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Chapter 4. Immunotoxic Effects

Health Assessment for
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
and Related Compounds

NOTICE

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
Ah	Aryl hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
ALT	L-alanine aminotransferase
AST	L-asparate aminotransferase
BDD	Brominated dibenzo-p-dioxin
BDF	Brominated dibenzofuran
BCF	Bioconcentration factor
BGG	Bovine gamma globulin
bw	Body weight
cAMP	Cyclic 3,5-adenosine monophosphate
CDD	Chlorinated dibenzo-p-dioxin
cDNA	Complementary DNA
CDF	Chlorinated dibenzofuran
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DCDD	2,7-Dichlorodibenzo-*p*-dioxin
DHT	5α-Dihydrotestosterone
DMBA	Dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin-responsive enhancers

LIST OF ABBREVIATIONS (cont.)

DTG	Delayed type hypersensitivity
DTH	Delayed-type hypersensitivity
ED_{50}	Dose effective for 50% of recipients
ECOD	7-Ethoxycoumarin-0-deethylase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EROD	7-Ethoxyresurofin 0-deethylase
EOF	Enzyme altered foci
FSH	Follicle-stimulating hormone
GC-ECD	Gas chromatograph-electron capture detection
GC/MS	Gas chromatograph/mass spectrometer
GGT	Gamma glutamyl transpeptidase
GnRH	Gonadotropin-releasing hormone
GST	Glutathione-S-transferase
HVH	Graft versus host
HAH	Halogenated aromatic hydrocarbons
HCDD	Hexachlorodibenzo-p-dioxin
HDL	High density lipoprotein
HxCB	Hexachlorobiphenyl
HpCDD	Heptachlorinated dibenzo-p-dioxin

LIST OF ABBREVIATIONS (cont.)

HpCDF	Heptachlorinated dibenzofuran
HPLC	High performance liquid chromatography
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
HxCDD	Hexachlorinated dibenzo-p-dioxin
HxCDF	Hexachlorinated dibenzofuran
ID_{50}	
I-TEF	International TCDD-toxic-equivalency
LD_{50}	Dose lethal to 50% of recipients (and all other subscripter dose levels)
LH	Luteinizing hormone
LDL	Low density liproprotein
LPL	Lipoprotein lipase activity
LOAEL	Lowest-observable-adverse-effect level
LOEL	Lowest-observed-effect level
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
MFO	Mixed function oxidase
mRNA	Messenger RNA
MNNG	*N*-methyl-*N*-nitrosoguanidine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NK	Natural killer
NOAEL	No-observable-adverse-effect level

LIST OF ABBREVIATIONS (cont.)

NOEL	No-observed-effect level
OCDD	Octachlorodibenzo-p-dioxin
OCDF	Octachlorodibenzofuran
PAH	Polyaromatic hydrocarbon
PB-Pk	Physiologically based pharmacokinetic
PCB	Polychlorinated biphenyl
OVX	Ovariectomized
PBL	Peripheral blood lymphocytes
PCQ	Quaterphenyl
PeCDD	Pentachlorinated dibenzo-p-dioxin
PeCDF	Pentachlorinated dibenzo-p-dioxin
PEPCK	Phosphopenol pyruvate carboxykinase
PGT	Placental glutathione transferase
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
ppm	Parts per million
ppq	
ppt	Parts per trillion
RNA	Ribonucleic acid
SAR	Structure-activity relationships
SGOT	Serum glutamic oxaloacetic transaminase

LIST OF ABBREVIATIONS (cont.)

SGPT	Serum glutamic pyruvic transaminase
SRBC	Sheep erythrocytes (red blood cells)
$t_{½}$	Half-time
TCAOB	Tetrachloroazoxybenzene
TCB	Tetrachlorobiphenyl
TCDD	Tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalency factors
TGF	Thyroid growth factor
tPA	Tissue plasminogen activator
TNF	Tumor necrosis factor
TNP-LPS	lipopolysaccharide
TSH	Thyroid stimulating hormone
TTR	Transthyretrin
UDPGT	UDP-glucuronosyltransferases
URO-D	Uroporphyrinogen decarboxylase
VLDL	Very low density lipoprotein
v/v	Volume per volume
w/w	Weight by weight

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4. IMMUNOTOXICITY

4.1. INTRODUCTION

Concern over the potential toxic effects of chemicals on the immune system arises from the critical role that the immune system plays in maintaining health. It is well recognized that suppressed immunological function can result in increased incidence and severity of infectious diseases as well as some types of cancer. Conversely, the inappropriate enhancement of immune function or the generation of misdirected immune responses can precipitate or exacerbate the development of allergic and autoimmune diseases. Thus, suppression as well as enhancement of immune function are considered to represent potential immunotoxic effects of chemicals.

The immune system consists of a complex network of cells and soluble mediators that interact in a highly regulated manner to generate immune responses of appropriate magnitude and duration. Consequently, comprehensive evaluation of immunotoxicity must include specific assessments of multiple functional parameters on a kinetic basis. In addition, because an immune response develops in a time-dependent manner relative to antigen exposure, the immunotoxicity of a chemical can be profoundly influenced by the timing of chemical exposure relative to antigen challenge. Consideration of these levels of complexity involved in immunotoxicology assessment are critical for interpretation of the effects of chemical exposure on immune function.

Extensive evidence has accumulated over the past 20 years to demonstrate that the immune system is a target for toxicity of TCDD and structurally related HAH, including the CDFs, PCBs and PBBs. This evidence has derived from numerous studies in various animal species, primarily rodents, but also guinea pigs, rabbits, monkeys, marmosets and cattle. Epidemiological studies also provide evidence for the immunotoxicity of HAH in humans. In animals, relatively high doses of HAH produce lymphoid tissue depletion, except in the thymus where cellular depletion occurs at lower doses. Alterations in specific immune effector functions and increased susceptibility to infectious disease have been identified at doses of TCDD well below those which cause lymphoid tissue depletion. Both cell-mediated and humoral immune responses are suppressed

following TCDD exposure, suggesting there are multiple cellular targets within the immune system that are altered by TCDD. Evidence also suggests that the immune system is indirectly targeted by TCDD-induced changes in nonlymphoid tissues. In addition, in parallel with increased understanding of the cellular and molecular mechanisms involved in immunity, studies on TCDD are beginning to establish biochemical and molecular mechanisms of TCDD immunotoxicity. These advances will be highlighted in this document.

There is an enormous literature based on descriptive studies on the immunotoxic effects of TCDD and related HAH in laboratory animals. Unfortunately, due to widely differing experimental designs, exposure protocols, and immunologic assays used, it has been very difficult to define a "TCDD-induced immunotoxic syndrome" in a single species, let alone across species. For example, there is only one report that directly compared the effects of TCDD on the immune system of rats, mice, and guinea pigs, and, even then, different immunologic parameters were assessed and different antigens were used in the different species (Vos et al., 1973). In that study, the DTH response to tuberculin was evaluated in guinea pigs and rats for assessment of cell-mediated immunity, while the GVH response was measured in mice. A decreased DTH response to tuberculin was observed in guinea pigs following 8 weekly doses of 40 ng/kg TCDD (total dose, 320 ng/kg), while the DTH response of rats to tuberculin was unaffected by 6 weekly doses of 5 μg/kg TCDD (total dose, 30,000 ng/kg TCDD). The GVH response in mice was suppressed by 4 weekly doses of 5 μg/kg TCDD (total dose, 20,000 ng/kg TCDD). The greater sensitivity of guinea pigs compared to rats and mice to the immunosuppressive effects of TCDD is consistent with the greater sensitivity of guinea pigs to other toxic effects of TCDD (McConnell et al., 1978; Poland and Knutson, 1982). Although these results appear to suggest that cell-mediated immunity in mice is more sensitive to TCDD than in rats, no studies have directly compared rats and mice using the same antigens and endpoints. In another study in mice, the DTH response to oxazolone was suppressed by 4 weekly doses of 4 μg/kg TCDD (total dose, 16,000 ng/kg), while the DTH response to SRBC was unaffected by a 10-fold higher dose of TCDD (Clark et al., 1981), illustrating that DTH responses to different antigens are not equally sensitive to TCDD-induced suppression, even in the same species. When

PCB and PBB studies are considered, variable effects on DTH and other immune reactions are also apparent (Fraker, 1980; Vos and Van Driel-Grootenhuis, 1972; Luster et al., 1980a; Thomas and Hinsdill, 1978). Because the exact basis for the inter-study variability is not known, it would serve no useful purpose in terms of risk assessment to catalogue all of the effects of TCDD and other HAH on the immune system that have been reported. Several comprehensive reviews have been published on the immunotoxic effects of HAH in general (Kerkvliet, 1984; Vos and Luster, 1989), and TCDD in particular (Holsapple et al., 1991a,b). The reader is also referred to the previous EPA TCDD-Risk assessment document, Appendix E (Sonawane et al., 1988) for another perspective on TCDD immunotoxicity. The present document will not reiterate this extensive literature, but rather, will emphasize more recent developments in the field of HAH immunotoxicity that may assist in the risk assessment process. Gaps in our knowledge that require further research will also be identified.

4.2. ROLE OF THE AH LOCUS IN HAH IMMUNOTOXICITY

One of the most important advances in the study of HAH toxicity in recent years has been the elucidation of a genetic basis for sensitivity to the toxicity of these chemicals, which may ultimately provide a logical explanation for much of the controversial data in the literature regarding HAH toxicity in different species and in different tissues of the same species. In this regard, many of the biochemical and toxic effects of HAH appear to be mediated via binding to an intracellular protein known as the Ah or TCDD receptor, in a process similar to steroid hormone receptor-mediated responses (Poland and Knutson, 1982; Cuthill et al., 1988). Ah receptor activation follows stereospecific ligand binding; interaction of the receptor-ligand complex with DREs in the genome induces the transcription of the structural genes encoding mRNA for CYP1A1 enzyme activity (i.e., cytochrome P_1450), as well as the expression of additional unidentified genes, the products of which are hypothesized to mediate HAH toxicity (Whitlock, 1990). Differences in toxic potency between various HAH congeners generally correlate with differences in Ah receptor binding affinities. The most toxic HAH congeners are approximate stereoisomers of 2,3,7,8-TCDD and are halogen-

substituted in at least three of the four lateral positions in the aromatic ring system.

