Immunological Effects of Chlorinated Dibenzo-p-dioxins

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and structurally similar halogenated aromatic hydrocarbons cause a broad range of immunologic effects in experimental animals including decreased host resistance to infectious disease and suppressed humoral and cell-mediated immune responses. In the mouse, TCDD immunotoxicity has been shown to be an aryl hydrocarbon (Ah) receptor-dependent process. However, despite considerable research, the biochemical and molecular alterations that occur subsequent to Ah receptor activation that lead to altered immune reactivity remain to be elucidated. In addition to immune suppression, TCDD promotes inflammatory responses. This effect may result from upregulation of the production of inflammatory cytokines such as interleukin-1 and tumor necrosis factor. Nonhuman primates exposed to TCDD show suppressed antibody responses and changes in lymphocyte subsets in the peripheral blood. The immunotoxic effects of TCDD in humans are poorly characterized, and few studies have examined the immune status of individuals with known, documented exposure to TCDD. It is important for laboratory research to focus on defining TCDD-sensitive immunologic biomarkers in animal models that can also be used in human subjects. Understanding the mechanisms that underlie species differences in TCDD immunotoxicity is also of critical importance for extrapolation of effects seen in laboratory animals to man. — Environ Health Perspect 103(Suppl 9):47-53 (1995)

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Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has posed much concern to both the public and the scientific community because of its toxic potency and widespread distribution in the environment. TCDD is the most toxic member of a large class of planar halogenated aromatic hydrocarbons (HAH) that include other environmental contaminants such as polychlorinated biphenyls (PCBs) and dibenzofurans (PCDFs). These chemicals are unusual in that most of their toxicity is elicited through their initial binding to a specific intracellular protein, the aryl hydrocarbon (Ah) receptor (AhR) (1,2). In a process similar to steroid hormone receptor-mediated responses, the receptor–ligand complex is translocated from the cytoplasm to the nucleus where it binds to DNA at specific sequences called dioxin-response elements (DREs) to modify transcription of the DRE-containing genes (3,4). A widely held hypothesis is that altered transcription leads to over production or underproduction of specific protein products that mediate the ultimate biochemical and toxic effects of TCDD and related HAH. In support of the AhR hypothesis, differences in toxic potency between various chlorinated congeners of dioxins, furans, and biphenyls generally correlate well with differences in their binding affinity for the Ah receptor (5).

Immunotoxicity Studies

Laboratory Animal Studies

The immune system appears to be one of the most sensitive targets for the toxicity of TCDD and related HAH. Studies in the 1970s demonstrated that exposure of laboratory rodents to low doses of TCDD by diet or by gavage resulted in involution of the thymus, increased susceptibility to various infectious diseases, and suppression of both cell-mediated and humoral immune functions (6,7). Subsequently, many different animal models in addition to rodents have been used to demonstrate the immunotoxicity of TCDD. Unfortunately, due to differences in experimental design and outcomes, a defined TCDD-induced immune deficiency syndrome has not emerged. Likewise, because of the difficulties associated with the measurement of many immune functions in some species of animals, there is no clear ranking of species sensitivity to TCDD immunotoxicity.

The type of immune response that is most sensitive to suppression following TCDD exposure is also difficult to generalize from animal studies. For example, the antibody response to sheep red blood cells (SRBC) in C57Bl/6 mice is very sensitive to suppression by TCDD, with a single dose of TCDD of 0.7 μg/kg sufficient to suppress this response to 50% of the control level (8–10). In contrast, a dose of TCDD as high as 30 μg/kg does not suppress this response in rats (11). However, even in mice, antibody responses to different antigens may differ more than 10-fold in sensitivity to suppression by TCDD as compared to the response to SRBC (8,9), which indicates that the nature of the antigenic stimulus is also an important factor. This is not unexpected given that different types of antigens are known to evoke different cellular interactions (e.g., antigen-presenting cells for soluble vs particulate antigens, requirement for different T-helper cell subtypes for humoral and cell-mediated responses, sensitivity to suppressor T-cell regulation, etc.). Understanding the mechanism(s) of TCDD toxicity to these different subsets of cells of the immune system will be necessary for understanding why certain immune responses are more sensitive to TCDD than others.

