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EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD) ON THE IN VITRO ANTIBODY RESPONSE: Differential effects on the B lymphocyte depending on the state of in vivo activation and the modulation by serum-derived growth factors

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia/Virginia Commonwealth University

by

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DEDICATION

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LIST OF ABBREVIATIONS

3-MC	3-methylcholanthrene
^{3}H-TdR	^{3}H-thymidine deoxyribonucleic acid
2,7-DCDD	2,7-dichlorodibenzo-*p*-dioxin
2,8-DCDD	2,8-dichlorodibenzo-*p*-dioxin
2,3,7-TCDD	2,3,7-trichlorodibenzo-*p*-dioxin
2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
2,3,7,8-TCDF	2,3,7,8-tetrachlorodibenzofuran
Ab	antibody
AFC	antibody forming cell
Ag	antigen
Ah-R	*Ah*-receptor
AHH	aryl hydrocarbon hydroxylase
ANF	alpha-napthoflavone
APC	antibody producing cell
BL-CFU-C	B lymphocyte colony forming units in culture
BNF	beta-napthoflavone
BSA	bovine serum albumin
Ca^{++}_i	intracellular calcium
CD	cluster designation
CFU-E	colony forming units erythroid
CFU-GM	colony forming units granulocyte/monocyte

CFU-S	colony forming units spleen
CMI	cell-mediated immunity
CO_2	carbon dioxide
Con A	Concanavalin A
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DHR	delayed type hypersensitivity
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNP-Ficoll	dinitrophenyl haptenated Ficoll
DRE	"dioxin responsive element(s)"
EBSS	Earle's balanced salt solution
EC_{50}	effective concentration 50
EGF	epidermal growth factor
EROD	ethoxyresorufin-O-deethylase
FACS	flourescence activated cell sorter
FBS	fetal bovine serum
FES	fetal equine serum
FITC	fluorescein isothiocyanate
G-IFN	gamma-interferon
gd	gestational day
H_2O_2	hydrogen peroxide
HAH	halogenated aromatic hydrocarbon
HBSS	Hank's balanced salt solution
HCDD	hexachlorodibenzo-*p*-dioxin
HI	humoral immunity

HxCB	hexachlorobiphenyl
Ig	immunoglobulin
IgA	immunoglobulin-A
IgE	immunoglobulin-E
IgG	immunoglobulin-G
IgM	immunoglobulin-M
IL-2	interleukin-2
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
KCl	potassium chloride
KPO_4^-	potassium phosphate
L-Gln	L-glutamine
LD_{50}	lethal dose 50
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NBCS	newborn calf serum
NK	natural killer
NMS	normal mouse serum
OCDD	octachlorodibenzo-p-dioxin
PBB	polybrominated biphenyl
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PCP	pentachlorophenol

PFC	plaque forming cell
PHA	phytohemagglutinin
PK-C	protein kinase-C
PL-C	phospholipase-C
PMN(s)	polymorphonuclear leukocyte
poly I:C	poly inosine:cytosine
ppb	parts per billion
RES	reticuloendothelial system
RNase	ribonuclease
SAC	Staphylococcus aureus Cowan strain I
SAR	structure-activity-relationship
sIg	surface immunoglobulin
SRBC(s)	sheep red blood cell
T_c	cytotoxic T cell
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCR	T cell receptor
TD	T-dependent
TdT	terminal deoxyribonucleotidal transferase
T_{dth}	delayed type hypersensitivity T helper cell
T_h	T helper cell
TI	T-independent
TNP-LPS	trinitrophenyl haptenated lipopolysaccharide
TRF	T cell replacing factor
T_s	T suppressor cell

EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD) ON THE IN VITRO ANTIBODY RESPONSE: Differential effects on the B lymphocyte depending on the state of in vivo activation and the modulation by serum-derived growth factors

Abstract

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia/Virginia Commonwealth University

Dale L. Morris

Medical College of Virginia/Virginia Commonwealth University, December, 1991

Major Professor: Michael P. Holsapple, Ph.D.

Previous reports have indicated a dichotomy in the actions of TCDD on humoral immunity, both in vivo and in vitro, in which enhancements and suppression have been identified. The latter effect has been correlated with induction of liver P4501A1 enzyme activity, a response which is regulated by the *Ah-gene* locus. Additionally, the primary alteration in suppression of antibody responses is in the differentiation of the B cell. Therefore, the current investigation was undertaken to determine the relationship between these dual actions of TCDD on humoral immunity as related to its direct actions on B lymphocyte function. Specific emphasis was placed on determining the potential role of the *Ah-gene* locus in the modulation of the B cell and in vitro antibody responses.

Initial observations determined that: 1) the degree of suppression of in vitro antibody responses in B6C3F1 mouse (an *Ah*-high responder strain) splenocytes is dependent on both the lot, concentration, and type (i.e., fetal bovine versus newborn bovine) of serum used in culture; and 2) there is a similarity in the actions of TCDD and Staphylococcus aureus Cowan strain I (SAC); a polyclonal B cell activator. These observations prompted the study of the direct effects of TCDD on B cells in different stages of in vivo activation

and in the presence of different serum environments for which SAC has been reported to have opposing actions. TCDD was found to increase background levels of proliferation and differentiation in resting B cells (Go); thereby suggesting that resting B cells are a primary target in enhancement of immune responses by TCDD. However, no effect was seen on either response when the cells were stimulated with lipopolysaccharide (LPS). In cycling B cells (G1), TCDD caused suppression of both background and LPS-stimulated proliferation and differentiation and demonstrated a serum dependency that paralleled its actions in whole lymphocyte antibody responses; thereby demonstrating that it is the cycling B cell that is the primary target in suppression of antibody responses by TCDD and that both proliferation and differentiation are affected.

The modulatory role of serum was also determined in primary hepatocyte cultures using P4501A1 enzyme induction as the endpoint. Sera which supported either no effect or enhancements in both whole lymphocyte and purified B cell responses did not support induction of P4501A1 activity above BSA, a protein control. Conversely, sera which supported a consistent and dose-related suppression of in vitro antibody responses were found to enhance the induction of P4501A1 activity. More importantly, normal mouse serum was found to allow for the full expression of the *Ah*-dependent phenomena in vitro, where primary hepatocytes and splenocytes from *Ah*-high responder (B6C3F1) and *Ah*-low responder (DBA/2) mouse strains were affected by TCDD in a manner that parallels the effects seen in the two strains following in vivo exposures. The latter results using mouse sera are consistent with a role by the *Ah-gene* locus in the direct effects of TCDD on whole lymphocyte and purified B cell antibody responses. However, the results with other, more traditional sources of sera, indicate that the ultimate expression of the TCDD-induced responses can be modulated by serum factors, which are at present unidentified. Furthermore, the results of this investigation indicate that the susceptibility of the cell, as related to its stage in the cell cycle, can contribute to the complex effects that are seen in the alteration of antibody responses following TCDD exposure.

I. INTRODUCTION

One of the primary focuses of this laboratory has been to investigate the mechanism by which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces the suppression of humoral immunity. Specifically, this investigation has centered around the effects of TCDD on the T-dependent antibody response, a highly sensitive target for toxicity by TCDD and other members of the halogenated aromatic hydrocarbons [Thomas and Hinsdill, 1979; Hinsdill, et al., 1980; Clark, et al., 1981; Thomas and Faith, 1985]. It has been well documented that many of the toxicities induced by TCDD in vivo are mediated through binding to a specific steroid-like cytosolic protein known as the *Ah*-receptor. As modeled in the hepatocyte, this TCDD/*Ah*-receptor complex has been further shown to translocate to the nucleus and bind to specific "dioxin-responsive-elements" (DRE) of the DNA and to induce the transciptional activation of a number of genes, including those which code for the P4501A1 and 1A2 cytochrome P450 enzymes. [Poland, et al., 1974; Poland and Glover, 1975; Poland and Glover, 1980; Poland and Knutson, 1982]. Similarly, the impairment of immune cell and organ functions by TCDD have also been implicated as being regulated via the *Ah*-receptor and its associated gene locus [Vecchi, et al., 1983; Nagarkatti, et al., 1984; Blank, et al., 1987]. However, work from this laboratory has demonstrated that suppression of the T-dependent antibody response by TCDD has a component which is at least partially independent of effects mediated through the *Ah-gene* locus. This conclusion is based on two specific lines of evidence. The first is the report by Holsapple, et al. (1986) which demonstrated that splenocytes derived from

B6C3F1 (*Ah*-high responder) and DBA/2 (*Ah*-low responder) mice had comparable dose-response curves for the TCDD-induced suppression of LPS-stimulated in vitro antibody responses when compared under identical culture conditions [Holsapple, et al., 1986a]. The second was the report by Holsapple, et al. (1986) which demonstrated that subchronic exposure of B6C3F1 mice to 2,7-DCDD, a low-affinity *Ah*-receptor ligand, caused suppression of the in vivo T-dependent antibody response which was comparable to that produced by TCDD [Holsapple, et al., 1986b]. Therefore, it appears that both *Ah*- and non-*Ah*-receptor mediated processes are involved in the effects of TCDD on humoral immunity, the relative contributions of these two actions to the overall effects on immune cell function however, are still unresolved.

In terms of the specific effects of TCDD on the T-dependent antibody response, previous studies have demonstrated that the primary target for suppression is the B lymphocyte [Tucker, et al., 1986; Dooley and Holsapple, 1988]. Uniquely, the effects of TCDD on this cell have been characterized as immunomodulatory, in which TCDD exposure selectively alters the normal function and differentiation of these cells during the antibody response without any sign of overt toxicity [Tucker, et al., 1986; Holsapple, et al., 1986a; Kramer, et al., 1987; Dooley and Holsapple, 1988; Luster, et al., 1988b]. Additionally, TCDD was shown to cause no detectable alteration in mitogenically-induced proliferation of lymphocytes, and it was concluded that the ultimate effect of TCDD was to cause a specific alteration in the differentiation of the B cell during normal progression through the cell cycle [Tucker, et al., 1986; Dooley and Holsapple, 1988; Luster, et al., 1988b]. This conclusion is consistent with the work in other tissue systems in which alterations in differentiation have been reported [Poland and Knutson, 1982; Hudson, et al., 1985; Hudson, et al., 1986]. In contrast, several lines of evidence still suggest that activation and/or proliferation in the B cell can be altered by TCDD exposure. First, although TCDD seems to manifest its effects by arresting the differentiation of the B lymphocyte following antigen stimulation, it was recognized by several investigators that

this compound must be present during the first 24 hours of a T-dependent antibody response to SRBC (or within the first 3 hours of an LPS-induced polyclonal antibody response) to produce it's suppressive effects [Tucker, et al., 1986; Holsapple, et al., 1986a]. This is an important observation since all of the B cells that are committed to differentiation during an in vitro antibody response to SRBC have been determined to be activated and proliferating within 27 hours of culture, whereas T cell help is not needed until 48 hours [Dutton, 1975]. Secondly, addition of TCDD to naive (unsensitized) splenocytes was found to increase the basal antibody forming cell (AFC) response, which peaked on day 3 of culture [Kramer, et al., 1987]. This finding is in agreement with earlier work which demonstrated that in vivo exposure of naive animals to low doses of TCDD caused an increase in the level of serum antibodies and background antibody forming cell responses in the absence of antigen stimulation [Sharma and Gehring, 1979; Clark, et al., 1981]. It was further noted in the work by Clark, et al. (1981) that TCDD also increased the number of spleen colony-forming cells in bone marrow by 25-39%, an additional indication that under selective exposure conditions, TCDD can act at specific site(s) within hematopoietic cells to initiate an "activation-like" signal. Moreover, in the report by Kramer, et al. (1987), it was determined that addition of TCDD to purified B cells produced an increase in the level of basal kinase activity, produced after only 30 minutes of incubation. This kinase activity was further shown not to involve PK-C; a kinase which has been shown to be functionally activated during antigen stimulation through the immunoglobulin receptor of the B cell [Kramer, et al., 1986]. However, many growth factor receptors have associated kinase activity (tyrosine kinases in particular), suggesting the possible alteration in B cell growth at the level of a growth factor receptor. This hypothesis is supported by the previous work of Hudson, et al. (1986) which demonstrated an alteration in the EGF receptor on epidermal cells following exposure to TCDD [Hudson, et al., 1986]. Lastly, it has recently been established by our laboratory that the pleiotropic cytokine, gamma-interferon (G-IFN), can inhibit the TCDD-

induced suppression of the T-dependent antibody response, the TCDD-induced kinase activation in purified B cells and the TCDD-induced EROD activity in primary hepatocytes (Dooley, Yang, Snyder and Holsapple; unpublished observations). Most interesting about this finding was the fact that this cytokine, although it can affect many different cell types, had to be added early into culture to reverse the effects of TCDD on the in vitro antibody response (first 18 hours of culture) which correlated precisely with the time of TCDD addition that was needed to produce suppression. Moreover, G-IFN (or immune interferon) has been shown by a number of investigators to enhance both B cell activation and proliferation, as well as differentiation, to a number of activating stimuli; thereby suggesting a direct interaction between this cytokine and TCDD at the molecular level of the B cell during early activation by antigen [Defrance, et al., 1986; Romagnani, et al., 1986; Boyd, et al., 1987; Karray, et al., 1987; Morikawa, et al., 1987; Xia and Choi, 1988; Punnonen and Viljanen, 1989a; Punnonen and Viljanen, 1989b].

Collectively, these observations have suggested that early activation events in the B lymphocyte are targeted by TCDD and that both enhancements and/or suppression can result depending on the conditions of exposure. Therefore, the primary objective of this investigation was to determine the relationship between the immune enhancing and immunosuppressive effects of TCDD on humoral immunity and to determine whether these two opposing actions of TCDD on in vitro antibody responses is the result of differential effects on separate B lymphocyte subpopulations within the spleen (i.e., dense resting and cycling B cells). In addition, studies were conducted to determine if these two opposing actions of TCDD are correlated with responses mediated by the *Ah*-receptor and the *Ah-gene* locus.

Results of the current investigation demonstrate that TCDD has a differential pattern of effects on the B lymphocyte depending on their state of in vivo activation - potentiation of resting (Go) and a generalized suppression of cycling (primarily G1) B cell function; where suppression of cycling B cell responses was found to result from effects on both

proliferation and differentiation into antibody secreting cells. Furthermore, these two opposing actions of TCDD on the B cell were found to be modulated by the presence of either serum-derived growth factors (and/or hormones) and T cell-derived cytokines which were found to potentiate the direct effects of TCDD on the B cell to either increase or decrease the enhancements or suppression in B cell function seen in vitro. Moreover, it was determined that the effects of TCDD on both in vitro antibody responses and modulation of B cell function correlate with responses mediated by the *Ah*-receptor and associated gene locus. This effect was most apparent when lymphocytes and primary hepatocytes from both *Ah*-low (DBA/2) and *Ah*-high (B6C3F1) responsive mouse strains were exposed to TCDD in the presence of normal mouse sera; serum conditions which do not appear to alter the genotypic expression of *Ah*-receptor-mediated responses to TCDD in the lymphocyte and which are normally encountered in vivo. Additionally, it was determined that suppression of in vivo antibody responses by TCDD in DBA/2 (*Ah*-low responsive) mice can be enhanced following subchronic (14 day repetitive) exposures to TCDD. This enhanced suppression of the antibody response was accompanied by an increase in splenic, but not liver, P450 activity and a coordinate loss in a non-B/non-T cell population within the spleen; indicating that the effects of TCDD on lymphocyte function in vivo is a dynamic process that can be altered by changes in the conditions of TCDD exposure.

II. LITERATURE REVIEW

A. Review of Immunology and the Humoral Immune Response:

Although a generalized overview of the immune system and effector mechanisms will be described, this review of the immunology literature will focus primarily on humoral, or antibody-mediated immunity, and the role of T cells in this response. A more detailed characterization of other T cell-mediated mechanisms of immunity, while being equally important, is beyond the scope of this discussion and the current work for which it precedes. However, because of the potential cooperativity that may occur between T cells and B cells during humoral as well as other immune responses to infectious agents, a more detailed description of the effects of TCDD on both cell-mediated and humoral immunity will be included in the following section.

1. General Concepts of Immunity:

The essential function of the immune system is to defend against infection. As such, it comprises all of the physiological mechanisms which enables an animal (or host) to recognize materials as foreign to itself and to neutralize, eliminate or metabolize them with or without injury to its own tissues [Paul, 1984; Roitt, et al., 1985]. Lower animal forms possess innate immune mechanisms which protect them against infecting organisms. Higher animal forms have evolved to possess, in addition to innate immunity,

acquired immune mechanisms which provide a more customized, specific and efficient defense against infectious agents.

Innate immunity represents the first line of defense during infections and is characterized as being non-specific (or non-selective) and as not having immunological memory (i.e., the resistance is not improved upon secondary challenge or repeated infection). The mechanisms involved in innate immunity are comprised of specialized physical and biochemical barriers outside or within the body (i.e., skin, cilia lining the trachea, stomach acid, sebaceous gland secretions, lysozymes and mucous), commensal organisms of the gut, non-specific soluble factors (i.e., the complement proteins, acute phase proteins and interferons) and specialized leukocytes (i.e., neutrophils, monocytes/macrophages, natural killer cells, Kupffer cells and synovial A cells). The latter patrol the tissues of the body, non-specifically recognize a foreign substance (via cell surface receptors for complement proteins and antibodies bound to the surface of the infectious agent) and then phagocytize or secrete soluble factors such as lysozyme which destroys the organism. Other mechanisms of innate immunity include inflammation, fever and bodily reflexes such as coughing and sneezing [Paul, 1984; Roitt, et al., 1985].

Acquired or adaptive immunity is the second line of defense, and is activated if innate immune mechanisms fail to rid the body of the infection. This line of defense is characterized as producing a specific reaction to each infectious agent (i.e., specific recognition of self versus non-self via specialized cell surface molecules encoded in the major histocompatibility gene complex) and as producing immunological memory of the experience which improves the resistance of the host to that agent upon secondary challenge. The cellular mediators of this response are the T and B lymphocytes which form two divisions of acquired immunity known as cell mediated (CMI) and humoral (HI) immunity, respectively. CMI is acquired immunity that is dependent upon T cells which, upon activation, differentiate into distinct subsets of T effector cells that function to attack and rid the body of a wide spectrum of infectious agents. HI is acquired immunity which

is dependent on the B cells which, upon activation, differentiate into effector B cells (also called plasma cells) that secrete specific antibodies that can bind to and either neutralize or mediate the destruction of microorganisms. Although the two arms of acquired immunity can act independent of one another, there is considerable interaction between them, and it is this cooperativity that enables the immune system to develop a very diversified reaction to most infectious agents [Paul, 1984; Roitt, et al., 1985].

2. Specificity of the Immune Response: Self Versus Non-Self.

In order to mount an immune response against any infectious agent, the immune system must be able to distinguish between "self" and "non-self". "Self" to the immune system, as the term implies, comprises all of the tissues within the body, including normal flora. Privileged sites are the exception (i.e., cornea of the eye, testes and brain) which are considered to be outside the bodies realm of surveillance.

In innate immune mechanisms (i.e, phagocytosis), "non-self" recognition is mediated by way of cell surface receptors on the phagocyte that are specific for a number of various immune factors (i.e., complement and antibodies) that are activated or secreted during an immune response. These factors bind to the surface of an infectious agent and mediate their recognition and subsequent engulfment and/or destruction by the phagocyte. In acquired immune mechanisms, specialized molecules on the surface of all nucleated cells of the body help to distinguish "self" from "non-self". These molecules are cell surface proteins that are encoded by the major histocompatibility gene complex (MHC) and are known as the MHC antigens [Paul, 1984; Roitt, et al., 1985]. This region of the genome in the mouse is referred to as the H-2 complex (located on chromosome 17) or in the human as the HLA gene cluster (located on chromosome 6). Two classes of MHC molecules exist and are distinguished from one another by structure, function and cellular distribution. Class I antigens consist of 2 polypeptides, a larger protein which is encoded

by the H-2 complex, and a smaller protein, known as ß2-microglobulin, which is encoded outside the H-2 complex. Class I antigens are found on all nucleated cells and platelets and function to present endogenously produced foreign antigens (i.e., such as viral gene products) at the cell surface. Class II antigens consist of 2 polypeptide chains (*a* and ß), both of H-2 complex origin, and are expressed on specific types of immune cells - B cells, macrophages, monocytes, dendritic and Langerhans cells, epithelial cells and activated T cells (identified in the human only). These molecules function in immune responses to mediate the presentation of exogenously encountered antigens on the surface of antigen presenting cells (APC) following internal processing. It is the latter system that allows for more specific immune responses to occur and is further governed by antigen specific T cells that recognize foreign antigens only when bound to or in context with the MHC antigen on the surface of APC [Paul, 1984; Roitt, et al., 1985].

3. Cells of the Immune Response:

The cells of the immune system are a diversified group, each having specialized functions that enables them to attack and rid the host of a multitude of organisms and transformed cells (i.e., virally infected or tumor cells). These cells include, among others, the lymphocytes, monocytes/macrophages, neutrophils, dendritic and Langerhans cells and specialized epithelial cells such as those formed within the thymus. These cells occur in organized tissues and organ systems including the spleen, lymph nodes, Peyer's patches, tonsils, thymus and bone marrow. In addition, a trafficking population of these cells are found in blood and lymph.

All cells of the immune system share a common precursor known as the pleuripotent stem cell. This cell undergoes hematopoietic differentiation down two distinct pathways within the primary immune organs (i.e., fetal liver, fetal spleen and fetal bone marrow or bone marrow of adult mammals) to give rise to two generalized populations of cells [Paul,

1984; Roitt, et al., 1985]. The first is the myeloid pathway which begins with the development of a myeloid progenitor cell. The myeloid progenitor then undergoes hematopoietic differentiation to produce the mononuclear phagocytes (i.e., monocytes/macrophages), the polymorphonuclear granulocytes (i.e., neutrophils, basophils and eosinophils), mast cells and megakaryocytes (i.e., which produce and release the blood platelets upon maturity). The second pathway consists of the lymphoid lineage and begins with the development of the lymphoid progenitor. These cells undergo further differentiation to form the various populations of immature lymphocytes (i.e., the pre-T and pre-B cells). Lymphocytes undergo additional development within a second set of primary immune organs, the thymus and Bursa of Fabricius (and/or Bursa-equivalent). Pre-T cells develop into mature T cells within the thymus whereas pre-B cells develop within a specialized organ known as the Bursa of Fabricius (in birds) or in the bone marrow or Bursal-equivalent of mammals. It is the developmental origin within these two tissues from which their names are derived. Both pathways (i.e., myeloid and lymphoid) give rise to mature immune cells which then migrate to secondary immune organs, such as the spleen and lymph nodes (i.e., lymphocytes), or to sites surrounding all tissues of the body (i.e., monocytes/macrophages and neutrophils), where they can survey the tissues and secretions of the body for infectious agents.

Functionally, the mononuclear phagocytes have two important tasks, phagocytosis and antigen presentation, each carried out by distinctly different forms of the same cell [Paul, 1984; Roitt, et al., 1985]. Phagocytic macrophages are dispersed throughout the body and play a key role in the engulfment and destruction of particulate antigens. Destruction takes place within the cell when phagosomes containing the microorganism are fused with degradative enzyme containing lysosomes. APC are found primarily in the skin, lymph nodes, spleen and thymus and include, in addition to the mononuclear phagocytes [Mosier, 1967], the Langerhans cells [Stingl, et al., 1978], dendritic cells [Inaba, et al., 1983] and B cells [Chestnut, et al., 1982]. APCs are especially rich in Class

II MHC antigens, and following incorporation by phagocytosis and/or pinocytosis (i.e., macrophages) or receptor-mediated endocytosis (i.e., B cells), process and present soluble antigens to antigen-responsive lymphocytes.

Polymorphonuclear granulocytes act non-specifically, and together with other soluble factors play an important role in acute inflammation and protection against microoganisms [Paul, 1984; Roitt, et al., 1985]. Their predominant function, like the macrophage, is phagocytosis, where ingested microbes contained within the phagosomes are fused with granules that contain degradative enzymes. Eosinophils, basophils and mast cells make up a group of specialized granulocytes which play a more specific role during helminth infections and more selective forms of infammation. Another myeloid lineage cell, the megakaryocyte, does not leave the bone marrow upon maturation, but rather functions to produce and release platelets into the circulatory system of the host. Platelets are important in blood clotting and inflammatory responses. They not only release factors that can increase the permeability of the vascular system, thus allowing for entry of immune cells into infected areas, but release other factors such as PDGF, EGF and TGF which can locally modulate immune responses mediated by other immune cells in the area.

Lymphocytes are comprised of subsets of cells that are morphologically very similar but functionally very distinct. They can be distinguished from one another by cell surface proteins or surface markers, many of which are important in their responses to infectious agents. For example, T cells and B cells can be distinguished via their antigen receptor complexes, T cells possessing the CD3 signalling complex of the T cell receptor (TCR) and B cells the surface immunoglobulin (sIg) receptor. Whereas B cells primarily function to produce specific antibodies during HI responses, T cells are additionally separated into 4 functionally distinct subsets - helper cells (T_h), cytotoxic cells (T_c), suppressor cells (T_s) and delayed type hypersensitivity helper cells (T_{dth}). T_h cells, of which there are two subtypes based on their spectrum of lymphokine release (i.e., T_{h1} and T_{h2}) [Mosmann, et al., 1986], are distinguished by the mouse cell surface marker L3T4 (or CD4 in the

human) and function in providing help to B cells during HI responses and to other effector T cells during CMI responses. Th_1 cells predominantly secrete IL-2 and G-IFN whereas Th_2 cells have been shown to secrete IL-4, IL-5 and IL-6 [Mosmann and Coffman, 1987]. T_{dth} cells are a specific type of helper cell that resides in the skin and aids in immune responses during dermal attack by pathogens. T_s cells, are distinguished in the mouse by possessing the Lyt-2 surface marker (or CD8 in the human) and play an active role in controlling the immune response once the antigen has been successfully destroyed or in immune tolerance to the hosts own tissues. T_c (or CTL) also possess the Lyt-2 or CD8 marker but function primarily in the attack against intracellular parasites (i.e., viruses) and against transformed tumor cells. NK cells, which possess characteristics of both T and B lymphocytes, fulfill a similar function to the CTL, but act in a more non-selective, as opposed to specific, manner.

4. The T-Dependent Humoral Immune Response:

The work of Mishell and Dutton (1967) [Mishell and Dutton, 1967] was key in establishing the cells and events necessary to produce specific antibodies to an antigen. This was accomplished by the development of an in vitro culture system which models the events during a HI response. In this system, mouse splenocytes were cultured in the presence of a red blood cell (RBC) antigen (i.e., a particulate T-dependent antigen). Under controlled atmospheric conditions, these cells gave rise to specific antibodies to the antigen that could be detected by a plaque formation technique developed by Jerne and Nordin (1963) [Jerne and Nordin, 1963]. Using this technique, Mosier and co-workers (1967) [Mosier, 1967] later separated spleen cells into 2 main populations, adherent (monocyte/macrophages) and non-adherent (lymphocytes), and determined that both populations were required for maximal antibody formation in vitro. Therefore, the T-dependent antibody response, so named for its dependence on thymus-derived or T cells as

earlier determined by Claman, et al. (1966) [Claman, et al., 1966], requires the cooperative interaction of 3 major immune cell types - i.e., the B cell , the T cell and the macrophage (or APC). Additionally, Melchers and Andersson (1986) [Melchers and Andersson, 1986] further determined that the interaction of these cells in production of specific antibodies to an antigen can be divided into 3 distinct phases. As reviewed elsewhere [Vitetta, et al., 1989], the first phase is the specific recognition and endocytosis of the antigen by the APC, processing of the antigen and presentation of the antigen on the cell surface for recognition by antigen-specific T_h cells. Simultaneously to these events, APC secrete soluble factors, such as IL-1 [Vitetta, et al., 1989], that aid in the initiation of T cell activation and growth [Durum, et al., 1985]. In the second phase, B cells become activated and, in combination with soluble factors secreted by the helper T cell (i.e., IL-4 or BSF-1), would move into the cell cycle [Mosmann and Coffman, 1987]. The third phase is the specific regulation of B cell growth and differentiation by T cell factors (i.e., IL-2, G-IFN, IL-4, IL-5 and IL-6) which control both proliferation and differentiation of the B cell into an antibody secreting cell [Mosmann and Coffman, 1987]; the T_h2 cell clone being the most important T_h cell involved in the promotion of growth and differentiation of T-dependent antigen-stimulated B cells.

5. T-Independent Antigens:

As opposed to T-dependent antigens (i.e., SRBC, soluble proteins and hapten-carrier complexes) which require T cell help, T-independent antigens can cause the direct activation of B cells without the apparent need for T cells. Most T-independent antigens are high molecular weight polymers with repeating subunits that enable them to cross-link cell surface receptors on the B cell. T-independent antigens have been classified according to two types - Type I (TI-1) and Type II (TI-2). This classification is based on the ability of a TI antigen to activate B cells from the CBA/N mouse strain which has the *xid* gene

mutation [Mosier and Subbarao, 1982] and lacks the Lyb 5 subset of mature B cells. TI-1 antigens, such as TNP-LPS and TNP-*B. abortus*, can effectively stimulate CBA/N B cells [Mond, et al., 1978] whereas TI-2 antigens, such as TNP-Ficoll and TNP-dextran, can not. Moreover, TI-1 antigens such as LPS have the ability to polyclonally activate B cells to differentiate into antibody forming cells with a wide spectrum of specificities whereas TI-2 antigens generally do not. TI antigens have also demonstrated differences in accessory cell help, where both can be modulated by the presence of either macrophages or T cells. TI-2-stimulated B cells have been shown to require either macrophage-like adherent accessory cell help or T cell factors to produce an optimal response whereas TI-1 antigens seem to be less dependent on these accessory cells [Mond, et al., 1980; Mond, et al., 1983].

6. B Cell Activation and Lymphocyte Signalling:

The activation of resting B cells results in clonal expansion and subsequent differentiation into antibody producing cells. This process is regulated in a complicated manner by exogenous molecules such as antigens and bacterial cell wall products, as well as by cytokines that are produced by the T lymphocyte and macrophage APC [Kishimoto, 1985; Melchers and Andersson, 1986]. In developing an understanding of this process, various agents have been used to determine the mechanisms of signal transduction that are important in cell-surface-receptor-mediated responses of the B cell (or T cell), and it is this collection of signals which transduces information from the external environment to the cell interior and ultimately produces the functional response.

As described in later sections of the dissertation, the TI antigen LPS can be used as an excellent tool in the study of B cell activation. This is in light of the direct activating potential of LPS on B cells in the absence of either T cells, macrophage accessory cells or their respective secretory factors. However, although a great deal is known about the

cellular effects of LPS on B cells, the molecular mechanisms associated with activation of B cells by LPS is still unresolved. In as much, LPS has been shown to activate a class of membrane signalling components known as G proteins [DeFranco, et al., 1987]. As activation of these components is associated with a number of receptors for hormones and neurotransmitters, it is therefore likely that LPS also utilizes a specific cell surface receptor [Dziarski, 1989]. Furthermore, signal transduction mediated by LPS has been shown to be somewhat different with respect to signals mediated through the sIg receptor, demonstrating a distinction between these two types of cell surface "antigen" receptors in the activation of the B cell. As opposed to the sIg signalling cascade, LPS has been shown to cause the translocation of protein kinase-C (PK-C) [Chen, et al., 1986], without the activation of PL-C or the release of IP3 [Wrightman and Raetz, 1984; Bijsterbosch, et al., 1985]. Consistent with these findings, LPS has been further shown to induce the influx of extracellular calcium through membrane calcium channels [Freedman, 1979; Amigorena, et al., 1990a]. This latter effect has been further correlated with the proliferative capacity of B cells activated by LPS and may be the key second messenger signal in the activation process induced by LPS. Other signals that have been identified as early events following LPS stimulation include membrane depolarization [Kiefer, et al., 1980], the activation of a voltage-dependent K^+ channel [Freedman, 1979; Amigorena, et al., 1990a; Amigorena, et al., 1990b], the activation of a Na^+/H^+ exchange operation at the membrane [Gaidano, et al., 1989] and a decrease in intracellular cAMP [Cambier and Ransom, 1987; Defranco, 1987].

As opposed to the more "traditional" T-independent antigens as described above, a second class of T-independent antigens have been selectively used to study the cellular events which occur during T-dependent antigen stimulation of the B cell. These agents are the sIg-cross-linking agents and include anti-IgM and anti-IgD antibodies and Staphylococcus aureus Cowan strain I (SAC). At low concentrations, anti-Ig (≤ 5 μg/ml) has been found to transmit signals to the B cell which mimic the activation by antigen

[Cambier and Ransom, 1987; Imboden, 1988]. This signal induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by PL-C [Coggeshall and Cambier, 1984], which subsequently results in the release of both diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG, in combination with Ca^{++} and phospholipids, is able to activate PK-C [Nishizuka, 1984], whereas IP3 is responsible for the release of Ca^{++} from intracellular stores (Ca^{++}_i). These two second messengers therefore interact to transmit the antigen signal from the membrane to the nucleus. However, this signal is inefficient to produce DNA synthesis and proliferation of these cells. Entry of these cells into active cell cycle requires an additional signal which has been shown to be provided by IL-4 (BSF-1) [Kishimoto, 1985]. Additionally, these independent signals can be mimicked in the B cell by the addition of a phorbol ester, such as PMA (i.e., direct PK-C activator), and a calcium ionophore, such as ionomycin [Cambier and Ransom, 1987]. This combination, much like antigen and IL-4, can induce proliferation but not immunoglobulin secretion. Additional factors, secreted by the T cell, are required for differentiation of these cells into antibody producing cells [Mosmann and Coffman, 1987].

Conversely to the cellular events that occur following cross-linking of sIg on the B cell by low concentrations of anti-Ig, a secondary effect has been identified when B cells are exposed to higher concentrations of anti-Ig ($\geq$ 25 μg/ml) or SAC. SAC has been described as a T cell-independent polyclonal B cell activator, in that it can bind to (by way of protein A immunoglobulin binding molecules on the bacterial cell surface) and cause the direct activation and proliferation of resting B cells [Kehrl, et al., 1984]; the bacterial cell wall acting as a support or catalyst in transmitting a much greater intracellular signal to the B cell than is achieved by low concentrations of anti-Ig. However, although proliferation is induced, the signal is still inefficient in causing the differentiation of these cells in the absence of T cell-derived growth and differentiation factors. In this respect, activation by SAC (and high concentrations of anti-Ig) is unique, in that it can stimulate the expression of the high-affinity IL-2 receptor on the surface of resting B cells [Tanaka, et al., 1988],

and it is IL-2, as opposed to IL-4, which is the primary B cell growth factor for B cells activated in this manner. More importantly, proliferation of SAC activated B cells is modulated by serum-derived growth factors, which can increase or decrease the ability of these cells to proliferate and/or differentiate [Choi and Xia, 1987]. In fact, it has been speculated that it is the activation of B cells in an IL-2 dependent manner, as compared to IL-4, which may render these cells susceptible to modulation by the serum-derived factors. Moreover, G-IFN and TNF-alpha, as opposed to IL-4, IL-5, and IL-6, have been shown to be important modulators of SAC-induced proliferation and differentiation of B cells; where both have been shown to augment the effects of IL-2 [Karray, et al., 1987; Kehrl, et al., 1987]. It is therefore apparent that B cells can be activated and modulated by several different pathways, and thus demonstrates another level of diversity in the immune response which occurs at the molecular level of the B lymphocyte.

Analogous to the events occurring in the T-dependent antigen-stimulated B cell, antigen-specific T cells recognize antigen (in conjunction with class II MHC molecules on the APC) through specific binding to the T cell receptor (TCR). The TCR is a heterodimeric glycoprotein made up of an *a* and ß chain held together by disulfide bonds. Signals are transduced by this receptor through associated proteins known as the CD3 complex [Clevers, et al., 1988]. Much like the cross-linking of sIg on the B cell, triggering of this receptor in vitro (i.e., cross-linking antibodies to the TCR complex) leads to the activation of PL-C and hydrolysis of PIP2 to IP3 and DAG. IP3 and DAG in turn, cause the release of Ca^{++}_i and the activation of PK-C, respectively [Imboden, et al., 1987; Weiss and Imboden, 1987]. These events subsequently lead to the specific activation of the T cell and the expression of cell surface receptors and lymphokine secretion which are important in autocrine growth and in co-activation of the B cell [Isakov, et al., 1986].

B. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and Immunotoxicology:

As opposed to the review of the immunology literature, this section of the literature review will give a broad overview of the effects of TCDD on both cell-mediated and humoral immunity. This is to give the reader a full understanding of the effects of TCDD on both T cell and B cell functional responses and on the cooperativity that occurs between these two cells during the process of an immune response. Additionally, it is designed to demonstrate the potential for synergism between the effects on these two populations of cells in suppression of T-dependent antibody responses, as well as other immune responses mediated by the various lymphocyte effector cells which are responsible for host resistance to infection.

1. General Mechanism of TCDD Action:

While the overall focus of this section of the review is on the immunotoxicological effects of TCDD and related compounds, it is important to emphasize that exposure to these chemicals produces a wide spectrum of toxic responses (for a comprehensive review see Poland and Knutson, 1982 ([Poland and Knutson, 1982]). Besides immunosuppression, these responses include a generalized wasting syndrome, pancytopenia, hepatomegaly and hepatotoxicity, chloracne and hyperkeratosis, gastric lesions, urinary tract hyperplasia, edema, tumor promotion, teratogenicity and embryotoxicity, decreased spermatogenesis and lymphoid involution (especially of the thymus). The spectrum of toxicity observed following TCDD exposure is characterized by wide variations in potency among different species of laboratory animals. For example, the LD_{50} varies approximately 5000-fold between the most sensitive species, guinea pigs (0.6 - 2 μg/kg), and the least sensitive species, hamsters (5 mg/kg). Moreover, the specific features of the spectrum of toxicity can vary markedly between species. On the

other hand, certain toxic effects, including the generalized wasting syndrome, thymic involution, immunosuppression and hepatomegaly, have been observed as correlates to exposure in almost all species tested.

As described below, it was the ability of this chemical to cause lymphoid involution, which prompted the original interest in determining the effects of TCDD on immune function. However, it was the effects on the liver, most notably the induction of drug metabolizing enzymes, especially the $P450_{1a}$ isozyme, while not a toxic response in itself, which led to the most accepted and best supported model for the mechanism of action associated with exposure to HAH [Safe, 1986; Goldstein and Safe, 1989]. This model is centered around the binding of TCDD to a cytosolic receptor and has many similarities to the classical steroid receptor model. Early work demonstrated that the expression of this receptor is controlled by the *Ah-gene* locus and that various mouse strains differ in their expression of the receptor (i.e., there are highly responsive mouse strains, such as C57BL/6 mice, and less responsive mouse strains, such as DBA/2 mice). TCDD is thought to bind to this *Ah-gene* locus encoded cytosolic receptor (*Ah*-R), and the bound ligand-receptor complex then translocates to the nucleus where it binds to specific "dioxin regulatory elements" (DRE). It is this latter interaction which is thought to precipitate a pleiotrophic gene response which culminates in a measurable increase in mRNA specific for the various enzymes whose levels are controlled at the gene level by the *Ah-gene* locus [Whitlock, 1987; Nebert, 1989; Whitlock, 1990]. Differences in potency among different HAH congeners are explained by structure-activity-relationships (SAR) and differences in affinity for the *Ah*-R, where a strong correlation has been shown between receptor binding affinity and enzyme induction for all HAH families [Goldstein and Safe, 1989]. The SAR established for the PCDD indicates that maximal activity occurs when only the 4 lateral ring positions of the dibenzo-*p*-dioxin molecule are halogenated. This point is demonstrated in Figure 1, where the most active congener of the PCDD, TCDD, has 4 chlorines which are substituted at the 2, 3, 7, and 8 positions of

the parent ring structure. Active congeners must also have at least 3 of the 4 lateral positions occupied, with at least 1 position adjacent to the lateral positions remaining unsubstituted. Deviations from this structure are associated with decreased activity. Investigations with other families of HAH, especially PCB and PCDF, have demonstrated a parallel SAR for the same measured endpoints, although with decreased activity relative to the analogous PCDD congeners. In the case of PCB and PBB, the active congeners must be able to achieve the planar conformation which exists for PCDD and PCDF [Silkworth and Grabstein, 1982]. Therefore, the model centered around a role by the *Ah*-R is well established for enzyme induction, and either a parallel SAR or a correlation with *Ah*-R functionality in responsive and non-responsive strains, or both, have been demonstrated.

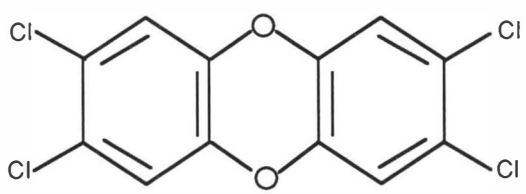


Figure 1. Chemical Structure of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD).

While differences in potency and the spectrum of toxic effects between species have been putatively explained by differences in gene activation and the presence, distribution and functional capacity of the cytosolic *Ah*-R, the association between the *Ah*-R and toxicity is not conclusive. Most importantly, a causal relationship between specific genes activated by *Ah*-R binding and the manifestation of toxic symptoms following TCDD

exposure has not been directly established. The most extensively used approaches to determining a role for the *Ah*-R in the profile of toxicity of TCDD, including immunotoxicity, have been somewhat indirect. These approaches have capitalized on either the definite SAR associated with HAH binding to the *Ah*-R, or on the genetics associated with the expression of the *Ah*-R. The possible mechanisms of action of TCDD on immunocompetence have been recently reviewed [Holsapple, et al., 1991a]. In particular, this review discusses a number of reports in which the same kinds of indirect approaches have been used as described above, and present conflicting data that are not readily explained by *Ah*-R-based mechanisms. Therefore, while the ultimate mechanism has not yet been elucidated, this earlier review extensively discusses the possibility that both *Ah*-R-dependent and *Ah*-R-independent processes may be involved. Because possible mechanism(s) have been extensively discussed elsewhere, the focus of this review will be to present an update on the profile of immunological effects by TCDD and related compounds.

2. Approaches to TCDD-Induced Immunotoxicity:

As discussed above, an immune response is mediated by the specific cooperative interaction of immunocompetent cells and the non-specific soluble factors produced by these cells. The effector systems are interrelated, complementary, and possess considerable redundancy. An infected host is able to marshall one or more of a number of defenses against a particular parasite or pathogen. The redundancy of effect and the fact that there are overlapping effector processes have contributed to the complexity associated with the evaluation of xenobiotic-induced changes in immunocompetence. As described by Luster, et al. (1988) [Luster, et al., 1988a], there are well characterized assays which can be applied to assess the responsiveness and functionality of immunocompetent cells, the effective cooperative interactions of these cells to produce CMI or HI, and the general

immunocompetence of the host when challenged by infectious agents or pathogens (i.e., host resistance capabilities). One of the most important conclusions reached in this paper is that when the immunological protocols are standardized, there can be excellent agreement between several laboratories regarding the effects of specific chemicals. In actuality, this type of standardization in immunotoxicology is rare. A given immune parameter can be elicited with several different antigens and/or protocols. More importantly, the precise experimental conditions regarding the length of chemical exposure and the frequency of chemical administration (i.e., daily or weekly), can vary markedly from one study to the next. A second factor which contributes to the problems in comparing results from several labs is that the immune response is not an event which can be approached with a discrete time point. Rather, it is an orchestrated series of distinct events which may have differential sensitivity to a given xenobiotic. Therefore, results from studies in which the xenobiotic is administered before antigen may be different from studies in which the xenobiotic and antigen are administered simultaneously, which in turn may be different from studies in which the xenobiotic is administered after the antigen. The consequence is that sometimes it is difficult to compare the results of two or more studies. This difficulty is particularly apparent regarding the immunological actions of TCDD, especially as it relates to attempts to compare the sensitivity of the various animal species in which it has been evaluated. As discussed briefly above and more extensively in several reviews [Poland and Knutson, 1982; Safe, 1986; Goldstein and Safe, 1989; Nebert, 1989], the acute toxicity (i.e., LD_{50} values) and liver enzyme induction (i.e., also generally measured by a standardized protocol after acute exposure) have been compared in several species by a number of laboratories. These results indicate a distinct species-specificity regarding sensitivity to these effects of acute exposure to TCDD and related compounds, with guinea pigs especially sensitive and hamsters much more resistant. Such comparisons of the immunotoxicity by TCDD are difficult, partially because individual laboratories have used a variety of immunological protocols, and no single laboratory has

made comparisons between several species using a single standardized immunological parameter. In one report where the effects of TCDD on the immunocompetence of multiple species were determined, Vos, et al. (1973) did not measure a comparable immune parameter in each of the three species studied [Vos, et al., 1973]. Their results did indicate a suppression of a tuberculin DHR in Hartley guinea pigs (i.e., at doses as low as 0.04 μg/kg/week for 8 weeks) but not in randomly bred CD rats (i.e., at doses up to 5 μg/kg/week for 6 weeks). A DHR was not measured in C57BL/6 mice in this study, but they did show that a graft-vs-host reaction was decreased (i.e., at doses as low as 1.0 μg/kg/week for 4 weeks). Humoral immunity was only assessed in the guinea pigs as both a primary and a secondary antibody titer in response to tetanus toxin. Exposure to low doses (0.008-0.04 μg/kg/week for 5 weeks) significantly enhanced the primary antibody response; but slightly reduced the secondary antibody response (i.e., 7 week exposure). Exposure to the high dose (0.2 μg/kg/week) had no effect on the primary response (i.e., 5 week exposure) and significantly reduced the secondary response (i.e., 7 week exposure). Surprisingly, this early study by Vos and co-workers represents the only report of the immunological effects by TCDD in guinea pigs, historically, the most sensitive species, and there are no studies on the effects of TCDD on immune function in hamsters, historically, the most resistant species. As discussed at several places in this review, the most effective comparisons in animal sensitivity to the immunological effects of TCDD have been made within one species, between highly responsive and low responsive mouse strains.

