AN ABSTRACT OF THE THESIS OF

Wentian Huang for the degree of Doctor of Philosophy in Toxicology presented on June 26, 1997. Title: Effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on Staphylococcal Enterotoxin B (SEB)-Induced Alterations in T-Cell Activation and Cytokine Production

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Loren D. Koller

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is an environmental contaminant that is a potent immunotoxicant. To date, the cellular mechanisms of immune dysfunction induced by TCDD are unknown. However, it is known that the sensitivity of laboratory animals to TCDD varies between species. In these studies, the effects of TCDD on alterations in T-cell activation and cytokine production were investigated using a superantigen rat model. The original hypothesis of this research was that TCDD would inhibit staphylococcal enterotoxin B (SEB)-induced T-cell activation based on reports of impaired T cell function by TCDD exposure in animal experiments. In the initial study to characterize the superantigen model, the production of interleukin-1 (IL1), interleukin-2 (IL2), interleukin-6 (IL6), interferon-γ (IFNγ) and tumor necrosis factor (TNF) as well as the percent of $CD4^+$ cells and $CD4^+$ cells in S/G2M phase of the cell cycle were increased in rats exposed to SEB. These results suggested that the SEB rat model would provide a useful tool for studying the effects of xenobiotics on alteration of T cell activation. The effects of TCDD exposure on T cell activation and cytokine production were investigated using this model. Unexpectedly, TCDD resulted in an increase in the serum levels of

IL2. TCDD also enhanced IL1 production by peritoneal cells and IL2 production by spleen cells from SEB-primed rats in culture co-stimulated with SEB in vitro. An increased percentage of both splenic $CD4^+$ and $CD8^+$ cells in S/G2M phase were also noted in rats co-exposed to SEB and TCDD. These results demonstrated that TCDD exposure could enhance production of IL1 and IL2 as well as the percent of $CD4^+$ and $CD8^+$ cells cycling in SEB exposed rats. The results showed that the percent and number of $CD4^+$ or $CD8^+$ subpopulations as well as percentage of $CD4^+$ or $CD8^+$ cells in S/G2M phase were similar in all single dose treatment groups (25μg TCDD/kg). In contrast, a repeated cumulative dose of TCDD (5μg/kg/day for 5 days) significantly decreased the percent of $CD4^+$ cells and $CD4^+$ cells in S/G2M phase. These findings indicate that repeated doses of TCDD had a different effect from an equivalent single dose of TCDD on changes in the percent of $CD4^+$ cells and $CD4^+$ cells in S/G2M phase.

Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin(TCDD) on Staphylococcal Enterotoxin B(SEB)-Induced Alterations in T-Cell Activation and Cytokine Production

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CHAPTER I

INTRODUCTION

THE IMMUNE SYSTEM

Overview

The immune system is the main body of defense against foreign materials and biologic agents, such as bacteria, viruses, chemicals, and foreign cells and tissues. The immune system has two functional divisions: 1) innate and 2) acquired immunity.

Innate Immunity

Innate immunity (also called natural or non-specific) is conferred by elements which are always present and with which an individual is born. These elements consist of exterior (such as the skin, the cilia lining, trachea and the mucous membranes.) and internal (such as preformed complement protein, interferon, phagocytes, natural killer (NK) cells and lysozyme.) components. Innate immunity acts as a first line of defense against foreign materials and potential pathology. Previous exposure or sensitization to a foreign antigen is not required to mount an innate immune response.

Acquired Immunity

In contrast to innate immunity, acquired immunity (also termed adaptive) is a more specialized form of immunity. The first contact with a foreign invader induces an immune response. The second contact with the same offending agent will trigger a more rapid and more intense response. Acquired immunity is found only in vertebrates.

Although the acquired immune response originates in cells, it is convenient to consider the effector mechanism as consisting of two major arms, the humoral and cellular, the function of which are mediated by B and T lymphocytes, respectively. B lymphocytes are so-named because they originate in the bursa of Fabricius in birds and bone marrow of adult mammals (Roitt et al., 1969). B lymphocytes synthesize and secrete serum globulins that are called antibodies which possess specificity against the foreign material. The activity of antibodies can be divided into two separate functions: recognition and effect. Antibodies recognize antigen via their variable regions and accomplish the effector functions via their constant regions interacting with a variety of other molecules and/or cells.

The important biological properties of antibodies include neutralization of toxins and viral activity, immobilization of microorganisms, agglutination of antigenic particles, and activating serum complement to facilitate the lysis of microorganisms or phagocytosis and destruction. B lymphocytes produce antibodies with (T-dependent response) or without (T-independent response) the help of T lymphocytes. There is a marked change in the type and quality of antibody (e.g., higher concentrations of IgG antibodies) after the second injection of a T-dependent antigen. Unlike T-dependent responses, a second injection of T-independent antigen generates a similar level of

production of IgM as the first, with no significant increase in level, speed of onset, or class switch.

T lymphocytes are so called because of their differentiation in the thymus. T cells follow a different path in ontogeny and migrate from the bone narrow to the thymus, where they acquire specific markers and differentiate into cells with a variety of functions. T cells also undergo rearrangement of the genes that encode the two chains that form an antigen-specific receptor. The T-cell receptor with $\alpha\beta$ chain is on the majority of T cells, while T cell receptor with $\gamma\delta$ is on a small number of T cells. The T cell receptor has a unique binding specificity. This property permits each T cell to recognize a different antigen.

Cell surface expression of $CD4^+$ and $CD8^+$ glycoprotein molecules divides mature T cells into two distinct subsets. $CD4^+$ cells have a "helper" function for B cells (which enables B cells to differentiate into antibody forming cells). $CD8^+$ cells can act as regulatory cells (e.g. they can suppress immune responses) and lyse other cells.

T cell activation and proliferation

In general, T cells, unlike B cells, are not activated by free antigen. T cell activation is a complex process that leads to the generation of mature effector cells (reviewed by Gajewski et al., 1989; Bath et al., 1989; Abbas et al., 1991). It is believed that T cell activation requires at least two signals. The first signal is the engagement of T cell receptors (TCR) with antigen peptide associated with the major histocompatibility complex (MHC), followed by further stabilization by the cellular interaction of adhesion molecules such as CD2, LFA-1 and CD28 on T cells, and LFA-3, ICAM-1 and B7 on

antigen presenting cells (APC). The second signal is received from lymphokines, such as interleukin-1, which are produced by the APC or by adhesion co-stimulatory molecules.

For production of the first signal, antigen must be processed and presented by the APC, such as macrophages, dendritic cells and infrequently, B cells. Antigen processing is a stage during which large antigen moieties, cells or proteins are degraded to fragments more recognizable by T cells. Some of these fragments become associated with either class I or class II MHC molecules. These complexes of peptides and MHC molecules are transported to the surface of the cell and presented to T cells by the APC. The complex is recognized by the antigen receptors on the $CD8^+$ cells (with $CD8^+$ molecules on the surface only binding to MHC I molecules) or $CD4^+$ cells (with $CD4^+$ molecules on the surface only binding to MHC II molecules). Meanwhile, the T cells receive a second signal through the cognate interaction of co-stimulatory molecules or in the form of lymphokines, such as IL1, which is produced by the APC to complete the process of activation.

As a consequence of T-cell activation, many different cytokines are produced and lymphokine receptors (such as interleukin 2 receptors (IL2R)) are expressed at the surface of the activated T cells. In the mouse, two activated Th-lymphocytes have been delineated, a suppressor-inducer (cytotoxic) Th1 subset and a helper-inducer (non-cytotoxic) Th2 subset. Th1 synthesizes IL2 and interferon-γ (IFN-γ), which activate cytotoxic $CD8^+$ T cells, natural killer cells (NKC) and macrophages, Th2 synthesizes interleukin-4 (IL4) and interleukin-5 (IL5), Interleukin-6 (IL6), Interleukin-9 (IL9) and interleukin-13 (IL13) which activate B cells. T cell proliferation following T cell

activation is accomplished by tightly regulating the production of certain lymphokines (such as IL2) and by regulating the expression of IL2R.

SUPERANTIGENS

Overview

A typical conventional antigen usually stimulates a very small portion of T cells, perhaps less than 1 in 10,000 cells. However, some microbial proteins are unique in that they can stimulate as many as 1 in 5 T cells. These powerful antigens were named "superantigen" by Kappler and Marrack about 9 years ago. Superantigens described thus far fall into two categories, bacterial (Kotzin et al., 1993; Swaminathan et al., 1992) and viral (Acha-orba, 1993; Kotzin et al., 1993; Abe and Hodes, 1982). The former are products mainly of virulent pathogenic bacteria. The best characterized superantigens are the Staphylococcal enterotoxins (SE). Viral superantigens are products of endogenous or exogenous mouse mammary tumor viruses. The consequences of superantigen exposure range from T cell activation and proliferation with massive cytokine release to apoptosis and T cell anergy. In addition, most of the bacterial superantigens have been implicated in the pathogenesis of immunodeficiency and autoimmunity.

Characteristics of superantigens

In general, superantigens differ from conventional peptide antigens in four features. 1). They can elicit a strong primary response. In contrast to conventional antigens, priming and boosting in vivo are not necessary to detect T-cell activation and proliferation in vitro in response to superantigens. 2). In contrast to the nature of T cell

recognition of a conventional antigen, superantigen recognition is mediated predominantly by the TCR Vβ chain (Kappler et al., 1989; MacDonald et al., 1993; Callahan et al., 1990). Superantigens interact with the external face of the TCR Vβ chain in a region of variability that is sometimes called the fourth hypervariable region (Choi et al., 1991; Mollik et al., 1993). Since there are about 15 to 20 Vβ elements expressed by mice and 60 Vβ elements expressed by man, 10-30% of T cells are available to mount a response to any superantigen. However, only approximately 1 in 10,000 T cells are stimulated by a conventional antigen since the region binding to a conventional antigen is made up of the joining elements of both Vjα and VDJβ. 3). All superantigens are presented by MHC class II that mainly provides a docking structure for the superantigens. However, the presentation is not classically MHCII-restricted because superantigen can bind to most MHC class II alleles and isotopes (Acharya et al., 1994; Jardetzky et al., 1994). Class II alleles only contribute different affinities to the interaction of superantigen and TCR Vβ chain and are not recognized per se by the TCR. 4). In contrast to the processing of conventional antigen in which antigen is processed into small peptides that are then loaded into the MHC-binding groove for presentation on the cell surface, superantigens do not require conventional processing, and bind as intact molecules on the external face of the MHC molecule (Mollick et al., 1989). The combination of these four characteristics unequivocally defines a superantigen.

Sources of superantigens

Superantigens are now classified into two groups. The first group is mainly products of pathogenic bacteria. The best characterized superantigens are the Staphylococcal enterotoxins (SE), proteins of molecular weights of approximately 24 to 30 kd, which include staphylococci, streptococci, and mycoplasma. SE are divided into five major serological types, SEA, SEB, SEC, SED & SEE. SEA, D, and E are most similar to each other, as are SEB, C1, and C3. For binding to MHC class II molecules, SEA and SEB probably bind to the same site on the molecule because they cross-compete for binding. It has been shown that binding of SEA and SEB was detected in DR^+ and DQ^+ human lymphocytes but not in $I\text{-}A^+$ murine lymphocytes (Saito et al., 1991; Scholl et al., 1989). The patterns of Vβ specificity of the different enterotoxins correspond loosely with their groupings by sequence similarity. For example, SEA, SED and SEE stimulate mouse T cells Vβ11. In contrast, SEB and SECs stimulate human T cells bearing Vβ 12 and mouse T cells bearing members of the Vβ8 family. There are species-specific TCR Vβ selectivity of superantigen-reactive T cells. For example, SEB stimulates human Vβ3, 12, 14, 15, 17 and 20 T cells (Kappler et al., 1989), mouse Vβ3, 7, 8.1, 8.2, 8.3 and 17 (Janaway et al.,1989; White et al., 1989;) and rat Vβ7, 11, 17 and 19 (Sellins et al., 1992).

The second group of superantigens are the minor lymphocyte stimulatory (Mls) determinants that are encoded by an open reading frame in the 3' long terminal repeat of mammary tumor virus (MMTV) (Acha-orbea and Palmer, 1991; Acha-orbea, 1993; Choi et al., 1991). Mls can stimulate a strong primary mixed lymphocyte reaction (MLR) between cells from mice of the same MHC haplotype (Festenstein et al., 1973). T cells

bearing particular Vβ elements respond to Mls presented by MHC class II molecules on the surface of a variety of cell types.

Superantigen-induced T cell activation and proliferation

A simultaneous binding of the superantigen to the TCR Vβ and to the MHC class II molecules on the APC delivers a powerful signal to T cells and APCs. An early measurable event is the induction of expression of several cytokine genes and cytokine production in both T cells and monocytes. The expression of IL2 mRNA, for example, was detected in lymph nodes and spleen within 1-2h and serum IL2 levels were enhanced within 2h following in vivo exposure to SEB in mice (Baschieri et al., 1993; Miethke, 1992). Subsequently a parallel decline in IL2 protein and steady state mRNA levels suggested transcriptional control of IL2 production in this system. The IL2 mRNA was found in $CD4^+$ cells than $CD8^+$ cells in those animals. The accumulation of IFN-γ was increased within 18h after SEB administration. In contrast to IL2 mRNA, IFN-γ mRNA was found mainly in the $CD8^+$ cells, although it was also detected in $CD4^+$ cells (Herrman et al., 1992). It has been shown that rapid (within 1-4h) appearance of IL1, IL2, IL6, tumor necrosis factor (TNF) and IFN-γ occurs in the serum of SEB-injected mice (Blackman and Woodland, 1995). Superantigen can either stimulate MHC class II^+ cells directly in the absence of T cells and/or may stimulate MHC^+ cells in the context of a T cell-superantigen-APC interaction. For example, toxic shock syndrome toxin-1 (TSST-1) can bind to and directly activate monocytes resulting in the production of IL1 and TNFα (Trede et al., 1991; Parsonnet et al., 1988).

Changes in expression of adhesion and activation molecules in lymphocytes were also found in SEB-treated mice. L-selectin expression, for example, was down-modulated within an hour and IL2R expression was induced within 18h post SEB administration (Miethke et al., 1993). Superantigens can induce $CD8^+$ T cells to mediate staphylococcal enterotoxin-dependent cell-mediated cytoxicity of MHC class II bearing targets, including B cells and APC (Herrman et al., 1991). This phenomenon peaks at 2-3days after superantigen exposure and decreases to background levels thereafter (Kalland et al., 1991).

Superantigen-induced T- cell anergy and deletion

Superantigen exposure not only induces T cell activation but also induces T cell anergy and deletion. The anergic cells are characterized by the inability of T cells to make IL2 and proliferate to a subsequent exposure to superantigen (MacDonald et al., 1991).. There are several features of superantigen-induced anergy: 1). anergic cells are only unresponsive to the priming superantigen. It has been reported that after the initial phase of SEB-induced T cell activation and proliferation, the remaining T cell subsets are only unresponsive to SEB restimulation and retain their ability to respond to another non cross-reactive enterotoxin (SEA). 2). $CD4^+$ cells seem to be more sensitive to anergy than $CD8^+$ cells. For example, Baschieri et al. (1993) indicated that $CD4^+$ cells from SEB-primed mice were hyporesponsive to SEB as assessed by IL2 and IFN-γ production at both the mRNA and protein levels. In contrast, $CD8^+$ cells from such anergic mice were actually slightly hyperresponsive to SEB in terms of IFN-γ production. 3). Superantigen-primed cells are more anergic in vitro than in vivo. In parallel studies, IL2 production was

lower following secondary SEB stimulation only by factor of 3-5 in vivo compared to a factor of 20-50 in vitro. Both $CD4^+$ and $CD8^+$ cell cycling were reduced following SEB injection. However, the reduction was considerably less than the 10-12 fold reduced proliferation observed for anergic cells in vitro. 4). Anergy may be reversible. Studies showed that anergy could be reversed in vitro by the addition of exogenous IL2 and via stimulation of anti-TCR antibodies (MacDonald et al., 1991). It has also been reported that anergy is reversible in vivo after removal of the superantigen (Ramsdell and Fowlkers, 1992). However, other studies showed that SEB-induced anergy could not be overcome in vitro (Perkins, 1993).

Two models describing the fate of superantigen-reactive T cells in vivo have been hypothesized. In model 1, all reactive specific Vβ cells initially undergo clonal expansion. Further progression to cell death or anergy may be dependent upon the influence of microenvironmental factors such as superantigen presentation by different cell types or the presence of different cytokines in different concentration. In model 2, strongly stimulated specific Vβ cells undergo clonal expansion leading to cell death. In contrast, more weakly stimulated specific Vβ cells progress to the anergic state in the absence of proliferation. However, the mechanism of the fate of superantigen-reactive T cells remains unclear.

Superantigen associated diseases

1) Food poisoning and toxic shock

It has been shown that SEB exfoliating toxin and TSST-1 cause food poisoning, toxic shock and/or scalded skin syndrome (Harris,1993). SE causes almost a quarter of

the food poisoning that occur in the United States (Spero et al., 1988). Superantigen can cause systemic shock in several species, including mouse and man. T cells are required for superantigen-mediated toxic shock and are associated with high serum cytokine levels of TNF, IL1 and IL6 (Herber-Katz and Acha-orbea, 1989).

2). Autoimmune diseases

The hypothetical mechanisms by which superantigen may contribute to autoimmunity include activation of dormant autoreactive cytotoxic T cells, anergy or deletion of T cell populations suppressing other autoimmune cells, or an indirect stimulation of populations of autoreactive B cells. An example is the rodent arthritis caused by mycoplasma arthritides superantigen, MAM, that causes both T cell activation and T-dependent B cell activation (Blackman and Woodland, 1995). However, direct responsibility of superantigen for the arthritis has not been confirmed.

3) Immunodeficiency

Several studies have suggested that AIDS virus contains a superantigen (Imberti et al., 1991; Dalgleish et al., 1992). It has been reported that a deficiency in specific Vβ T cells was found in HIV-infected individuals. Other studies showed that $CD4^+$ T cells bearing Vβ12 TCR support HIV replication more than $CD4^+$ T cells expressing other Vβ receptors that are found in HIV-infected patients (Laurence et al., 1992). However, the correlative association between HIV infection and superantigens, if any, is still unclear.

DIOXIN TOXICOLOGY

Overview

The halogenated dibenzo-p-dioxins, dibenzofurans, biphenyls, brominated biphenyls, azo(xy)benzenes, and naphthalenes are collectively termed halogenated aromatic hydrocarbons (HAHs) (figure I-1). They have received much attention in the scientific literature and popular press because of their ubiquitous occurrence, persistence in the environment, relatively high toxicity in human and laboratory animals and possible magnification in the food chain. In addition to commercial production (Safe et al., 1990; Lang et al., 1992), they have been unintentionally produced as byproducts of industrial or chemical processes such as combustion or photolysis (Brinkiman et al., 1980; Pomerantz et al., 1978; De Voogt and Brinkiman, 1989).

This group of chemicals is usually considered collectively because of their similar chemical structures, a similar and characteristic pattern of toxic responses, and a common mechanism of action. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the prototype of the polychlorinated dibenzo-p-dioxins (PCDD), has been demonstrated to be the most potent and the most biologically active congener among the HAHs (Poland and Knutson, 1982).

Sources and environment contaminants of dioxin

Natural sources such as forest fires and volcanic eruption can yield traces of dioxin. However, almost all dioxin contamination occurs from 1) byproducts in the synthesis of industrial chemicals; 2) byproducts of chlorine bleaching processes; 3) incomplete combustion of wastes (Olie et al., 1977); 4) emissions from automobiles using leaded gasoline (Muller and Buser, 1986); and 5) home heating systems using both coal

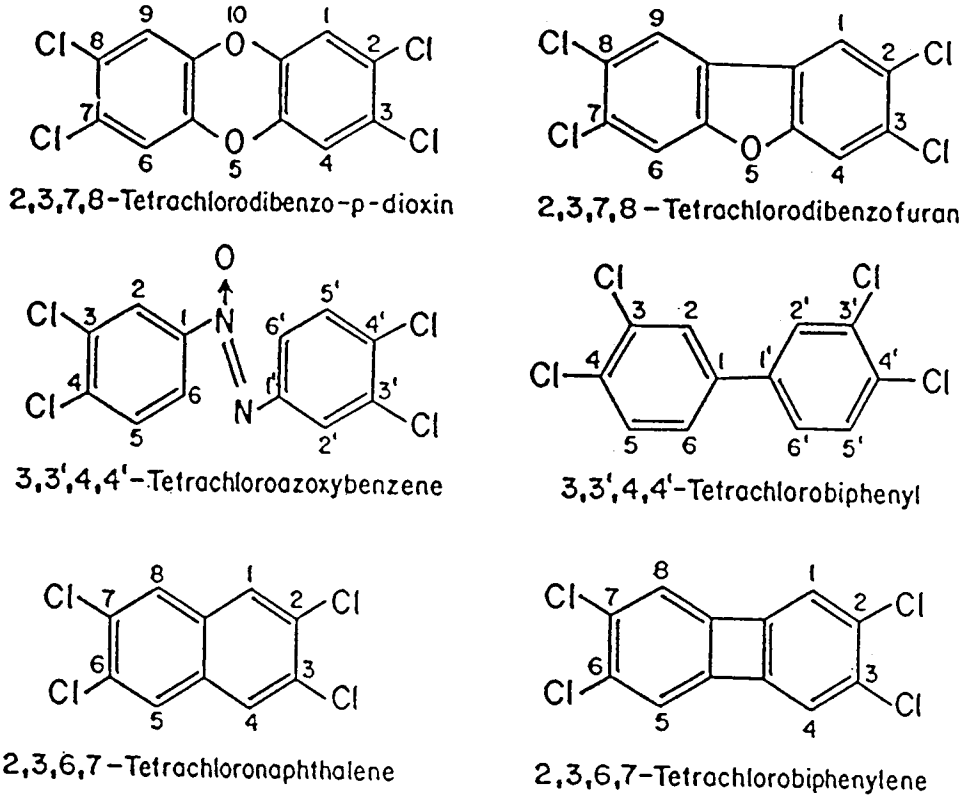

Figure I-1. The isosteric tetrachloro-congeners and ring numbering systems of various halogenated aromatic hydrocarbons.

The toxic isomers have halogen atoms in three or four of the lateral ring position and may or may not have halogen atoms in the other ring position.

and wood as fuel sources (Thomas et al., 1988; Hutzonger and Fiedler, 1988). Most TCDD was formed as a contaminant in the synthesis of 2,4,5-trichlorophenol, which is used to manufacture 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), a broad spectrum herbicide and defoliant.

