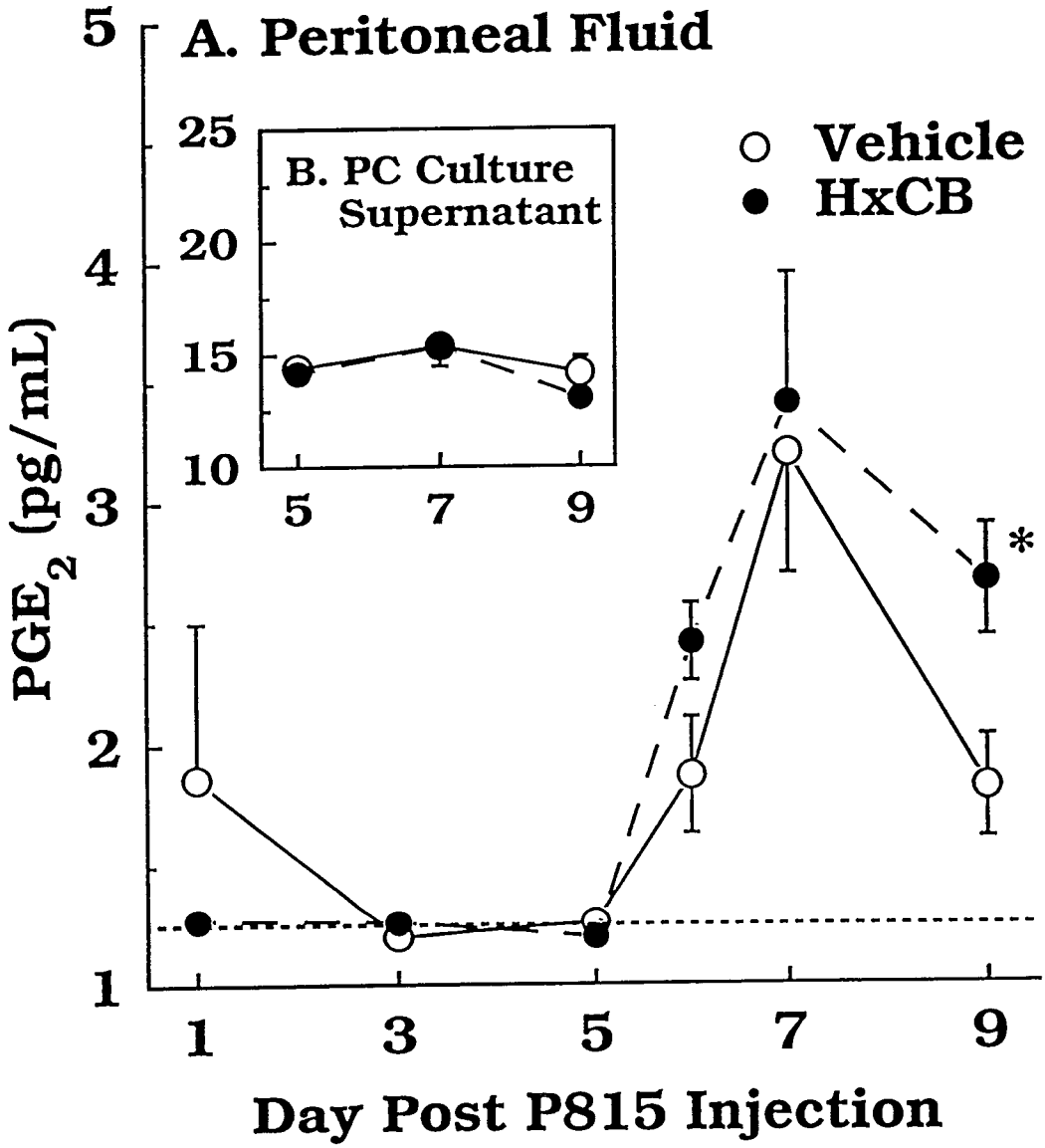

A. Peritoneal Fluid
B. PC Culture Supernatant
Vehicle
HxCB
PGE2 (pg/mL)
Day Post P815 Injection
*

**Figure VI-2.**

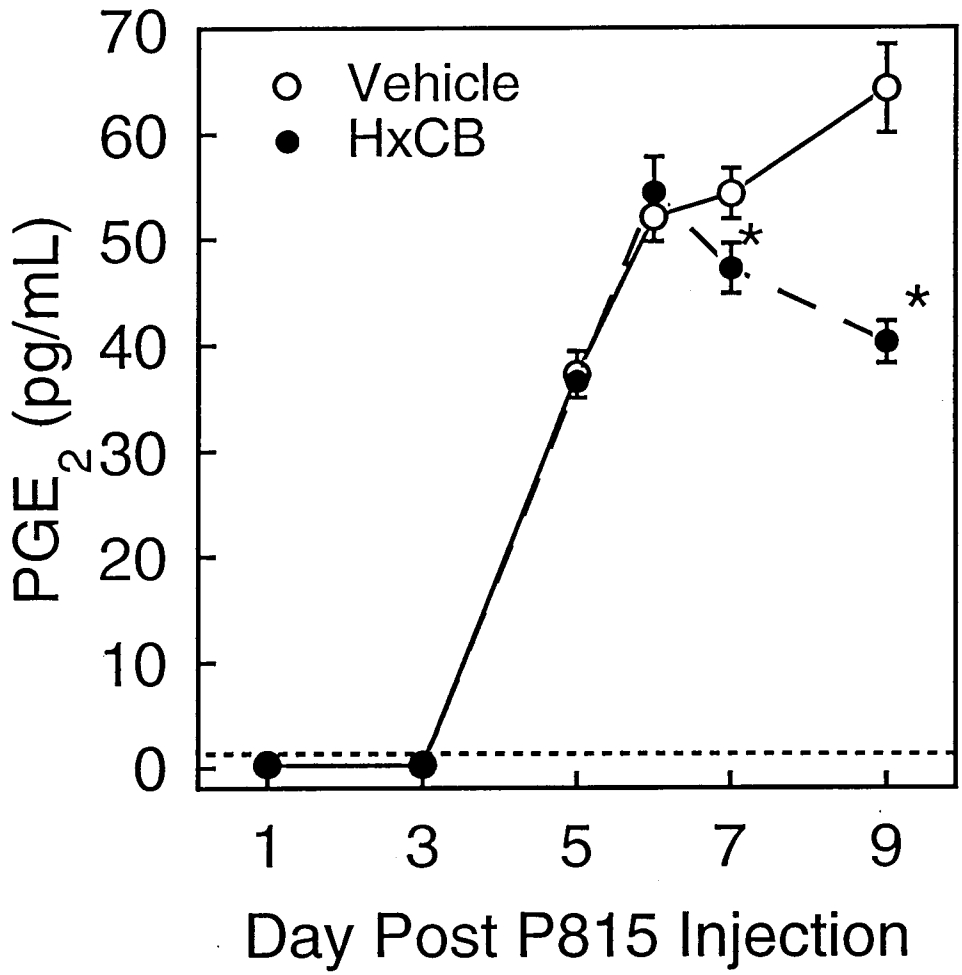
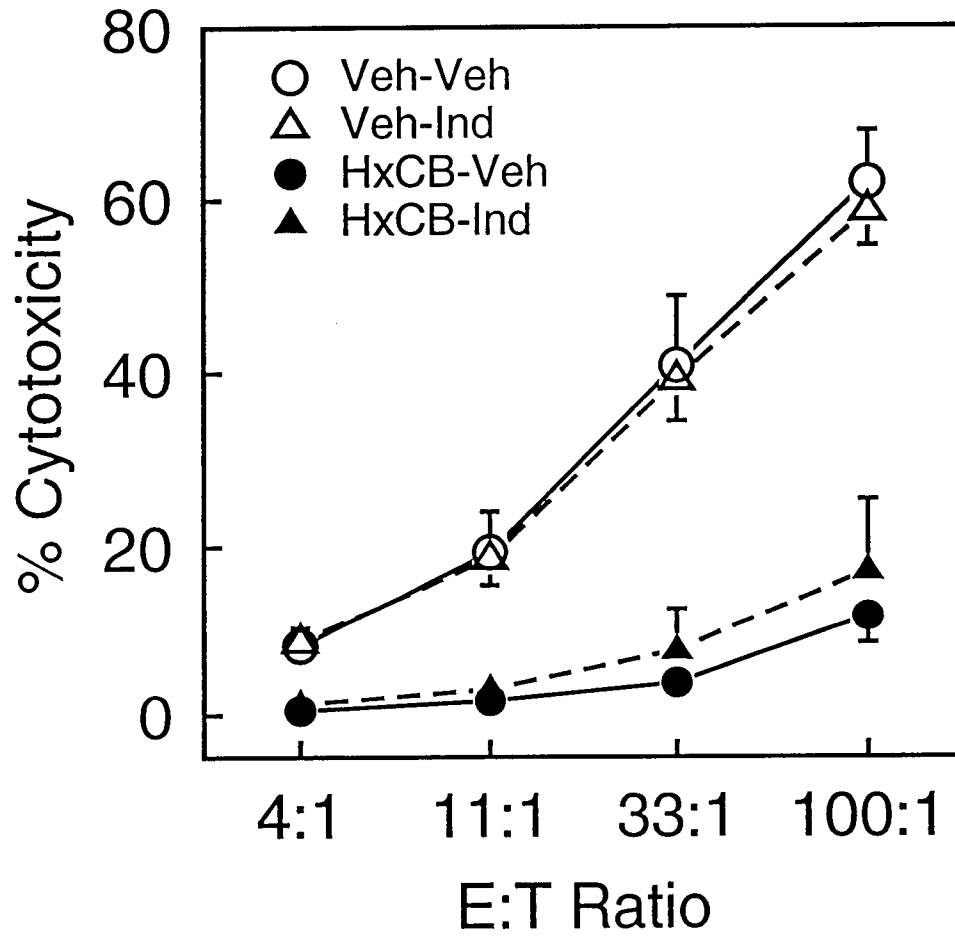