In mice, allelic variation at the Ah locus has been described (Poland et al., 1987; Poland and Glover, 1990). The different alleles code for Ah receptors that differ in their ability to bind TCDD, and thus help to explain the different sensitivities of various inbred mouse strains to TCDD toxicity. Ah^{bb} C57Bl/6 (B6) mice represent the prototype "responsive" strain and are the most sensitive to TCDD toxicity, while Ah^{dd} DBA/2 (D2) mice represent the prototypic "non-responsive" strain and require higher doses of TCDD to produce the same toxic effect. Recently, congenic Ah^{dd} mice on a B6 background were derived that differ from conventional B6 mice primarily at the Ah locus. The spectrum of biochemical and toxic responses to TCDD exposure was similar in both strains but the doses needed to bring about the responses were significantly higher in congenic mice homozygous for the Ah^{d} allele compared to mice carrying two Ah^{b} alleles (Birnbaum et al., 1990; Kerkvliet et al., 1990a).

Two lines of evidence have been used to investigate the Ah receptor-dependence of the acute immunotoxicity of TCDD and related HAH: (1) comparative studies using CDD, CDF and PCB congeners that differ in their binding affinity for the Ah receptor; and (2) studies using mice of different genetic background known to differ at the Ah locus. Vecchi et al. (1983) was the first to report that the antibody response to SRBC was differentially suppressed by TCDD in B6 mice compared to D2 mice, with D2 mice requiring ~10 times higher dose to produce the same degree of suppression. Immunosuppression in F_1 and backcross mice supported the role of the Ah locus in the expression of TCDD immunotoxicity. 2,3,7,8-TCDF was significantly less potent than TCDD and showed a similar differential immunosuppressive effects in B6 and D2 mice. At the same time, Silkworth and Grabstein (1982) reported a B6 versus D2 strain-dependent difference in sensitivity to suppression of the antiSRBC response by 3,4,3',4'-TCB, a ligand for the Ah receptor. In comparison, the 2,5,2',5'-TCB isomer, which lacks affinity for the Ah receptor, was not immunosuppressive in either B6 or D2 mice. Structure-activity relationships were extended by Kerkvliet et al. (1985) in studies that compared the immunosuppressive potency of the chlorinated

dioxin and furan isomers that contaminate technical grade pentachlorophenol. The 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD and 1,2,3,4,6,7,8-HpCDF isomers, which bind the receptor, were all significantly immunosuppressive. The dose of each isomer that produced 50% suppression of the antiSRBC response (ID_{50}) was 7.1, 85 and 208 μg/kg for HxCDD, HpCDD and HpCDF, respectively (Figure 4-1). The ID_{50} for TCDD was 0.65 μg/kg based on the data of Vecchi et al. (1980). In contrast, OCDD, which does not bind the receptor, was not immunosuppressive at a dose as high as 500 μg/kg (Kerkvliet et al., 1985). More extensive structure-dependent immunosuppressive activities of technical grade PCB mixtures (Davis and Safe, 1990), PCB congeners (Davis and Safe, 1989), and CDF congeners (Davis and Safe, 1988) have also been reported. Results of these studies using different HAH congeners are summarized in Table 4-1.

The role of the Ah receptor in suppression of the antiSRBC response has recently been verified in studies using B6 mice congenic at the Ah locus (Kerkvliet et al., 1990a). As expected, congenic Ah^{dd}-B6 mice were significantly less sensitive to TCDD-induced immune suppression compared to wild-type Ah^{bb}-B6 mice. Unexpectedly, however, the dose-response in congenic B6-Ah^{dd} mice appeared to be bimodal, with a portion of the response sensitive to suppression by low doses of TCDD. Because of the bimodal response, the data did not permit extrapolation of an ID_{50} dose in the congenic mice. The results were interpreted to suggest potential non-Ah receptor mediated immunosuppressive effects. It should be noted, however, that recent studies by Dr. Jay Silkworth using re-derived congenic Ah^{dd}-B6 mice have thus far not corroborated a bimodal dose-response (Silkworth, personal communication). The issue of Ah receptor-independent immunotoxicity will be discussed in detail in a subsequent section of this document.

Ah receptor dependency of HAH immunotoxicity has also been demonstrated in mice using other immunologic responses. For example, Kerkvliet et al. (1990a) reported that the ID_{50} for suppression of the antibody response to TNP-LPS in Ah^{bb} B6 mice was 7.0 μg/kg compared to a significantly higher ID_{50} of 30 μg/kg in congenic Ah^{dd} B6 mice. Since the antibody response to TNP-LPS shows little

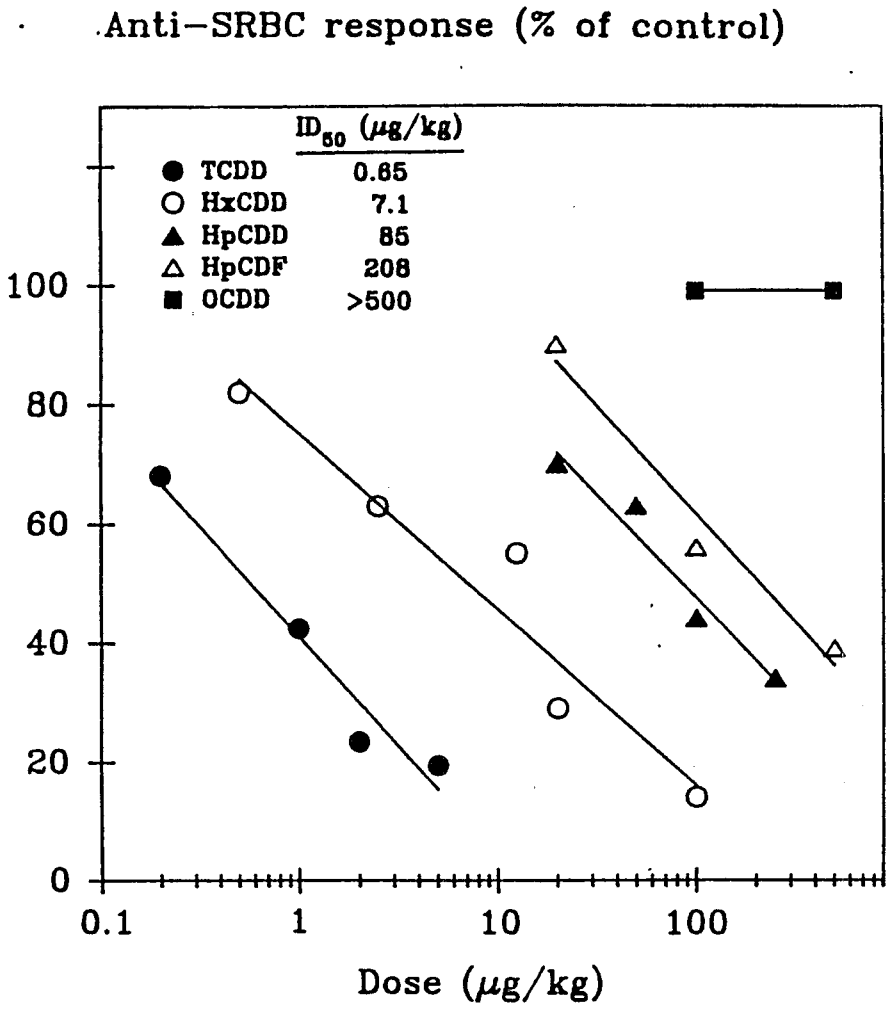

FIGURE 4-1

Structure Dependent Immunotoxicity of Some CDD and CDF Isomers. Immunotoxicity Assessed by Suppression of the Splenic Antibody Response to SRBC

Source: Kerkvliet et al., 1985

TABLE 4-1

TEFs for CDDs, CDFs and PCBs on the Acute Single Dose ID_{50} for Suppression of the PFC Response to SRBC in Ah Responsive B6 Mice

Congener	ID_{50}	TEF	Reference
2,3,7,8-TCDD	0.74 µg/kg	1.0[a]	Kerkvliet and Brauner, 1990
2,3,7,8-TCDD	0.65 µg/kg	1.0[a]	Vecci et al., 1980
2,3,7,8-TCDD	0.77 µg/kg	1.0[a]	Davis and Safe, 1988
2,3,7,8-TCDD	0.60 µg/kg	1.0[a]	Kerkvliet et al., 1990a
1,2,3,6,7,8-HxCDD	7.1 µg/kg	0.1	Kerkvliet et al., 1990a
1,2,3,4,6,7,8-HpCDD	85.0 µg/kg	8.2×10^{-3}	Kerkvliet et al., 1985
OCDD	>500 µg/kg	$>1.4 \times 10^{-3}$	Kerkvliet et al., 1985
2,3,4,7,8-TCDF	1.0 µg/kg	0.7	Davis and Safe, 1988
2,3,7,8-TCDF	4.3 µg/kg	1.6×10^{-1}	Davis and Safe, 1988
1,2,3,4,6,7,8-HpCDF	208 µg/kg	3.4×10^{-3}	Kerkvliet et al., 1985
1,2,3,7,9-PeCDF	239 µg/kg	2.9×10^{-3}	Davis and Safe, 1988
1,3,6,8-TCDF	11 µg/kg	6.4×10^{-5}	Davis and Safe, 1988
3,4,3',4'-TCB	28 mg/kg[b]	2.5×10^{-5}	Silkworth and Grabstein, 1982
2,3,4,5,3',4'-HxCB	0.7 mg/kg	1.0×10^{-3}	Davis and Safe, 1990
2,3,4,5,3',4'-HxCB	36 mg/kg[b]	1.9×10^{-5}	Silkworth et al., 1984
2,4,3',4',5',6'-HxCB	43 mg/kg	1.6×10^{-5}	Davis and Safe, 1990
2,3,4,3',5'-PeCB	65 mg/kg	1.1×10^{-5}	Davis and Safe, 1990
2,3,4,5,3',5'-HxCB	72 mg/kg	9.7×10^{-6}	Davis and Safe, 1990
2,4,2',4'-TCB	>100 mg/kg	$>7.0 \times 10^{-6}$	Silkworth et al., 1984
2,4,5,2',4',6'-HxCB	>360 mg/kg	$>1.9 \times 10^{-6}$	Davis and Safe, 1990
2,4,6,2',4',6'-HxCB	>360 mg/kg	$>1.9 \times 10^{-6}$	Davis and Safe, 1990
2,4,5,2'4',5'-HxCB	>360 mg/kg	$>1.9 \times 10^{-6}$	Biegel et al., 1989

TABLE 4-1 (cont.)