Dioxins have been identified in ambient air samples, fly ash samples, human milk, serum and adipose tissue, a variety of foods including fish, cow's milk, butter, chickens, and eggs (Safe et al., 1985; Oeheme et al., 1988; Miyata et al., 1987; Beck et al., 1989). Release of dioxin into the environment has occurred incidentally (e.g. application of herbicides), accidentally (e.g. the rupture of an industrial chemical reaction vessel in Seveso, Italy) and through negligence (e.g. spreading of waste oil in Times Beach, Missouri) (Tierman et al., 1985; Menzer 1991; Dickson and Buzik, 1993).

An aromatic hydrocarbon receptor model

The aromatic hydrocarbon receptor (AhR)-mediated action for TCDD was first proposed in 1976 in Alan Poland's laboratory (Poland et al., 1976). The AhR is an about 90,000 MW cytosolic protein that has been found in several cell types derived from human and animals (Safe et al., 1986). In the absence of ligand, the inactive AhR resides in the cytoplasm of target cells in a soluble complex with the heat shock protein Hsp 90 and possibly other proteins. TCDD and similar compounds diffuse across the plasma membrane of target cells and bind to the receptor. Two molecules of the chaperon protein Hsp 90 are displaced during the binding process. The AhR-ligand complex associated with AhR transporter (ARNT) translocates to the nucleus. The nuclear form of AhR binds with high affinity to specific elements (DREs), or xenobiotic response elements (XREs)

located in the 5'-flanking region responsive genes. Interaction of the AhR-ligand complex with upstream DREs has been shown to alter transcription of downstream genes. Alterations in transcription change the amount of mRNA and the synthesis of specific gene products. Cytochrome P450 (CYP1A1) is by far the most extensively studied TCDD-responsive gene. In addition to induction of CYP1A1 and other hepatic enzymes such as CYP1A2 and menadione oxidoreductase, UDP glucuronosyltransferas, HAH-induced thymic involution and immunotoxicity have been correlated with an AhR-mediated mechanism (Poland and Knutson, 1982). It has been suggested that most toxicity induced by TCDD is mediated by the AhR (Lucier et al., 1993).

Toxicity of dioxin

Dioxins have been of major toxicologic concern because of their widespread environmental contamination and high toxicity in humans and laboratory animals.

Humans

Human have been inadvertently exposed to dioxin in their occupations and living environments. In the general population, concentrations of range on the average from a non-detectable level to 30ppt (ηg/kg) in adipose tissue, human milk fat and human blood lipids (Patterson et al., 1986; Ryan et al., 1985; Schecter et al., 1987). In contrast, TCDD levels have been found in blood lipids to be as high as 12,000ppt in production workers (Schector and Ryan, 1988) and 56,660ppt in children who were exposed to TCDD following an accident explosion at a pesticide plant in Seveso, Italy.

A number of investigations have been conducted to study the toxic effects of dioxin in humans (Kimbrough, 1985, 1990; Moccarelli et al., 1991; Johnson, 1992;

Dickson and Buzik, 1993). The most noteworthy dioxin exposures include: 1) U.S troops exposed in Vietnam to Agent orange, a TCDD-tainted herbicide; 2) people exposed to TCDD following an accidental explosion at a pesticide plant in Seveso, Italy in 1976; and 3) TCDD contaminated oil spread on roadways in Time Beach, Missouri in 1971. An increased risk of skin, genito-urinary and othopharyngeal cancers and a tendency to develop underactive thyroid and diabetes were reported in veterans who were exposed to Agent orange (Kang et al., 1987). In people exposed to higher levels of TCDD in Seveso, no abnormal findings other than chloracne were identified in this population in spite of extensive and repeated studies. Johnson (1992) reported that TCDD exposure resulted in an increased risk of human lung and thyroid cancers. A wide variety of toxic symptoms have been reported following TCDD exposure including chloracne, hyperkeratosis, weight loss, eye irritation, sexual dysfunction, abnormal liver function and increased risk of cancers. However, up to now, the only adverse human health effects that have been conclusively associated with TCDD exposure are chloracne and hyperkeratosis.

Laboratory animals

A variety of toxic syndromes have been reported in laboratory animals with dioxin including a wasting syndrome, lymphoid involution, hepatotoxicity, chloracne, gastric lesions, urinary tract hyperplasia, teratogenicity, changes in immune function, alterations of enzyme activities, changes in hormone levels, and carcinogenesis (reviewed by Poland and Knutson, 1982; Safe, 1990; Dickson and Buzik, 1993; Lucier et al., 1993; James et al., 1993). The sensitivity of animals to dioxin varies between species. The acute oral LD50 (μg/kg) of TCDD varies over a 5000-fold range in different species:

Guinea pig 1, Long/Evans rat (female) 9.8, Sprague Dawley rat (female) 45, monkey <70, mouse 114, rabbits 115, dog >300, hamster 5000 (McConnell et al., 1978; Poland and Knutson, 1982). Even within a particular species, the toxic effects of dioxin can vary with strain, sex and age such as LD50 (μg/kg) which is 9.8 for the Long/Evans rat (female) and >7200 for the Han/Wistar (female) (Pohjanvirta et al., 1993).

Immunotoxicity of dioxin

Humans

The effects of TCDD on the immune system of humans have been derived from epidemiological studies associated with human exposure. For example, a cohort from an industrial accident at a British herbicide plant manufacturing 2,4,5-T showed that the numbers of circulating NK cells were elevated in plant workers. However, the total number of both B and T cells and the T-helper/T-suppressor ratio were unaltered (Jenning et al., 1988). Suppression of the delayed type hypersensitivity reaction (DHR) was found in high risk individuals in Time Beach, Missouri (Knutson, 1984); whereas the result of a follow-up study showed no change of the DHR. Pocchiari et al. (1979) reported that no abnormalities in serum immunoglobulin concentrations, levels of circulating complement, or lymphoproliferative response of T and B cells following mitogen stimulation were found in 44 children (20 had chloracne) in Seveso, Italy. Six years later, a second study involving a different set of subjects found elevation of complement protein levels in the exposed children which correlated with the incidence of chloracne (Tognoni and Bonaccorsi, 1982). There are few studies in which the direct effects of TCDD have been determined in cultured human lymphocytes. Wood et al. (1992)

demonstrated that TCDD had no effects on PWM-induced responses by human lymphocytes. However, a year later, they reported that toxic shock syndrome toxin (TSST-1)-induced B-cell differentiation, as manifested by IgM secretion, was significantly suppressed by TCDD (Wood and Holsapple, 1993). Despite the fact that several cohort studies of exposed populations and the direct effects of TCDD on cultured human lymphocytes have been conducted, the results suggest that the effects of TCDD on human immunocompetence are equivocal.

Laboratory animals

Immune dysfunction has been demonstrated to be among the earliest and most sensitive indicators of exposure to dioxin. Recent evidence indicates that dioxin exposure causes alterations in cell-mediated and humoral immunity.

Effects of dioxin on cell-mediated immunity

Severe thymus atrophy has been reported as an early immunotoxic effect in animals exposed to TCDD (Buu-Hoi et al., 1972). This effect by TCDD was shown to occur in almost all laboratory animals exposed to lethal or sublethal doses of TCDD (Vos et al., 1980; Poland and Knutson, 1982; Kociba et al., 1976). Greenlee et al. (1985) demonstrated that the thymic atrophy is due to a direct action on the thymic epithelium. They reported that thymic epithelial cells are pushed toward terminal differentiation by TCDD exposure so that these cells can no longer produce the necessary factors for Lymphocyte maturation. Clark et al. (1981, 1983) reported that alteration of T lymphocyte function in adult mice exposed to TCDD in vivo was associated with activation of T suppressor cells and loss of T lymphocyte cytoxicity for tumor target

cells. In addition, mitogenic responses of splenic lymphocytes to PHA and ConA, graft-vs-host reaction, DHR and prolongation of allograft rejection have also been described in the in vivo exposure of adult animals to TCDD (reviewed by Kerkvliet, 1994; Vos and Luster, 1989; Holsapple et al., 1991a). However, a direct effect of dioxin on T cell antigen-driven responses in vitro has not been found (Clark et al., 1981; Kerkvliet and Baecher-stappan, 1988). These findings suggest that dioxin indirectly affects T cell function.

Dioxin exposure via maternal dosing during either the perinatal or postnatal period has been shown to cause effects on cell-mediated immunity. A dosage of 5 μg/kg in mice and rats on various days of gestation and weaning suppressed a variety of cell-mediated immune responses including DHR, mitogen response of splenic and thymic lymphocytes to PHA and ConA, graft-vs-host reactivity of spleen cells and ability to reject skin allografts (Vos and Moore, 1974; Faith and Moore, 1977; Luster et al., 1979; Thomas and Hinsdill, 1979). The effects of TCDD on cell-mediated immunity are more severe in animals exposed to TCDD during the pre- and postnatal periods than when exposed to TCDD only postnatally (Luster et al., 1979).

One well-studied effect of TCDD on cell-mediated immunity is suppression of cytolytic T lymphocyte (CTL) function. Clark et al. (1981) reported that C57BL/6 mice exposed to 0.4μg/kg TCDD did not appear to produce a consistent or time-dependent suppression of the antibody response to sheep red blood cells (SRBC) and TNP-Brucella abortus (TNP-BA); whereas the CTL response to alloantigen was shown to be consistently affected at a dose as low as 0.04μg/kg. Therefore, they concluded that the CTL response is more sensitive to the effects of TCDD than the antibody response. It has

been shown that treatment of C57BL/6 mice with TCDD results in a dose-dependent suppression of the in vivo CTL response to alloantigen (Clark et al., 1981; Kerkvliet and Baecher-steppan, 1988; Kerkvliet et al., 1990). Further, it has been suggested that suppression of the CTL response is mediated through the AhR by using mice congenic for the Ah locus and polychlorinated biphenyl congeners with different affinities for the AhR (Clark et al., 1983; Kerkvliet et al., 1990).

A number of studies have been conducted to examine effects of TCDD on alteration of T lymphocyte subsets. Acute exposure to TCDD in C57BL/6 mice caused significant reduction in both the percentage and absolute number of most of the immature $CD4^+$ $CD8^+$ double positive cell population in the thymus (Kerkvliet and Brauner, 1987). Several studies showed that reduction in the percentage or absolute number of $CD4^+$ or $CD8^+$ cell subsets in the thymus or spleen were noted in mice exposed to TCDD (Prell and Kerkvliet, 1995; Kerkvliet et al., 1996; Rhil et al., 1996). In contrast, it has been reported that no significant alteration of $CD4^+$ or $CD8^+$ cells or even an increase of $CD4^-$ $CD8^+$ cells occurs in rats following TCDD exposure (Smialowicz, 1994, 1996; Badesha et al., 1995). The decrease in $CD4^+$ and increase in $CD8^+$ subsets were not only shown in non-human primates exposed to TCDD in vivo but also in peripheral lymphocytes from those animals cultured with TCDD in vitro (Neuber et al., 1992). Recently, Lang et al. (1996) reported that the percentages of $CD4^+$ and $CD8^+$ subsets in human peripheral blood lymphocytes were not affected by TCDD treatment in vitro.

Effects of TCDD on humoral immunity

The most frequent studies of humoral immunity and dioxin use procedures of specific antigen challenge. The effects of TCDD on alteration of antibody to specific antigens have been shown in a variety of animal models including guinea pig, mice, rats, monkeys and rabbits (Vos and Luster, 1989; Holsapple et al., 1991b; Kerkvliet and Burleson, 1994; Smialowicz, 1994). The first indication of a differential sensitivity to the suppression of humoral antibody production in strains of mice exposed to TCDD was described by Vecchi et al. (1983). They reported that acute exposure of C57BL/6 mice and C3H/HeN mice (i.e. AhR-responsive) to TCDD significantly suppressed the antibody response to SRBC; whereas a greater concentration of TCDD produced only moderate effects in the DBA/2 and AKR strains (i.e. AhR-nonresponsive). In addition, a biphasic dose-response to a T-dependent antibody occurred in Ah^{dd} congenic mice but not in Ah^{bb} congenics when exposed to TCDD (Kerkvliet et al., 1990). Kerkvliet et al. (1987) demonstrated that dioxin can suppress the antibody response to both T-dependent (SRBC) and T-independent (TNP-LPS and DNP-Ficoll) antigens, but there was a significantly higher degree of suppression (by 10-fold) in the response to SRBC than TNP-LPS. These findings suggest that dioxin-induced humoral immune suppression in mice is linked to an Ah-mediated mechanism and alteration of T cell function in vivo.

It has been reported that dioxin congeners could suppress in vitro humoral immunity by direct addition of dioxin to cell cultures (Holsapple et al., 1984, 1986; Tucker et al., 1986). Holsapple et al. (1986) reported that the humoral response to both SRBC and LPS were suppressed at doses which caused neither cellular changes or effects on mitogen-induced proliferation. It has also been demonstrated that the polyclonal

response to LPS, the T-independent response to TNP-LPS, and the T-dependent response to SRBC are characterized by comparable dose-response curves that are approximately parallel (Dooley and Holsapple, 1988; Chastain-Jr and Pazdernik, 1985). Holsapple et al. (1986) demonstrated that comparable suppression of the in vitro antibody response could be obtained in both responsive and non-responsive mouse strains. These findings suggest that the B cell is the primary target for the direct suppressive effects of dioxin and that the in vitro suppressive effects of dioxin are dissociated from the AhR activity which occurs in vivo.

A number of studies have been conducted to determinate TCDD-induced alteration of cytokines. Decreased IL2 levels in plasma or cell cultures has been noted in mice and rats following TCDD exposure in vivo (Lundberg et al., 1990, 1992; Prell and Kerkvliet, 1995; Bedesha et al., 1995; Kerkvliet et al., 1996). Interestingly, IL2 mRNA and production of IL2 was initially elevated but later suppressed in TCDD-treated mice following P815 tumor cell injection (Kerkvliet et al., 1996). Several laboratories have reported that TCDD exposure enhanced IL1 mRNA and the production of IL1, TNF, and inflammatory mediators (Steppan and Kerkvliet, 1991; Clark et al., 1981; Moos et al., 1994). In addition, the hyperimflammatory responses were reduced by pretreating TCDD-exposed mice with anti-TNF antibodies or TNF-soluble binding protein (Taylor et al., 1992; Moos et al., 1994). These results suggest that TCDD may enhance responses to inflammatory stimuli. The production of IL4, IL6 and IL10 seems to be unaffected by TCDD exposure (Kerkvliet et al., 1996).

Species-specific responses to TCDD-induced alteration of immune system

A number of studies have shown species difference in the effects of TCDD on immune function. For example, influenza virus titers were enhanced while virus-augmented NK activity was suppressed in virus infected-rats exposed to 10μg/kg or 3μg/kg TCDD, respectively (Yang et al., 1994). In contrast, increased mortality to influenza virus was observed in mice exposed to 0.1μg/kg (House et al., 1990) or 0.01μg/kg TCDD (Burleson et al., 1996). These results suggest that mice are more sensitive to TCDD-induced immune suppression than rats. Lubeke et al. (1994, 1995) reported that proliferative responses of lymphocytes cultured with Trichinella spiralis antigen were enhanced in F344 rats exposed to TCDD; whereas suppression of the proliferative responses to this antigen was noted in B6C3F1 mice exposed to TCDD. TCDD suppressed the plaque forming cell (PFC) responses to SRBC in mice but enhanced it in rats (Smialowicz et al., 1994). However, to date, the exact mechanism of species-specific responses to TCDD-induced alteration of immune system is unclear.

PURPOSE OF THESIS

This dissertation describes the effects of TCDD on T cell activation and cytokine production using a superantigen rat model and provides new information concerning the underlying cellular mechanism(s) of TCDD-induced immune dysfunction in rats.

The studies described herein include 1) development of a superantigen model in the Long/Evans rat; 2) examination of the effects of TCDD on circulating cytokines, production of cytokines by cultured spleen and peritoneal cells, and alteration of T cell

subsets; 3) examination of the effects of a single or repeated doses of TCDD on T cell subsets.

CHAPTER II

XENOBIOTIC AND SUPERANTIGEN ASSOCIATIONS I.IMMUNOCYTOKINE KINETICS OF A SUPERANTIGEN MODEL IN THE LONG/EVANS RAT

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ABSTRACT

The kinetics of selected cytokines (interleukin 2 [IL2], interleukin 6 [IL6], interferon γ [IFN-γ] and tumor necrosis factor [TNF]) and phenotype and cell cycle analysis of T lymphocytes were determined in Long/Evans rats administered a single intraperitoneal (ip) dose of either 50 μg or 500 μg of staphylococcus entotoxin B (SEB). Rats injected with 50 μg SEB had significantly elevated levels of IL2, IL6 and IFN-γ in their serum two hours post injection. IL2 serum levels peaked at two hours and returned to near control values by 12 hours while both IL6 and IFN-γ peaked at six hours but remained significantly increased at 24 hours post SEB exposure.

A 500 μg dose of SEB did not further enhance these cytokine responses. When spleen cells were collected for culture two hours after rats were injected ip with 50 μg SEB and co-cultured with SEB, TNF levels were significantly increased after two hours incubation, while IL2 and IL6 were significantly elevated at six hours. Production of all these cytokines continued to increase over the 24 hours sampled. Peritoneal cells were collected for culture either at one hour or two hours after injection of either 50 μg or 500 μg of SEB. Interleukin 6 was significantly increased after one hour in culture while TNF was significantly increased by two hours regardless of whether the cells were harvested one or two hours after SEB injection. The greatest response for both IL6 and TNF occurred when cells from animals injected with 50 μg SEB were restimulated in vitro with SEB. The peak levels for IL6 were 26at 12 hours post SEB exposure while TNF peaked at six hours. The percent of $CD4^+$ cells was significantly increased at 48 and 72 hours post SEB (50 μg) administration while the percentage of $CD8^+$ cells remained similar to control values for the 168-hour test period. A similar pattern was observed in

cell cycling where the $CD4^+$ cells proliferated up to two days post SEB injection and then were suppressed from day three to day seven. The $CD8^+$ cells were comparable to control values. These studies demonstrate that the cytokine response in Long/Evans rats exposed to a superantigen are similar to those in humans, e.g., a rapid short increase in the production of IFN-γ and TNF that was accompanied by an increase in the production of IL2 and IL6 as well as an early increase in the number and cycling of $CD4^+$ cells followed by a suppression of these events. These activities occurred in the absence of notable histopathological alteration of lymphoid organs.

INTRODUCTION

Superantigens are a family of bacterial and viral proteins which activate a large proportion of T lymphocytes in a MHC class II-dependent, but unrestricted, fashion as a result of binding to T-cell receptor (TCR) Vβ regions (Fleischer and Schrezenmeier, 1988; Marrack and Kappler, 1990; Jardetzky *et al.*, 1994; Acharya *et al.*, 1994). The processing of antigens is not necessary for binding of superantigens to the MHC class II, and the sequences on the MHC class and TCR Vβ chains involved are distinct from the parts involved in interaction with conventional peptide antigens.

The staphylococcal enterotoxins are a group of proteins secreted by *Staphylococcal aureus* (Herman *et al.*, 1991; Goodglick and Braun 1994; Blackman and Woodland, 1995; Johnson *et al.*, 1996). These proteins encoded by bacteriophages are the cause of staphylococcal food poisoning and severe shock in humans and experimental animals (Herman *et al.*, 1991; Goodglick and Braun, 1994; Uchiyama *et al.*, 1994; Webb and Gascoigne, 1994; Blackman and Woodland, 1995). Staphylococcal enterotoxin B

(SEB) activates Vβ selective T cells and macrophages to induce high levels of interleukin 2 (IL2), interferon γ (IFN-γ) and tumor necrosis factor (TNF) (Herrman *et al.*, 1992; Miethke *et al.*, 1992; Nagelkerken *et al.*, 1993; Bette *et al.*, 1993; Litton *et al.*, 1994; Florquin *et al.*, 1995; Blackman and Woodland, 1995). It is believed that the secretion of these cytokines plays a critical role in the pathogenesis of the symptoms of endotoxin-induced shock (Miethke *et al.*, 1993). The initial massive T-cell activation, secretion of cytokines, and expansion of the stimulated T-cell subsets is followed by death and development of anergy in these cells (White *et al.*, 1989, Kawabe and Ochi, 1991; Webb and Gascoigne, 1994; Schols *et al.*, 1995).

There are several reports of SEB effects on cytokine production in the mouse (Mietake *et al.*, 1992; Pfaffer *et al.*, 1993; Nagaki *et al.*, 1994; Matthys *et al.*, 1995; Florquin *et al.*, 1995), but the syndrome has not been characterized in the rat. It has been demonstrated that SEB preferentially stimulates mouse T cells bearing Vβ3, 7, 8.1, 8.2, 8.7 and 17 gene products (Janaway *et al.*, 1989; White *et al.*, 1989), human T cells bearing Vβ3, 12, 14, 15 and 17 gene products (Kappler *et al.*, 1989) and rat T cells bearing Vβ7, 11, 17 and 19 gene products (Sellins *et al.*, 1992). Although SEB stimulates different T cell Vβ gene products in the mouse, rats and humans, it is unknown if the cytokine responses are comparable in these three species following exposure to SEB.

This investigation was conducted to identify and quantitate, over time, selected cytokine responses in Long/Evans rats following exposure to SEB. The purpose was to develop an appropriate superantigen model in the rat in which to subsequently evaluate the interaction of concomitant exposure of superantigens and xenobiotics. In the present

study, we investigated the cytokine responses in the serum and cultured spleen and peritoneal cells of rats after a single injection of different doses of SEB. The time course of *in vitro* SEB restimulation was also assessed, as was phenotype analysis and cell cycling of $CD4^+$ and $CD8^+$ T cells and histopathology of the lymphoid organs.

METHODS

Animals and SEB Treatment. Female Long/Evans rats (Simonson Laboratories, Inc., Gilroy, CA) 10-11 weeks of age were housed in polycarbonate cages with hardwood bedding and maintained on a 12-hour light/dark cycle at 22 ± 2° C. Harlan Teklad rodent diet and tap water were given *ad libitum.* The rats were injected intraperitoneally (ip) with 50 μg or 500 μg of staphylococcal enterotoxin B (SEB) (Sigma, St. Louis, MO) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) (GIBCO-BRL, Grand Island, NY). Controls were injected ip with DPBS. Animals were killed 1, 2, 6, 12, 24, 48, 72, 120 or 168 hours post SEB injection by CO_2 asphyxiation. There were four rats per respective sampling time for each treatment and control group. Blood was collected via heart puncture while the animal was anesthetized and the plasma was promptly separated by centrifugation and stored at -70° C until used in the IL2, IL6, IFN-γ and TNF assays. Spleen and peritoneal cells were collected and processed for cytokine production or flow cytometric analysis.