70
60
50
40
30
20
10
0
PGE2 (pg/mL)
Vehicle
HxCB
*
*
1
3
5
7
9
Day Post P815 Injection

**Figure VI-3.**


80
60
40
20
0
% Cytotoxicity
Veh-Veh
Veh-Ind
HxCB-Veh
HxCB-Ind
4:1
11:1
33:1
100:1
E:T Ratio

## DISCUSSION

$PGE_2$ is a potent immunomodulator that has been shown to suppress CTL responses in vitro (Henney et al., 1972; Leung and Mihich, 1982; Wolf and Droege, 1982; Skinner et al., 1989). The objective of the studies reported here was to determine the role of $PGE_2$ in suppression of CTL activity after exposure to an immunosuppressive dose of HxCB.

In vehicle-exposed mice, the levels of $PGE_2$ in PF taken from the site of P815 injection varied significantly over time from a low on days three and five to a high on day seven post P815 injection. As previously reported (Kerkvliet and Baecher-Steppan, 1988a), the numbers and type of cells found in the peritoneal cavity of P815-injected C57Bl/6 mice (macrophages, neutrophils, lymphocytes, P815 cells) changed markedly over time. Interestingly, however, in these studies the production of $PGE_2$ by cultured PC was invariant from day five through day nine suggesting that the variation of $PGE_2$ levels in PF was due to production by a cell type not found in PC (for example, fibroblasts or endothelial cells). Unlike cultured PC, the production of $PGE_2$ by cultured spleen cells was elevated in a time dependent-fashion and correlated with the development of CTL activity in the spleen (Kerkvliet and Baecher-Steppan, 1988a).

In mice exposed to HxCB, $PGE_2$ levels in PF were significantly elevated on day nine post P815 injection, whereas $PGE_2$ production by cultured PC was unchanged. This supports the above conclusion that the variation in $PGE_2$ levels in PF was not due to production by PC. The mechanism of HxCB-induced $PGE_2$ elevation on day nine is unknown, but enhanced $PGE_2$ production is more likely than a reduction of $PGE_2$ clearance since HAH (Aroclor 1254 and Firemaster BP-6) have been shown to enhance prostaglandin metabolism (Theoharides and Kupfer, 1981). In contrast to peritoneal $PGE_2$, HxCB exposure significantly decreased the production of $PGE_2$ by cultured spleen cells on days seven and nine. The mechanism for reduced splenic $PGE_2$ production is unknown.

Previous studies in vitro have shown that $PGE_2$ does not affect CTL effector function (Leung and Mihich, 1980; Jordan et al., 1987) or the proliferation of CTL clones (Jordan et al., 1987; Skinner et al., 1989). In addition, $PGE_1$ suppresses in vitro CTL generation only if added within the first 48 hours post culture initiation (Leung and Mihich, 1980, 1982). These results suggest that prostaglandins may only affect an early step in the CTL response. Interestingly, the in vivo response to P815 injection included an elevation of $PGE_2$ but not until day five. HxCB effects on $PGE_2$ levels were not

observed until day seven. Thus, it is unlikely that HxCB-induced changes in $PGE_2$ levels influence CTL activity in this model.

Indomethacine was used in these studies to investigate the role of prostaglandin in HxCB-induced CTL suppression. Since $PGE_2$ levels were measurable beginning on day five post P815 injection, daily indomethacine treatments were begun on day four. The dose of indomethacine administered was sufficient to reduce day nine peritoneal $PGE_2$ levels to below the limit of detection. However, indomethacine treatment did not alter CTL activity in vehicle-exposed mice, and, importantly, it also did not alleviate CTL suppression in HxCB-exposed mice. These results suggest that elevated $PGE_2$ does not influence CTL activity late in the response, and, furthermore, we conclude that altered $PGE_2$ production is not involved in suppression of CTL activity by HxCB.

The levels of $PGE_2$ in both vehicle- and HxCB-exposed mice were less than 6 pg/mL in PF and less than 70 pg/mL in spleen cell supernatants. These levels were markedly lower than the plasma $PGE_2$ levels correlated with immune suppression in mice bearing the Lewis lung sarcoma (900-1200 pg/mL) (Young and Hoover, 1986) and were also lower than the concentrations of $PGE_2$ required to suppress the generation of CTL in vitro (>3 ng/mL) (Leung and Mihich, 1982; Wolf and Droege, 1982; Parhar and Lala, 1988; Skinner et al., 1989). This supports the conclusion that $PGE_2$ is not involved in suppression of CTL activity by HxCB.

# CHAPTER VII

## POLYCHLORINATED BIPHENYL-INDUCED IMMUNE SUPPRESSION: SUPPRESSED SPLENIC CYTOTOXIC T LYMPHOCYTE ACTIVITY CORRELATES WITH REDUCED SPLENIC $CD8^+$ CELL NUMBERS AND ALTERED IL-2, IL-6 AND IFN-$\gamma$ PRODUCTION IN RESPONSE TO ALLOANTIGEN

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## ABSTRACT

Following ip. challenge of C57Bl/6 mice with allogeneic P815 tumor cells, a vigorous allograft rejection response is observed. In the spleen, a significant increase in cell number is observed concurrent with the peak of alloantigen-specific cytotoxic T lymphocyte (CTL) activity on days 9-10. Pretreatment of mice with 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB), an aromatic hydrocarbon (Ah) receptor-binding polychlorinated biphenyl, results in dose-dependent suppression of CTL activity. In this study, HxCB-induced suppression of CTL activity was correlated with suppressed spleen cell proliferation and a reduced frequency of $CD8^+$ spleen cells. Since proliferation and differentiation of CTL are dependent on appropriate cytokine signalling, production of selected cytokines by spleen cells was assessed ex vivo. In vehicle-treated mice, both interleukin (IL)-2 and interferon (IFN)-$\gamma$ production increased over days 5-9 after P815 tumor cell challenge, coincident with development of splenic CTL activity. IL-6 production increased on days 1-7 and was maintained through day nine. When compared to vehicle-treated mice, HxCB treatment led to 1) an early (day five after alloantigen challenge) enhancement followed by a late (day 7-9) reduction of interleukin (IL)-2 production, 2) a highly significant suppression of interferon-$\gamma$ production on days 6-9, and 3) an enhancement of IL-6 production on days six and nine. These results suggest that altered cytokine production plays a role in HxCB-induced CTL suppression.


## INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs), including polychlorinated biphenyls (PCBs), dibenzofurans and dibenzodioxins, are ubiquitous environmental contaminants. Exposure to HAHs can induce numerous toxic responses which vary depending on the species studied (see reviews by Poland and Knudsen, 1982; Vickers et al., 1985; Safe, 1986; Goldstein and Safe, 1989). HAH-induced immunotoxicity is among the most sensitive toxic indicators described and is exceptional because it is observed in nearly all species examined (see reviews by Holsapple et al. [1991], Vos and Luster [1989], and Kerkvliet and Burleson [1994]). In mice, HAH-induced suppression of both humoral and cellular immune responses has been shown to be dependent on HAH binding with high affinity to the intracellular aromatic hydrocarbon (Ah) receptor (Kerkvliet et al., 1990a,b; Clark et al., 1983; Harper et al., 1993). The prototype Ah receptor ligand, and the most potent HAH is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). 3,3',4,4',5,5'-Hexachlorobiphenyl (HxCB) is among the most potent of the immunosuppressive PCBs.

The cytotoxic T lymphocyte (CTL) response has been used as a model to study the effects of both TCDD (Clark et al., 1981, 1983; Nagarkatti et al., 1984; Kerkvliet et al., 1990b; DeKrey and Kerkvliet, 1994) and HxCB (Kerkvliet and Baecher-Steppan, 1988a,b; Kerkvliet et al., 1990b; DeKrey et al., 1993a) on cellular immunity in mice. Alloantigen-specific CTL activity is generated by ip. injection of P815 mastocytoma cells (H-$2^d$) into allogeneic (H-$2^b$) C57Bl/6 mice. Splenic CTL activity can be measured by day six after alloantigen challenge and peak CTL activity is observed on day 10 (Kerkvliet and Baecher-Steppan, 1988a). As CTL activity increases in the spleen over days 6-10, a concurrent increase in splenic cellularity is also observed (Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b). This increase in splenic cellularity following alloantigen challenge likely reflects antigen-driven clonal proliferation.

In mice treated with HxCB, alloantigen-specific splenic CTL activity is dose-dependently suppressed (Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b). Treatment of mice with HxCB at 10 mg/Kg body weight reduces the number of spleen cells and suppresses CTL activity by ~80% when measured on day 10 after alloantigen challenge (Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b). CTL suppression is not due to altered alloantigen exposure because intraperitoneal P815 cell proliferation is unchanged by HxCB treatment (Kerkvliet and Baecher-Steppan, 1988a). Also, the kinetics of the CTL response is unaltered by HxCB treatment indicating true immune suppression rather than a delay in the CTL response (Kerkvliet and Baecher-Steppan, 1988a).

Antigen-driven clonal proliferation and development of mature CTL effector activity is dependent on production of a variety of cytokines. Interleukin (IL)-2, interferon (IFN)-$\gamma$ and IL-6 have been shown to play an important role in the generation of CTL; other cytokines such as IL-4 and IL-10 have also been linked with CTL development or suppression (see reviews by Smith, 1988; Bach et al., 1989; Ruscetti, 1990; Bertagnolli et al., 1991; Howard and O'Garra, 1992; Houssiau and Van Snick, 1992). Both TCDD and PCBs have been shown to modulate the activity of certain cytokines (Sutter et al., 1991; Taylor et al., 1992; Hoglen et al., 1992). Therefore, we hypothesized that HxCB suppresses the production of key cytokines leading to a reduction of CTL activation, proliferation and/or differentiation.

The intent of this study was 2-fold: 1) to examine the proliferation, phenotypes and cytokine production of spleen cells over time in response to in vivo alloantigen challenge, and 2) to examine the effects of HxCB exposure on the former as a possible mechanism for the suppression of CTL activity observed in HxCB-treated mice. The

production of IL-2, IL-4, IL-6, IL-10, and IFN-γ was determined by measuring the concentration of cytokines in spleen cell culture supernatants after short-term (six hours) culture. *Ex vivo* spleen cell proliferation was determined by measuring incorporation of $^{3}$H-thymidine, and the phenotypes of spleen cells ($CD4^{+}$, $CD8^{+}$, $Ig^{+}$) were determined by flow cytometry.

## METHODS

### Animals

Male C57Bl/6 mice, 6 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed individually in polycarbonate shoe-box cages in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experimentation. Cages were randomly assigned to positions in a cage rack, and animals were provided with Bed-O-Cob bedding (The Andersons, Maumee, OH). Food (Wayne Rodent Blox, Harlan Sprague Dawley Co., Bartonville, IL) and tap water were available *ad libitum*. Animal rooms were maintained with a 12 hr light/dark cycle (fluorescent, 7:30 AM lights on) and constant temperature (72 ± 1 [°F]) and 50% humidity. The mice remained seronegative for all common murine pathogens as assessed by Charles River Professional Services, Wilmington, MA.

### Animal Treatment

The P815 mastocytoma cell line was propagated in ascites form by weekly passage of 6 x $10^{5}$ cells in syngeneic DBA mice (H-$2^{d}$). P815 cells were harvested from DBA mice after 6-8 days of in vivo growth. Allogeneic C57Bl/6 mice (H-$2^{b}$) were injected ip. with 1.0 x $10^{7}$ viable P815 cells in 0.5 mL of Hanks Balanced Salt Solution (HBSS) (BioWhittaker, Walkersville, MD). Environmental standard grade (99% purity) 3,3',4,4',5,5'-hexachlorobiphenyl (HxCB) was obtained from Ultrascientific, Hope, RI. HxCB was dissolved in acetone and mixed with peanut oil (Nabisco Brands Inc., East Hanover, NJ); the acetone was evaporated under a stream of nitrogen for a final concentration of 1.0 mg/mL. Animals were given 0 or 10 mg/kg HxCB (0.1 mL per 10 g body weight) by gavage. A dose of HxCB at 10 mg/Kg body weight has been previously shown to cause reproducible and statistically significant suppression of CTL activity (Kerkvliet and Baecher-Steppan, 1988a, DeKrey et al., 1993a).

## Experimental Design

Animals were exposed to HxCB or vehicle one day prior to alloantigen challenge. Animals were killed with an overdose of $CO_2$ between 8:30 AM and 11:00 AM on various days after alloantigen challenge. Spleens were removed and single cell suspensions were prepared in HBSS supplemented with 20 mM HEPES buffer and 50 µg/mL gentamicin. Erythrocytes were lysed by incubating with water for 10 seconds.

## Proliferation Assay

Potential contamination by P815 cells was eliminated by treatment of spleen cells with anti-P815 antibody and Low-Tox rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) prior to culture. The anti-P815 antiserum was obtained from C57Bl/6 mice injected 10-15 days previously with 1 x $10^7$ P815 cells. The antiserum was heat-inactivated and titrated for complement-dependent cytotoxic activity on P815 cells. Spleen cells were treated with antiserum and complement, washed, and cultured at 1 x $10^6$ cells/0.2 mL in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS (Hyclone, Orem, UT), 10 mM HEPES and 5 x $10^{-5}$ M 2-ME in wells of flat-bottom 96-well plates. $^3$H-Thymidine (0.5 µCi) was added to each well at the initiation of culture. After 18 hours, cells were collected onto glass fiber filter paper using a cell harvester (Skatron, Inc., Sterling, VA). The filter discs were placed in minivials with 2 mL scintillation fluor (Ecolume®, ICN Biomedicals Inc., Irvine, CA) and analyzed for radioactivity by liquid scintillation spectroscopy (Packard Instruments, Downers Grove, IL). Each sample was counted for 10 minutes or to a preset count of 10,000. Counts per minute (CPM) of triplicate samples were averaged.

## Flow Cytometry

Phycoerythrin (PE)-conjugated rat anti-mouse CD4, CD8 and Ig-kappa monoclonal antibodies and streptavidin were purchased from Becton-Dickenson, Mountainview, CA. Biotinylated rat IgG (Accurate Chemical and Scientific Corp., Westbury, NY) was used as a control reagent. Spleen cells were labelled with fluorochrome-conjugated antibodies, fixed with 0.7% paraformaldehyde and analyzed using an EPICS V flow cytometer (Coulter Electronics, Hiahleah, FL) as described in Neumann et al. (1992).