Congener	ID_{50}	TEF	Reference
Aroclor 1260	104 mg/kg	$6.7x10^{-6}$	Davis and Safe, 1989
Aroclor 1254	118 mg/kg	$5.9x10^{-6}$	Davis and Safe, 1989
Aroclor 1254	207 mg/kg	$3.4x10^{-6}$	Lubet et al., 1986
Aroclor 1248	190 mg/kg	$3.7x10^{-6}$	Davis and Safe, 1989
Aroclor 1242	391 mg/kg	$1.8x10^{-6}$	Davis and Safe, 1989
Aroclor 1016	408 mg/kg	$1.7x10^{-6}$	Davis and Safe, 1989
Aroclor 1232	464 mg/kg	$1.5x10^{-6}$	Davis and Safe, 1989

[a]Based on mean ID_{50} of 0.7±0.07 µg/kg

[b]Interpolated from two data points

requirement for macrophages or T helper cells (Jelinek and Lipsky, 1987), these results suggest an Ah receptor dependent B cell response. In terms of cytotoxic T cells, Clark et al. (1983) was first to report data suggesting that TCDD and PCB isomers suppressed *in vitro* CTL responses of B6 and D2 mice through an Ah receptor-dependent mechanism. Subsequently, Kerkvliet et al. (1990b) reported that B6 mice congenic at the Ah locus showed Ah-dependent sensitivity to suppression of the CTL response following exposure to either TCDD or 3,4,5,3',4',5'-HxCB. Furthermore, the potency of TCDD and of three HxCB congeners to suppress the CTL response of Ah^{bb}-B6 mice directly correlated with their relative binding affinities for the Ah receptor (Table 4-2). The ID_{50} of TCDD for suppression of the CTL response in B6 mice was 7.0 μg/kg[1].

In summary, the data relating HAH immunotoxicity, at least in part, to Ah receptor-dependent events are convincing. However, it should be emphasized that all of the data have been obtained from studies in inbred mice using an acute or subacute exposure regimen. Except for thymic atrophy, structure-immunotoxicity relationships in other species including rats have not been established, and the availability of inbred strains of other species with defined Ah genotype are not currently available. The importance of Ah receptor mediated events in chronic, low-level HAH immunotoxicity also remains to be established. Morris et al. (1992) have recently reported that the sensitivity of D2 mice to TCDD-induced suppression of the antiSRBC response increased significantly when TCDD was administered daily over two weeks rather than as an acute single dose. Unfortunately, in these studies, the lowest dose of TCDD produced near-maximum suppression of the antiSRBC response of B6C3F1 mice in the acute exposure model, precluding the detection of any similar increase in sensitivity of the B6C3F1 mice to chronic dosing. In contrast to these findings, Vecchi et al. (1983) reported that multiple exposures to TCDD (2 μg/kg for 5 weeks or 0.5 μg/kg for 8 weeks) did not increase the sensitivity of D2 mice to suppression of the antiSRBC response. Thus, the basis for any change in potency resulting from

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[1]The dose of TCDD required to suppress the CTL response reported by Kerkvliet et al. (1990b) is significantly greater than that reported by Clark et al. (1981), who reported CTL suppression following four weekly doses of 0.1 μg/kg TCDD. Clark et al (1983) also reported that doses of TCDD as low as 4 ng/kg to B6 mice suppressed the *in vitro* generation of CTL and that the suppression was Ah dependent. The potency of TCDD described in the Clark et al. (1981, 1983) studies has not been corroborated by other laboratories.

TABLE 4-2

TEFs Based on the ID_{50} for Suppression of Alloantigen (P825)-Specific CTL Response in Ah Responsive B6 Mice[a]

Congener	ID_{50}	TEF
TCDD	7 μg/kg	1.0
3,4,5,3',4',5'-HxCB	7 mg/kg	1000
2,3,4,5,3',4'-HxCB	70 mg/kg[b]	10,000
2,4,5,2',4',5'-HxCB	>300 mg/kg	>42,857

[a]Source: Kerkvliet et al., 1990b

[b]Interpolated from two data points

multiple treatment or chronic exposure to TCDD and the role of Ah receptor-mediated events in the phenomenon remain to be elucidated.

4.3. TOXIC EQUIVALENCY FACTORS (TEFs) FOR IMMUNOTOXICITY

Based on the available data from mice, the majority of the immunotoxic effects of HAH appear to be mediated via the Ah receptor. Thus, the toxicity of different HAH congeners can be compared by calculating TEFs. TEFs based on acute, single dose exposure (oral or intraperitoneal) of B6 mice to various HAH for suppression of the antiSRBC response and the CTL response are presented in Table 4-1 and 4-2, respectively. As shown in Table 4-1, the potency of TCDD to suppress the antibody response to SRBC has been reported by several laboratories, with remarkable agreement[2] in the ID_{50} value of 0.7 μg/kg in B6 mice. The ID_{50} of B6C3F1 mice is similar (<1 μg/kg) (House et al., 1990) or slightly higher (1.2 μg/kg) (Holsapple et al., 1986a) in comparison to B6 mice. This data thus provides a well-defined base value to use in calculating TEFs for other HAH congeners in the context of suppression of the antiSRBC response.

However, in contrast to the reproducible data on TCDD, the accuracy of the derived TEFs for other HAH congeners shown in Table 4-1 is difficult to evaluate since few congeners have been examined in more than one study. In the few cases where the same congener has been evaluated independently, discrepancies in the data exist. For example, both Davis and Safe (1990) and Silkworth et al. (1984) evaluated the potency of the 2,3,4,5,3',4'-HxCB congener in the antiSRBC response. Based on the ID_{50}'s from the two data sets, the respective TEFs differ by almost two orders of magnitude ($1x10^{-3}$ versus $2x10^{-5}$). When the same congener was compared to TCDD for suppression of the CTL response, the TEF was $1x10^{-4}$ (Table 4-2). The basis for these discrepancies is unknown. Thus, the data base

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[2]Several laboratories have reported that the antibody response to SRBC is sensitive to suppression following acute exposure to TCDD, either intraperitoneally or orally, at doses <1 μg/kg. In contrast, Clark et al. (1981) reported that 4 weekly ip doses of 10 but not 1 or 0.1 μg/kg TCDD significantly suppressed the antiSRBC response in B6 mice. The chronic dosing protocol used by Clark does not readily explain his decreased potency since Vecchi et al (1983) reported that five weekly doses of 2 μg/kg or eight weekly doses of 0.5 μg/kg TCDD significantly suppressed the antiSRBC response. Likewise, when total doses of 0.2 or 1 μg/kg TCDD were given as a single dose or divided into five daily doses, the divided dose produced slightly more suppression that the single dose (Table 4-3) (Kerkvliet and Deyo, unpublished data). In addition, the route of antigen challenge [intravenous (used by Clark) versus intraperitoneal (used by Vecchi)] does not appear to greatly influence the degree of suppression of the antiSRBC response produced by TCDD (Table 4-4) (Kerkvliet and Deyo, unpublished data). Thus, the basis for the discrepancies between the data of Clark et al. (1981, 1983) and other laboratories regarding the potency of TCDD to suppress the antiSRBC response is unknown.

TABLE 4-3

Effect of Single Versus Multiple Dosing with TCDD on Suppression of the Antibody Response to SRBC in C57Bl/6 Mice

Total Dose (μg/kg)	Plaque-Forming Cells/10^6 Spleen Cells (mean ± SD)	
	Single[a]	Multiple[b]
0	2460±657	3846±1618
0.2	1879±445 (76)	2356±592 (61)
1.0	1293±285 (52)[c]	1143±208 (30)[c]

[a]Total dose of TCDD given once two days prior to SRBC injection

[b]Total dose of TCDD divided into five equal doses administered on days -7 to -2 prior to SRBC injection

[c]$p<0.01$

TABLE 4-4

Influence of Route of Antigen Challenge on Suppression of the Antibody Response to SRBC in C57Bl/6 Mice[a]

Dose of TCDD (μg/kg)	Plaque-Forming Cells/10^b Spleen Cells (mean ± SD)	
	Intravenous	Intraperitoneal
0	1151±367	1812±872
0.2	623±324 (54)	1197±519 (66)
1.0	466±212 (40)[b]	697±163 (38)[b]

[a] 2.5×10^8 SRBC were injected intravenously or intraperitoneally 2 days after oral dosing with TCDD; plaque-forming cells were measured 5 days later.

[b] $p < 0.01$

for TEF comparisons using immunotoxicity data must be expanded considerably before TEFs can be used with confidence in risk assessment.

4.4. INTERACTIONS BETWEEN HAH

If the immunotoxicity of TCDD and structurally related HAH depend on Ah-receptor mediated mechanisms, then co-exposure to subsaturating levels of more than one Ah receptor ligand should produce additive effects. An additive interaction has been demonstrated in mice co-exposed to 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD, two relatively strong Ah receptor ligands (Kerkvliet et al., 1985). On the other hand, Davis and Safe (1988, 1989) have reported that co-exposure of mice to an immunotoxic dose of TCDD and a subimmunotoxic dose of different commercial Aroclors or different PCB congeners resulted in partial antagonism of TCDD suppression of the antiSRBC response. In limited studies, an apparently similar antagonism was observed following co-exposure to 2,3,7,8-TCDF (10 μg/kg) and TCDD (1.2 μg/kg) (Rizzardini et al., 1983). The mechanism for the antagonism has not been fully elucidated, but the effects are consistent with competition for binding at the Ah receptor, since the weaker agonist was administered in excess compared to TCDD. Furthermore, Silkworth et al. (1988) and Silkworth and O'Keefe (1992) have shown that the immunotoxicity of TCDD can be modified by coexposure to other HAH present as co-contaminants of actual environmental samples from Love Canal. Such interactions complicate hazard assessment of mixtures based on TEFs and may preclude dependence on TEFs without biological response evaluation for risk assessment.