Serum, Spleen Cell and Peritoneal Cell Culture Preparation. Single spleen cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Red blood cells were removed by hypotonic lysis. Spleen cells were washed two times and resuspended to $5x10^6$ cells/ml in 1640 media (RPMI)

supplemented with 10% fetal calf serum (FCS), 1mM penicillin-streptomycin and 50μM 2-mercaptoethanol (Sigma, St. Louis, MO). Spleen cells were cultured in duplicate wells without or with 10μg/ml of SEB in vitro in 12-well plates. Plates were incubated at 37° C in an atmosphere of 5% CO_2 for respective time until sampled post SEB injection. Supernatants were collected and stored at -70° C until used in the IL2, IL6, or TNF assay. Peritoneal cells were collected by carefully inserting a 20-gauge needle through the abdominal wall and injecting 35 ml of cold PBS containing 10 U/ml of heparin. The abdomen was massaged for one minute and the lavage fluid (30-32 ml) was slowly aspirated. The red blood cells were removed by hypotonic lysis. The peritoneal cells were washed two times and resuspended to $1x10^6$ cells/ml in RPMI supplemented with 10% FCS, 1mM penicillin-streptomycin and 50μM 2-mercaptoethanol. The peritoneal cells were cultured in duplicate wells with or without 10μg/ml of SEB *in vitro* in 12-well plates. Plates were incubated at 37° C in an atmosphere of 5% CO_2 for their respective times after SEB exposure. Supernatants were collected and stored at -70° C until analyzed.

Bioassay for IL2. IL2 activity in the serum and supernatants from spleen cell cultures was measured by the CTLL-2 cell assay. Briefly, CTLL-2 cells were cultured in a 96-well plate with $5x10^3$ cells/well in RPMI supplemented with 10% FCS, 1mM penicillin-streptomycin and 50μm 2-mercaptoethanol. Samples were used at a serial two-fold dilution and measured in triplicate. After 28 hours at 37° C and in 5% CO_2, cells were added to 1-μci of [3H] thymidine for 24 hours, harvested, and thymidine incorporation was measured by liquid scintillation counting.

Bioassay for IL6. IL6 activity in the serum and supernatants from spleen cell and peritoneal cell cultures was measured by the B9 cell assay. Briefly, B9 cells were cultured in a 96-well plate with 2,000 cells/ml in RPMI supplemented with 10% FCS, 1mM penicillin-streptomycin and 50μm 2-mercaptoethanol. Samples were used at a serial four-fold dilution and measured in triplicate. After incubating 84 hours at 37° C in 5% CO_2, the cells were added to 0.5 uCi of [^{3}H] thymidine for six hours, harvested, and thymidine incorporation was measured by liquid scintillation counting.

Bioassay for TNF. TNF activity in the serum and supernatants from spleen cell and peritoneal cell cultures was assayed as cytotoxic activity against murine L-929 fibroblasts. Briefly, L-929 cells were seeded at 25,000 cells/well in a flat-bottomed 96-well plate in RPMI supplemented with 10% FCS, 2mM L-Glutamine, 1mM penicillin-streptomycin, and 50 μm 2-mercaptoethanol and incubated overnight, after which non-adherent cells were removed and replaced with 100 μl fresh media containing 6 μg/ml Actinomycin D for 30 minutes at 37° C. The samples were added to the plates in duplicate at a serial five-fold dilution. After 18 hours at 37° C, supernatants were discarded and adherent cells stained with 200 μl of 0.2% crystal violet for 10-15 min. Plates were rinsed with double distilled water and allowed to dry, after which the stained cells were lysed with 200 μl of 10% SDS for 1-2 hours, and optical density read at 595nm.

Enzyme-Linked Immunosorbant Assay (ELISA) for IFN-γ. IFN-γ activity in the plasma was measured using an ELISA kit from Biosource International (Camarillo, CA, USA) at an optical density of 450nm using a microplate reader (Biotek, Winooski, VT, USA).

Flow cytometric analysis. Spleens were removed after SEB injection. Single cell suspensions of spleen cells were prepared as described previously. Spleen cells ($2x10^6$) were incubated on ice in V-bottomed 96 well plates in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Plates were blocked with excess mouse IgG (Sigma Chem. Co., St., Louis, MO) prior to staining with FITC-conjugated anti-$CD4^+$ or anti-$CD8^+$. Immediately after staining, cells were passed through a 40 μm nylon mesh to remove cell clumps. The cells were stained with propidium iodide for 30 min. on ice. The samples were analyzed for S phase DNA content on an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL, USA).

Histopathology. The rats were necropsied and sections of the lung, liver, kidney, spleen, lymph nodes and thymus were collected for histopathological examination. Samples were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin.

Statistics. ANOVA modeling was performed to determine significant change in cytokine levels over time. The student's t test was used to compare treatment effects. Differences were considered statistically significant at $p<0.05$.

RESULTS

Administration of 50 μg SEB increased the serum levels of IL2, IL6 and IFN-γ at 2, 6, 12 and 24 hours post injection. There was a significant increase in the serum IL6 and IFN-γ levels in the SEB treated groups compared to the controls at 2, 6, 12 and 24 hours post SEB treatment (Figure 1). Serum IL2 peaked earlier (two hours post SEB treatment) than did serum IL6 and IFN-γ, which peaked at six hours. The serum TNF

level in the SEB treatment group was comparable to the controls (Figure 1). When rats were injected ip with 500 μg of SEB, the serum levels of IL2, IL6, IFN-γ, or TNF one hour post SEB treatment were similar to the 50 μg treatment group. At two hours post SEB, there was a significant increase in IL2, IL6 and IFN-γ activity in both the 50 μg and 500 μg groups compared to controls (Figure 2).

SEB enhanced the production of IL2 in the 6, 12 and 24 hour spleen cell cultures, but not in the one and two hour cell cultures (Figure 3). The IL2 levels increased as the time in culture increased. The splenic IL2 levels from the SEB-primed animals co-cultured for 24 hours with SEB were significantly increased compared to the SEB-primed (*in vivo*) but non-SEB restimulated (*in vitro*) groups (Figure 3).

SEB treatment enhanced the production of IL6 at 6, 12 and 24 hours in spleen cell cultures (Figure 3) and at 1, 2, 6, 12 and 24 hours in peritoneal cultures (Figure 4). The IL6 levels in the spleen cells increased as the time in culture increased (Figure 3). In the spleen cells, SEB in vivo combined with SEB in vitro resulted in the largest IL6 response (Figure 3). In the peritoneal cell cultures, the largest IL6 responses were in the SEB in vitro and the 50 μg or 500 μg in vivo plus SEB in vitro groups (Figure 4).

SEB treatment significantly stimulated the production of TNF at 2, 6, 12 and 24 hours in spleen cell cultures (Figure 3) and at 2, 6, 12 and 24 hours in peritoneal cell cultures (Figure 4). In the spleen cell cultures, the TNF levels increased as the time inculture increased (Figure 3). However, in the peritoneal cell cultures, the peak of TNF production was at six hours (Figure 4).

Phenotypic analysis of T cells as performed by flow cytometry demonstrated that the percentage of $CD4^+$ cells was significantly increased at 48 and 72 hours after ip

Figure II-1. Effect of SEB on serum cytokine profiles from rats injected intraperitoneally with 50 μg SEB. Rats were killed 1, 2, 6, 12 and 24 hour post SEB injection and serum cytokine levels were measured. P<0.05*, 0.01**, or 0.001*** between treatment groups.

Figure II-2. Effect of SEB on serum cytokine profiles from rats injected intraperitoneally with 50 μg or 500 μg of SEB. Rats were killed one or two hours post SEB injection and serum cytokine levels were measured. p<0.05* or 0.01** between control and treatment.

Figure II-3. Effect of SEB on cytokine profiles in spleen cell cultures from rats injected intraperitoneally with 50 μg of SEB. Spleens were removed two hours post SEB injection. Spleen cells ($5x10^6$ cells/ml) were cultured without or with 10 μg/ml of SEB in vitro for 1, 2, 6, 12 and 24 hours. Cytokine levels in culture supernatants were measured. p<0.05* or 0.01** between control and treatment unless specified.

Figure II-4. Effect of SEB on cytokine profiles in peritoneal cell cultures from rats injected intraperitoneally with 50 µg or 500 µg of SEB. Peritoneal cells were harvested in one hour (Figure A and Figure C) or two hours (Figure B and Figure D) post SEB injection. Peritoneal cells ($1x10^6$ cells /ml) were cultured without or with 10 µg/ml of SEB in vitro for 1, 2, 6, 12 and 24 hours. Cytokine levels in culture supernatants were measured. P<0.05* or 0.01** between without and with SEB in vitro treatment groups. d-f, p<0.05*.

Figure II-5. Cell cycle analysis of CD4 and CD8 cells in SEB-injected rats. Spleens were removed from Long/Evans rats at 1, 2, 3, 5 and 7 days after injection with 50 μg SEB. Spleen cells were stained with FITC-conjugated anti-CD4 or anti-CD8 and then stained with propidium iodide. The DNA content of CD4 and CD8 population was measured (Neumann et al, 1993). Data points represent the mean percentage of cells in the S&G2/M phases of the cell cycle. *p<0.05 between treatment groups.

Figure II-1

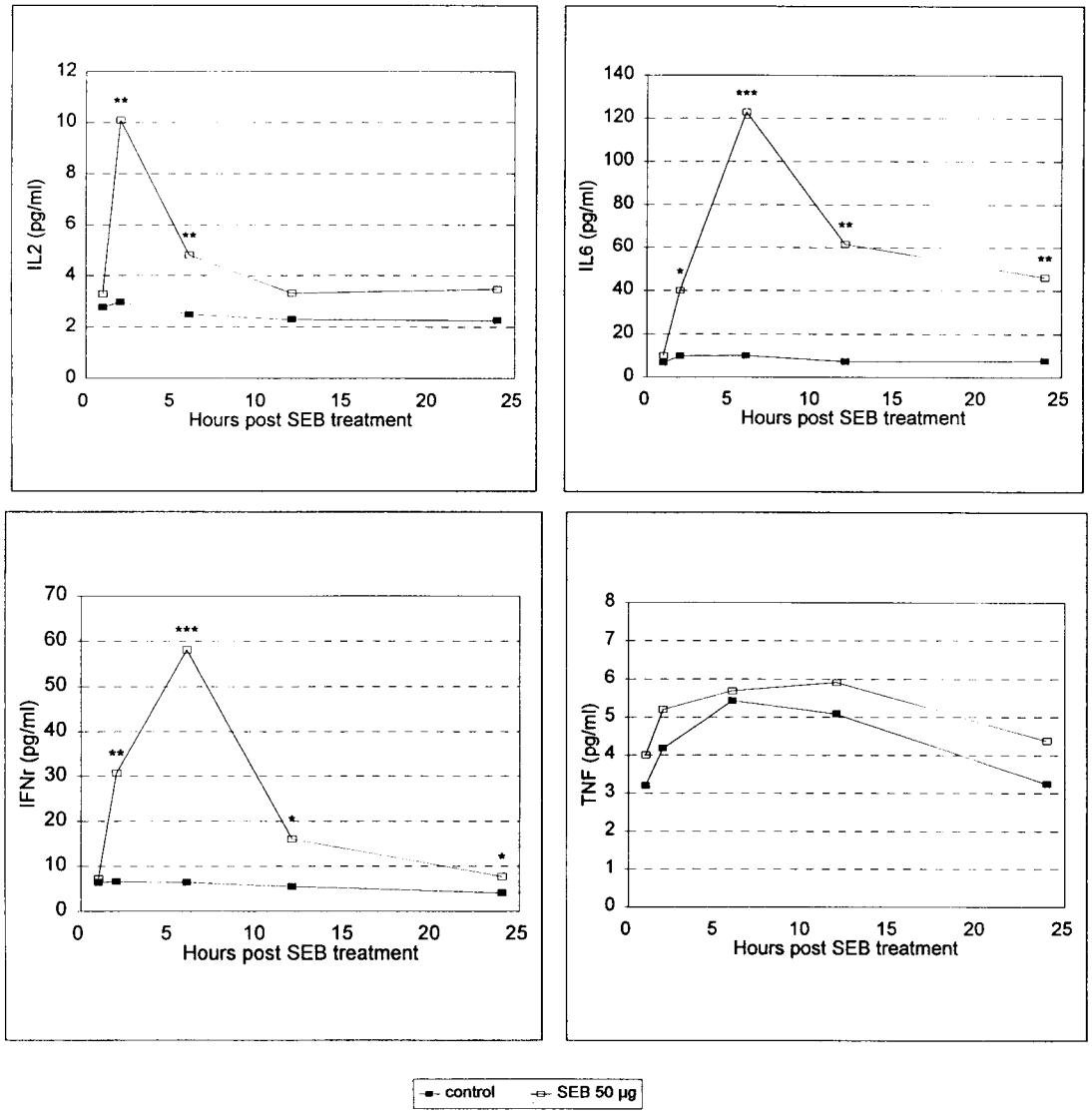
IL2 (pg/ml)
Hours post SEB treatment
IL6 (pg/ml)
Hours post SEB treatment
IFNr (pg/ml)
Hours post SEB treatment
TNF (pg/ml)
Hours post SEB treatment
control
SEB 50 µg

Figure II-2

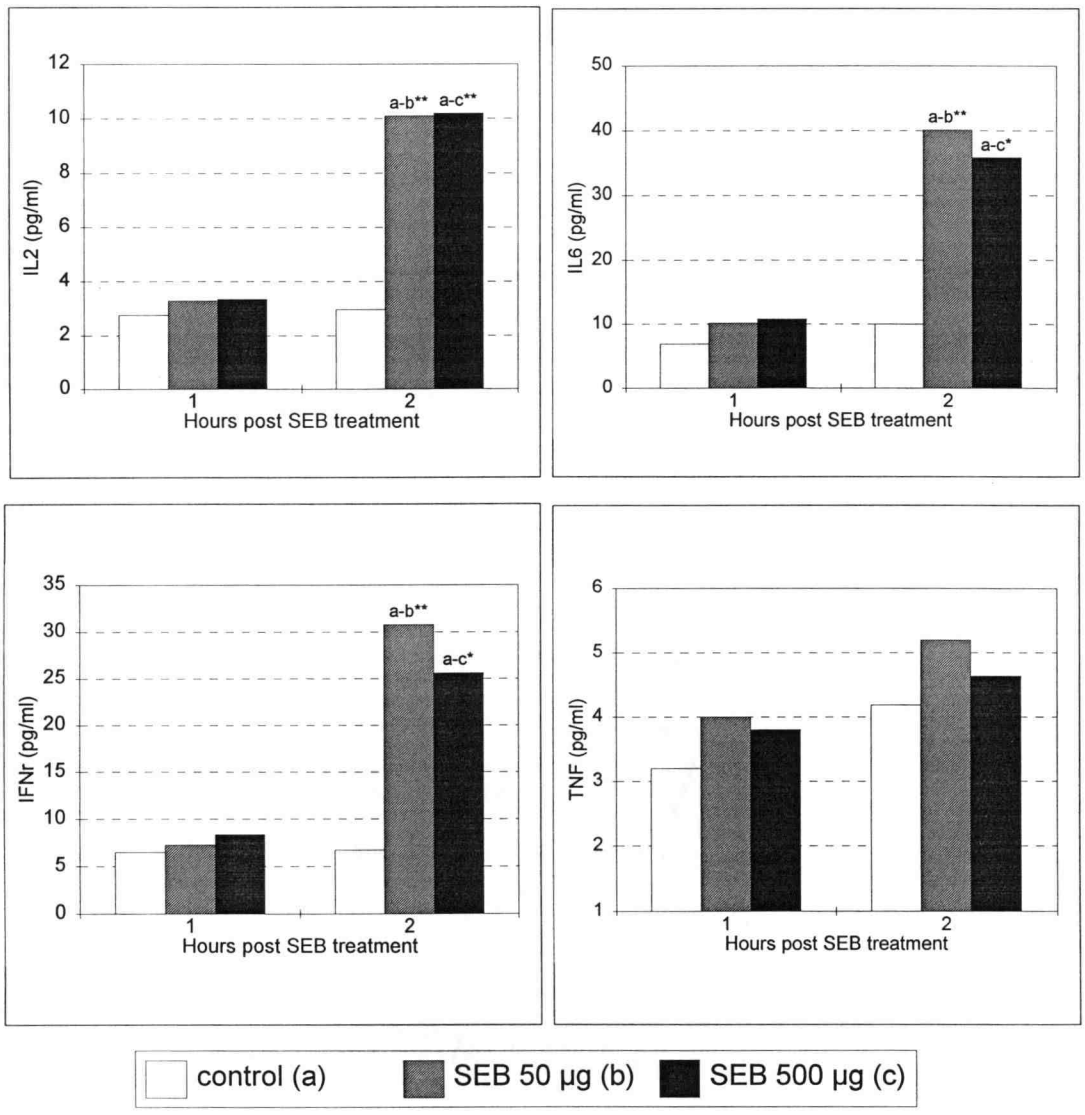
IL2 (pg/ml)
12
10
8
6
4
2
0
a-b** a-c**
1
2
Hours post SEB treatment
IL6 (pg/ml)
50
40
30
20
10
0
a-b**
a-c*
1
2
Hours post SEB treatment
IFNr (pg/ml)
35
30
25
20
15
10
5
0
a-b**
a-c*
1
2
Hours post SEB treatment
TNF (pg/ml)
6
5
4
3
2
1
1
2
Hours post SEB treatment
control (a)
SEB 50 µg (b)
SEB 500 µg (c)

Figure II-3

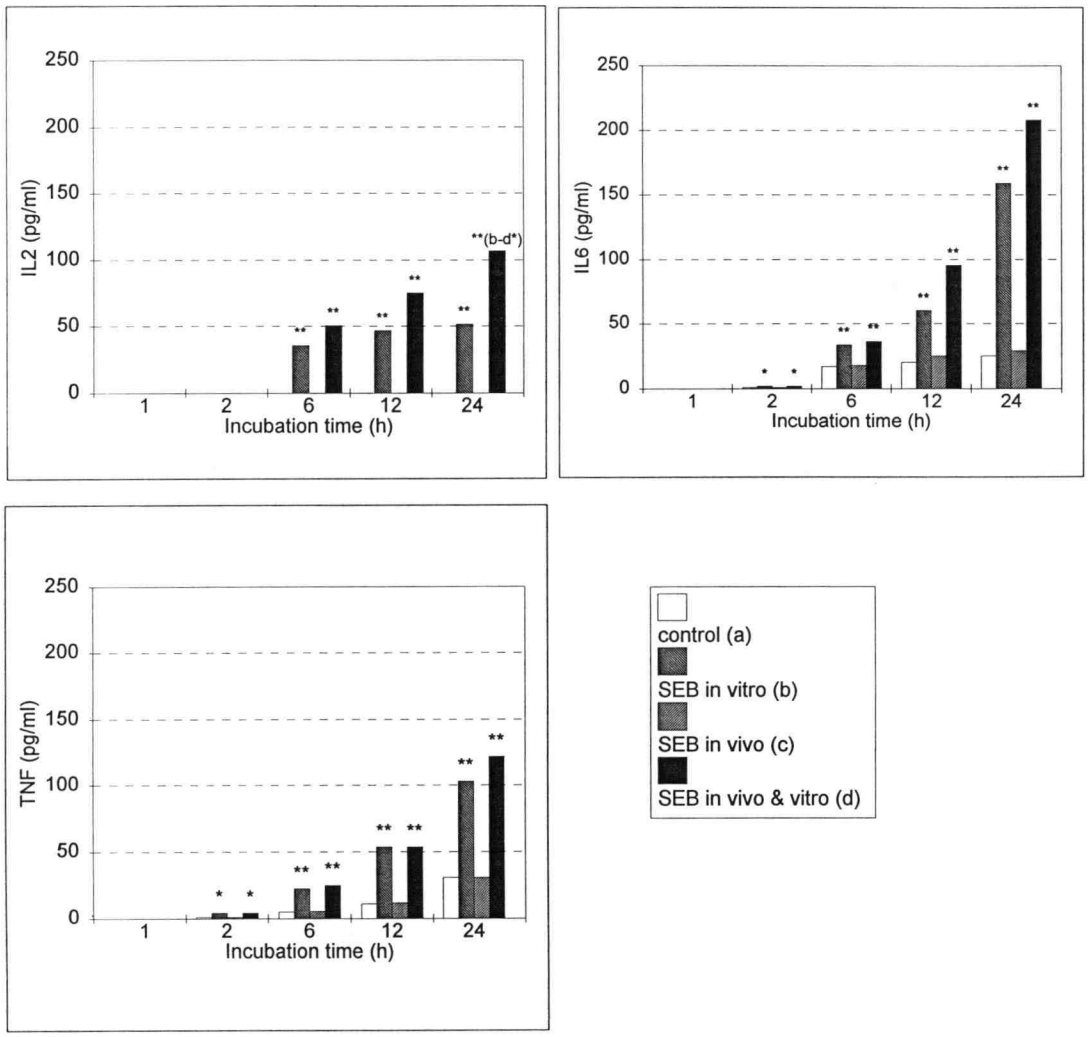
IL2 (pg/ml)
250
200
150
100
50
0
1
2
6
12
24
Incubation time (h)
**(b-d*)
IL6 (pg/ml)
TNF (pg/ml)
control (a)
SEB in vitro (b)
SEB in vivo (c)
SEB in vivo & vitro (d)