## Cytokine Measurement

Spleen cells ($1.0 \times 10^7$) were cultured with 2 mL serum free Ultraculture (Bio-Whittaker) supplemented with 2 mM L-glutamine, 20 mM HEPES buffer and $5 \times 10^{-5}$ M 2-mercaptoethanol in 24-well tissue culture plates (Corning, Corning, NY) for six hours at 37°C with 5% $CO_2$ in the presence of $1 \times 10^6$ P815 cells. All culture supernatants were stored at -70°C until analyzed. Culture supernatants were analyzed for multiple cytokines using antibody sandwich ELISAs. Commercial ELISA kits (Endogen Inc., Boston, MA; Genzyme, Cambridge, MA; Collaborative Biomedical Research, Bedford, MA) were used to measure murine cytokines: IL-2, IL-4, IL-6, IL-10, IFN-γ. Each ELISA was performed using protocols optimized by the manufacturers. Performance characteristics for each ELISA kit are listed in table VII-1.

**Table VII-1. ELISA kit specifications as provided by manufacturers**

| | | Assay %CV | | |
|---|---|---|---|---|
| Cytokine | Detection Range | Inter | Intra | Manufacturer |
| IL-2 | 15-240 pg/mL | ≤8.9 | ≤7.0 | Genzyme |
| IL-2 | 200-10,000 pg/mL | ≤11.1 | ≤8.2 | Collaborative |
| IL-4 | 15-375 pg/mL | ≤10.0 | ≤10.0 | Endogen |
| IL-6 | 50-1250 pg/mL | ≤10.0 | ≤10.0 | Endogen |
| IL-10 | 1.5-13.5 U/mL[a] | ≤10.0 | ≤10.0 | Endogen |
| IFN-γ | 125-4100 pg/mL | ≤10.0 | ≤10.0 | Genzyme |

[a]1.0 Unit of IL-10 is equivalent to 160 pg IL-10.

## Statistical Analysis

Statistical analyses were performed using the SAS statistical software database (version 6.03, SAS Institute Inc., Cary, NC) for the IBM personal computer. Significant treatment effects were determined by analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of SAS. Comparisons between more than two means were performed using LSD multiple comparison *t* tests (GLM of SAS). Comparisons between two means were performed using *t* tests (TTEST of SAS). Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### Splenic CTL activity on days 6-10 after alloantigen challenge.

As shown if figure 1, the cytotoxic activity of spleen cells toward allogeneic P815 cells on days 7-10 post alloantigen challenge was significantly suppressed by pre-treatment with HxCB at a dose of 10 mg/Kg body weight. Suppression of CTL activity by HxCB is a highly consistent observation between studies (Kerkvliet and Baecher-Steppan, 1988a; Kerkvliet et al., 1990b; DeKrey et al., 1993a).

### Spleen cell numbers and spleen cell phenotypes on days 0-10 after alloantigen challenge.

As shown in figure 2, the number of spleen cells in vehicle-treated animals had significantly increased (>1.5-fold) by day 10 in response to alloantigen challenge. Concurrently, there was a significant increase in the percentage (2.4-fold) and total number (3.5-fold) of $CD8^+$ spleen cells (figures 3A and 3D). In contrast, there was no significant alteration in the percentage or total number of $CD4^+$ spleen cells (figures 3B and 3E). Alloantigen challenge did not alter the percentage of $Ig^+$ spleen cells (figure 3C), however the total number of $Ig^+$ cells was significantly increased on day 10 (figure 3F).

In HxCB-treated mice, the increase in spleen cell numbers following alloantigen challenge was significantly suppressed (figure 2). Concurrently, HxCB treatment also suppressed an increase in the percentage and total number of $CD8^+$ spleen cells. The percentage of $CD4^+$ spleen cells was not altered by HxCB treatment (figure 3B), but the total number of $CD4^+$ cells was significantly decreased on day 10 (figure 3E). Both the percentage and total number of $Ig^+$ spleen cells were significantly decreased by HxCB treatment on day 10 (figures 3C and 3F).

### Ex vivo spleen cell proliferation on days 2-10 after alloantigen challenge.

As shown in figure 4, $^3$H-thymidine incorporation by spleen cells increased over time after alloantigen challenge. While the level of proliferation on days 2-4 after alloantigen challenge was not different from that observed in alloantigen-naive spleen cells (figure 4), by day six, the degree of proliferation significantly increased (>3-fold) and continued to increase through day 10. In HxCB-treated mice, spleen cell proliferation was significantly suppressed on all days examined. The background level of proliferation in alloantigen-naive mice was not altered by HxCB treatment (data not shown).

**Splenic cytokine production on days 1-9 after alloantigen challenge.**

Four cytokines were detected in spleen cell culture supernatant: IFN-γ, IL-2, IL-6 and IL-4. In contrast, IL-10 was not detected on any day examined. In cultures of spleen cells from vehicle-treated mice, increased IL-6 levels were first observed on day one after alloantigen challenge (figure 5C), whereas increased levels of IL-2 and IFN-γ were first observed on day five (figures 5A and 5B). Levels of each cytokine continued to rise over time to a maximum on day nine. In cultures of spleen cells from HxCB-treated mice, increased cytokine levels were first observed at the same time as in vehicle-treated mice. Thereafter, each cytokine was affected differently. When compared to vehicle-controls, IL-2 levels were significantly higher on day five after alloantigen challenge, peaked on day six, and declined to significantly lower levels on days 7-9 in HxCB-treated mice (figure 5A). IFN-γ levels were nearly undetectable on days 6-9, and markedly lower than vehicle controls (figure 5B). IL-6 levels paralleled those of vehicle controls but were significantly higher on days six and nine (figure 5C). IL-4 production by spleen cells was highly variable; the only consistent finding between studies was significantly elevated production on day nine post alloantigen challenge in HxCB-treated mice (data not shown).

## DISCUSSION

The allograft response to P815 mastocytoma cells was characterized in this study by a significant increase in spleen cell production of IL-2 and IFN-γ which occurred coincident with an increase in the number of $CD8^+$ spleen cells and splenic CTL activity. An increase in the production of both IL-2 and IFN-γ preceded, by one day, an increase in the *ex vivo* proliferation of spleen cells on day six, which in turn preceded, by approximately one day, an increase in both $CD8^+$ spleen cells and splenic CTL activity on day seven. The increase in $CD8^+$ cells is likely reflective of the increase in CTL activity since alloantigen-specific CTL activity is predominantly mediated by $CD8^+$ cells (Moscovitch et al., 1986; Hutchinson, 1991; Sherman and Chattopadhyay, 1993). Spleen cell production of IL-2 and IFN-γ continued to rise through day nine roughly paralleling the continued time-dependent increase in spleen cell proliferation, $CD8^+$ spleen cell numbers and CTL activity. This is the first report of which we are aware that describes production of lymphokines in association with CTL activity in the P815 mastocytoma model. These correlative results suggest that IL-2 and IFN-γ play a role in the

**Figure VII-1. HxCB treatment causes suppression of alloantigen-specific splenic CTL activity.**

Alloantigen-specific CTL activity is shown as adapted from Kerkvliet and Baecher-Steppan, 1988a. Percent cytotoxicity (mean ± S.E.) is given for an E:T ratio of 33:1. Male mice (4-5 per group) were treated with HxCB at 0 or 10 mg/Kg one day prior to ip. injection with 1 x $10^7$ P815 cells. * indicates a significant difference between treatment groups on the day specified ($p < 0.05$).

**Figure VII-2. HxCB treatment inhibits ex vivo spleen cell $^3$H-thymidine incorporation.**

Data are presented as mean ± S.E. of triplicate wells for 5-6 animals per group. Spleen cell $^3$H-thymidine incorporation (proliferation) was analyzed as described in Methods. Proliferation by spleen cells from alloantigen-naive mice is represented by a dotted line. *Indicates a significant difference between treatment groups on the specified day ($p < 0.05$).

**Figure VII-3. HxCB treatment inhibits the increase in spleen cell numbers following alloantigen challenge.**

Data are presented as total number of spleen cells (mean ± S.E. for 5-6 animals per group). Spleen cells were analyzed as described Methods. * indicates a significant difference between treatment groups on the specified day ($p < 0.05$).