Involution of the thymus gland following TCDD exposure is a hallmark of TCDD toxicity in several animal species (2,12); however, no direct relationship between the effects of TCDD on the thymus and effects on immune function have been demonstrated following exposure of animals to TCDD (13,14). In adult animals, immune suppression occurs at doses of TCDD below that required for thymic atrophy (15–17), suggesting that thymus toxicity and systemic immune toxicity are independent. On the other hand, the effects of prenatal exposure to TCDD on the fetal...
However, the specific cellular defects induced by TCDD have not been fully elucidated despite considerable research. One of the problems has been a difficulty in demonstrating direct effects of TCDD on in vitro responses of lymphoid cells (35,36). This is especially true with T cells; direct effects of TCDD on T cells in vitro have not been observed even though T-cell functions in vivo are clearly altered by TCDD (14,32,33,36–38). Furthermore, when in vitro effects of TCDD are observed, they may be inconsistent with the in vivo immunotoxic effects of TCDD. For example, in vitro suppression of the antibody response to SRBC has been reported by some laboratories to be independent of the AhR (39,40), whereas suppression of this response in vivo is clearly AhR-dependent. Although the basis for the discrepancies between in vivo and in vitro effects is not known, differences in culture conditions may be partially responsible since the in vitro effects of TCDD on lymphoid cells appear to be influenced by unknown factors present in serum-supplemented tissue culture media (41,42). Interestingly, serum was also shown to modulate the induction of H3501A1-dependent enzyme activity by TCDD in primary cultures of hepatocytes (42), demonstrating that the serum phenomenon is not restricted to effects on lymphocytes.

While T-cell responses appear to be resistant to direct effects of TCDD, several laboratories have reported that TCDD directly alters B-lymphocyte functions in vitro (13,40,43,44). Studies using both murine and human B cells suggest that TCDD alters the terminal differentiation of B cells into antibody-secreting plasma cells without altering B-cell proliferation (43–45). The induction of protein kinase activity (46–48) and altered calcium homeostasis (49) have been implicated in the immunotoxic effects of TCDD on B cells. Snyder et al. (48) reported that TCDD induced the phosphorylation of 29, 45, 52, and 63 kDa proteins in B cells, which by density gradient were characterized as activated B cells. Interferon-γ (IFNγ) was shown to antagonize the TCDD-induced phosphorylation and to reverse the TCDD-induced suppression of the antibody response to SRBC in vitro. Interestingly, suppression of IFNγ production has recently been correlated with the in vitro suppression of cytotoxic T-lymphocyte activity in TCDD- and PCB-treated mice [NI Kerkvliet, unpublished data; (50)]. Thus, changes in IFNγ production may represent an underlying mechanism for TCDD-induced immunotoxicity.

Activities associated with innate immune function have also been examined following TCDD exposure and have generally been found to be resistant to suppression when assessed ex vivo. Macrophage-mediated phagocytosis, macrophage-mediated tumor cell cytosis or cytostasis, oxidative reactions of neutrophils and macrophages, and natural killer (NK) cell activity were not suppressed following TCDD exposure, with doses as high as 30 μg/kg failing to suppress NK and macrophage functions (51,52). A potentially important exception is the reported inhibition of phorbol ester-activated antitumor activity of neutrophils by TCDD (34). On the other hand, the pathology associated with TCDD toxicity often includes neutrophilia and an inflammatory response in certain tissues (e.g., liver and skin) characterized by activated macrophage and neutrophil accumulation (53–55). While these observations may simply reflect a normal inflammatory response to tissue injury, there is increasing experimental evidence to suggest that inflammatory cells may be activated by TCDD exposure. For example, TCDD exposure has been shown to produce an enhanced inflammatory response in the peritoneal cavity of mice following SRBC injection (56). This effect of TCDD was characterized by a 2- to 4-fold increase in the number of neutrophils and macrophages locally infiltrating the intraperitoneal site of SRBC injection. However, the time course of the cellular influx was not altered by TCDD exposure. Likewise, the expression of activation markers (I-A and F4/80) and the antigen-presenting function of the peritoneal exudate cells was unaltered by TCDD exposure. Thus, the effect of TCDD appeared to reflect a qualitative rather than a quantitative change in the inflammatory response. Interestingly, although enhanced antigen clearance/degradation caused by the increased numbers of phagocytic cells could result in a decreased antibody response in TCDD-treated mice, increasing the amount of antigen used for sensitization did not alter the immunosuppressive effect of TCDD (56). Thus, a relationship between the inflammatory and immunosuppressive effects of TCDD in the SRBC model was not apparent.