4.5. SENSITIVE TARGETS FOR HAH IMMUNOTOXICITY

Despite considerable investigation, the cells that are altered by HAH exposure leading to suppressed immune function have not been unequivocally identified. The main reason for the lack of definitive progress in this area is the conflicting data reported from different laboratories regarding the ability of TCDD to suppress lymphocyte functions when examined "*ex vivo*" or *in vitro*. As discussed in a subsequent section of this document, the *in vitro* effects of TCDD are greatly influenced by the *in vitro* culture conditions, which may explain the discrepancies in effects observed in different laboratories.

In contrast to *in vitro* studies, the *in vivo* immunotoxicity of TCDD, expressed in terms of suppression of the antiSRBC response of B6 or B6C3F1 mice,

is highly reproducible between laboratories. Since the magnitude of the antiSRBC response depends on the concerted interactions of antigen-presenting cells (APC), regulatory T cells (helper and suppressor), and B cells, this response has been used most widely to evaluate target cell sensitivity to HAH. In addition, the antibody response to SRBC can be modulated by many non-immunological factors, including hormonal and nutritional variables, and HAH are known to affect numerous endocrine and metabolic functions. These latter effects will be apparent only in *in vivo* studies, while only direct effects of HAH on APC and lymphocyte functions would be evident following *in vitro* exposure to HAH. To date, direct *in vitro* effects of TCDD on purified B cell activity have been reported (Holsapple et al., 1986a; Morris and Holsapple, 1991; Luster et al., 1988), while direct effects on macrophages and T cells *in vitro* have not been described. (The *in vitro* effects of TCDD will be discussed in more detail in a subsequent section of this document.)

Kerkvliet and Brauner (1987) compared the sensitivity of antibody responses to antigens that differ in their requirements for APC and T cells as an *in vivo* approach to evaluate the cellular targets of 1,2,3,4,6,7,8-HpCDD humoral immunotoxicity. The T-helper cell independent (TI) antigens, DNP-Ficoll and TNP-LPS, were used in these studies. These TI antigens differ from each other in their requirement for APC (higher for DNP-Ficoll) and their sensitivity to regulatory (amplifier and suppressor) T cell influence (DNP-Ficoll is sensitive, TNP-LPS is not) (Braley-Mullen, 1982). Obviously, all antibody responses require B cell differentiation into antibody-secreting plasma cells. Although HpCDD produced dose-dependent suppression of the antibody response to all three antigens, sensitivity to suppression directly correlated with the sensitivity of the response to T cell regulation. The ID_{50} values were 53, 127 and 516 μg/kg for SRBC, DNP-Ficoll, and TNP-LPS, respectively. These results were interpreted as follows: If one assumes that B cell function is targeted in the TNP-LPS response, then regulatory T cells and/or APC may represent the more sensitive target in the SRBC and DNP-Ficoll responses. The difference in sensitivity between the SRBC and DNP-Ficoll responses suggest that the T helper cell may be a particularly sensitive target. The differential sensitivity of the antibody

responses to TNP-LPS versus SRBC has been corroborated in TCDD-treated mice (House et al. 1990; Kerkvliet et al., 1990a). Thus, the exquisite *in vivo* sensitivity of the antibody response to SRBC would appear to depend on the T cell and/or APC components of the response rather than the B cell, unless the B cells that respond to SRBC are different from the B cells that respond to TNP-LPS. Currently, evidence for such a difference is lacking. However, this interpretation conflicts with the "*ex vivo*" data of Dooley and Holsapple (1988). Using separated spleen T cells, B cells and adherent cells from vehicle- and TCDD-treated mice, they reported that B cells from TCDD-treated mice were functionally compromised in *in vitro* antibody responses but T cells and macrophages were not. The basis for these discrepant findings has not been established. However, it is possible that the effects of TCDD on T cells are indirectly induced following antigen exposure such that removal of the cells from the TCDD environment of the host prior to antigen challenge would preclude detection of T cell dysfunction. This interpretation is supported by the findings of Tomar and Kerkvliet (1991) that spleen cells taken from TCDD-treated mice were not compromised in their ability to reconstitute the antibody response of lethally irradiated mice. This interpretation is also consistent with the reported lack of direct effects of TCDD and other HAH on T cells *in vitro* (Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988a).

While the direct effects of TCDD on T cells *in vitro* have not been demonstrated, it is clear that functional T cell responses generated *in vivo* are compromised following *in vivo* exposure. Nude mice that are congenitally T cell deficient are significantly less sensitive to HpCDD-induced immunotoxicity when compared to their T cell-competent littermates (Kerkvliet and Brauner, 1987) Likewise, exposure to TCDD or HxCB suppresses the development of CTL activity following alloantigen challenge (Kerkvliet et al., 1990b). Interestingly, the sensitivity of the CTL response to suppression by TCDD is approximately the same as the sensitivity of the antibody response to TNP-LPS (both have an ID_{50} of approximately 7 μg/kg in B6 mice) (Kerkvliet et al., 1990a,b). Since the B cell response to TNP-LPS and the CTL response to I-A^- P815 alloantigen have little requirement for antigen-presenting cells or classic $CD4^+$ T helper cells, these

results suggest that the differentiation of B cells and $CD8^+$ CTLp to effector cells have an equivalent "functional" sensitivity to TCDD.

The influence of TCDD exposure on regulatory T cell functions has been addressed in a limited number of studies. Clark et al. (1981) first proposed that T suppressor cells were induced by TCDD in the thymus that were responsible for the suppressed CTL response. However, increased suppressor cell activity in peripheral lymphoid tissue was not observed in mice exposed to TCDD (Dooley et al., 1990) or 3,4,5,3',4',5'-HxCB (Kerkvliet and Baecher-Steppan 1988b). In terms of T helper cell activity, Tomar and Kerkvliet (1991) reported that a dose of 5 μg/kg TCDD suppressed the *in vivo* generation of carrier-specific T helper cells. Lundberg et al. (1990) reported that thymocytes from B6 mice treated with TCDD (50 μg/kg) were less capable of providing help for an *in vitro* antiSRBC response. However, Clark et al. (1983) reported in *ex vivo* studies that T cells from TCDD-treated mice produced normal levels of IL-2. The *in vivo* effect of TCDD on the production of IL-2 as well as other lymphokines important in the development of an antibody response (e.g., IL-4, IL-5) have not been reported.

The influence of TCDD exposure on B cell function has been addressed primarily in *in vitro* studies. The issue is difficult to address *in vivo* given that most B cell responses (except perhaps anti-LPS responses) are dependent on interactions with T cells and macrophages. *In vitro* studies have described the direct effects of TCDD on the activation and differentiation of purified B cells (Luster et al., 1988; Morris et al., 1991). These studies suggest that TCDD inhibits the terminal differentiation of B cells via alteration of an early activation event (Luster et al., 1988). Increased phosphorylation and tyrosine kinase activity in TCDD-treated B cells may underlie this B cell dysfunction (Kramer et al., 1987; Clark et al., 1991a).

Macrophage functions have also been examined following TCDD exposure and generally found to be resistant to suppression by TCDD when assessed *ex vivo*. Macrophage-mediated phagocytosis, macrophage-mediated tumor cell cytolysis or cytostasis, oxidative reactions of neutrophils and macrophages, and NK (cell activity) were not suppressed following TCDD exposure, with doses as high as 30 μg/kg failing to suppress NK and macrophage functions (Vos et al., 1978;

Mantovani et al., 1980). A potentially important exception is the reported selective inhibition of phorbol ester-activated antitumor cytolytic and cytostatic activity of neutrophils by TCDD (Ackermann et al., 1989).

On the other hand, it is interesting to note that the pathology associated with TCDD toxicity often includes neutrophilia and an inflammatory response in liver and skin characterized by activated macrophage and neutrophil accumulation (Weissberg and Zinkl, 1973; Vos et al., 1973; Vos et al., 1974; Puhvel et al., 1988). While these observations may simply reflect a normal inflammatory response to tissue injury, there is some preliminary experimental evidence that suggests inflammatory cells may be activated by TCDD exposure. For example, Alsharif et al. (1990) recently reported that TCDD increased superoxide anion production in rat peritoneal macrophages. In addition, it has been shown that TCDD exposure results in an enhanced inflammatory response following SRBC challenge (Kerkvliet and Brauner, 1990b). This effect of TCDD was characterized by a 2-4 fold increase in the number of neutrophils and macrophages locally infiltrating the intraperitoneal site of SRBC injection. However, the kinetics of the cellular influx was not altered by TCDD. Likewise, the expression of macrophage activation markers (I-A and F4/80) and the antigen-presenting function of the peritoneal exudate cells was unaltered by TCDD. Thus, the effect of TCDD appeared to reflect a quantitative rather than a qualitative change in the inflammatory response. Importantly, TCDD-induced suppression of the antiSRBC response could not be overcome by increasing the amount of antigen used for sensitization, suggesting that enhanced antigen clearance/degradation by the increased numbers of phagocytic cells (e.g., decreased antigen load) was not responsible for the decreased antibody response in TCDD-treated mice. Thus, the relationship, if any, between the inflammatory and immune effects of TCDD remain to be elucidated.

One mechanism by which TCDD and related HAH may augment inflammatory responses is via enhance production of inflammatory mediators such as interleukin 1 (IL-1) and TNF. Recent evidence suggests that the long-recognized hypersusceptibility of TCDD- and PCB-treated animals to endotoxin (LPS) (Thomas and Hinsdill, 1978; 1979; Vos et al., 1978; Loose et al., 1979) may be related to an increased production of TNF and/or IL-6 in the chemically treated animals (Clark

et al., 1991b; Taylor et al., 1990; Hoglen et al., 1992). The ability of methylprednisolone to reverse the mortality associated with TCDD/endotoxin treatment is also consistent with an inflammatory response (Rosenthal et al., 1989). Similarly, increased inflammatory mediator production may underlie the enhanced rat paw edema response to carrageenan and dextran in TCDD-treated rats (Theobald et al., 1983; Katz et al., 1984). Limited, preliminary data is available to indicate that the production of inflammatory mediators such as TNF (Taylor et al., 1990; Clark et al., 1991b) and IL-6 (Hoglen et al., 1992) may be increased in HAH-treated animals. Serum complement activity, on the other hand, has been reported to be suppressed in dioxin-treated mice (White et al., 1986), although enhanced activity was reported at the lowest exposure level when 1,2,3,6,7,8-hexachlorodioxin was tested. A primary effect of TCDD on IL-1 is supported by the recent findings of Sutter et al. (1991) that the IL-1β gene contains a DRE. Likewise, Steppan and Kerkvliet (1991) have reported that under some exposure conditions TCDD increased the level of mRNA for IL-1 in TCDD-treated IC21 cells, a macrophage cell line derived from B6 mice. On the other hand, House et al. (1990) reported that inflammatory macrophages obtained from TCDD-treated mice produced control levels of IL-1 when examined *ex vivo*. Thus, the effect of TCDD on inflammatory mediator production may be a "priming effect" and require co-exposure to antigen or LPS. The influence of TCDD on inflammatory mediator production and action is an important area for further study.