**Figure VII-4. HxCB treatment inhibits the increase in $CD8^+$ and $Ig^+$ spleen cell numbers following alloantigen challenge.**

Data for $CD8^+$, $CD4^+$ and $Ig^+$ cells are presented as percentage of total cells (A-C) and as total number of spleen cells (D-F). The mean ± S.E. of 5-6 animals per group is shown for vehicle- (*circle*) and HxCB-treated (*diamond*) mice. Spleen cells were analyzed as described Methods. * indicates a significant difference between treatment groups on the specified day ($p < 0.05$).

**Figure VII-5. HxCB treatment alters cytokine production by spleen cells in culture.**

Data for production of IL-2 (A), IFN-γ (B) and IL-6 (C) by spleen cells are presented. The mean ± S.E. for 5-6 animals per group is shown for vehicle- (*circle*) and HxCB-treated (*diamond*) mice. Open symbols indicate that all samples at that time point had levels below the limit of detection which is represented by a dotted line. Spleen cells were cultured and supernatants were analyzed as described in Methods. * indicates a significant difference between treatment groups for the specified day at ($p < 0.05$).

**Figure VII-1.**

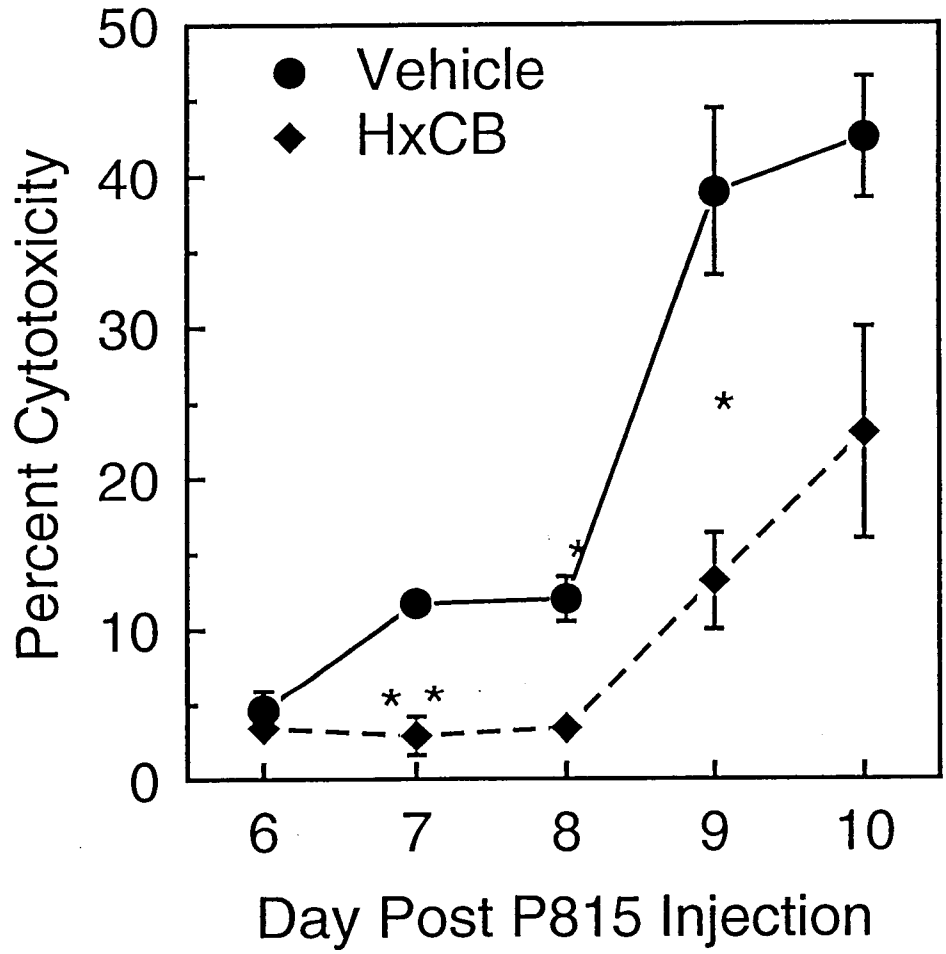

Vehicle
HxCB
Percent Cytotoxicity
50
40
30
20
10
0
6
7
8
9
10
Day Post P815 Injection
*
*
*
*

**Figure VII-2.**

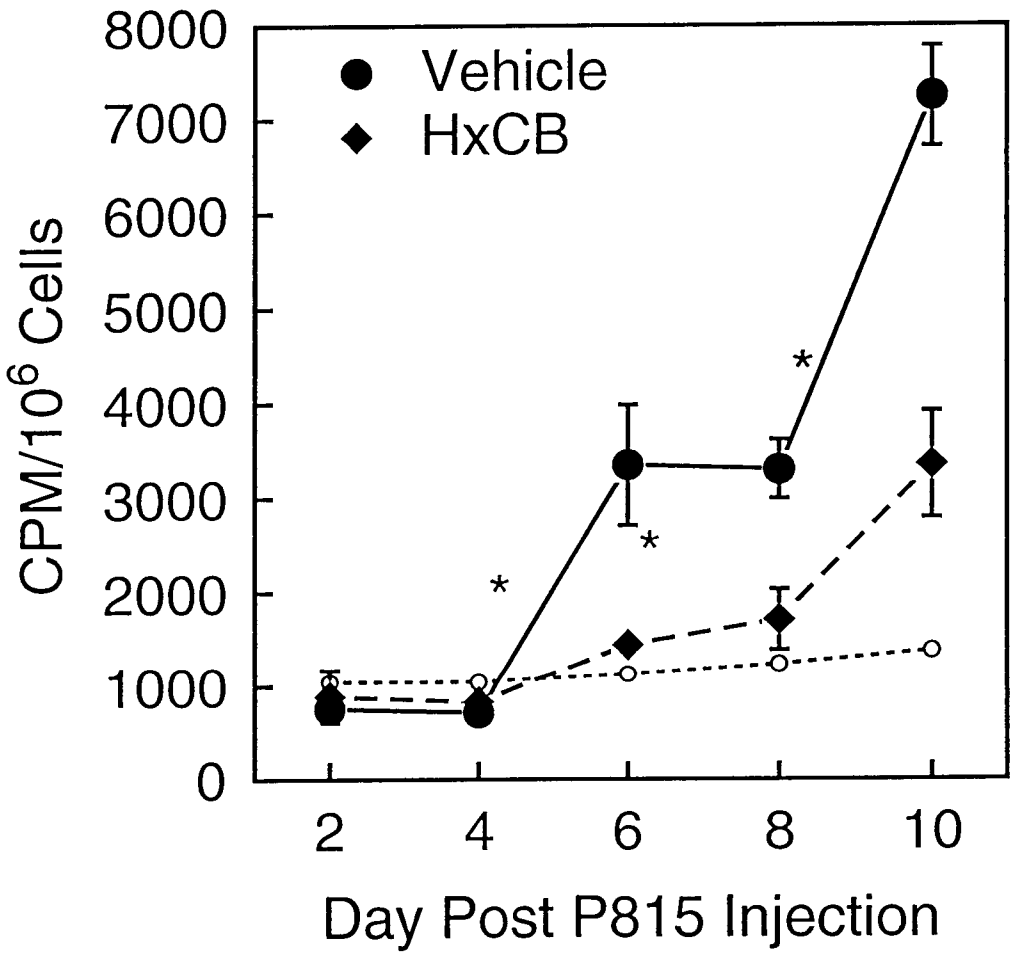

8000
7000
6000
5000
4000
3000
2000
1000
0
CPM/10^6 Cells
Vehicle
HxCB
*
*
*
2
4
6
8
10
Day Post P815 Injection