Overview: Immunotoxicity in animal models. Immunotoxicity testing with TCDD was initially undertaken to extend the findings of general toxicity studies. These earlier investigations were important in demonstrating that exposure of animals to TCDD produced a prominant lymphoid involution, especially of the thymus. Since the thymus represents the central organ for maturation of T lymphocytes, initial immunotoxicity studies concentrated on T cell-mediated immunity. Among the different species tested,

suppression of CMI was shown to be dependent upon the age at which the animal was exposed. Exposures in younger animals produced a more extensive and longer lasting suppression, where in utero exposures during organogenesis were required to induce maximal effects in some species. Consistent with these findings, it has been reported that TCDD induces the terminal differentiation of thymic epithelial cells, thus indirectly preventing the maturation of thymocytes to mature T cells because of the absence of the proper microenvironment within the thymus [Greenlee, et al., 1984b; Greenlee, et al., 1985]. Because of the prominant effect by TCDD on the thymus, it was assumed that CMI would be much more sensitive than HI, and that immune responses which are mediated by antibody would be largely unaffected in perinatally exposed animals. In actuality, antibody responses were not consistently measured in these early studies which were centered around perinatal or in utero exposure. In one study where both CMI and HI were measured in 5-week old offspring from mice fed chow containing TCDD, Thomas and Hinsdill showed that the anti-SRBC PFC response (HI) was significantly reduced at both 2.5 ppb and 5.0 ppb, while the contact hypersensitivity response (CMI) to dinitrofluorobenzene was only significantly reduced at 5.0 ppb. Moreover, as discussed below in the section on lymphocyte development and homing, effects on both B- and T cells have been described. Therefore, it is not surprising that both HI and CMI are targeted when exposure occurs in younger animals. In contrast with these studies, several laboratories have shown that exposure in adult animals produces a profound suppression of antibody responses under conditions where T cell-mediated immunity appears to be less affected. As described below, TCDD-induced suppression of humoral immune responses appears, at least in part, to be a direct effect on the B lymphocyte. Although the functional deficit has not been identified, studies suggest that the differentiative processes of these cells are altered. Thus, current data suggests that TCDD either indirectly (i.e., in the case of T cells) or directly (i.e., in the case of B cells) affects the maturational or differentiative processes of immunocompetent cells, and that such cells are sensitive to the effects of this

xenobiotic during periods of activity associated with these events. This observation is consistent with findings in other cell systems in which the basis for the actions of TCDD were reported to be the consequence of altered differentiation or growth [Greenlee, et al., 1984b; Greenlee, et al., 1985].

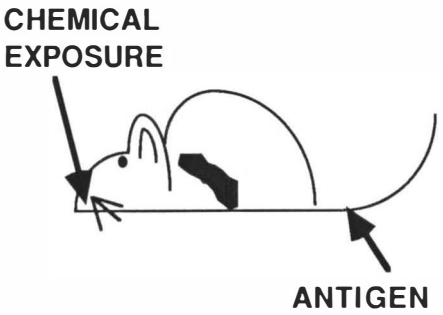
Overview: Immunotoxicity in humans. To what extent significant human health effects are associated with environmental levels of exposure to TCDD is controversial. The only adverse human health effects which have been conclusively associated with dioxin exposure are chloracne and hyperkeratosis, which are oftentimes accepted as hallmarks for human exposure to TCDD and related HAH. Various cancers, embryotoxic and teratogenic effects, and immune dysfunction are alleged to result from both environmental and occupational exposure. As noted, these toxic effects have been demonstrated in various animal species following exposure to TCDD. In the animal species studied, atrophy of lymphoid tissue, especially the thymus, and suppressed immune function are among the earliest and most sensitive indicators of HAH toxicity, and significant suppression of both T cell- and B cell-mediated immune function occurs with doses which do not produce overt signs of toxicity. It is reasonable, therefore, to assume that the chronic, low level human exposures associated with HAH contamination, might adversely affect immune function. Studies in exposed human populations and in non-human primates have shown that HAH do produce measurable alterations in immune parameters associated with both innate and acquired immunity. Significant deficits in immunocompetence, however, have not been conclusively associated with these changes and the interpretation of these data is difficult. For further review of the effects of TCDD on human and non-human primate immunity, please refer to the recent reviews by Holsapple, et al. (1990) [Holsapple, et al., 1991a; Holsapple, et al., 1991c].

3. Immunotoxicity Studies of 2,3,7,8-TCDD in Animal Models:

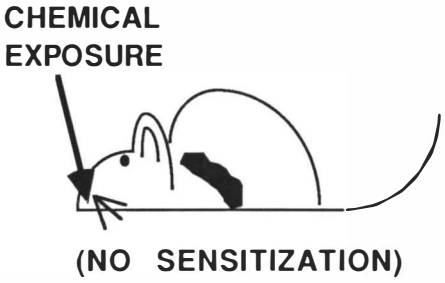
Immune dysfunction is an early and sensitive indicator of exposure to TCDD and is manifested to some extent in every species in which it has been measured. Figure 2 illustrates that the effects of a xenobiotic on immunocompetence can be approached in animal studies by three separate but related protocols which differ in regards to chemical exposure and antigen sensitization. Approach #1 is the most "real world" approach because both the chemical and the antigen are given to the animal. This is the approach most often employed in immunotoxicological studies. However, as described above, there are several ways in which these two components of a protocol can be integrated. The ultimate effects associated with Approach #1 are due to a combination of direct actions on immunocompetent cells and any of a number of indirect effects - for example, a change in an endocrine system of an animal which results in a change in the immune status. Approach #3 is an important mechanistic approach in which both the chemical and the antigen are added to cultured cells derived from naive (i.e., previously unexposed and unimmunized) animals. This approach is the most definitive way to assess the direct effects of a chemical on immunocompetent cells in the absence of other systems which can affect immune status in the whole animal. Approach #2 is another important mechanistic approach in which antigen is added to cultured cells derived from animals previously exposed to the chemical. This approach is an integral component of separation/reconstitution studies which are employed to identify the primary cellular targets affected by a given xenobiotic. The ultimate effects associated with Approach #2 would indicate that exposure to a chemical can precede exposure to the antigen in order to produce the defect. The results summarized in this review indicate that all three approaches have been used successfully to study TCDD, which is yet another reason why this xenobiotic is unique in immunotoxicology.

Figure 2. Mechanistic Approaches to Study Drug/Chemical-Induced Modulation of the Immune Response.

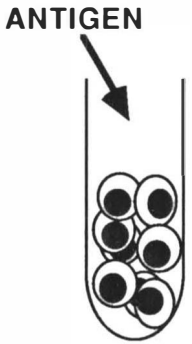
APPROACH #1
in vivo/in vivo



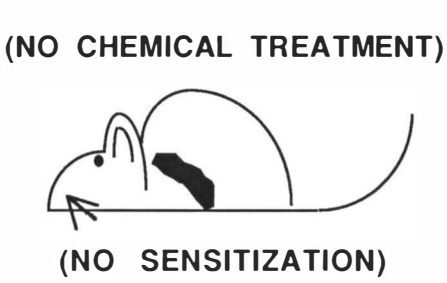
APPROACH #2
in vivo/in vitro



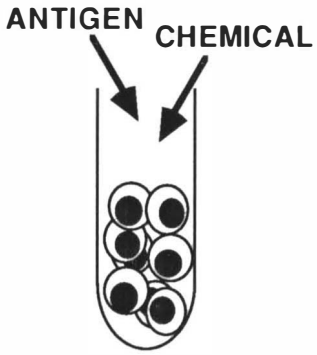
ISOLATE SPLEEN CELLS



APPROACH #3
in vitro/in vitro



ISOLATE SPLEEN CELLS



In this section, the effects of TCDD on various specific or selective immune parameters reflecting cell-mediated immunity, humoral immunity, myelotoxicity, lymphocyte development and homing, and innate immunity will be described. Effects on more holistic indicators of immunocompetence, as measured in host resistance models, will also be presented.

4. Effects of 2,3,7,8-TCDD on Cell-Mediated Immunity:

One of the first indications of immunotoxicity observed in an animal model was described by Buu-Hoi and co-workers (1972), in which exposure to TCDD caused a dramatic involution of the thymus [Buu-Hoi, et al., 1972]. This acute effect by dioxin was later confirmed by numerous investigators and was shown to occur in all animal species tested, including the rat, mouse, guinea pig, hamster, cow, chicken, and monkey [Harris, et al., 1972; Gupta, et al., 1973; Vos, et al., 1973; Vos and Moore, 1974a; Vos, et al., 1974b; Kociba, et al., 1976; Moore and Faith, 1976; Vos, 1977; Vos, et al., 1980; Clark, et al., 1981; Poland and Knutson, 1982]. This observation was extended by several laboratories to show that TCDD and other HAH could cause the involution of the spleen and, to a lesser degree, the lymph nodes of rats, mice, and guinea pigs [Harris, et al., 1972; Gupta, et al., 1973; Vos, et al., 1973]. Lymphoid involution was shown to be even more pronounced if the animals were exposed either peri- or postnatally, suggesting that TCDD could have a more selective effect on the developing immune system than on that of the adult animal [Vos and Moore, 1974a]. These effects were further shown to occur in the absence of any major pathological changes in the liver; although a pronounced wasting syndrome was observed.

In light of the dramatic effects on the thymus and other lymphoid organs, numerous investigators began to characterize the functional consequences associated with the TCDD-induced atrophy of lymphoid tissue by defining the acute and chronic effects on

the two divisions of acquired immunity. The earliest studies focused primarily on cell-mediated immunity, in which thymus-derived cells (i.e., T cells) play an integral role in tumor surveillance and the defense against infecting microorganisms. In addition to the severe thymic atrophy and lymphoid involution, mitogenic responses of splenic and thymic lymphocytes of young mice and rats to PHA and Con A [Vos and Moore, 1974a; Faith and Luster, 1979; Luster and Faith, 1979a; Luster, et al., 1979b], graft *vs* host reactions [Vos, et al., 1973; Vos and Moore, 1974a], delayed type hypersensitivity responses (DHR) [Moore and Faith, 1976; Faith, et al., 1978; Faith and Luster, 1979; Luster and Faith, 1979a; Luster, et al., 1979b], and prolongation of allograft rejection [Vos, et al., 1973; Vos and Moore, 1974a] have all been described as being decreased following exposure to TCDD; although the suppression of mitogenic responses has generally been the least consistently observed, and was the smallest in terms of magnitude. These effects have been further shown to exclude specific alterations in helper T cell function [Faith, et al., 1978], to be especially pronounced in either peri- or postnatally exposed animals (as previously noted) [Vos and Moore, 1974a; Faith, et al., 1978; Luster and Faith, 1979a; Luster, et al., 1979b] and to be prevalent for an extended period of time following initial exposure [Faith and Moore, 1977; Faith, et al., 1978; Luster and Faith, 1979a; Luster, et al., 1979b]. More recently, Lundberg, et al. (1990) [Lundberg, et al., 1990a] described a slightly different effect on thymocytes taken from 15-day-old mice (C57BL/6) which were pretreated with TCDD (50 μg/kg; i.p.) 4 days prior to sacrifice. When placed in culture, these cells demonstrated a heightened response to Con A stimulation which was characterized by an earlier onset of response and a higher maximal cell proliferation. Levels of IL-2 were coordinately reduced on day 2 of culture but were postulated to be due to an increased cellular IL-2 consumption. An increase in cells of the $CD8^+$ subpopulation was also observed on days 2, 3 and 4 of these cultures.

A structure-activity-relationship was revealed for the effects on the thymus in a comparison study between 9 week old C57Bl/6 male mice and 3 to 4 week old Hartley

male guinea pigs. In looking at the acute effects of a single oral dose of a series of PCDD, it was determined that the most potent congener was TCDD, causing an extensive atrophy of the thymus in both species [McConnell, et al., 1978; Vos, et al., 1980; Poland and Knutson, 1982]. It is interesting to note however, that whereas the C57Bl/6 mice showed a higher LD_{50} value for TCDD than the guinea pig, they displayed a greater sensitivity to the thymic involution, indicating the potential for this compound to produce a complex profile of toxicity. In this same report [McConnell, et al., 1978], it was determined that the thymic involution was the direct result of the loss of thymocytes from the cortical region of the thymus, thus confirming the earlier findings of Vos and Moore (1974) and that of Kociba and co-workers (1976), in which both peri- or postnatal exposure of rats and mice produced an effect on the thymus which resulted in an almost complete loss of the cortical-derived thymocytes [Vos and Moore, 1974a; Kociba, et al., 1976]. In addition, it was further recognized that these effects were not directly correlated with changes in peripheral lymphocyte and monocyte counts [Vos and Moore, 1974a]; although an earlier study by Zinkl, et al. (1973) [Zinkl, et al., 1973] had demonstrated that exposure to TCDD could result in a reduction in the numbers of peripheral lymphocytes in both guinea pigs and mice. No apparent effect was seen in the rat.

Extending these and other observations, it was later determined that the mechanism for the effect of TCDD on the cortical thymocytes was not due to a decrease in food intake [van-Logten, et al., 1980], to lymphocyte destruction [Vos, 1977], to stress-induced release of glucocorticoids [van-Logten, et al., 1980], to a reduction in growth hormone levels [van-Logten, et al., 1980], to an increase in serum alpha-fetoprotein levels [Vos, 1977], to decreased thymosin activity [Vos, 1977], to a reduction in serum zinc levels [Vos, et al., 1978], or to an increase in the release of pituitary hormones (as evidenced by studies in hypophysectomized animals which had in fact increased the involution produced by TCDD exposure) [van-Logten, et al., 1980]. In addition, Greenlee and co-workers have since demonstrated that the thymic atrophy is due primarily to a direct action on the

thymic epithelium [Greenlee, et al., 1985]. These investigators have reported that exposure to TCDD can cause a "push" in the thymic epithelial cell population toward terminal differentiation so that the necessary factors which are critical for lymphocyte maturation can no longer be produced by these cells. This finding is also consistent with a recent report by Kerkvliet and Brauner (1990) in which acute exposures to an immunosuppressive dose of TCDD (i.e., 0.74 μg/kg) in C57BL/6 mice caused significant reductions in both the percentage and absolute number of the most immature $CD4^+CD8^+$ double-positive cell population of the thymus [Kerkvliet and Brauner, 1990d]. The absolute numbers of $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were not significantly altered.

Perhaps the most frequently studied alteration in CMI induced by TCDD exposure, and possibly the most sensitive, is the specific reduction in the generation of cytolytic T lymphocytes (CTLs). Clark and co-workers have reported that TCDD at doses ranging from 0.004 to 40 μg/kg could significantly affect the generation of CTLs in C57Bl/6 mice, in the abscence of any significant alteration in cellularity of the spleen or lymph nodes or a suppression of the DHR (≥4 μg/kg) [Clark, et al., 1981; Clark, et al., 1984]. The antibody responses to SRBC and TNP-*Brucella abortus* (TNP-BA) were also reduced in a dose-related manner on days 3 (TNP-BA; 0.4 and 40 μg/kg) and 4 (SRBC; 0.4-40 μg/kg) after sensitization of the exposed animals. However, the lowest doses tested in these antibody responses (i.e., 0.4 and 4 μg/kg) did not appear to produce a consistent, or time-dependent, suppression of the antibody response to either antigen whereas the CTL response was shown to be consistently affected at doses as low as 0.04 μg/kg. It was therefore concluded that the CTL response is more sensitive to the effects of TCDD than the antibody response; although doses of TCDD below 0.4 μg/kg were not shown to have been tested for effects on the antibody response in either of these reports. It was further shown by limiting dilution analysis that TCDD did not deplete the number of CTL precursors but induced a population of suppressor cells which prevented the generation of

functional CTLs in vitro. T-helper cell function, as assessed by the ability to secrete IL-2, appeared normal [Clark, et al., 1983].

Several laboratories have further studied the role of the *Ah-gene* locus, and its associated receptor, in the suppression of the CTL response by TCDD. Using different strains of mice (C57Bl/6 and DBA/2) and bone marrow chimeras, Clark, et al. (1983) have demonstrated that the effects of TCDD and related PCDD on CTL generation segregates with the *Ah-gene* locus and the sensitivity to induction of ethoxyresorufin-o-deethylase (EROD) activity [Clark, et al., 1983; Nagarkatti, et al., 1984]. Additionally, it was found that 3,3',4,4'-TCB and Aroclor 1254, PCB congeners which have also been found to bind to the *Ah*-R, suppress the CTL response, whereas 2,2',4,4',6,6'-HCB, which has a much lower affinity for the *Ah*-R, did not [Clark, et al., 1983]. More recently, Kerkvliet, et al. [Kerkvliet, et al., 1990b] presented additional support for the specific role of the *Ah-gene* locus in the direct modulation of the effects of TCDD on the CTL response. They demonstrated that the potency of HxCB congeners - specifically, 3,4,5,3',4',5'-HxCB, 2,3,4,5,3',4'-HxCB and 2,4,5,2',4',5'-HxCB - and TCDD for suppression of the CTL response, as well as their effects on spleen cellularity, thymic atrophy and elevation of serum corticosterone levels, was directly correlated with the binding affinities of these congeners for the *Ah*-R. In addition, *Ah*-nonresponsive (Ah^{dd}) C57BL/6 congenic mice were significantly more resistant to alterations induced by either 3,4,5,3',4',5'-HxCB or TCDD when compared to *Ah*-responsive (Ah^{bb}) C57BL/6 congenic mice. Another recent study has further demonstrated that the TCDD-induced effects on Con A stimulated proliferation of splenic lymphocytes were more pronounced in the C57BL/6 mouse than in the DBA/2 mouse [Pavlyak, et al., 1989]. Phenobarbital did not modify the effects of TCDD, suggesting that suppression of these responses were mediated via specific binding to the *Ah*-R.

5. Effects of 2,3,7,8-TCDD on Humoral Immunity:

The other division of acquired immunity is humoral immunity and is defined as the production of specific antibody (Ab) to a specific antigen challenge. Because of the dramatic effect seen on the thymus and cell-mediated immunity as previously noted, the effects of TCDD on T-dependent humoral immune responses requiring T cell help, were first determined. Although some agreement in terms of deficits in cellular function are evident, understanding the specific mechanism involved in the suppression of humoral responsiveness has met with some discrepancies, especially with regards to effects in the whole animal versus effects in vitro, as related to differences in the biological activity of specific dioxin congeners and the genotypic differences between various mouse strains.

The first studies were centered around the effects of pre- or postnatal exposure to TCDD on humoral immune responsiveness. Thomas and Hinsdill (1979) found that young Swiss-Webster mice from mothers exposed to 1, 2.5, 5, 10 or 20 ppb TCDD in feed (from 4 weeks prior to conception to 3 weeks after birth) had significantly suppressed humoral responses [Thomas and Hinsdill, 1979]. Mice from mothers fed 2.5 and 5 ppb TCDD had atrophy of the thymus and a significantly reduced splenic plaque forming cell (PFC) response to sheep erythrocytes (SRBC). Serum anti-SRBC antibody titers and lymphocyte responses to Con A and LPS were not affected at these doses. Similar results were obtained in a comparison study between juvenile (4 week old) and adult (7 week old) female Swiss-Webster mice [Hinsdill, et al., 1980]. Animals exposed to $\geq$ 10 ppb TCDD in the feed had significantly suppressed primary and secondary humoral responses to both tetanus toxoid and SRBCs, as well as a decrease in circulating IgG levels and tetanus toxoid-induced IgE production in the exposed animal (i.e., at 10 and 100 ppb TCDD in feed). This effect was further noted to be dose-related and more extensive in the younger animals. The suppression of humoral PFC formation was further characterized as an effect on the production of all immunoglobulin classes including IgM, IgG, and IgA [Clark, et

al., 1981]. More recently, House, et al. (1990) demonstrated in B6C3F1 mice that a single exposure to TCDD caused a dose-related suppression (0.1-10 μg/kg) in the numbers of IgM and IgG PFC in response to SRBC [House, et al., 1990].

Vecchi and co-workers (1983) were the first to describe a differential sensitivity to the suppression of humoral antibody production in strains of mice which differ with respect to induction of aryl hydrocarbon hydroxylase (AHH) activity by TCDD [Vecchi, et al., 1983]. Using an acute exposure regimen and Approach #1 as described in Figure 2, they reported that the C57BL/6 and C3H/HeN mouse strains were highly susceptible strains to both the enzyme induction and immunosuppression, and that the DBA/2 and AKR strains were less sensitive, in that greater concentrations of TCDD were required to produce only moderate effects in these animals. Longer exposures (i.e., once per week for 8 weeks) did not increase the sensitivity of the DBA/2 strain and established a correlate to humoral toxicity with the degree of binding of TCDD to the cytosolic *Ah*-R. This observation was later extended to show that other inducers of AHH (3-MC and BNF) could cause an additive effect on the suppression of both humoral antibody production and responsiveness to Con A, but not PHA or LPS, when combined with TCDD [Vecchi, et al., 1986]. However, subchronic exposure (i.e., once per day for 14 consecutive days) of mice to 2,7-DCDD, which has no or weak binding capacity for the *Ah*-R, caused a suppression of <u>in vivo</u> antibody responses, which was comparable to that observed with TCDD [Holsapple, et al., 1986b]. More recently, we have compared acute and subchronic (i.e., again once per day for 14 consecutive days) exposures to identical total doses of TCDD in B6C3F1 mice and DBA/2 mice [Holsapple, et al., 1991b]. Following acute single exposure to TCDD at doses of 4.2, 14.0 and 42.0 μg/kg, we confirmed the results of earlier studies by demonstrating a greater suppression in the responsive strain. However, following subchronic exposure to doses of 0.3, 1.0 and 3.0 μg/kg/day for 14 consecutive days, there was at least a 10-fold shift in the sensitivity of the DBA/2 mice, which resulted in a comparable suppression of the antibody response in responsive and

non-responsive mice. Taken together, the available results indicate that the length - i.e., acute vs subchronic - and conditions - i.e., repeated weekly vs repeated daily - of exposure can affect the suppression of the antibody response by TCDD, and that repeated weekly exposures appears to produce the same profile as that observed after an acute single exposure. Moreover, these results may be indicative of a bifunctional effect of these compounds on the immune response as related to the length of exposure and the role played by the *Ah*-R.

Holsapple and co-workers [Holsapple, et al., 1984] were the first to report that any dioxin congener could suppress in vitro humoral immunity by demonstrating that direct addition of 1,2,3,6,7,8-HCDD suppressed several models of the antibody response (i.e., Approach #3 as described in Figure 2). Subsequent studies by Holsapple, et al. (1986) and Tucker, et al. (1986) suggested that the primary defect elicited by direct exposure to TCDD is the selective inhibition of B lymphocyte differentiation into antibody producing cells [Tucker, et al., 1986; Holsapple, et al., 1986a]. This conclusion was based primarily on the finding that the humoral response to both SRBCs (T-dependent antigen) and LPS (T-independent) were suppressed at doses which caused neither cellularity changes nor effects on mitogen-induced proliferation. Time of addition studies have further demonstrated that TCDD must be present early in culture (within 24 hours of a T-dependent response [Tucker, et al., 1986] and within 3 hours of an LPS response [Holsapple, et al., 1986a]) to suppress these PFC responses.

Studies comparing the direct effects of TCDD on various mouse strains using Approach #3 (Figure 2) and a limited number of *Ah*-R antagonists and dioxin congeners over a narrow concentration range, have also suggested that the suppression of the in vitro humoral immune response was correlated with binding to the *Ah*-R [Tucker, et al., 1986; Blank, et al., 1987]. However, these results are in contrast with the work of Holsapple, et al. (1986) [Holsapple, et al., 1986a] and more recently, Davis and Safe (1990) [Davis and Safe, 1990], which indicated that the direct suppression of the in vitro antibody response

did not segregate with the *Ah*-R. Both of these studies demonstrated the lack of segregation with the *Ah*-R by showing that congeners with weak binding affinity were suppressive, and by showing that comparable suppression could be obtained in both responsive and non-responsive mouse strains. Of particular importance to a consideration of the structure-activity relationship are the results from the earlier study which demonstrated the suppression by direct addition of 2,7-DCDD [Holsapple, et al., 1986a], the congener with weak binding affinity which was also shown to be effective in vivo when administered daily for 14 days [Holsapple, et al., 1986b]. Of particular importance to a consideration of the comparison between various mouse strains is a recent report from Nemoto, et al. (1990) which apparently represented the first comparative study of the P450 gene expression and AHH inducibility in adult primary hepatocytes from responsive and non-responsive mice [Nemoto, et al., 1990]. The results indicated that although AHH induction was genetically determined, appreciable activity could be induced in hepatocytes of non-responsive, as well as responsive mouse strains by treatment with TCDD and several polyaromatic hydrocarbons (i.e., including benz(a)anthracene, benzo(a)pyrene and 3-methylcholanthrene) following transfer of the cells to primary culture. Additional studies are required to more fully characterize the nature of the *Ah*-R in the suppression of the antibody response by TCDD and the basis for the non-*Ah*-R-dependent component. The available results suggest that the effects of direct addition of TCDD and related compounds to cultured cells may be similar to the profile of activity associated in vivo with repeated daily exposures to these chemicals in that both types of studies demonstrated a dissociation from an exclusive role by the *Ah*-R.

Utilizing Approach #2 (Figure 2), Dooley and Holsapple (1988) and Chastain and Pazdernik (1985) have provided convincing evidence that the B lymphocyte is the primary target for suppression of the T-dependent humoral immune response by demonstrating that the suppression of several humoral responses: the polyclonal response to LPS [Dooley and Holsapple, 1988], the T-independent response to TNP-LPS [Chastain-Jr. and

Pazdernik, 1985], the T-independent response to DNP-Ficoll [Dooley and Holsapple, 1988], and the T-dependent response to SRBCs [Dooley and Holsapple, 1988] are characterized by comparable dose-response-effect curves that are approximately parallel. Dooley and Holsapple subsequently used separation/reconstitution assay techniques (i.e., Approach #2; Figure 2) to show that the suppression of antibody responses by TCDD is the predominant result of a specific effect on the functional capabilities of the B cell and not to effects on the functionality of either the adherent macrophage population or the T cell [Dooley and Holsapple, 1988]. The absence of cellularity changes following acute exposures to TCDD has been recently confirmed by flow cytometry studies which showed that splenic Ig^+, Thy 1.2^+ and $CD4^+$ lymphocyte populations remain unaltered in either the absence or presence of an antigen challenge to the animal [Kerkvliet and Brauner, 1990d]. The lack of effect by TCDD on macrophage antigen-presenting cells has also been confirmed in a preliminary study by Kerkvliet, et al. (1990) [Kerkvliet and Brauner, 1990a]. Moreover, Chastain and Pazdernik (1985) compared the functional deficits of both bone marrow-derived (immature) or splenic-derived (mature) B cells to find that TCDD had a selectively greater effect on immature B cells than on the more mature B cells of the exposed animal [Chastain-Jr. and Pazdernik, 1985]. This finding, as in the case with immature T cells (i.e., thymocytes), further suggests that susceptibility of lymphocytes to the effects of TCDD is related to the early stages of development either within the bone marrow or thymus or as a consequence of antigen stimulation.

The most recent report by Dooley and co-workers [Dooley, et al., 1990] has also confirmed earlier reports that effects of TCDD on the T-dependent humoral immune response does not appear to be due to an alteration in helper T cell function [Faith, et al., 1978; Clark, et al., 1983]. These results were derived from experiments in which T-dependent antibody responses were measured using cultured T cells from TCDD-treated mice that were titrated into B cells from vehicle-treated controls, and on experiments which demonstrated the absence of an effect on IL-2 release following mitogen stimulation of

spleen cell suspensions and isolated T cells derived from TCDD-treated mice. Additional support for a lack of effect by in vivo exposure to TCDD on T-helper cell function comes from the results by House, et al. (1990) which demonstrated a comparable dose-related suppression of both the T-dependent antibody response to SRBC and the T-independent antibody response to DNP-Ficoll [House, et al., 1990]. The report by Dooley, et al. (1990) also demonstrated that the effects of TCDD on humoral responsiveness, specifically the effects on the B cell, were not mediated by the activation of a suppressor T cell [Dooley, et al., 1990]. Therefore, these results differ from the earlier report by Clark, et al. (1983) [Clark, et al., 1983] in which the suppression of the CTL response was attributed to the induction of a T-suppressor cell, and support the interpretation for a separation in the mechanisms for the functional suppression of these two cellular targets of dioxin toxicity.

Additional support for an effect on B cells comes from the results of Luster, et al. (1988) who used an in vitro model of B cell activation to assess the direct effects of TCDD on the proliferation and differentiation of isolated B lymphocytes [Luster, et al., 1988b]. In this report, TCDD caused the specific suppression of IgM secretion in B cells that were activated by anti-Ig and T cell replacing factor (TRF), but had no effect on proliferation; further suggesting a selective effect on B cell differentiation. This conclusion was further supported by the finding that neither the expression of Ia nor the proliferative marker, 7D4, were affected, whereas the expression of the plasma cell specific marker, PC.2, was significantly reduced. Collectively, it has been concluded by this and other laboratories, that TCDD causes an alteration in the cellular programming, either prior to or during antigen stimulation, which results in a specific alteration in the capacity of activated B cells to differentiate into antibody producing cells [Tucker, et al., 1986; Holsapple, et al., 1986a; Luster, et al., 1988b].

However, while several laboratories have ruled out that T-helper cells are targeted by TCDD [Faith, et al., 1978; Clark, et al., 1983; Dooley, et al., 1990; House, et al., 1990],

there are at least two reports that have indicated an effect on T cell regulation of immune responses. In the first report, Kerkvliet, et al. (1987) demonstrated that 1,2,3,4,6,7,8-HpCDD 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 in the response to SRBC [Kerkvliet and Brauner, 1987]. In addition, nu/nu mice were shown to be significantly more resistant to the immunosuppressive effects of HpCDD to DNP-Ficoll (at doses of ≤ 100 μg/kg) as compared with their nu/+ littermates, an observation which further indicates a primary immunological defect by this compound on regulatory T cells. In the second report, Kerkvliet, et al. (1990) [Kerkvliet, et al., 1990c] described an apparent biphasic dose-response curve for TCDD-induced suppression of the in vivo T-dependent antibody response in Ah^{dd} congenic C57BL/6 mice which was not seen in Ah^{bb} congenics, and have suggested that these data represent an *Ah*-independent component of the observed suppression. The authors suggest that the T-helper cell is the likely target for *Ah*-independent toxicity induced by low levels of TCDD because they observed linear, approximately parallel dose-response curves, showing an approximately 4-fold difference in sensitivity between Ah^{dd} and Ah^{bb} congenics for the suppression of the in vivo antibody response to TNP-LPS. However, an influential role by thymic-derived hormones in the suppression of humoral immune responses has not been ruled out by these studies. Thymic humoral factor(s) (THF) have been documented to modulate both T- and B cell responses both in vitro and in vivo [Trainin, et al., 1985; Kouttab, et al., 1988]. Moreover, Greenlee and co-workers [Greenlee, et al., 1985] have shown that the primary cellular targets responsible for thymic atrophy following TCDD exposure were the thymic epithelial cells and not the thymocytes, which were shown to lack sensitivity to the direct effects of TCDD.

In addition to demonstrating that exposure to TCDD has an effect on the antibody response to specific antigens, a number of laboratories have reported effects on serum

immunoglobulins. However, whereas the specific antibody responses were suppressed, in general, the results have indicated that exposure to TCDD elevates serum immunoglobulin levels. Sharma and Gehring (1979) have reported increases in total serum immunoglobulins at both two and four weeks following exposure of mice to doses of 0.01-1.0 μg/kg TCDD [Sharma and Gehring, 1979]; a significant decrease was noted at higher doses (i.e., 10 μg/kg). Sharma, et al. (1984) showed that a similar effect occurred in New Zealand rabbits - almost a 2-fold enhancement in total serum IgG levels at 8 weeks of exposure to 0.01 and 0.1 μg/kg/week; and a 50% decrease in total serum IgG levels at 8 weeks of exposure to 10.0 μg/kg/week [Sharma, et al., 1984]. A more recent paper by Moran and co-workers showed in rats that a single exposure to 30 μg/kg caused an increase in total serum IgA with no effect on total serum IgG [Moran, et al., 1986]. While they suggested that the increase in serum IgA levels in exposed rats was attributable to generalized TCDD-induced liver damage, the possible contribution by the ability of TCDD to cause an aberrant lymphocyte activation, as described below, cannot be discounted.

6. Myelotoxicity and Effects on Lymphocyte Development and Homing:

As previously mentioned, TCDD has been shown to alter the immunocompetence of rodents more effectively when administered during pre- or postnatal life, a time during the maturational development of the immune system. These results have suggested that TCDD could be acting to alter normal maturational processes involved in lymphocyte development and has led several investigators to determine if exposure can alter hematopoietic stem cell function or lymphocyte homing so as to indirectly affect the proper immunological development and subsequent immunocompetence of the animal.

One report [Luster, et al., 1980] has demonstrated that exposure to female C57Bl/6N mice (crossed with male C3H mice) on day 14 of gestation and days 1, 7 and 14 post

parturition at doses of 1, 5, and 15 μg/kg, resulted in severe alterations in fetal development of immunocompetence as measured in the 3-week old neonates. In addition to general toxicity changes (decreased body weight and thymic atrophy), maternal exposure to 15 μg/kg showed significant hematological changes which included decreased red blood cell counts, hematocrits, hemoglobin levels, and absolute numbers of lymphocytes. Platelets were also decreased at all exposure levels. In contrast, there was a slight increase in white blood cell counts and neutrophils and an absence of effect on the number of circulating monocytes. When bone marrow was examined, there was a decrease in cellularity which occurred at both 5 and 15 μg/kg, in which the latter was accompanied by a significant decrease in CFU-GM. Exposures to 1 and 5 μg/kg gave a small increase in the CFU-GM. In addition, exposure to TCDD reduced the proliferative capacity of pluripotent stem cells in the bone marrow at both 5 and 15 μg/kg, as measured by a decrease in CFU-S formation in these animals.

Of relevance to this latter finding are the results of the effects of TCDD on lymphocyte homing patterns following pre- or postnatal exposure in the rat [Faith and Luster, 1979]. When inbred Fisher rats were exposed to TCDD via maternal dosing (5 μg/kg) on gestational day 18 and on days 0, 7 and 14 postpartum, significant effects were detected in the homing patterns of splenic-, thymic-, and bone marrow-derived lymphocytes obtained from 45 day old rats. These effects were hallmarked by differences in the patterns of homing of exposed thymus cells to both the spleen and thymus of unexposed animals and of unexposed cells to the spleen or thymus of exposed animals.

Additional studies were initiated to determine if acute exposure of young adult mice (6 to 8 weeks old) could cause a similar pattern of myelotoxicity and alterations in stem cell function as that seen in the pre- and postnatally exposed animal [Luster, et al., 1985]. In addition, a comparison was made between B6C3F1 and DBA/2 mice to determine if differences existed in terms of their sensitivity to the myelotoxic effects of dioxin as related to differences in their susceptibility to the induction of aryl hydrocarbon hydroxylase

activity. Mice were exposed to a single dose of 0.2, 1, 2, 5 or 10 μg/kg TCDD and their bone marrow was removed and examined on day 5 for cellularity and stem cell alterations. In the B6C3F1 strain, bone marrow cellularity was significantly decreased only in the 10 μg/kg dosage group. Likewise, CFU-S (a measure of stem cell proliferative capacity) was only moderately decreased over this dose range. However, CFU-GM was decreased at doses of ≥ 1 μg/kg, a dose 5-fold below that producing thymic involution, and seemed to be a more sensitive indicator of the myelotoxic effects in these animals. Kinetic studies were performed using the 10 μg/kg acute dose to disclose that the effects on cellularity and CFU-GM had occurred within 24 hours after exposure. During a 10 day observation period however, cellularity changes had recovered within 96 hours of exposure whereas CFU-GM did not recover until day 10. In comparison, the DBA/2 strain remained insensitive to the effects on CFU-GM even at doses as high as 50 μg/kg. In addition, Chastain and Pazdernik [Chastain-Jr. and Pazdernik, 1985] have also demonstrated a significant reduction in the number of B lymphocyte colony forming units in culture (BL-CFU-C) derived from femoral bone marrow cells of TCDD exposed animals (acute doses of 30-120 μg/kg).

In vitro experimentation was utilized to further investigate the myelotoxic effects of dioxin as a function of the direct effects on progenitor cell function [Luster, et al., 1985]. Using a sensitive (B6C3F1) and a less-sensitive (DBA/2) strain of mouse, it was determined that CFU-GM and CFU-E colony formation was significantly suppressed in the bone marrow cells derived from the B6C3F1 strain, at concentrations as low as 5×10^{-10} M. Cells derived from the DBA/2 strain were only affected at the highest concentration tested, 10^{-7} M. In further characterizing the effects on the B6C3F1-derived cells, a significant effect on DNA synthesis was observed in cells stimulated with mouse lung conditioned medium in the presence of 10^{-9} to 10^{-7} M TCDD, thus exposing an effect on these progenitor cells which either selectively affects cells already in S phase or prevents them from entering S phase. It is important to note that these effects were determined to be

more a function of the direct effects of TCDD on the nonadherent progenitor cells than an effect on the feeder cells; although some suppression was acquired by exposure to feeder cells alone. In this same report [Luster, et al., 1985], these investigators further studied the relationship between the myelotoxicity and the *Ah*-R. Using B6D2F1/J mice, heterozygous at the *Ah-gene* locus (Ah^bAh^d), it was determined that the heterozygous responsive mice were more sensitive to the myelotoxic effects of TCDD (as measured by suppression of CFU-GM colony formation) than were the homozygous nonresponsive mice; suggesting that these effects segregate with the *Ah-gene* locus. This finding was further supported by the observation that congeners with low affinities for the *Ah*-R such as OCDD and 2,8-DCDD did not inhibit progenitor cell proliferation whereas those that bound with at least moderate affinities, 2,3,7,8-TCDF and 2,3,7-TCDD, directly suppressed this activity.

Fine, et al. (1988 and 1990) [Fine, et al., 1988; Fine, et al., 1990] have also examined the acute effects of TCDD on lymphocyte stem cell function in young adult BALB/c mice following either direct or perinatal exposures to determine if alterations in the early events of T cell lymphopoiesis can contribute to the TCDD-induced thymic atrophy. Following perinatal exposures to 10 μg/kg TCDD on gestational day (gd) 14, lymphocyte stem cell populations in the fetus and neonate were examined for alterations. In both the fetus and the neonates, significant reductions were found in both the biosynthesis and mRNA levels of the lymphocyte stem cell-specific DNA polymerase terminal deoxyribonucleotidal transferase (TdT). In contrast, thymic TdT biosynthesis appeared relatively unaffected at this dose; even though an extensive involution of the thymus was produced. A slight reduction in the Lyt-2$^+$L3T4$^+$ immature thymocyte population was also noted [Fine, et al., 1988]. In young BALB/c mice, direct exposures to doses of 5 to 120 μg/kg TCDD caused a dose-dependent decrease in thymic weight which closely correlated with a dose-dependent suppression in both the biosynthesis and mRNA levels of TdT in the bone marrow and thymus [Fine, et al., 1990]. The absolute number of thymocyte

subpopulations were only reduced at doses ≥ 30 μg/kg TCDD. The relative percentage of $CD4^+/CD8^+$ and IL-2 receptor expressing cells were also altered on a cell-to-cell basis and led to the conclusion that thymocytes are more refractory to the actions of TCDD than are pre-T cells. Additionally, prothymocytes were removed from the bone marrow of exposed animals and analyzed for their ability to replenish the thymus of irradiated recipients. This activity by prothymocytes was severely impaired by exposure and occurred at low tissue levels of TCDD. They found no change in the number of granulocyte/monocyte colony forming units (CFU-GM) and only moderate effects on the number of stem cell colony forming units (CFU-S) at doses which significantly affected prothymocyte function. These studies suggest that thymic atrophy induced by TCDD is partially due to a decreased seeding of the thymus by prothymocytes emigrating from the bone marrow.

d'Argy, et al. (1989) [d'Argy, et al., 1989] have described the use of fetal thymus organ cultures (day 14 of gestation) to characterize the effects of TCDD and a number of polycyclic hydrocarbons on lymphoid development. In this assay system, TCDD was shown to exhibit an EC_{50} of ~5 x 10^{-10}M for decreased thymocyte development whereas polycyclic hydrocarbons were shown to have relatively low toxicities (i.e., EC_{50} values of 10^{-5} to 10^{-4}M). It was further noted in this report that ^{3}H-thymidine incorporation into the thymuses of TCDD-treated mice was almost completely suppressed at 48 hours after exposure, suggesting that TCDD can impair cellular proliferation in the thymus. A similar report has been documented by Lundberg, et al. (1990) [Lundberg, et al., 1990a] in which 2 to 4-week old mice and fetal thymus cultures were exposed to TCDD (5, 25 and 50 μg/kg and 10^{-9}M, respectively) and used to monitor thymocyte subpopulations with respect to their differentiation antigens (i.e., CD4 and CD8). They found that cell numbers of all thymocyte populations, except CD8 single positive ($CD8^+$), were significantly decreased as compared to controls. Following in vivo exposure, the most marked decrease occurred in the $CD4^-/CD8^-$ (double negative) and the $CD4^+/CD8^+$ (double positive) populations [Lundberg, et al., 1990b]. In addition, proliferation in the thymus was

decreased at 24, 48 and 72 hours after exposure, but returned to near normal levels at 96 hours. Marked enhancements were seen on days 7 and 8. Following in vitro exposure, the $CD4^+/CD8^+$ population was the most severely affected (i.e., decreased), although cell numbers of all populations recovered from the fetal thymus cultures were decreased.

Nikolaidis, et al. (1990) [Nikolaidis, et al., 1990] have described similar effects of TCDD on the support of B cell development by the Bursa of Fabricious. Bursae of 10-day-old chick embryos were cultured in the presence of TCDD for 24 hours and were then transplanted into the chorioallantoic membrane of 10-day-old eggs of the same strain or of a strain expressing a different B cell surface alloantigen. After 5 days, the number of B cells were determined and the grafts were sectioned for immunocytochemistries. Lower numbers of lymphoid cells were observed in the TCDD-treated transplants as compared to controls, with a 40-50% decrease noted at 10^{-9}M. Bursal epithelium showed relatively normal development, even in cases of impaired B cell development. Because lymphoid cells recovered from grafted bursae originated from the embryo of the host egg, these findings suggest that TCDD and related congeners have a direct effect on the bursa which leads to an inhibition of lymphoid development. It was specifically concluded that the result of such actions by these compounds was an alteration in the microenvironment of the bursal tissue which resulted in a decrease in the attraction of stem cells and/or in the capacity of the affected tissue to induce proliferation of the colonizing cells. These findings are consistent with those of Greenlee and co-workers [Greenlee, et al., 1985], in relation to the effects of TCDD on thymic epithelial cells and the ultimate alterations in the development of mature T cells in the thymuses of exposed mice, as discussed in an earlier section of this review.

7. Effects of TCDD on Innate Immunity:

The results presented in the previous sections have indicated that exposure to TCDD can affect the antigen-driven processes associated with lymphocytes involved in acquired immunocompetence. There is a limited number of studies which have evaluated the effects of TCDD on components of innate immunocompetence. Using peritoneal macrophages, Mantovani, et al. (1980) [Mantovani, et al., 1980] reported that TCDD did not affect spontaneous macrophage-mediated cytotoxicity per unit number of effector cells or the endotoxin-stimulated increase in cytolytic and cytostatic activities. In the same report, they showed that TCDD also did not affect NK cell-mediated lysis of YAC-1 lymphoma cells when calculated based on the number of effector cells. However, because there were lower numbers of peritoneal macrophages and splenocytes recovered from exposed mice, the total number of lytic units recovered from animals exposed to TCDD was lower than controls, and they concluded that these changes were enough to contribute to a potential decrease in resistance. More recently, House, et al. (1990) reported no significant alterations by TCDD in splenic NK-cell activity; in the numbers of resident or elicited macrophages obtained from the peritoneal cavity; or in a number of assays reflecting the functional capabilities of thioglycollate-elicited macrophages including; cytostasis, cytolysis, phagocytosis, IL-1 production or H_2O_2 production [House, et al., 1990]. In contrast, Ackermann, et al. (1989) [Ackermann, et al., 1989] have reported that exposure to TCDD did reduce the cytolytic and cytostatic activity of activated polymorphonuclear neutrophils (PMNs) taken from the peritoneal cavity, but had no effect on the production of superoxide or hydrogen peroxide, or on degranulation. As these effects were greater in B6C3F1 mice than DBA/2 mice, the authors concluded that this action segregated with the *Ah*-R. Because examination of bone marrow stem cells revealed that granulocytic, but not monocytic colonies, were reduced after exposure, the authors speculated that TCDD may

affect PMNs at the level of hematopoiesis, an effect which is consistent with the myelotoxicity discussed in the preceding section.

Luster and co-workers have also investigated the effects of TCDD on the fixed cells associated with the reticuloendothelial system (RES). Rosenthal, et al. (1989) [Rosenthal, et al., 1989] demonstrated that the clearance of endotoxin by the liver was decreased by exposure to TCDD, an effect which could contribute to the TCDD-induced hypersensitivity to endotoxin reported by others [Vos, et al., 1978]. Taylor, et al. (1990) [Taylor, et al., 1990] have also speculated that an increase in the sensitivity to LPS may be due to an increase in the release of tumor-necrosis factor by Kupffer cells isolated from exposed rats. Therefore, exposure to TCDD seems to produce a complex profile of activity on fixed cells of the RES, with both a decrease in the uptake of bacterial fragments and an increase in the release of cytokines in response to bacterial stimuli.