Since the rapid influx of phagocytic cells to the site of pathogen invasion is an important factor in host resistance to infection, the ability of TCDD to augment the production of inflammatory chemoattractive mediators would imply that TCDD exposure could result in enhanced host resistance. However, since TCDD exposure is, at the same time, immunosuppressive, resulting in decreased specific immune responses generated by T and B lymphocytes, the overall impact of TCDD exposure on disease susceptibility will likely vary depending on the nature of the pathogen and the major mode of host response to the specific infectious agent. Such effects may in fact help to explain the disparate effects of TCDD in different host resistance models that have been previously reported.

4.6. INFLUENCE OF TCDD ON HOST RESISTANCE TO DISEASE

The ability of an animal to resist and/or control viral, bacterial, parasitic and neoplastic diseases is determined by both nonspecific and specific immunological functions. Decreased functional activity in any immunological compartment may result in increased susceptibility to infectious and neoplastic diseases. In terms of risk assessment, host resistance is often accorded the "bottom line" in terms of relevant immunotoxic endpoints. Animal host resistance models that mimic human disease are available and have been used to assess the effect of TCDD on altered host resistance.

TCDD exposure increases susceptibility to challenge with the gram negative bacterium *Salmonella*. TCDD was given *per os* at 0.5 to 20 μg/kg once a week for 4 weeks to male 4-week-old C57Bl/6Jfh (J67) mice and challenged 2 days after the fourth dose (when mice were 8 weeks old) with either *Salmonella bern* or *Herpesvirus suis* (also known as pseudorabies virus). Results with *S. bern* indicated there was an increased mortality at 1 μg TCDD/kg (total dose of 4 μg/kg) and a reduced time to death after bacterial challenge with 5 μg TCDD/kg (total dose of 20 μg/kg). In contrast, the same doses of TCDD did not alter the time to death or the incidence of mortality following *H. suis* infection (Thigpen et al., 1975). A TCDD feeding study by Hinsdill et al. (1980) also demonstrated increased susceptibility of 7-week-old Swiss Webster outbred female mice to *Salmonella typhimurium* var. Copenhagen. Mice were fed control feed or feed containing 10, 50, or 100 ppb TCDD for 8 weeks, after which they were injected intravenously with $10^{3.5}$ *S. typhimurium* var. Copenhagen. Results indicated that 50 and 100 ppb TCDD increased mortality from *Salmonella* and shortened the time to death while 10 ppb caused an increased bacteremia. Vos et al. (1978) reported that TCDD resulted in a reduced resistance to endotoxin (*Escherichia coli* O 127:B 8 lipopolysaccharide) and suggested that the increased susceptibility to *Salmonella* caused by TCDD may be due to the lipopolysaccharide or endotoxin present on this gram negative bacterium. Vos et al. (1978) demonstrated reduced resistance to endotoxin with a single oral dose of 100 ¶g TCDD/kg using 3- to 4-week-old outbred female mice and challenged with endotoxin 5 days later. Vos et al. (1978) also reported enhanced mortality from the intravenous injection of

endotoxin 2 days after the final oral dose of TCDD (1.5, 5, 15 or 50 μg/kg once a week for 4 weeks) in 3- to 4-week-old male outbred Swiss mice. These studies indicate a reduced resistance to endotoxin after single or multiple doses of TCDD. Thomas and Hinsdill (1979), using *S. typhimurium* lipopolysaccharide, demonstrated a reduced resistance to endotoxin in the offspring of female Swiss Webster mice fed TCDD prior to mating, during gestation and between parturition and weaning. Rosenthal et al.(1989) used female B6C3F1, DBA/2, as well as congenic mice to demonstrate that acute doses of 50, 100 or 200 μg TCDD *per os* increased endotoxin-induced mortality in B6C3F1 mice, associated with hepatotoxicity and decreased clearance of the endotoxin. D2 and Ah^{dd} congenic mice were relatively resistant to this effect implicating Ah receptor dependent mechanisms in endotoxin hypersensitivity.

White et al. (1986) reported that *Streptococcus pneumoniae*, a gram positive bacterium that does not contain endotoxin, caused increased mortality in 5- to 6-week-old female B6C3F1 mice after subchronic oral administration of TCDD (1 μg/kg for 14 days) and challenged with *S. pneumoniae* intraperitoneally 1 day after the last treatment. The 1,2,3,6,7,8-HCDD isomer also resulted in a dose-dependent increase in susceptibility to *S. pneumoniae*.

Enhanced susceptibility to viral disease has also been reported after TCDD administration. Clark et al. (1983) injected TCDD intraperitoneally once a week for 4 weeks and challenged mice 7-22 days later with *Herpes simplex type II strain 33* virus. Mice receiving TCDD at 0.04, 0.4, or 4.0 μg/kg weekly (total dose of 160, 1600 and 16,000 ng/k) all had significantly enhanced mortality to Herpesvirus type II infection. House et al. (1990) also reported an enhanced susceptibility to viral infection following low level single dose TCDD administration intraperitoneally. B6C3F1 female mice, 6-8 weeks of age, were challenged with Influenza/A/Taiwan/1/64 (H2N2) virus 7-10 days following TCDD. TCDD administration at 10, 1.0 and 0.1 μg/kg decreased resistance to virus.

TCDD exposure also results in more severe parasitic diseases. Tucker et al. (1986) studied the effects of TCDD administration on *Plasmodium yoelii* 17 XNL, a nonlethal strain of malaria, in 6- to 8-week-old B6C3F1 female mice. A single dose of TCDD at 5 μg/Kg or 10 μg/kg per os resulted in increased susceptibility

to *P. yoelii*. The peak parasitemia was greater and of longer duration in TCDD-treated animals than in controls, the difference being significant at 5 μg/kg on day 10 and at 10 μg/kg on days 12 and 14.

Luster et al. (1980b) demonstrated enhanced growth of transplanted tumors in mice treated with TCDD at doses of 1.0 or 5.0 μg/Kg in B6C3F1 mice. Mothers were given TCDD by gavage at day 14 of gestation and again on days 1, 7 and 14 following birth; host resistance studies were performed 6-8 weeks after weaning. This exposure protocol resulted in an increased incidence of PYB6 tumors in pups from dams receiving repeated doses of 1.0 but not 5.0 μg TCDD/Kg.

While it is clear that TCDD adversely affects numerous host resistance models detailed above, the effects of TCDD on susceptibility to *Listeria monocytogenes* infections are ambiguous. The disparate results may reflect different study designs including dose, route, single versus multiple administrations, mouse strain, age or sex. However, it is clear that TCDD, under certain conditions, results in increased susceptibility to *Listeria*. Hinsdill et al. (1980) reported the increased susceptibility of 7-week-old Swiss Webster outbred female mice to *Listeria*. Mice were fed control feed or feed containing 10 or 50 ppb TCDD for 8 weeks, after which they were injected intravenously with 10^5 *Listeria*. Results indicated that the 50 ppb diet increased bacteremia and mortality. Luster et al. (1980b) used doses of 1.0 or 5.0 ug TCDD/Kg in B6C3F1 mice. Mothers were given TCDD by gavage at day 14 of gestation and again on days 1, 7 and 14 following birth and host resistance studies were performed 6-8 weeks after weaning. This exposure protocol resulted in an increased susceptibility to *Listeria* in pups from dams receiving repeated doses of 5.0 μg TCDD/Kg. However, Vos et al. (1978) reported that oral administration of 50 μg TCDD/kg once a week for 4 weeks to 3- to 4-week-old male Swiss mice followed by intravenous challenge 4 days after the last dose with Listeria had no affect on nonspecific phagocytosis and killing of *Listeria*. House et al. (1990) used B6C3F1 female mice, 6-8 weeks of age, and challenged intravenously with *Listeria* 7-10 days following a single dose of TCDD at 10, 1.0 and 0.1 μg/kg. TCDD did not enhance mortality to *Listeria*.

In summary, results from host resistance studies provide evidence that exposure to TCDD results in increased susceptibility to bacterial, viral, parasitic and neoplastic disease. These effects are observed at low doses and likely result from TCDD-induced suppression of immunological function. However, it is interesting that the role of the Ah receptor has not been addressed in terms of host resistance models except in studies on endotoxin hypersensitivity by Rosenthal et al. (1989). Furthermore, the specific immunological functions targeted by TCDD in each of the host resistance models remain to be fully defined.