**Figure VII-3.**

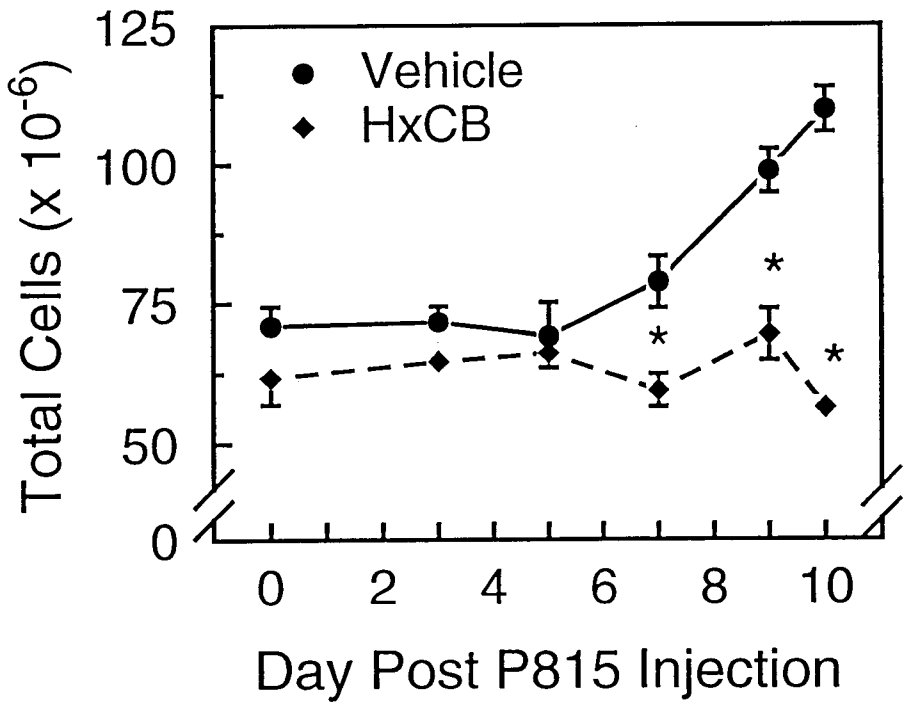

125
100
75
50
0
Total Cells (x 10-6)
Vehicle
HxCB
*
*
*
0
2
4
6
8
10
Day Post P815 Injection

**Figure VII-4.**

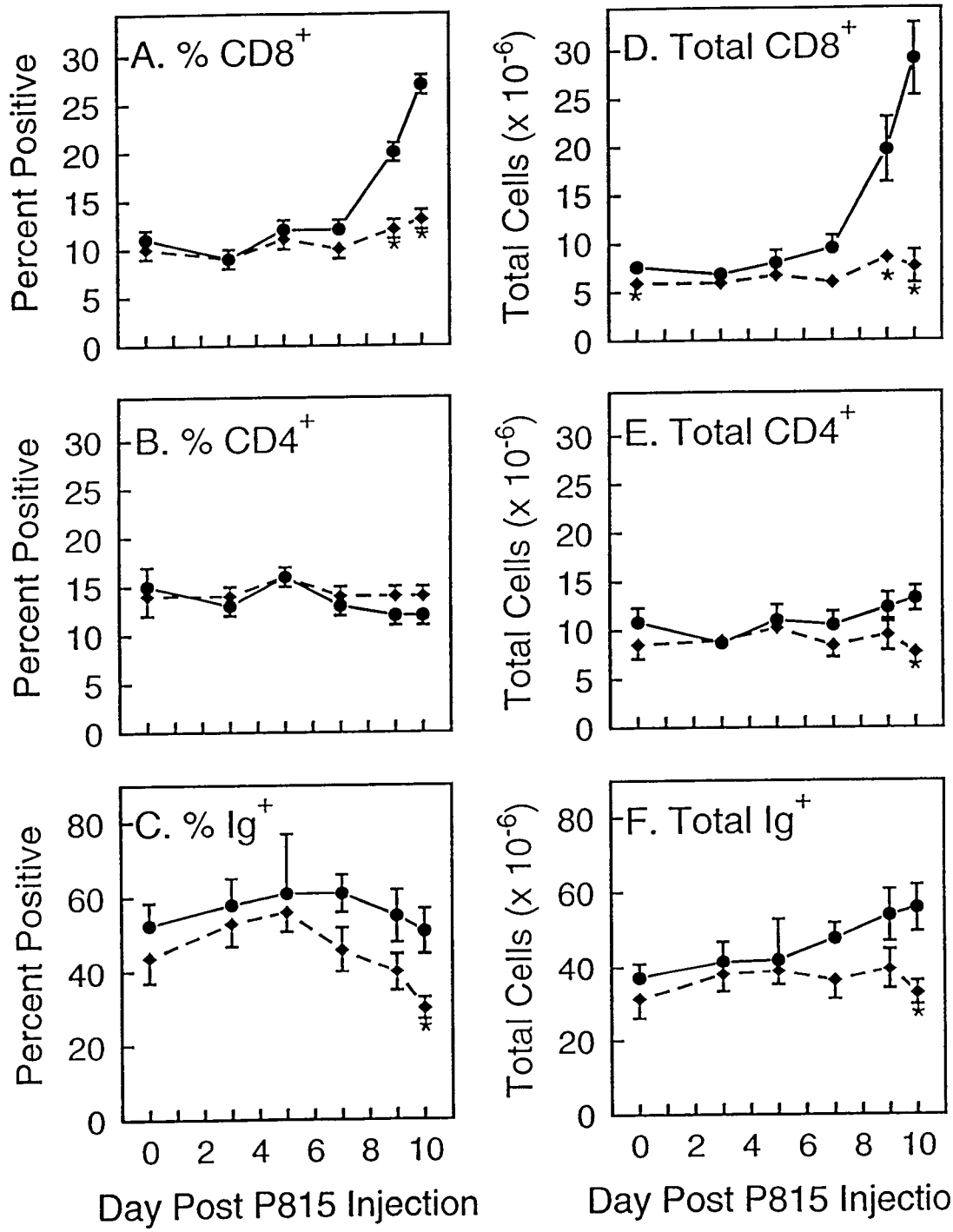
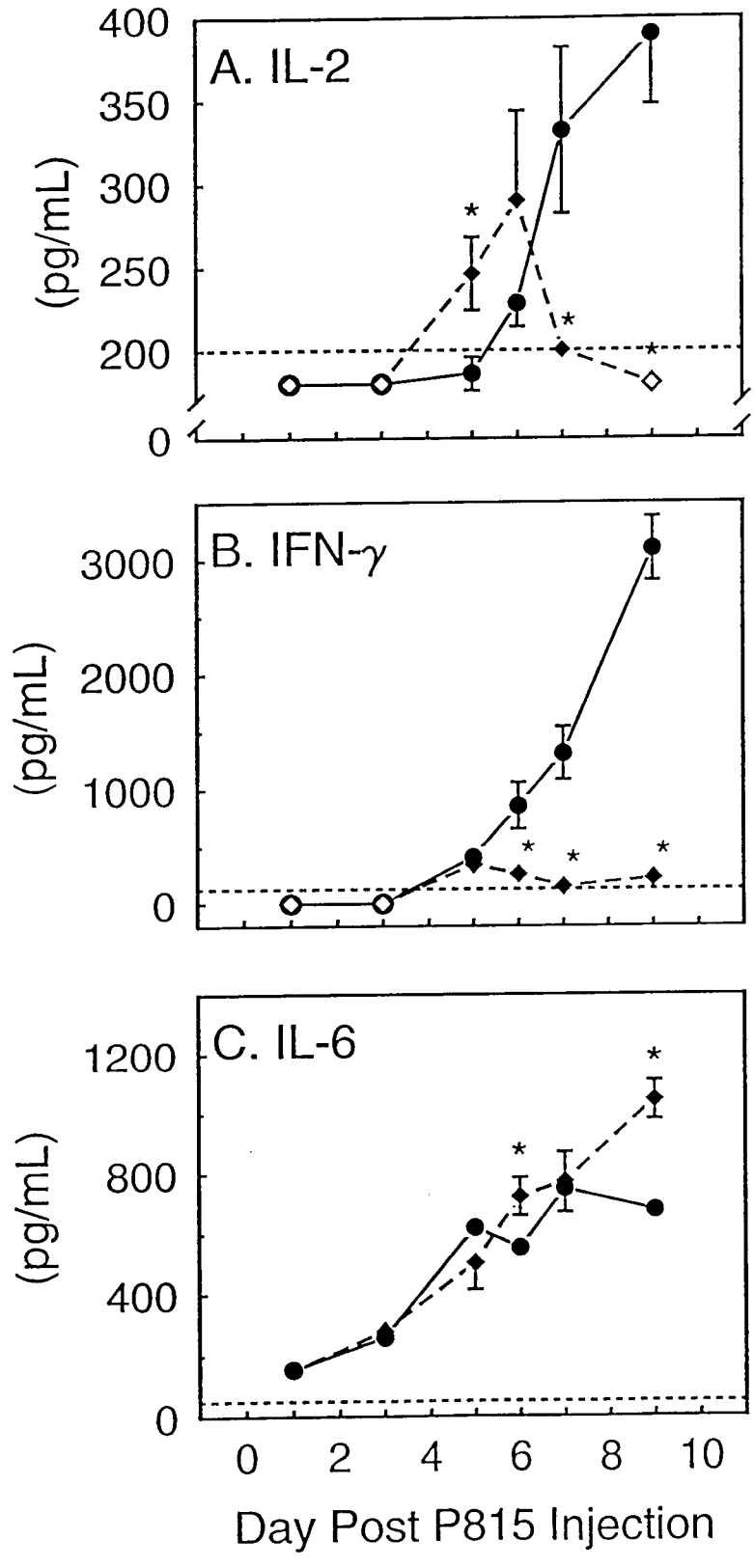