Results by White and co-workers have demonstrated that exposure to TCDD and related PCDD suppressed the complement pathway indicating that soluble mediators associated with innate immunity are also targeted by the dioxins. White and Anderson (1985) [White and Anderson, 1985b] initially demonstrated that technical grade pentachlorophenol (PCP), but not pure PCP, suppressed the classical complement pathway, the spontaneous C1 autoactivation, the alternate pathway and the levels of complement component, C3. Confirmation that the PCDD's, which contaminated the PCP, principally 1,2,3,6,7,8-HCDD, were responsible for the actions on the complement system, was provided in a subsequent report [White, et al., 1986]. TCDD was included as a comparative control in these studies (i.e., TCDD is not a major contaminant of PCP). Recovery studies demonstrated that complement activity in mice treated with TCDD (1 μg/kg) and the high dose of 1,2,3,6,7,8-HCDD (10 μg/kg) was suppressed until 50 days post-treatment. Interestingly, treatment with the lower doses of 1,2,3,6,7,8-HCDD (0.1 and 1.0 μg/kg) produced an elevation in complement activity. In contrast with these results with complement, House, et al. (1990) have reported that exposure to TCDD had

no effect on a second type of soluble mediator, serum interferon, as triggered by the administration of poly I:C [House, et al., 1990].

The effects of TCDD on innate immunity, although limited, are important from a number of perspectives. First, they demonstrate that the effects on immunocompetence are complex and are not restricted to a given cell type. Second, they provide an important perspective for the discussion in the following section, in that innate immune processes are an important component of the repertoire of host resistance. Finally, the effects on innate immunity observed in animal studies have been corroborated in some human studies, as discussed below. This latter point is particularly true in regards to changes in the complement system.

8. Effects of TCDD on Host Resistance Models:

In response to studies which had demonstrated that exposure to TCDD could induce thymic atrophy and suppress cell-mediated immunity in laboratory animals, Thigpen, et al. (1975) investigated whether or not mice exposed to TCDD were more susceptible to *Salmonella bern* (*S. bern*) and *Herpesvirus suis*, or pseudorabies virus [Thigpen, et al., 1975]. Following exposures as low as 1 μg/kg administered once per week for 4 consecutive weeks beginning at 4 weeks of age, an exposure which did not produce any clinical or pathological evidence of toxicity, male C57Bl/6 mice infected with *S. bern* had a significantly higher rate of mortality and significantly shorter time to death when compared to control mice. In contrast to the findings with *S. bern*, TCDD exposure did not increase mortality following pseudorabies virus infection at any of the dose levels tested. Subsequent studies by Vos, et al. (1978) and Thomas and Hinsdill (1979) indicated that the increased susceptibility to *Salmonella* infection is likely due to the endotoxin content of the bacteria [Vos, et al., 1978; Thomas and Hinsdill, 1979]. Using an exposure regimen similar to that which was used by Thigpen and co-workers [Thigpen, et al., 1975], both

male Swiss mice [Vos, et al., 1978] and the offspring of female Swiss-Webster mice, fed TCDD-containing chow prior to mating, during gestation, and between partuition and weaning [Thomas and Hinsdill, 1979], demonstrated increased mortality following bacterial endotoxin (*E. coli* and *S. typhimurium* LPS, respectively) administration relative to controls. In neither case was resistance to *Listeria monocytogenes* (*L. monocytogenes*), a gram-positive bacteria lacking endotoxin, affected by TCDD exposure. Because the phagocytic activity of peritoneal macrophages was not affected, Vos, et al. (1978) [Vos, et al., 1978] concluded that a suppression of CMI was not mediated by a combined effect on T cells and macrophages. However, as noted by Thigpen, et al. (1975) [Thigpen, et al., 1975], studies which have dealt with circulating macrophages do not address the potential effects on tissue (i.e., fixed) macrophages. Moreover, as discussed in the preceding section, changes in cells of the RES induced by TCDD tend to at least partially explain the increase in endotoxin sensitivity observed in animal models (i.e., this point is further discussed below).

Support for the lack of effect on *L. monocytogenes* host resistance model by exposure to TCDD comes from the results of House, et al. (1990) [House, et al., 1990]. However, this study did demonstrate one of the most sensitive effects of exposure to TCDD on a host resistance model. Exposure to as little as a single injection of 0.1 μg/kg caused a significant increase in the mortality associated with challenge to a mouse-adapted influenza A/Taiwan/1/64 (H2N2) virus. Following viral infection, a cascade of immune responses occurs, including interferon production, enhanced macrophage and NK-cell activity, induction of cytotoxic T lymphocyte (CTL) activity and antibody production. As described above, in this same study, House, et al. (1990) showed that exposure to TCDD had no effect on interferon, NK cell activity or macrophage function. While they did not measure the effects of TCDD on CTL activity, they did show an effect on the antibody responses to both T-dependent and T-independent antigens, although the effects of the low dose (0.1 μg/kg) of TCDD were modest. Moreover, a dose of 10 μg/kg was required to

cause suppression of the specific antibody titers to inhibit viral hemagglutination. Further work is necessary to understand the basis for the sensitivity of this influenza model to TCDD.

In contrast to some of the earlier observations, Hinsdill, et al. (1980) [Hinsdill, et al., 1980] reported increased susceptibility to both *S. typhimurium* var. copenhagan and *L. monocytogenes* in outbred female Swiss Webster mice fed chow containing up to 50 ppb TCDD for 8 weeks prior to infection; although the data was quite variable. In addition to an increased mortality and shortened time to death, TCDD exposure was associated with an increase in the number of viable bacteria recovered from the blood. This effect was interpreted by the authors to suggest that TCDD compromises the ability of Kupffer cells to clear bacteria from the blood. As discussed above, a recent report by Rosenthal, et al. (1989) [Rosenthal, et al., 1989] confirms that the ability of these cells to clear endotoxin is reduced. Changes seen with *L. monocytogenes* were less pronounced than those observed following *S. typhimurium* infection. An increased susceptibility to *L. monocytogenes* in B6C3F1 progeny of C57Bl/6N mice administered TCDD by gavage on Day 14 of gestation and Days 1, 7, and 14 post-partum has also been reported by Luster, et al. (1980) [Luster, et al., 1980]. A dose-related increase in mortality was observed with statistically significant differences from control mice reported in mice from dams receiving 5 μg/kg TCDD. In this study, however, time to death was not affected by TCDD treatment. A modest increase in susceptibility to PYB6 tumor cells was also reported. The latter observation was a further indication that TCDD can compromise CMI.

As previously described in an earlier section, Clark and co-workers have argued through a number of research communications that TCDD-induced suppression of cell-mediated immunity is mediated through the induction of suppressor cells which target cytotoxic T cells (CTL). Since it has been proposed that CTL play a role in the resolution of certain viral infections, Clark, et al. (1983) [Clark, et al., 1983] evaluated the susceptibility of male C57Bl/6 mice to Herpes simplex type II strain 333 infection to test

for an in vivo effect on immunosuppression by low doses of TCDD. Treatment via gavage once per week for 4 weeks with 0.04, 0.4, or 4 μg/kg TCDD significantly increased mortality among infected mice. The increased mortality was greatest at the lowest dose tested, with the effect less pronounced and similar at both 0.4 and 4 μg/kg TCDD. Despite this lack of a clear dose-response relationship, the data suggest that susceptibility to certain viral infections can be increased by exposure to TCDD.

In contrast to the suppression of CMI most often associated with perinatal exposure to TCDD, exposure in adult rodents is characterized by the suppression of humoral immunity. Tucker, et al. (1986) [Tucker, et al., 1986] have reported that parasitemia following infection with *Plasmodium yoelii*, a non-lethal murine malarial parasite believed to be defended against by humoral immune responses, was greater and of longer duration in female BCF mice administered a single dose of 10 μg/kg TCDD by gavage as compared to vehicle-treated controls.

As discussed in the previous section, White, et al. (1986) [White, et al., 1986] have reported a suppression of serum complement and C3 levels in B6C3F1 mice following exposure to PCDD. Exposure to both TCDD (1 μg/kg by i.p. injection for 14 days) and 1,2,3,6,7,8-HCDD (10 μg/kg) were shown to significantly increase mortality following infection with *Streptococcus pneumoniae*. Host defense against this bacterium is believed to be mediated by complement activation, and thus requires functional C3.

Susceptibility to infectious agents, including bacteria, viruses, parasites and transplantable tumors, has been used as an integrative measure of the effect of TCDD exposure on the immunocompetence of laboratory animals in vivo. Collectively, these studies demonstrate that at least in mice: (1) exposure to TCDD can suppress host defense mechanisms leading to a greater susceptibility to mortality and morbidity following infection; (2) both innate and acquired (and both cell-mediated and humoral) immune functions can be compromised; and (3) there is a good correlation between alteration of

functional assays of individual components of the immune system and suppression of host resistance mediated by these components.

III. MATERIALS AND METHODS

A. Animals:

(C57BL/6 X C3H)F1 (B6C3F1) female mice were purchased through the National Cancer Institute (Frederick, MD). DBA/2 female mice were purchased from Charles River Laboratories (Raleigh, NC). Mice arrived at between 5 and 7 weeks of age and were housed 4/cage in a humidity and temperature regulated atmosphere with alternating 12 hour light/dark cycles. Food and water were available ad libitum. Particular care was exerted to guarantee that both strains of animal were age and weight matched at between 19-22 grams at the time of experimentation.

B. Chemicals:

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) that was used in in vivo experiments was obtained through the National Toxicology Program and Bio-Rad Laboratories (Rockville Centre, NY) and kept as a 100 μg/ml solution in corn oil (vehicle; Sigma). TCDD that was used in in vitro experiments was obtained from Chemsyn Science Laboratories (Lenexa, Kansas) and kept at 25°C as a 600 μM stock solution in 100% DMSO. The purity of the latter was assessed by Chemsyn laboratories using gas chromatography/mass spectrophotometry and was found to be ≥98%. No contamination by dibenzofurans was detected. Dimethylsulfoxide (DMSO), glucose-6-phosphate,

NADPH, NADH, glucose-6-phosphate dehydrogenase, dicumorol, bovine serum albumin (BSA), collagenase type I and trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxyresorufin and resorufin were obtained from Pierce Chemical Co. (Rockville, IL).

C. Culture Media and Reagents:

Media, media supplements, and balanced salt solutions were obtained from the following sources: RPMI 1640 medium without $NaHCO_3$, Waymouth's MB751/1 medium, EBSS without $NaHCO_3$, HBSS without $NaHCO_3$, $CaCl_2$, $MgCl_2.6H_2O$, and $MgSO_2.7H_2O$, and L-Glutamine (Gibco, Grand Island, N.Y.); KC 2000 Serum-independent medium (Dulbecco's Modified Iscove's MEM), Penicillin-Streptomycin-Fungizone solution, and MEM Spinners without L-Gln (Hazelton Research Products, Inc., Denver, PA); 2-Mercaptoethanol (Bio-Rad Laboratories, Richmond, CA); Nutrient Mixture F-12 (HAM) with HEPES, without L-Gln, without $NaHCO_3$ (Sigma Chemical Co., St. Louis, MO); Fetal Bovine Serum (FBS; Hyclone Laboratories, Inc., Logan, Utah); New Born Calf Serum (NBCS) and Fetal Equine Serum (FES; Flow Laboratories, McLean, VA). Complete RPMI media was prepared as a 1X solution by the addition of 2 mM L-Gln, 1% (v/v) Penicillin-Streptomycin-Fungizone solution, 5×10^{-5} M 2-Mercaptoethanol, and the appropriate concentration of serum (5 or 10%; see results) to the RPMI stock solution. KC 2000 serum-independent media was prepared as directed, except for the addition of Nutrient Mixture F-12 (HAM) (Sigma) to a final concentration of 0.5% (w/v). Vitrogen 100 was purchase from Collagen Corporation (Palo Alto, CA).

D. Antibodies and Immunoreagents:

FITC-conjugated rat anti-mouse Thy 1.2 and goat anti-mouse Ig, L3T4 and Lyt-2 were purchased from Becton-Dickinson (San Jose, CA). FITC-conjugated hamster anti-mouse CD3-epsilon was purchased from Boehringer-Mannheim Biochemicals. HO-13-4 mouse anti-mouse Thy 1.2 culture supernatant was produced in our own laboratory. The HO-13-4 hybridoma was obtained through the American Tissue Culture Bank. Goat anti-mouse IgM $F(ab')_2$, mouse IgM, and peroxidase-conjugated goat anti-mouse IgA, G, and M were purchased from Pel-Freez (Rogers, AR), Sigma (St. Louis, MO), and Organon Teknika/Cappel Laboratories (Malvern, PA), respectively. Recombinant mouse interleukin-2, interleukin-4 and gamma-interferon were purchased from Genzyme Corporation (Boston, MA). Staphylococcus aureus Cowan strain I (SAC; Boehringer-Mannheim Biochemicals, Indianapolis, IN) was obtained and frozen as a 10% solution.

E. Chemical Treatments:

For in vivo dosing the following regimens were used: In acute single exposure studies, animals were administered either corn oil or 4.2, 14, or 42 µg/kg TCDD via oral gavage on day 0 of the study and were sacrificed either on day 2 or day 15 of the study. In acute 5-day regimens, corn oil (vehicle) and TCDD (1, 10 or 30 µg/kg) were administered daily for 5 consecutive days to give cumulative doses of 5, 50 and 150 µg/kg. Two days rest were allowed prior to experimentation or sensitization during the acute 5 day regimens. For subchronic (14 day) exposures, animals were administered either corn oil or 0.3, 1.0, or 3.0 µg/kg TCDD/day via oral gavage for 14 consecutive days (cumulative doses of 4.2, 14, and 42 µg/kg). In subchronic studies, organ necropsies and antibody responses were performed 24 hours after the last chemical treatment. Figure 3 illustrates the scheduling for

the dosing regimens used in these experiments, including the various times at which the animals were dosed, sensitized and sacrificed.

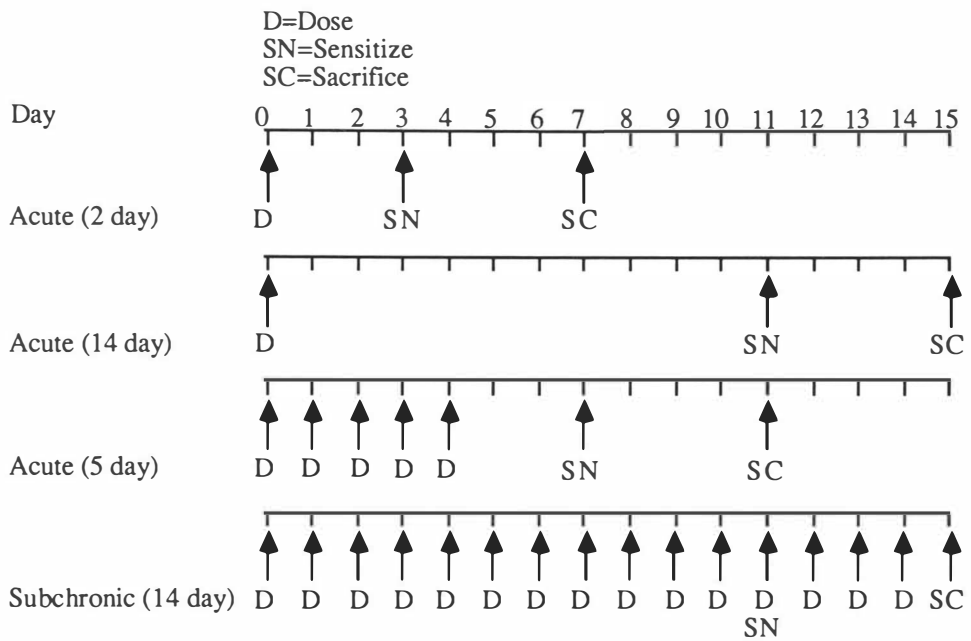


Figure 3. Dosing Regimens for <u>In Vivo</u> TCDD Administration.

F. Splenocyte Preparation:

Animals were sacrificed by cervical dislocation. Spleens were aseptically removed, placed in a 3X volume of sterile EBSS or RPMI media, mashed into single cell suspensions, and washed once to remove residual cell debris. For all <u>in vitro</u> T-dependent assays, cells were resuspended in RPMI complete media before addition to cultures. For B cell and T cell isolations, cells were resuspended in 1 ml of HBSS (5% FBS)/spleen.

Red blood cells were removed by hypertonic lysis using 3 mls of Gey's working solution/spleen and incubating on ice for 5 minutes with occasional mixing. Gey's working solution was prepared as previously described [Mishell, et al., 1980]. Splenocytes (minus red blood cells) were then adjusted to 1 X 10^7 cells/ml in HBSS (5% FBS) before removal of T cells (B cell isolation procedure) or separated on percoll density step gradients (T cell isolation procedure) as described below.

G. Necropsies and Antibody Forming Cell Responses:

Animals were sensitized to the T-dependent antigen, sheep red blood cell (SRBC), by administration of 5 X 10^8 SRBC, via i.p. injection in 1 ml of saline, on either day 8 for acute (5-day) or on day 11 for both acute (14-day) and subchronic (14-day) exposed animals. On days 2 (acute 2 day), 12 (acute 5-day) or 15 (acute 14-day and subchronic 14-day), animals were sacrificed, body and organ weights were recorded, and anti-SRBC antibody forming cell (AFC) responses were determined as previously described [Holsapple, et al., 1986b]. We have historically observed that this antibody response peaks 4 days after sensitization. In studies comparing the effects of TCDD on the antibody response following acute (14 days rest) and subchronic (14 day repetitive dosing) exposures, background IgM AFC from exposed, but unsensitized, spleens were also determined. Refer to Figure 3 which illustrates the scheduling for the dosing regimens used in these experiments, including the various times at which the animals were dosed, sensitized and sacrificed. Background IgM AFC were enumerated by the reverse plaque assay as described by Wood, et al. (1991) [Wood, et al., 1991] using a modified version of a previously described method [VanOudenaren, et al., 1981] and were done in direct collaboration with Dr. Steve Wood. Briefly, cells were resuspended in HBSS and added to 96 microtiter plates. Rabbit anti-mouse IgM (Accurate Chemicals, Westbury, NY) and detoxified (i.e., removal of immunoglobulin) guinea pig complement were then added and

the mixture pipetted into Cunningham chambers. The chambers were then incubated for 2 hours at 37°C (5% CO_2) and the plaques enumerated.

H. In Vitro Assay of the T-Dependent Antibody Response:

Assays using either bovine serum supplemented RPMI media or KC2000 serum-independent media were carried out as previously described [Dooley and Holsapple, 1988] except for minor modifications. SRBCs (Colorado Serum Co., Denver, CO) were added directly to splenocyte suspensions, at a final concentration of 1.3×10^7 cells/ml, prior to addition of cells to 48 well flat bottom culture plates (Costar, Cambridge, MA). Vehicles, TCDD (in 0.5% DMSO) and/or SAC (diluted in RPMI medium) were added to each well (10 µl/well) to attain the appropriate final concentrations. Cultures were incubated for 5 days under the same conditions as previously described. When serum supplemented media was used, cultures were fed on day 1 with 30 µl of a feed mixture prepared as follows: 0.64% dextrose (w/v), 6.41% essential amino acids (v/v; Gibco), 3.21% non-essential amino acids (v/v; Gibco), 3.21% L-Gln (v/v), 44.9% MEM Spinners (v/v; Hazelton), 9.62% $NaHCO_3$ (v/v), 0.64% Penicillin-Streptomycin-fungizone solution (v/v), and 32.05% FBS or NBCS (prepared individually for each lot of serum used). For serum-independent media and mouse serum supplements, cultures were fed as above except that a 1% BSA solution in KC 2000 was used in place of the designated serum supplement. For assays comparing bovine and normal mouse serum supplemented RPMI media, the following modifications were made. Cells were adjusted to 1.1×10^7/ml in RPMI complete media (including L-Gln, Penicillin-Streptomycin-Fungizone solution and 2-mercaptoethanol) without serum. SRBC were added to the splenocyte suspension and then added to appropriate wells in 48 well culture plates. Bovine sera and/or normal mouse serum were then added directly to the wells to achieve the appropriate concentrations (i.e., 5% normal mouse serum was used in these studies unless otherwise

noted). All other culture conditions were the same as described above. Cells were removed from culture on day 5 and plaqued against SRBCs to detect the number of antibody forming cells (AFC)/10^6 recovered cells by a modified version of the Jerne hemolytic plaque assay [Dooley and Holsapple, 1988].

I. Blood Collection and Serum Preparation:

Blood was collected from anesthetized mice via cardiac puncture, immediately placed into 12 X 75 mm glass tubes and allowed to clot at room temperature. Tubes were centrifuged at 1000 X g for 30 minutes and serum was collected. Pooled sera were then centrifuged again at 1000 X g for 10 minutes to ensure that all red cells were removed. Serum was then sterilized by centrifugation through 0.2 μm microfuge filters (Costar) and stored at -70°C. Prior to use in in vitro cultures, sera were heated at 56°C for 30 minutes to deactivate endogenous complement. Protein determinations were carried out by the Bio-Rad protein assay method (Bio-Rad Laboratories, Rockville Centre, NY) using BSA as the protein control.

J. B Cell and T Cell Isolations:

In the present studies, two different methods of isolation were employed to isolate highly purified B and T cells of different stages of in vivo activation. In both methods, three major subpopulations of cells (resting Go, cycling G1 and differentiated B and T cells) were isolated on percoll density step gradients using a modification of previously established methods [DeFranco, et al., 1982; Layton, et al., 1985; Monroe, 1988]. To obtain purified B cell subpopulations, as opposed to T cells, methods used to isolate resting and cycling B cells depended on whether adherence to plastic was used to remove residual macrophages/monocytes from the splenocyte preparation prior to the isolation

procedure. For the isolation of both T cell subpopulations, adherence to plastic was used in all experiments. For the purification of cycling B cells, freshly prepared splenocytes were resuspended in RPMI complete media containing 5% NBCS at 1 X 10^7 cells/ml and plated on plastic petri dishes (10 ml/plate) for 10 hours prior to T cell removal and percoll separation. Isolation of resting B cells did not require adherence since no macrophages/monocytes were detected at this density; although a small percentage of contaminating PMNs were detected at this density on occasion (refer to results and Table 5 for details). In addition, adherence resulted in significant losses in this population of cells and was therefore counterproductive in the isolation procedure to purify this population of cells. For the isolation of both populations (resting and cycling), splenocytes were collected, treated to remove autologous red blood cells (i.e., hypertonic lysis with Gey's solution; see above) and incubated (37°C for 1 hour) with a 1:50 dilution of HO-13-4 mouse anti-mouse Thy-1.2 supernatant plus a 1:100 dilution of DNase I (Sigma) and a 1:10 dilution of Low-Tox rabbit complement (Accurate Chemical Co., Westbury, NY) to remove viable T cells. Various densities of percoll (1.10, 1.092, 1.079, and 1.070 g/ml; Pharmacia, Piscataway, NJ) were isoosmotically prepared and adjusted to pH 7.2. Two mls of each of the first 3 densities of percoll were then gently layered into pre-chilled Falcon 16 ml round bottom tubes on ice (Thomas Scientific, Swedesboro, NJ). Cells were washed once in HBSS without FBS and reconstituted in 2 mls of the 1.070 density percoll (2 mls/2 spleens) and layered on top of the 1.079 density percoll. Gradients were capped with 2 mls of HBSS (d=1.058 g/ml) and centrifuged at 500 X g for 30 minutes (4°C) without braking. Cells layering at the interface of the 1.092/1.079 densities have been characterized as resting (G_0) B cells (as indicated by both previously established methods and by FACScan, Wright-Giemsa stained cytospin preparations and Cell Cycle analysis in this laboratory). Those cells layering at the interface of the 1.079/1.070 densities contain B cells in various stages of activation and differentiation (cycling; predominantly G1 cells but containing a majority of the total S and G2/M cells). Cells at

the interface of 1.070/1.058 (HBSS) contain residual macrophages/monocytes, differentiated B cells, and dead cell debris (refer to results and Figures 4 and 5 and Tables 6 and 7 for details). A reference gradient, using density marker beads (Pharmacia) in place of cells, was prepared and centrifuged under identical conditions to estimate the densities of recovered cell populations. Immunofluorescence FACScan analysis for the presence of B cells and T cells in the various cell populations revealed resting and cycling B cell purities of 95-98% and 97-99%, respectively, as determined by specific staining with goat-anti-mouse Ig (refer to results and Tables 4 and 5 for details). To obtain the two major subpopulations of T cells (resting and cycling cells) a modified version of the B cell isolation procedure (described above) was employed using percoll density centrifugation combined with an immunomagnetic negative selection procedure as described by Ladics, et al. (1991) [Ladics, et al., 1991a]. It should be noted that the method for isolation of purified T cells in various states of in vivo activation were worked out in direct collaboration with Dr. Greg Ladics. Briefly, splenocytes were prepared and plated for 10 hours as described above and treated to remove red blood cells. Cells were then washed and immediately separated by centrifugation on percoll density step gradients at the same densities used in the B cell isolation procedure (i.e., 1.10, 1.092, 1.079 and 1.07 g/ml). Cells banding at the interfaces of 1.092/1.079 (resting Go lymphocytes) and 1.079/1.07 (cycling G1 lymphocytes) were collected (see results and Figure 5), washed and adjusted to 2 X 10^7 cells/ml in RPMI media containing 5% FBS and 5 mM EDTA. FITC-conjugated goat-anti-mouse Ig, B220 (CD45), and granulocyte antibodies were added to the cell suspensions (6 µg antibody/ml of cells) and incubated at 4°C for 1 hour. Cells were then washed to remove excess antibody and incubated with the magnetic beads (with anti-FITC antibodies bound to the surface; Advanced Magnetics, Cambridge, MA) at a bead:cell ratio of 100:1 for 45 minutes at 4°C in round bottom tubes. Tubes were then placed in a magnetic particle concentrator (Dynal) to remove B cells from the splenocyte suspensions. Resulting T cells were analyzed by flow cytometry and Wright-Giemsa

stained cytospin preparations (as described for isolated B cells above) with routine purities of ≥95% (refer to results and Tables 4 and 5 for details). Cell populations were removed from the gradients by aspiration (B cells) or from tubes following removal of magnetic beads (T cells), washed in RPMI stock media (without FBS), and analyzed (cell number, viabilities, etc.) or used for further experimentation. B and T cell yields were determined as the percentage of total lymphocytes recovered from the gradients for each B cell subpopulation. Recoveries were determined as the number of B and T cells recovered from each band as a percentage of the total B cells at the beginning of the isolation as determined by fluorescence FACScan analysis (see results and Figures 2.2 and Table 3 for details).

K. Immunofluorescence Staining and Flow Cytometry:

Whole splenocytes and purified lymphocyte populations (recovered from Percoll density gradients) were washed 4 times in HBSS and 2 times in phosphate azide buffer and added to 96-well U bottom microtiter plates (Costar); 1 X 10^6 cells/well. Phosphate azide buffer was prepared by adding 1% BSA and 0.1% sodium azide to a 1X PBS stock solution. Cells were incubated for 20 min. with FITC-conjugated monoclonal antibodies (5 μg/10^6 cells) to either T cell or B cell markers and analyzed by flow cytometry to determine the percentage of T cells or B cells present in each cell preparation. Viable cells were detected by prior incubation of all preparations with propidium iodide (5 μg/ml; Sigma). All flow cytometric analyses of splenocytes and enriched lymphocyte populations were carried out with the help of Dr. Malvin L. Stern (Department of Pharmacology and Toxicology, MCV/VCU, Richmond, VA) using a Becton-Dickinson FACScan™ Flow Cytometer; equipped with a Hewlett Packard HP 9000 series computer with applications software allowing for single and multiparameter analysis of the data.

L. Cell Cycle Analysis:

Cell cycle analyses of unseparated splenocytes and purified lymphocytes were carried out by the method of Nusse, et al. (1990) [Nusse, et al., 1990]. Briefly, cell nuclei were prepared and stained by incubating 2 X 10^6 cells for 30 minutes in a 0.0584% (w/v) Na-citrate/0.1% (w/v) NaCl solution containing 0.03% Nonidet P40, 10 µg/ml RNase and 25 µg/ml ethidium bromide. After 30 minutes, a second solution was added containing 1.5% citric acid, 0.25 M sucrose and 40 µg/ml ethidium bromide. Cells were then refrigerated at 4°C until analyzed. Isolated nuclei were washed and analyzed by flow cytometry as described by Nusse, et al. (1990). Where indicated, the cell cycle distribution of some cell populations were further analyzed using fixed whole cell preparations combined with the use of CellFit Cell Cycle analysis software (Becton-Dickinson) and flow cytometry. For fixed whole cell analyses, 5 X 10^6 cells were washed and fixed on ice in 95% ethanol. Fixed cells were then washed in PBS containing 38 mM sodium citrate and 1.25 mg/ml RNase and stained with 10 µg/ml propidium iodide. In both procedures, cells in the 3 major stages of cell cycle (i.e., Go/G1, S and G2/M) were determined by DNA content and expressed as a percentage of the total fluorescing cells.

M. Lymphocyte Proliferation Assays:

For direct addition studies in the absence of mitogens (i.e., LPS or Con A), splenic-derived dense resting (Go) and cycling (G1) B cells and T cells from naive B6C3F1 mice were isolated as described above and adjusted to the proper cell density in RPMI complete media containing either FBS 717 or NBCS at various concentrations as described in the results. These serum lots were shown to either not support or support, respectively, a suppression of the T-dependent antibody response by TCDD (see results). Cells were added to 96 well flat bottom plates (Costar) (1 X 10^5 cells in 150 µl) and incubated in the

presence of vehicle (0.01% DMSO) or increasing concentrations of TCDD (0.3, 3, 10, 30 and 60 nM depending on the study) under the same atmospheric conditions as previously described for in vitro antibody responses to SRBC. For experiments involving in vivo treatment and in vitro proliferation, female B6C3F1 mice were treated with either corn oil (vehicle) or TCDD (1 μg/kg/day) for 5 days and their splenic-derived dense B cells isolated and adjusted and incubated as described above for in vitro exposures. Proliferation was assessed at various time points after treatment (i.e., 24, 48, 72, and 96 hours depending on the study - see results) as ^{3}H-TdR incorporation (addition of 1 μCi/well) following a 16 hour pulse, except for B cell proliferations that were measured following in vivo exposures or LPS stimulation, in which only a single measurement was taken at 48 or 72 hours, respectively. For direct addition studies involving mitogens, lipopolysaccharide (LPS; *S. Typhosa*) and Concanavalin A (Con A) were added at the beginning of the culture period (i.e., simultaneous with vehicle or TCDD treatments) at final concentrations of 10 and 5 μg/ml, respectively. Cells were harvested and the amount of ^{3}H-TdR incorporation was determined using liquid scintillation counting as previously described [Dooley and Holsapple, 1988].

N. IgM and IgG ELISAs:

96 well flat bottom microtiter plates (Dynatech, Chantily, VA) were coated overnight with goat anti-mouse IgM F(ab')$_2$ or goat anti-mouse IgG (Fc specific) primary antibodies (Pel-Freez and Jackson Laboratories) at 1 μg/ml and 0.5 μg/ml, respectively, and incubated overnight at 4^{o}C in PBS. Plates were washed once with PBS containing 0.05% Tween-20 (v/v; Sigma) and coated with blocking buffer (PBS+Tween-20+5% powdered milk) for 2 hours at room temperature to reduce nonspecific Ig binding. Mouse IgM or IgG standards were prepared in blocking buffer and standards or 7 day culture supernatants (10-75 μl) were added to the appropriate wells. Following a 2 hour

incubation, plates were washed and incubated in the presence of peroxidase-conjugated goat anti-mouse IgM, G, and A (Cappel) for 1 hour at room temperature. Plates were washed and the amount of IgM or IgG present in the standards and culture supernatants was determined by the addition of peroxidase substrate (Bio-Rad) and spectrophotometric detection of absorbance changes at 414 nm using a Bio-Rad model 2550 EIA plate reader. Data was calculated using linear regression analysis of the samples as based on the semi-log plot of log μg standard/well versus absorbance.

O. Preparation of Liver and Splenic Microsomes:

The studies to assess enzyme induction were done in direct collaboration with Mr. Hye Gwang Jeong, a visiting student scientist from the Korea Advanced Institute of Science and Technology (KAIST), Seoul, Korea. Livers and spleens were removed from both B6C3F1 and DBA/2 mice following either acute or subchronic exposures to TCDD, and placed in ice cold 0.15 M KCl. Livers were rinsed to remove excess blood and homogenized in 3 ml of KCl using a teflon glass homogenizer. Spleens were smashed into single cell suspensions, washed, and then homogenized, with the splenic capsule intact, in 1 ml KCl by sonication using a Vibra Cell sonic cell disrupter (Sonics and Materials Inc., Danbury, CT). S-9 homogenates were prepared as previously described [Johnson, et al., 1987] by centrifugation of an aliquot of each liver supernatant and spleen cell supernatants at 9000 X g for 10 minutes. Microsomes were then prepared from both liver and spleen S-9 fractions by differential centrifugation as previously described [Yang, et al., 1977]. S-9 fractions were centrifuged at 105,000 X g for 60 minutes under vacuum in an ultracentrifuge. Pellets were rinsed once with 0.15 M KCl and 10 mM EDTA (pH 7.4), resuspended in 0.25 M sucrose and stored at -70°C until assayed (see below).

P. Isolation and Culture of Primary Hepatocytes:

Primary hepatocytes were were isolated using the collagenase perfusion technique of Klaunig, et al. (1981) [Klaunig, et al., 1981] with minor modifications as previously described [Yang, et al., 1986; Johnson, et al., 1987; Kim, et al., 1987]. Briefly, the liver was perfused with 100 ml of Ca^{++}- and Mg^{++}-free HBSS containing 100 U/ml collagenase and 100 U/ml trypsin inhibitor. Cells were centrifuged at 50 X g for 5 minutes, resuspended in Waymouth's AB stock media (without hormone supplements), and checked for viabilities using trypan blue exclusion. Viabilities were routinely ≥80%. Cells were adjusted to 0.5 X 10^5 cells/ml and added (3 mls/well; 1.5 X 10^5 cells) to Vitrogen 100 pre-coated 6 well culture plates (Costar, Cambridge, MA). Plates were incubated at 37°C (humidified atmosphere with 5% CO_2) for 3 hours to allow for adherence of viable cells to the plates. Cells were then washed twice in warm stock AB media before adding 2 mls of AB media (without hormones and supplemented with L-Gln and penicillin-streptomycin) to each well. BSA or serum was added directly to appropriate wells at a final protein concentration of 5 mg/ml, and swirled to mix. Vehicle (0.01% DMSO) or TCDD were then added (10 µl/well) and the plates were incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO_2. At 24 hours, media containing unattached cells was removed and fresh AB media (with antibiotics), serum and TCDD (or vehicle) were added. At the end of the 48 hour culture period, cell monolayers were collected by first rinsing each well twice with ice cold PBS (pH 7.2). Two mls of PBS were then added and the cells collected by scraping the cultures with a rubber policeman. Cell suspensions were centrifuged at 50 X g for 5 minutes, resuspended in 1 ml 0.1 M $KP0_4^-$ buffer (pH 7.4), and stored at -70°C until assayed for enzyme activity. On the day of the enzyme assay, cells were thawed and homogenates prepared by sonication using a Vibra Cell sonic cell disrupter (Sonics and Materials Inc., Danbury, CT).

Q. Measurement of Ethoxyresorufin-O-deethylase (EROD) Activity in Microsomes and Cell Homogenates:

Total 7-Ethoxyresorufin-O-deethylase (EROD) activity in microsome aliquots and cell homogenates was measured by the method of Blank, et al. (1987) [Blank, et al., 1987] with minor modifications. The buffer used in the assay consisted of 0.1 M $KPO4^-$ (pH 7.4) containing 2 mg/ml BSA, 10 μM dicumorol, 5 mM glucose-6-phosphate, 20 U/ml of glucose-6-phosphate dehydrogenase and 0.5 μM NADPH. 2.5 μM ethoxy resorufin was added and the formation of resorufin was measured spectrofluorometrically (Perkin and Elmer LS 50) with excitation and emission wavelengths of 550 and 585 nm, respectively. Protein determinations were carried out by the Bio-Rad protein assay method using BSA as the protein control (Bio-Rad Laboratories, Rockville Centre, NY).

R. Statistical Analysis:

All results were evaluated using a one-way analysis of variance for vehicle and TCDD-treated responses. When significant treatment effects were observed, a Dunnett's *T*-test was used to determine which groups were significantly different from the controls.

IV. RESULTS

A. Isolation and Characterization of Dense Resting (Go/G1) and Cycling (G1, S, and G2/M) B and T Cells for In Vitro Immunotoxicity Testing:

1. Isolation and Analysis of Purities.

As opposed to the isolation of T cells, B cells can be efficiently isolated by a method involving antibody-mediated complement lysis of T cells, coupled with the separation of the remaining cells over percoll density step gradients [DeFranco, et al., 1982; Layton, et al., 1985; Monroe, 1988]. Conversely, a method to isolate highly enriched T cells was developed which involves the separation of whole splenocytes (minus autologous red blood cells) by percoll density gradient centrifugation coupled to immunomagnetic negative selection using FITC-conjugated antibodies, directed against cell surface antigens specific for either B or T cells, and anti-FITC antibodies, bound to magnetic beads, and subsequent removal of the unwanted cell population; as described by Ladics, et al. (1991) [Ladics, et al., 1991a]. These two different, but related, procedures yield two populations of B and T cells that have been characterized as dense resting (Go/G1) and cycling cells (as described by Layton, et al. for the isolation of B cells [Layton, et al., 1985]), based primarily on their size and DNA:cytoplasmic ratios (i.e., cell density). In addition, to obtain highly enriched populations of the two B cell subtypes for the use in functional

testing, it was found that slightly different methods of splenocyte preparation had to be used. For the isolation of dense resting B cells, lysis of T cells from freshly prepared splenocytes and separation on percoll density step gradients (see Figures 4 and 5), yielded a population of cells which separated at the interface of 1.079/1.092 percoll (band 3) and consisted of approximately ≥95% Ig^+ cells (Table 3). These cells were also found to contain only 2% T cells, <1% macrophages/monocytes, and approximately 3-5% neutrophils as assessed by both flow cytometry and cytospin analyses (Tables 3 and 4). Cell yields ranged from 2-5% of the total number of original splenocytes whereas % recoveries, as a percentage of the total number of B cells at the start of the isolation, averaged 6-10% (Table 5). Conversely, cycling B cells and T cells isolated from freshly prepared splenocytes, at the interface of 1.07/1.079 percoll (band 2), were invariably contaminated with an excess number of residual macrophages. This point is demonstrated in Figure 4 and Tables 1, 2, 3 and 4, in which isolation of either whole splenocytes before the isolation of T cells, by immunomagnetic negative selection (Figure 4 and Tables 1 and 2), or B cells, after complement mediated lysis (Tables 3 and 4), resulted in macrophage/monocyte contaminated (and occasionally PMN contaminated) cycling B and T cell preparations (see materials and methods). Therefore, to obtain highly enriched cycling cells, a 10 hour adherence step to plastic petri dishes was employed in order to remove a significant proportion of macrophages and to induce the spreading and enlargement of the remaining monocytes for subsequent removal during percoll centrifugation. As shown in Figure 5 and Tables 1, 2, 3 and 4, adherence of whole splenocytes to plastic resulted in substantial increases in the purities of cells isolated in band 2 of either percoll separated splenocytes (for T cell isolation) or B cells (following T cell lysis). No improvements in purities were seen in band 3 of either cell preparation except for the removal of residual PMNs (which occurred only on occasion) which resulted in only slight increases in Ig^+ B cells in this band (i.e., 95 to 98%; data not shown); no macrophages/monocytes were found to separate at this density in either

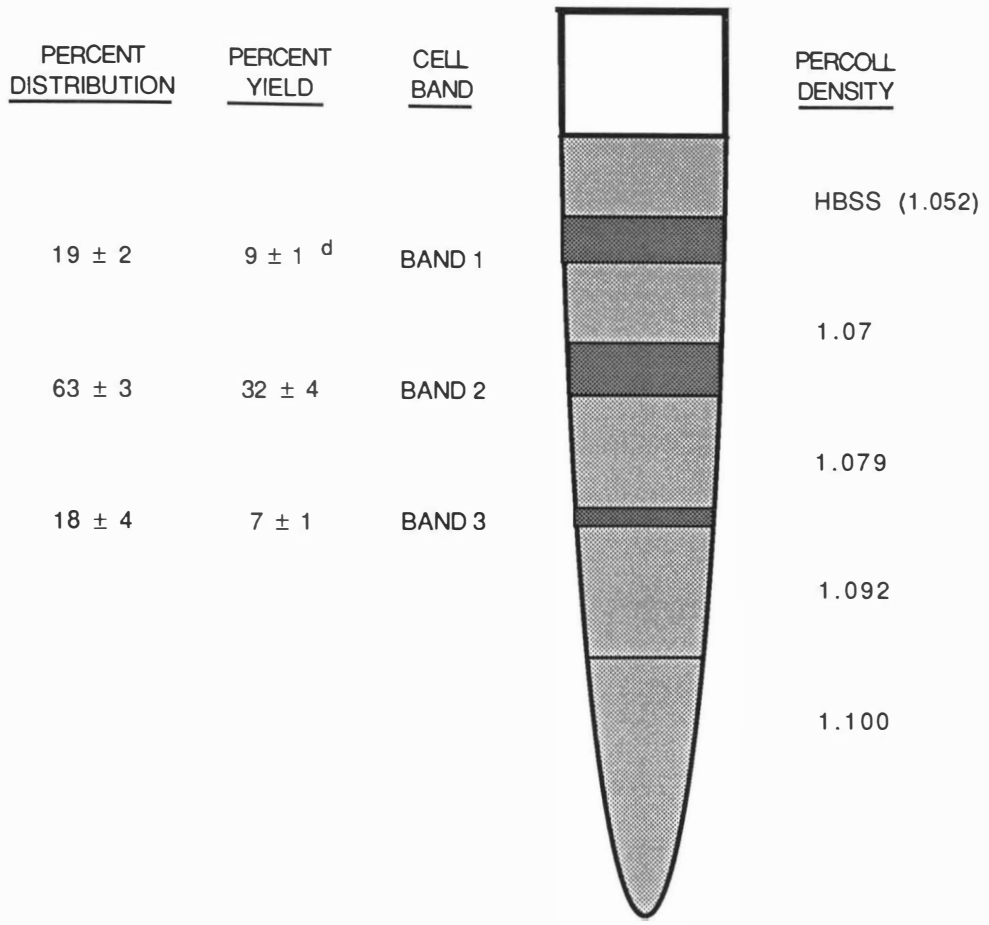


Figure 4. Percent Distributions and Yields of Splenocyte Subpopulations Isolated on Percoll Density Gradients Without Adherence to Plastic. Whole splenocytes were separated into 3 distinct subpopulations of cells based on bouyant cell density by centrifugation over percoll density step gradients. Red blood cells were removed by hypertonic lysis with Gey's solution prior to isolation (see materials and methods). Data represents the mean ± SE (n=4) percent distributions (i.e., number of cells collected in each band as a percentage of the total cells recovered from the gradients) and percent yields (i.e., the number of cells collected in each band as a percentage of the total number of splenocytes before isolation) of the 3 major subpopulations of splenocytes as based on cell density. The representative cell types collected in bands 1, 2 and 3 are fully differentiated lymphocytes and macrophages/monocytes, cycling (G1) lymphocytes and resting lymphocytes, respectively. d = Cell yields in band 1 do not represent actual yields due to variable amounts of monocytic clumping during collection of these cells.

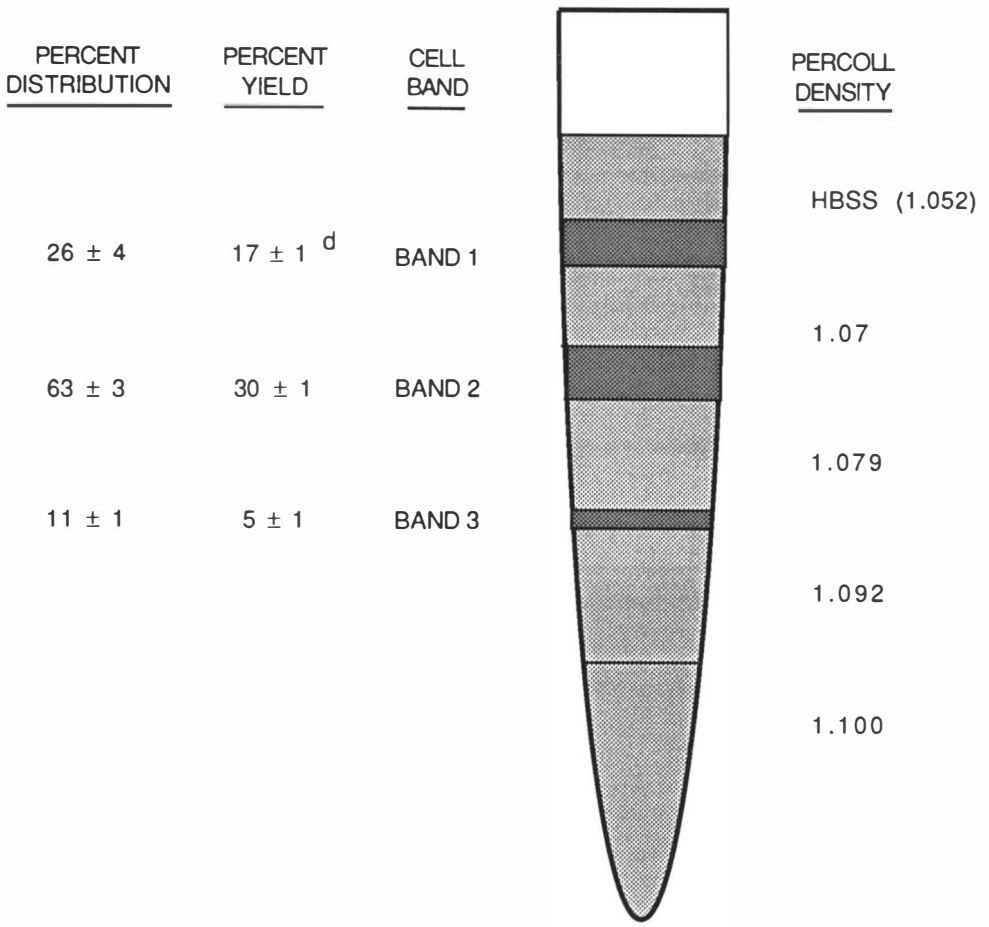


Figure 5. Percent Distributions and Yields of Splenocyte Subpopulations Isolated on Percoll Density Gradients Following a 10 Hour Adherence to Plastic. Whole splenocytes were cultured in plastic petri dishes for 10 hours in RPMI complete media containing 5% serum and separated into 3 distinct subpopulations of cells based on bouyant cell density by centrifugation over percoll density step gradients. Red blood cells were removed by hypertonic lysis with Gey's solution prior to isolation (see materials and methods). Data represents the mean ± SE (n=4) percent distributions (i.e., number of cells collected in each band as a percentage of the total cells recovered from the gradients) and percent yields (i.e., the number of cells collected in each band as a percentage of the total number of splenocytes before isolation) of the 3 major subpopulations of splenocytes as based on cell density. The representative cell types collected in bands 1, 2 and 3 are fully differentiated lymphocytes and macrophages/monocytes, cycling (G1) lymphocytes and resting lymphocytes, respectively. d = Cell yields in band 1 do not represent actual yields due to variable amounts of monocytic clumping during collection of these cells.