4.7. *IN VITRO* IMMUNOTOXIC EFFECTS OF HAH

Investigators in the field of TCDD immunotoxicity have long acknowledged the difficulties in consistently demonstrating the immunotoxicity of TCDD when cells from treated animals are tested *ex vivo* as well as when TCDD is added to culture *in vitro*. While effects following *in vitro* and *ex vivo* exposure to TCDD on lymphocyte functions have been reported (Tucker et al., 1986; Luster et al., 1988; Dooley and Holsapple, 1988), other laboratories have failed to observe suppression with *in vitro* or *ex vivo* exposure to dioxin (Lundberg et al., 1990; Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988b; Kerkvliet, unpublished data). In addition, the effects of TCDD seen *in vitro* are sometimes inconsistent with those observed after *in vivo* assessment of immunotoxicity. For example, the rank order of sensitivity to suppression of T helper cell-dependent and T helper cell-independent antibody responses seen *in vivo* (Kerkvliet and Brauner 1987; 1990a; House et al., 1990) is not seen *in vitro* (Holsapple et al., 1986a; Tucker et al., 1986) suggesting different cellular targets may be affected following *in vitro* exposure to TCDD. More importantly, some data suggest that suppression of the *in vitro* antibody response may occur independent of the Ah receptor. Tucker et al. (1986) and Holsapple et al. (1986a) reported that direct addition of TCDD *in vitro* suppressed the antibody response to SRBC. However, based on the response of cells from congenic mice as well as a limited structure-activity study, the data of Tucker et al. (1986) supported an Ah receptor-dependent suppression while the data of Holsapple et al. (1986a) did not. In the latter study, the magnitude of suppression was comparable using cells from responsive

B6C3F1 or congenic heterozygous (B6-Ah^{bd}) mice compared to nonresponsive D2 or homozygous B6-Ah^{dd} mice. In addition, they reported that the 2,7-dichloro-dibenzo-p-dioxin congener which lacks affinity for the Ah receptor, was equipotent with TCDD in suppressing the *in vitro* response.

In other studies, Davis and Safe (1991) directly compared the *in vitro* structure-immunotoxicity relationships for a series of HAH congeners which show >14,900 fold-difference in *in vivo* immunotoxic potency. Results of these studies indicated that all of the congeners were equipotent *in vitro* and produced a similar concentration-dependent suppression of the *in vitro* antiSRBC response using cells from either B6 or D2 mice. Co-exposure to the Ah-receptor antagonist α-napthoflavone antagonized the immunosuppression induced by either TCDD or 1,3,6,8-TCDF (a weak Ah receptor agonist). Collectively, the results supported a mechanism of suppression *in vitro* that was independent of the Ah receptor.

The basis for these variable effects of TCDD *in vitro* are currently not known. However, recent studies by Morris et al. (1991) demonstrated that the *in vitro* effects of TCDD on the antiSRBC response were critically dependent on the type and concentration of the serum used in the *in vitro* culture. Only 3 of 23 lots of serum were able to support a full dose-responsive suppression, and, in serum-free cultures, TCDD caused a 15-fold enhancement of the antiSRBC response. Thus, differences in media components used in *in vitro* cultures may account for the different effects seen *in vitro* in different laboratories. Other factors such as the TCDD carrier/solvent used, the calcium content of the media, or procedures used for preparation of spleen cell suspensions may all contribute to variable effects of TCDD *in vitro*.

The obvious question relates to the relevance of the *in vitro* findings to the *in vivo* immunotoxicity. In this respect, it is important to note that the concentrations of TCDD required for *in vitro* suppression of immune function ($1\text{-}30\times10^{-9}$ M) of murine lymphocytes is several orders of magnitude higher than the concentration found in lymphoid tissues following exposure *in vivo* to an immunotoxic dose of TCDD (Neumann et al., 1992). The amount of TCDD associated with isolated spleen cells obtained from mice 2 days following treatment with 5 μg/kg ^{3}H-TCDD was 2×10^{-15} M per 10^{7} spleen cells. Importantly, as much as 50% of

the radioactivity associated with whole spleen tissue was recovered in the stromal and/or capsular material (i.e., splenic tissue that resisted passage through the mesh screens used for preparation of spleen cell suspensions). These findings suggest that the most potent effects of TCDD on immune function *in vivo* may be induced indirectly by effects on nonlymphoid cells, or that based on the delivered dose of TCDD, this molecule is more toxic than previously thought. Alternatively, TCDD effects *in vivo* on nonlymphoid cells may amplify the direct effects of TCDD on lymphoid tissue. Certainly, additional studies are needed to elucidate the serum components that are permissive for suppression or enhancement of immune responses *in vitro* and to determine their relevance to *in vivo* conditions. Such studies are also likely to provide insight into the mechanisms of TCDD interaction with lymphoid cells.

4.8. INDIRECT MECHANISMS OF HAH IMMUNOTOXICITY

The difficulty in demonstrating consistent, direct effects of TCDD *in vitro* on lymphocytes, the dependence of those effects on serum components, and the requirement for high concentrations of TCDD are all consistent with an indirect mechanism of TCDD on the immune system. One potentially important indirect mechanism is via effects on the endocrine system. Several endocrine hormones have been shown to regulate immune responses, including glucocorticoids, sex steroids, thyroxine, growth hormone and prolactin. Importantly, TCDD and other HAHs have been shown to alter the activity of all of these hormones (see chapter on endocrine system, this document).

Kerkvliet et al. (1990b) reported that exposure of mice to 3,4,5,3',4',5'-HxCB followed by injection of P815 allogeneic tumor cells induced a dose-dependent elevation of serum corticosterone concentrations which correlated with the dose-dependent suppression of the antiP815 CTL response. However, since adrenalectomy or treatment with the glucocorticoid receptor antagonist RU38486 failed to protect mice from the immunosuppressive effect of HxCB (DeKrey et al., 1990), a role for the elevated CS in the suppression of the CTL response seems unlikely. Adrenalectomy and hypophysectomy also failed to prevent TCDD-induced thymic atrophy in rats (Van Logten et al., 1980).

Using the P815 allogeneic tumor model, Kerkvliet and Baecher-Steppan (1988a) reported that male mice were more sensitive than female mice to suppression of

the CTL response by HxCB (Kerkvliet and Baecher-Steppan, 1988a). Castration of male rats partially ameliorated the immunosuppressive effects of HxCB (DeKrey et al., 1992), suggesting a role for testosterone in suppression of the CTL response.

Pazdernik and Rozman (1985) suggest that thyroid hormones may play a role in TCDD immunotoxicity based on the finding that radiothyroidectomy prevented the suppression of the antiSRBC response in rats treated with TCDD. However, since thyroidectomy alone suppressed immune function, the significance of the findings require further study.

4.9. ROLE OF THE THYMUS IN HAH IMMUNOTOXICITY

Thymic involution is one of the hallmarks of exposure to TCDD and related HAH in all species examined. In mice, thymic involution occurs by an Ah receptor dependent mechanism (Poland and Knutson, 1982). Because the thymus plays a critical role in the ontogeny of T lymphocytes, thymic involution is often referred to as an immunotoxic effect. However, while an intact thymus is crucial to the developing immune system during the prenatal and early postnatal period of rodents as well as during the prenatal period of humans, the physiological role played by the thymus in adult life has not been established. In animal models, adult thymectomy has little affect on the quantity or quality of T lymphocytes, which have already matured and populated the secondary lymphoid organs (Benjamini and Leskowitz, 1991). Likewise, in humans, childhood and adult thymectomy produces no clearly identifiable adverse consequences in terms of altered immune function, although some might argue that such studies have not been done. Based on this knowledge, it is not surprising that a direct relationship between the effects of TCDD on the thymus and immune suppression has not been established in studies using adult animals. In fact, adult thymectomy prior to HAH exposure did not modify TCDD- or HpCDD-induced suppression of the antiSRBC response (Tucker et al., 1986; Kerkvliet and Brauner, 1987). Furthermore, suppression of immune responses occurs at dose levels of HAH significantly lower than those required to induce thymic atrophy (Vos et al., 1978; Silkworth and Antrim, 1985; Holsapple et al., 1986b; Tucker et al., 1986; Kerkvliet and Brauner, 1990a). Thus, it is clear that thymic involution does not represent a surrogate marker for TCDD immunotoxicity in adult animals. On the

other hand, it is possible that chronic exposure to TCDD resulting in a chronic thymic atrophy may produce more delayed, subtle effects on immune function not yet identified (Clarke and MacLennan, 1986).

In contrast to adult animals, congenital thymic aplasia or neonatal thymectomy results in severe reduction in the number and function of T lymphocytes, and produces a potentially lethal wasting disease (Benjamini and Leskowitz, 1991). Similarly, there is evidence from studies carried out in the 1970's that rodents exposed to TCDD or PCBs during the pre/neonatal period are more sensitive to immune suppression compared to rodents exposed as adults, and that the prenatal effects are more selective for cell-mediated immunity (Vos and Moore, 1974; Faith et al., 1978; Luster et al., 1980b). TCDD has also been shown to alter thymocyte differentiation *in vitro* in cell cultures (Greenlee et al., 1985; Cook et al., 1987) and organ cultures (Dencker et al., 1985; d'Argy et al., 1989) as well as *in vivo* following prenatal exposure to TCDD (Blaylock et al., 1992). These observations suggest that altered thymic T cell maturation induced by TCDD in the thymus may play an important role in the suppressed immune function of prenatally exposed animals. However, since TCDD also influences B cell development in the bursa of chick embryos (Nikolaidis et al., 1990) as well as lymphocyte stem cells in the fetal liver and bone marrow of mice (Fine et al., 1989; 1990), other mechanisms of immunotoxicity are also likely to be important.

4.10. IMMUNOTOXICITY FOLLOWING PRE/NEONATAL EXPOSURE TO HAH

The reported increase in susceptibility of very young animals to HAH immunotoxicity necessitates a close examination of the available literature on prenatal/neonatal immunotoxic effects. Several studies have examined immune function in mice, rats and/or guinea pigs following exposure to TCDD or PCB during fetal development (Vos et al., 1973; Vos and Moore, 1974; Thomas and Hinsdill, 1979; Luster et al., 1980a).