Figure II-4

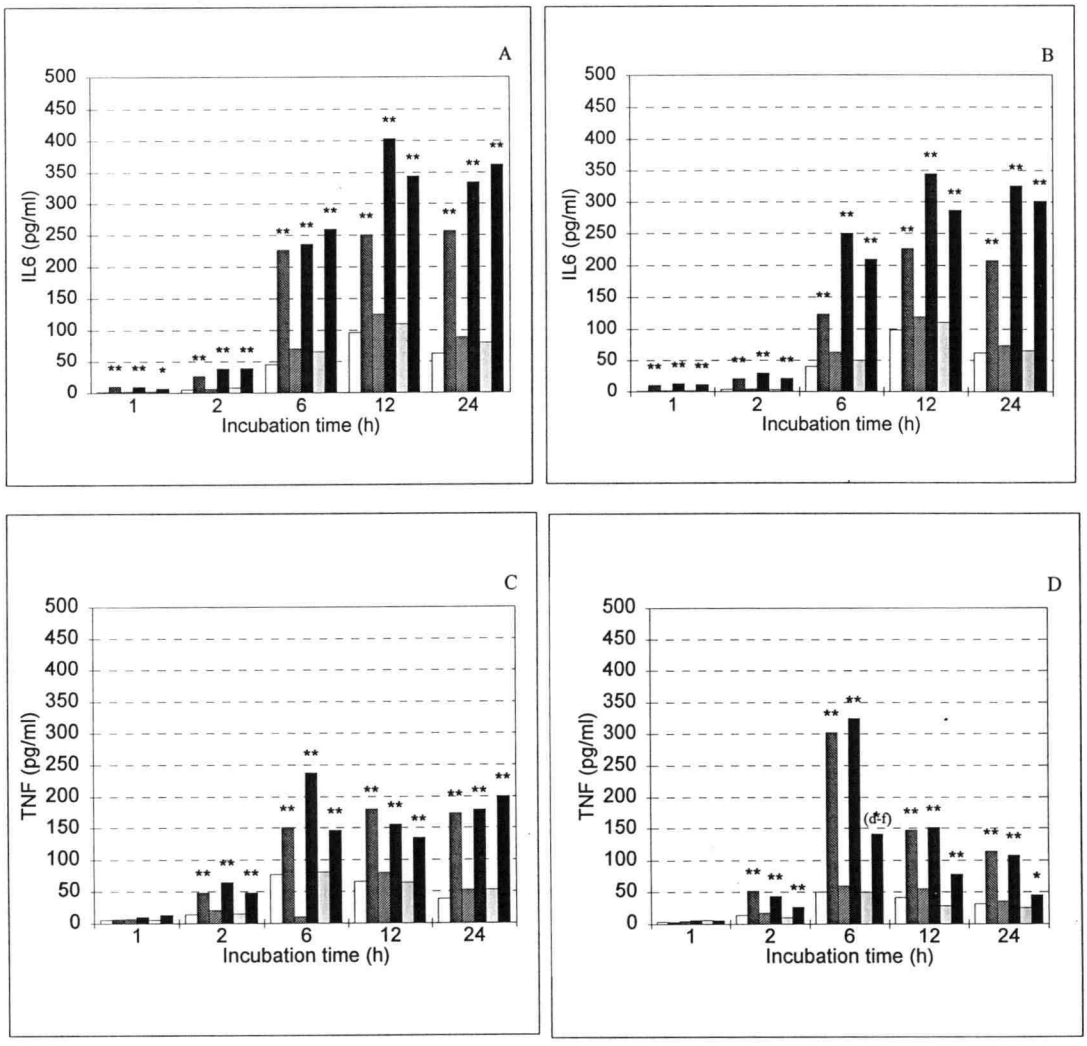
A
B
C
D
IL6 (pg/ml)
TNF (pg/ml)
Incubation time (h)
1
2
6
12
24
0
50
100
150
200
250
300
350
400
450
500
control (a)
SEB in vitro (b)
SEB in vivo 50ug (c)
SEB in vitro & vivo 50ug (d)
SEB in vivo 500ug (e)
SEB in vitro & vivo 500ug (f)

Figure II-5

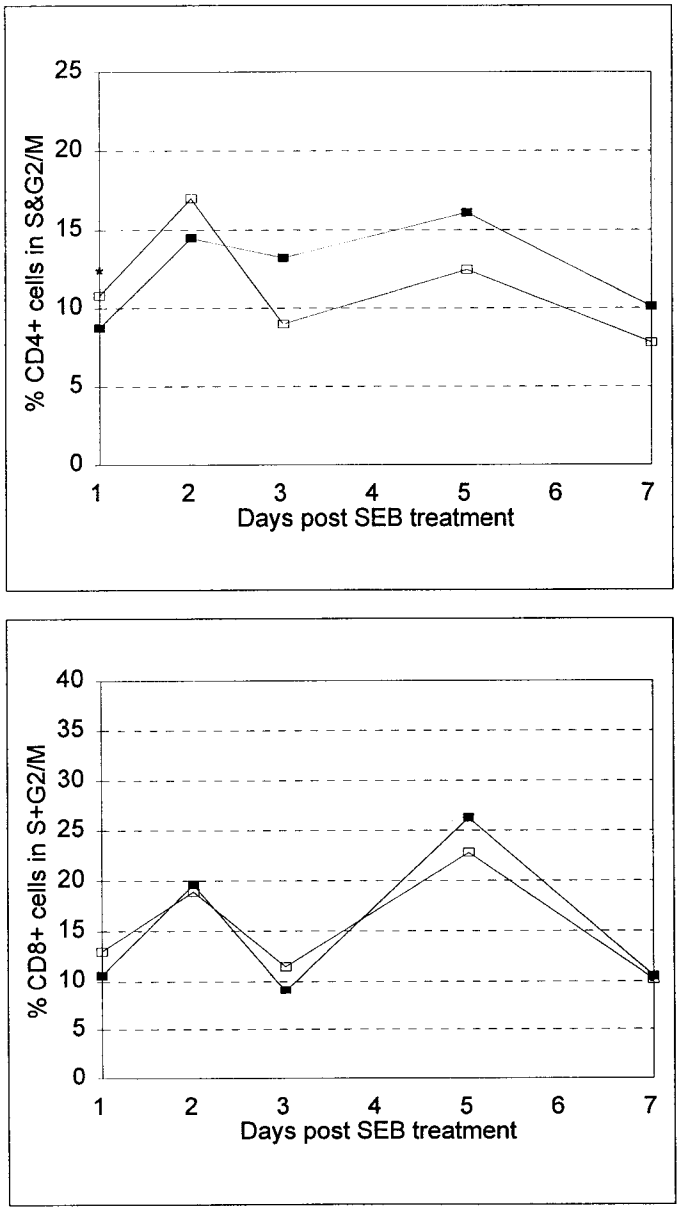
25
20
15
10
5
0
% CD4+ cells in S&G2/M
1
2
3
4
5
6
7
Days post SEB treatment
40
35
30
25
20
15
10
5
0
% CD8+ cells in S+G2/M
1
2
3
4
5
6
7
Days post SEB treatment
control
SEB 50 µg

Table II-1. Phenotypic analysis of T cells in the spleens of rats injected intraperitoneally with 50 μg SEB

Time (h) post SEB	%CD4 Control	%CD4 SEB	%CD8 Control	%CD8 SEB
24	30.96±1.56	32.09±1.16	33.68±0.72	36.96±2.53
48	31.33±0.40	34.93±0.51**	36.18±2.64	40.72±2.82
72	25.38±2.63	32.18±0.54*	38.89±1.04	39.29±3.09
120	34.20±3.13	36.91±2.23	32.22±1.17	34.11±1.59
168	35.22±3.01	33.72±0.33	33.02±1.53	34.91±2.04

Data represent mean ± SEM of four rats/treatment.
Significantly different than control for respective treatment group, P<0.05 or 0.01**

administration of SEB while there was no significant change in the $CD8^+$ cells compared to the non-SEB controls (Table 1). When the percentage of $CD4^+$ and $CD8^+$ cells in the S&G2/M phase were evaluated, there was a significant proliferation of the $CD4^+$ cells at 24 hours and a non-significant suppression at three, five and seven days (Figure 5). The percentage of $CD8^+$ cells was similar between the control and SEB treated groups.

Histopathologically, there were no observable effects of SEB on the lymphoid organs examined; e.g., spleen, thymus and lymph nodes.

DISCUSSION

Superantigens are a class of immunostimulating molecules that are produced by bacteria and viruses. Superantigens can induce an initial massive T cell activation which is expressed as a transient expansion of stimulated T cell subsets that is followed by death and development of anergy; e.g., functional inactivation of the activated cells. Cell death is primarily due to apoptosis that results in a long-lasting specific non-responsiveness in these cells to subsequent challenge to the superantigen (Kawabe and Ochi, 1991; MacDonald *et al.*, 1991; Hamel *et al.*, 1995; Lee *et al.*, 1995; Renno *et al.*, 1996). The symptoms of "toxic shock syndrome" are due to a burst-like expression of different cytokines (Bette *et al.*, 1993).

The cytokines actively involved in the pathogenesis of superantigen-induced shock appear to be IL2, IFN-γ and TNF (Herrman *et al.*, 1992; Miethke *et al.*, 1992; Nagelkerken *et al.*, 1993; Bette *et al.*, 1993; Litton *et al.*, 1994; Florquin *et al.*, 1995; Blackman and Woodland, 1995). In this study, these three cytokines and IL6 were evaluated following SEB exposure to develop a superantigen model in the Long/Evans

rats. The cytokine response was assessed in the serum, spleen cells and peritoneal cells at intervals up to 24 hours post exposure to SEB.

Significant levels of IL2, IL6 and IFN-γ appeared in the serum as early as two hours post SEB injection. IL2 peaked by two hours, declined sharply by six hours, and was no longer significantly elevated by 12 hours (Figure 1). Both IL6 and IFN-γ peaked at six hours, declined more than one-half by 12 hours, but remained significantly elevated at 24 hours post SEB exposure (Figure 1). Thus, there was a rapid co-stimulation of IL2, IL6 and IFN-γ in rats treated with SEB. The quick decline in serum IL2 was expected since transcription of IL2 is inhibited in animals re-exposed to SEB (Schwartz 1996). A 10-fold increase in the dose (500 μg) of SEB did not result in an additional increase in the response of these cytokines to SEB (Figure 2).

TNF was not significantly increased in the serum following exposure to SEB although elevated levels occurred in the spleen and peritoneal cell cultures that were co-cultured with SEB. TNF values did increase at one hour post SEB injection and peaked at 12 hours, but a similar pattern occurred in the control animals which was not expected and cannot be explained at this time. Further studies are necessary to better understand this response.

IL2, IL6 and TNF production was evaluated in spleen cells that were collected and cultured two hours after the rats were injected with 50 μg SEB. SEB significantly stimulated TNF levels by two hours and IL2 and IL6 levels by six hours (Figure 3). The levels of all three cytokines continued to increase with time for the 24 hours tested. The trend was somewhat different for production of IL6 andTNF from peritoneal cells that were cultured two hours after SEB injection. The IL6 levels were significantly increased

at one hour in culture while TNF was significantly increased at two hours (Figure 4B and 4D). The IL6 levels continued to increase and peaked at 12 hours with a slight decrease noted by 24 hours (Figure 4B). TNF, on the other hand, peaked at six hours with a marked decline by 12 hours that continued downward to 24 hours (Figure 4D). Thus, the production of IL6 and TNF occurred more rapidly in the cultured peritoneal cells than the spleen cells and the rapid decline in TNF from peritoneal cells could account for the low levels of TNF in the serum. TNF also was elevated earlier in the spleen cell cultures (two hours) than was IL2 or IL6 (six hours).

The IL6 and TNF response in peritoneal cell cultures was also assessed when the cells were cultured one hour after injection with 50 or 500 μg SEB (Figure 4A and 4C). The response was similar to SEB that was injected two hours before collecting the peritoneal cells for culture. In the spleen cell cultures, the greatest increase in IL2, IL6 and TNF production occurred upon restimulation of the cultured spleen cells with SEB in culture (Figure 3). The next largest response was the in vitro stimulation of cytokine production with SEB while cytokine induction via SEB exposure in vivo was comparable with the non-SEB exposed control values. A similar response occurred in cultured peritoneal cells. The largest values occurred with SEB exposure both in vivo and in vitro followed by in vitro only exposure, while the in vivo exposure to SEB was comparable to control values (Figure 4). IL6 was the first cytokine to be stimulated by SEB in culture (Figure 4). This occurred in the peritoneal cell cultures that are rich in macrophages, the primary source of IL6 production. Although IFN-γ and TNF are the principal cytokines involved in toxic shock (MacDonald *et al.*, 1993; Miethke *et al.*, 1992; Nagaki *et al.*, 1994), IL6 is involved in regulating the levels of IFN and TNF in the serum (MacDonald

et al., 1993). Thus IL6 could be an important secondary cytokine in the pathogenesis of the toxic shock syndrome.

Interleukin 1 is also stimulated by SEB. IL1 concentrations in the spleen and peritoneal cell cultures were significantly elevated at two hours after rats were injected with 50 μg SEB but had returned to near control levels by six hours (unpublished data). Thus, macrophages are activated by SEB to increase production of at least three monokines, e.g., IL1, IL6 and TNF.

In mice, IFN-γ and TNF are the predominant cytokines involved in the pathogenesis of SEB-induced shock (Miethke *et al.*, 1992; Pfeffer *et al.*, 1993; Nagaki *et al.*, 1994; Florquin *et al.*, 1995; Matthys *et al.*, 1995). Anti-TNF antibody prevented lethality in SEB-sensitized mice (Miethke *et al.*, 1992) and mice deficient in the TNF receptor 55-KDa were resistant to shock (Pfaffer *et al.*, 1993). Mice sensitized to SEB had five-fold higher levels of IFN-γ than controls while mice pre-treated with anti-IFN-γ were protected from the toxic effects of SEB (Florquin *et al.*, 1995). Thus the levels of both TNF and IFN-γ have been shown to be significantly increased in SEB-treated mice (Miethke *et al.*, 1992; MacDonald *et al.*, 1993; Florquin *et al.*, 1995; Matthys *et al.*, 1995).

Interleukin 2 levels are also increased in mice exposed to superantigens (Herrmann *et al.*, 1992; Florquin *et al.*, 1995; Hamel *et al.*, 1995). Elevated levels of IL1, IL2, IL6, IFN-γ and TNF appeared in the serum of mice injected with SEB (Blackman and Woodland, 1995). Further, IL2, IFN-γ and TNF were the principal cytokines produced in lymph nodes of SEB-treated mice (Litton *et al.*, 1994). These data are supported by stimulation of the messenger RNAs for IL2, IFN-γ and TNF of

superantigen-induced cytokine production *in situ* in the spleens of BALB/c mice (Bette *et al.*, 1993). MRL-lpr/lpr fas-defective mice administered a single dose of SEB had elevated serum levels of TNF and IL6 (Edwards *et al.*, 1996). When these mice were exposed to anti-TNF monoclonal antibody before SEB exposure, the serum TNF levels were reduced, as were the clinical signs of inflammatory arthritis and autoantibody formation, a spontaneous disease in these trangenic mice.

The response in the Long/Evans rats to a superantigen was similar to that which occurs in mice. There are elevated levels of IL2, IL6, IFN-γ and TNF in both species. Further, in a companion rat study, a significant increase in the percent of $CD4^+$ cells expressing the IL2 receptor occurred at 24 hours in spleen cell cultures (unpublished data). Although elevated levels of TNF were not detected in the serum of SEB-sensitized rats, a significant increase in production of TNF occurred in both the spleen and peritoneal cells. Additional studies are necessary to determine if TNF has a role in the clinical expression of toxic shock in rats. The doses used in these investigations were insufficient to induce toxic shock in the rats.

IFN-γ and TNF are also considered to be important mediators of the toxic shock syndrome in humans. SEB stimulation of human T cells *in vitro*-induced Type 1 T cells to produce high levels of IFN-γ and lower levels of IL4, IL5 and IL10, which was a result of early induction of TNF (Nagelkerken *et al.*, 1993). Human $CD4^+$ T cells produced larger amounts of TNF than $CD8^+$ T cells when stimulated with toxic shock syndrome toxin-1 (Akatsuka *et al.*, 1994).

The murine model has advanced the knowledge and understanding of the pathogenesis of superantigen-induced shock. The rat data from these studies would

indicate that the pattern of response of the most reactive cytokines following exposure to a superantigen are similar in rats, mice and humans. The rat, therefore, appears to be an acceptable animal model to further investigate the mechanisms of toxic shock syndrome in humans. The model does, however, require further development to fully characterize the disease process in this species.

Superantigens predominantly induce a Th1-type immune response in $CD4^+$ T cells (Dohlsten *et al.*, 1988; Holden and Moller, 1991; Herrmann *et al.*, 1992; Cardell *et al.*, 1993; Gollob *et al.*, 1993; Nagelkerken *et al.*, 1993). In the murine model, restimulation of SEB-primed animals induces anergy more severely in $CD4^+$ T cells than $CD8^+$ cells (Kawabe and Ochi, 1990; MacDonald *et al.*, 1991; Williams *et al.*, 1993). Further, anergy has been shown to be induced in Th1 clones but not Th2 clones (Quill *et al.*, 1992; Williams *et al.*, 1992; Cho *et al.*, 1993; Gajewski *et al.*, 1994). In this study, there was an initial increase in the percent and proliferation of $CD4^+$ cells in the S&G2/M cell cycle followed by suppression while there was no effect on $CD8^+$ cells (Figure 5 and Table 1). This decrease in $CD4^+$ cell cycling could have been a result of apoptosis of the $CD4^+$ subset.

In conclusion, Long/Evans rats respond to superantigens similar to the response observed in the mouse and humans. In particular, there is a rapid, rather short burst in the production of IL2, IL6, IFN-γ and TNF with an initial increase in percent and proliferative cycling of $CD4^+$ cells followed by a suppression of these events. The optimum *in vivo* priming dose in this study was 50 μg SEB since 500 μg generally resulted in a lower response. Although these basic studies would indicate that the Long/Evans rat is an acceptable model to study the mechanisms of action of

superantigen-induced disease in humans, additional investigations are warranted to further develop and characterize this animal model system.

CHAPTER III

XENOBIOTIC AND SUPERANTIGEN ASSOCIATIONS II. EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ON STAPHYLOCOCCAL ENTEROTOXIN B (SEB)-INDUCED CHANGES IN T-CELL ACTIVATION AND CYTOKINE PRODUCTION

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an environmental contaminant that is a potent immunotoxicant. In the present study, we examined the effect of TCDD on T-cell activation and cytokine production stimulated with a superantigen, Staphylococcal Enterotoxin B (SEB), which can active T-cells to produce and secrete cytokines. TCDD increased the serum levels of interleukin-2 (IL2) but did not affect the serum levels of interleukin-1 (IL1), interleukin-6 (IL6) or tumor necrosis factor (TNF) 2h post intraperitoneal injection of SEB. The ability of spleen cells and peritoneal cells to produce cytokines in response to SEB restimulation in vitro was also evaluated. TCDD exposure significantly enhanced IL2 production by spleen cells from SEB-primed rats after 6h or 24h in cultures co-stimulated with SEB in vitro. However, TCDD treatment did not alter the production of IL1, IL6 and TNF in these cultures. Although TCDD did not influence the production of IL6 and TNF in peritoneal cells from SEB-primed rats with SEB restimulation in vitro, IL1 production was significantly increased at 2h. TCDD did not significantly alter the percentage or the total numbers of $CD4^+$ and $CD8^+$ subpopulations at various times after SEB injection. However, flow cytometric analysis showed that TCDD exposure increased the percentage of both $CD4^+$ and $CD8^+$ cell cycling in S+G2M. Collectively, these results showed that TCDD can enhance production of IL1 and IL2 and the percentage of $CD4^+$ and $CD8^+$ cells cycling in SEB-exposed rats.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the prototype of the polychlorinated dibenzo-p-dioxins (PCDD), has been demonstrated to be the most potent and the most biologically active congener among the halogenated aromatic hydrocarbons (HAH) that are widely dispersed in the environment. Exposure to TCDD produces a wide spectrum of toxic responses, among them anorexia, a wasting syndrome (Christian *et al.*, 1986), thymus involution (Faith & Luster, 1979; Poland & Knutson, 1982; Clark *et al.*, 1983) and a variety of alterations in the integrity of the immune system (reviewed by Thomas & Faith, 1985; Vos & Luster, 1989; Holsapple *et al.*, 1991a; Kerkvliet & Burleson, 1994). Of the many organs/systems affected by TCDD, one of the most sensitive is the immune system.

Most of the immunotoxicological studies involving TCDD have been conducted in mice over the past two decades. It has been demonstrated by many investigators that the consequences of TCDD exposure include suppression of the antibody plaque-forming cell (PFC) response to the T cell-dependent antigen sheep red blood cells (SRBC) (Kerkvliet & Brauner, 1990; Kerkvliet & Oughton, 1993), inhibition of T-helper cell function (Tomar & Kerkvliet, 1991; Holsapple *et al.*, 1991b; Rhile *et al.*, 1996) and inhibition of cytotoxic T-lymphocyte activity (Clark *et al.*, 1981; Kerkvliet *et al.*, 1990). In addition, in the P815 tumor model, TCDD suppressed the production of tumor necrosis factor (TNF) and interferon-γ (IFN-γ), initially enhanced but later suppressed the production of interleukin-2 (IL2) (Kerkvliet *et al.*, 1996) and down-regulated the expression of B7 on B cells and macrophages (Prell & Kerkvliet, 1997).

However, compared to investigations in mice, few studies have been conducted in rats to assess the effects of TCDD on the humoral and cell-mediated immune response, especially on alteration of cytokines. It has been reported that humoral immunity was not affected in the offspring of rats dosed with TCDD during pregnancy and lactation (Faith & Moore, 1977). However, more recently, it has been shown that TCDD enhanced the PFC response to SRBC and affected cell mediated immunity in an inverted u-shaped dose-response curve with the low doses enhancing and high doses suppressing cell-mediated immunity (Fan *et al.*, 1996). In a study on the immunotoxic effects of prolonged dietary exposure of male Wistar rats to a low dose of TCDD, production of interleukin-1 (IL1) by splenic macrophages and IL2 by spleen cells were suppressed by TCDD (Badesha *et al.*, 1995). Direct comparison of the effects of TCDD on the PFC response in mice and rats revealed that the PFC response to a T-dependent antigen (SRBC) was suppressed in mice but enhanced in rats (Smialowicz *et al.*, 1994), while the PFC response to a T cell-independent antigen (TNF-LPS) was suppressed in both mice and rats (Smialowicz *et al.*, 1996).

Studies using anti-CD3 (Neumann *et al.*, 1993; Prell *et al.*, 1995) and ovalbumin models (Lundberg *et al.*, 1992) indicated that TCDD appeared to affect T-cells that were undergoing activation rather than residing in a resting state. The exact mechanism by which TCDD alters the immune system, especially species-specific responses, has yet to be elucidated (Holsapple, 1991; Kerkvliet & Burleson, 1994).