Table 1

Flow Cytometric Analysis of Lymphocyte Subpopulations in Spleen Cells Isolated on Percoll Density Step Gradients

Separation Without Adherence				
Recovered Band [c]	Ig+	Thy 1.2+	L3T4+	Lyt-2+
Unfractionated Splenocytes	49±2 [d]	35±1	18±1	11±1
Band 1 Splenocytes	63±2	15±1	14±1	5±1
Band 2 Splenocytes	51±2	44±2	19±1	13±2
Band 3 Splenocytes	22±6	65±5	28±1	21±1
Separation After 10 Hour Adherence to Plastic				
Recovered Band	Ig+	Thy 1.2+	L3T4+	Lyt-2+
Unfractionated Splenocytes	53±2	43±2	23±3	13±1
Band 1 Splenocytes	73±4	13±3	11±2	3±1
Band 2 Splenocytes	43±1	58±3	32±1	21±1
Band 3 Splenocytes	29±6	65±4	25±1	31±1

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Data represents the mean ± SE (n=3) specific fluorescent antibody labelling from three separate experiments.

Table 2

Identification of Leukocyte Distributions Within Splenocyte Subpopulations Isolated on Percoll Density Step Gradients Using Giemsa Stained Cytospin Preparations

Separation Without Adherence				
Recovered Band c	Lymphocytes	PMNs	Macs/Monos	Eosinophils
Unfractionated Splenocytes	91±2 d	4±1	4.5±1	0.5±0.1
Band 1 Splenocytes	76±2	2±1	22±1	0.2±0.1
Band 2 Splenocytes	93±2	2±0.5	5±1	0.8±0.4
Band 3 Splenocytes	88±4	11±5	0.3±0.1	0.5±0.2
Separation After 10 Hour Adherence to Plastic				
Recovered Band	Lymphocytes	PMNs	Macs/Monos	Eosinophils
Unfractionated Splenocytes	95±3	3±1	2±0.5	0.5±0.2
Band 1 Splenocytes	78±4	2±1	20±1	0.5±0.5
Band 2 Splenocytes	98±1	1±0.5	1±0.4	0.3±0.3
Band 3 Splenocytes	97±1	3±1	0±0	0.4±0.3

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Data represents the mean ± SE (n=3) of slides read from three separate experiments.

Table 3

Flow Cytometric Analysis of Purified Splenic Lymphocyte Subpopulations Isolated on Percoll Density Step Gradients

Separation Without Adherence				
Recovered Band c	Ig+	Thy 1.2+	L3T4+	Lyt-2+
Band 2 B Cells	93±1 d	0.4±0.1	0.5±0.1	0.4±0.1
Band 3 B Cells	96±2	--	(CD3+ = 2.0 ± 0.1%) e	
Separation After 10 Hour Adherence to Plastic				
Recovered Band	Ig+	Thy 1.2+	L3T4+	Lyt-2+
Band 2 B Cells	97±1	0.3±0.2	0.5±0.1	0.1±0.1
Band 2 T Cells	3±1	96±1	58±2	36±2
Band 3 T Cells	2±1	96±1	53±3	43±4

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Data represents the mean ± SE (n=3) specific fluorescent antibody labelling from three separate experiments. e = Specific staining with anti-CD3-epsilon was used as the selective T cell marker in these experiments.

Table 4

Identification of Leukocyte Distributions Within Purified Lymphocyte Subpopulations Isolated on Percoll Density Step Gradients Using Giemsa Stained Cytospin Preparations

Separation Without Adherence				
Recovered Band c	Lymphocytes	PMNs	Macs/Monos	Eosinophils
Band 2 B Cells	94±1 d	2±0.1	4±2	0.5±0.5
Band 3 B Cells	93±1	5±1	0.5±0.5	2±0.5
Separation After 10 Hour Adherence to Plastic				
Recovered Band	Lymphocytes	PMNs	Macs/Monos	Eosinophils
Band 2 B Cells	98±1	0.2±0.2	1±0.5	0.3±0.1
Band 3 B Cells	98±1	1±0.1	0.6±0.6	0.4±0.1
Band 2 T Cells	97±1	0.5±0.4	1.5±0.4	0.7±0.6
Band 3 T Cells	94±3	5±2	0.6±0.6	0.4±0.4

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Data represents the mean ± SE (n=3) of slides read from three separate experiments.

Table 5

Percent Yields and Recoveries of Purified Lymphocyte Subpopulations Isolated on Percoll Density Step Gradients

Recovered Band c	Percent Yields	Percent Recoveries
Separation Without Adherence		
Band 2 B Cells	27±2 d	54±3
Band 3 B Cells	4±1	8±3
Separation After 10 Hour Adherence to Plastic		
Band 2 B Cells	31±2	64±4
Band 3 B Cells	<1	<1
Band 2 T Cells	14±1	30±3
Band 3 T Cells	3±1	7±1

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Data represents the mean ± SE (n=3) of three separate experiments.

procedure. It should also be noted that plating was found to greatly interfere with the recoveries of the dense resting B cells (band 3) and resulted in significant losses in cell yields (Table 5). In fact, this effect was found to complicate the use of plating for the isolation of the resting B cell population and made analysis of these cells impossible, given the small number of cells that were recovered (Table 5). Figure 5 and Table 5 show the percentage of cycling splenocytes and B and T cells (band 2) recovered after a 10 hour adherence to plastic, which resulted in an increase in purity as compared to no adherence (Figure 4 and Tables 1, 2, 3 and 4). More specifically, cytospin analyses of these cell preparations demonstrated that the number of contaminating macrophages/monocytes had been greatly reduced (i.e., 5% to 1% in the splenocyte band 2 and 4% to 1% in B cell band 2), as were the numbers of contaminating neutrophils (Tables 2 and 5). % yields and % recoveries were also found to be increased for isolated cycling B cells (i.e., 31% and 64% versus 27% and 54%, respectively) following the adherence step. This effect was reflective of a loss in adherent cells during the plating procedure and not to a change in the separation of these cells during isolation.

2. Cell Cycle Analysis and Mitogen Responses.

Table 6 illustrates the cell cycle distribution of unfractionated and cycling (band 2) whole splenocytes, resting (band 3) and cycling (band 2) B cells, and cycling (band 2) T cells isolated on percoll density step gradients. Cycling cells of all 3 cell populations isolated with or without adherence showed a similar pattern of cell cycle distribution regardless of which approach was used. In addition, the pattern of cell cycle distribution closely paralleled the cycle distribution of whole splenocytes and demonstrates that neither adherence nor the isolation procedure alters the overall distribution of the cycling cells. Moreover, cycling lymphocytes make up the majority of cells within the spleen, as demonstrated by the percent distributions as shown in Figures 4 (without adherence)

Table 6

Cell Cycle Distribution of Splenocyte and Purified Lymphocyte Subpopulations Isolated on Percoll Density Step Gradients

Separation Without Adherence			
Recovered Band c	Go/G1	S	G2/M
Unfractionated Splenocytes	76	18	6
Band 2 Splenocytes	72	21	7
Band 2 B Cells	67	23	10
Band 2 B Cells	79 d	13	7
Band 3 B Cells	85 d	2	14
Separation After 10 Hour Adherence to Plastic			
Recovered Band	Go/G1	S	G2/M
Unfractionated Splenocytes	73	19	8
Band 2 Splenocytes	68	22	10
Band 2 B Cells	67	22	11
Band 2 T Cells	71	20	9

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Cell cycle analyses of these cell populations were determined using fixed intact cells as opposed to isolated nuclei (see methods).

and 5 (with adherence); further supporting the primary use of this population of cells in in vitro functional assays as a reflection of lymphocyte populations in whole splenocyte responses. In comparison to unseparated splenocytes, some decrease in Go/G1 cells were seen, whereas S and G2/M cells were slightly increased. This latter effect was also seen following adherence alone (Table 6; unseparated splenocytes and splenocyte cell band 2), and is speculatively caused by the removal of interfering macrophages/monocytes and/or neutrophils.

Resting B cells (band 3), from no adherence only, were analyzed for cell cycle distribution by two methods, one using isolated nuclei and the other using fixed whole cell preparations (see materials and methods). It was found that analysis of the dense resting cells (both T and B cells) by the method of Nusse, et al. [Nusse, et al., 1990], yielded distorted values for cycle distribution which were unlike either freshly prepared splenocytes or isolated cycling cells (i.e., cycling B cells were also analyzed using both methods; Table 6). Analysis of this B cell population using fixed cells showed an increase in Go/G1 cells and a decrease in S phase cells as opposed to either splenocyte cell band 2 or band 2 B cells (isolated by either method described above). Some differences were also detected between the two methods of analysis for band 2 B cells but were not as dramatic as for the resting B cell population. However, cytospin analyses of these two populations of cells visually confirmed the larger nuclear:cytoplasmic ratios of the resting cells in band 3 as compared to the smaller ratio that was seen in cytospins of the cycling cells in band 2. An increase in G2/M cells were also found in band 3 B cells. Interestingly, this observation was consistent with the findings of Layton, et al. (1985) [Layton, et al., 1985] who concluded that a small number of G2/M cells have a density that would make them indistinguishable from the Go/G1 cells. Alternatively, this finding may also indicate an interference by contaminating neutrophils which make up the majority of non-B cells in this band (i.e., up to 5%; Table 4). The multi-lobed nature of the neutrophil nucleus may

by sufficient to increase their fluorescence during DNA analysis. This effect however, has not been determined.

To further demonstrate the enrichment and functional nature of the B and T cells purified using the isolation procedures described above, mitogen responses for both unseparated and separated splenocytes and isolated cycling (band 2) and resting (band 3) B and T cells were performed. Table 7 illustrates the degree of cellular transformation in the presence of lipopolysaccharide (LPS; B cell selective mitogen) and Concanavalin A (Con A; T cell selective mitogen) as measured by ^{3}H-thymidine deoxyribose (3H-TdR) incorporation over 96 hours in culture. The proliferative responses of cells obtained with and without adherence are provided. As compared with either unseparated or separated band 2 splenocytes, band 2 B cells were found to be fully functional following the isolation procedure, with little difference between cells obtained with or without adherence. This is an important observation in that the 10 hour plating step did not appear to alter the functional capacity of these cells for mitogen transformation and supports the usefulness of adherence to remove residual macrophages/monocytes to obtain highly enriched cycling B cells at no expense to B cell function. Moreover, these cells were found to have no capacity to proliferate above background levels in the presence of Con A, further indicating the relative purities of B cells obtained by the method described. Likewise, resting and cycling T cells were found to selectively respond to Con A versus LPS in the mitogen responses, with differences also noted between T cells isolated from bands 3 (resting) and 2 (cycling) in response to Con A as were seen in the same populations of B cells (Table 7). Cycling T cells responded to a much greater degree than resting T cells but neither population attained the degree of proliferation that was seen in unfractionated or separated splenocytes. Moreover, the background proliferation of cycling T cells was much greater than resting T cells, further distinguishing this population as at least partially consisting of more mature or pre-activated cells than are found in the cells of band 3.

Table 7

Mitogen Responses of Splenocyte and Purified Lymphocyte Subpopulations Isolated on Percoll Density Step Gradients

Recovered Band c	LPS (CPM±SE) 48	72	96	Con A (CPM±SE) 48	72	96
Mitogen Response Without Adherence						
Unfractionated Splenocytes	88389 d ±3055	54458 ±966	32563 ±2360	51993 ±5638	55113 ±4435	17382 ±1271
Band 2 Splenocytes	119878 ±1723	49100 ±2005	34944 ±899	53913 ±3090	44944 ±1017	17607 ±454
Band 3 Splenocytes	19814 ±531	50598 ±1968	53542 ±778	3223 ±930	2485 ±222	3477 ±1555
Band 2 B Cells	131090 ±4085	50973 ±3883	39171 ±2367	413 ±139	497 ±36	1019 ±280
Band 3 B Cells	14116 ±656	42011 ±1906	88328 ±1734	-- e	--	--
Mitogen Response After 10 Hour Adherence to Plastic						
Recovered Band	48	72	96	48	72	96
Unfractionated Splenocytes	84917 ±5516	48717 ±2345	40383 ±1376	16744 ±824	46092 ±2926	39027 ±943
Band 2 Splenocytes	112299 ±5128	49523 ±1079	45982 ±1531	6062 ±497	12449 ±1156	10843 ±1176
Band 3 Splenocytes	45770 ±5137	57184 ±2127	25044 ±8351	1373 ±585	593 ±288	447 ±176
Band 2 B Cells	152977 ±6075	46979 ±3896	39898 ±2307	849 ±165	1156 ±413	318 ±33
Band 3 B Cells	-- e	--	--	--	--	--
Band 2 T Cells	1499 ±133	1947 ±239	3066 ±794	3590 ±751	5256 ±1032	164 ±3210
Band 3 T Cells	523 ±156	499 ±46	451 ±93	1227 ±288	1542 ±459	636 ±218

c = Refer to Figures 4 and 5 for population designations according to cell density. d = Cell proliferation was determined by ^{3}H-TdR incorporation. Values represent the mean ± SE of absolute CPM of quadruplicate cultures harvested at 48, 72 and 96 hours (See materials and methods for details). e = Not determined.

Because of the limitation in the number of cells obtained for band 3 (dense resting) B cells, mitogen responses were only assessed in the presence of LPS (as opposed to LPS and Con A). However, as consistent with our other observations (i.e., cell cycle analysis) and with other reports [Layton, et al., 1985], these cells responded differently than the cycling B cells to this mitogen. Both the peak day and magnitude of proliferation was found to be slightly different than in the cycling B cells. Differences were also found in the degree of background proliferation, in which the band 3 B cells had a much lower average proliferation than was found for either unseparated splenocytes or enriched cycling B cells (data not shown). Band 3 B cells isolated following adherence were not assessed for their response to either mitogen because of the lower yields that were obtained.

B. Effects of TCDD on the In Vitro T-Dependent Antibody Response:

1. Serum Dependency for Suppression.

One of the first observations made concerning the effects of TCDD on the in vitro T-dependent antibody response, was the dependency not only on the lot, but on the concentration of serum present in the culture media. To verify and further characterize this effect, we obtained 23 lots of serum (21 lots of fetal bovine serum, 1 lot of newborn calf serum, and 1 lot of fetal equine serum; FBS, NBCS, and FES respectively), all of which could support a good in vitro antibody response to SRBC, and determined their ability to support the suppressive effects of 30 nM TCDD (0.01% DMSO) on this response at serum concentrations of both 5 and 10%. Of these 23 lots, only 5 lots of serum (3 lots of FBS, the one lot of NBCS, and the one lot of FES) could support the suppression by TCDD on the peak day of the response (day 5). The remaining lots (all FBS) seemed to demonstrate a "protective-like" effect against TCDD exposure (i.e., did not support the suppression by TCDD), and in several cases showed enhancements in the AFC response following TCDD

addition; as compared to vehicle-treated controls. The latter effect is depicted most notably in Figure 6, in which TCDD addition to cultures containing 5% FBS of lot 890 caused a >2-fold enhancement in the AFC response on day 5 of culture (at 30 nM TCDD). At 10% FBS lot 890, no enhancements were seen (but rather suppression at 3 nM) and further demonstrated the complex interaction between TCDD and growth factors present in FBS. We further examined the serum-dependency of the suppression produced by TCDD by looking at the dose responsiveness of TCDD in each of the 5 remaining lots at concentrations of both 5 and 10%. Shown in Figure 7 (Figures A and B) is a comparison of the effects of TCDD in each of 5 representative lots of serum at both 5 and 10%, respectively. As noted above, these lots were selected based upon their ability to support a suppression at a concentration of 5% in the presence of 30 nM TCDD. As seen in Figure 7 (A), when log dose response curves were established using 0.3, 3.0, and 30 nM TCDD (0.01% DMSO) in a 5% concentration of each serum, we found that only 1 lot of FBS (lot 847), the single lot of NBCS (lot 453), and the single lot of FES could support a full dose response suppression. In the remaining two lots (FBS 717 and 856) TCDD did not produce a dose-related suppression. In comparing this profile of effects with what is seen at 10% serum (Figure A versus B), only the NBCS (lot 453) continued to support a full dose response suppression by TCDD. The remaining 4 lots demonstrated either a loss in dose responsiveness or an enhancement (FBS 717, at 0.3 and 3 nM) in the presence of TCDD. With exception of lot 856 (1 trial only), all sera were tested at least 2 times for dose related effects by TCDD. Collectively, it can be seen that, whereas the FBS (lot 847) and FES support a suppression of the T-dependent antibody response only at a concentration of 5% serum, the NBCS could support a suppression at either 5 or 10%. Subsequent to these experiments, two additional lots of NBCS (lots 512 and 313; Armour Pharmaceuticals) were analyzed for support of the suppressive effects of TCDD on the <u>in vitro</u> antibody response. As seen in Figure 8, NBCS lot 512 was found to also support an equivalent suppression of the antibody response at both concentrations. The same effect

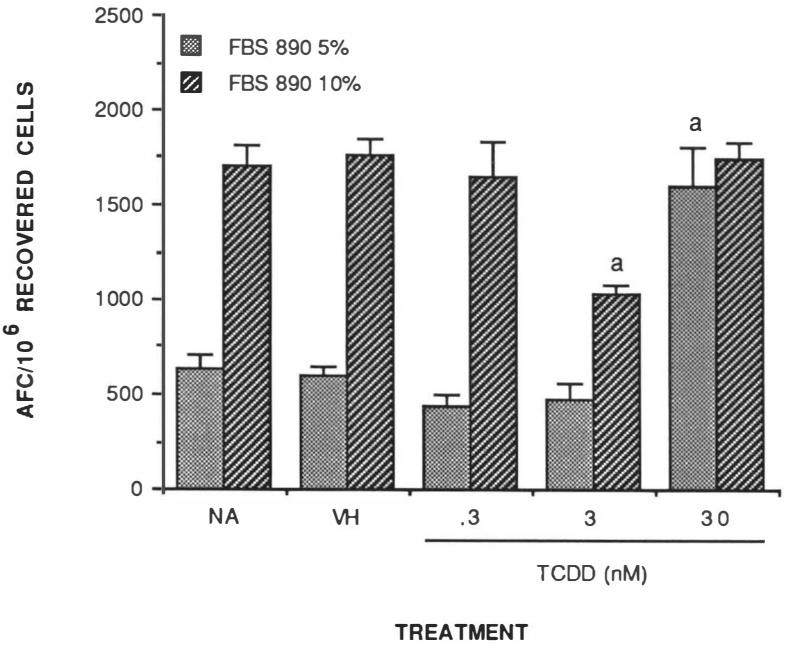


Figure 6. Enhancement of the In Vitro T-Dependent Antibody Response by TCDD in the Presence of Fetal Bovine Sera is Dependent on the Lot and Concentration of Serum. Data represents the effect of TCDD on the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with either 5% or 10% fetal bovine serum lot 890. Abbreviations are defined as follows: NA=naive (media only); VH=vehicle control (0.01% DMSO); FBS=fetal bovine serum. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the vehicle control are defined as follows: a=p≤0.01.

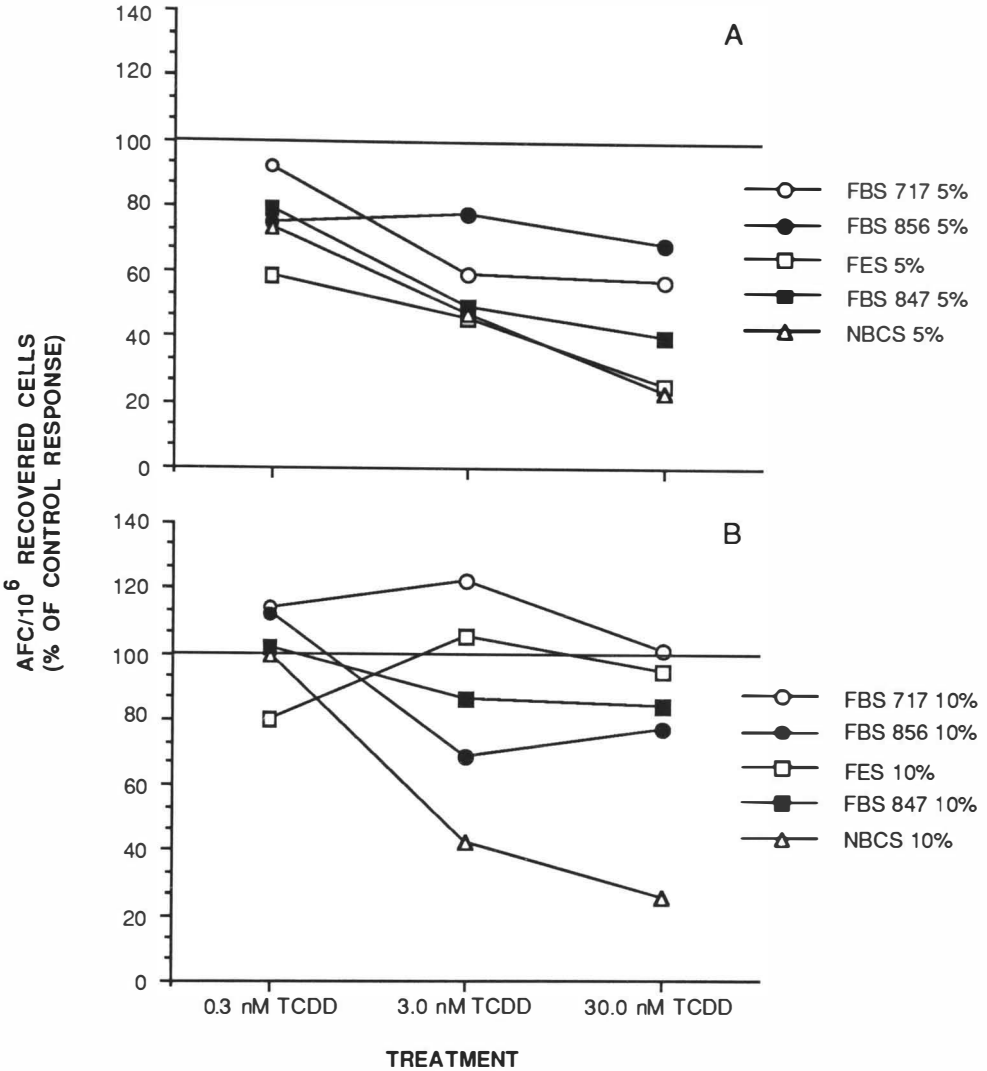


Figure 7. Serum Dependency for Suppression of the In Vitro T-Dependent Antibody Response by TCDD. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in culture and is presented as a percentage of the control AFC response in the presence of increasing concentrations of TCDD. Figures A (top) and B (bottom) are the results of cultures containing media supplemented with either 5% or 10%, respectively, of the different lots serum tested. Abbreviations are defined as follows: FBS=fetal bovine serum; FES=fetal equine serum; NBCS=newborn calf serum. Data presented is representative of at least 2 separate experiments.

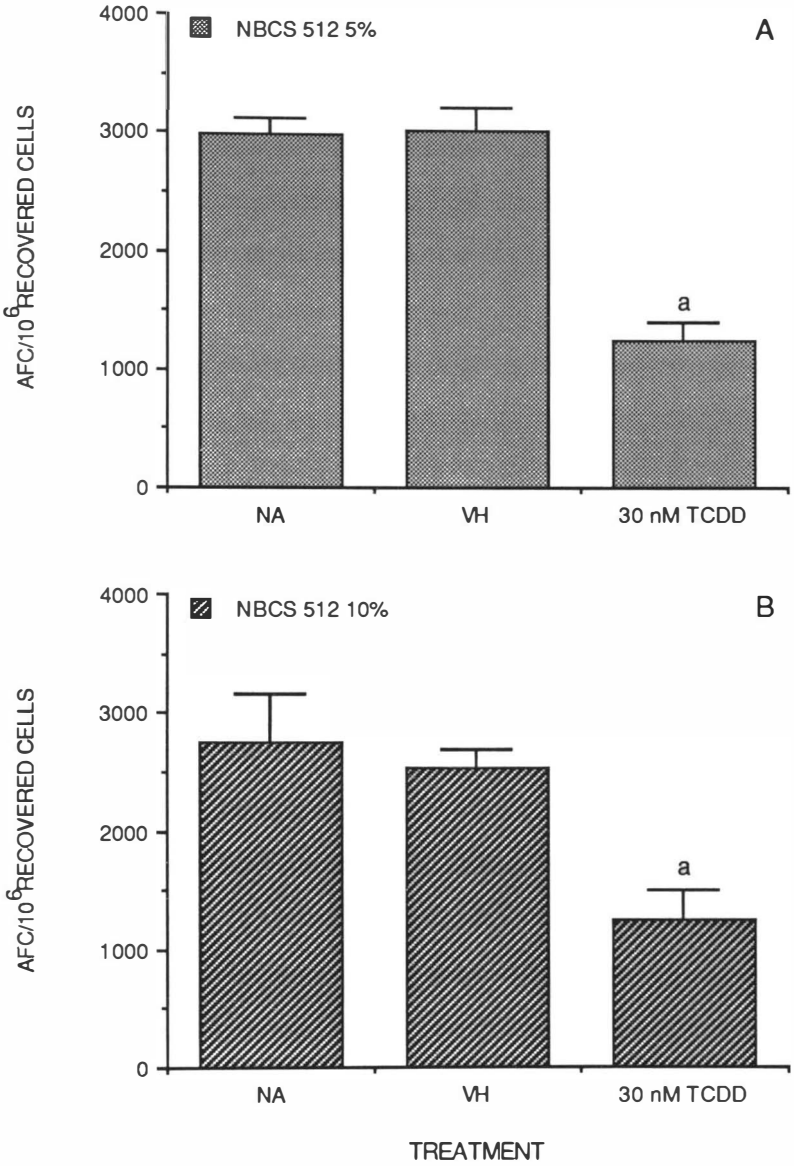


Figure 8. Suppression of the In Vitro T-Dependent Antibody Response by TCDD in the Presence of Newborn Calf Serum Is Not Dependent Upon the Lot or Concentration Used in Culture. Data represents the effect of TCDD on the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with either (figure A) 5% or (figure B) 10% newborn calf serum 512. Abbreviations are defined as follows: NA=naive (media only); VH=vehicle (0.01% DMSO); NBCS=newborn calf serum. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the vehicle control are defined as follows: a=p≤0.01.

was seen in NBCS lot 313 and supports the conclusion that the synergistic serum mediator of the TCDD-induced suppression is a generalized component of all newborn calf sera; as opposed to FBS which demonstrated an enormous degree of lot to lot variation in this response. It should also be noted that these effects did not correlate with the level of endotoxins found in the five representative lots of serum presented in Figure 7. Endotoxin levels were determined by the serum suppliers (see materials and methods) using the LAL Gel-Clot method with endotoxin levels for each of the 5 representative lots being described as follows: lot 717 - 0.2 ng/ml, lot 847 - 0.05 ng/ml and lot 856 - 0.025 ng/ml; NBCS (lot 453) - 11.1 ng/ml; FES (lot 163) - 0.3 ng/ml.

2. Enhancements Following In Vivo Exposure.

The enhancements in the in vitro T-dependent antibody response to SRBC that were seen following direct addition of TCDD to cultures containing FBS were not found to be a unique characteristic of in vitro exposures. Figure 9 illustrates the effect of in vivo exposure on the in vitro antibody response. Following a 5 day repetitive exposure of B6C3F1 female mice to 1, 10 and 30 μg/kg TCDD, splenocytes were removed and stimulated with SRBC in cultures supplemented with either 5% (Figure 9 A) or 10% (Figure 9 B) of the various lots of FBS and NBCS as indicated (see materials and methods). As can be seen in Figure 9, enhancements were found in the AFC responses of cultures containing cells exposed in vivo at levels of either 10 or 30 μg/kg TCDD. Some suppression was seen at the 1 μg/kg exposure level but was not a consistent effect. Interestingly, a slight difference was seen in the enhancements following in vivo exposures as opposed to in vitro, in which the enhancements seen following in vivo exposure were not dependent on the serum lot. This difference is demonstrated in Figure 9, where exposure of mice to the 50 μg/kg cumulative dose of TCDD produced enhancements in the antibody responses of the exposed splenocytes when cultured in the presence of 5%

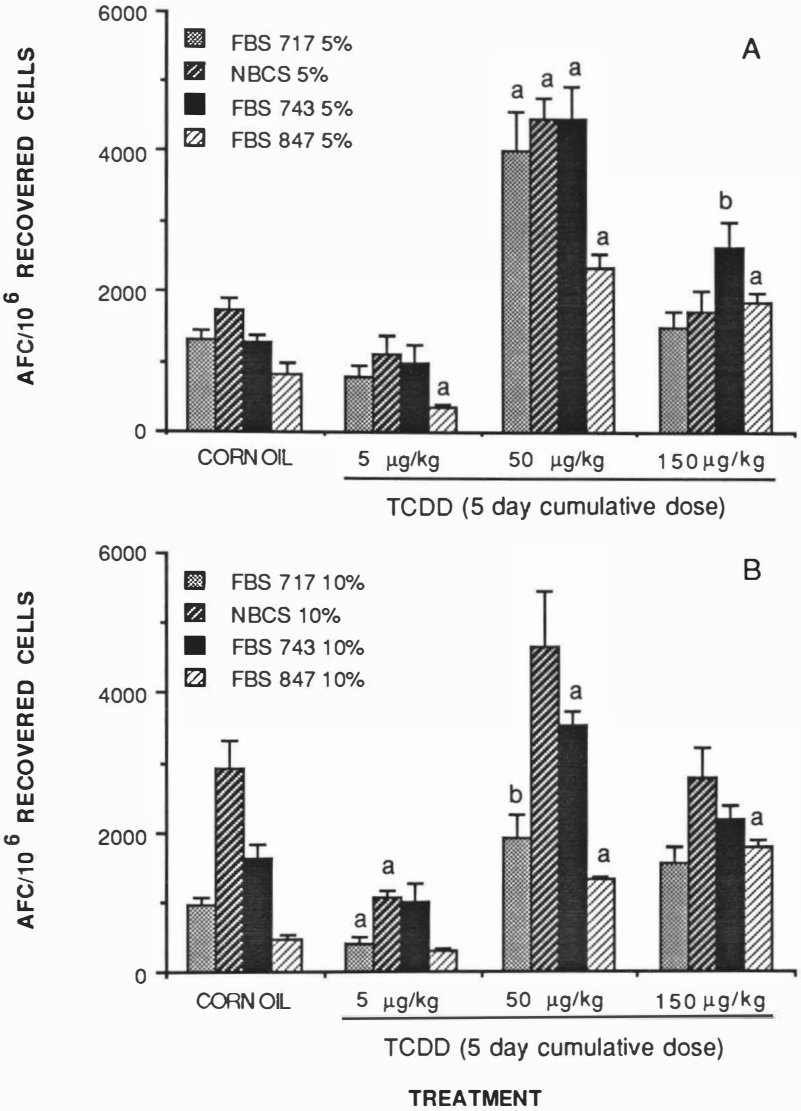


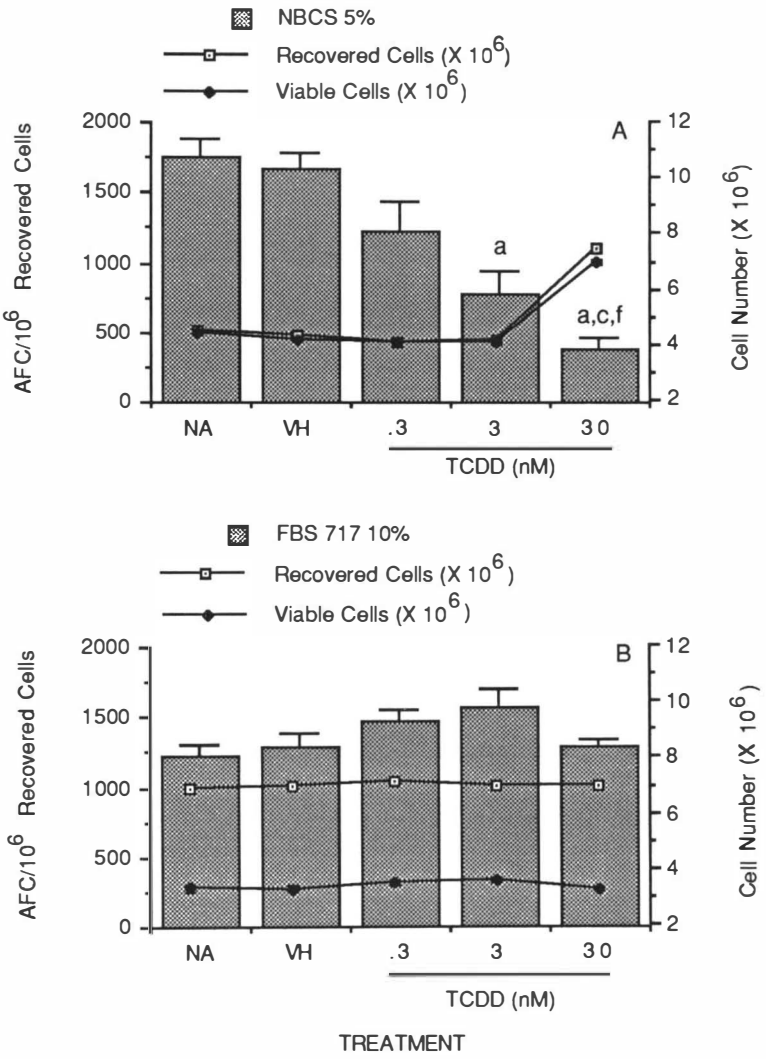
Figure 9. Enhancement of the In Vitro T-Dependent Antibody Response Following In Vivo Exposure of Splenocytes to TCDD. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with either (figure A) 5% or (figure B) 10% of various lots of serum following in vivo exposure of mice to TCDD for 5 consecutive days (see methods). Abbreviations are defined as follows: FBS=fetal bovine serum; NBCS=newborn calf serum. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the corn oil (vehicle)-treated control cell response are defined as follows: a=$p \leq 0.01$; b=$p \leq 0.05$.

serum, even in the presence of NBCS. However, this effect was not seen at 10% serum, where enhancements were seen in all 3 lots of FBS, but not in the NBCS. More consistent with the effects during in vitro exposures, there was a concentration dependency for these effects, in which differences were seen in the magnitude of the enhancements when comparing the responses obtained using 5 versus 10% serum (Figures 9 A and B).

3. Comparison of the Effects of TCDD and SAC Under Serum-Supplemented Culture Conditions.

When compiling the previous findings indicating that TCDD appears to act primarily on the B lymphocyte [Dooley and Holsapple, 1988] with the current observation that the effects of TCDD on the antibody response to SRBC is dependent on serum-derived growth factors to produce either enhancements or suppression of the in vitro antibody response, we were struck by the unique profile of activity. It was through the use of these key observations that we began to look at the effects of the polyclonal B cell activator, Staphylococcus aureus Cowan strain I (SAC), on the in vitro antibody response. Because the literature (i.e., as described above in the literature review) suggests that there might be similarities between TCDD and SAC, a comparison was made between the actions of SAC and TCDD on the in vitro T-dependent antibody response in 2 separate lots of serum (one which supported the suppression by TCDD - 5% NBCS lot 453, and one which did not - 10% FBS lot 717). It should also be noted that protein determinations were conducted (Bio-Rad protein assay) on serum lots NBCS 453 and FBS 717 and revealed that media supplemented with 5% NBCS (5 mg/ml protein) was approximately equivalent to the protein content of the media supplemented with 10% FBS (5.5 mg/ml). Therefore, differences in total protein cannot explain the immunomodulatory effects of the serum and support the role by a specific serum-derived growth factor. As can be seen in Figure 10 (A and B), TCDD, at concentrations of 0.3, 3.0, and 30 nM, caused a dose dependent

Figure 10. Effect of TCDD on the In Vitro T-Dependent Antibody Response in the Presence of Fetal Bovine and Newborn Calf Serum. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with either (figure A) 5% NBCS or (figure B) 10% FBS (lot 717) in the presence of increasing concentrations of TCDD. Abbreviations are defined as follows: NA=naive (media only); VH=vehicle control (0.01% DMSO); FBS=fetal bovine serum; NBCS=newborn calf serum. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the vehicle-treated control are defined according to the parameter measured as indicated: AFC response/10^6 recovered cells - a=$p \leq 0.01$; Recovered cell number - c=$p \leq 0.01$; Viable cell number - f=$p \leq 0.05$.


NBCS 5%
Recovered Cells (X 10^6)
Viable Cells (X 10^6)
AFC/10^6 Recovered Cells
Cell Number (X 10^6)
2000
1500
1000
500
0
12
10
8
6
4
2
A
a
a,c,f
NA
VH
.3
3
30
TCDD (nM)
FBS 717 10%
Recovered Cells (X 10^6)
Viable Cells (X 10^6)
AFC/10^6 Recovered Cells
Cell Number (X 10^6)
B
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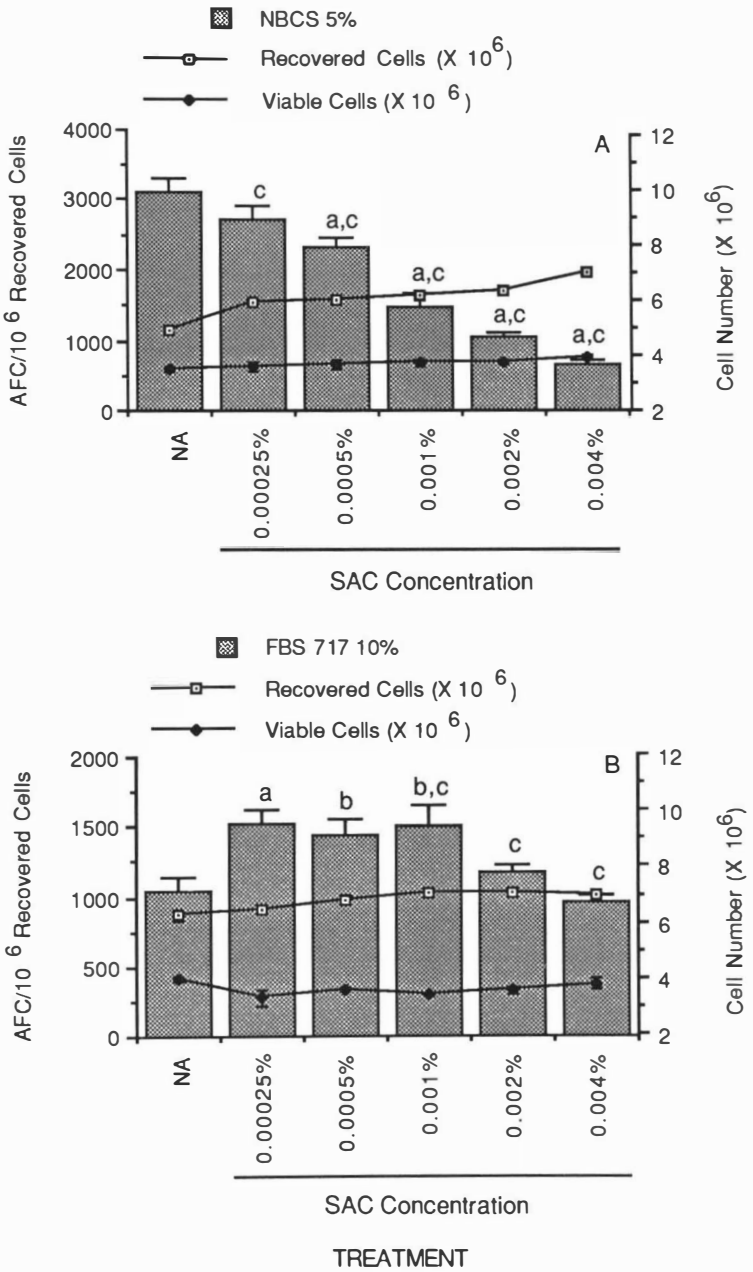
suppression of the in vitro T-dependent response in media containing 5% NBCS (lot 453; Figure 10 A) whereas the same concentrations of TCDD in media containing 10% FBS (lot 717; Figure 10 B) showed no suppression, but instead produced a small enhancement at concentrations of 0.3 and 3.0 nM. Vehicle-treated controls (0.01% DMSO) demonstrated no effect. Likewise, when SAC was added to identically prepared cultures, at final concentrations ranging from 0.00025 to 0.004%, it showed the same profile of effects (Figure 11). In media containing 5% NBCS (lot 453), a dose dependent suppression of the T-dependent response was observed, whereas in media containing 10% FBS (lot 717), there was a loss of suppressive effects by SAC on this response; with the 3 lowest concentrations (0.00025, 0.0005, and 0.001%) producing a slight enhancement. Both TCDD and SAC suppressed this response to a near equivalent degree in the presence of 10% NBCS (30 nM TCDD and 0.001% SAC) as demonstrated in Figure 12; which further demonstrates that neither TCDD nor SAC produce a serum-concentration-dependent suppression of the antibody response in NBCS. Because of the labor intensity of these studies, and the non-dose-dependent effects by TCDD, comparisons were not carried out at 5% FBS 717 (see Figure 7). Although these data appear to show identical profiles between the effects of TCDD and SAC on the T-dependent response, there were also some differences. In the case of SAC's effects on the T-dependent response in the presence of 5% NBCS, there was a dose dependent increase in recovered cell numbers which were significant at all concentrations of SAC ($p \leq 0.01$; consistent throughout 3 separate experiments; Figure 11). Concomitantly, this increase in cell number was accompanied by a dose dependent decrease in cellular viability, again significant at all concentrations ($p \leq 0.01$). However, no significant change in viable cell numbers occurred even though the total numbers of plaques were reduced by the treatment. In the presence of 10% FBS, as was the case with 5% NBCS, there was a dose dependent increase in cell number which plateaued at a concentration of 0.001% SAC, but the effects on cell viabilities were not as prominent. Only 1 out of 3 separate experiments, each showing

Figure 11. Effect of SAC on the In Vitro T-Dependent Antibody Response in the Presence of Fetal Bovine and Newborn Calf Serum. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with either (figure A) 5% NBCS or (figure B) 10% FBS (lot 717) in the presence of increasing concentrations of Staphylococcus aureus Cowan strain I (SAC). Abbreviations are defined as follows: NA=naive (media only); FBS=fetal bovine serum; NBCS=newborn calf serum. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the vehicle-treated control are defined according to the parameter measured as indicated: AFC response/10^6 recovered cells - a=$p \leq 0.01$, b=$p \leq 0.05$; Recovered cell number - c=$p \leq 0.01$; Viable cell number - no significant differences were detected.


NBCS 5%
Recovered Cells (X 10 6)
Viable Cells (X 10 6)
AFC/10 6 Recovered Cells
Cell Number (X 10 6)
A
c
a,c
a,c
a,c
a,c
NA
0.00025%
0.0005%
0.001%
0.002%
0.004%
SAC Concentration
FBS 717 10%
Recovered Cells (X 10 6)
Viable Cells (X 10 6)
AFC/10 6 Recovered Cells
Cell Number (X 10 6)
B
a
b
b,c
c
c
NA
0.00025%
0.0005%
0.001%
0.002%
0.004%
SAC Concentration
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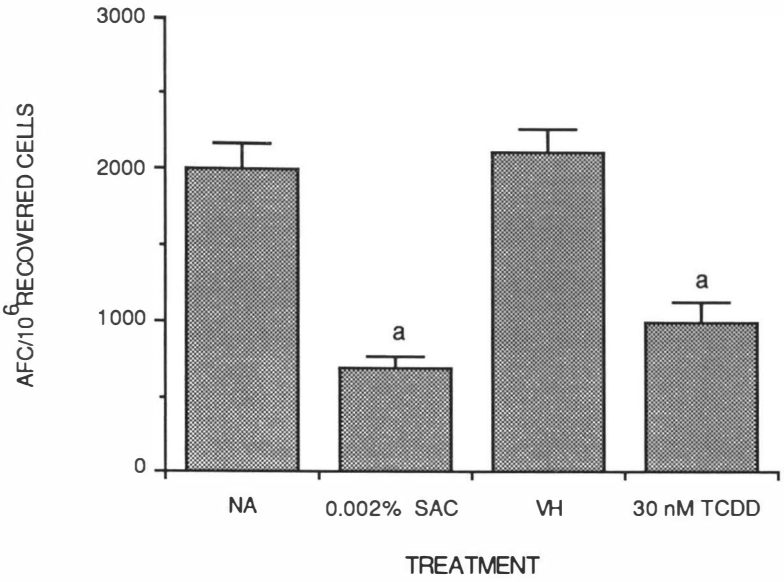


Figure 12. Suppression of the In Vitro T-Dependent Antibody Response by SAC in the Presence of Newborn Calf Serum Is Not Dependent Upon the Concentration Used in Culture. Data represents the effect of either TCDD or SAC on the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with 10% newborn calf serum. Abbreviations are defined as follows: NA=naive (media only); VH=vehicle (0.01% DMSO); SAC=Staphylococcus aureus Cowan strain I. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the media or vehicle controls are defined as follows: a=$p \leq 0.01$.

almost identical effects on the T-dependent response, showed any effect on cell viability in the presence of 10% FBS (lot 717), and only at concentrations ≥ 0.005% SAC. In comparison, TCDD, in the presence of 5% NBCS, caused significant increases in cell number ($p \leq 0.01$) only at a concentration of 30 nM (consistent throughout 3 separate experiments) which was accompanied by only a slight decrease in cellular viability (unlike what was seen with SAC; Figures 10 and 11). Again in contrast to SAC, TCDD, in the presence of 10% FBS (lot 717), showed no significant effects on cell number or cell viability.