The results of three major studies in which exposure of the progeny occurred via placental transfer and lactation are summarized in Table 4-5. The most sensitive indicator of TCDD immunotoxicity in these studies was an increase in the growth of transplanted tumor cells in the offspring of B6C3F1 mice (Ah responsive strain) treated with 1 μg/kg TCDD at 4 weekly intervals. (Total TCDD dose to dam was 4 μg/kg; dose to offspring was not determined.) The offspring

TABLE 4-5

Immunotoxic Effects of TCDD in the Offspring Following Pre/Neonatal Exposure to TCDD

Protocol	Endpoints	Effect	LOAEL	Reference
Study #1: Pregnant B6 or B6C3F1 mice given 1, 2, 5 or 15 µg/kg TCDD orally on day -7, 0, +7, +14 relative to parturition on day 0	PYB6 tumor incidence	increased	1 µg/kg x 4	Vos and Moore, 1974; Luster et al., 1980
	allograft rejection time	increased	2 µg/kg x 4	
	body, thymus, spleen weight	decreased	5 µg/kg x 4	
	bone marrow cellularity	decreased	5 µg/kg x 4	
	Con A, PHA blastogenesis	decreased	5 µg/kg x 4	
	mortality following *Listeria monocytogenes* challenge	decreased	5 µg/kg x 4	
	bone marrow CFU-S colonies	decreased	5 µg/kg x 4	
	mortality	increased	15 µg/kg x 4	
	LPS blastogenesis	--	15 µg/kg x 4	
	anti-SRBC serum titers	--	15 µg/kg x 4	
Study #1: Pregnant Swiss mice fed diets containing 1.0, 2.5 or 5.0 ppb TCDD for 7 weeks (4 weeks prior to and 3 weeks after birth)	endotoxin	increased	1.0 ppb diet[a]	Thomas and Hinsdill, 1979
	thymus weight	decrease	2.5 ppb diet	
	PFC response to SRBC	decreased	5.0 ppb diet	
	DTH response	decreased	5.0 ppb diet	
	anti-SRBC serum titers	--	>5.0 ppb diet	
	LPS and Con A blastogenesis	--	>5.0 ppb diet	
	Listeria-induced mortality	--	>5.0 ppb diet	
Study #3: Pregnant Fischer 344 rats given 1 or 5 µg/kg TCDD orally on days -3, 0, +7 and +14 relative to parturition on day 0	allograft rejection time	increased	5 µg/kg x 4	Vos and Moore, 19774; Faith and Moore, 1977
	PHA blastogenesis	decreased	5 µg/kg x 4	
	DTH response	decreased	5 µg/kg x 4	
	mortality following *Listeria* challenge	increased	5 µg/kg x 4	
	body and thymus weight	decreased	5 µg/kg x 4	
	anti-BGG serum titers	--	>5 µg/kg x 4	

[a]A 1.0 ppb diet consumed by a 20 g mouse at a rate of 5 g/day = 0.25 ng TCDD/kg/day.

LOAEL = Lowest-observed-adverse-effect level; BGG = bovine gamma globulin; LPS = lipopolysaccharide; PHA = phytohemagglutinin; Con A = Concanavalin A; SRBC = sheep red blood cells; DTH = delayed-type hypersensitivity; PFC = plaque-forming cell; CFU-S = colony-forming units-spleen

of Swiss mice fed a diet containing 1 ppb TCDD for 7 weeks showed enhanced mortality following endotoxin challenge, while the plaque-forming cell response to SRBC and delayed hypersensitivity response were suppressed in offspring of mice fed 5.0 ppb TCDD diets. (Estimated daily dose to 20 g dam consuming 5 g of 5 ppb TCDD diet is equivalent to 1.25 μg/kg TCDD/day.) Rats appeared to be more resistant to the immunotoxic effects of pre/neonatal exposure to TCDD based on the finding that 5 but not 1 μg/kg TCDD given four times at weekly intervals produced immunotoxicity in the offspring. Immunotoxic endpoints that were unaffected by the highest exposure levels in these studies included blastogenesis induced by LPS and serum antibody titers to SRBC and BGG.

Two recent studies have examined immune function in offspring of female mice exposed to TCDD (Holladay et al., 1991) or PCB (Kanechlor 500) (Takagi et al., 1987) but that were cross-fostered to unexposed lactating mice at birth. Thus, exposure was limited to *in utero* exposure. (It is important to recognize that rodents are born with an immature immune system that matures in the first few weeks following birth. In contrast, the human immune system is considered to be more mature at birth.) B6 mice exposed to 3.0 μg/kg TCDD on gestational days 6-14 gave birth to offspring that had significant thymic atrophy and hypoplasia measured on gestational day 18 or on day 6 postnatally. The thymic effects were no longer apparent by day 14. At 7-8 weeks postnatally, mitogen responses and antibody plaque forming cell response to SRBC were unaltered while the CTL response was significantly suppressed compared to controls (Holladay et al., 1991). These results suggest a selectivity of prenatal TCDD on the CTL and not the T helper cells involved in the antibody response to SRBC. In contrast to these results, Takagi et al. (1987) exposed female C3H mice *per os* to 50 mg/kg Kanechlor 500 twice per week for 4 weeks, at which time steady state tissue levels were noted. The offspring derived from mating to unexposed males had an unaltered antibody response to the T-independent antigen DNP-dextran. On the other hand, carrier-primed T helper cell activity assessed by adoptive transfer was significantly suppressed by PCB exposure when assessed 4 and 7 weeks after birth, but fully recovered by 11 weeks. Together, these studies confirm prior studies to indicate that T cell function is selectively altered by HAH when

exposure is prenatal. While both T helper cells and CTL show altered function, T helper cell activity may recover faster than CTL function.

Fine et al. (1990) reported on TCDD levels in offspring following maternal treatment with TCDD (10 μg/kg) on gestational day 14. The fetal liver had the highest concentration on gestational day 18 (235 fg/mg) which declined slightly by postnatal day 6 to around 100 fg/mg. The concentration of TCDD in the thymus on gestation day 18 was 140 fg/mg, which declined to 20 fg/mg on day 6 after birth. (These thymic TCDD concentrations are equivalent to 60 to 425 pM assuming 1 kg of tissue is equivalent to 1 L of water.) TCDD concentrations in the spleen remained constant at about 40 fg/mg during the same time frame, while bone marrow concentrations were very low (~3 fg/mg). These concentrations of TCDD were associated with thymic atrophy (Fine et al., 1989) and significant reduction in the ability of prothymocytes in liver and bone marrow to repopulate an irradiated thymus (Fine et al., 1990).

4.11. IMMUNOTOXICITY OF HAH IN NON-HUMAN PRIMATES

A limited number of studies using nonhuman primates as surrogate models for humans have been conducted to assess HAH immunotoxicity. Immunological effects were described in Rhesus monkeys and their offspring chronically exposed to TCDD at levels of 5 or 25 ppt for 4 years (Hong et al., 1989). In the mothers, the total number of T cells increased in monkeys fed 25 ppt TCDD, with a selective increase in $CD8^+$ cells and a decrease in $CD4^+$ cells. However, no significant effect on T cell function was established when assessed as proliferation response to mitogens, alloantigens, or xenoantigens. Natural killer cell activity and production of antibodies to tetanus immunization were normal. In the offspring of TCDD exposed dams examined 4 years after exposure, a significantly increased antibody response to tetanus toxoid immunization was observed which correlated with TCDD tissue levels. The body burden of TCDD in the offspring ranged from a low of 290 ppt to a high of 1400 ppt. Interestingly, there was no strict correlation between exposure levels and resulting body burden.

In other TCDD studies, a single injection of TCDD in marmosets (*Callithrix jacchus*) resulted in a delayed decrease in the percentage of $CD4^+$ T cells and $CD20^+$ B cells in the blood and an increase in the percentage of $CD8^+$ cells

(Neubert et al., 1990). The total number of T cells was not significantly altered by TCDD exposure. The $CD4^+$ subset most affected was the $CDw29^+$ "helper-inducer" or "memory" subset, with significant effects observed after a TCDD dose of 10 ng/kg. The NOEL for this effect was 3 ng/kg TCDD. Concomitant with suppression of the CDw29 subset in TCDD treated animals, the percentage of $CD4^+CD45RA^+$ cells increased. This subset has been classified as "suppressor-inducer" or "naive" cells. The changes in the T cells subsets were intensified following *in vitro* culture of the cells with mitogen (Neubert et al., 1991). Interestingly, however, a recent study from the same laboratory reported that chronic exposure of young marmosets to very low levels of TCDD (0.3 ng/kg/week for 24 weeks) produced the opposite effect on the $CD4^+CDw29^+$ subset, resulting in a significant increase in this population (Neubert et al., 1992). Concomitantly, the $CD4^+CD45RA^+$ subset decreased. Upon transfer of the animals to a higher dose of TCDD (1.5 ng/kg/week) for 3 weeks, the enhancement effect was reversed, and suppression of the $CD4^+CDw29^+$ subset was observed, with maximum suppression after 6 weeks of exposure to the higher dose. In addition, the $CD8^+$ $CD56^+$ T cytotoxic T cell subset was transiently increased, but which normalized even though TCDD dosing continued. After discontinuation of dosing, the reduction in the percentage and absolute number of $CD4^+CDw29^+$ cells persisted for 5 weeks, reaching normal range 7 weeks later. These results led the authors' to conclude that "extrapolations of the results obtained at higher doses to very low exposures is not justified with respect to the effects induced by TCDD on the immune system of marmosets."

The immunomodulatory effects of chronic low level PCB exposure in monkeys has also been investigated. In early studies, Thomas and Hinsdill (1978) reported that rhesus monkeys fed diets containing 2.5 or 5 mg/kg of Aroclor 1248 had significantly suppressed antibody response to SRBC but not to tetanus toxoid (TT). These monkeys also had chloracne, alopecia and facial edema. Similarly, exposure of cynomolgus monkeys to Aroclor 1254 (100 or 400 μg/kg/day) for 3 months suppressed antibody responses to SRBC but not TT (Truelove et al., 1982).

Suppressive effects on antiSRBC responses were more severe in cynomolgus monkeys when the PCB mixture contained PCDFs (Hori et al., 1982).

Tryphonas et al. (1989; 1991a,b) have recently reported results of studies in rhesus monkeys exposed chronically to Aroclor 1254 (5-80 μg/kg/day) for 23 or 55 months. These exposures resulted in steady state blood PCB levels that ranged from a mean low of 0.01±0.001 ppm in the 5 μg/kg group to a mean high of 0.11±0.01 ppm in the 80 μg/kg group. The only consistently altered immune parameter was the primary and anamnestic antibody responses to SRBC which were suppressed in a dose-dependent manner. In contrast, the antibody response to pneumococcus vaccine antigen measured at 55 months of exposure was not significantly altered. At 23 months, the percentage of T helper cells in the blood was significantly decreased in the 80 μg/kg group, and the percentage and absolute number of T suppressor cells was increased; however, these effects were not apparent at 55 months of exposure (Tryphonas, et al., 1991b). Lymphoproliferative responses to PHA and Con A were not significantly altered at 23 months but were dose-dependently suppressed at 55 months. Proliferation to alloantigens was not significantly altered. Likewise, serum immunoglobulin and hydrocortisone levels did not differ between treatment groups. After 55 months, the chemiluminescent response (time to peak) of monocytes was slower in PCB exposed cells. Also noted at 55 months was a significant elevation in serum hemolytic complement levels, a dose-related increase in natural killer cell activity, and a dose-related increase in thymosin alpha-1 levels but not thymosin beta-4 levels (Tryphonas et al., 1991a). Effects on interferon levels were inconsistent, and TNF production was not altered.