One mechanism by which TCDD could augment inflammatory responses is through enhanced production of inflammatory mediators. For example, recent evidence suggests that the hypersusceptibility of
TCDD- and PCB-treated animals to endotoxin (57, 58) and the increased inflammatory response to SRBC (59) may be related to an increased production of tumor necrosis factor (TNF). The ability of methylprednisolone to reverse the mortality associated with TCDD/endotoxin treatment is also consistent with a proinflammatory mechanism (60). Similarly, increased inflammatory mediator production may underlie the enhanced rat paw edema response to carrageenan and dextran in TCDD-treated rats (61, 62). A primary effect of TCDD on inflammatory mediator production is supported by the recent findings that keratinocytes exposed to TCDD in vitro have increased mRNA for interleukin-1β, plasminogen activator inhibitor-2, and transforming growth factor α and decreased mRNA for transforming growth factor β (63, 64). Interestingly, mRNA for TNF was not altered by TCDD treatment (64). The effects of TCDD on keratinocytes are similar to the effects of TCDD on the macrophage cell line IC-21 in that TCDD treatment increased endotoxin induction of mRNA for interleukin-1 but not TNF (65).

The influence of TCDD exposure on inflammatory mediator production and action is an important area for further study. In this regard, it is relevant to note that treatment of mice with a soluble TNF-binding protein, under conditions that resolved the hyperinflammatory response to SRBC induced by TCDD exposure, did not affect TCDD-induced suppression of the anti-SRBC antibody response (66). Similarly, daily treatment of mice with aminoguanidine, an inhibitor of inducible nitric oxide synthase, did not influence the suppression of the anti-SRBC antibody response by TCDD (66). Thus, the relationships, if any, between the proinflammatory and immunosuppressive effects of TCDD remain to be elucidated.

The ability of TCDD to augment the production of certain inflammatory chemoaactive mediators suggests that TCDD exposure could result in enhanced host resistance to pathogenic infection since the rapid influx of phagocytes to the site of pathogen invasion is an important factor in host resistance. However, since TCDD exposure is at the same time immunosuppressive, which results in decreased specific immune responses generated by T and B lymphocytes, the overall impact of TCDD exposure on disease susceptibility will probably vary depending on the nature of the pathogen and on the major mode of host response to the specific infectious agent. These divergent effects of TCDD on inflammation and immunity may, in fact, help to explain the disparate effects of TCDD in different host resistance models that have been previously reported (20, 52, 67, 68).

**Studies in Nonhuman Primates**

A limited number of studies using nonhuman primates have been conducted to assess TCDD immunotoxicity. Few immunologic effects were found in rhesus monkeys and their offspring that were chronically exposed to TCDD in food at levels of 5 or 25 parts per trillion (ppt) for 4 years (69). Although T-cell numbers decreased in the TCDD-fed mothers (with a selective decrease in CD4+ cells), T-cell function as measured by proliferation to mitogens, allogeneic cells, or xenogeneic antibodies was not affected. NK cell activity and the antibody response to tetanus toxoid were also normal. Interestingly in the offspring, T-cell numbers were increased as was the antibody response to tetanus toxoid. (It is relevant to note that the antibody response to SRBC was not measured in these studies because the antibody response to SRBC but not to tetanus toxoid was decreased in monkeys exposed to much higher levels of PCB (70).)

In other studies, a single injection of TCDD in marmosets (Callithrix jacchus) resulted in a decrease in the percentages of CD20+ B cells and CD4+ T cells and an increase in the percentage of CD8+ T cells in the blood without affecting the total numbers of these cells (71). The CD4+ subset that was most affected was the CD4+CD829+ helper-inducer or memory subset, with significant effects observed after a TCDD dose of 10 ng/kg but not after a dose of 3 ng/kg. The changes in the T-cell subsets were intensified following culture of the cells with mitogens (72). Paradoxically, however, chronic exposure of young marmosets to lower levels of TCDD (0.3 ng/kg/week for 24 weeks) produced the opposite effect of acute exposure on the CD4+CD829+ subset, with TCDD treatment resulting in a significant increase in this population (73). Upon transfer of the animals to a higher dose of TCDD (1.5 ng/kg/week) for 3 weeks, the enhancing effect was reversed and suppression of the CD4+CD829+ subset was observed. After discontinuation of dosing, the reduction in the percentage and absolute number of CD4+CD829+ cells persisted for 5 weeks, reaching normal range 7 weeks later. Based on these results the authors concluded 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" (73). The relevance of these changes in subset distributions to immune function in the marmoset have not been determined. Interestingly, a similar reduction in the "memory" CD4+ T-cell subset was observed in C57Bl/6 mice treated once a week for 60 weeks with 0.2 μg/kg TCDD (74), suggesting that the memory CD4+ T-cell may represent a very sensitive biomarker of exposure to TCDD. A reduction in the memory T-cell population is consistent with the immunosuppressive effects of TCDD.

**Human Studies**

The immunotoxicity of TCDD in humans has been the subject of a limited number of studies in which cohorts were exposed to TCDD either occupationally or as a result of residence in a TCDD-contaminated area. Mocarelli et al. (75) reported 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 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 (76). No specific health problems were correlated with dioxin exposure in these children.