Because of the similarities between the actions of TCDD and SAC on the in vitro antibody response, experiments were also conducted to determine the potential interaction between these two agents when administered in combination to in vitro cultures stimulated with SRBC. As shown in Figure 13, the combination of TCDD + SAC caused a greater degree of suppression of the antibody response than either of the two agents administered alone at suboptimal concentrations (TCDD=0.3 nM; SAC=0.001%). This effect was produced in two separate experiments (Figures 13 A and B) and demonstrates the interactive potential of TCDD and SAC in suppression of the in vitro T-dependent antibody response.

4. Comparison of the Effects of TCDD and SAC Under Serum-Free Culture Conditions.

Having demonstrated that TCDD and SAC can suppress the primary T-dependent antibody response in a manner which is dependent on the lot and concentration of serum present in the culture media, it was of interest to develop a serum-free culture system to investigate the effects of TCDD on this response in the absence of serum growth factors (sensitization with SRBC; day 5 response). In preliminary studies, it was determined that serum-free conditions did not support a very pronounced T-dependent response (≤10

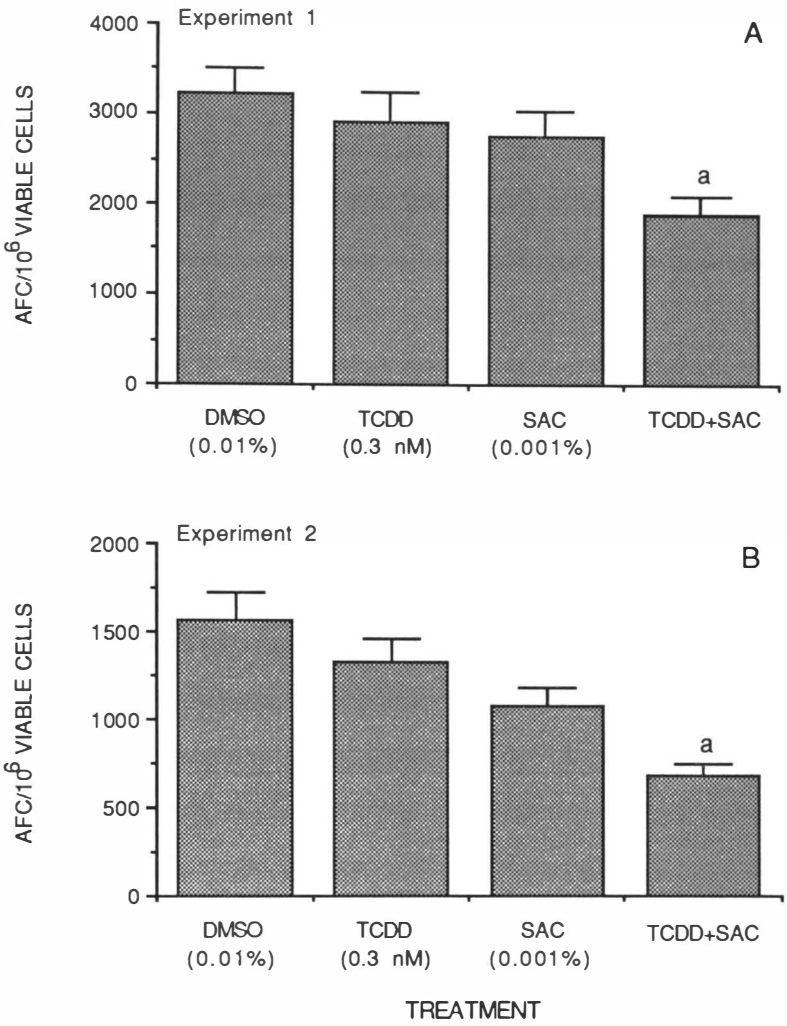


Figure 13. Potentiation of Effects Between TCDD and SAC in Suppression of the In Vitro T-Dependent Antibody Response. Data represents the effects of a combination of suboptimal concentrations of TCDD and SAC on the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 viable B6C3F1 splenocytes in cultures containing media supplemented with 5% newborn calf serum. Abbreviations are defined as follows: DMSO=dimethylsulfoxide (vehicle); SAC=Staphylococcus aureus Cowan strain I. Figures A (top) and B (bottom) represent two separate experiments as indicated. Data is presented as the mean ± SE of 6 replicate cultures/treatment group. Letter codes for significance as compared to the vehicle control are defined as follows: a=$p \leq 0.01$.

AFC/Culture). Although this finding may seem to hinder its usefulness in the study of many agents which can interfere (i.e., suppress) with humoral immune responses, it can be very useful in the study of agents which can cause the activation of lymphocytes. In this type of antigen driven (T-dependent) system, however, T cell-derived growth and differentiation factors would be available so that the effect elicited by agents that potentiate the activation of lymphocytes would be manifested as an enhancement in the AFC response; as compared to either media or vehicle-treated controls. As depicted in Figure 14 (Figure A), 30 nM TCDD, added to cultured splenocytes stimulated with SRBCs in a serum-independent environment, increased the T-dependent AFC response/culture 15-fold over the vehicle-treated controls. These results were obtained in the absence of any significant effects on either cell number or viabilities and were consistent throughout 5 separate experiments. Following identical methods as those used for TCDD, we administered a dose responsive range of SAC concentrations to whole splenocytes which were cultured under the same serum-independent conditions. As demonstrated (Figure 14 B), SAC, at concentrations ranging from 0.0005 to 0.002%, caused a dose related increase in the AFC response which culminated in as high as an 18-fold increase. However, as was the case under serum supplemented conditions, SAC differed from TCDD in that significant increases in recovered cell numbers were indicated. Likewise, there was a slight decrease in the number of viable cells recovered, indicating again that TCDD and SAC have similar but slightly different modes of action on the in vitro T-dependent response. Additional studies have shown that the increases in the AFC response by TCDD and SAC under serum-free culture conditions only occurs in the presence of antigen stimulation (i.e., SRBC), suggesting a possible requirement for antigen stimulated soluble mediators in the enhanced response by TCDD and SAC, and

that the AFC responses measured in earlier studies were specific anti-SRBC AFCs and not non-specific plaques formed as an artifact of the serum-free culture system (Figure 15).

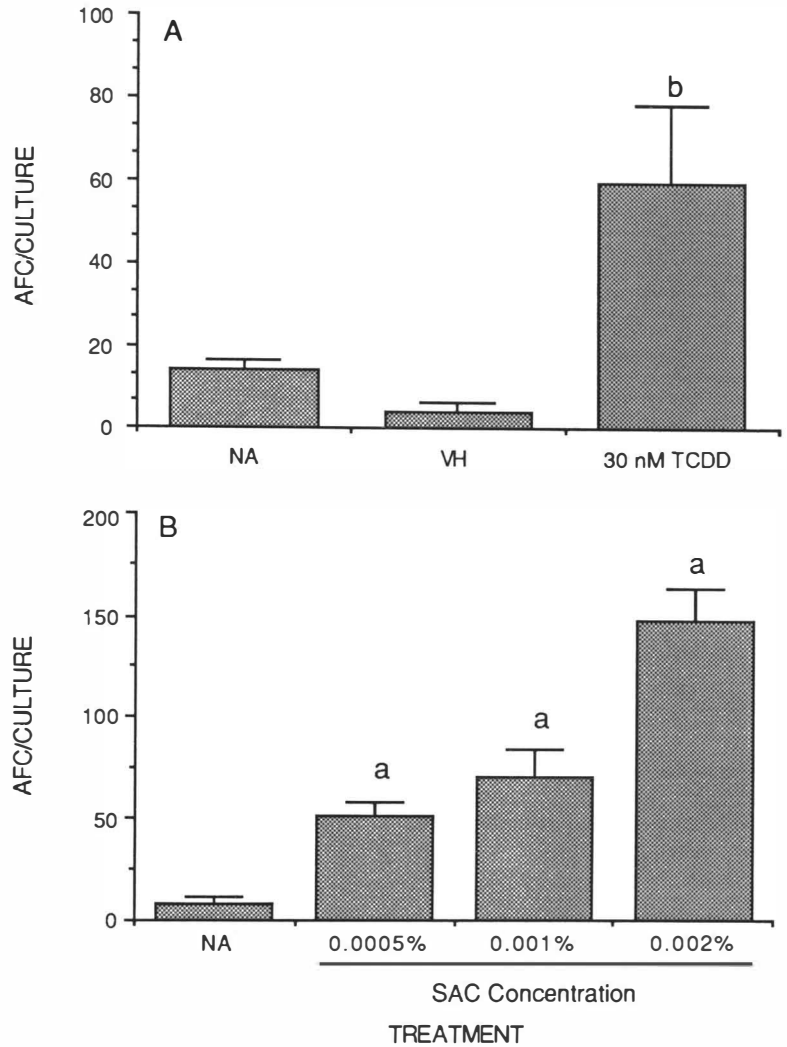


Figure 14. Effect of TCDD and SAC on the In Vitro T-Dependent Antibody Response Under Serum-Free Culture Conditions. Data represents the effect of either (figure A) TCDD or (figure B) SAC on the day 5 anti-SRBC antibody forming cell (AFC) response/ B6C3F1 splenocyte in cultures containing KC2000 serum-independent media (see materials and methods). Abbreviations are defined as follows: NA=naive (media only); VH=vehicle (0.01% DMSO); SAC=Staphylococcus aureus Cowan strain I. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the media or vehicle controls are defined as follows: a=p≤0.01, b=p≤0.05.

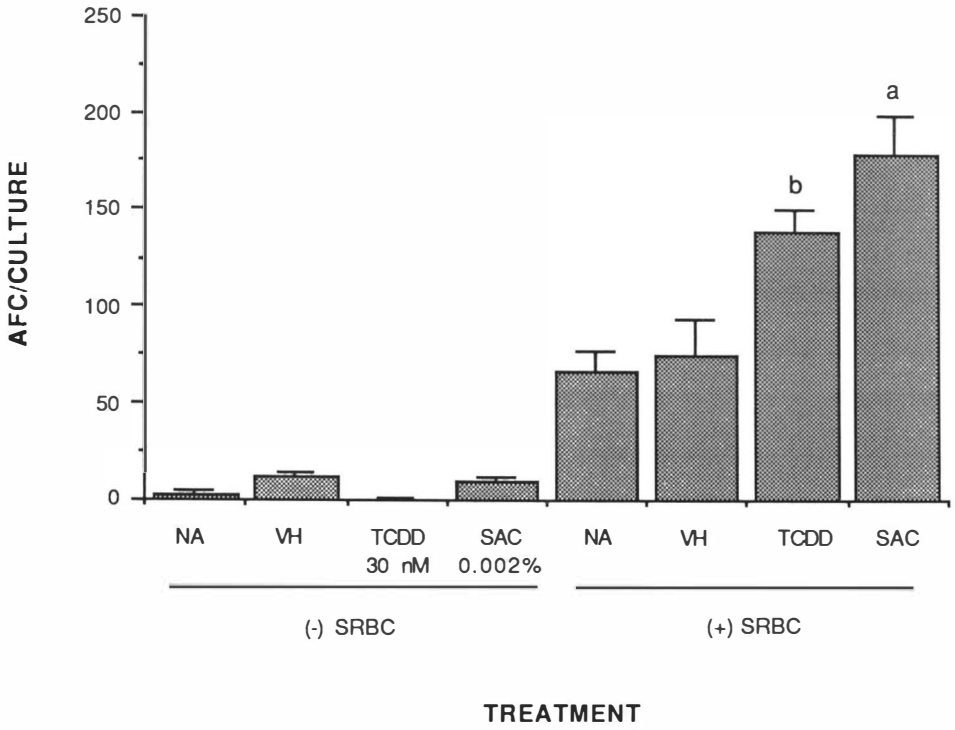


Figure 15. Antigen Dependency for Enhancement of the In Vitro T-Dependent Antibody Response by TCDD and SAC Under Serum-Free Culture Conditions. Data represents the effect of either TCDD or SAC on the day 5 antibody forming cell (AFC) response/ B6C3F1 splenocyte in cultures containing KC2000 serum-independent media in the presence or absence of the sheep red blood cell (SRBC) antigen. Abbreviations are defined as follows: NA=naive (media only); VH=vehicle (0.01% DMSO); SAC= Staphylococcus aureus Cowan strain I. Data is presented as the mean ± SE of 6 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the media or vehicle controls are defined as follows: a=$p \leq 0.01$, b=$p \leq 0.05$.

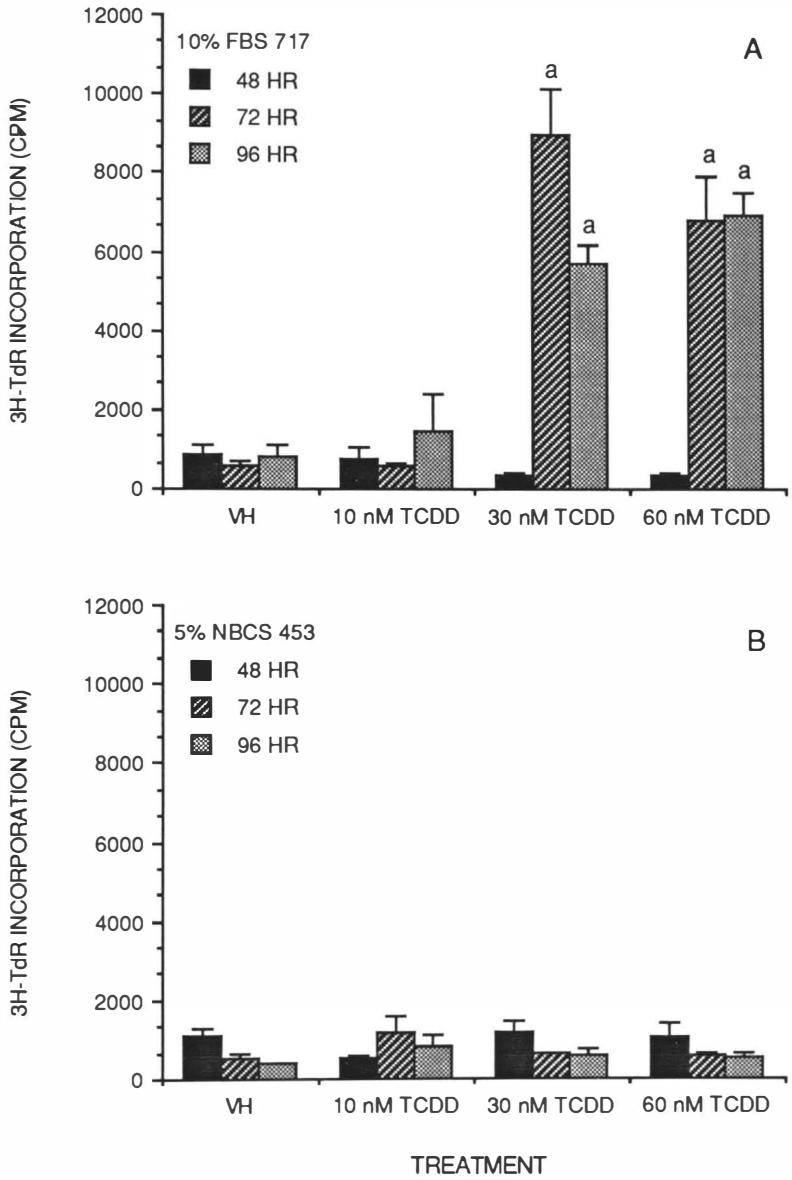
C. Effects of TCDD on Splenic-Derived Dense Resting (Go) B Cells:

1. Serum Dependency for Proliferation and Differentiation Into IgM Secreting Cells Following Direct Addition of TCDD.

Given the apparent enhancements in the in vitro AFC response and the similarities in effects between TCDD and SAC, studies were performed to determine if TCDD could cause the activation and differentiation of dense resting B lymphocytes upon direct addition to culture. Splenic-derived dense B cells were isolated from naive B6C3F1 mice (see material and methods) and placed into cultures containing media supplemented with sera that were shown to either support the suppression of the T-dependent antibody response by TCDD (5% NBCS lot 453) or show a "protective-like" action to the effects of TCDD (10% FBS lot 717). As demonstrated in Figure 16 A, 30 and 60 nM TCDD added to cultures of dense resting B cells in the presence of 10% FBS caused a significant increase in the proliferation of these cells, as compared to the vehicle-treated cultures. This effect was most apparent at 72 hours after treatment and continued to be significantly increased up to 96 hours. No effect was seen at 48 hours. In contrast, cultures prepared in 5% NBCS demonstrated no proliferative effects by TCDD even up to 96 hours of culture. The vehicle used in these experiments (0.01% DMSO) demonstrated no effect on the naive response of these cells regardless of the serum used (data not shown). In subsequent experiments, it was further determined that 30 nM was the lowest concentration of TCDD that was found to enhance the proliferative capacity of isolated resting B cells in culture (Figure 17). No effects were seen at concentrations up to 10 nM within a 96 hour culture period.

Additionally, as shown in Figure 18, TCDD (30 nM; a concentration giving maximal proliferation in serum lot 717) caused an increase in the level of IgM secretion in isolated dense resting B lymphocytes on day 7 of culture as compared to either media or vehicle-

Figure 16. Serum Dependency for Proliferation of Splenic-Derived Dense Resting B Cells Following Direct Addition of TCDD. Highly purified dense resting B cells were isolated as described (see materials and methods) and cultured (1 X 10^5/well) with media supplemented with either (figure A) 10% fetal bovine serum lot 717 or (figure B) 5% newborn calf serum in the presence of increasing concentrations of TCDD (i.e., 10, 30 and 60 nM). Cellular proliferation was measured at 48, 72 and 96 hours after culture initiation as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Abbreviations are defined as follows: FBS=fetal bovine serum; NBCS=newborn calf serum; VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.


12000
10000
8000
6000
4000
2000
0
3H-TdR INCORPORATION (CPM)
10% FBS 717
48 HR
72 HR
96 HR
A
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a
a
a
VH
10 nM TCDD
30 nM TCDD
60 nM TCDD
5% NBCS 453
B
TREATMENT

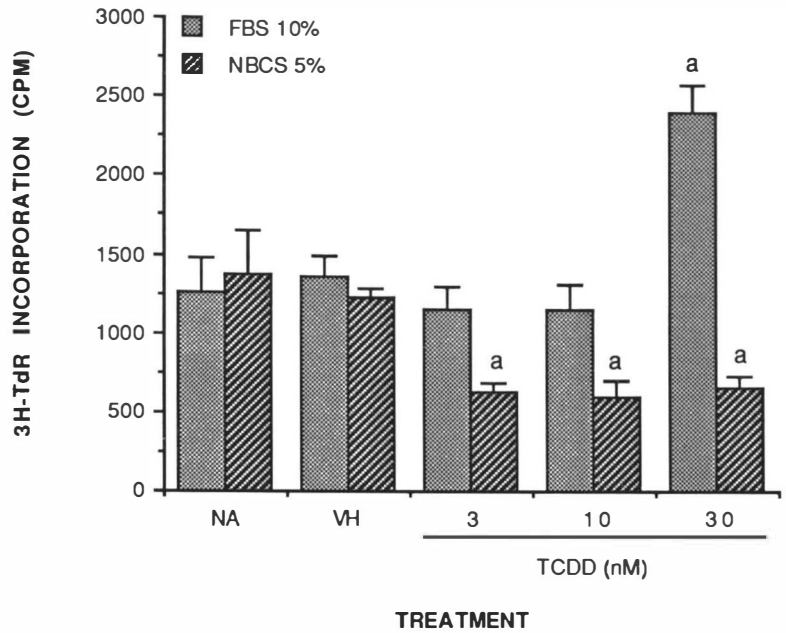


Figure 17. Activation of Dense Resting B Cells Requires the Maximal Suppressive Concentration of TCDD. Highly purified dense resting B cells were isolated as described (see materials and methods) and cultured (1 X 10^5/well) with media supplemented with either 10% fetal bovine serum lot 717 or 5% newborn calf serum in the presence of increasing concentrations of TCDD (i.e., 3, 10 and 30 nM). Cellular proliferation was measured at 72 hours after culture initiation as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Abbreviations are defined as follows: FBS=fetal bovine serum; NBCS=newborn calf serum; NA=naive (media only); VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.

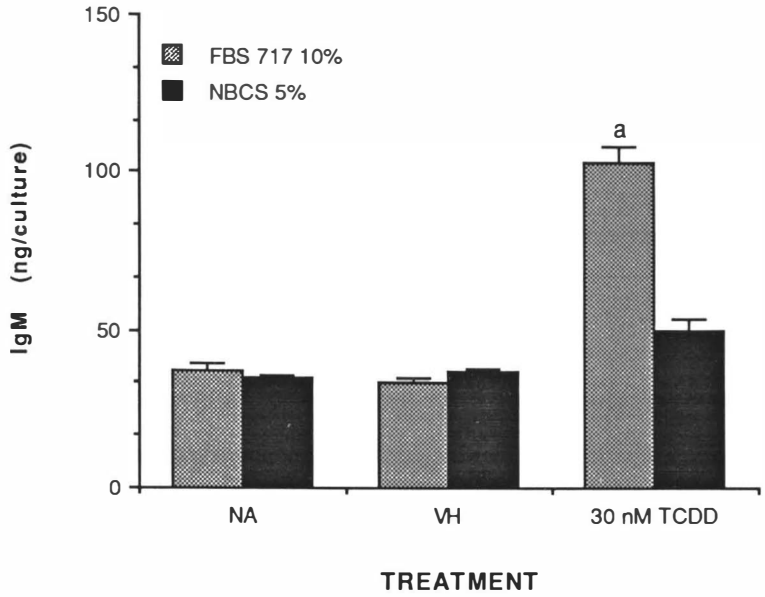


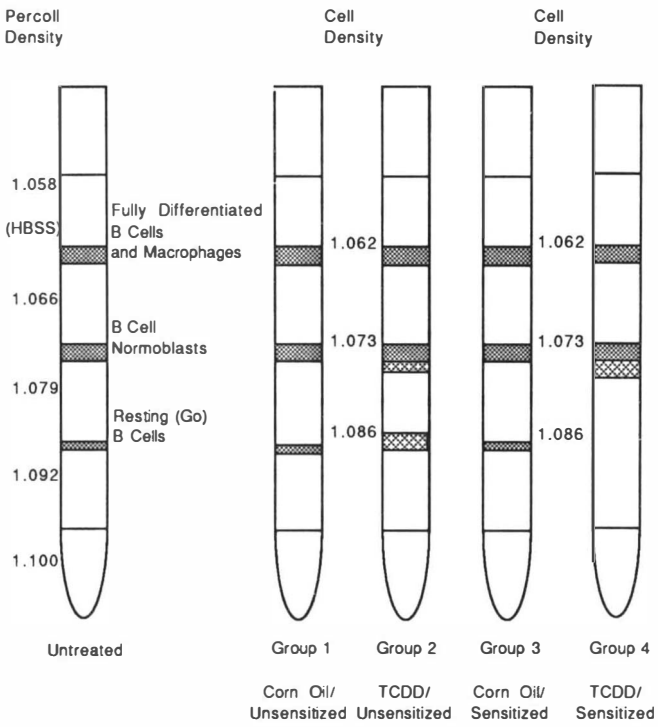
Figure 18. Serum Dependency for Stimulation of IgM Secretion From Splenic-Derived Dense Resting B Cells Following Direct Addition of TCDD. Highly purified dense resting B cells were isolated as described (see materials and methods) and cultured (5 X 10^5/well) with media supplemented with either 10% fetal bovine serum lot 717 or 5% newborn calf serum in the presence of 30 nM TCDD. Supernatants were collected on day 7 of culture and assayed for total murine IgM (see materials and methods). Data is presented as the mean ± SE ng IgM/culture of 6 replicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.

treated controls. Like the effects seen on proliferation, this increase in immunoglobulin production did not occur in the presence of 5% NBCS; further supporting the serum dependency for the enhancements of the antibody response by TCDD.

2. Depletion of Dense Resting B Cells Following In Vivo Sensitization of TCDD Exposed Mice.

B cells in different stages of the cell cycle (particularly resting Go and cycling G1 cells) have distinct buoyant densities. This characteristic therefore allows for the isolation of B cell subpopulations using the well established method of density centrifugation on percoll density step gradients (see materials and methods). To determine if TCDD has the capacity to alter these populations in vivo (in the absence or presence of antigen challenge) an experiment was designed to monitor any changes in these B cell subpopulations. The rationale follows that if TCDD caused an arrest in the differentiation of normally activated B cells (the presumed consequence of TCDD's actions on the B lymphocyte; [Holsapple, et al., 1986a; Dooley and Holsapple, 1988; Luster, et al., 1988b]), then it seemed possible that this type of effect would be manifested on the density gradients by new band formations at densities near to, or less than, that of the cycling B cell (an undifferentiated phenotype). Accordingly, an experiment was devised in which four groups of 8-12 week old mice (3/group) were treated with either 1 μg/kg TCDD (groups 2 and 4) or corn oil (vehicle; groups 1 and 3) for 5 days and rested for 2 days. Animals in groups 1 and 2 (unsensitized) were sacrificed on day 7, their spleens removed and pooled, and the various B cell populations separated on percoll density gradients. As shown in Figure 19 (groups 1 and 2), changes occurred in both the resting (band 3) and cycling (band 2) cell populations isolated from the TCDD-treated mouse spleens as compared to the corn oil-treated controls. These changes were manifested as the formation of a less distinct resting cell band, and the formation of a population of cells which produced a very non-compact

Figure 19. Effect of TCDD Exposure on Splenic B Cell Subpopulations Isolated From Female B6C3F1 Mice. Eight to twelve week old B6C3F1 mice were separated into 4 treatment groups (3 mice/group). Mice were administered either vehicle (corn oil; groups 1 and 3) or 1 μg/kg TCDD (groups 2 and 4) for 5 days, rested for 2 days, and either sacrificed (day 7 after initial dosing; groups 1 and 2) or sensitized by i.p. injection with sheep red blood cells (SRBCs; see materials and methods) and then sacrificed on the peak of an in vivo T-dependent antibody response (day 4). B cell subpopulations were isolated as described in materials and methods. Data are presented as observed on either day 7 after TCDD treatment (groups 1 and 2) or on day 4 following sensitization of TCDD-treated animals with SRBCs (groups 3 and 4). A table is provided to include recovered cell numbers and yields of each respective subpopulation. The data presented are representative of two separate experiments giving almost identical results.



Cell Number and % Yields for Splenic B Cell Subpopulations Isolated from Female B6C3F1 Mice Following In Vivo Exposure to 2,3,7,8-TCDD

Population	Corn Oil/ Group 1 - Unsensitized Cell No. ($X 10^6$)	% Yield	TCDD/ Group 2 - Unsensitized Cell No. ($X 10^6$)	% Yield
Plasma Cells; Macrophages	10.25	4.40	9.85	4.40
B Cell Blasts	23.50	10.00	30.00	13.60
Resting B Cells (Go)	16.25	7.00	24.00	10.90
	Total B Cell Yield=17.00 %		Total B Cell Yield=24.50 %	

Population	Corn Oil/ Group 3 - Sensitized Cell No. ($X 10^6$)	% Yield	TCDD/ Group 4 - Sensitized Cell No. ($X 10^6$)	% Yield
Plasma Cells; Macrophages	19.40	15.60	16.20	7.86
B Cell Blasts	63.10	39.00	57.10	22.33
Resting B Cells (Go)	11.20	8.75	N.D.	N.A.
	Total B Cell Yield=61.64 %		Total B Cell Yield=30.10 %	

Abbreviations: N.D.=Not Detectable; N.A.=Not Applicable

band at a density that was slightly less than that of the matched corn oil-treated resting cells. In addition, there was a second band of cells formed, which was also less compact and appeared at a density just higher than the density of the matched corn oil-treated blasting cells. When the various populations were collected and analyzed for cell number and % yields (based on cells recovered as a percentage of the total lymphocytes present in the original splenocyte preparation) some additional findings were observed (Table/Figure 19). As compared to the corn oil-treated B cell populations, the overall B cell yield had increased in the spleens of the TCDD-treated animals (17% vs 24.5%); indicating the possibility that these cells had been activated in the absence of antigen challenge. This effect was characterized by increases in the number and % yield of cells banding at or near the densities which are recognized as the resting and the blasting cell populations. No change in cell number or yield was evident in the population of cells characterized as containing fully differentiated plasma cells (although other cell types are present; i.e., macrophages). These effects were observed in the absence of any apparent alteration in either spleen weight or total number of lymphocytes recovered from the spleen. The second phase of this study was initiated by sensitizing groups 3 and 4 (day 7 after initial dosing) i.p. with 5×10^8 SRBCs (in 0.2 ml saline) and allowing these mice to respond to this antigen for 4 days prior to isolating the various B cell populations as described above. Interestingly, whereas the three B cell subpopulations from the corn oil-treated animals again appeared normal on the percoll gradients, the isolated B cell populations from the TCDD-treated splenocytes were marked by several distinct changes, the most obvious being a complete loss of the resting B cell pool. This was further characterized by the lack of detection of any cells banding at a buoyant density ≥ 1.082 g/ml on the percoll step gradient. An additional finding was the formation of an even more distinct and denser band, at a density just slightly higher than that of the cycling B cells, which was similar to that seen in the cells isolated from the unsensitized TCDD-treated animals (Figure 19; groups 3 and 4). When the cell number and % yields of each population were analyzed,

we observed a decrease in the % yield of total B cells recovered (Table; Figure 19). Although we have not investigated this effect fully, we feel that because these yields are assessed according to the total number of lymphocytes recovered from the spleen, T cell proliferation following antigen stimulation (in the absence of appreciable B cell proliferation) has skewed the yields accordingly. Thus, the data would appear to be more a function of altered B cell responses in these animals than a loss in total B cells recovered (i.e., this is in comparison to a normal T-dependent antigen driven response where increases in the B cell yields are expected to occur). However, some B cell loss is evidenced by the fact that the number of recovered cells for both the cycling (band 2) and differentiated (band 1) cells are only slightly decreased below controls while the resting B cells have been completely depleted.

3. Serum Dependency for Proliferation Following In Vivo Exposure to TCDD.

To correlate the effects of direct exposure of TCDD on splenic-derived dense B lymphocytes (i.e., activation; as evidenced by an increase in proliferation and production of IgM) with the alterations found in the splenic B cell subpopulations isolated from TCDD-treated mice (as described above), further experiments were conducted to determine whether dense B lymphocytes isolated from TCDD exposed animals, like those exposed directly in culture, would show: 1) a capacity to spontaneously proliferate in culture; and 2) a serum dependency for this effect. As shown in Figure 20, splenic-derived dense B lymphocytes isolated from female B6C3F1 mice exposed to 1 μg/kg/day of TCDD for 5 days (with 2 days rest), showed a 10-fold increase in proliferative capacity as compared to the matched corn oil-treated control cells. This effect was observed when cells were cultured in media containing 10% FBS but not 5% NBCS, and further demonstrates the importance of serum-derived growth factors in the modulation of splenic-derived dense B

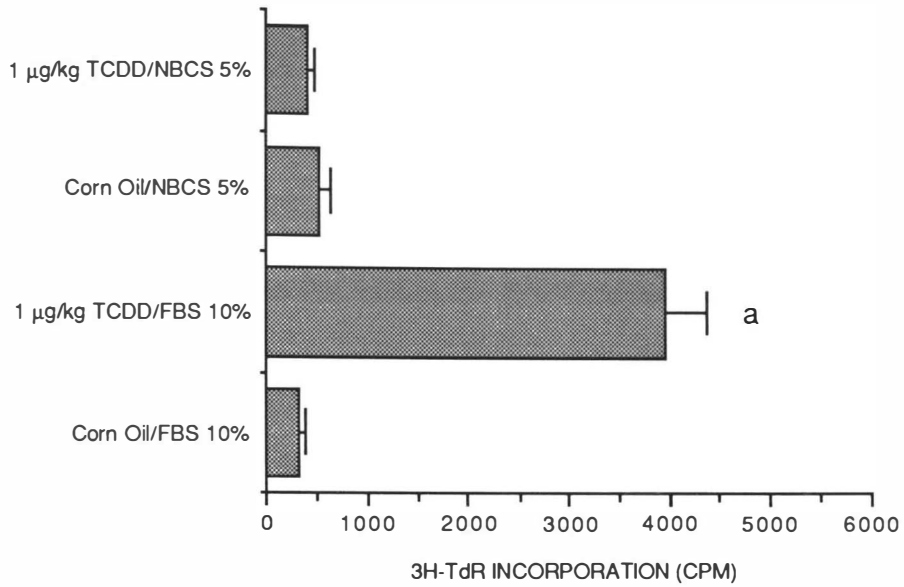


Figure 20. Serum Dependency for Proliferation of Splenic-Derived Dense B Cells Following In Vivo exposure of Female B6C3F1 Mice. Mice were administered either corn oil (vehicle) or 1 µg/kg TCDD for 5 consecutive days before isolation of splenic dense resting B cells. Cells were cultured (1 X 10^5/well) with media supplemented with either 10% fetal bovine serum or 5% newborn calf serum under conditions as described in materials and methods. Cellular proliferation was measured at 48 hours after culture initiation as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.

lymphocyte responses to the effects of dioxin. Cycling B cells, when assayed under comparable conditions, were not found to spontaneously proliferate in culture, but rather showed no effect following the in vivo exposure regimen described above (data not shown).

4. Gamma-Interferon Potentiates the Proliferation Induced by TCDD.

Previous studies in this laboratory have shown that the pleiotropic cytokine, gamma-interferon (G-IFN), can block the immunosuppressive effects of TCDD on the in vitro T-dependent antibody response (Dooley, Holsapple, Snyder and Yang, unpublished observations). Therefore, experiments were conducted to determine the potential interactive effect of G-IFN on the TCDD-induced increase in proliferation of dense resting B cells when administered simultaneously to cultured cells. In addition, the effects of interleukin-2 (IL-2) on both SAC and TCDD-exposed cells were also investigated because of the potentiating effects of this cytokine on the proliferation of SAC activated resting B cells and to determine if the "activation-like" effect of TCDD on dense resting B cells was related to the activation induced by SAC. Moreover, potential cytokine interactions were also determined by adding both IL-2 and G-IFN in combinations to TCDD-treated cultures, in which the time of IL-2 and G-IFN additions were switched between 0 and 24 hours of culture as indicated (Figure 21; t=0 and t=24). A 48 hour time point was chosen to assess the effects of both agents since preliminary studies had determined that only low amounts of proliferation occurred within this time period when cells were exposed to either agent alone (see Figure 16). As demonstrated in Figure 21, neither TCDD (30 nM) and SAC (0.001%) nor IL-2 and G-IFN (or the combination; data not shown) caused any appreciable proliferation of the dense resting B cells above naive or vehicle-treated controls when administered alone within the 48 hour culture period. IL-2 was also shown to have

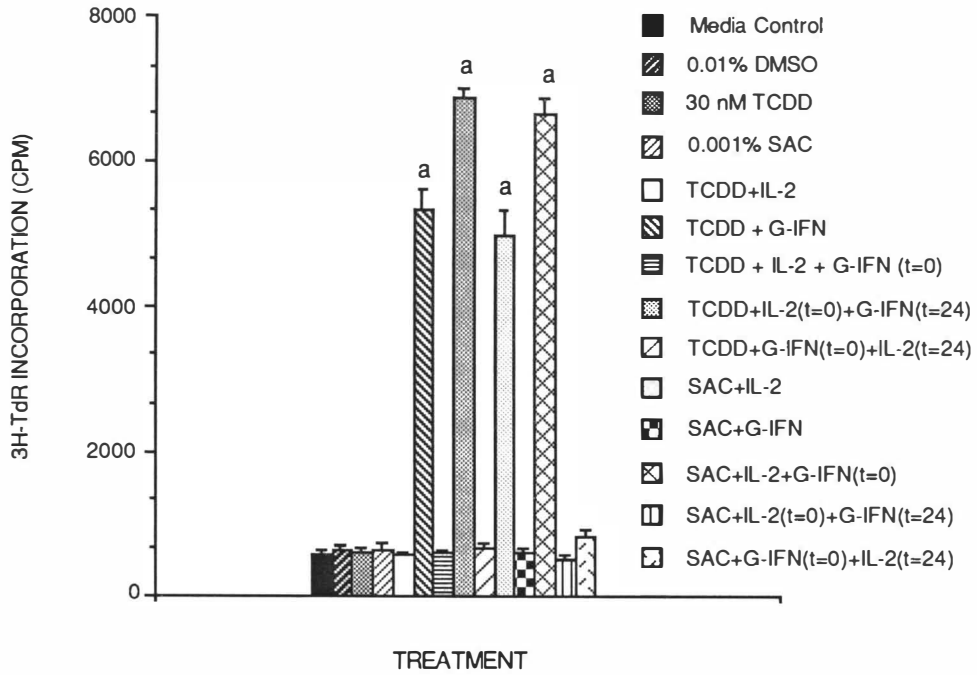


Figure 21. Gamma-Interferon Potentiates the Activation of Dense Resting B Cells by TCDD. Highly purified dense resting B cells were isolated as described (see materials and methods) and cultured (1 X 10^5/well) with media supplemented with 10% fetal bovine serum (lot 717). SAC and TCDD were added at the start of culture (t=0) whereas IL-2 (25 U/ml) and G-IFN (50 U/ml) were added at either the start of (t=0) or at 24 hours after (t=24) culture initiation as indicated in the legend. Cellular proliferation was measured at 48 hours after culture initiation (24 hours after last cytokine addition) as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Controls for the combinations of IL-2 and G-IFN at t=0 and t=24 are not shown in the figure but gave the same response as the media control. Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Abbreviations are defined as follows: DMSO=dimethylsulfoxide; SAC= Staphylococcus aureus Cowan strain I; IL-2=interleukin 2; G-IFN=gamma-interferon. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.

no effect on potentiating the TCDD-induced effects on proliferation. Interestingly however, G-IFN (50 U/ml) added in combination with TCDD, potentiated the activation of the dense resting B cells, demonstrating further that TCDD and G-IFN have an interaction at the level of the B lymphocyte. For SAC-induced effects on the proliferation of resting B cells, the opposite effect from TCDD was seen. SAC in combination with IL-2 caused a proliferation of the B cells to a level comparable with that of the TCDD + G-IFN combination. However, SAC + G-IFN did not produce proliferation of these cells. These findings further suggest that the actions of TCDD and SAC on the B lymphocyte are similar but distinct. Other interesting observations were determined when the combinations of IL-2 and G-IFN were added to TCDD and SAC treated cells. In particular, IL-2 given in combination with G-IFN at t=0 blocked the increased proliferation induced by G-IFN + TCDD alone. However, if cells were exposed to the combination of TCDD + IL-2 for 24 hours and then exposed to G-IFN the proliferative response was reestablished. Conversely, the same combination that suppressed TCDD induced proliferation (i.e., TCDD + IL-2 + G-IFN at t=0) enhanced the proliferation of SAC stimulated B cells above the response by SAC + IL-2 alone; this effect however, was only seen when these cytokines were added simultaneously to culture and not when added at different times (see Figure 21). This study further demonstrates the capacity of TCDD to induce an "activation-like" signal in the resting B lymphocyte and that complex cytokine interactions are important in modulating this effect (particularly IL-2 and G-IFN; products of the T_h 1 subtype of helper T cells).

5. Effects on LPS-Stimulated Proliferation and Differentiation.

To determine whether the TCDD-induced immunoenhancing (or stimulatory) effects in resting B cells (both in vivo and in vitro) and the immunosuppressive effects in whole lymphocyte responses are causally related, studies were conducted to characterize the

effects of TCDD on resting B cells following stimulation with Lipopolysaccharide (LPS). LPS-induced B cell proliferation and differentiation was chosen as the in vitro model system for a number of reasons: 1) LPS is a selective B cell mitogen and can stimulate B cells to proliferate and differentiate in the absence of macrophages and/or T cells; 2) differentiation of B cells in response to LPS does not require the addition of soluble T cell factors and insures that responses mediated by TCDD are due to direct effects on the B cell and not to interactions with soluble mediators that are secreted by the macrophage or T cell; 3) the whole lymphocyte response to LPS has been previously shown to be a sensitive in vitro system for the suppressive effects of TCDD and demonstrates a parallel relationship with the effects of TCDD on the T-dependent antibody response to SRBC [Holsapple, et al., 1986a]. Table 8 shows the effects of increasing concentrations of TCDD (0.3, 3 and 30 nM) on both proliferation (^{3}H-TdR incorporation) and differentiation (IgM and IgG secretion; day 7) of LPS-stimulated dense resting B cells. As demonstrated, TCDD did not have any significant effect on either response in the presence of either 10% FBS (lot 717) or 5% NBCS (lot 453) to either enhance or suppress the function of resting B cells. Vehicle exposure had no effect on either response as compared to the naive control (data not shown). In fact, NBCS alone was found to be suppressive on LPS-induced proliferation and immunoglobulin secretion as compared to FBS, thus suggesting that resting B cells play little role in whole lymphocyte responses to antigen in the presence of suppressive serum conditions. The importance of this study was to demonstrate that, although TCDD appears to enhance resting B cell responses (under specified culture conditions), resting B cells are insensitive to the suppressive effects of TCDD in a model system that has been previously shown to be sensitive to this effect. Therefore, it is concluded from this finding that the immunoenhancing (in either resting B cells or whole lymphocytes in the presence of appropriate FBS) and immunosuppressive effects of TCDD (as seen in the T-dependent antibody response to SRBC) are not causally related.

Table 8

Effect of TCDD on LPS-Stimulated Resting B Cell Proliferation and Differentiation in Fetal Bovine versus Newborn Calf Serum

	3H-TdR Incorporation (mean cpm ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	53905 ± 2568 c	336 ± 25
0.3 nM TCDD	57327 ± 3558	435 ± 54
3.0 nM TCDD	52271 ± 1201	399 ± 60
30 nM TCDD	50836 ± 1799	473 ± 61
	μg IgM/Culture (mean ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	1.35 ± 0.14 d	0.01 ± 0.001
0.3 nM TCDD	1.48 ± 0.17	0.01 ± 0.001
3.0 nM TCDD	1.31 ± 0.22	0.01 ± 0.001
30 nM TCDD	1.37 ± 0.16	0.01 ± 0.001
	ng IgG/Culture (mean ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	1.04 ± 0.09	--- e
0.3 nM TCDD	1.03 ± 0.08	---
3.0 nM TCDD	0.85 ± 0.12	---
30 nM TCDD	0.76 ± 0.05	---

c = Values represent the mean ± SE of absolute CPM of quadruplicate cultures harvested at 72 hours in the presence of increasing concentrations of TCDD and 10 μg/ml LPS (See materials and methods for details). d = Supernatants from 6 cultures/treatment group were collected on day 7 and measured for total murine IgM and IgG. e = No IgG was detected.

D. Effects of TCDD on Cycling (G1) B Cells:

1. Serum-Dependent Effects on Background and LPS-Stimulated Proliferation and Differentiation.

Since TCDD was shown to cause enhancements in resting B cell background responses but to have no effect on LPS-stimulated proliferation or differentiation, further studies were conducted using cycling B cells to determine if this population of cells (primarily G1 cells) are the sensitive cells for the immunosuppressive effects of TCDD. In line with studies conducted in resting B cells (as above), studies were conducted to look at the direct effects of TCDD on both background and LPS-stimulated proliferation and differentiation in cycling B cells. Table 9 shows the effects of increasing concentrations of TCDD (0.3, 3 and 30 nM) on the background proliferation of cycling B cells cultured in the presence of either 10% FBS (lot 717; conditions which did not support suppression of the T-dependent antibody response) or 5% NBCS (lot 453; conditions shown to support suppression of the T-dependent antibody response). As demonstrated, TCDD had a consistent suppressive effect on background proliferation at all concentrations, and in the presence of both serum environments, that was most noted at 72 and 96 hours of culture. However, there was a serum dependency for the degree of suppression produced in the two serums in which the suppression of proliferation was more severe in the presence of NBCS than was seen in the presence of the FBS; the same pattern that was seen in the whole lymphocyte responses to SRBC. It is also noteworthy to point out that NBCS was again shown to support a lower proliferation of these cells than FBS and was similar to what was seen in the resting B cell responses to LPS. IgM secretion was also decreased on day 7 of culture in the presence of the FBS but not NBCS (Table 10). No IgG was detected in either culture supernatant (data not shown). This selective effect on IgM secretion is probably due to the low amounts of background proliferation, and thus

Table 9

Effect of TCDD on Background Proliferation of Cycling (G1) B Cells in the Presence of Fetal Bovine versus Newborn Calf Serum

FBS	^{3}H-TdR Incorporation (mean cpm ± SE)		
Treatment	48 HR	72 HR	96 HR
0.01% DMSO	6898 ± 435 c	14475 ± 395	27266 ± 1313
0.3 nM TCDD	7149 ± 318	12144 ± 237 [a]	22595 ± 221 [a]
3.0 nM TCDD	4568 ± 109 [a]	11280 ± 242 [a]	19242 ± 824 [a]
30 nM TCDD	4540 ± 182 [a]	10615 ± 478 [a]	19993 ± 1192 [a]
NBCS	^{3}H-TdR Incorporation (mean cpm ± SE)		
Treatment	48 HR	72 HR	96 HR
0.01% DMSO	990 ± 484	3513 ± 711	2998 ± 418
0.3 nM TCDD	1460 ± 478	2546 ± 114	2156 ± 122 [a]
3.0 nM TCDD	764 ± 136	657 ± 31 [a]	896 ± 192 [a]
30 nM TCDD	601 ± 65	596 ± 43 [a]	689 ± 52 [a]

c = 1 X 10^5 B cells/well were cultured in the presence of increasing concentrations of TCDD (see materials and methods) and measured for proliferation at 48, 72 and 96 hours of culture as determined by ^{3}H-TdR incorporation. Values represent the mean ± SE of absolute CPM of quadruplicate cultures. Data represents at least 3 separate experiments giving almost identical results. Letter codes for significance as compared to the vehicle control (0.01 % DMSO) are defined as follows: a = $p \leq 0.01$.