Staphylococcal Enterotoxin B (SEB), one of a group of staphylococcal enterotoxins causing food poisoning in the United States (Spero *et al.*, 1988), binds to class MHC-II molecules on macrophages or B-cells and to the Vβ region of the T-cell

receptor (TCR) . SEB activates Vβ specific T cells and macrophages to induce transient high levels of IL-2, IFN-γ and TNF (Herrman *et al.*, 1992; Nagelkerken *et al.*, 1993; Litton *et al.*, 1994; Florquin *et al.*, 1995; Blackman & Woodland, 1995). SEB can induce early activation signals, cytokine production, and proliferation of T cells expressing the appropriate TCR Vβ chains, followed by anergy and apoptosis (Renno *et al.*, 1996).

Our previous study revealed that the production of IL1,IL2, IL6, TNF and INF-γ was increased in rats exposed to SEB and indicated that the SEB model would provide a useful tool for studying the events that follow T-cell activation (Huang & Koller, 1997). In the current study, we evaluated the effects of TCDD on T cell activation and cytokine production in SEB primed rats.

METHODS

Animals. Female Long/Evans rats (Simonson Laboratories, Inc., Gilroy, CA) 10-11 weeks of age were housed in polycarbonate cages with hardwood bedding and maintained on a 12-hour light/dark cycle at 22+2° C. Harlan Teklad rodent diet and tap water were given ad libitum.

Animal Treatment. TCDD (Cambridge Isotope Laboratories, Woburn, MA. U.S.A. >90% pure) dissolved in anisole and diluted in peanut oil, was administrated by gavage at 5 or 25 μg/kg two days prior to Staphylococcal Enterotoxin B (SEB) treatment. Controls were treated with peanut oil by gavage. SEB (Sigma, St. Louis, MO) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) (GIBCO-BRL, Grand Island, NY), was injected intraperitoneally (ip) 50 μg/rat. Body weights were recorded daily throughout the studies. Animals were killed by CO_2 asphyxiation at 2, 24, 48, 72, 120 or 168 hours

post SEB injection. There were four to six rats per respective sampling time for each treatment and a control group. Blood was collected via heart puncture while the animal was anaesthetized and the serum was separated by centrifugation and stored at -70° C until used in the IL1, IL2, IL6 and TNF assays. Spleen and peritoneal cells were collected and processed for cytokine production or flow cytometric analysis.

Spleen Cell and Peritoneal Cell Culture Preparation. Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Red blood cells were removed by hypotonic lysis. Spleen cells were washed two times and resuspended to $5x10^6$ cells/ml in RPMI 1640 media (RPMI) supplemented with 10% fetal calf serum (FCS), 1mM penicillin-streptomycin and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO.). Spleen cells were cultured in duplicate wells without or with 10 μg/ml of SEB stimulation in vitro in 12-well plates. Plates were incubated at 37° C in an atmosphere of 5% CO2 for 2, 6, and 24h. Supernatants were collected and stored at -70° C until tested in the IL1, IL2, IL6 and TNF assays. Peritoneal cell were collected by carefully inserting a 20-gauge needle through the abdominal wall and injecting 35ml of cold DPBS containing 10 μ/ml of heparin. The abdomen was massaged for one minute and the lavage fluid (30-32 ml) was slowly aspirated. The red blood cells were removed by hypotonic lysis. The peritoneal cells were washed two times and resuspended to $1x10^6$ cells/ml in RPMI supplemented with 10% FCS, 1mM penicillin-streptomycin and 50 μM 2-mercaptoethanol. The peritoneal cells were cultured in duplicate wells with or without 10 μg/ml of SEB in vitro in 12-well plates. Plates were incubated at 37° C in an atmosphere of 5% CO_2 for 2, 6, and 24h. Supernatants were collected and stored at -70° C until analyzed.

Bioassay for Interleukin 1. IL1 activity in the serum and supernatants from spleen cell and peritoneal cell cultures was measured by the NOB-1 cell (generous gift from Dr. N.I. Kerkvliet's laboratory) assay described by Gearing *et al.* (1987). Briefly, the standard or samples used at a serial two-fold dilution and measured in duplicate were added to a flat-bottom 96 well plate. NOB-1 cells were resuspended at $2x10^6$/ml in RPMI supplemented with 10% FCS and added to each well with $2x10^5$ cells. After incubating 20-26 hours at 37° C in 5% CO2, the plate was centrifuged at 1000 rpm for 10 minutes to pellet the cells. The supernatant was harvested carefully and added to a 96 well plate in duplicate. IL2 levels in the supernatants were measured using the CTLL-2 cell bioassay. The IL2 standard curve was plotted and the average cpm values for the IL1 standard was converted to IL2 concentration. Then the IL1 standard concentration vs. the calculated IL2 concentration was plotted. The assay standard was used to determined IL1 in samples.

Bioassay for Interleukin 2. IL2 activity in the serum and supernatants from spleen cell cultures was measured by the CTLL-2 cell assay. Briefly, CTLL-2 cells were cultured in a 96-well plate with $5x10^3$ cells/well in RPMI supplemented with 10% FCS, 1mM penicillin-streptomycin and 50μM 2-mercaptoethanol. Samples were used at a serial two-fold dilution and measured in triplicate. After 28h at 37° C, 5% CO2, cells were added 1 μci of [^{3}H] thymidine for 24h, harvested and incorporation was measured by liquid scintillation counting.

Bioassay for Interleukin 6. IL6 activity in the serum and supernatants from spleen cell and peritoneal cell cultures was measured by using the B9 cell method. Briefly, B9 cells were cultured in a 96-well plate with 2,000 cells/ml in RPMI supplemented with

10% FCS, 1mM penicillin-streptomycin and 50 μM 2-mercaptoethanol. Samples were used at a serial four-fold dilution and measured in triplicate. After 84h at 37° C, 5% CO2, cells were added 0.5 μci of [^{3}H] thymidine for 6h, harvested and incorporation was measured by liquid scintillation counting.

Bioassay for Tumor Necrosis Factor. TNF activity in the serum and supernatants from spleen cell and peritoneal cell cultures was assayed as cytotoxic activity against murine L-929 fibroblasts. Briefly, L-929 cells were seeded at 25,000 cells/well in a flat-bottomed 96-well plate in RPMI supplemented with 10% FCS, 2mM L-Glutamine, 1mM penicillin-streptomycin and 50 μM 2-mercaptoethanol and incubated overnight, after which non-adherent cells were removed and replaced with 100 μl fresh media containing 6 μg/ml Antimycin D for 30 minutes at 37° C. The samples were added to the plates in duplicate at a serial five-fold dilution. After 18 hours at 37° C, supernatants were discarded and adherent cells stained with 200 μl of 0.2% crystal violet for 10-15 min. Plates were rinsed with DDH_2O and allowed to dry, after which the stained cells were lysed with 200 μl of 10% SDS for 1-2h, and optical density read at 595nm.

Flow Cytometric Analysis. Spleens were removed after SEB injection. A single cell suspension of spleen cells was prepared as described above. Spleen cells ($2x10^6$) were incubated on ice in V-bottomed 96 well plates in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Plates were blocked with excess mouse IgG (Sigma Chem. Co., St. Louis, MO) prior to staining with FITC-conjugated anti-CD4 or anti-CD8. Immediately after staining, cells were passed through a 40 μm Nylon mesh to remove cell clumps. The cells were stained with propidium iodide for 30 minutes on ice.

The samples were analyzed for S phase DNA content on an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL, USA).

Histopathology. The rats were necropsied and sections of the lungs, liver, kidneys, spleen, lymph nodes and thymus were collected for histopathological examination. Samples were fixed in 10% buffered formalin, sectioned, and stained with hemotoxylin and eosin.

Statistics. Data were analyzed for statistical significance using ANOVA or Student's T test. In all analyses, $p<0.05$ was considered statistically significant. The data presented in this paper are representative of data that were validated in at least two independent studies.

RESULTS

Effect of TCDD on body weight. One injection of 25 μg/kg of TCDD significantly impaired body weight gain compared to non-TCDD exposed rats (Fig. 1). However, rats injected with SEB alone did not lose body weight. Co-exposure of rats to TCDD and SEB did not further reduce the body weight produced by TCDD alone.

Effect of TCDD on spleen weight and cellularity. As shown in Fig. 2, there was no significant change in spleen weight, normalized to body weight, in SEB-treated rats. Spleen weight in TCDD-treated rats showed no change except an increase at 168h. Co-exposure of rats to TCDD and SEB showed a similar change in spleen weight compared to treatment with TCDD alone. The increased spleen weight, normalized to body weight, in the TCDD alone and co-exposed to SEB and TCDD animals at 168h could be due to

the body weight loss in those animals since absolute spleen weights were unaffected by these two groups.

Significant differences among the groups were the significant increases of the cell numbers in the spleens of rats co-exposed to TCDD and SEB at 2h and 120h post SEB injection compared to the control or the control/SEB alone animals, respectively. There were no significant changes in the spleen cell numbers in all treatment groups compared to the controls at other time points.

Effect of TCDD on serum cytokine levels. To investigate the dose-dependent effect of TCDD on SEB induced cytokine production, serum cytokine levels were measured at 2h post SEB injection. In our previous study, the results of a time-course study demonstrated that the peak SEB-induced increase in serum IL-2 was at 2 hours post SEB injection and serum cytokines were measured at 2 hours post SEB injection to investigate if TCDD abrogated the production of cytokines. As shown in Fig.3, treatment with SEB alone resulted in elevated serum IL2 and IL6 levels but not serum IL1 levels. There were no significant changes compared to the non-SEB control in the IL1, IL2 and IL6 levels in the TCDD alone animals. TCDD treatment did significantly alter the magnitude of SEB induced change in serum IL2 levels by enhancing the serum IL2 levels by about 10-fold in the 5 μg/kg TCDD treated rats and about 8-fold in the 25 μg/kg TCDD treated rats. TCDD did not significantly increase the serum IL6 levels in the SEB treated animals (Fig. 3). The IL1 levels were similar in all treatment groups and serum TNF levels were not detected in any of the treatment groups (data not shown).

Effect of TCDD on cytokine production by culture spleen cells. The spleen cells collected from animals 2h post SEB injection were stimulated with SEB in vitro for 2, 6

or 24h and cytokine levels in culture supernatants were measured. As shown in Fig. 4, the production of IL1, IL2, IL6 & TNF in all treatment groups increased as the time in culture increased. There were no significant changes in the levels of IL1, IL6 & TNF in the different treatment groups when compared to the control or each other. The IL-2 production in spleen cells from SEB-primed animals was increased at 24 hours. TCDD exposure significantly enhanced IL-2 production in the 5 μg/kg TCDD treated-SEB primed rats by 2.4-fold ($p<0.01$) at 6 hours , 1.8-fold ($p<0.01$) at 24 hours and in the 25 μg/kg TCDD treated-SEB primed rats by 2.2 fold ($p<0.01$) at 6 hours and 2.5-fold ($p<0.01$) at 24 hours in cultures.

Effect of TCDD on cytokine production by culture peritoneal cells. The production of IL1 & IL6 in peritoneal cell culture supernatants increased as the time in culture increased (Fig. 5). However, TNF peaked at six hours with a sharp decline by 24 hours. This phenomenon suggested that production of TNF for only a short period of time could account for the low levels of TNF in the serum. There were no significant changes in TNF between treatment groups for respective times in culture. The only significant change in IL6 was an increase in the 6h cell cultures in the SEB alone group compared to the control. TCDD exposure significantly increased the production of IL-1 by cultured

peritoneal cells from SEB-primed rats in 2h cell cultures (Fig. 5). IL1 production in cells from rats co-exposed to SEB and 5 μg/kg TCDD was significantly increased in 6h cell cultures compared to the control but not in other groups.

Effect of TCDD on IL2R and TCR expression. The dose-dependent effect of TCDD on $CD4^+$ cells expressing the interleukin-2 receptor (IL2R) in spleen cells stimulated with SEB in vitro 2h post SEB injection was investigated. As shown in

Figure III-1. The effect of TCDD on body weight of SEB-treated rats. Rats were treated with peanut oil or 25 μg/kg TCDD 2 days prior to SEB injection and body weight was recorded daily for 9 days. Each point represents the mean±S.E.M. of four rats per treatment group per timepoint. Significant difference between treatment groups: c-a,c-b, d-a, d-b, $p<0.05$* or 0.01**.

Figure III-2. The effect of TCDD on spleen weight to body weight ratio and number of spleen cells in SEB-treated rats. Rats were treated with peanut oil or 25 μg/kg TCDD prior to SEB treatment and killed at various times following SEB injection. Each bar represents the mean±S.E.M. of four to six rats per treatment group per timepoint spleen/body weight: c-a,d-a; spleen cell recovery: 2h d-a; 120h d-a, d-b; $p<0.05$* or 0.01**.

Figure III-3. The dose-dependent effects of TCDD on serum cytokine profiles in SEB-treated rats. Rats were treated with peanut oil or 5 μg/kg or 25 μg/kg TCDD 2 days prior to SEB treatment. Rats were killed 2 hour after SEB injection and serum cytokine levels were measured as described in Experiment Procedures. Each bar represents the mean+S.E.M. of six rats per treatment. IL2 b-a,b-c,d-a,d-b,d-c,e-a,e-b,e-c; IL6 b-a, b-c, d-a, d-c, e-a, e-c; $p<0.05$* or 0.01**.

Figure III-4. The effect of TCDD on cytokine profiles in spleen cell cultures from SEB-treated rats. Spleens were removed 2h post SEB injection. Spleen cells($5x10^6$ cells/ml) were cultured with 10ug/ml of SEB in vitro for 2, 6 & 24h. cytokine levels in culture supernatants were measured as described under Experimental Procedure. IL2: 6h d-a,d-b,d-c,e-a,e-b,e-c; 24h b-a,d-a, d-c, e-a, e-b, e-c $p<0.05$* or 0.01**.

Figure III-5. The effect of TCDD on cytokine profiles in peritoneal cell cultures from SEB-treated rats. Peritoneal cells were harvested 2h post SEB injection. Peritoneal cells ($1x10^6$ cells/ml) were cultured with 10 μg/ml of SEB in vitro for 2, 6&24h. Cytokine levels in culture supernatants were measured as described under Experimental Procedure. IL6 b-a, IL1: 2h d-a,d-b,d-c,e-a,e-b and e-c; IL1: 6h d-a; $p<0.05$* or 0.01**.

Figure III-6. The effect of TCDD on the kinetics of IL2R and TCR expression on $CD4^+$ cells from SEB-treated rats. Rats (n=4) were treated with TCDD 2 days prior to SEB treatment. Spleens were removed 2h post SEB injection. Spleen cells ($5x10^6$ cells/ml) were cultured with 10 μg/ml of SEB in vitro for 0, 6 & 24h and analyzed by flow cytometry as described in Experimental Procedures. Data reported as mean channel fluorescence. Each point represents the mean+S.E.M. per treatment.

Figure III-7. The effect of TCDD on the percentage of $CD4^+$ or $CD8^+$ cells cycling in S/G2M in SEB-treated rats. Spleens were removed from rats at 1, 2, 3, 5 & 7 days post SEB injection. Spleen cells were stained with propidium iodide. The DNA content of $CD4^+$ and $CD8^+$ population was measured. Data points represent the mean percentage of cells in the S+G2M phases of the cell cycle $CD4^+$: day 1 b-a,d-a,d-c; day 2 d-a; day 5 d-b; $CD8^+$: day 1 d-a,d-b,d-c; day 5 d-b, $p<0.05$*.

Figure III-1

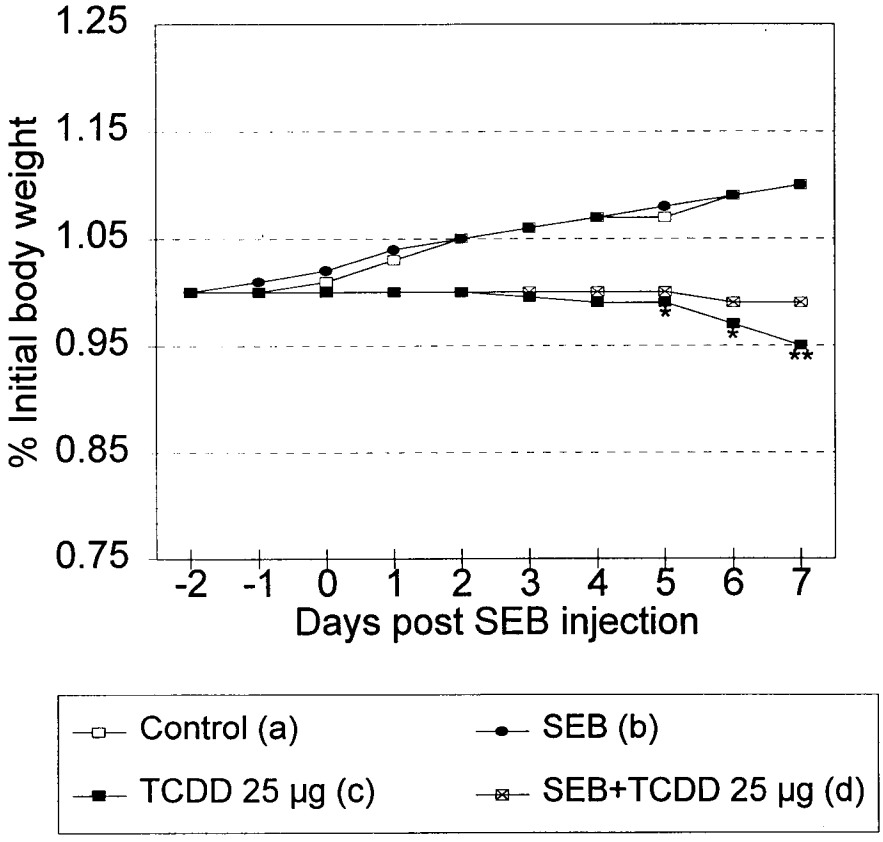

1.25
1.15
1.05
0.95
0.85
0.75
% Initial body weight
-2 -1 0 1 2 3 4 5 6 7
Days post SEB injection
*
*
**
Control (a)
SEB (b)
TCDD 25 µg (c)
SEB+TCDD 25 µg (d)

Figure III-2

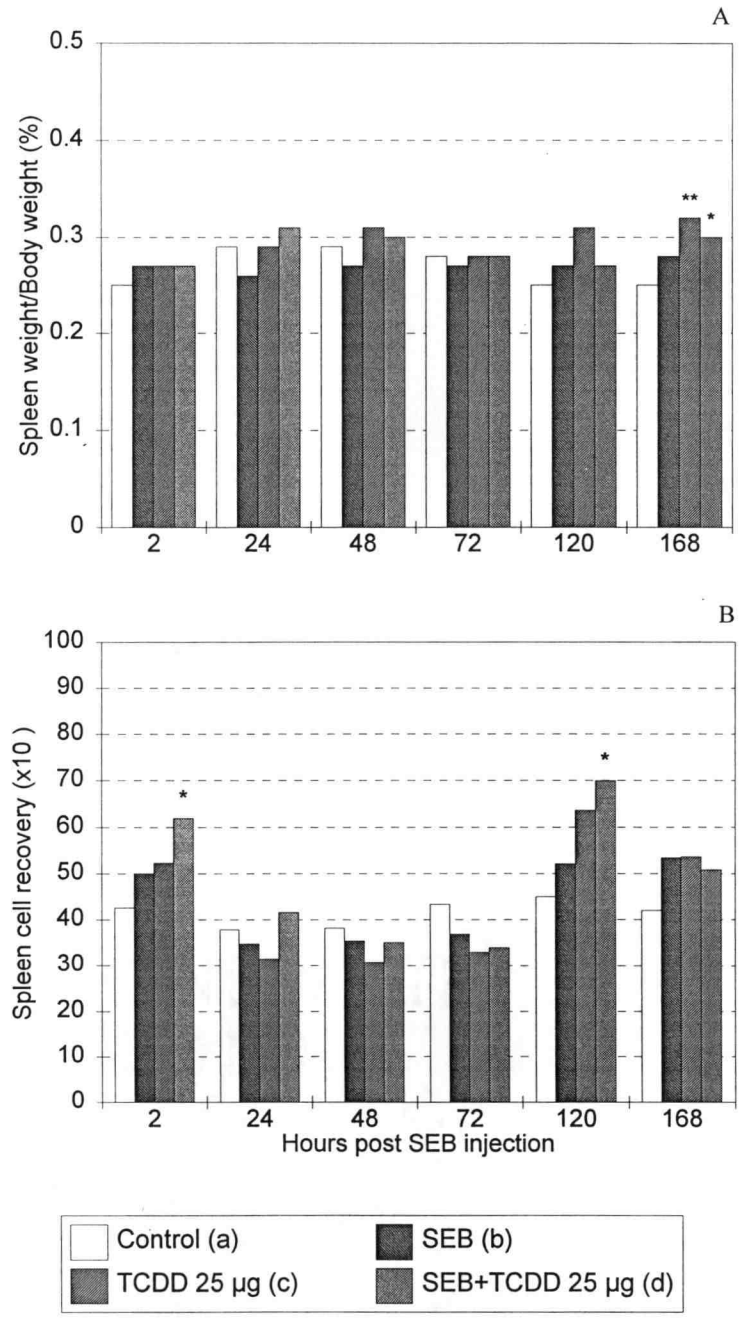
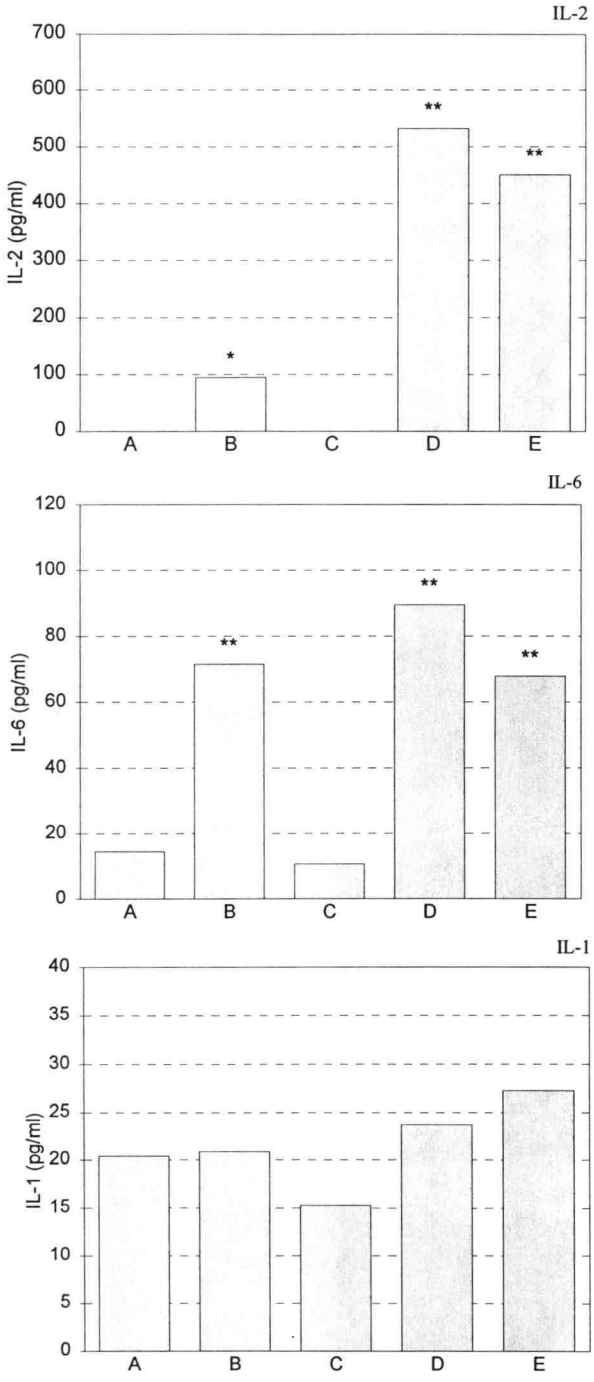