Webb et al. (77) 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 less than 20 ppt, 13 had levels between 20 and 60 ppt, and 12 had levels greater than 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 T-cell mitogens or tetanus toxoid were not
altered nor was the cytotoxic T-cell response. Serum immunoglobulin A (IgA) was increased but IgG was not. No adverse clinical disease was associated with these 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 (78). However, subsequent retesting of these anergic subjects failed to confirm the suppressed immunity (79). On the other hand, when sera from some of these individuals were tested for levels of the thymic peptide thymosin α-1, the entire frequency distribution for the TCDD-exposed group was shifted toward lower thymosin α-1 levels (80). A 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 α-1 levels as the number of years of residence in the TCDD-contaminated residential area increased. The thymosin α-1 levels were not correlated with changes in other immune system parameters or with any increased incidence of clinically diagnosed immune suppression. The decrease in thymosin α-1 levels in this cohort contrasts with the increase in thymosin α-1 seen in PCB-treated monkeys (81).

Two studies have evaluated the immunologic function of Vietnam veterans exposed to TCDD via use of the pesticide Agent Orange. When U.S. Army ground troops were matched with a comparison population, no differences in lymphocyte subsets or serum immunoglobulins were found (82). In the U.S. Air Force Ranch Hand Study, comprehensive immunologic profiles were developed for each participant and correlated with serum TCDD concentrations (83). The only significant positive association with TCDD exposure was increased serum IgA level. Roegner et al. (83) suggested that the increase in serum IgA was consistent with a subclinical inflammatory response, but no other evidence for an inflammatory response was obtained.

The basis for the lack of consistent or significant exposure-related effects to TCDD in these human populations is unknown and may be dependent on several factors. Most notable in this regard is the inherent difficulties in assessing subclinical immunomodulation in an outbred human population. Most immunologic assays have a very broad range of normal responses that reduce the sensitivity to detect small changes. Similarly, the assays used to examine immune function in humans exposed to TCDD have unfortunately been based to a greater extent on what was clinically feasible (e.g., lymphocyte phenotype, mitogen responsiveness) rather than on assays that have been shown to be sensitive to TCDD in animal studies (e.g., antibody response to SRBC). Thus, the lack of consistent 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 TCDD. 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 (77). Likewise, the important role that Ah phenotype plays in TCDD immunotoxicity has not been considered when addressing human sensitivity. Finally, in most studies, the assessment of immune function in exposed populations was carried out long after exposure to TCDD ceased. Thus, recovery from any immunotoxic effects of TCDD may have occurred by the time of testing.

As an alternate approach to evaluating the sensitivity of the human immune system to TCDD, several laboratories have recently reported on the direct in vitro effects of TCDD on human lymphocytes. Neubert et al. (72) reported that TCDD reduced the percentage of CD20 B cells and CD4/CD8 T cells in pokeweed mitogen-stimulated cultures of peripheral blood lymphocytes at concentrations as low as 10^{-12} to 10^{-14} M TCDD. These results, however, were not corroborated in similar studies reported by Lang et al. (35) in which concentrations of TCDD ranging from 10^{-7} to 10^{-11} M were tested. In another model, Wood and Holsapple (84) reported that proliferation and antibody secretion by pokeweed mitogen-stimulated human tonsillar lymphocytes were not altered by exposure to TCDD at concentrations ranging from 3 \times 10^{-8} to 10^{-10} M. Yer these same concentrations of TCDD significantly suppressed the ability of human tonsillar B cells of some donors to produce antibodies in response to toxic shock syndrome toxin (85). Because of the limited amount of data available and the lack of corroboration between laboratories, no conclusions can yet be drawn regarding the relative sensitivity of human lymphoid cells to TCDD.

**Research Needs**

For the field of immunotoxicology in general, there is a strong need to establish a broad database of normal values for the clinical immunology end points that may be of use as biomarkers of immune function in immunotoxicity assessments. To validate these biomarkers, there is a parallel need for animal research to identify TCDD-sensitive immune end points in animals that can also be measured in humans in order to establish 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. Also limited at the present time are good correlative data between changes in immune function measurements and changes in host resistance to specific disease challenges induced by xenobiotic exposure. 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 and characterize immune 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. Until then, risk assessment must be based on the best available data derived from well-controlled animal studies on TCDD immunotoxicity. Because the antibody response to SRBC has been widely studied and has been shown to be dose-dependently suppressed by TCDD and related HAH in several animal species, including nonhuman primates, this database would appear to be best suited for current application to risk assessment. The approaches used to establish acceptable exposure levels for humans for immunotoxicity should be based on the same procedures that are used for other noncarcinogenic toxic end points.
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