Table 10

Effect of TCDD on Background IgM Secretion From Cycling (G1) B Cells in the Presence of Fetal Bovine versus Newborn Calf Serum

Experiment 1	μg IgM/Culture (mean ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	1.35 ± 0.18 c	0.08 ± 0.01
0.3 nM TCDD	1.27 ± 0.13	0.08 ± 0.01
3.0 nM TCDD	1.09 ± 0.01	0.08 ± 0.01
30 nM TCDD	0.93 ± 0.05	0.06 ± 0.01
Experiment 2	μg IgM/Culture (mean ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	0.84 ± 0.06	0.05 ± 0.003
0.3 nM TCDD	0.76 ± 0.06	0.05 ± 0.002
3.0 nM TCDD	0.63 ± 0.03 b	0.04 ± 0.001
30 nM TCDD	0.52 ± 0.03 a	0.04 ± 0.001

c = 5 X 10^5 B cells/well were cultured in the presence of increasing concentrations of TCDD for 7 days. Supernatants were collected and assayed for total murine IgM (see materials and methods). Data is presented as the mean ± SE μg IgM/culture of 6 replicate cultures. Two representative experiments are shown. Letter codes for significance as compared to the vehicle control (0.01% DMSO) are defined as follows: a = $p \leq 0.01$; b = $p \leq 0.05$.

secreted antibody, that is achieved in the presence of the NBCS as compared to FBS, where the difference between the responses in the two sera is reflective of higher levels of new antibody secreted in the presence of FBS during the culture period. In either case, the findings of this study are similar to those of Sharma and Gehring (1979) [Sharma and Gehring, 1979], where effects on background proliferation were seen following direct addition of TCDD to whole splenocyte cultures in the presence of FBS, and further supporting the notion that the primary effects of TCDD on lymphocyte function are effects on their ability to obtain optimal proliferation following antigen stimulation.

Table 11 shows the effect of TCDD on LPS-stimulated proliferation of cycling B cells (at 72 hours of culture) in the presence of the two opposing serum environments. As demonstrated, TCDD had a significant effect on LPS-stimulated proliferation at concentrations as low as 0.3 nM (NBCS). Most notable of these effects was again the serum dependency for the suppression of proliferation which was similar to the effects seen on background proliferation. Specifically, NBCS was shown to support a more dramatic and more consistent effect on LPS-stimulated proliferation than did the FBS, further supporting the effects of TCDD on B cell proliferation and the correlation between the suppression of cycling B cell responses and the effects of TCDD on the in vitro whole lymphocyte responses to SRBC. It is again noteworthy to point out that NBCS was shown to suppress the proliferation of these cells by LPS as compared to responses in the presence of FBS, similar to what was seen in the resting B cell responses to LPS and background responses of the cycling B cells, and further suggests that some interactive effect of serum components within the NBCS are important for suppression of these responses by TCDD. In close parallel to the effects of TCDD on proliferation of the cycling B cells, total IgM secretion following LPS-stimulation (day 7) was suppressed in a dose-related fashion (Figure 22). IgG secretion was also measured in these responses, but showed some variability in the effects by TCDD. As shown in Table 12, two experiments showed a slight but significant suppression of IgG secretion (two of four separate

Table 11

Effect of TCDD on LPS-Stimulated Proliferation of Cycling (G1) B Cells in the Presence of Fetal Bovine versus Newborn Calf Serum

Experiment 1	3H-TdR Incorporation (mean cpm ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	49257 ± 3717 c	6873 ± 199
0.3 nM TCDD	40978 ± 2538	5524 ± 144 b
3.0 nM TCDD	36356 ± 2368 b	5108 ± 318 a
30 nM TCDD	40099 ± 1720	5394 ± 257 b
Experiment 2	3H-TdR Incorporation (mean cpm ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	162939 ± 4162	12400 ± 578
0.3 nM TCDD	151945 ± 3144	9852 ± 629 b
3.0 nM TCDD	136257 ± 2847 a	9070 ± 265 a
30 nM TCDD	139056 ± 3293 a	8749 ± 250 a

c = 1 X 10^5 B cells/well were cultured in the presence of increasing concentrations of TCDD and 10 μg/ml LPS (see materials and methods). Cell proliferation was measured at 72 hours as determined by ^{3}H-TdR incorporation. Values represent the mean ± SE of absolute CPM of quadruplicate cultures. Two representative experiments are shown. Letter codes for significance as compared to the vehicle control (0.01% DMSO) are defined as follows: a = $p \leq 0.01$; b = $p \leq 0.05$.

Figure 22. Serum Dependency for Suppression of LPS-Stimulated IgM Secretion From Splenic-Derived Cycling (G1) B Cells Following Direct Addition of TCDD. Highly purified cycling B cells were isolated as described (see materials and methods) and cultured (5 X 10^5/well) with media supplemented with either (figure A) 10% fetal bovine serum (lot 717) or (figure B) 5% newborn calf serum in the presence lipopolysaccharide (LPS; 10 μg/ml) and increasing concentrations TCDD. Supernatants were collected on day 7 of culture and assayed for total murine IgM (see materials and methods). Data is presented as the mean ± SE μg IgM/culture of 6 replicate cultures. Two representative experiments are shown for each culture condition. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=$p \leq 0.01$; b=$p \leq 0.05$.

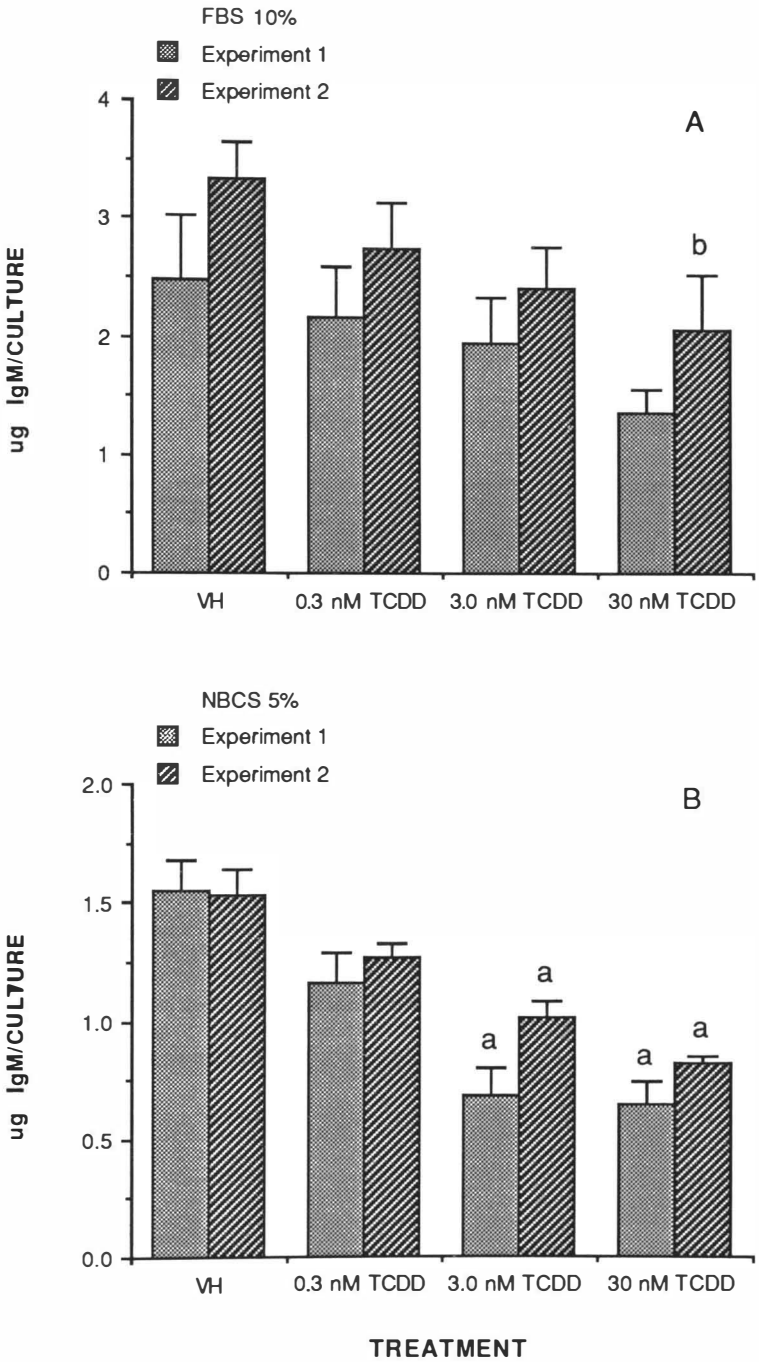

FBS 10%
Experiment 1
Experiment 2
A
b
ug IgM/CULTURE
4
3
2
1
0
VH
0.3 nM TCDD
3.0 nM TCDD
30 nM TCDD
NBCS 5%
Experiment 1
Experiment 2
B
a
a
a
a
ug IgM/CULTURE
2.0
1.5
1.0
0.5
0.0
VH
0.3 nM TCDD
3.0 nM TCDD
30 nM TCDD
TREATMENT

Table 12

Effect of TCDD on LPS-Stimulated IgG Secretion From Cycling (G1) B Cells in the Presence of Fetal Bovine versus Newborn Calf Serum

Experiment 1	ng IgG/Culture (mean ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	6.52 ± 0.23 c	1.01 ± 0.10
0.3 nM TCDD	6.39 ± 0.20	1.20 ± 0.23
3.0 nM TCDD	7.11 ± 0.43	0.87 ± 0.03
30 nM TCDD	6.45 ± 0.18	0.90 ± 0.04

Experiment 2	ng IgG/Culture (mean ± SE)	
Treatment	FBS	NBCS
0.01% DMSO	22.04 ± 1.65	8.07 ± 0.25
0.3 nM TCDD	20.19 ± 0.50	6.33 ± 0.59
3.0 nM TCDD	20.54 ± 0.79	5.92 ± 0.49 b
30 nM TCDD	15.12 ± 0.74 a	5.42 ± 0.26 a

c = 5 X 10^5 B cells/well were cultured for 7 days in the presence of increasing concentrations of TCDD and 10 µg/ml LPS. Supernatants were collected and assayed for total murine IgG (see materials and methods). Data is presented as the mean ± SE ng IgG/culture of 6 replicate cultures. Two representative experiments are shown. Letter codes for significance as compared to the vehicle control (0.01% DMSO) are defined as follows: a = $p \leq 0.01$; b = $p \leq 0.05$.

experiments) following LPS-stimulation in the presence of TCDD, but was not a consistent effect. However, enhancements in either IgM or IgG were never seen in these studies. From these latter studies several interesting observations were made concerning the effects of TCDD on the B lymphocyte, however the most important was the establishment of a direct correlation between the serum dependency for suppression of IgM antibody secretion following LPS-stimulation of cycling B cells and the serum dependency for suppression of the in vitro T-dependent antibody response to SRBC. This finding is important by conclusively identifying the cycling (G1) B cell as the sensitive target cell in suppression of antibody responses by TCDD.

2. Effect of Pre-exposure on IL-2- and IL-4-Mediated Proliferation.

Because of the similarities to SAC, and because of the stimulatory effect on resting B cells, additional studies were carried out to determine if either recombinant IL-2 or IL-4 (both being B cell activation factors) could synergistically act along with the TCDD treatment to activate cycling B cells as TCDD treatment alone activated resting B cells. In preliminary experiments cycling B cells were exposed to either TCDD or the combination of TCDD and recombinant cytokines and was found not to cause proliferation of the cycling B cells within a 72 hour time period. However, this did not rule out the possibility that pre-treatment of the cells with TCDD was required in order to have the proper sequence of stimuli that would normally take place in a whole splenocyte response.
Therefore, experiments were carried out in which purified cycling B cells were cultured for 24 hours in the presence of TCDD (30 nM) and 10% FBS, washed and then reincubated for an additional 48 hours in the presence of increasing concentrations of either IL-2 or IL-4. Proliferation of the cells was measured at 72 hours following a 16 hour pulse with ^{3}H-TdR. As shown in Figure 23, IL-2 seemed to potentiate the effects of the TCDD and resulted in significant increases in proliferation of the cycling B cells at the 50 U/ml

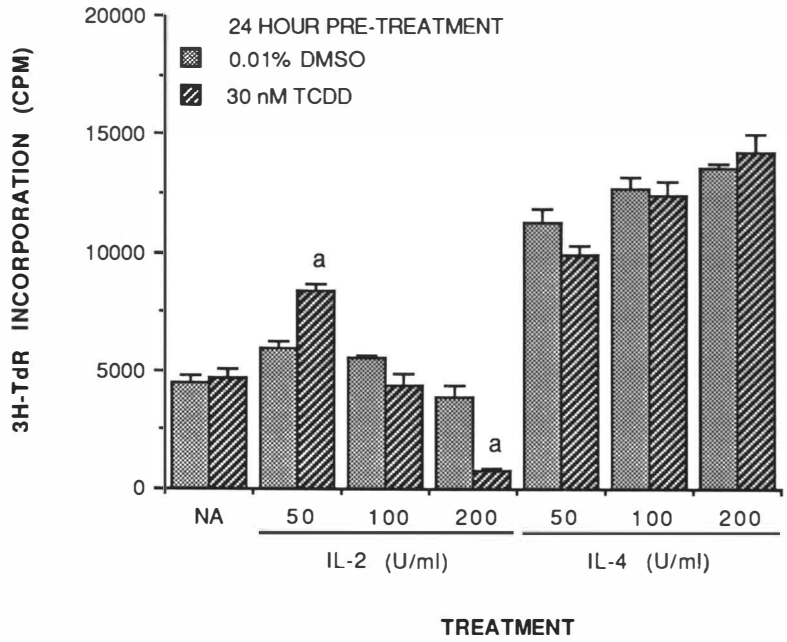


Figure 23. Effect of TCDD Pre-Exposure on Cycling (G1) B Cell Proliferation in the Presence of IL-2 and IL-4. Highly purified cycling B cells were isolated as described (see materials and methods) and cultured (5 X 10^6/well) with media containing 10% fetal bovine serum and either vehicle (0.01% DMSO) or TCDD (30 nM) for 24 hours. Cells were washed and replated in microtiter wells (1 X 10^5/well) in the presence of increasing concentrations of IL-2 and IL-4. Cellular proliferation was measured 24 hours after cytokine additions as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.

concentration as compared to the vehicle-treated control. However, the effects of IL-2 on the proliferative response was demonstrated to be biphasic, in which higher concentrations of IL-2 suppressed proliferation as compared to the vehicle control. Recombinant IL-4 did not alter the background proliferation of these cells at any concentration, and had a normal proliferative pattern as compared to control. These results demonstrate that the stimulatory actions of TCDD on cycling B cells can be manifested if the proper cytokines are available following initial exposure. In addition, the selectivity for the potentiation of the actions by IL-2 (as opposed to IL-4) again finds similarity to the actions of SAC on the B cell, in which IL-2 is the primary growth factor during SAC activation, and further suggests the interactive role of IL-2 in the modulation of TCDD's effects on the B lymphocyte.

E. Effects of TCDD on Background and Con A-Stimulated T Cell Proliferation:

As determined from previous studies looking at suppression of the T-dependent antibody response to SRBC, and from previous and the present studies characterizing the effects on the LPS response, the B lymphocyte appears to be the primary target for suppression of antibody responses whereas the T cell appears to express a relative refractoriness [Holsapple, et al., 1985; Tucker, et al., 1986; Holsapple, et al., 1986a; Dooley and Holsapple, 1988; Luster, et al., 1988b; Dooley, et al., 1990]. Studies were therefore conducted in isolated T cells to determine if effects on either background or Con A-stimulated proliferation of these cells would indicate a similar pattern of effects as the whole cell responses (described above) when compared to the effects of TCDD on the LPS-stimulated response in cycling B cells. Con A-stimulated proliferation has been shown to be a selective model for T cell activation and is a selective stimulus for the T cell, as LPS is for the B cell. It should be noted that the cycling B and T cells as isolated in these studies showed equivalency at all levels of their morphological and functional

characteristics (see materials and methods and results above). Resting T cells were first probed for effects by TCDD on either background or Con A-stimulated proliferation. In these studies, no effect was seen by TCDD to either enhance or suppress proliferation in either system (data not shown). It should be noted however, that background and Con A-stimulated proliferation in these cells was very low and made it difficult to make any conclusive arguments based on the results. Figure 24 shows the effects of TCDD on background (Figure A) and Con A-stimulated (Figure B) proliferation that were seen in the presence of 10% FBS (lot 717). As is demonstrated in this figure, TCDD (3 and 30 nM) caused little effect on background proliferation of these cells whereas Con A-stimulated proliferation was significantly enhanced by the TCDD (3 and 30 nM) treatment in a dose-related manner. The effect was most evident at the 72 hour time point and showed little to no effect at either 24 or 48 hours. A second experiment did show a decrease in background proliferation that was significant at all time points and enhancements in Con A-stimulated proliferation were again observed (data not shown). Lastly, and most important in these studies, was to determine the effects of TCDD on background and Con A-stimulated proliferation of cycling T cells in the presence of 5% NBCS; serum conditions that are selective in supporting suppression of the whole lymphocyte T-dependent and LPS-stimulated cycling B cell antibody responses. As shown in Figure 25 A, TCDD showed a slight suppression of background proliferation at the 72 hour time period. Conversely, cultures stimulated with Con A showed no effect by the TCDD treatment to either enhance or suppress the proliferation of these cells (Figure 25 B). Collectively, the results of these studies using T cells, in comparison to cycling B cells, demonstrate that the T cell is less sensitive to the effects of TCDD when mitogen-stimulated proliferation is used as an endpoint. Not only was there an absence of suppression, even in the presence of the suppressive serum environment (i.e., 5% NBCS), but significant enhancements were seen. This latter effect is interesting from the standpoint that the stimulation of enhancements in the T-dependent antibody response by TCDD is probably due not only to

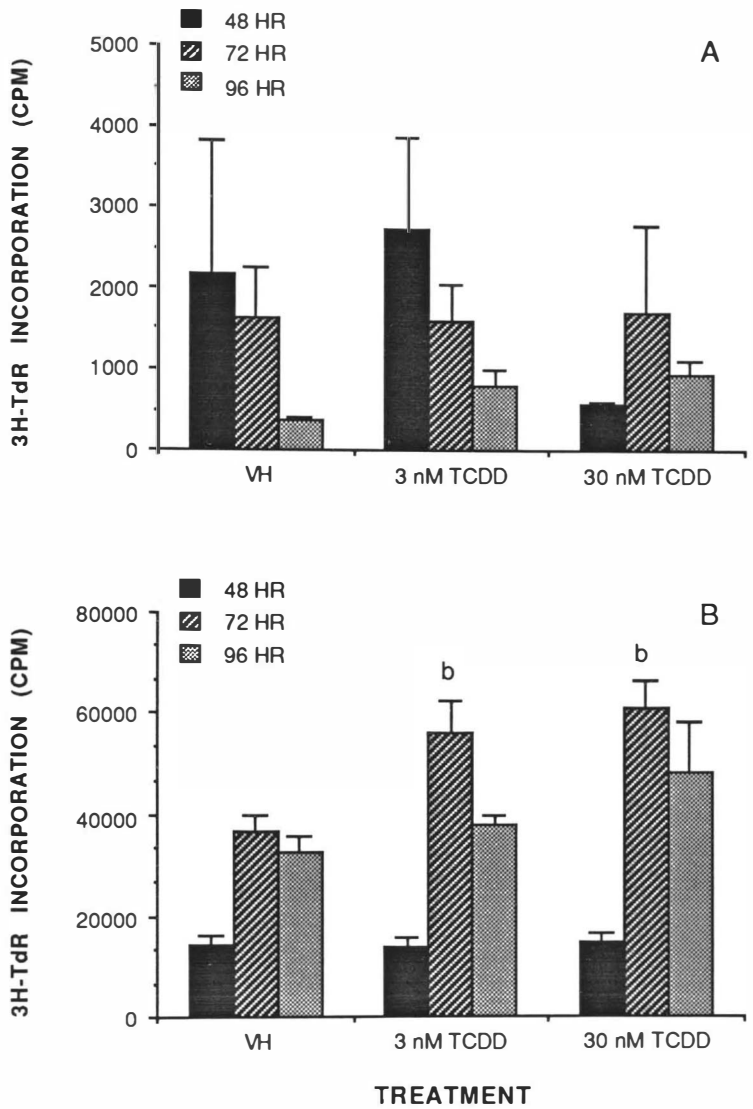


Figure 24. Effect of TCDD on Background and Con A-Stimulated Proliferation of Cycling (G1) T Cells in the Presence of Fetal Bovine Serum. Highly purified cycling T cells were isolated as described (see materials and methods) and cultured (1 X 10^5/well) with media supplemented with 10% fetal bovine serum (lot 717) +/- Con A (5 µg/ml) in the presence of either vehicle or 30 nM TCDD. Background (figure A) and Con A-stimulated (figure B) proliferation were measured at 24, 48, 72 and 96 hours after culture initiation as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Abbreviations are defined as follows: NA=naive (media only); VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01.

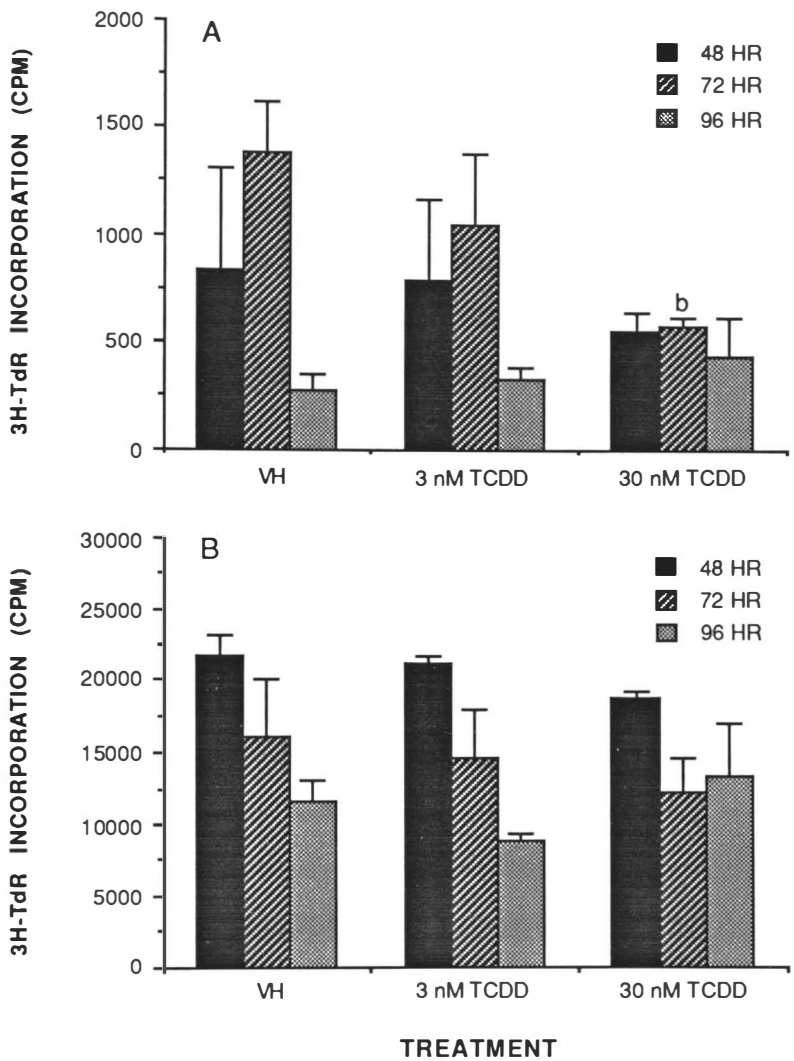


Figure 25. Effect of TCDD on Background and Con A-Stimulated Proliferation of Cycling (G1) T Cells in the Presence of Newborn Calf Serum. Highly purified cycling T cells were isolated as described (see materials and methods) and cultured (1 X 10^5/well) with media supplemented with 10% fetal bovine serum (lot 717) +/- Con A (5 µg/ml) in the presence of either vehicle or 30 nM TCDD. Background (figure A) and Con A-stimulated (figure B) proliferation were measured at 48, 72 and 96 hours after culture initiation as determined by ^{3}H-TdR incorporation following a 16 hour pulse time. Abbreviations are defined as follows: VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of absolute CPM of quadruplicate cultures. Letter codes for significance as compared to the vehicle-treated control are defined as follows: a=p≤0.01; b=p≤0.05.

the stimulatory effects on the resting B cell, but to enhancements of stimulation in the T cell as well. In terms of suppression, although proliferation was unaffected by TCDD direct exposure, there is still the possibility that cytokine production by these cells is altered in a manner which would not provide the necessary help to antigen stimulated B cells. However, in light of the dramatic effects on the cycling B cell (or B cells in general), and previous reports that have found the T cell to be a less sensitive target in this response [Dooley and Holsapple, 1988; Dooley, et al., 1990], it is unlikely that this effect would be of any consequence on the suppression of the antibody response.

F. Role of the *Ah-Gene* Locus in Suppression of the In Vitro Antibody Response and the Modulation of B Cell Function by TCDD:

As reported by Holsapple, et al. (1986) [Holsapple, et al., 1986a] and by Davis and Safe (1991) [Davis and Safe, 1990], suppression of in vitro antibody responses by TCDD, as opposed to in vivo, does not appear to correlate with a role by the *Ah*-receptor gene locus. However, in light of the apparent modulatory role of calf sera on both the TCDD-induced effects on in vitro antibody responses and B cell responses in general (i.e., direct modulation of proliferation and differentiation), it was of interest to determine the effects of TCDD on the in vitro antibody response in the presence of normal mouse serum; the natural serum environment in which mouse spleen cells are exposed in vivo. In addition, splenocytes from both *Ah*-high responder (B6C3F1) and *Ah*-low responder (DBA/2) mice were compared. Specifically, these studies were three tiered in composition. In the first set of studies, the effects of increasing concentrations of TCDD (0.3, 3 and 30 nM) on the in vitro antibody responses to SRBC of splenocytes derived from both B6C3F1 and DBA/2 mice were compared in the presence of increasing concentrations of NBCS (3, 4 and 5%). In the second set of studies, the effects of increasing concentrations of TCDD (0.3, 3 and 30 nM) on the in vitro antibody responses to SRBC in splenocytes derived

from both B6C3F1 and DBA/2 mice were compared in the presence of increasing concentrations of normal mouse sera (3, 4 and 5%). The latter were also compared in cross-over experiments in which the effects of TCDD on splenocyte responses from each strain were tested in cultures containing mouse serum from each of the two strains. These studies were to specifically determine if the *Ah*-phenotype of the two strains could influence the composition of the sera to enhance or block the direct actions of TCDD on this response. Lastly, studies were conducted to determine the modulatory role of serum (i.e., FBS, NBCS, B6C3F1 and DBA/2) on the TCDD-induced ethoxyresorufin-O-deethylase (EROD; P4501A1) activity in primary hepatocytes cultured under comparable in vitro conditions as splenocytes in the AFC responses. Collectively, the objective of these studies was to determine if the role of the *Ah-gene* locus in the modulation of in vitro antibody responses by TCDD was dependent upon the presence of an appropriate serum environment.

Figure 26 shows the effects of increasing concentrations of TCDD (0.3, 3 and 30 nM) on the in vitro T-dependent antibody response to SRBC of B6C3F1 (Figure A) and DBA/2 (Figure B) splenocytes in the presence of increasing concentrations of NBCS (3, 4 and 5%). As can be seen under these culture conditions, TCDD causes a comparable suppression of the antibody response in both B6C3F1 and DBA/2 splenocytes. This finding is consistent with previous reports [Holsapple, et al., 1986a] and would suggest that the direct effects of TCDD on this response is mediated through an *Ah*-independent mechanism.

In the second series of experiments, a model of the in vitro T-dependent antibody response in the presence of normal mouse sera was developed. Two interesting findings however, should be noted: 1) suppressive macrophages (as evidenced by the cytolytic morphology of the cells in culture) complicated the use of freshly isolated splenocytes in the studies where NMS was used and may account for the historical perception that NMS is immunosuppressive. Therefore, a 3 hour adherence to plastic was used to reduce the

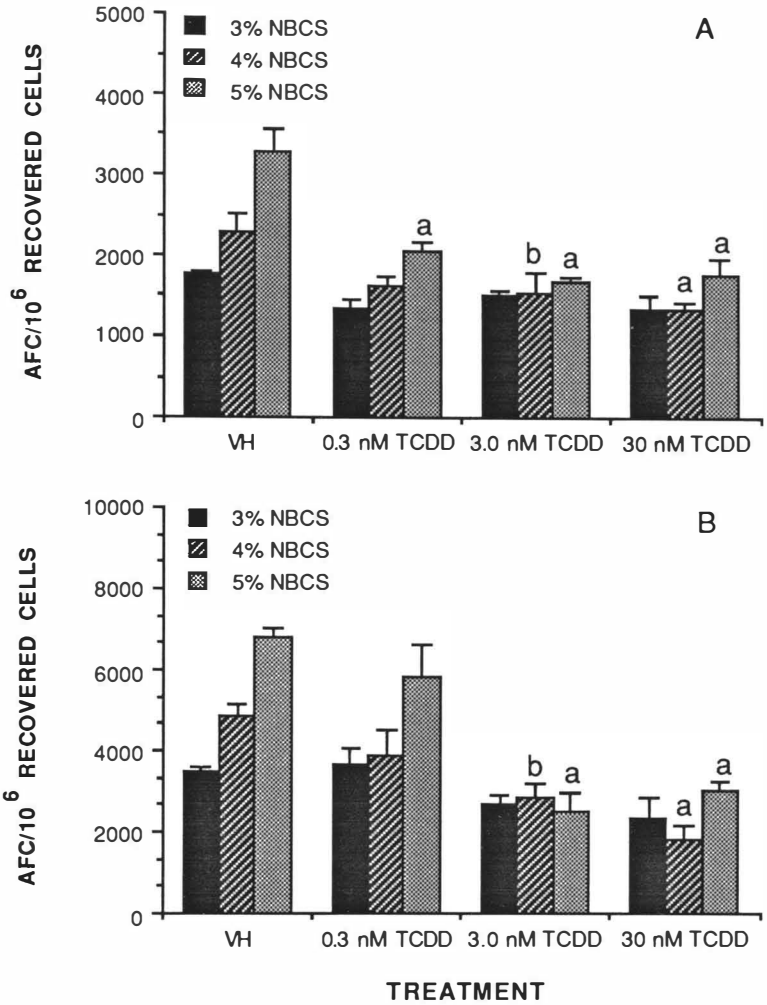


Figure 26. Effect of TCDD on the In Vitro T-Dependent Antibody Response of B6C3F1 and DBA/2 Splenocytes in the Presence of Newborn Calf Serum. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 (figure A) or DBA/2 (figure B) splenocytes in cultures containing media supplemented with increasing concentrations of newborn calf serum (3 to 5%) and TCDD (0.3 to 30 nM). Abbreviations are defined as follows: NBCS=newborn calf serum; VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of 4 replicate cultures/treatment group and is representative of at least 2 separate experiments. Letter codes for significance as compared to the vehicle-treated control cell response are defined as follows: a=p≤0.01; b=p≤0.05.

number of macrophages that were present in the spleens of naive animals, which in turn allowed for a strong antibody response to occur in the presence of normal mouse serum (see materials and methods for details); 2) the serum titration curve for support of growth and antibody responses was much sharper in normal mouse sera than in calf sera with concentrations greater than 5% significantly suppressing both growth and antibody production. As illustrated in Figure 27, when B6C3F1 splenocytes are adhered and cultured in the presence of B6C3F1 normal mouse sera, at concentrations up to 5% of the culture media, antibody responses were obtained that were comparable to those obtained in either FBS or NBCS. It should also be noted that the experiments conducted as presented in Figure 26 (antibody responses in NBCS) were carried out using the adherence procedure to ensure that direct comparisons between responses in calf and mouse sera could be made.

Figure 28 shows the effect of increasing concentrations of TCDD (0.3, 3 and 30 nM) on the antibody responses of both B6C3F1 and DBA/2 splenocytes when cultured in the presence of increasing concentrations of B6C3F1 normal mouse serum (3, 4 and 5%). In contrast to the effects seen in NBCS, responses of the two splenocyte populations to the effects of TCDD addition demonstrated an *Ah*-dependency which parallels the effects that are seen following acute in vivo exposure [Vecchi, et al., 1980; Vecchi, et al., 1983]. Specifically, the responses of B6C3F1 splenocytes were strongly suppressed at all concentrations of TCDD whereas the responses of DBA/2 splenocytes were not suppressed. In fact, the responses in the DBA/2 splenocytes were significantly enhanced (at 0.3 and 3 nM) in a similar manner as was shown to occur in the B6C3F1 splenocytes when cultured in the presence of either TCDD or SAC and 10% FBS (see Figures 10 and 11). In subsequent experiments, it was determined that the *Ah*-dependent phenomena was not dependent upon the strain-derived type of sera that was used to supplement the culture media. As shown in Figure 29, the responses of both B6C3F1 (Figure A) and DBA/2 (Figure B) splenocytes to TCDD direct addition were the same regardless of whether

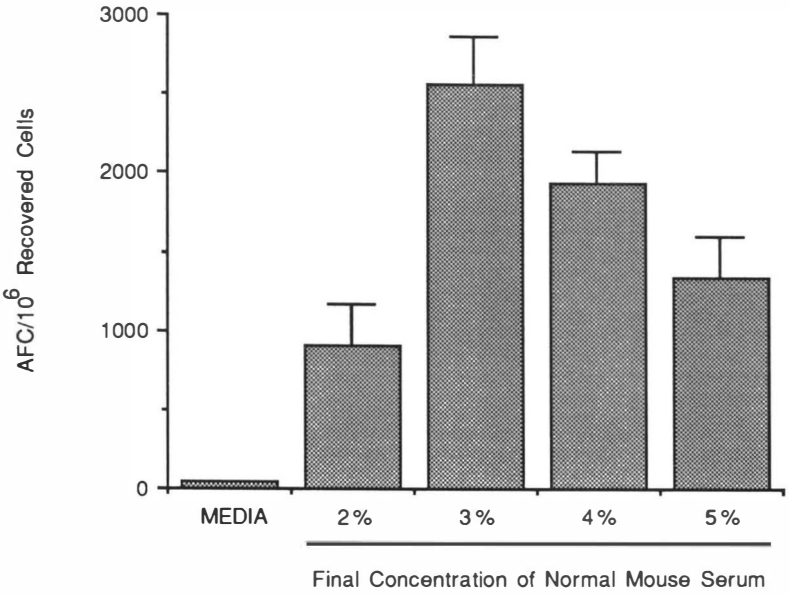


Figure 27. In Vitro T-Dependent Antibody Response in the Presence of Increasing Concentrations of Normal Mouse Serum. Data represents the concentration-dependent support of the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 splenocytes in cultures containing media supplemented with increasing concentrations of B6C3F1 normal mouse serum. Splenocytes were adhered to plastic (see materials and methods) for 3 hours in media containing 5% NBCS and then washed prior to culture in the presence of normal mouse serum and SRBC antigen.

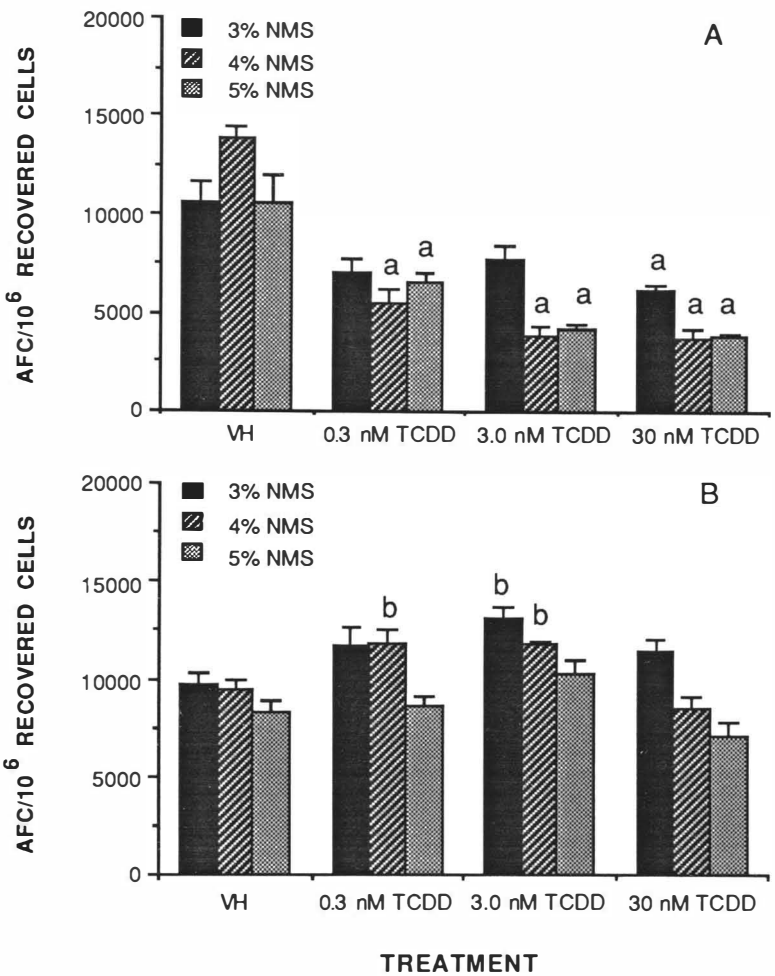
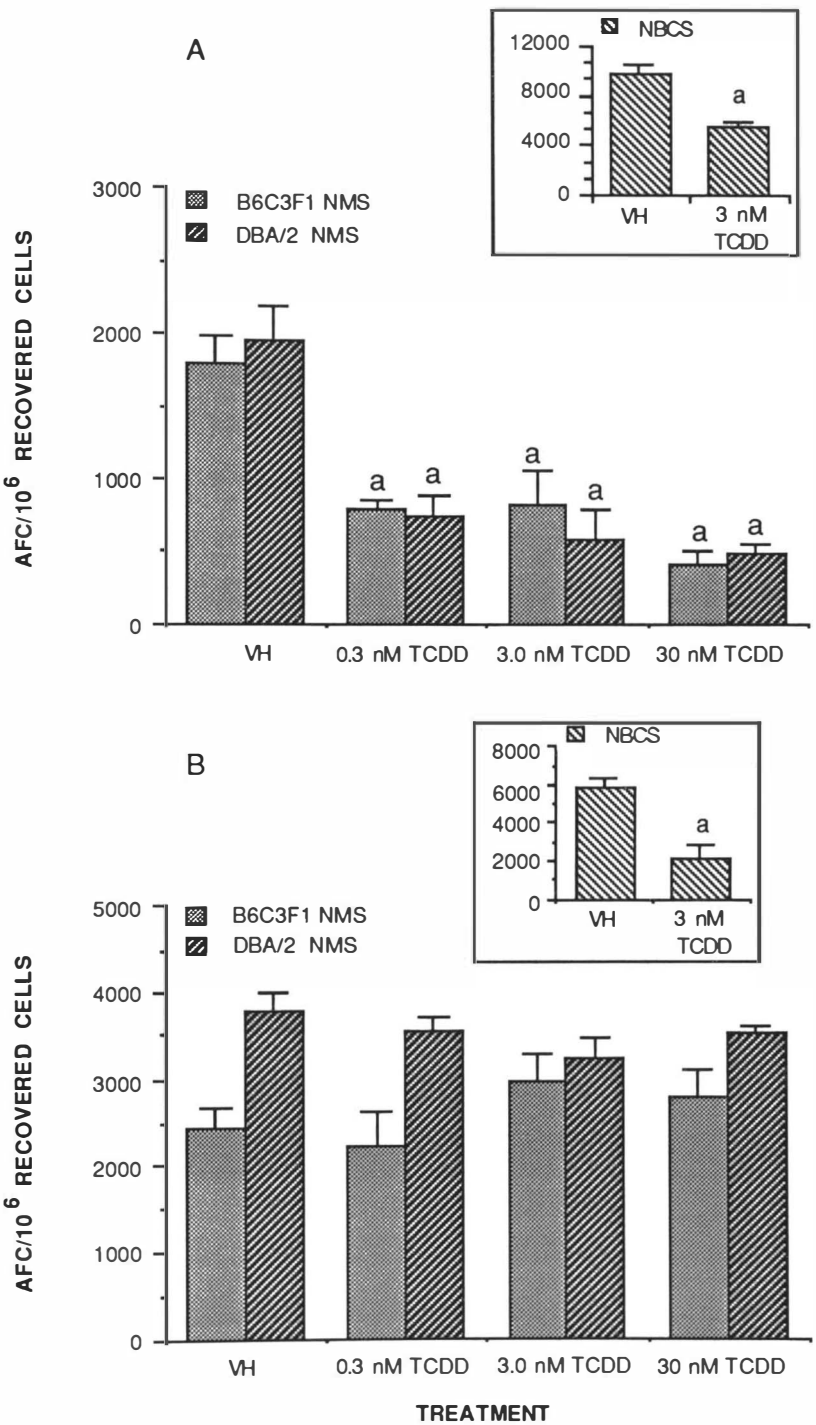


Figure 28. *Ah*-Dependent Suppression of the <u>In Vitro</u> T-Dependent Antibody Response in the Presence of Nomal Mouse Serum. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 (figure A) or DBA/2 (figure B) splenocytes in cultures containing media supplemented with increasing concentrations of B6C3F1 normal mouse serum (3 to 5%) and TCDD (0.3 to 30 nM). Abbreviations are defined as follows: NMS=normal mouse serum; VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of 4 replicate cultures/treatment group. Letter codes for significance as compared to the vehicle-treated control cell response are defined as follows: a=p≤0.01; b=p≤0.05.

Figure 29. *Ah*-Dependent Suppression of the In Vitro T-Dependent Antibody Response is Dependent on the Genotype of the Responding Splenocytes. Data represents the day 5 anti-SRBC antibody forming cell (AFC) response/10^6 recovered B6C3F1 (figure A) or DBA/2 (figure B) splenocytes in cultures with media supplemented with either 5% B6C3F1 or 5% DBA/2 normal mouse serum (as indicated) in the presence of increasing concentrations of TCDD (0.3 to 30 nM). Abbreviations are defined as follows: NMS=normal mouse serum; VH=vehicle (0.01% DMSO). Data is presented as the mean ± SE of 4 replicate cultures/treatment group and is representative of 2 separate experiments. Letter codes for significance as compared to the vehicle-treated control cell response are defined as follows: a=$p \leq 0.01$.


A
NBCS
12000
8000
4000
0
a
VH
3 nM TCDD
3000
2000
1000
0
B6C3F1 NMS
DBA/2 NMS
AFC/10^6 RECOVERED CELLS
a
a
a
a
a
a
VH
0.3 nM TCDD
3.0 nM TCDD
30 nM TCDD
B
NBCS
8000
6000
4000
2000
0
a
VH
3 nM TCDD
5000
4000
3000
2000
1000
0
B6C3F1 NMS
DBA/2 NMS
AFC/10^6 RECOVERED CELLS
VH
0.3 nM TCDD
3.0 nM TCDD
30 nM TCDD
TREATMENT

B6C3F1 or DBA/2 serum was used. It should also be pointed out that responses of the two splenocyte populations in the presence of 3 nM TCDD and 5% NBCS (Figure 29; inserts), as a comparison control, were suppressed equivalently between the two strains as previously shown (see Figure 26).