A
Spleen weight/Body weight (%)
0.5
0.4
0.3
0.2
0.1
0
**
*
2
24
48
72
120
168
B
Spleen cell recovery (x10)
100
90
80
70
60
50
40
30
20
10
0
*
*
2
24
48
72
120
168
Hours post SEB injection
Control (a)
SEB (b)
TCDD 25 µg (c)
SEB+TCDD 25 µg (d)

Figure III-3



Control(A) SEB (B) TCDD 25ug (C)
SEB+TCDD 5ug (D) SEB+TCDD 25ug (E)

Figure III-4

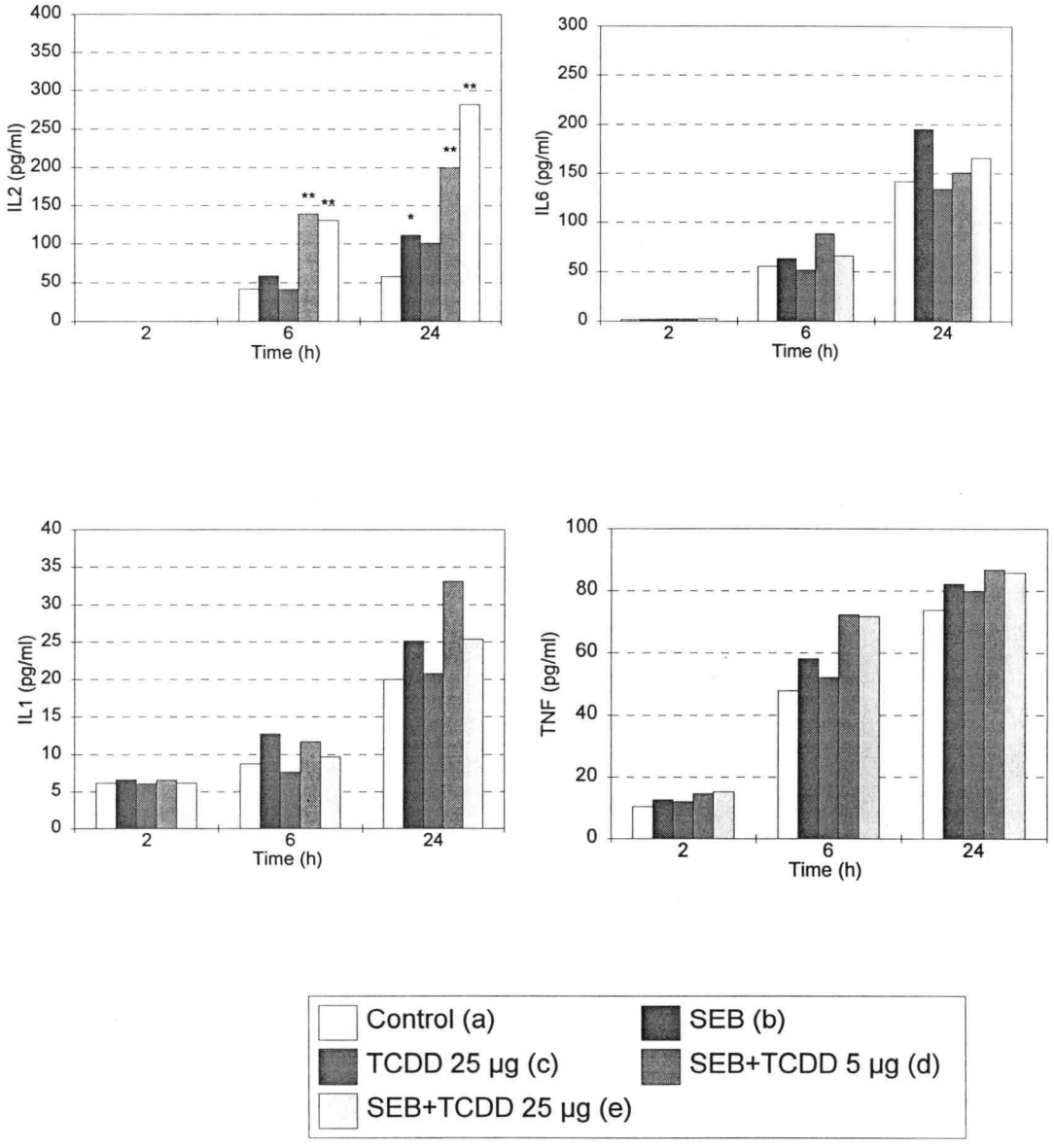

IL2 (pg/ml)
400
350
300
250
200
150
100
50
0
2
6
24
Time (h)
IL6 (pg/ml)
300
250
200
150
100
50
0
2
6
24
Time (h)
IL1 (pg/ml)
40
35
30
25
20
15
10
5
0
2
6
24
Time (h)
TNF (pg/ml)
100
80
60
40
20
0
2
6
24
Time (h)
Control (a)
SEB (b)
TCDD 25 µg (c)
SEB+TCDD 5 µg (d)
SEB+TCDD 25 µg (e)

Figure III-5

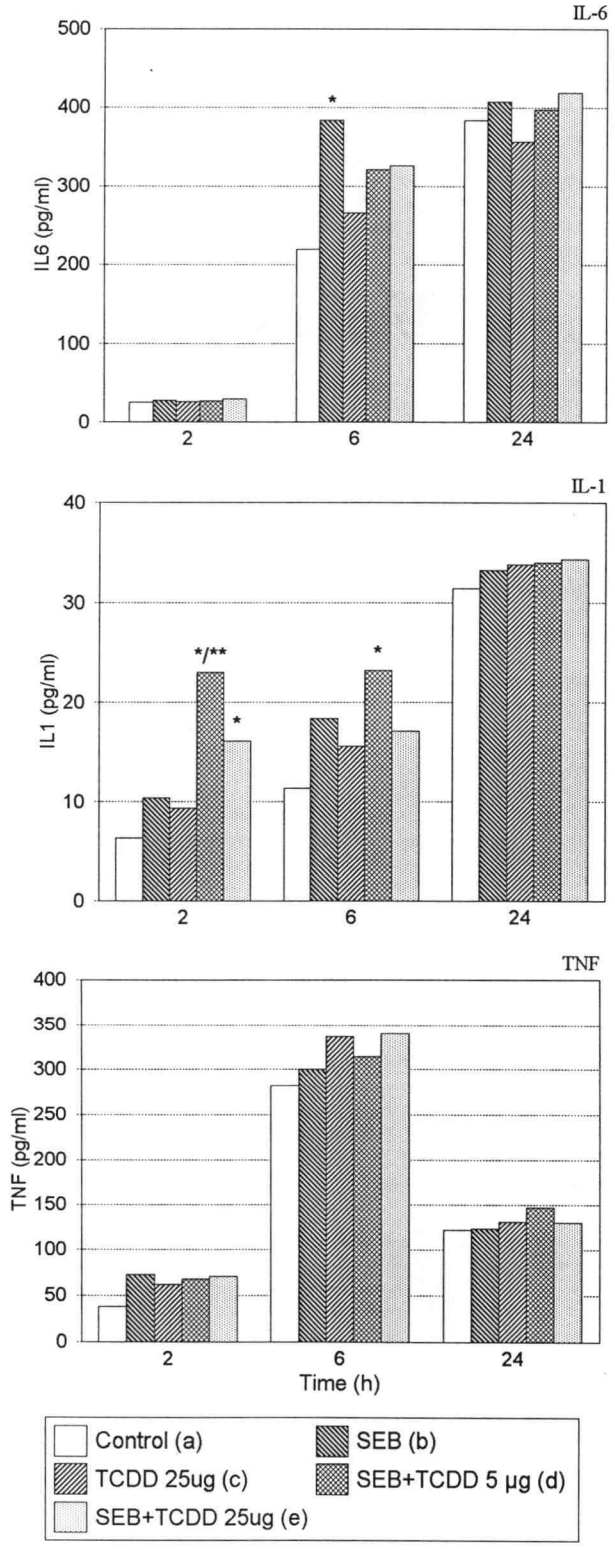

IL-6
500
400
300
200
100
0
IL6 (pg/ml)
*
2
6
24
IL-1
40
30
20
10
0
IL1 (pg/ml)
*/**
*
*
2
6
24
TNF
400
350
300
250
200
150
100
50
0
TNF (pg/ml)
2
6
24
Time (h)
Control (a)
SEB (b)
TCDD 25ug (c)
SEB+TCDD 5 µg (d)
SEB+TCDD 25ug (e)

Figure III-6

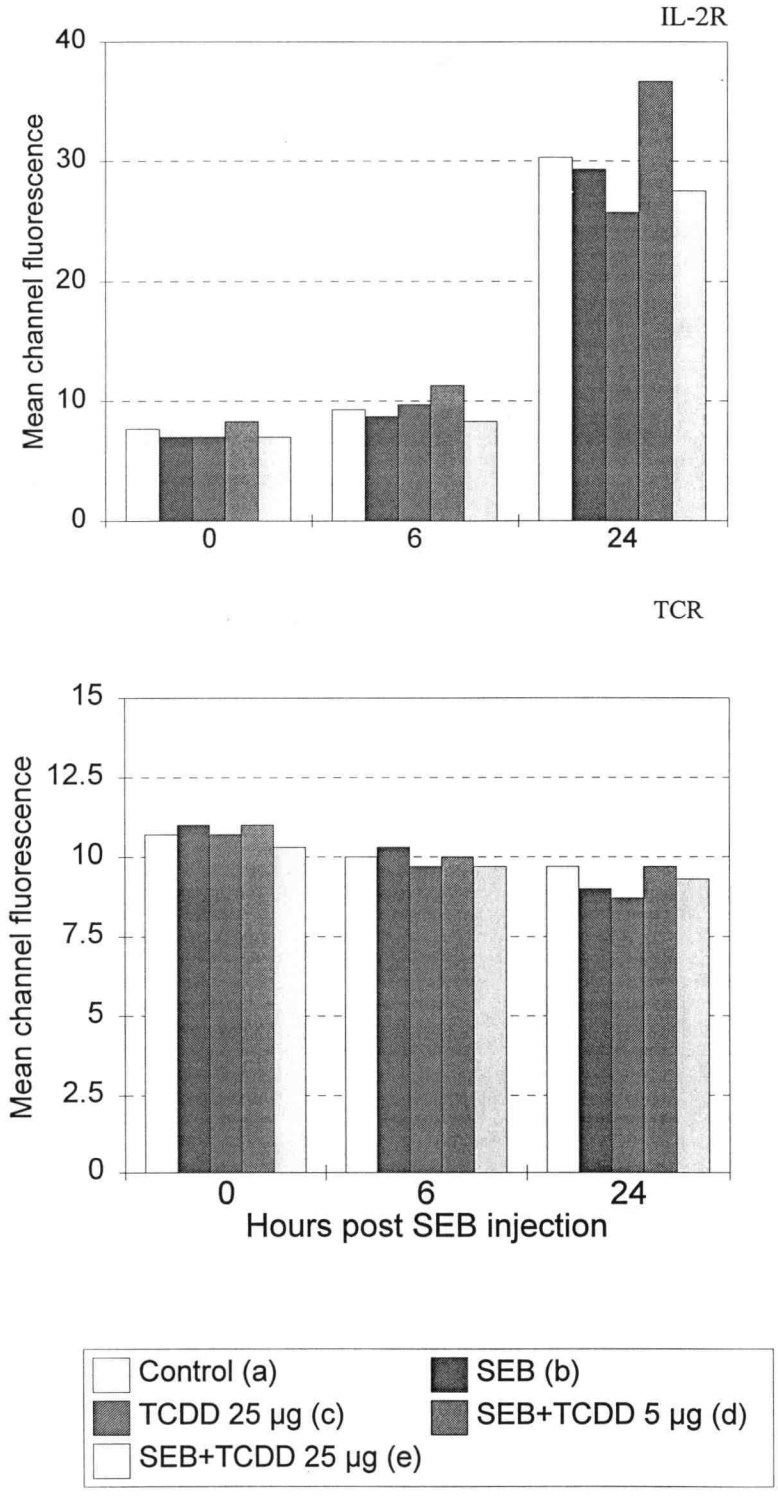

IL-2R
40
30
20
10
0
Mean channel fluorescence
0
6
24
TCR
15
12.5
10
7.5
5
2.5
0
Mean channel fluorescence
0
6
24
Hours post SEB injection
Control (a)
SEB (b)
TCDD 25 µg (c)
SEB+TCDD 5 µg (d)
SEB+TCDD 25 µg (e)

Figure III-7

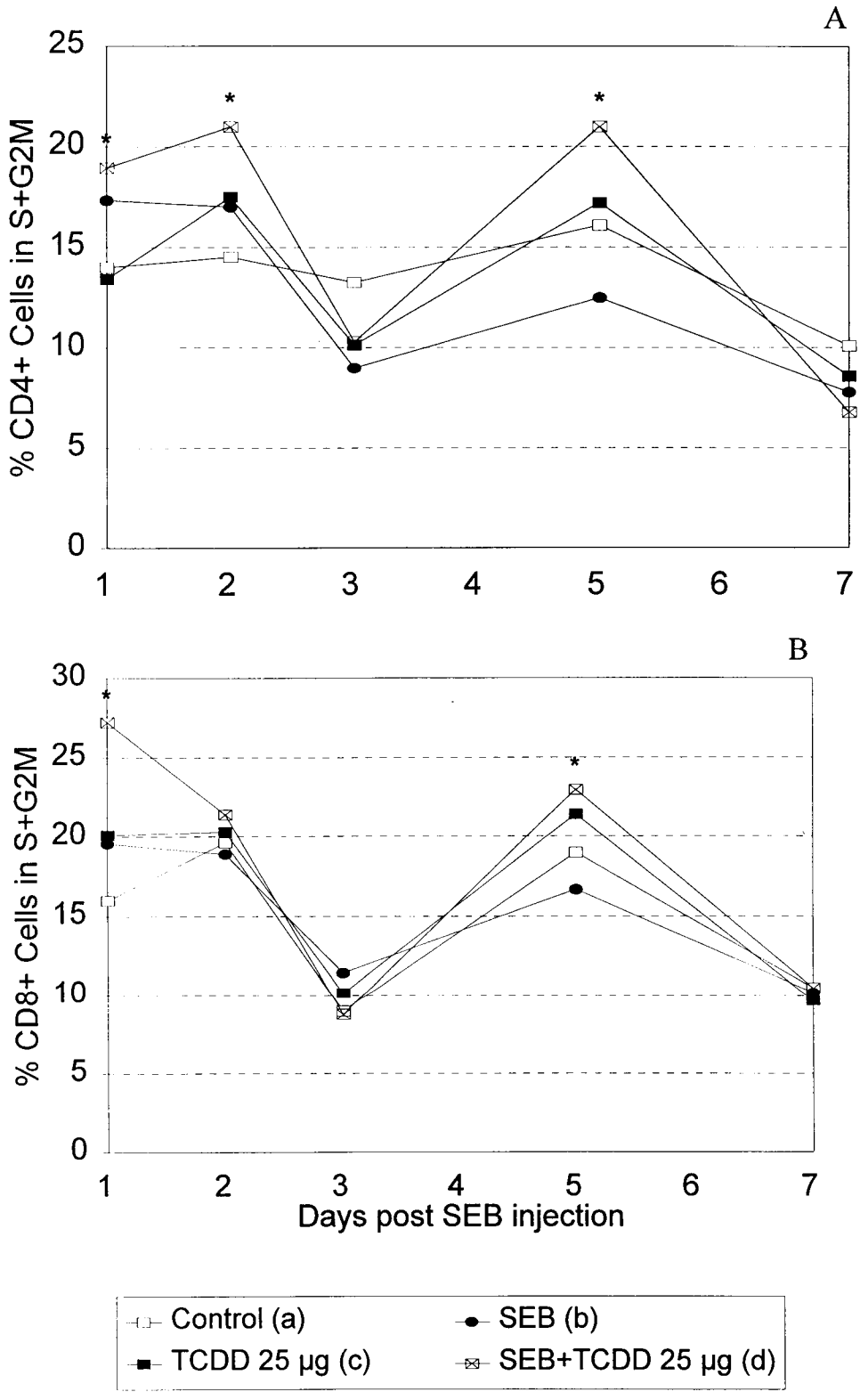

A
% CD4+ Cells in S+G2M
25
20
15
10
5
0
1
2
3
4
5
6
7
B
% CD8+ Cells in S+G2M
30
25
20
15
10
5
0
Days post SEB injection
Control (a)
SEB (b)
TCDD 25 µg (c)
SEB+TCDD 25 µg (d)

Table III-1. Phenotypic analysis of T cells in the spleens of SEB-treated rats (percentage of cell subsets).

Time (h) post SEB	%CD4 Control (a)	SEB (b)	TCDD (c)	TCDD+SEB (d)	%CD8 Control (a)	SEB (b)	TCDD (c)	TCDD+SEB (d)
24	30.96±1.56	32.09±1.16	33.65±2.08	33.04±1.83	33.86±0.72	36.96±2.53	33.18±0.85	32.80±2.45
48	31.33±0.40	34.93±0.51**	33.38±2.66	30.21±3.00	36.18±2.64	40.72±2.82	37.41±2.50	38.41±2.48
72	25.38±2.63	32.18±0.54*	28.43±2.48	28.80±2.17	38.89±1.04	39.29±3.09	40.61±1.05	34.68±3.05
120	34.20±3.13	36.91±2.23	33.80±2.27	36.80±0.35	32.22±1.17	34.11±1.59	33.90±3.73	30.90±4.93
168	35.22±3.01	33.72±0.33	33.01±1.13	33.78±3.08	33.02±1.53	34.91±2.04	37.70±4.21	35.70±2.40

Data represent the mean±S.E.M. of four rats/treatment.
Significantly different between treatment groups, b-a, $P<0.05$* or 0.01**

Table III-2. Phenotypic analysis of T cells in the spleens of SEB-treated rats (total number of cell subsets).

Time (h) post SEB	CD4(x10-7) Control (a)	SEB (b)	TCDD (c)	TCDD+SEB (d)	CD8(x10-7) Control (a)	SEB (b)	TCDD (c)	TCDD+SEB (d)
24	9.85±0.782	11.45±0.797	10.63±0.813	13.75±1.63*	10.83±0.85	12.85±0.88	10.45±0.81	13.88±1.66
48	11.8±2.03	12.43±3.44	9.45±1.77	10.62±0.89	13.8±2.35	14.38±4.0	10.88±1.96	13.45±1.14
72	10.98±0.95	8.65±1.49	9.38±1.36	9.83±1.60	16.83±1.48	11.58±1.82	13.38±1.95	11.78±1.91
120	15.4±2.60	19.27±1.18	21.53±3.80	25.73±6.92*	14.5±2.45	17.8±1.10	21.53±3.80	21.60±5.7
168	14.73±0.50	18.0±3.32	16.6±1.61	16.67±1.52	13.8±0.48	18.63±3.45	20.20±1.96	18.13±1.69

Data represent the mean±S.E.M. of our four rats/treatment.
Significantly different between treatment groups, d-a, $p<0.05$*

Fig. 6A and 6B, induction of IL2R expression on $CD4^+$ cells increased as the time in culture increased. However, IL-2R expression on $CD4^+$ cells from SEB-primed rats was not altered by exposure of rats to a single dose of 5 μg/kg or 25 μg/kg TCDD compared to the control.

Induction of TCR expression on $CD4^+$ cells was similar for all treatment groups for 0, 6 and 24 hours in culture.

Effect of TCDD on CD4 or CD8 cell population and CD4 or CD8 cells in the S+G2M phase. Phenotypic analysis of T cells was performed by flow cytometry to determine if TCDD altered $CD4^+$ or $CD8^+$ cell populations in spleens of rats exposed to SEB. The percentage of $CD4^+$ cells was significantly increased at 48 and 72h in the SEB alone group compared to the control (Table 1), the percentage of $CD8^+$ cells was similar for all groups. The total number of $CD4^+$ cells in the spleen was increased in the rats co-exposed to SEB and TCDD at 24h & 120h post SEB injection compared to the control but not in other groups (Table 2). No significant change was noted in the number of $CD4^+$ or $CD8^+$ cells in the other time and treatment groups.

As shown in Fig. 7A, the percentage of $CD4^+$ cells cycling in S+G2M was significantly increased in the SEB alone group at 24 h with a non-significant suppression at 3, 5, and 7 days compared to the control. There was no significant change in the percentage of $CD4^+$ cells in S+G2M in the TCDD alone group compared to the control. However, there were significant increases in the percentage of $CD4^+$ cells in S+G2M at 1, 2 and 5 days after SEB injection in rats co-exposed to SEB and TCDD compared to the control, TCDD alone or SEB alone groups. The percentage of $CD8^+$ cells in S+G2M were similar in the control , SEB alone and TCDD alone groups. The percentage of $CD8^+$

cells in S+G2M in animals co-exposed to SEB and TCDD increased at days 1 and 5 post SEB injection compared to the control, TCDD alone or SEB alone groups.