The studies in nonhuman primates are important from the standpoint that the antibody response to SRBC emerges as the only immunological parameter consistently suppressed by HAH in several different animal species. Other immunological endpoints such as total T cell numbers, percentages of T cell subsets, lymphoproliferative responses, and DTH responses are inconsistently increased or decreased in various studies. At the present time, it is not clear why the antibody response to SRBC is most consistently altered by HAH exposure in different species. The sensitivity of the antiSRBC response does not appear to be due solely to the T-cell dependency of the response since antibody responses

to other T-dependent antigen (e.g., TT, BGG) are not suppressed and may be enhanced following HAH exposure. It is possible that the particulate nature of the SRBC antigens is an important factor even though a mechanistic basis for this is not readily apparent. The sensitivity of the technique used to quantify the antibody response may also contribute to apparent increased sensitivity of the SRBC model, which is most often measured as the PFC response rather than serum antibody titers which are usually more variable. Nonetheless, the finding that the SRBC response is also suppressed in nonhuman primates exposed to PCB lends support to the use of the antiSRBC data generated in mice to calculate TEFs for immunotoxicity.

4.12. IMMUNOTOXICITY OF HAH IN HUMANS

The immunotoxicity of TCDD and related HAH in humans has been the subject of several studies derived from accidental and/or occupational exposures to PCBs, PBBs, and TCDD. Immunological assessment was carried out on patients who consumed acnegenic and hepatotoxic doses of PCDF-PCB contaminated rice oil in Taiwan in 1979. Clinical symptoms were primarily related to increased frequency of various kinds of infection, especially of the respiratory tract and skin (Lu and Wu, 1985). Immunologic effects included decreased serum IgA and IgM but not IgG, decreased percentage of T cells in blood related to decreased $CD4^+$ T helper cells and increased $CD8^+$ T suppressor cells, and suppressed dermal delayed type hypersensitivity responses to streptokinase/-streptodornase and tuberculin antigens (Lu and Wu, 1985). The percentage of anergic patients increased and the degree of induration decreased with increased PCB concentration in the blood. In contrast, lymphoproliferative responses of PBL to PHA, PWM and tuberculin but not Con A were significantly augmented in PCB-exposed patients. PCB concentrations in the blood ranged from 3-1156 ppb with a mean of 89±6.9 ppb. The oil was contaminated at PCB concentrations of 4.8-204.9 ppm with a mean of 52±39 ppm.

Immunotoxic effects were also described in Michigan dairy farmers exposed to PBBs via contaminated dairy products and meat in 1973 (Bekesi et al., 1979). Like PCB-exposed patients, the percentage and absolute numbers of T cells in peripheral blood of PBB-exposed farmers were significantly reduced compared to a control group. However, in contrast to PCB, lymphoproliferation responses to

PHA, PWM and allogeneic leukocytes were significantly decreased in PBB exposed persons. Also in contrast to PCB, skin testing using standard recall antigens indicated that PBB-exposed Michigan dairy farmers had significantly increased responses, particularly to candida and varidase. Tissue levels of PBB in the subjects were not determined in these studies.

Webb et al. (1989) reported the findings from immunologic assessment of 41 persons from Missouri with documented adipose tissue levels of TCDD resulting from occupational, recreational or residential exposure. Of the participants, 16 had tissue TCDD levels <20 ppt, 13 had levels between 20 and 60 ppt and 12 had levels >60 ppt. The highest level was 750 ppt. Data were analyzed by multiple regression based on adipose tissue level and the clinical dependent variable. Increased TCDD levels were correlated with an increased percentage and total number of T lymphocytes. $CD8^+$ and $T11^+$ T cells accounted for the increase, while $CD4^+$ T cells were not altered in percent or number. Lymphoproliferative responses to Con A, PHA, PWM or tetanus toxoid were unaltered as was the cytotoxic T cell response. Serum IgA but not IgG was increased. No adverse clinical disease was associated with TCDD levels in these subjects. Only 2 of the 41 subjects reported a history of chloracne. These findings differ from those reported for the Quail Run Mobile Home Park residents (tissue levels unknown) in which decreased T cell numbers (T3, CD4 and T11) and suppressed cell-mediated immunity was reported (Hoffman et al., 1986). However, subsequent retesting of these anergic subjects failed to confirm the anergy (Evans et al., 1988). On the other hand, when serum from some of these individuals was tested for levels of the thymic peptide, thymosin alpha-1, the entire frequency distribution for the TCDD-exposed group was shifted toward lower thymosin alpha-1 levels (Stehr-Green et al., 1989). The statistically significant difference between the TCDD-exposed persons and controls remained after controlling for age, sex, and socioeconomic status, with a trend of decreasing thymosin alpha-1 levels with increasing number of years of residence in the TCDD-contaminated residential area. The thymosin alpha-1 levels were not correlated with changes in other immune system parameters nor with any increased incidence of clinically diagnosed immune suppression. The decrease in thymosin alpha-1 levels in humans contrasts

with the increase in thymosin alpha-1 seen in PCB treated monkeys (Tryphonas et al., 1991b).

Finally, Mocarelli et al. (1986) reported studies on the immune status of 44 children, 20 of whom had chloracne, that were exposed to TCDD following an explosion at a herbicide factory in Seveso, Italy. No abnormalities were found in the following parameters: serum immunoglobulin concentrations, levels of circulating complement, or lymphoproliferative responses to T and B cell mitogens. Interestingly, in a study conducted 6 years after the explosion, a different cohort of TCDD-exposed children exhibited a significant increase in complement protein levels, which correlated with the incidence of chloracne, as well as increased numbers of peripheral blood lymphocytes, and increased lymphoproliferative responses (Tognoni and Bonaccorsi, 1982). However, no specific health problems were correlated with dioxin exposure in these children.

It is readily apparent that no clear pattern of immunotoxicity to HAH emerges from these studies in humans. In some cases T cell numbers increase; in others, they decrease. The findings are not unlike the varied and often conflicting reports found in the literature regarding animal studies of HAH immunotoxicity. The basis for the lack of consistent, significant exposure-related effects is unknown and may be dependent on several factors. Most notable in this regard is the generic difficulties in assessing subclinical immunomodulation, particularly in outbred human populations. Most immunological assays have a very broad range of normal responses reducing the sensitivity to detect small changes. Similarly, the assays used to examine immune function in humans exposed to TCDD and related HAH have unfortunately been based to a greater extent on what was clinically "doable" (e.g., mitogen responsiveness) rather than on assays that have been shown to be sensitive to TCDD in animal studies. Thus, the lack of consistent and/or significant immunotoxic effects in humans resulting from TCDD exposure may be as much a function of the assays used as the immune status of the cohort. In addition, few studies have examined the immune status of individuals with known, documented exposure to HAH. Rather, cohorts based on presumption of exposure have been studied. There is some evidence to suggest that the lack of consistent, significant effects may sometimes be due to the inclusion of subjects that had little or no actual exposure to TCDD (Webb et al., 1989). Likewise, the

important role that Ah phenotype plays in TCDD immunotoxicity has not been considered when addressing human sensitivity. Whether there are human equivalents of murine Ah^{bb} and Ah^{dd} types is not known. Finally, in most studies, the assessment of immune function in exposed populations was carried out long after exposure to TCDD ceased. Thus, recovery from the immunotoxic effects of TCDD may have occurred.

In summary, one might speculate that any future study to determine HAH immunotoxicity in humans should evaluate their antibody response to SRBC. However, it should be emphasized that even the relatively low exposure levels that have been shown to suppress the antiSRBC response in nonhuman primates resulted in blood and tissue PCB or TCDD levels that far exceed the levels measured in humans in most studies published to date involving environmental exposure. Thus, even the antiSRBC response may not have been sensitive enough to demonstrate immune suppression in these cohorts. Given the current lack of data correlating clinical immunological endpoints with immune status in humans (except in cases of overt immune deficiencies), massive retrospective studies of poorly defined exposure groups cannot be justified to try to "prove" that immune modulation has occurred in these people. Rather, such efforts would be better directed toward the establishment of a broad data base of normal values for the clinical immunology endpoints that may be of use in immunotoxicity assessments. In conjunction with this effort, research must focus on the definition of sensitive endpoints (i.e., biomarkers) of immune dysfunction in humans so that, in the future, emergency response teams could respond rapidly to accidental exposures to assess the immunological status of the exposed persons. To validate these biomarkers, there is a parallel need for animal research to identify TCDD-sensitive immune endpoints in animals that can also be measured in humans in order to establish in correlative changes in the biomarker and immune function. In particular, it will be important to determine in animal models how well changes in immune function in the lymphoid organs (e.g., spleen, lymph nodes) correlate with changes in the expression of lymphocyte subset/activation markers in peripheral blood. Until such correlations are established, the interpretation of changes observed in subsets/activation markers in human peripheral blood

lymphocytes in terms of health risk will be limited to speculation. Research must also continue to develop well-defined animal models using multiple animal species that will lead to an understanding of the underlying mechanisms of HAH immunotoxicity. For example, there is a clear need to document Ah receptor involvement in the immunotoxicity of TCDD and related HAH in species other than mice. These studies need to go beyond descriptive immunotoxicity assessment to determine the mechanistic basis for differences in species sensitivity to TCDD immunotoxicity following both acute and chronic exposure. In the interim, the available data base derived from well-controlled animal studies on HAH immunotoxicity can be used for establishment of no effect levels and acceptable exposure levels for human risk assessment of TCDD using the same procedures that are used for other noncarcinogenic toxic endpoints. Because the antibody response to SRBC has been shown to be dose-dependently suppressed by TCDD and related HAH in several animal species, this data base is best suited for current application to risk assessment. The validity of using TEFs to extrapolate from one HAH to another, however, remains to be established.

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