Lastly, experiments were designed to determine if the modulatory effects of the serum on the in vitro responses of splenocytes to the effects of TCDD were the result of the modulation of responses mediated through the *Ah*-receptor; as has been previously shown in reports where *Ah*-responsiveness was measured in human leukocytes [Kouri, et al., 1979]. Specifically, these studies were to determine if the serum environment could influence the induction of ethoxyresorufin-O-deethylase (EROD; P4501A1) activity in cultured primary hepatocytes. Induction of this enzyme system has been shown to be mediated by the *Ah*-receptor and correlates with the *Ah*-receptor genotype following in vivo exposure of mice [Vecchi, et al., 1983]. Figure 30 demonstrates the effects of either 10% FBS (lot 717) or 5% NBCS (lot 453) on the TCDD-induced (0.3 and 3 nM) induction of EROD activity in primary hepatocytes over a 48 hour culture preiod. Bovine serum albumin (BSA at 5 mg/ml) was included in these experiments as a protein control, where NBCS and FBS, at their respective concentrations, were of approximately equal protein contents (i.e., 5 mg/ml). As is shown, NBCS was found to enhance the EROD activity above the BSA control in a dose related manner, whereas the FBS was the same as the BSA protein control and neither enhanced nor supported a dose-related response of the effects of TCDD. Additionally, Figure 31 demonstrates that NBCS enhances the induction of EROD activity (1 nM TCDD) regardless of concentration (3 and 5%) whereas the FBS shows some alteration in the responses depending on the concentration used in the culture media. Only 5 mg/ml BSA was used as a control in these experiments and it should be pointed out that the alteration in protein content was found to alter the EROD inductive response of hepatocytes in culture in a similar manner and could account for the difference that is seen between 5 and 10% FBS in this experiment (data not shown). Interestingly

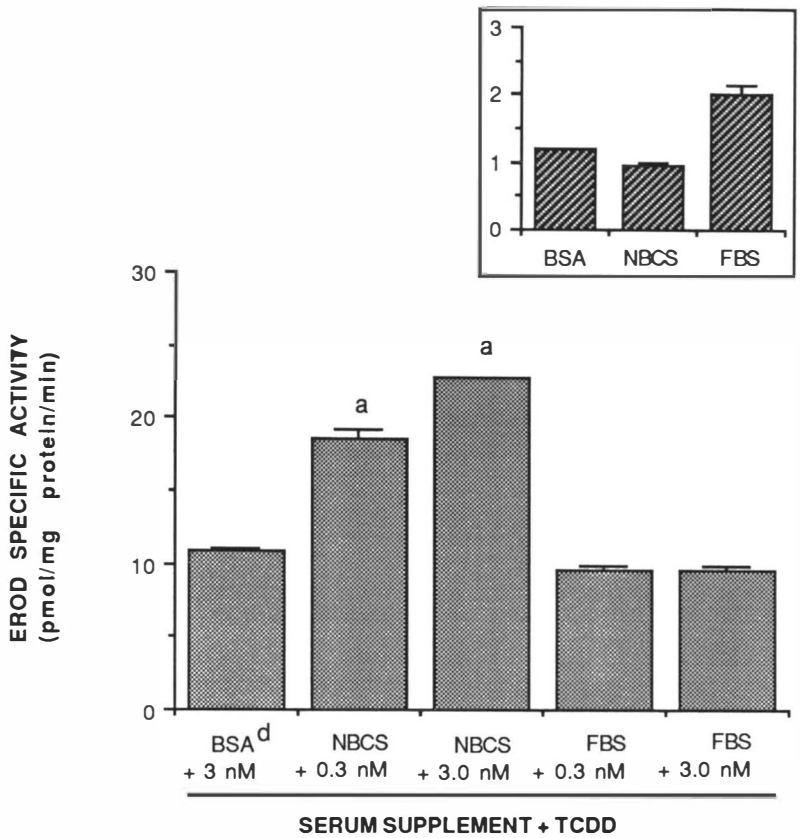


Figure 30. Serum Dependency for TCDD-Induced Ethoxyresorufin-O-deethylase (EROD) Activity in Primary Hepatocytes From B6C3F1 Mice. Data represents the total TCDD-induced EROD specific activity in B6C3F1 primary hepatocytes cultured in AB media for 48 hours in the presence of media supplemented with 5 mg/ml protein of either bovine serum albumin (protein control), newborn calf serum (lot 453) or fetal bovine serum (lot 717; see materials and methods). Basal EROD activity in the presence of the vehicle is indicated in the inserted figure. Data is presented as the mean ± SE of 3 replicate cultures/treatment group and is representative of 2 separate experiments. Abbreviations are defined as follows: BSA=bovine serum albumin; NBCS=newborn calf serum; FBS=fetal bovine serum. Letter codes for significance as compared to the BSA basal or TCDD-treated control response are defined as follows: a=p≤0.01. d = BSA + 3 nM TCDD was used as a maximal induction control at 5 mg/ml protein for both NBCS and FBS.

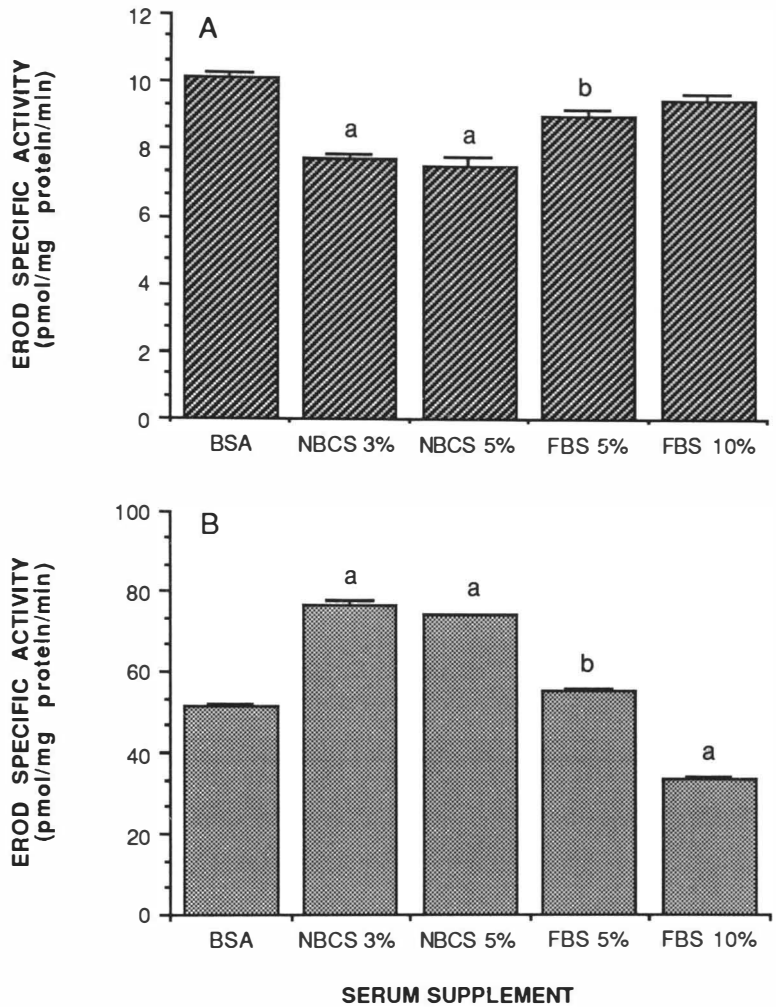


Figure 31. Effect of Serum Concentration on TCDD-Induced Ethoxyresorufin-O-deethylase (EROD) Activity in Primary Hepatocytes From B6C3F1 Mice. Data represents the basal (figure A) and total TCDD-induced (figure B) EROD specific activity in B6C3F1 primary hepatocytes cultured in AB media supplemented with increasing concentrations of newborn calf and fetal bovine serum (see materials and methods). 5% newborn calf serum and 10% fetal bovine serum represent approximately 5 mg/ml protein contents of these respective sera. The BSA control was represents 5 mg/ml protein. Data is presented as the mean ± SE of 3 replicate cultures/treatment group and is representative of 2 separate experiments. Abbreviations are defined as follows: BSA=bovine serum albumin; NBCS=newborn calf serum; FBS=fetal bovine serum. Letter codes for significance as compared to the BSA basal or TCDD-treated control response are defined as follows: a=$p \leq 0.01$; b=$p \leq 0.05$.

however, the in vitro T-dependent antibody responses of splenocytes cultured in media containing 5% FBS (lot 717) were found to support suppression of the antibody response by TCDD (although it did not demonstrate a dose-related effect) and suggests that it is not just the response that is mediated by the *Ah*-receptor, but the level which is important (i.e., different thresholds for enhancements and suppression of the antibody response). Subsequently, experiments were carried out to determine the relative influences of calf and mouse sera on the EROD inductive response of primary hepatocytes. Figure 32 shows the EROD activity generated in B6C3F1 primary hepatocyte cultures containing either FBS, NBCS or normal mouse sera (from B6C3F1 or DBA/2 mice), at equal protein contents, and in the presence of increasing concentrations of TCDD (0.1 and 1 nM). As demonstrated, both NBCS and normal mouse sera from both strains enhanced the EROD activity generated in B6C3F1 hepatocytes above FBS (as the control in this experiment). This finding is consistent with the antibody responses in B6C3F1 splenocytes, in which normal mouse sera from either strain supported a similar suppression of the response as was demonstrated in NBCS (see Figures 26 and 29). This experiment was important from the standpoint that the profile of activity at 0.1 and 1 nM TCDD were the same, where additional experiments comparing the effects of calf and mouse sera on hepatocyte EROD activity were carried out at the single TCDD concentration of 1 nM. In a second experiment, the effects of calf and normal mouse sera on TCDD-induced (1 nM) EROD activity in B6C3F1 primary hepatocyte cultures were compared to the BSA protein control. Figure 33 demonstrates that the same pattern of effects were obtained as compared to the BSA control and further correlates the effects of TCDD on hepatocyte EROD activity with effects on splenocyte antibody responses. Additionally, experiments were carried out to determine the modulatory role of both calf and normal mouse sera on the TCDD-induced (1 nM) EROD activity in hepatocytes from the DBA/2 *Ah*-low responder mouse strain. As previously noted, normal mouse sera was not found to support suppression of the in vitro antibody response by TCDD in DBA/2 splenocytes when directly compared to effects in

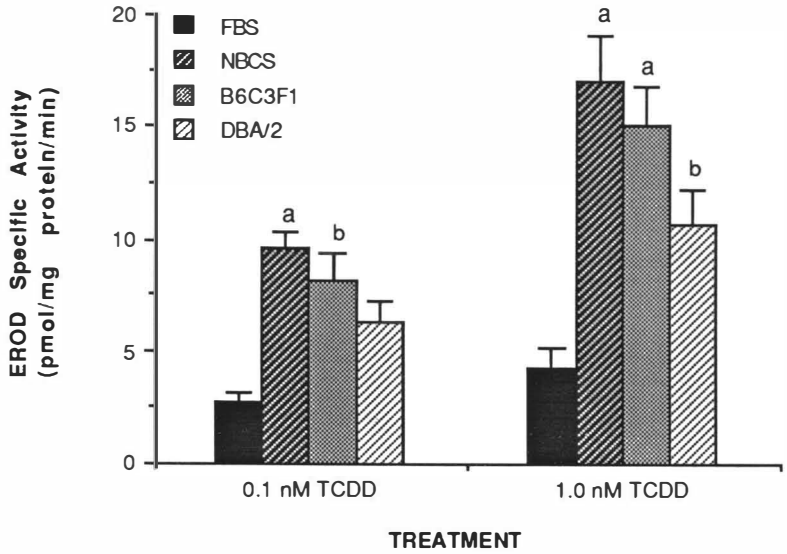


Figure 32. Comparative Effects of FBS, NBCS and NMS on Ethoxyresorufin-O-deethylase (EROD) Activity in Primary Hepatocytes From B6C3F1 Mice Cultured in the Presence of Increasing Concentrations of TCDD. Data represents the absolute TCDD-induced EROD specific activity (i.e., TCDD-induced minus basal activity) in B6C3F1 primary hepatocytes cultured in AB media supplemented with 5 mg/ml protein of either fetal bovine serum (lot 717), newborn calf serum, B6C3F1 normal mouse serum or DBA/2 normal mouse serum in the presence of increasing concentrations of TCDD (0.1 and 1 nM). Data is presented as the mean ± SE of 3 replicate cultures/treatment group and is representative of 2 separate experiments. Abbreviations are defined as follows: FBS=fetal bovine serum; NBCS=newborn calf serum; NMS=normal mouse serum. Letter codes for significance as compared to the FBS control response at each concentration of TCDD are defined as follows: a=$p \leq 0.01$; b=≤ 0.05.

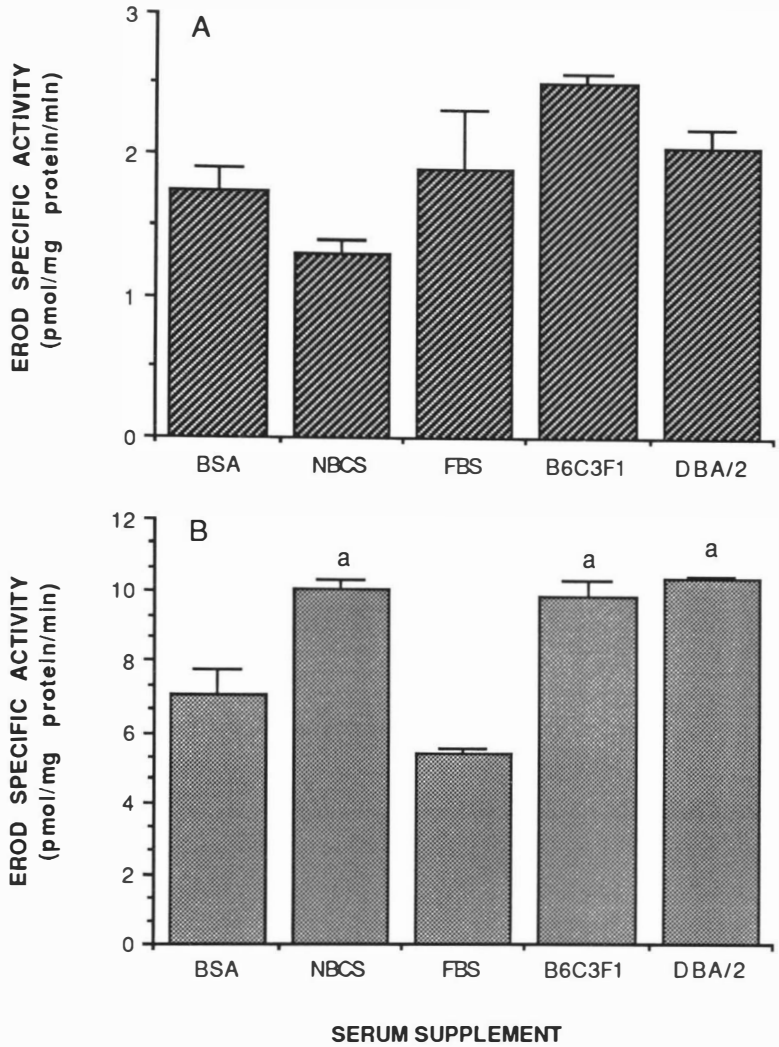


Figure 33. Comparative Effects of BSA, FBS, NBCS and NMS on the TCDD-Induced Ethoxyresorufin-O-deethylase (EROD) Activity in Primary Hepatocytes From B6C3F1 Mice. Data represents the basal (figure A) and total TCDD-induced (figure B) EROD specific activity in B6C3F1 primary hepatocytes cultured in AB media supplemented with 5 mg/ml protein of either bovine serum albumin, fetal bovine serum (lot 717), newborn calf serum, B6C3F1 normal mouse serum or DBA/2 normal mouse serum in the presence of 1 nM TCDD. Abbreviations are defined as follows: BSA=bovine serum albumin; FBS=fetal bovine serum; NBCS=newborn calf serum; NMS=normal mouse serum. Data is presented as the mean ± SE of 3 replicate cultures/treatment group and is representative of 2 separate experiments. Letter codes for significance as compared to the vehicle or TCDD-treated BSA control response are defined as follows: a=$p \leq 0.01$.

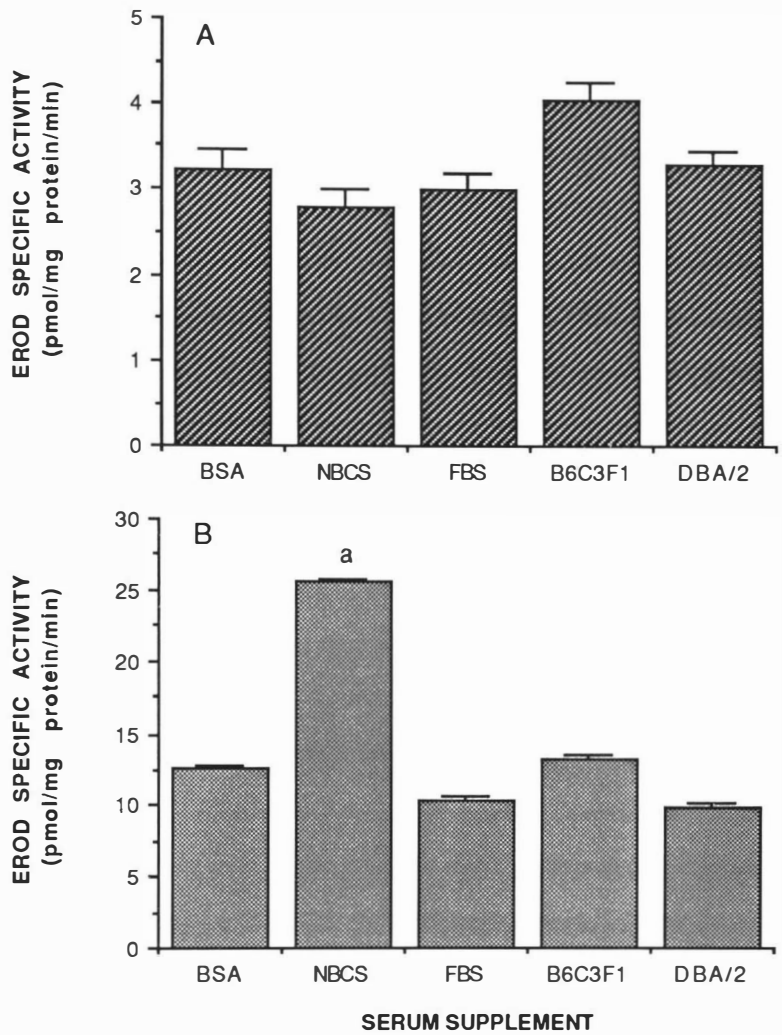


Figure 34. Comparative Effects of BSA, FBS, NBCS and NMS on the TCDD-Induced Ethoxyresorufin-O-deethylase (EROD) Activity in Primary Hepatocytes From DBA/2 Mice. Data represents the basal (Figure A) and total TCDD-induced (Figure B) EROD specific activity in DBA/2 primary hepatocytes cultured in AB media supplemented with 5 mg/ml protein of either bovine serum albumin, fetal bovine serum (lot 717), newborn calf serum, B6C3F1 normal mouse serum or DBA/2 normal mouse serum in the presence of 1 nM TCDD. Abbreviations are defined as follows: BSA=bovine serum albumin; FBS=fetal bovine serum; NBCS=newborn calf serum; NMS=normal mouse serum. Data is presented as the mean ± SE of 3 replicate cultures/treatment group and is representative of 2 separate experiments. Letter codes for significance as compared to the vehicle or TCDD-treated BSA control response are defined as follows: a=$p \leq 0.01$.

the presence of NBCS. As demonstrated in Figure 34, when the sera were again compared, on an equal protein bases, in the DBA/2 (as opposed to the B6C3F1) hepatocytes, only the NBCS continued to support an enhanced induction of EROD activity. The FBS and the normal mouse sera from both strains did not support induction of EROD activity by TCDD (1 nM) above the BSA protein control.

Collectively, these studies establish a direct correlation between the suppression of both whole splenocyte T-dependent and LPS-stimulated cycling B cell antibody responses and responses mediated by the *Ah-gene* locus. Both the lymphocyte antibody responses and EROD activity in primary hepatocytes are influenced by TCDD in a similar manner. Moreover, to reiterate a point that was mentioned above, it appears that it is not simply whether or not an *Ah*-receptor mediated response is elicited by TCDD, but whether or not the level of responsiveness reaches an appropriate threshold to produce either the enhancements or suppression of antibody responses when exposed to TCDD. For example, both 10% FBS and BSA (the protein constituent contained in the serum-free media) support a lower induction of EROD activity in primary hepatocytes and enhancements in the T-dependent antibody response. Conversely, NBCS enhances the EROD activity, above either FBS or BSA, and supports suppression of the antibody response. Mouse sera, on the other hand, either supports a lower or enhanced EROD activity and suppression of the T-dependent antibody response, relative to either BSA and FBS or NBCS, depending upon the *Ah*-genotype of the hepatocytes or splenocytes used for in vitro experimentation.

G. Effects of TCDD on the In Vivo T-Dependent Antibody Response Following Acute and Subchronic Exposures of B6C3F1 (*Ah*-high responder) and DBA/2 (*Ah*-low responder) Mice:

As indicated above, acute exposure of mice to TCDD leads to changes in organ weights (i.e., specifically liver and thymus, reflecting hepatomegaly and involution, respectively) and a suppression of humoral and cell-mediated immunity that is consistent with a role by the *Ah-gene* locus [Sharma and Gehring, 1979; Vecchi, et al., 1983]. However, while these findings are fairly descriptive of acute dosing regimens, one report has indicated that differences may exist in the toxicities produced by TCDD, and closely related congeners, depending on the length of exposure. Specifically, Holsapple, et al. (1986) [Holsapple, et al., 1986b] have reported that subchronic exposure of B6C3F1 mice to the low affinity *Ah*-receptor ligand, 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD), caused a comparable suppression of the in vivo antibody forming cell (AFC) response to SRBC to that seen with TCDD; even in the absence of significant induction of liver P450 activity (i.e., aryl hydrocarbon hydroxylase activity; AHH) [Holsapple, et al., 1986b]. This finding was one of the first to demonstrate that low affinity agonists for the *Ah*-receptor, while having little or no effect on immunity when given acutely, could have dramatic effects on these immune parameters when administered sequentially over a longer period of time.

Studies were therefore conducted to determine if a similar pattern of effects could be induced following subchronic administration of TCDD to DBA/2 (*Ah*-low responder) mice. These mice are less responsive to liver P450 enzyme induction and toxicity induced by TCDD following acute exposures, than are B6C3F1 mice. The reasoning follows the premise that if sequential dosing can enhance the suppression of humoral immunity in B6C3F1 mice by what appears to be non-*Ah*-receptor mediated mechanisms, as previously observed during the subchronic exposure to 2,7-DCDD, then subchronic exposures of

DBA/2 mice to TCDD may likewise be able to increase suppression of the antibody response without significant increases in liver weight or liver ethoxyresorufin-O-deethylase (EROD; P4501A1) activity. Experiments were therefore conducted to compare the TCDD-induced effects on organ weights, both background and T-dependent antigen (SRBC)-stimulated antibody responses, and the microsomal P450 activities generated in both the liver and spleen of B6C3F1 and DBA/2 female mice following acute (single dose; 14 days rest) and subchronic (repetitive daily exposure for 14 days) exposures to the same cumulative doses of TCDD. In addition, flow cytometric analyses were performed to determine if the effects of subchronic TCDD exposure on splenic lymphocyte subpopulations, relative to either acute (2 days rest) or acute (14 days rest), correlate with the effects on splenic microsomal EROD induction or antibody responses.

1. Effects Following Acute (Single Dose; 14 Days Rest) Exposures:

Table 13 illustrates the changes that occurred in body and organ weight parameters following acute (single dose; 14 days rest) exposure of B6C3F1 (*Ah*-high responder) and DBA/2 (*Ah*-low responder) mice to TCDD. Of particular importance are the abrupt changes in liver (hepatomegaly), thymus (involution), and spleen (involution) weights in the B6C3F1 strain which occurred in a dose-related manner (i.e., when normalized to % of body weight). In contrast, DBA/2 mice demonstrated little change in liver, thymus or spleen weights except at the highest dose tested (42 μg/kg). Table 14 shows the acute (14 days rest) effects of TCDD on both the background (IgM AFC) and T-dependent antigen (SRBC)-stimulated antibody responses (presented as AFC/10^6 recovered cells) in B6C3F1 and DBA/2 mice. In the B6C3F1 strain, highly significant reductions in both background and antigen-stimulated antibody responses, as well as spleen cell numbers, were obtained which dose-dependently followed the changes in both liver and thymus weights. Moreover, microsomal EROD activity in the liver was also greatly enhanced in a dose-

Table 13

Body and Organ Weights of SRBC-Sensitized B6C3F1 and DBA/2 Mice Following Acute (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)

	B6C3F1 Mice			
	Vehicle	2,3,7,8-TCDD (µg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	21.08 ± 0.67 c	21.31 ± 0.71	22.06 ± 0.68	21.70 ± 0.47
Liver Weight (g)	1.15 ± 0.06	1.25 ± 0.07	1.37 ± 0.04	1.47 ± 0.09 b
% Body Weight	5.46 ± 0.18	5.85 ± 0.13	6.21 ± 0.12 b	6.75 ± 0.25 a
Thymus Weight (mg)	82.50 ± 3.93	73.75 ± 2.66	66.50 ± 3.52 b	53.25 ± 2.36 a
% Body Weight	0.40 ± 0.03	0.35 ± 0.02	0.30 ± 0.02 b	0.24 ± 0.01 a
Spleen Weight (mg)	105.75 ± 5.76	79.25 ± 5.59	86.25 ± 4.77 b	73.75 ± 7.97 a
% Body Weight	0.51 ± 0.02	0.37 ± 0.02 a	0.39 ± 0.03 b	0.34 ± 0.03 a
	DBA/2 Mice			
	Vehicle	2,3,7,8-TCDD (µg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	20.56 ± 0.46	22.36 ± 0.65	22.01 ± 0.57	20.35 ± 0.73
Liver Weight (g)	0.96 ± 0.03	1.00 ± 0.04	1.06 ± 0.07	0.99 ± 0.03
% Body Weight	4.68 ± 0.16	4.47 ± 0.09	4.80 ± 0.28	4.89 ± 0.18
Thymus Weight (mg)	56.60 ± 4.13	56.80 ± 8.19	52.80 ± 6.93	61.40 ± 8.25
% Body Weight	0.27 ± 0.02	0.26 ± 0.04	0.24 ± 0.03	0.30 ± 0.04
Spleen Weight (mg)	113.00 ± 3.44	126.20 ± 16.90	105.00 ± 5.11	80.60 ± 1.03
% Body Weight	0.55 ± 0.03	0.56 ± 0.07	0.48 ± 0.02	0.40 ± 0.02 b

c = Values represent the mean ± SE of organ weights derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

Table 14

Day 4 IgM Antibody Forming Cell Response in B6C3F1 and DBA/2 Mice Following Acute (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)

	B6C3F1 Mice			
		2,3,7,8-TCDD (µg/kg)		
Parameter	Corn Oil	4.2	14	42
Cells/SRBC-Sensitized Spleen	12.33 ± 0.69 c	8.09 ± 0.50 a	6.80 ± 0.58 a	8.08 ± 0.69 a
Anti-SRBC AFC/Spleen (X 10^3)	151 ± 22	25 ± 6 a	23 ± 6 a	19 ± 8 a
Anti-SRBC AFC/10^6 Recovered Cells	1209 ± 122	322 ± 91 a	344 ± 109 a	219 ± 88 a
Cells/Unsensitized Spleen	9.50 ± 0.60	6.80 ± 0.20 b	8.20 ± 0.26	8.30 ± 0.19
IgM AFC/Spleen (X 10^3)	120 ± 10	75 ± 7 a	50 ± 10 a	29 ± 9 a
IgM AFC/10^6 Recovered Cells	1299 ± 184	1101 ± 22 a	714 ± 147 a	355 ± 94 a
	DBA/2 Mice			
		2,3,7,8-TCDD (µg/kg)		
Parameter	Corn Oil	4.2	14	42
Cells/SRBC-Sensitized Spleen	11.37 ± 0.80	10.98 ± 0.58	10.00 ± 0.95	8.75 ± 1.42
Anti-SRBC AFC/Spleen (X 10^3)	86 ± 11	80 ± 6	34 ± 6 a	32 ± 7 a
Anti-SRBC AFC/10^6 Recovered Cells	781 ± 129	729 ± 52	363 ± 82 b	370 ± 73 b
Cells/Unsensitized Spleen	12.40 ± 0.84	12.50 ± 0.78	11.00 ± 0.26	11.20 ± 0.34
IgM AFC/Spleen (X 10^3)	41 ± 3	36 ± 1	34 ± 2	23 ± 2 a
IgM AFC/10^6 Recovered Cells	339 ± 50	327 ± 19	287 ± 15	200 ± 22 a

c = Values represent the mean ± SE of the day 4 IgM AFC response derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

dependent manner, and further correlated with the changes in both organ weights and antibody responses (Table 15). Conversely, splenic microsomal EROD activity was not induced, but rather reduced, following TCDD exposure (Table 15). Flow cytometric analyses of the splenocytes, derived from treated but unsensitized B6C3F1 mice, revealed slight increases in the percentage and slight decreases in the absolute numbers of both Ig^+ B cells and Lyt-2^+ and L3T4^+ T cells (Table 16). However, the effects on the lymphocyte populations were not found to differ from the effects seen following an acute exposure with only 2 days rest (Table 17); which was used as a length of exposure control in these experiments. In contrast to the B6C3F1 strain, antibody responses (AFC/10^6 recovered cells) in the DBA/2 strain were less affected by acute exposure. Likewise, liver microsomal EROD activity was lower in the DBA/2 mice and showed significant levels of activity only at the two highest doses of TCDD (14 and 42 μg/kg). Microsomal EROD activity in the spleen was undetectable. Flow cytometric analyses of splenocytes, derived from treated but unsensitized DBA/2 mice, demonstrated slight effects on the absolute numbers of both Ig^+ B cells and Lyt-2^+ and L3T4^+ T cells; similar to what was seen in the B6C3F1 strain (Table 16). As in the analysis of B6C3F1 splenocytes, the effects on the lymphocyte populations were not found to differ from the effects seen following an acute exposure followed by only 2 days rest (Table 17); the length of exposure control in these experiments.

2. Effects Following Subchronic (Repetitive 14 Day) Exposures:

In close parallel to acute exposures, body and organ weights in the B6C3F1 strain were similarly affected following the subchronic (repetitive 14 day) exposures to TCDD (Table 18). These effects were characterized by significant and dose-related increases in liver weight parameters, and coordinate decreases in both thymus and spleen weights. Likewise, both the background and antigen-stimulated antibody responses were

Table 15

Hepatic and Splenic Microsomal Parameters in B6C3F1 and DBA/2 Mice Following Acute (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)

	B6C3F1 Mice			
	Vehicle	2,3,7,8-TCDD (μg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	21.90 ± 0.66 c	21.28 ± 0.40	22.29 ± 0.58	22.66 ± 1.50
Liver Weight (g)	1.13 ± 0.06	1.20 ± 0.06	1.39 ± 0.09	1.39 ± 0.10
% Body Weight	5.14 ± 0.12	5.62 ± 0.21	6.24 ± 0.25 a	6.15 ± 0.19 a
Hepatic Microsomal Protein (mg/g liver)	11.68 ± 0.66	15.54 ± 0.31 a	17.70 ± 0.69 a	19.42 ± 0.61 a
Hepatic EROD Activity (pmol/mg protein/min)	15.21 ± 0.58	569.04 ± 33.59 a	763.89 ± 22.77a	1087.93 ± 33.72 a
Spleen Weight (mg)	85.50 ± 6.54	71.25 ± 4.50	74.75 ± 4.21	65.00 ± 3.29 b
% Body Weight	0.39 ± 0.02	0.33 ± 0.02	0.33 ± 0.01	0.29 ± 0.03 b
Splenic Microsomal Protein (μg/mg spleen)	12.02 ± 0.09	18.39 ± 0.12 a	20.34 ± 0.12 a	20.39 ± 0.14 a
Splenic EROD Activity (pmol/g protein/min)	160.00 ± 10.00	70.00 ± 10.00 a	110.00 ± 10.00 a	20.00 ± 10.00 a

	DBA/2 Mice			
	Vehicle	2,3,7,8-TCDD (μg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	20.37 ± 0.60	20.59 ± 0.43	20.10 ± 0.31	21.49 ± 0.61
Liver Weight (g)	0.86 ± 0.02	0.92 ± 0.03	0.90 ± 0.03	1.05 ± 0.01 a
% Body Weight	4.25 ± 0.11	4.57 ± 0.15	4.71 ± 0.23	4.92 ± 0.13 b
Hepatic Microsomal Protein (mg/g liver)	8.88 ± 0.55	9.40 ± 0.70	12.24 ± 0.48 a	13.93 ± 0.74 a
Hepatic EROD Activity (pmol/mg protein/min)	17.36 ± 0.99	53.66 ± 8.98	243.81 ± 14.07 a	496.72 ± 41.46 a
Spleen Weight (mg)	77.80 ± 4.22	74.50 ± 7.31	82.75 ± 10.52	78.20 ± 2.04
% Body Weight	0.38 ± 0.02	0.36 ± 0.03	0.41 ± 0.06	0.37 ± 0.01
Splenic Microsomal Protein (μg/mg spleen)	21.11 ± 0.09	17.26 ± 0.12 a	19.79 ± 0.13 a	24.21 ± 0.14 a
Splenic EROD Activity (pmol/mg protein/min)	----- d	-----	-----	-----

c = Values represent the mean ± SE of organ weights derived from 4-5 unsensitized animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$. d = No measurable EROD activity was detected.

TABLE 16

Flow Cytometric Analysis of Splenic Lymphocyte Populations in B6C3F1 and DBA/2 Mice Following Acute (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Strain/Treatment	Cells/Spleen (X 10^7)	% of Total Splenocytes				Absolute Number of Cells/Spleen (X 10^7)			
		Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$	Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$
B6C3F1									
Corn Oil	7.23 c ± 2.13	49.70 ± 1.60	35.30 ± 1.80	9.70 ± 0.50	18.50 ± 0.70	4.74 ± 0.24	3.35 ± 0.13	0.92 ± 0.02	1.41 ± 0.36
4.2 µg/kg TCDD	6.89 ± 0.24	56.10 a ± 1.00	39.80 ± 1.30	10.80 ± 0.60	20.40 ± 1.20	3.86 b ± 0.07	2.73 ± 0.06	0.74 ± 0.05	1.13 ± 0.30
14.0 µg/kg TCDD	8.21 ± 0.37	54.10 ± 1.40	39.30 ± 1.90	10.40 ± 0.40	21.20 ± 1.20	4.45 ± 0.31	3.24 ± 0.29	0.79 ± 0.09	1.05 ± 0.44
42.0 µg/kg TCDD	8.31 ± 0.20	58.20 a ± 0.80	37.90 ± 1.90	10.40 ± 0.50	21.00 ± 0.60	4.84 ± 0.14	3.15 ± 0.19	0.86 ± 0.04	1.75 ± 0.07
Strain/Treatment	Cells/Spleen (X 10^7)	% of Total Splenocytes				Absolute Number of Cells/Spleen (X 10^7)			
		Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$	Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$
DBA/2									
Corn Oil	12.50 ± 0.84	58.80 ± 1.90	13.80 ± 0.90	3.80 ± 0.30	10.60 ± 0.50	7.37 ± 0.65	1.73 ± 0.21	0.48 ± 0.05	1.32 ± 0.10
4.2 µg/kg TCDD	12.25 ± 0.76	65.20 ± 0.50	15.00 ± 0.60	4.00 ± 0.30	15.50 a ± 1.00	8.00 ± 0.47	1.84 ± 0.14	0.49 ± 0.05	1.90 a ± 0.16
14.0 µg/kg TCDD	11.00 ± 0.27	58.00 ± 1.50	12.10 ± 2.70	2.60 b ± 0.30	11.90 ± 0.70	6.37 ± 0.15	1.34 ± 0.31	0.29 b ± 0.03	1.30 ± 0.09
42.0 µg/kg TCDD	11.30 ± 0.32	63.20 ± 2.10	11.20 ± 0.70	3.00 ± 0.30	11.90 ± 0.50	7.14 ± 0.40	1.27 ± 0.08	0.34 ± 0.03	1.34 ± 0.06

c Values represent the mean ± SE specific staining of splenocytes derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

TABLE 17

Flow Cytometric Analysis of Splenic Lymphocyte Populations in B6C3F1 and DBA/2 Mice Following Acute (2 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Strain/Treatment	Cells/Spleen (X 10^7)	% of Total Splenocytes Ig$^+$	Thy-1.2$^+$	Lyt-2$^+$	L3T4$^+$	Absolute Number of Cells/Spleen (X 10^7) Ig$^+$	Thy-1.2$^+$	Lyt-2$^+$	L3T4$^+$
B6C3F1									
Corn Oil	9.39 c ± 0.34	55.20 ± 1.40	47.20 ± 0.80	10.90 ± 0.10	26.40 ± 0.90	5.20 ± 0.30	4.41 ± 0.12	1.02 ± 0.04	2.47 ± 0.09
4.2 µg/kg TCDD	8.01 ± 0.36	56.30 ± 1.10	47.60 ± 2.70	11.30 ± 0.50	26.00 ± 1.20	4.59 ± 0.27	3.80 ± 0.22	0.90 ± 0.05	2.08 ± 0.13
14.0 µg/kg TCDD	8.75 ± 0.57	57.80 ± 1.40	44.50 ± 1.70	10.30 ± 0.60	26.00 ± 1.10	5.05 ± 0.28	3.90 ± 0.32	0.91 ± 0.11	2.27 ± 0.19
42.0 µg/kg TCDD	8.92 ± 0.28	57.70 ± 0.60	47.90 ± 1.50	10.50 ± 0.30	26.10 ± 0.70	5.15 ± 0.20	4.27 ± 0.18	0.93 ± 0.03	2.40 ± 0.10
Strain/Treatment	Cells/Spleen (X 10^7)	% of Total Splenocytes Ig$^+$	Thy-1.2$^+$	Lyt-2$^+$	L3T4$^+$	Absolute Number of Cells/Spleen (X 10^7) Ig$^+$	Thy-1.2$^+$	Lyt-2$^+$	L3T4$^+$
DBA/2									
Corn Oil	9.97 ± 0.83	68.60 ± 2.00	21.10 ± 1.20	5.30 ± 0.60	16.40 ± 0.40	7.00 ± 0.30	2.15 ± 0.07	0.53 ± 0.02	1.67 ± 0.07
4.2 µg/kg TCDD	8.29 ± 0.84	67.50 ± 1.70	19.90 ± 2.20	5.10 ± 0.60	15.40 ± 1.30	5.56 ± 0.47	1.61 b ± 0.14	0.41 ± 0.04	1.26 b ± 0.11
14.0 µg/kg TCDD	7.10 ± 0.44	70.50 ± 1.10	24.70 ± 0.70	5.80 ± 0.30	18.70 ± 0.60	5.00 b ± 0.27	1.76 ± 0.12	0.41 b ± 0.02	1.32 ± 0.08
42.0 µg/kg TCDD	7.80 ± 1.26	69.70 ± 2.30	19.80 ± 2.90	4.80 ± 0.40	15.30 ± 1.20	5.40 ± 0.73	1.47 a ± 0.05	0.37 b ± 0.03	1.17 b ± 0.11

c Values represent the mean ± SE specific staining of splenocytes derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

Table 18

Body and Organ Weights of SRBC-Sensitized B6C3F1 and DBA/2 Mice Following Subchronic (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)

	B6C3F1 Mice			
	Vehicle	2,3,7,8-TCDD (µg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	21.21 ± 0.46 c	21.79 ± 0.99	21.94 ± 0.72	22.02 ± 0.41
Liver Weight (g)	1.13 ± 0.04	1.38 ± 0.12 b	1.51 ± 0.02 a	1.53 ± 0.02 a
% Body Weight	5.36 ± 0.26	6.32 ± 0.26 b	6.91 ± 0.18 a	6.96 ± 0.18 a
Thymus Weight (mg)	57.75 ± 2.25	62.50 ± 3.38	49.25 ± 2.66	34.00 ± 2.80 a
% Body Weight	0.27 ± 0.01	0.29 ± 0.01	0.23 ± 0.02 b	0.15 ± 0.01 a
Spleen Weight (mg)	97.50 ± 4.29	80.00 ± 6.57	81.00 ± 4.56	78.75 ± 4.13
% Body Weight	0.46 ± 0.02	0.37 ± 0.02 b	0.37 ± 0.03 b	0.36 ± 0.02 b

	DBA/2 Mice			
	Vehicle	2,3,7,8-TCDD (µg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	20.66 ± 0.65	20.60 ± 0.22	20.36 ± 0.62	20.20 ± 0.30
Liver Weight (g)	1.00 ± 0.05	1.01 ± 0.01	1.03 ± 0.03	1.10 ± 0.03
% Body Weight	4.82 ± 0.11	4.90 ± 0.06	5.07 ± 0.21	5.43 ± 0.13 b
Thymus Weight (mg)	66.00 ± 2.35	52.25 ± 5.20 b	42.80 ± 2.96 a	40.00 ± 2.86 a
% Body Weight	0.32 ± 0.02	0.25 ± 0.03	0.21 ± 0.02 a	0.20 ± 0.01 a
Spleen Weight (mg)	89.60 ± 2.62	82.50 ± 1.19	77.40 ± 2.62 b	81.75 ± 5.76
% Body Weight	0.43 ± 0.01	0.40 ± 0.01	0.38 ± 0.02 b	0.40 ± 0.02

c = Values represent the mean ± SE of organ weights derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

Table 19

Day 4 IgM Antibody Forming Cell Response in B6C3F1 and DBA/2 Mice Following Subchronic (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)

	B6C3F1 Mice			
		2,3,7,8-TCDD (µg/kg)		
Parameter	Corn Oil	4.2	14	42
Cells/SRBC-Sensitized Spleen	13.77 ± 0.38 c	11.76 ± 0.66 b	10.97 ± 0.44 a	10.69 ± 0.38 a
Anti-SRBC AFC/Spleen (X 10^3)	298 ± 44	45 ± 6 a	19 ± 1 a	22 ± 4 a
Anti-SRBC AFC/10^6 Recovered Cells	2468 ± 320	387 ± 41 a	176 ± 13 a	212 ± 45 a
Cells/Unsensitized Spleen	12.30 ± 1.50	10.20 ± 0.51	9.00 ± 0.27	8.40 ± 0.25
IgM AFC/Spleen (X 10^3)	48 ± 2	29 ± 2 a	24 ± 1 a	18 ± 2 a
IgM AFC/10^6 Recovered Cells	402 ± 56	283 ± 8	269 ± 8 b	207 ± 13 a

	DBA/2 Mice			
		2,3,7,8-TCDD (µg/kg)		
Parameter	Corn Oil	4.2	14	42
Cells/SRBC-Sensitized Spleen	15.62 ± 0.51	15.00 ± 0.46	13.02 ± 0.55	15.00 ± 1.32
Anti-SRBC AFC/Spleen (X 10^3)	151 ± 16	80 ± 7 a	48 ± 13 a	32 ± 8 a
Anti-SRBC AFC/10^6 Recovered Cells	962 ± 72	534 ± 100 a	377 ± 105 a	222 ± 61 a
Cells/Unsensitized Spleen	12.40 ± 0.84	12.50 ± 0.78	11.00 ± 0.26	11.20 ± 0.34
IgM AFC/Spleen (X 10^3)	85 ± 8	70 ± 4	46 ± 3 a	54 ± 6 a
IgM AFC/10^6 Recovered Cells	670 ± 70	601 ± 39	415 ± 28 a	493 ± 56 b

c = Values represent the mean ± SE of the day 4 IgM AFC response derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

Table 20

Hepatic and Splenic Microsomal Parameters in B6C3F1 and DBA/2 Mice Following Subchronic (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)

	B6C3F1 Mice			
	Vehicle	2,3,7,8-TCDD (µg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	21.74 ± 0.74 c	21.89 ± 0.93	20.29 ± 0.34	20.10 ± 0.51
Liver Weight (g)	1.13 ± 0.07	1.39 ± 0.06 a	1.35 ± 0.03 b	1.36 ± 0.03 a
% Body Weight	5.18 ± 0.15	6.35 ± 0.11 a	6.65 ± 0.04 a	6.79 ± 0.17 a
Hepatic Microsomal Protein (mg/g liver)	15.02 ± 0.88	18.52 ± 0.18.52 b	21.55 ± 0.72 b	22.35 ± 0.31 a
Hepatic EROD Activity (pmol/mg protein/min)	4.00 ± 2.00	821.00 ± 18.00 a	961.00 ± 81.00 a	1029.00 ± 6.00 a
Spleen Weight (mg)	72.75 ± 7.58	55.75 ± 7.97	50.75 ± 7.09	51.00 ± 5.73
% Body Weight	0.33 ± 0.03	0.25 ± 0.03	0.25 ± 0.04	0.25 ± 0.03
Splenic Microsomal Protein (µg/mg spleen)	11.24 ± 0.13	24.12 ± 0.58 a	8.21 ± 0.13 a	9.53 ± 0.07 b
Splenic EROD Activity (pmol/g protein/min)	90.00 ± 10.00	940.00 ± 18.0 a	1662.00 ± 50.0 a	531.00 ± 18.00a

	DBA/2 Mice			
	Vehicle	2,3,7,8-TCDD (µg/kg)		
Parameter	(corn oil)	4.2	14	42
Body Weight (g)	19.47 ± 0.50	19.79 ± 0.61	19.81 ± 0.91	20.71 ± 1.13
Liver Weight (g)	0.82 ± 0.07	0.92 ± 0.06	0.96 ± 0.09	1.08 ± 0.05 b
% Body Weight	4.18 ± 0.26	4.65 ± 0.23	4.87 ± 0.42	5.24 ± 0.16
Hepatic Microsomal Protein (mg/g liver)	12.04 ± 0.69	13.97 ± 0.63	15.47 ± 1.28 b	14.86 ± 0.21 b
Hepatic EROD Activity (pmol/mg protein/min)	10.81 ± 0.77	52.41 ± 7.90	267.15 ± 39.35 a	326.78 ± 71.37a
Spleen Weight (mg)	72.00 ± 9.27	70.00 ± 8.50	67.50 ± 6.67	62.25 ± 1.65
% Body Weight	0.37 ± 0.04	0.35 ± 0.04	0.34 ± 0.03	0.30 ± 0.02
Splenic Microsomal Protein (µg/mg spleen)	14.17 ± 0.07	16.50 ± 0.13 a	8.35 ± 0.03 a	7.93 ± 0.09 a
Splenic EROD Activity (pmol/g protein/min)	20.00 ± 10.00	80.00 ± 10.00 a	150.00 ± 10.00 a	30.00 ± 10.00

c = Values represent the mean ± SE of organ weights derived from 4-5 unsensitized animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

TABLE 21

Flow Cytometric Analysis of Splenic Lymphocyte Populations in B6C3F1 and DBA/2 Mice Following Subchronic (14 day) Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Strain/Treatment	Cells/Spleen (X 10^7)	% of Total Splenocytes				Absolute Number of Cells/Spleen (X 10^7)			
		Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$	Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$
B6C3F1									
Corn Oil	13.63 c ± 1.41	55.70 ± 0.60	32.70 ± 2.90	5.00 ± 0.30	16.10 ± 1.50	7.49 ± 0.68	4.38 ± 0.11	0.68 ± 0.09	1.51 ± 0.65
4.2 µg/kg TCDD	10.28 b ± 0.52	61.30 a ± 0.90	36.00 ± 1.90	5.00 ± 0.20	20.40 a ± 0.50	6.30 ± 0.34	3.72 ± 0.34	0.51 ± 0.03	2.07 ± 0.12
14.0 µg/kg TCDD	9.10 a ± 0.28	61.10 a ± 1.00	30.50 ± 0.80	6.10 ± 0.60	18.30 ± 0.50	5.55 b ± 0.16	2.77 b ± 0.03	0.55 ± 0.05	1.66 ± 0.07
42.0 µg/kg TCDD	8.62 a ± 0.27	63.70 a ± 0.60	37.20 ± 1.10	6.80 b ± 0.20	19.60 b ± 0.90	5.48 b ± 0.17	3.21 ± 0.18	0.59 ± 0.03	1.70 ± 0.12

Strain/Treatment	Cells/Spleen (X 10^7)	% of Total Splenocytes				Absolute Number of Cells/Spleen (X 10^7)			
		Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$	Ig^+	$Thy\text{-}1.2^+$	$Lyt\text{-}2^+$	$L3T4^+$
DBA/2									
Corn Oil	12.59 ± 0.26	60.70 ± 1.30	21.70 ± 0.90	3.40 ± 0.20	15.00 ± 0.70	7.71 ± 0.26	2.76 ± 0.16	0.44 ± 0.03	1.91 ± 0.11
4.2 µg/kg TCDD	12.11 ± 0.90	65.00 b ± 0.90	25.10 ± 1.00	3.60 ± 0.20	17.60 a ± 0.50	7.88 ± 0.64	3.01 ± 0.11	0.43 ± 0.01	2.12 ± 0.11
14.0 µg/kg TCDD	11.17 ± 0.33	65.10 a ± 0.90	24.90 ± 1.30	4.10 ± 0.40	17.40 b ± 0.60	7.27 ± 0.24	2.78 ± 0.21	0.45 ± 0.05	1.95 ± 0.11
42.0 µg/kg TCDD	10.93 ± 0.17	70.60 a ± 0.50	27.20* ± 1.70	4.00 ± 0.20	18.50 a ± 0.60	7.71 ± 0.09	2.98 ± 0.18	0.44 ± 0.02	2.03 ± 0.07

c Values represent the mean ± SE specific staining of splenocytes derived from 4-5 animals/treatment group. Significance, as determined by the Dunnett's T test, is indicated by asterisks: b = $p \leq 0.05$; a = $p \leq 0.01$.

significantly and dose-dependently suppressed and correlated with both the degree of hepatomegaly and induction of liver microsomal EROD activity (Tables 19 and 20). In addition, splenic microsomal EROD activity was enhanced at all exposure levels. Flow cytometric analyses of these splenocytes, again derived from treated but unsensitized B6C3F1 mice, demonstrated a greater degree of effects on both the percentage and the absolute numbers of both Ig^+ B cells and $Lyt\text{-}2^+$ and $L3T4^+$ T cells (Table 21). The most noted change however was in the Ig^+ cells, where the percentage of these cells were dose-relatedly increased while the absolute numbers were decreased. No significant change in the absolute numbers of T cells was determined. The interpretation of this finding would be the selective loss of a non-B/non-T cell from the spleens of these animals; most likely of the macrophage/monocyte lineage.