A. % CD8+
B. % CD4+
C. % Ig+
D. Total CD8+
E. Total CD4+
F. Total Ig+
Percent Positive
Total Cells (x 10-6)
Day Post P815 Injection
0
5
10
15
20
25
30
40
60
80
2
4
6
8

**Figure VII-5.**


A. IL-2
(pg/mL)
400
350
300
250
200
0
B. IFN-γ
(pg/mL)
3000
2000
1000
0
C. IL-6
(pg/mL)
1200
800
400
0
0
2
4
6
8
10
Day Post P815 Injection

development of CTL activity in vivo. These results support previous findings in other allograft models in vitro that both IL-2 and IFN-γ are important for the proliferation and maturation of CTL (Landolfo et al., 1985; Smith, 1988). Since $CD8^+$ cells have been shown to produce IL-2 (Ruscetti, 1990) and IFN-γ (Young and Hardy, 1990; Farrar and Schreiber, 1993), a likely source of these lymphokines is the expanding population of $CD8^+$ cells in the spleen.

$CD4^+$ cells can also produce IFN-γ (Farrar and Schreiber, 1993; Young and Hardy, 1990) and may be a more important source of IL-2 than $CD8^+$ cells (Street and Mosmann, 1991). However, $CD4^+$ cell numbers did not significantly increase over the course of the CTL response. Yet an essential role for $CD4^+$ cells in the generation of alloantigen-specific CTL activity has been suggested by preliminary results in this laboratory where depletion of $CD4^+$ cells prior to alloantigen challenge eliminated the development of alloantigen-specific splenic CTL activity. The specific role for $CD4^+$ cells in this model is unclear, but they are presumably an important source of lymphokine help.

Following alloantigen challenge, IL-6 levels in ex vivo spleen cell cultures increased ~4-fold over days 1-7, then leveled off through day nine. IL-6 was of interest in this model because, by itself, IL-6 can induce responsiveness of $CD8^+$ cells to IL-2 in vitro, and, in the presence of IL-1, IL-6 can enhance IL-2 production by $CD8^+$ cells (Renauld et al., 1989). IL-6 is produced by a number of cell types, but macrophages and T cells are the cells associated with the highest production (see reviews by Hirano [1992] and Van Snick [1990]). Increased IL-6 production did not correlate with an increase in the numbers of any known IL-6 producing spleen cell type examined. Preliminary results in this laboratory suggest that splenic macrophage numbers increase, but only slightly, following alloantigen challenge in mice (Kerkvliet and Oughton, unpublished results). Interestingly, Ford et al. (1991) has shown that peak IL-6 levels in sponge matrix allografts do not correlate with peak CTL activity.

In HxCB-treated mice, a significant decrease in the total number of spleen cells and $CD8^+$ spleen cells was observed concurrent with suppression of CTL activity. These data suggest that HxCB-mediated CTL suppression may involve suppression of alloantigen-specific $CD8^+$ cell proliferation. This is strongly supported by the observation that $^3$H-thymidine incorporation by spleen cells from HxCB-treated mice was significantly suppressed. Since lymphokines are important for CTL proliferation and maturation (Landolfo et al., 1985; Smith, 1988), and HAHs have been shown to modulate lymphokines (Taylor et al., 1992; Sutter et al., 1991; Kerkvliet and Burleson, 1994), lymphokine production by spleen cells from HxCB-treated mice was examined.

IL-2 production by spleen cells from HxCB-treated mice was significantly increased on day five post alloantigen challenge. These results have been confirmed by other studies in this laboratory and also shown to occur following TCDD exposure (data not shown). An explanation for the increased production is unclear. These results contrast those reported by Clark et al. (1983) who showed no alteration of Con-A stimulated IL-2 production by spleen cells from TCDD-treated mice. These contradictory results may reflect the difference between the mechanisms of antigen stimulation and mitogen stimulation, or the difference between an in vivo versus and in vitro generated response. Premature IL-2 production cannot be explained on the basis of altered cell number because neither $CD4^+$ nor $CD8^+$ spleen cell numbers were significantly altered as early as day five. However, since IL-2 is a product of activated T cells, these results may suggest that HxCB exposure can enhance T cell activation. Enhanced T cell activation in vivo as measured as cell cycling following exposure to TCDD has been reported by our laboratory in another model (Neumann et al., 1993).

In contrast to the enhanced production of IL-2 on day five, IL-2 production on days 7-9 was significantly reduced by HxCB exposure. Since $CD8^+$ cells have been shown to produce IL-2 (Ruscetti, 1990), this may be reflective of the reduced number of $CD8^+$ cells in HxCB-treated mice on these days. It is not clear if the enhanced IL-2 production on day five influences the reduction of $CD8^+$ cells on days 7-9. IL-2 production does not correlate with cytotoxic activity toward alloantigens (Fong and Mosman, 1990). However, HxCB-induced altered IL-2 production may impact the production of other cytokines such as IFN-$\gamma$ since IL-2 has been shown to enhance the production of IFN-$\gamma$ (Farrar and Schreiber, 1993).

IFN-$\gamma$ production by spleen cells from HxCB-treated mice was markedly reduced on days 6-9 and closely paralleled the suppressive effects of HxCB on $CD8^+$ cell numbers and CTL activity. Since $CD8^+$ cells can produce IFN-$\gamma$ (Farrar and Schreiber, 1993; Young and Hardy, 1990), the most likely explanation for reduced spleen cell production of IFN-$\gamma$ is the significantly reduced number of $CD8^+$ spleen cells in HxCB-treated mice. IFN-$\gamma$ production by $CD4^+$ T cells and NK cells has also been reported (Farrar and Schreiber, 1993; Young and Hardy, 1990). However, HAHs have not been shown to suppress NK cell activity (Holsapple et al., 1991; Kerkvliet and Burleson, 1994), suggesting that suppression of IFN-$\gamma$ production by NK cells is not likely to be important in this model. $CD4^+$ spleen cell numbers were significantly reduced by HxCB on day 10 after alloantigen challenge, but the influence of HxCB on IFN-$\gamma$ production by $CD4^+$ cells is not known.

In HxCB-treated mice, IL-6 production was significantly increased compared to vehicle-treated mice on days six and nine after alloantigen challenge. An explanation for enhanced splenic IL-6 production in HxCB-treated mice is unclear. Preliminary results in this laboratory have suggested that TCDD exposure causes a large increase in the number of splenic macrophages following alloantigen challenge (Kerkvliet and Oughton, unpublished results). Since macrophages are a major source of IL-6 (Van Snick, 1990; Hirano, 1992), an increased number of splenic macrophages in HxCB-treated mice could explain the increased IL-6 production observed.