Histopathology. The 5 μg/kg dose of TCDD affected the thymus while lesions were noted in the thymus and liver at the 25 μg/kg dose of TCDD. At 5 μg/kg TCDD, there was thinning of the cortex although cellular density remained similar to the controls. The medulla was slightly more hypercellular than the control and the margin between the medulla and cortex was less distinct than in the normal thymus. At the 25μg/kg dose, these lesions were much more pronounced with the cortex remaining primarily as a narrow band of densely packed cells. Most of the thymus consisted of medulla but it was frequently difficult to identify the margin between the medulla and cortex. There was a diffuse hepatocellular hypertrophy observed in the livers of animals exposed to 25 μg/kg TCDD.

DISCUSSION

Immunotoxicity is one of the hallmark features of toxicity observed in several animal models following exposure to TCDD (Holsapple *et al.*, 1991; Kerkvliet & Burleson, 1994). Besides B cells, T cells have been proposed as primary target cells of TCDD (Kerkvliet & Brauner, 1987).

Since T cell activation plays a pivotal role in determining both the nature and magnitude of subsequent immune responses and there are divergent effects of TCDD on T cell function in different species, it is necessary to understand the mechanisms by which TCDD alters T cell immunity.

The data from our previous study showed that the serum levels of IL-2, IL-6 and IFN-γ were increased at 2, 6, 12 & 24 hours post intraperitoneal (ip) administration of 50μg SEB (Huang & Koller, 1997). Serum IL-2 peaked earlier (2 hours post SEB treatment) than did serum IL-6 & INF-γ, which peaked at six hours. SEB enhanced the production of IL-2, IL-6 & TNF in spleen cell cultures and the production of IL-6 and TNF in peritoneal cell cultures. The percentage of $CD4^+$ cells was significantly increased at 48 & 72 hours after ip administration of SEB. When the percentage of $CD4^+$ cells in the S+G2M phase was evaluated, there was a significant proliferation of $CD4^+$ cells at 24 hours. These results suggest that SEB could be a useful tool for studying the events leading to T cell activation (Huang & Koller, 1997).

In the current study, we report the effects of TCDD on SEB-induced cytokine production and lymphocyte phenotypes. Our original hypothesis was that TCDD would inhibit SEB-induced T-cell activation based on reports of impaired T cell function by TCDD exposure in animal experiments, especially in the mouse (Faith *et al.*, 1977; Tomar & Kerkvliet, 1991; Lundberg *et al.*, 1992). Since our previous study demonstrated that serum IL-2 peaked at 2 hours post SEB treatment, we investigated in this study if TCDD exposure would abrogate the peak serum IL-2 response. Unexpectedly, TCDD failed to suppress the IL2 response and, in fact, further stimulated IL2 production that was detected in the sera and spleen cell cultures of SEB-treated animals.

In general, at least two signals are required for T cell activation and IL2 production. The first signal is the engagement of the TCR with antigen presented in association with the MHC complex, followed by further stabilization of the cellular interaction by adhesion molecules such as CD2, LFA1 and CD28 on T cells, and LFA-3,

ICAM1 and B7 on antigen processing cells (APC). Cytokines, such as IL-1, which are produced by the APC, deliver the second signals to complete the process of T cell activation. In order to understand the enhancement of SEB-induced IL-2 production by TCDD exposure, we attempted to investigate if TCR expression would be modulated by TCDD exposure. However, as expected, since there was no suppression of the cytokine responses, no changes in TCR expression occurred in T cells from rats co-exposure with SEB and TCDD or in the SEB alone group.

It has been shown that cytokines, such as IL1, IL6 & TNF, are associated with the production of IL2. We also measured those cytokine levels in sera, spleen cell cultures and peritoneal cell cultures. The results showed that TCDD did not alter the pattern of SEB-induced changes in TNF and IL6 levels. However, TCDD did significantly enhance the production of IL-1 by peritoneal cells with SEB restimulation in vitro at 2 hours and appeared to non-significantly elevate IL-1 levels in the serum. The elevation of IL1 may play a role in enhancement of IL2 in this study. An early time-course study of serum IL1 may clarify this relationship.

The results of this study suggested that TCDD may affect APC function. It has been shown that increased levels of B 7.1 and/or B 7.2 cell surface expression was associated with "a faster kinetics" of IL2 production in response to SEB (Muraille *et al.*, 1996) while blocking mAbs against LFA-1 & LFA-3 added to SEB-activated cultures of T cells decreased genetic expression of IL2 (Langoo *et al.*, 1995). The early modulation of these adhesion molecules by TCDD exposure could result in enhancement of IL-2 production in SEB-treated rats. However, Prell & Kerkvliet (1997) reported that TCDD exposure significantly decreased the expression of B7.2 on B220+ and Mac-1+ cells in

mice between days 6 and 10 after P815 tumor cell injection. Whether TCDD exposure initially increases and then decreases the expression of B7 remains to be determined.

It has been shown that the production of IL2 in lymph node cells or thymocytes cultured with ovalbumin or ConA were suppressed in mice exposed to TCDD (Lundberg *et al.*, 1992). Prell *et al.* (1995) also reported that TCDD treatment resulted in reduced IL2 production in spleen cell cultures at 90 min post anti-CD3 injection of mice. This would suggest a species difference in responses to TCDD. It has also been reported that the delayed-type hypersensitivity (DTH) response was enhanced after TCDD treatment in rats (Fan *et al.*, 1996) and suppressed in mice (Luster *et al.*, 1979). Other studies have also observed species differences in responses to infectious agents between rats and mice exposed to TCDD (Luebke *et al.*, 1994 and 1995; Yang et al., 1994). In a direct comparison of the effect of TCDD on these two species, Smialowicz *et al.* (1994) indicated that the PFC response to SRBC was enhanced in rats but suppressed in mice following TCDD exposure. However, recently it has been reported that LPS-induced IL-1 by splenic macrophages and conA-induced IL-2 by spleen cells were suppressed in rats fed a low dose of TCDD (Bedesha *et al.*, 1995). These discrepancies may be explained by species differences in responses to TCDD and different experimental designs, e.g., duration of exposure, dosage, in vivo versus in vitro exposure.

The effects of TCDD on SEB-induced IL2R expression, on $CD4^+$ and $CD8^+$, cells and percentage of $CD4^+$ and $CD8^+$ cell cycling was also investigated. The results indicated that TCDD did not alter SEB-induced IL2R expression or the kinetics of SEB-induced IL2R expression. However, TCDD did increase the percent of $CD4^+$ cells cycling at day 5 and the percent of $CD8^+$ cells cycling at days 1 and 5 post SEB treatment.

Similar effects of TCDD on the percent of $CD4^+$ and $CD8^+$ cells cycling occurred at day 1 in an anti-CD3 mouse model (Newmann *et al.*, 1993). In this study, the decrease in the percentage of $CD4^+$ and $CD8^+$ cells cycling after three days in SEB-treated rats might be associated with clonal anergy although the decreased percentage of those cells cycling was not significant compared to the control. The significant increase in percent of $CD4^+$ and $CD8^+$ cell cycling in rats exposed to SEB and TCDD compared to SEB alone, but not to the control, appeared to be related to TCDD protection of SEB-induced $CD4^+$ or $CD8^+$ from clonal anergy. However, the protection from SEB-induced clonal anergy appeared to be temporary because the percentage of $CD4^+$ or $CD8^+$ cell cycling decreased close to levels of the control and SEB alone group 7 days after SEB treatment. Recently, Kremer *et al.* (1995) reported that TCDD induced protection from apoptotic stimuli on mouse $CD4^+$ $CD8^+$ T cell receptor-thymocytes through upregulating expression of bcl-2 gene encoding a memberane associated protein which protects thymocytes from programmed cell death. Further research is required to determine if there is a relationship between expression of the bcl-2 gene upregulation by TCDD and the increased percentage of $CD4^+$ and $CD8^+$ cell cycling in SEB-treated rats. It has been shown that withdrawal of IL-2 resulted in T cell anergy and then apoptosis in SEB treated animals (Colotta *et al.*, 1992) and that SEB-induced anergy can apparently be reversed by IL-2 treatment (Gutierrez-Ramos *et al.*, 1992; Schols *et al.*, 1995). Baschieri et al. (1993) also reported that SEB-induced T cell proliferation *in vivo* was IL2-dependent. This current study showed that TCDD enhanced serum IL-2 levels and IL2 production in spleen cell cultures in SEB-treated rats and increased the percentage of $CD4^+$ and $CD8^+$ cell cycling. However, the relationship between the enhancement of IL2 production and the increased

percentage of $CD4^+$ and $CD8^+$ cell cycling in this study is still not clear. A time-course study of serum IL2 levels could possibly clarify this relationship.

TCDD may provoke T cell activation by activating pp60c-src tyrosine kinase in mouse thymocytes (Bombick & Matsumura, 1987) and increasing cytosolic Ca^{+2} levels as shown in mouse and rat thymocytes (McConkey & Orrenius, 1989). Alternatively, TCDD may indirectly affect T cell activation and proliferation by stimulating the production of cytokines, such as IL-1, IL6 and TNF. The production of IL-1 but not TNF or IL-6 was elevated in peritoneal cell cultures from TCDD-treated rats co-cultured with SEB for 2 hours in this study. It has also been reported that TCDD exposure increased message RNA for IL-1 but not TNF in macrophages (Steppan & Kerkvliet, 1991). Recently, Nakata *et al.* (1995), indicated that administration of recombinant human IL-1 in vivo interfered with tolerance induction in mice. However, even though TCDD enhanced the production of IL-1 in peritoneal cell cultures in this study, further work is needed to determine the relationship between IL-1 and $CD4^+$ and $CD8^+$ cell cycling in this model. Although the mechanism by which TCDD enhanced SEB-induced levels of IL-2 in the sera and spleen cell cultures, IL-1 production in peritoneal cell cultures, and percentage of $CD4^+$ and $CD8^+$ cell cycling is not clear, TCDD appears preferentially to target T-lymphocytes and APC that are undergoing activation following SEB treatment because TCDD alone does not appear to activate the T cells or APC. However, this study did not demonstrate a dose-dependent effect of TCDD on SEB-induced stimulation of IL-1, IL-2, or percentage of $CD4^+$ and $CD8^+$ cell cycling since these changes were not significantly different between rats co-exposed to SEB and a single dose of 5 μg/kg or 25 μg/kg TCDD.

In conclusion, these studies have described the application of a superantigen model to study the effects of TCDD on T-cell activation and proliferation in vivo. These results indicated that a single exposure of TCDD-enhanced SEB-induced activation and proliferation of T cells and noted that there is a species difference between rats and mice in their response to a single dose of TCDD. Further investigations are necessary to pursue the mechanisms of these observations.

CHAPTER IV

XENOBIOTIC AND SUPERANTIGEN ASSOCIATIONS III.EFFECT OF SINGLE AND REPEATED EXPOSURES OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ON SEB-INDUCED CHANGES IN T CELL SUBPOPULATIONS

Wentian Huang and Loren D. Koller

Submitted to *Toxicology*

ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exposure results in adverse effects on the immune system of experimental animals. Our previous study showed that a single dose exposure of TCDD increased the percentage of $CD4^+$ or $CD8^+$ cell cycling in the S and G2M phase on day 5 or days 1 and 5 respectively, post injection of rats with 50 μg of Staphylococcal Enterotoxin B (SEB). The purpose of this study was to compare the effects of a single and repeated dosing of TCDD on splenic T cell subpopulations in rats 7 days post exposure to SEB . A single dose (25 μg) of TCDD either alone or co-exposed with SEB resulted in reduced body weight. The percent and number of $CD4^+$ or $CD8^+$ subsets and percentage of $CD4^+$ or $CD8^+$ cell cycling in the S and G2M phase was similar in all single TCDD treatment groups. A repeated dose (5 μg/day for 5 days) of TCDD either alone or co-exposed with SEB also resulted in a significant reduction in body weight. However, multiple doses of TCDD significantly decreased the percent of $CD4^+$ cells and $CD4^+$ cells cycling in the S and G2M phase. Co-exposure of SEB and multiple doses of TCDD significantly decreased the number of $CD4^+$ cells and percentage of $CD4^+$ cell cycling in the S and G2M phase compared to non-SEB control but not to SEB alone. No significant change occurred after single or multiple dosing with TCDD in $CD8^+$ cell subpopulation. These results demonstrated that repeated dosing of TCDD decreased the percentage of $CD4^+$ cells and $CD4^+$ cell cycling in the S and G2M phase while an equivalent single dose of TCDD failed to affect the $CD4^+$ cell subpopulation.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most biologically potent halogenated aromatic hydrocarbons (HAH) that are widespread environmental pollutants of health concern (Schewetz et al., 1973; Poland and Knutson, 1982; Safe, 1986). TCDD has been shown to cause a generalized wasting syndrome, hepatotoxicity, thymus involution and suppression of humoral and cell-mediated immunity that appears to be mediated by specific binding to the Ah receptor (Sharma and Gehring, 1979; Silkworth and Grabstein, 1982; Vecchi et al., 1983; Kerkvliet et al., 1990; Holsapple et al., 1991). However, most of these findings were reported in animals exposed to a single dose of TCDD. It has been suggested that various toxicities produced by TCDD exposure depends on the dose (Clark et al., 1981; 1983), route (Pohjanvirta et al., 1989), and length of exposure (Holsapple et al., 1986; Morris et al., 1992; Yang et al., 1994). It has been demonstrated that the suppression of humoral immunity can be compounded approximately 10-fold in DBA/2 mice following subchronic versus acute exposure to the same cumulative doses of TCDD (Morris et al., 1992). Recently, Yang et al. (1994) reported a significant increase in lung weight and the lung/body weight ratio in rats concomitantly exposed to influenza virus and repeated doses of TCDD, suggesting an enhanced pulmonary inflammation due to infection in rats exposed to repeated versus an acute dose of TCDD. These findings suggested that the severity of the immunotoxic effect of TCDD was related to the length and repetitive nature of exposure.

Since T-lymphocytes play a pivotal role in determining both the nature and magnitude of subsequent immune responses, it is necessary to understand the effect of TCDD on changes of T cell subsets. Recently, we have investigated the effects of TCDD

on T cell activation and cytokine production using a superantigen model, Staphylococcal Enterotoxin B (SEB), that binds to MHC-II molecules on antigen presenting cells (APC) and to the Vβ region of the T cell receptor (TCR) (Huang and Koller, 1997). SEB also induces cytokine production that leads to activation and proliferation of T cells followed by apoptosis and T cell anergy (Miethke et al., 1992; Bette et al., 1993; Goodglick and Braun, 1994; Hubber et al., 1996). These studies showed that a single dose of TCDD-enhanced production of interleukin 1 (IL1) and interleukin 2 (IL2) and the percent of $CD4^+$ and $CD8^+$ cells cycling in the S and G2M phase in rats exposed to SEB (Huang and Koller, 1997). Since few studies have been conducted to investigate the effects of a single versus repeated dosing of TCDD on the immune system, especially on changes of T cell subsets, we examined the effects of a single and repeated administration of TCDD on alteration of T cell subsets in rats exposed to SEB.

METHODS

Animals

Female Long/Evans rats (Simonson Laboratories, Inc., Gilroy, CA) 10-11 weeks of age were housed in Polycarbonate cages with hardwood bedding and maintained on a 12-hour light/dark cycle at 22+2^{0}c. Harlan Teklad rodent diet and tap water were given ad libitum.

Animal Treatment

TCDD (Cambridge Isotope Laboratories, Auburn, MA., U.S.A. >90% pure) was dissolved and diluted in peanut oil. A single dose of TCDD was administrated by gavage

at 25 μg/kg body weight two days prior to Staphylococcal Enterotoxin B (SEB) treatment. For multiple dosing, the animals were administrated by gavage 5 μg TCDD/kg daily for 5 consecutive days (cumulative dose of 25 μg/kg), and then treated with SEB 2 days after the last exposure to TCDD. Controls in both studies were treated with peanut oil by gavage. SEB (Sigma, St. Louis, MO) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) (GIBCO-BRL, Grand Island, NY), was injected intraperitoneally (ip) 50 μg/rat. Animals were killed by CO2 asphyxiation 7 days post SEB injection. There were three or four rats per respective sampling time for each treatment and control groups. Spleen cells were collected and processed for flow cytometry.

Flow Cytometry Analysis

Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Red blood cells were removed by hypotonic lysis. Spleen cells were washed two times and resuspended to $2x10^7$ cells/ml in DPBS. Spleen cells ($2x10^6$) were incubated on ice in V-bottomed 96 well plates in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Plates were blocked with excess mouse IgG (Sigma Chem. Co., St. Louis, MO) prior to staining with FITC-conjugated anti-CD4 or anti-CD8. Immediately after staining, cells were passed through a 40 μM Nylon mesh to remove cell clumps. The cells were stained with propidium iodide for 30 minutes on ice. The samples were analyzed for S phase DNA content in an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL, U.S.A.).

Statistics

Data were analyzed for statistical significance using ANOVA or Student's t test. In all analyses, $p<0.05$ was considered statistically significant. The data presented in this paper are conducted in two separative studies.

RESULTS

Effects of single or repeated exposures to TCDD on body weight, spleen weight and spleen cellularity.

In the single exposure study, TCDD alone significantly reduced body weight compared to the non-TCDD exposed rats (Table 1). Rats exposed to SEB alone had body weights similar to the controls. Co-exposure of SEB and TCDD did not further reduce the body weight produced by TCDD alone. There were no significant changes in the spleen weights or spleen cell numbers in the treatment groups compared to the controls. However, spleen weight, normalized to body weight, was increased in the TCDD alone and the co-exposed SEB-TCDD groups (Table 1).

In the repeated exposure study, TCDD alone significantly reduced body weight compared to the non-TCDD exposed rats (table 2). Although rats exposed to SEB alone did not lose body weight, co-exposure of SEB and TCDD further reduced the body weight produced by TCDD alone. The spleen weights and spleen cell numbers were similar in all treatment groups compared to the control. However, increased spleen weight, normalized to body weight, was found in the TCDD alone and the co-exposed SEB-TCDD groups (table 2).

Figure IV-1. The effect of single or repeated exposures of TCDD on the percentage of $CD4^+$ or $CD8^+$ cell subsets in SEB-treated rats.

In the single exposure study, animals were treated with 25 μg TCDD/kg and injected with 50 μg SEB/rat 2 days later. In the repeated exposure study, animals were treated for 5 consecutive days to TCDD at 5ug/kg/day (cumulative dose of 25 μg/kg) and injected with 50 μg SEB/rat 2 days later. Spleens were removed 7 days post SEB treatment. Spleen cells were stained with FITC-conjugated anti-CD4 or anti-CD8 and analyzed by flow cytometry as described in Experimental Procedures. Each bar represents the mean±S.E.M. per treatment. c-a, $p<0.05$*.

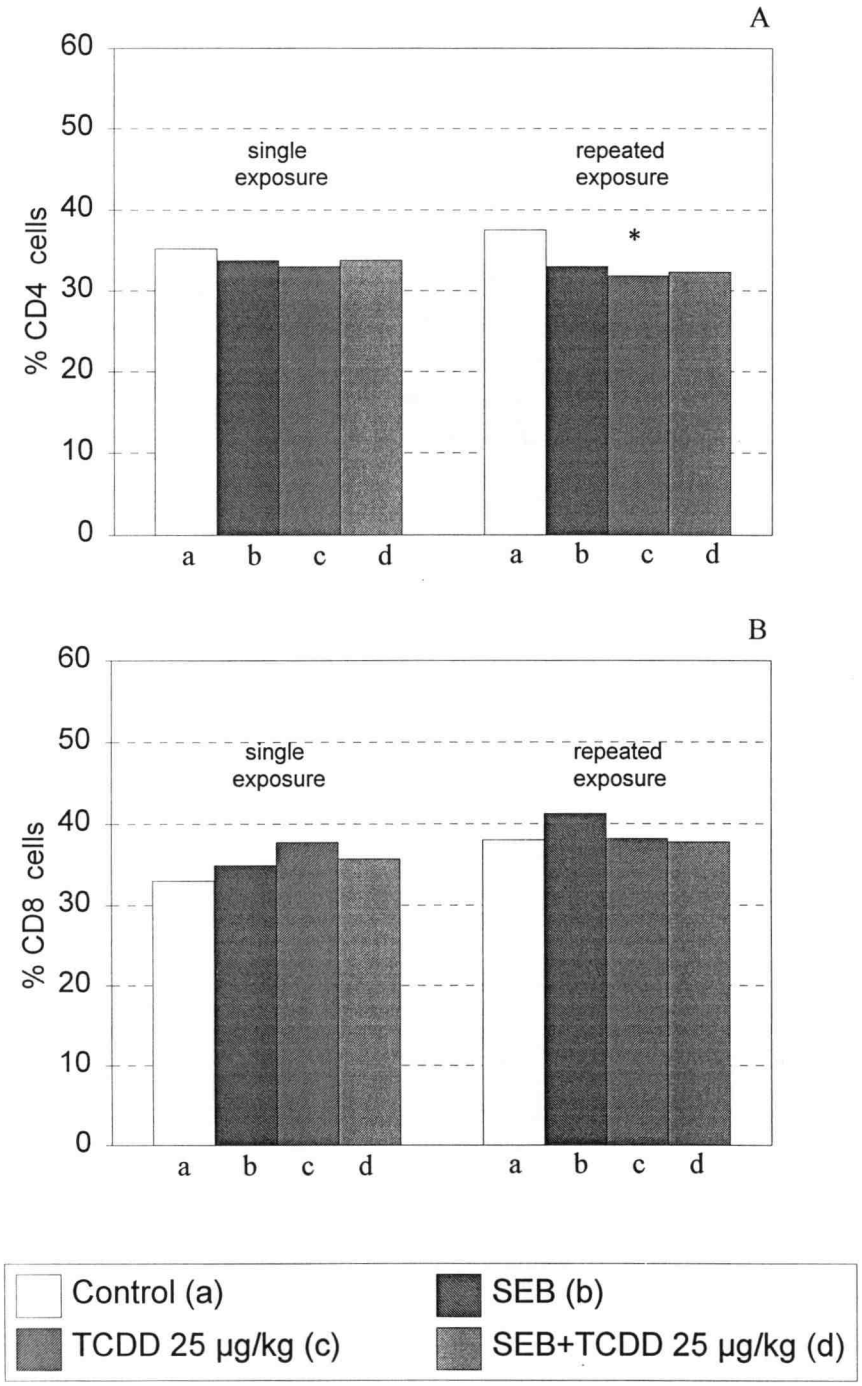
Figure IV-2. The effect of single or repeated exposures of TCDD on the number of $CD4^+$ or $CD8^+$ cell subsets in SEB-treated rats .