Subchronic exposures in DBA/2 mice produced changes in both lymphoid organ weight and humoral immune parameters which were inconsistent with either results obtained following acute exposures or with changes in the liver weight or liver microsomal EROD activity (Table 20). In addition, thymic involution was greatly enhanced in these mice. This effect on thymus weight was consistent throughout several experiments, where subchronic exposure in the DBA/2 strain usually caused a greater degree of thymic atrophy than was seen in the B6C3F1 mice when exposed under the same conditions (data not shown). More consistent with effects of TCDD on the thymus, as opposed to those on the liver, suppression of both the background and antigen-stimulated antibody responses in the DBA/2 mice were significantly enhanced following the repetitive 14 day exposure regimen (Table 18), and was consistent throughout several experiments. Coordinately, splenic microsomal EROD activity was enhanced at the 4.2 and 14 μg/kg exposure levels, and may be related to the enhanced suppression that is seen following subchronic exposures in DBA/2 mice (Table 20). Flow cytometric analyses of these splenocytes, again derived from treated but unsensitized DBA/2 mice, demonstrated a greater degree of effects; particularly with respect to the loss of a non-B/non-T cell (Table 21). As in the case of

splenocytes derived from the B6C3F1 strain following this treatment regimen, the most noted change was an increase in the percentage of Ig+ cells; without a change in the absolute numbers of any of the lymphocyte populations. The interpretation is the same, in which there appears to be the loss of a non-B/non-T cell from the spleens of these mice; most likely of the macrophage/monocyte lineage. Moreover, the loss in splenic EROD activity at the 42 μg/kg cumulative dose in both strains correlates with the loss of this non-B/non-T cell population, thus implicating this population of cells as the primary cell type involved with the TCDD-induced P450 activity in the spleen.

V. DISCUSSION

As indicated in the review of the literature, the compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) represents the most potent congener of a family of biologically active halogenated aromatic hydrocarbons [Poland, et al., 1979]. It has attracted much interest in recent years for both its frequent association with environmental contamination and its broad spectrum of toxic effects in numerous species, including man. Exposure in animals is most characterized by thymic atrophy, functional immunosuppression, liver porphyria, and induction of liver P450 enzymes [Poland, et al., 1979]. In contrast, the most notable effect in man is chloracne, a condition of the skin in which exposure to TCDD causes hyperplasia and hyperkeratinization of the epidermis [Poland and Kende, 1976]. These earlier studies have led various investigators, including this laboratory, to investigate the mechanism by which TCDD can cause such diverse effects in a wide variety of tissues and organ systems. Biological models are necessary in determining the biochemical and functional mechanism of dioxin's action and various non-lymphoid tissues and cell lines have been employed to address this issue [Poland and Knutson, 1982; Hudson, et al., 1985; Hudson, et al., 1986]. Additionally, several findings have suggested a role by TCDD in the alteration of either activation or proliferation of various cell types; including the B lymphocyte. Of these types of studies, two such observations deserve special mention. First is the finding by Knutson and Poland (1980) [Knutson and Poland, 1980] in which exposure of the murine teratoma cell line XB to TCDD caused a decrease in their capacity to proliferate. Second, and more important, was the observation by Tucker, et al. [Tucker, et al., 1986] in which a

significant suppression in DNA synthesis was noted following addition of TCDD to LPS-stimulated cultures, further suggesting an effect by TCDD on the early stages of B cell activation and proliferation.

In terms of more specific effects on humoral immunity, TCDD's profile of activity has been somewhat unclear and found to be rather unique in nature. This point is exemplified in reports from several laboratories which have demonstrated the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on humoral immunity to be comprised of dual components - immune enhancements and immune suppression. The manifestation of these effects however, has depended on the level of exposure that is used in rodent model systems. For example, Sharma and Gehring (1979) and Clark, et al. (1981) have reported that exposure of naive animals to lower doses of TCDD caused an increase in the level of spontaneous lymphocyte transformation (^{3}H-TdR incorporation), serum antibodies, and background splenic AFC formation in the absence of antigen stimulation, and at a level of dioxin which did not cause detectable induction of metabolic enzymes in the liver [Sharma and Gehring, 1979; Clark, et al., 1981]. Interestingly, suppression of mitogen responsiveness of whole splenocytes to both phytohemagglutinin (PHA) and pokeweed mitogen (PWM) directly correlated with the spontaneous proliferation detected in these cells at both 2 and 4 weeks post-exposure. Both had returned to control levels by 8 weeks. Additionally, Clark and co-workers [Clark, et al., 1981] further noted that low levels of TCDD (0.4 and 4 μg/kg), given once a week for four weeks, caused an increase in the number of spleen colony-forming cells in bone marrow by 25-39%, suggesting that TCDD could be acting at some specific site to initiate an "activation-like" signal within hematopoietic cells. These observations are also consistent with a similar profile of activity on lymphocyte function in humans. Lipson (1987) has demonstrated that either direct (in vitro) or in vivo exposures of human peripheral blood lymphocytes (PBL) to the TCDD-related compounds, polybrominated biphenyls (PBB), causes an increase in B cell activation [Lipson, 1987]. As reported, concentrations of PBB as low as 0.001 μg (3.1 X

10^{-6} μmol)/10^5 PBL decreased lymphocyte responses to pokeweed mitogen (PWM) whereas higher concentrations stimulated the in vitro synthesis and release of immunoglobulins; most notably IgG. Similarly, PBL obtained from PBB-exposed Michigan farmers demonstrated an enhanced release of IgG in culture following PWM stimulation. Moreover, as noted in the epidemiological reports of Tognoni and Bonaccorsi (1982), increases in spontaneous lymphocyte proliferation and mitogen responsiveness have also been found in PBL obtained from children exposed to TCDD in Seveso, Italy [Tognoni and Bonaccorsi, 1982]. This is also indicated in the present studies in which enhancements were detected in in vitro antibody responses following either in vivo or direct in vitro exposures under specific exposure and culture conditions. Conversely, higher doses of TCDD (i.e., those causing a detectable induction of the liver and producing atrophy of the thymus) were found to inhibit both mitogen-stimulated and primary antibody responses to SRBC for up to 42 days following exposure [Clark, et al., 1981; Vecchi, et al., 1983]. Furthermore, the degree of antibody response suppression was found to correlate with the ability of TCDD to induce liver P450 enzyme activity, as based on both the dose-responsive behavior of TCDD and the sensitivity of various strains of mice [Vecchi, et al., 1980; Vecchi, et al., 1983]. These studies therefore demonstrate a dose-related biphasic response to dioxin in the whole animal which suggests a segregation in the effects of exposure on lymphocyte responsiveness. Moreover, it was previously determined that the primary cell affected by TCDD in suppression of antibody responses is the B lymphocyte and that an alteration in early signalling events during B lymphocyte activation leads to improper differentiation of these cells and suppression in their ability to secrete antibody [Tucker, et al., 1986; Holsapple, et al., 1986a; Dooley and Holsapple, 1988; Luster, et al., 1988b]. Given these findings, it was the overall objective of the current investigation to determine the relationship between the immune enhancing and immunosuppressive effects of TCDD on humoral immunity as related to it's effects on the two main subpopulations of B cells within the spleen. Specific emphasis was placed on

determining whether these two phenomena are mechanistically related or mutually exclusive components of TCDD's profile of immunomodulatory activity. Additionally, studies were conducted to determine whether these two opposing actions of TCDD are correlated with responses that are mediated by the *Ah*-receptor and the *Ah-gene* locus.

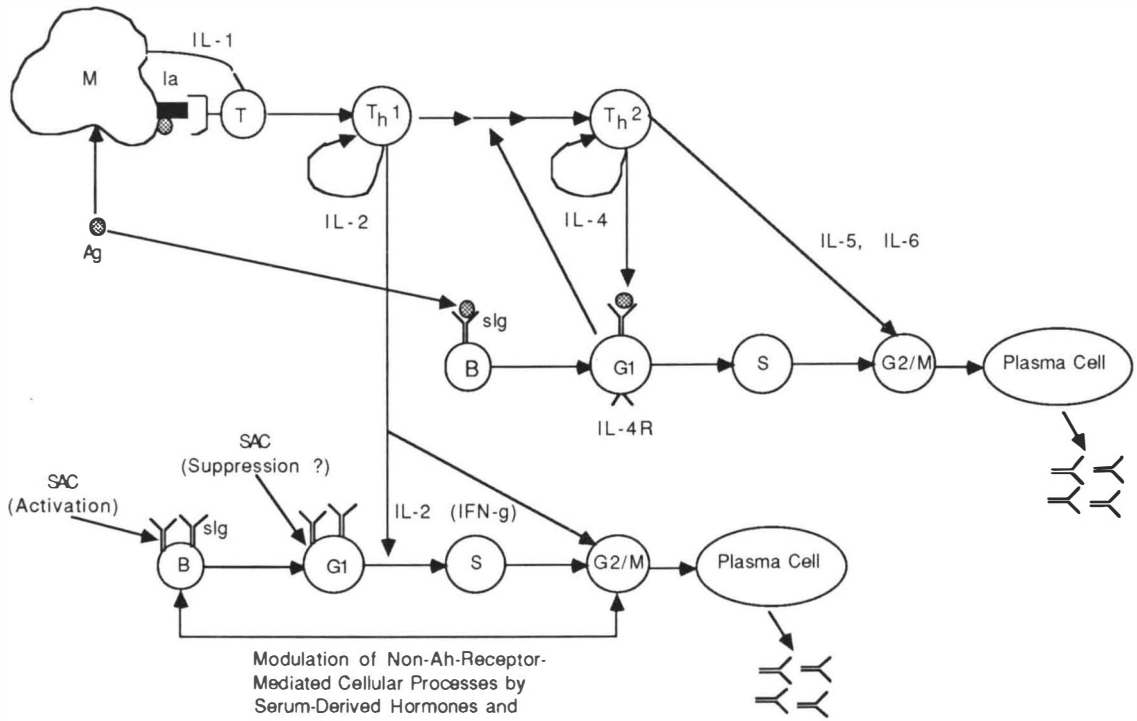
In the first series of experiments that were carried out in the laboratory, it was determined that a similar duality in effects could be induced by TCDD on the in vitro T-dependent antibody response under selective culturing conditions. Specifically, it was determined that the degree of suppression of in vitro antibody responses in *Ah*-high responder B6C3F1 female mouse splenocytes is dependent on both the lot and concentration of supplemental serum that is present during the time of exposure. In the presence of fetal bovine sera (FBS), both enhancements and suppression were seen but were dependent upon both the concentration of TCDD administered and the concentration of serum which was used in support of the response (i.e., 5% versus 10%; see Figure 7). Under totally serum-free conditions (i.e., bovine serum albumin - BSA - as an equivalent protein source), only enhancements in the anti-SRBC AFC response could be seen. Opposingly, newborn calf sera (all lots tested to date) supported a dose-related suppression of the anti-SRBC AFC response regardless of the concentration used (only 5 and 10% sera were tested in these studies). Therefore, in relation to the dual effects induced by TCDD on antibody responses, alteration of the serum environment during in vitro culture could be used as a tool to explore this phenomena. All subsequent experiments took full advantage of this effect by comparing the actions of TCDD on both whole lymphocyte and isolated B cell antibody responses in the presence of both a selectively suppressive and selectively enhancing serum environment. From these studies it is evident that these two modulatory actions of TCDD are separate entities and that the manifestation of these effects is determined by the lymphocyte cell type, stage of cell cycle and the presence of modulatory hormones present in serum.

Collectively, the observations above suggested that: 1) TCDD acts primarily on the B lymphocyte; 2) TCDD has characteristics of a polyclonal B cell activator; and 3) the suppression of the T-dependent antibody response is dependent on serum-derived growth factors. Therefore, the second series of experiments centered on the similarities between TCDD and the polyclonal B cell activator, Staphylococcus aureus Cowan strain I (SAC). SAC is described in the literature as a T cell-independent polyclonal B cell activator, in that it can bind to (via protein A immunoglobulin binding molecules on the bacterial cell surface) and cause the activation of resting B cells in the absence of T cells [Kehrl, et al., 1984]. However, it is inefficient at differentiating these cells in the absence of T cell-derived growth and differentiation factors. It is also interesting that the proliferation of resting B cells following SAC activation is modulated by serum-derived growth factors which can increase or decrease the ability of these cells to proliferate and/or differentiate [Choi and Xia, 1987; Wasik, et al., 1987]. Activation by SAC is further unique in that it stimulates the expression of the high-affinity IL-2 receptor on the surface of resting B cells [Tanaka, et al., 1988]. In fact, it has been speculated that it is the activation of B cells in an IL-2-dependent manner which may render these cells susceptible to modulation by the serum-derived growth factors. Moreover, it has recently been described that the early acting cytokine, IL-2, can directly modulate B cell activation and differentiation. However, this type of activation scheme seems to be dependent on the type of stimulus received by the B cell and its role in a "normal" T-dependent antibody response is still unclear [Jelinek and Lipsky, 1987; Abbas, 1988]. Since IL-2 would be included as one of the first major B cell modulatory cytokines to be released by the T cell during a T-dependent response (normally functioning as a T cell growth factor), it would seem logical to assume that B cells activated in such a way as to express the high-affinity IL-2 receptor, would be rendered susceptible to "apparent" suppression, due to the anti-proliferative effect of a non-supportive serum; without significant effects on response kinetics. It should also be noted that our laboratory has previously demonstrated that IL-2 secretion by

Con A activation of T cells is not affected by TCDD exposure [Dooley, et al., 1990]. Thus, a comparison was made between the actions of SAC and TCDD on the T-dependent response in 2 separate lots of serum (one which supported the suppression by TCDD - 5% NBCS lot 453, and one which did not - 10% FBS lot 717). From these studies it was determined that the effects of TCDD on the T-dependent antibody response by B6C3F1 splenocytes are similar to the effects of SAC. This similarity was based on the observation that under the same serum supplemented conditions which were shown to support either enhancements (i.e., FBS or BSA) or suppression (i.e., NBCS) of the in vitro anti-SRBC antibody response, direct addition of SAC to these cultures caused the same pattern of effects. Likewise, under totally serum-free culture conditions, SAC produced dose-dependent enhancements in the the AFC response of approximately equal magnitude as TCDD at equivalent suppressive doses. These studies are important from the standpoint that TCDD has actions that are similar to those of an in vitro polyclonal B cell activator and that hormonal influences can determine whether enhancements or suppression can be manifested by either of these two agents. More importantly, it demonstrates that the actions of TCDD on humoral immunity could be mediated outside the *Ah-gene* locus by cellular mechanisms that control lymphocyte activation, and which are hormonally regulated by their respective environment. The proposed actions of SAC on the in vitro T-dependent antibody response to SRBC is illustrated in Figure 35. This model was used as a working hypothesis for subsequent experiments to determine the direct actions of TCDD on lymphocyte function and the relationship between the actions of TCDD and SAC.

Given the dual nature of effects elicited by both TCDD and SAC on in vitro antibody responses, further investigations were initiated with several additional objectives in mind. First, because of the close parallel between the effects of TCDD and SAC, studies were preformed to determine the relative sensitivities of the two main populations of B cells within the spleen to direct TCDD exposure - i.e., resting (Go) and cycling (primarily G1) B cells as based on separation of these cells on discontinuous percoll density gradients.

Figure 35. Proposed Actions of Staphylococcus Aureus Cowan Strain I (SAC) on B Cell Activation and Differentiation in the In Vitro T-Dependent Antibody Response.



Modulation of Non-Ah-Receptor-Mediated Cellular Processes by Serum-Derived Hormones and Growth Factors

Specifically, these studies were carried out to determine if enhancements and suppression of B cell responses are a function of differential effects on these two populations of cells and were based primarily on reports that SAC and anti-immunoglobulin antibodies have opposing actions on these two cell types - i.e., activation of resting and a more generalized suppressive effect on activated, cycling, B cells [Defranco, et al., 1982; Kehrl, et al., 1984; Maruyama, et al., 1985; Symons, et al., 1985; Chen, 1988; Calvert, et al., 1990; Flores-Romo, et al., 1990]. The experimental model system used in these studies involved the effects of TCDD on both background and lipopolysaccharide (LPS)-induced proliferation and differentiation (i.e., IgM and IgG) of purified splenic resting and cycling B cells derived from *Ah*-high responder B6C3F1 female mice. The relative purities and functional capacities of these two populations of cells were carefully and extensively characterized as was described in the methods and results. This was important in validating the use of purified B cell populations in mechanistic studies related to the actions of TCDD on lymphocyte function in particular, and in in vitro immunotoxicity studies in general. LPS was selected as the immunological probe in these studies based on its ability to stimulate both proliferation and differentiation of purified B cells in the absence of either macrophages or T cells or their respective B cell modulatory cytokines. This was important in allowing the determination of the direct effects of TCDD on B lymphocyte proliferation and differentiation without the interference of secondary effects through macrophages or T cells. The second objective was related to the first, in that the studies to determine the actions of TCDD on different populations of B cells were carried out in the presence of two separate serum environments; one which selectively inhibited suppression of in vitro antibody responses to SRBC (i.e., 10% FBS) and one which selectively supported suppression in a significant and dose-related fashion (i.e., 5% NBCS). This added condition was to enable the determination of the direct modulatory role of serum-derived growth factors in the effects of TCDD on B lymphocyte function. Specifically, these studies were performed to determine if, in addition to a differential pattern of effects

of TCDD on splenic B cell populations, serum-born growth factors could be partially responsible for the dual behavior of B cells when directly exposed to TCDD. Moreover, T cell proliferation in these two serum environments was also compared to determine the relative sensitivities between B cells and T cells in suppression of whole lymphocyte antibody responses. In a similar manner to the B cell, both background and Con A-stimulated proliferation were monitored. The third objective of this investigation was to determine the potential role, and/or relationship, between the dual effects of TCDD on B lymphocyte function and the *Ah-gene* locus. These studies were conducted in a two-fold manner. The first series of studies were conducted to determine the modulatory effect of the various sera (i.e., fetal and newborn bovine sera and normal mouse sera from *Ah*-high responder - B6C3F1 - and *Ah*-low responder - DBA/2 - mouse strains) on the ability of TCDD to cause induction of P4501A1 (ethoxyresorufin-O-deethylase; EROD) activity in primary hepatocyte cultures derived from *Ah*-high responder, B6C3F1, mice. These studies were designed to determine if enhancements and/or no-effect responses of lymphocytes derived from *Ah*-high responder animals, to direct in vitro exposure to TCDD in the presence of the various sera, would correlate with the levels of P450 activity obtained in hepatocytes cultured under the same serum-supplemented conditions. The second series of experiments consisted of cross-over studies in which hepatocytes and splenocytes from both *Ah*-high responder (B6C3F1) and *Ah*-low responder (DBA/2) mice were directly exposed to TCDD under various serum-supplemented conditions, including their own natural sera environment (i.e., normal mouse serum). These studies were conducted to determine if natural components within the sera of *Ah*-high responder and *Ah*-low responder mouse strains could confer the phenotypic patterns of P450 activity and immunosuppression that is typically seen in these two strains of mice following acute exposures in vivo. The findings of these experiments are discussed individually, but in a manner which relates to the effects of TCDD on whole lymphocyte responses.

Results of the studies conducted using purified B cell subpopulations demonstrate that the dual nature of the effects of TCDD on antibody responses is a function of both the state of in vivo activation (i.e., stage of the cell cycle) and the presence of serum-derived hormones, each being required in the production of either effect. These actions are illustrated in Figure 36 and are modeled after the proposed actions of SAC on B lymphocyte responses as described above (Figure 35). For resting B cells, TCDD appears to induce an "activation-like" signal which can cause at least a proportion of these cells to proliferate and differentiate into immunoglobulin-secreting cells in the absence of any exogenously added B cell stimulus. This effect however, only occurs in the presence of fetal bovine serum (FBS; i.e., conditions which do not support suppression of in vitro antibody responses) and at TCDD concentrations of ≥30 nM. TCDD did not affect either response when resting B cells were stimulated by LPS. In cycling B cells a more generalized pattern of suppression was seen, which occurred more prevalently in the presence of the NBCS. TCDD caused a dose-related decrease in both background and LPS-stimulated proliferation, with significant effects at concentrations as low as 300 pM. Similarly, TCDD was found to inhibit differentiation of these cells by decreasing both background and LPS-stimulated IgM and IgG secretion in a dose-related manner, and across the same dose range shown to inhibit B cell proliferation. Most interestingly, there was a serum dependency for the effects of TCDD on LPS-stimulated cycling B cells which closely paralleled the effects seen on whole splenocyte responses to SRBC; thus confirming a major effect on the cycling B lymphocyte in suppression of humoral immunity by TCDD. Therefore, enhancements in immune function by TCDD are at least partially due to the direct activating effects of TCDD on the resting B cell. Conversely, the effects of TCDD on the background proliferation of cycling B cells are characterized as a generalized suppression; with a more consistent suppression occurring in the presence of the NBCS. These observations are therefore important in demonstrating the relationship between enhancements and suppression of humoral immune function, as related to both the

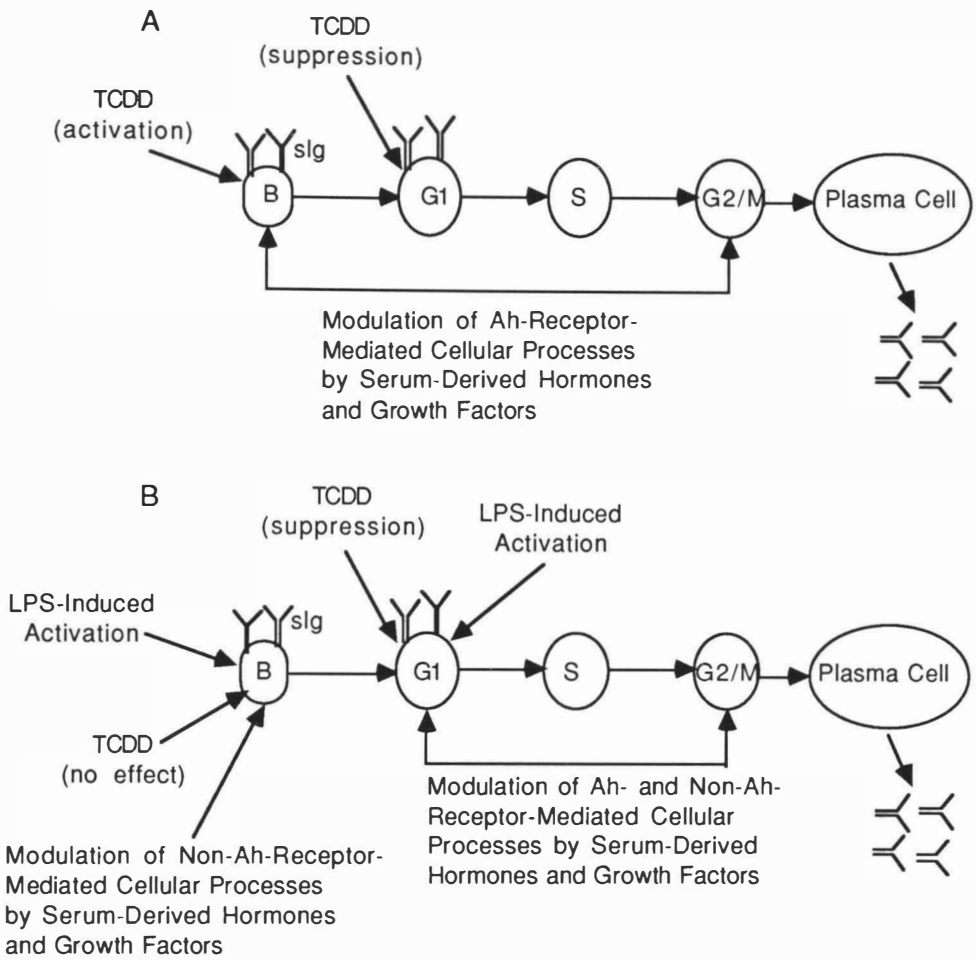


Figure 36. Model of the Actions of TCDD on Resting (Go) (Figure A) and Cycling (G1) (Figure B) B Cell Proliferation and Differentiation in the Absence and Presence of a B Cell Stimulus.

stage in the B cell cycle and the modulation of serum-derived growth factors. From these studies it can be concluded that the enhancements in resting B cell proliferation and differentiation has no consequence in either suppressing or enhancing the proliferative or differentiative effects of a B cell stimulus and would suggest that this population of cells is less sensitive to the suppressive effects of TCDD on B cell function. However, in contrast to this suggestion, it should be reemphasized that the direct enhancements in resting B cell proliferation results in the complete depletion of these cells following antigen stimulation of exposed mice, thereby contributing as much to the overall immunotoxicity in the whole animal as the direct functional suppression of B cell responses as described below for the cycling B cell population. As indicated, additional studies were key to determining that TCDD can interfere with both the proliferation and differentiation of both unstimulated and LPS-stimulated cycling B cells and that the same serum-dependent phenomena is observed in these cells as was found for the in vitro T-dependent antibody response (i.e., there were more dramatic effects in the presence of NBCS). Moreover, this latter finding further establishes this population of B cells as the sensitive cells to functional suppression by TCDD and that proliferation is significantly affected. In comparative studies using purified T cells, no significant suppression of either background or Con A-stimulated proliferation was detected regardless of the serum used. Interestingly however, significant and dose-related enhancements in Con A-stimulated cycling B cell proliferation were seen in the presence of FBS. This finding suggests that in addition to enhancement of resting B cell proliferation and differentiation, increases in antigen stimulated T cell proliferation may contribute to the enhancements seen in the in vitro, and even in vivo at low TCDD concentrations, antibody responses. In terms of relative sensitivities for suppression however, the T cell appears to be less affected by TCDD exposure. However, this data does not rule out the possibility that there is not an indirect influence by the T cell to suppress B cell responses. The potential role of T cell cytokines in modulation of either enhanced activation or proliferation of both resting and cycling B cells, as well as the

whole lymphocyte anti-SRBC response, is illustrated in Figure 15. These results suggest that enhancements in the AFC response under serum-free culture conditions requires antigen stimulation and the release of T cell-derived cytokines. In addition, Figures 21 and 23 demonstrate that both gamma-interferon (G-IFN) and IL-2 (both secretory products of the inflammatory T_h1 T cell subtype) can modulate the direct effects of TCDD on B cell proliferative responses in the absence of antigen challenge. The effects of G-IFN in enhancing the activation of resting B cells by TCDD is especially noteworthy given the many reports which have characterized the actions of this cytokine as having properties of a B cell growth and differentiation factor following stimulation by a number of B cell activating stimuli, including SRBC [Leibson, et al., 1984; Nakamura, et al., 1984; Sidman, et al., 1984; Brunswick and Lake, 1985; Defrance, et al., 1986; Romagnani, et al., 1986; Boyd, et al., 1987; Karray, et al., 1987; Morikawa, et al., 1987; Xia and Choi, 1988; Punnonen and Viljanen, 1989a; Punnonen and Viljanen, 1989b].

Results of experiments used to address the third objective have added the most compelling evidence that enhancements and suppression of both whole lymphocyte AFC and isolated B cell antibody responses is regulated in an *Ah*-dependent manner. In these studies it was determined that the influence of the serum on the ability of TCDD to either inhibit (and/or enhance) or suppress whole lymphocyte and purified cycling B cell antibody responses has a direct correlation with the modulation of TCDD-induced P4501A1 activity in primary hepatocytes. When comparing the direct effects of TCDD on the lymphocyte with the induction of ethoxyresorufin-O-deethylase (EROD; P4501A1) activity in primary hepatocytes it was found that calf serum conditions which inhibit and/or enhance antibody responses in the presence of TCDD (i.e., 10% FBS) support a lower induction of enzyme activity in the hepatocyte (as compared to a BSA protein control). Conversely, NBCS supports a more pronounced and dose-dependent suppression of antibody responses and a higher induction of P450 activity in the hepatocyte. It should be pointed out however, that NBCS does express a concentration dependency for the effects

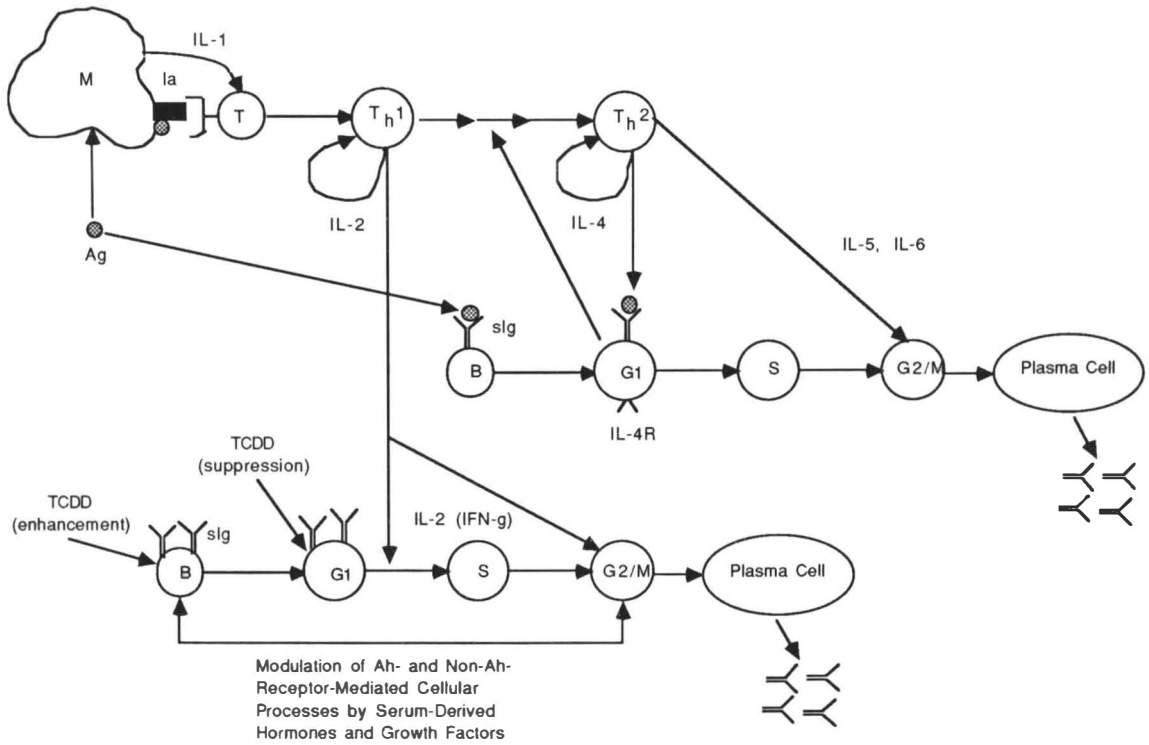
of TCDD on whole lymphocyte responses (see Figure 26) in which concentrations below 4% were not found to support suppression, thus suggesting that the active component in the serum must be at appropriate concentrations in order to potentiate the effects of TCDD on lymphocyte function. In contrast to the effects seen in the presence of calf sera, immune responses and induction of hepatocyte P450 enzyme activity in the presence of normal mouse sera display a more direct correlation to effects mediated through the *Ah-gene* locus and the *Ah*-receptor. In particular, antibody responses to SRBC mediated by the *Ah*-high responder B6C3F1 splenocyte are suppressed in a dose-related manner by TCDD in the presence of normal mouse serum whereas responses by the *Ah*-low responder DBA/2 splenocyte are not. In contrast, responses by DBA/2 splenocytes were actually enhanced in the presence of normal mouse serum, at TCDD concentrations of 0.3 and 3 nM (much like what is seen in B6C3F1 splenocyte responses in the presence of FBS). Coordinately, TCDD-induced EROD activity in the presence of normal mouse serum is selectively enhanced in the B6C3F1 hepatocyte, and not in the DBA/2 hepatocyte, as compared to the BSA protein control. Moreover, crossover studies determined that the responses of splenocytes and hepatocytes of either strain were no different in the presence of serum from either of the two strains and demonstrates that the *Ah*-dependency of the effects of TCDD are not related to the compositions of serum derived from these two strains of mice. These latter findings help to explain the dual pattern of effects of TCDD on lymphocyte function and correlate with several reports that have described the factors which can influence the induction of P4501A1 activity in human leukocytes [Busbee, et al., 1972; Kouri, et al., 1974; Kouri, et al., 1979]. In these reports, it has been described that both the serum and the activation state are important in inducing P4501A1 activity. More specifically, the higher the proliferative capacity of the cell the higher the inductive capacity. This in turn is related to the serum in support of cellular growth in <u>in vitro</u> culture systems. These earlier findings again correlate with the observations in the present study, in which differential effects were seen between resting and cycling B cells in

response to TCDD. Collectively, these results, combined with these earlier reports, would indicate that the dual nature of the effects of TCDD on lymphocyte function is related to the capacity of the cell to respond to the TCDD through *Ah*-receptor mediated cellular events. Resting B cells, which have a lower capacity for responses mediated through the *Ah*-receptor, demonstrate an enhancement or "activation-like" response, whereas cycling B cells, which have a higher capacity for *Ah*-mediated responses, demonstrate a generalized suppressive response following TCDD exposure.

To summarize the effects of TCDD on the B lymphocyte, Figure 37 illustrates the proposed actions of TCDD on the two major populations of B lymphocytes as related to the manifestation of effects on the whole lymphocyte T-dependent antibody response. As modeled from the proposed actions of SAC on antibody responses, TCDD has opposing, but equally toxic effects on the resting and cycling B cell. Each of these actions can disrupt lymphocyte responses to a T-dependent antigen and are influenced by hormonal factors. However, given the fact that TCDD causes the activation of cellular kinases within minutes of exposure [Kramer, et al., 1987] and the similarity in actions to SAC, these responses are most likely mediated by both *Ah*-receptor-dependent and *Ah*-receptor-independent mechanisms. Moreover, in keeping with a lower sensitivity to suppression of T cell function as previously reported [Dooley, et al., 1990], cycling T cells demonstrate only enhanced behavior following TCDD exposure and would be indicative of a lower capacity for direct suppressive effects on this cell in suppression of immune function. Therefore, it is not just the response that is mediated through the *Ah*-receptor, but also the level of the response, which is important in producing the ultimate effect - either enhancements or suppression; both however, being toxic responses that are mediated at different theshold levels of TCDD exposure within the cell.

In summary, the present studies demonstrate several key observations in the understanding of the actions of TCDD on B lymphocyte function. Experiments conducted using purified B cell subpopulations demonstrate that the dual effects of TCDD on humoral

Figure 37. Proposed Actions of TCDD on B Cell Activation and Differentiation in the In Vitro T-Dependent Antibody Response.



Modulation of Ah- and Non-Ah-Receptor-Mediated Cellular Processes by Serum-Derived Hormones and Growth Factors

immune responses are a function of both the stage of the cell cycle and the serum environment in which the cells are exposed. Cycling B cells appear to be the primary target for suppression by TCDD whereas resting B cells appear to be associated with immune enhancements that are seen following low dose exposures in the whole animal. Based on studies conducted on the modulatory role of sera on both hepatocyte P4501A1 activity and suppression of both in vitro AFC responses to SRBC and purified B cells to LPS, the effects of TCDD on lymphocyte responses are consistent with a role by the *Ah-gene* locus. Interestingly, lower levels of induction as seen in the presence of FBS, or the lower levels of P450 activity expected in resting B cells, appear to result in immune enhancements, whereas higher levels of induction as seen in the presence of NBCS, or previously shown in activated lymphocytes, appear to result in a more generalized suppressive activity on humoral immunity.

Lastly, experiments were conducted to investigate the immunotoxic effects of TCDD following either acute or subchronic exposures in mice. Acute exposure of mice to TCDD traditionally leads to changes in organ weights (i.e., specifically liver and thymus, reflecting hepatomegaly and involution, respectively) and a suppression of humoral and cell-mediated immunity that is consistent with a role by the *Ah*-locus [Sharma and Gehring, 1979; Vecchi, et al., 1983; Kerkvliet, et al., 1990b]. However, while these findings are fairly descriptive of acute dosing regimens, several reports have indicated that differences may exist in the toxicities produced by TCDD, and closely related congeners, depending on the dose [Clark, et al., 1981; Clark, et al., 1983], route [Pohjanvirta, et al., 1989] and length of exposure [Holsapple, et al., 1986b] used by various investigators. Of particular interest is this latter phenomena, in which a report from this laboratory has shown that subchronic exposure of B6C3F1 mice to the low affinity *Ah*-receptor ligand, 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD), caused suppression of the in vivo antibody forming cell (AFC) response to SRBC which was comparable to that seen with TCDD; even in the absence of significant induction of liver P450 activity (i.e., aryl hydrocarbon

hydroxylase activity; AHH) [Holsapple, et al., 1986b]. This finding was one of the first to demonstrate that low affinity agonists for the *Ah*-receptor, while having little or no effect on immunity when given acutely, could have dramatic effects on these immune parameters when administered sequentially over a longer period of time.

Results of this investigation demonstrate that the conditions of exposure are important in determining the immunotoxicological potential of TCDD in vivo. This conclusion is based on the finding that subchronic exposure in DBA/2 mice leads to an enhanced suppression of both the background IgM and stimulated (day 4 anti-SRBC) AFC responses in the absence of significant changes in hepatomegaly or liver P4501A1 (EROD) activity. These findings are consistent with previous reports which have demonstrated that subchronic exposure of mice (i.e., B6C3F1) to either 2,7-DCDD [Holsapple, et al., 1986b] or any of several polycyclic aromatic hydrocarbons (PAHs) [White, et al., 1985a] can produce suppression of the anti-SRBC AFC response that is comparable to that produced following acute exposures to TCDD. This latter study is especially noteworthy with respect to the current investigation because of the differential effects that were seen in DBA/2 mice, as opposed to the B6C3F1 strain. In particular, these results indicated that subchronic exposure to either 3-methylcholanthrene or 7,12-dimethylbenz(a)anthracene resulted in a higher degree of suppression of the day 4 antibody response in DBA/2 mice than was seen in B6C3F1 mice. Similar results were observed in one of several trials with DBA/2 mice in the present study. This finding is important from the standpoint that the PAHs are also thought to induce their immunosuppressive effects, at least in part, through *Ah*-receptor mediated mechanisms. Conversely, these findings instead suggest the involvement of additional, non-*Ah*-receptor mediated, mechanisms in suppression of humoral immunity by both the polycyclic and polyhalogenated aromatic hydrocarbon compounds. However, it is important to note that there was a small, but significant, enhancement in splenic P4501A1 activity in both strains following the subchronic

exposure regimen, and that our results cannot rule out the involvement of *Ah*-receptor-mediated responses in these effects. This finding is further discussed below.

Additionally, flow cytometric analyses of splenocyte subpopulations demonstrated that subchronic exposures resulted in the significant loss of a non-B/non-T lymphocyte which may be associated with the enhanced suppression of humoral immunity observed in these animals. Interestingly, there was a coordinated loss of splenic P450 activity with the loss of the non-B/non-T population. It is therefore likely that the non-B/non-T cell is of the macrophage/monocyte lineage, as based on the work of Ladics, et al. (1991) who have determined that the primary cell type associated with the induction of P450 activity by benzo(a)pyrene in the spleens of B6C3F1 mice is the macrophage [Ladics, et al., 1991b].

In terms of a mechanism which can account for the differential effects that are mediated by TCDD in the DBA/2 strain, there are no readily available explanations. For example, Curtis, et al. (1990) have determined that repeated exposures of C57BL/6J mice to TCDD can alter its distribution within the liver and other tissues of these mice (i.e., spleen, fat, etc.) [Curtis, et al., 1990]. This phenomena was attributable to an inducible, high affinity, low capacity system which affected the pharmacokinetics of TCDD. This observation is similar to the earlier findings of Leung, et al. (1988) who established that the greater accumulation of TCDD in the liver of C57BL/6J mice, as compared to DBA/2J mice, was not due to the higher fat content in DBA/2J mice or to cytosolic TCDD binding, but was more dependent on the presence and affinity of microsomal TCDD binding proteins [Leung, et al., 1988]. Therefore, while these findings may help to explain the enhanced toxic effects of repeated exposure in B6C3F1 mice, as demonstrated in the studies outlined in the results, they are insufficient in explaining the findings in the DBA/2 strain. This is in light of the fact that the redistribution of TCDD following repeated exposures, as identified by Curtis and co-workers, was accompanied by an increase in liver weight in the C57BL/6J mice, an effect that is clearly not seen in the DBA/2 strain in the present study. However, as indicated above, an enhancement in splenic EROD activity

was identified in the DBA/2 strain, thereby suggesting the involvement of *Ah*-receptor mediated effects in the enhanced suppression of humoral immunity in the DBA/2 strain. The enhancement in turn, may be the result of a selective redistribution of TCDD into the spleens of these animals. This effect is currently being investigated and may be the result of physiological differences which contribute to differences in the toxicokinetics of TCDD within these two strains.

In relation to the role of the *Ah*-locus in the mediation of effects on immunity by TCDD, Kerkvliet, et al. (1990) have recently demonstrated that *Ah*-receptor-independent mechanisms are likely to play some role in suppression of humoral immunity by TCDD [Kerkvliet, et al., 1990c]. This conclusion is based on the observation that a biphasic dose-response relationship for suppression of the anti-SRBC AFC response was produced in response to TCDD treatment in Ah^{dd} (low responsive) congenic mice, as opposed to Ah^{bb} (high responsive) mice, which did not correlate with induction of AHH activity in the liver. However, these results were obtained using acute exposure regimens which again are unlikely to represent an adequate comparison in explaining the differences identified within the present study. Therefore, further experimentation is necessary in order to uncover the differential mechanisms involved with humoral suppression in DBA/2 and B6C3F1 mice following subchronic exposures to TCDD. Moreover, this study, in present form, raises additional questions concerning the relevancy of subchronic exposures in immunotoxicity evaluations of TCDD. Previous investigators have demonstrated that a subchronic exposure regimen based on dosing once per week for several weeks produces a greater suppression in C57BL/6 mice than is observed in the DBA/2 strain [Vecchi, et al., 1983]. A comparison of these results with those obtained in the present study indicates that a subchronic regimen based on weekly exposures produces a profile of immunotoxicity that is more like the one associated with an acute (single) exposure [Clark, et al., 1981; Vecchi, et al., 1983]. Therefore, when considering human exposures specifically, a supposed *Ah*-low responder population, subchronic studies in low-

responsive animal models (i.e., DBA/2 mice) may better depict the dose regimen and body burden attained following repeated low level environmental exposures as would have occurred, for example, in Times Beach, Missouri [Knutson, 1984]. This is in contrast to exposures that have taken place following large scale industrial accidents as in Seveso, Italy [Pocchiari, et al., 1979; Tognoni and Bonaccorsi, 1982], in which acute exposures to large doses of TCDD may be more appropriately applied. In this regard, human populations exposed to low doses over an extended period of time (i.e., months to years) may be at increased risk to immunotoxic effects by these chemicals through presently unidentified *Ah*-receptor-independent mechanisms.

VI. LITERATURE CITED

LITERATURE CITED

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VII. VITA