Two antagonistic subclasses of T helper cell have been described in mice (Mosmann, 1991; Street and Mosmann, 1991). The Th1 cell type has been characterized by production of IL-2 and IFN-γ, is important for cell-mediated immune responses, and, through IFN-γ production, can inhibit Th2-mediated responses. The Th2 cell type has been characterized by production of IL-4 and IL-10, is important for humoral immune responses, particularly with help for production of IgM, IgG1, IgA and IgE, and, through IL-10 production, can inhibit Th1 responses. It was hypothesized that exposure to HxCB might predispose mice to a Th2 type of response following alloantigen challenge rather than a normal Th1 type of CTL response. This hypothesis stemmed from the implications of two separate observations: 1) in this laboratory, corticosterone (the major glucocorticoid in mice) was shown to be highly elevated in alloantigen-challenged, HxCB-treated mice (DeKrey et al., 1993; Kerkvliet et al., 1990), and 2) Daynes et al. (1991) showed that anti-CD3 stimulated spleen cells from corticosterone-treated mice produced less IL-2 and more IL-4 than those from untreated mice. Further, the production of all the cytokines measured in this study can be impacted by glucocorticoids (Arya et al., 1984; Grabstein et al., 1986; Zanker et al., 1990; Paliogianni et al., 1993), but changes in the production ratio of IL-2 and IL-4 have specifically been associated with altered T helper cell function. Importantly, predisposition to antigen-driven T helper cell responses has been linked to survival or susceptibility of mice to parasite infection (Heinzel et al., 1991; Pearse et al., 1991). However, the results of these studies do not support a predisposition to a Th2 type of response. Spleen cell production of both IL-2 and IFN-γ was suppressed by HxCB treatment but no similarly significant reciprocal increase in IL-4 and IL-10 production was observed. Further, an enhanced humoral response (measured as serum antibody titers) following alloantigen challenge in HxCB- or TCDD-treated mice has not been observed (Kerkvliet and Baecher-Steppan, unpublished results). To the contrary, suppression of $Ig^+$ spleen cell numbers was observed in this study, and suppression of antibody responses is a well established observation

following treatment of animals with HAHs (Vos and Luster, 1989; Holsapple et al., 1991; Kerkvliet and Burleson, 1994).

In conclusion, the results of these studies indicate that the production of IL-2, IL-6 and IFN-γ by spleen cells from alloantigen-challenged mice is altered by exposure to HxCB. The most outstanding finding was the marked suppression of IFN-γ production, an effect that has also been observed with cells from the peritoneal cavity, the site of antigen challenge (Steppan et al., 1993). In addition, ex vivo spleen cell proliferation was significantly suppressed by HxCB treatment, a result that suggests a mechanism for the reduced splenic $CD8^+$ cell numbers in HxCB-treated mice. Since coordinated lymphokine production is required for normal antigen-driven T cell proliferation and CTL responses, and lymphokine production preceded the development of both spleen cell proliferation and CTL activity in this model, these results suggest that altered lymphokine production plays a role in suppression of alloantigen-specific CTL activity by HxCB.

# CHAPTER VIII

## CONCLUSIONS

The findings of these studies indicate that exposure of male C57Bl/6 mice to HxCB caused significant elevation of plasma CS levels on days 3-10 after alloantigen challenge. The fact that CS elevation was only observed in mice that were both HxCB-treated and alloantigen-challenged suggests an interaction between the two treatments, the nature of which is unknown. In alloantigen-challenged ADX mice supplemented with exogenous CS, treatment with HxCB led to significantly higher plasma CS levels suggesting that clearance of CS is reduced by HxCB treatment. Reduced clearance of circulating CS could explain the elevation of CS observed in HxCB-treated alloantigen-challenged mice. Elevation of CS levels following treatment of alloantigen-challenged mice with TCDD was also observed. Elevation of circulating GC levels following exposure to HAHs has been reported by others (Sanders et al., 1977 and 1974; Gorski et al., 1988a; Jones et al., 1987; DiBartolomeis et al., 1987), although in none of those studies was HAH exposure coupled with antigen challenge. Thus, antigen challenge is not required for HAH-induced CS elevation.

The findings of these studies strongly suggest that CS elevation in HxCB-treated mice does not play a significant role in the suppression of alloantigen-specific CTL activity. The influence of CS on alloantigen-specific CTL activity was examined using three approaches: 1) ADX mice, 2) RU 38486 treatments, and 3) treatments with exogenous CS. ADX mice had a poor survival rate following HxCB exposure and alloantigen challenge and were therefore inadequate for use in this model. For the most part, RU 38486 treatment had no effect on CTL activity in normal mice or in mice treated with HxCB. The only exception to this was that RU 38486 treatment on days 5-7 post alloantigen challenge led to an enhanced mortality rate in HxCB-treated mice, and CTL activity could not be assessed in those animals that died. These results suggested that the effects of CS promote survival in HxCB-treated mice, at least on days 5-7 post alloantigen challenge. However, these results suggest that plasma CS elevation on days 3-5 and 7-9 post alloantigen challenge does not cause suppression of CTL activity in HxCB-treated mice. Infusion of exogenous CS into mice on days 3-6, 4-7, 5-8, and 6-9 after alloantigen, at a rate sufficient to elevate plasma CS to peak HxCB-like levels, had no effect on CTL activity.

Exposure of mice to TCDD significantly suppressed alloantigen-specific CTL activity in a dose dependent manner and in the absence of significant CS elevation. Since both TCDD- and HxCB-induced CTL suppression has been shown to be mediated through an Ah receptor mechanism, these results suggest that the CTL-suppressive mechanism down-stream of Ah receptor binding is not dependent on elevation of circulating CS levels. These results strongly suggest that CS is not the mediator of HxCB-induced CTL suppression.

Circulating TT and prolactin levels were decreased in alloantigen-challenged mice following HxCB exposure. Reduction of both TT and prolactin levels has been reported by others (Moore et al., 1985; Moore et al., 1989; Russell et al., 1988; Jones et al., 1987). The mechanism behind reduction of TT and prolactin levels in HxCB-treated mice was not examined here. Prolactin levels have been shown to be inversely regulated by CS in some animals. Although it is not know if CS levels influenced prolactin levels in this model, the time-dependent increase of circulating CS levels correlated roughly with the time-dependent decrease of circulating prolactin levels suggesting a possible interaction between the levels of these two hormones. Reduction of circulating prolactin levels, over the same time-frame and to the same degree as observe in HxCB-treated mice, had no effect on CTL activity in alloantigen-challenged mice. These results suggest that reduction of prolactin levels alone in HxCB-treated mice is not sufficient to cause suppression of CTL activity.

The only manipulation that significantly reduced the degree of CTL suppression in HxCB-treated male mice was castration (ODX). CTL activity in HxCB-treated ODX mice was increased by 2.4-fold. Since the degree of HxCB-induced CTL suppression in ODX male mice was approximately the same as that observed in HxCB-treated female mice, these results suggest that male mice are more sensitive to the CTL-suppressive effects of HxCB because of an increase in the production of, or sensitivity to, testes-derived immunosuppressive factors. Although TT is a known immunosuppressive testes-derived factor, the circulating levels of TT were reduced in HxCB-treated mice and, therefore, it is unlikely that TT is responsible for the enhanced sensitivity of male mice to HxCB-induced CTL suppression.

The findings of these studies indicate that exposure of alloantigen-challenged C57Bl/6 male mice to HxCB caused significant elevation of $PGE_2$ levels in PF and reduction of $PGE_2$ production by spleen cells. Indomethacine treatment of either vehicle- or HxCB-exposed, alloantigen-challenged mice with indomethacine reduced peritoneal $PGE_2$ to below detectable levels but had no effect on CTL activity. These

results suggest that $PGE_2$ does not play a role in HxCB-induced CTL suppression. These results are supported by the fact that all the $PGE_2$ levels measured in these studies were below the levels reported to alter CTL activity in vitro (Leung and Mihich, 1982; Wolf and Droege, 1982; Parhar and Lala, 1988; Skinner et al., 1989).

Exposure of mice to HxCB significantly altered the production of cytokines by spleen cells after alloantigen challenge (IL-2, IL-4, IL-6, IFN-γ). Both IL-2 and IFN-γ have been shown to play an important role in the development and maturation of CTL activity. IL-2 in particular has been shown to be essential for T cell clonal proliferation. The coincident findings of reduced *ex vivo* spleen cell proliferation and $CD8^+$ cell numbers in HxCB-treated animals, when compared to vehicle-treated controls, suggests that the alterations of IL-2 and IFN-γ production play a major role in mediating the suppression of CTL activity by HxCB. The biological significance of altered IL-4 and IL-6 production to development of CTL activity is unknown. Additional studies will be required to fully explain the mechanism behind HxCB-induced CTL suppression.

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