In the single exposure study, animals were treated with 25 μg TCDD/kg and injected with 50 μg SEB/rat 2 days later. In the repeated exposure study, animals were treated for 5 consecutive days to TCDD at 5 μg/kg/day (cumulative dose of 25 μg/kg) and injected with 50 μg SEB/rat 2 days later. Spleens were removed 7 days post SEB treatment. Spleen cells were stained with FITC-conjugated anti-CD4 or anti-CD8 and analyzed by flow cytometry as described in Experimental Procedures. Each bar represents the mean±S.E.M. per treatment. d-a, $p<0.05$*.

Figure IV-3. The effect of single or repeated exposures of TCDD on the percentage of $CD4^+$ or $CD8^+$ cell cycling in the S/G2M phase in SEB-treated rats.

In the single exposure study, animals were treated with 25 μg TCDD/kg and injected with 50 μg SEB/rat 2 days later. In the repeated exposure study, animals were treated for 5 consecutive days to TCDD at 5 μg/kg/day(cumulative dose of 25 μg/kg) and injected with 50 μg SEB/rat 2 days later. Spleens were removed 7 days post SEB treatment. Spleen cells were stained with FITC-conjugated anti-CD4 or anti-CD8 and propidium iodide and analyzed by flow cytometry as described in Experimental Procedures. Each bar represents the mean±S.E.M. per treatment. b-a, c-a & d-a, $p<0.05$*.

Figure IV-1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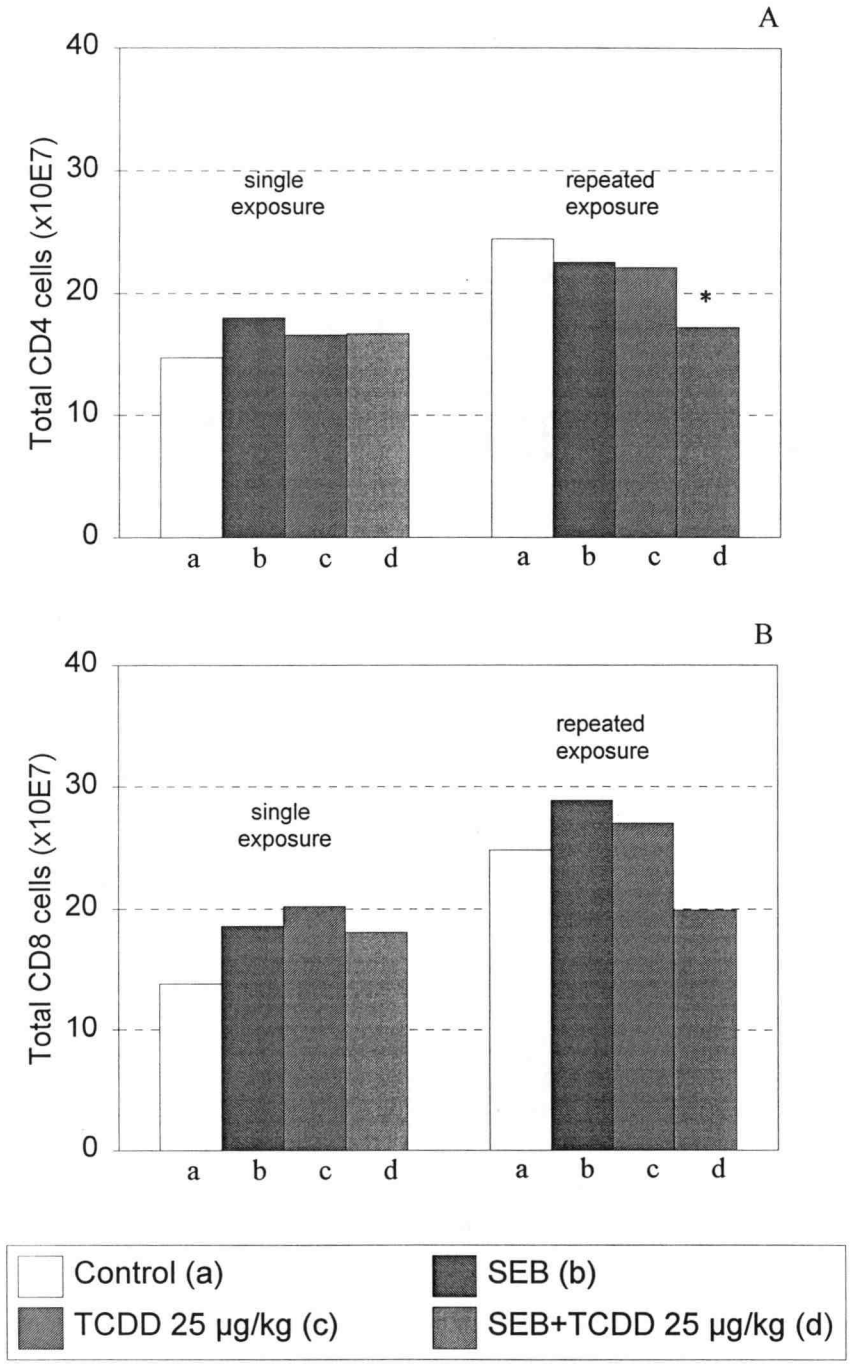
Control (a)
SEB (b)
TCDD 25 µg/kg (c)
SEB+TCDD 25 µg/kg (d)

Figure IV-2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Total CD8 cells (x10E7)
single exposure
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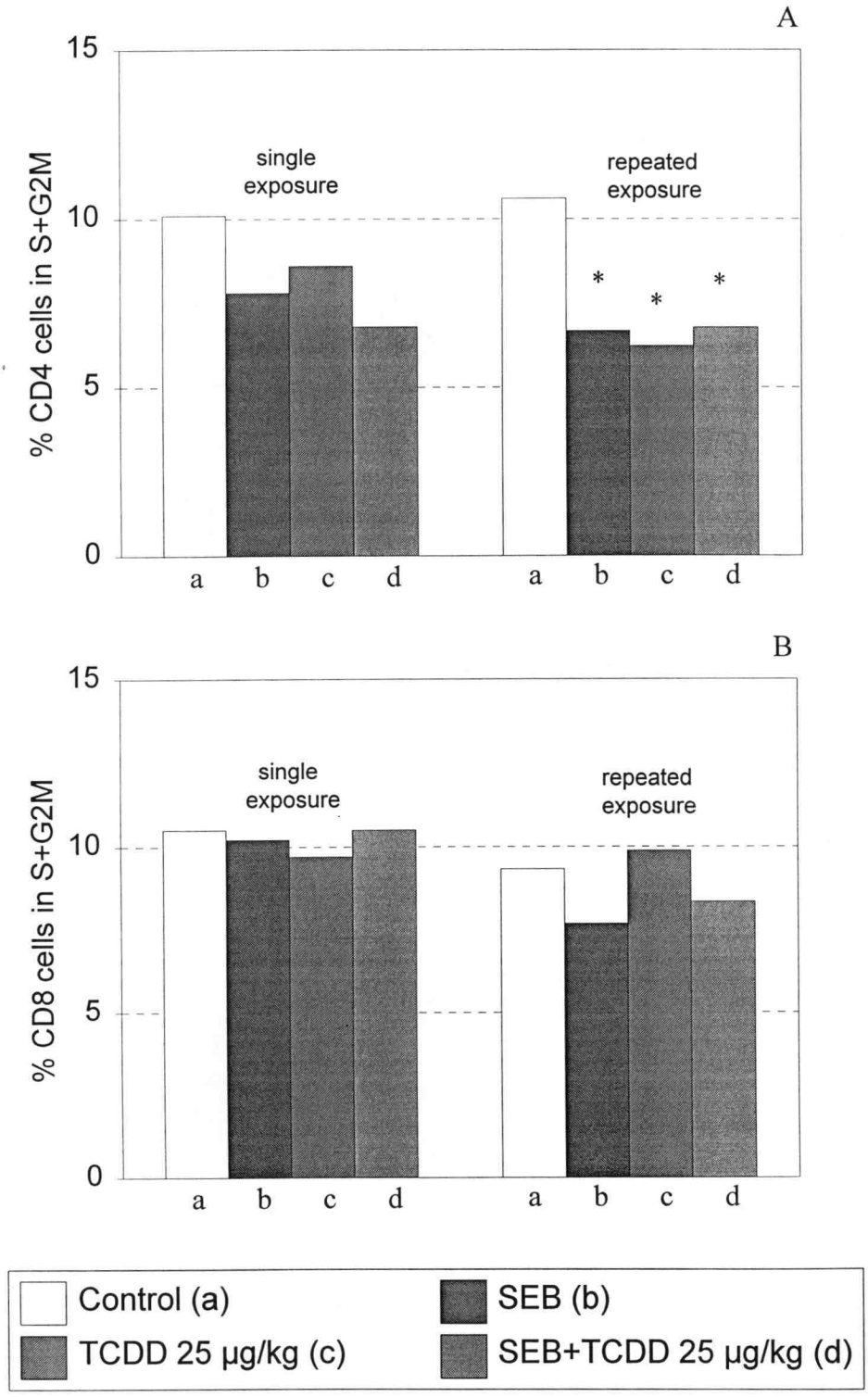
Control (a)
SEB (b)
TCDD 25 µg/kg (c)
SEB+TCDD 25 µg/kg (d)

Figure IV-3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Control (a)
SEB (b)
TCDD 25 µg/kg (c)
SEB+TCDD 25 µg/kg (d)

Table IV-1. The effect of a single dose of TCDD on body and spleen weight and cellularity in the spleens of SEB-treated rats

Treatment	Body weight (g)	Spleen weight (g)	Spl wt/Bd wt (%)	Cell recovery (X10-7)
control (a)	238±3.20	0.595±0.03	0.25±0.01	42.0±1.40
SEB[1] (b)	230±3.01	0.644±0.03	0.28±0.01	53.53±9.92
TCDD[2] (c)	c-a* 203±3.81c-b*	0.649±0.02	0.32±0.01c-a*	53.6±5.21
SEB[1]+TCDD[2] (d)	d-a* 205±4.20d-b*	0.615±0.03	0.30±0.003d-a*	50.8±4.68

Data represent the mean±S.E.M. of three or four rats/treatment.
Significantly different between treatment group, c-a&c-b etc., $p<0.05$
[1]50 μg SEB injected intraperitoneally
[2]5 μg/kg TCDD given orally for 5 consecutive days

Table IV- 2. The effect of repeated doses of TCDD on body and spleen weight and cellularity in the spleens of SEB-treated rats.

Treatment	Body weight (g)	Spleen weight (g)	Spl wt/Bd wt (%)	Cell recovery (X10-7)
control (a)	250±8.66	0.675±0.03	0.27±0.02	64.8±5.35
SEB (b)	257±4.41	0.722±0.03	0.28±0.02	68.5±8.96
TCDD (c)	c-a* 233.3±3.15c-b*	0.727±0.02	0.31±0.01c-a*	69.5±9.95
SEB[1]+TCDD[2] (d)	d-a* 215.9±4.27d-b*	0.691±0.03	0.32±0.01d-a*	52.8±5.38

Data represent the mean±S.E.M. of three or four rats/treatment.
Significantly different between treatment group, c-a & b-a etc., $p<0.05$*
[1] 50 μg SEB injected intraperitoneally
[2] 5 μg/kg TCDD given orally for 5 consecutive days

Effects of single or repeated exposures of TCDD on $CD4^+$ and $CD8^+$ cell subpopulations.

Phenotypic analysis of T cells was performed by flow cytometry to determine if TCDD altered the $CD4^+$ or $CD8^+$ cell subpopulations in the spleens on day 7 following SEB injection. As shown in Fig. 1 and Fig. 2, the percentage and total numbers of $CD4^+$ or $CD8^+$ cells did not show any significant change in all treatment groups compared to the control in the single exposure study. Although the percentage and total numbers of $CD8^+$ cells were similar for all treatment groups in the repeated exposure study, repeated exposure to TCDD alone significantly decreased the percent of $CD4^+$ cells (Fig. 1A) while co-exposure of SEB and multiple doses of TCDD significantly decreased the number of $CD4^+$ cells compared to their respective control (Fig. 2A).

Effects of single or repeated exposures to TCDD on CD4+ or CD8+ cell cycling.

Flow cytometry was utilized to determined if TCDD altered the percentage of $CD4^+$ or $CD8^+$ cell cycling. As shown in Fig. 3, in the single exposure study, the percentage of $CD4^+$ cell cycling in all treatment groups appeared to be decreased but was not significantly different from the control. The percentage of $CD8^+$ cell cycling was similar for all treatment groups. In the repeated exposure study, although no significant change was noted in the percent of $CD8^+$ cell cycling in all treatment groups, the percent of $CD4^+$ cell cycling was significantly decreased in the SEB alone, TCDD alone and the co-exposed SEB-TCDD groups compared to the control (Fig. 3).

DISCUSSION

Studies involving effects of TCDD on T cell function have been conducted over the past two decades. However, few investigations have compared T cell activity following exposure to a single or repeated doses of TCDD. This study reports the direct comparison of the effects of a single or repeated dosing of TCDD on T cell subpopulations in SEB-induced rats. We previously showed that a single exposure of TCDD enhanced production of IL1 by peritoneal cells in 2h cultures costimulated with SEB in vitro, enhanced IL2 levels in the sera and in 6h & 24h spleen cell cultures, and increased the percent of $CD4^+$ and $CD8^+$ cells cycling on day 5 or days 1 and 5 post SEB injection, respectively (Huang and Koller, 1997). These results suggested that a single dose of TCDD could alter cytokine production and $CD4^+$ and $CD8^+$ cell cycling in rats exposed to SEB in early events. Renno et al. (1996) reported that SEB exposure resulted in an initial increase in the percentage of $CD4^+$ Vβ T cells followed by a decrease in the number of these cells 7 days post SEB injection. Therefore, in this study, we investigated the change of T cell subpopulations 7 days post SEB injection.

The results from a single exposure of TCDD showed that TCDD alone caused body weight loss while co-exposure of SEB and TCDD did not further reduced the body weight loss that was produced by TCDD. However, in the repeated exposure study, co-exposure of SEB and TCDD did further reduced the body weight loss that occurred following 5 days of exposure to TCDD. These results suggested that general toxicity was more overt in rats co-exposed to SEB and multiple doses of TCDD than co-exposed to SEB and a single dose of TCDD. The increased spleen weight, normalized by body weight occurred in rats exposed to both a single and repeated doses of TCDD. This effect

was secondary to the body weight loss since absolute spleen weights were unaffected by these treatments.

After a single exposure to TCDD, the percent and number of $CD4^+$ or $CD8^+$ cell subsets in all treatment groups were comparable to the control. Although the percentage of $CD4^+$ cell cycling in the S and G_2M phase was decreased in the animals exposed to a single dose of TCDD, the change was not significantly different from the control. Our findings are consistent with a recent report that phenotypic analysis of $CD4^+$ or $CD8^+$ subsets present in the spleen of rats exposed to single dose of TCDD and trinitrophenyl-lipopolysaccharide (TNP-LPS) failed to demonstrate any change compared to controls (Smialowicz, 1996). However, Kerkvliet et al. (1996) demonstrated that a single exposure of TCDD decreased the number of $CD4^+$ and $CD8^+$ cell subsets but not the percentage of $CD4^+$ and $CD8^+$ cell subsets in mice challenged with P815 tumor cells. Prell et al. (1995) also showed a decreased percentage of $CD4^+$ cells in mice that were exposed to a single dose of TCDD in an anti-CD3 model. These different results may be associated with species differences in responses toTCDD and/or different experimental models.

In the repeated exposure study, SEB exposure decreased the percentage of $CD4^+$ cell cycling, but did not significantly decrease the percent or number of $CD4^+$ cells. This phenomenon might be related to $CD4^+$ clonal anergy. Repeated exposure to TCDD did not potentiate the decreased percentage of $CD4^+$ cell cycling that occurred in the SEB-exposed rats. The decreased percentage of $CD4^+$ cell cycling in co-exposure of SEB and multiple dosing of TCDD may be associated with the $CD4^+$ clonal detection because the number of $CD4^+$ cells was significantly decreased in this group compared to the control.

Repeated exposure of TCDD significantly decreased the percentage of $CD4^+$ cell cycling. This phenomenon may also be due to $CD4^+$ clonal deletion or lack of $CD4^+$ clonal expansion since repeated exposure of TCDD resulted in a decreased percent of the $CD4^+$ cell subset. Although there was a decrease in the percentage of $CD4^+$ cell cycling in the S and G2M phase in all multiple TCDD treatment groups, there were no significant changes in the $CD8^+$ cells in those animals. These results showed that $CD4^+$ cells were more sensitive to repeated exposure of TCDD than were $CD8^+$ cells in Long/Evan rats. We attempted to examine apoptosis of T cells. Unfortunately, the technique was not sensitive enough to detect apoptotic T cells. Whether the $CD4^+$ clonal delection in rats co-exposed to SEB and TCDD is related to $CD4^+$ cell apoptosis remains to be determined by more sensitive techniques.

It has been reported that repeated exposure of TCDD for 11 days did not only result in depletion of $CD4^+$, but also $CD8^+$ thymocytes in mice (Rhile et al., 1996). In another study (Badesha et al., 1995), there were no significant change of the $CD4^+/CD8^+$ cell ratio in Wistar rats exposed to TCDD for 30 days. In monkeys exposed to 1.5 ng TCDD/kg body weight for 3 weeks, there was a transient increase in the percent and absolute number of the $CD8^+$ $CD56^+$ cell population("cytotoxic T cells"), while in monkeys exposed to 0.3ng TCDD/kg body weight weekly for 24 weeks, there was an increase in the percent and absolute number of the $CD4^+$ $CDw29^+$ cells ("helper inducer" or "memory" cells) and a decrease in the percent of $CD4^+$ $CD45^+$ RA cells ("suppressor-inducer" or "naive" cells) (Neubert et al., 1992). These discrepancies may be related to strains and species differences in response to TCDD and different experimental design, e.g. length of exposure, dose and species.

This study demonstrated that there was a decrease in the percent and number of $CD4^+$ cells and $CD4^+$ cell cycling in the S and G2M phase in rats exposed to cumulative repeated but not to an equivalent single dose of TCDD. Morris et al. (1992) reported that the TCDD induced suppression of the IgM antibody response to sheep red blood cells (SRBC) can be enhanced in DBA/2 mice approximately 10-fold following subchronic versus acute exposure to the same cumulative doses of TCDD. Recently, it has also been shown that a significant increase of lung weight and lung/body weight ratio was observed in rats exposed to repeated doses of TCDD, suggesting an enhanced pulmonary inflammation due to infection in rats exposed to repeated doses of TCDD (Yang et al., 1994). This current study is consistent with those results suggesting that the immunotoxic effects of TCDD are associated with the length and repetitive nature of exposure. The present study raises a concern about the immunotoxic relevancy of using data from a single dose of TCDD in risk assessment analysis of humans exposed to repeated low environmental levels of TCDD, such as repeated exposure in Times Beach, Missouri (Knutson, 1984) compared to acute exposure to TCDD, such as the large scale industrial accident in Seveso, Italy (Pocchiari et al., 1979; Tognoni and Bonaccorsi, 1982). Further work is warranted to better characterize the biological differences that occur in rats following exposure to single versus multiple exposures of TCDD.

CHAPTER V

CONCLUSIONS

In these studies, rats exposed to staphylococcal enterotoxin B (SEB) had significantly elevated levels of interleukin-2 (IL2), interleukin-6 (IL6) and interferon-γ (IFNγ) in their serum with an increase in the percentage of $CD4^+$ cells in the spleen and the percent of $CD4^+$ cells cycling in S/G2M phase. The levels of IL2, IL6 and tumor necrosis factor (TNF) from spleen cell cultures or peritoneal cell cultures were significantly increased when these cells from SEB-primed animals were restimulated in vitro with SEB. These findings indicate that SEB model may be a useful model for evaluating the interaction of concomitant exposure of superantigen and xenobiotics in assessing T cell activation and cytokine production in the rat.

This SEB rat model was developed to investigate the effects of TCDD on T cell activation and cytokine production. The results revealed that a single TCDD exposure significantly increased the serum levels of IL2 2h post SEB injection and IL2 production by spleen cells from SEB-primed rats after 6h or 24h in cultures co-stimulated with SEB in vitro. Significantly increased IL1 production was also noted in peritoneal cells from SEB-primed rats with SEB restimulation in vitro for 2h. The alteration of IL1 and IL2 was not seen in the animals only exposed to TCDD. These results indicate that a single exposure of TCDD enhanced SEB-induced T cell activation and suggested that TCDD preferentially targets T-lymphocytes and antigen presnting cells (APC) since TCDD alone did not appear to activate the T cells or APC.

It has been reported that the initial superantigen activation of T-cells, secretion of cytokines, and expansion of the stimulated T-cell subsets is followed by T cell deletion and anergy (White et al., 1989; Kawabe and Ochi, 1991; Webb and Gascoigne, 1994; Schols et al., 1995). In this study, the decrease in the percentage of $CD4^+$ and $CD8^+$ cells cycling after three days in rats only exposed to SEB might be associated with clonal anergy. However, TCDD exposure significantly increased the percent of $CD4^+$ cells cycling at day 5 and the percent of $CD8^+$ cells cycling at days 1 and 5 post SEB treatment compared to SEB alone. These results suggest that TCDD protects against SEB-induced clonal anergy. However, this protection appeares to be temporary because the percent of $CD4^+$ or $CD8^+$ cells cycling decreased to close to levels of the control and SEB alone groups 7 days after SEB treatment. Additional studies will be required to clarify this phenomenon.

It has been suggested that various toxicities produced by TCDD exposure depend on the dose (Clark et al., 1981; 1983), route (Pohjanvirta et al., 1989), and length of exposure (Holsapple et al., 1986; Morris et al., 1992; Yang et al., 1994). These findings indicate that repeated dosing of TCDD decreased the percentage of $CD4^+$ cells and $CD4^+$ cells cycling in S/G2M phase while an equivalent single dose of TCDD failed to affect the $CD4^+$ cell subpopulation. Thus, these results suggest that the severity of the immunotoxic effect of TCDD was related to the length and repetitive nature of exposure.

In summary, the underlying cellular mechanism(s) of immune dysfunction induced by TCDD in the rat are unclear. Further investigations are necessary to pursue the mechanism of action of this chemical.

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