Role of Altered Arachidonic Acid Metabolism in 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Immune Suppression in C57Bl/6 Mice

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The systemic immune system has been shown to be a particularly sensitive target of halogenated aromatic hydrocarbon (HAH) toxicity. For example, exposure of mice to low doses of HAH causes thymic atrophy, suppression of both humoral and cell-mediated immune responses, and hyperinflammation (Kerkvliet and Burleson, 1994). Studies using various HAH congeners and mice congenic at the Ah locus have shown that the immunotoxic potency of HAH is directly correlated with their binding affinity for the Ah receptor, a ligand-dependent transcription factor (Silkworth et al., 1984; Kerkvliet et al., 1985, 1990a,b; Davis and Safe, 1988; Harper et al., 1993). Furthermore, several recent studies have demonstrated that macrophages, T cells, and B cells express the Ah receptor (Hayashi et al., 1995; Lawrence et al., 1996; Matsen and Shiverick, 1996). However, despite a rather large body of research, the biochemical and cellular mechanisms underlying the immunotoxic effects of HAH remain to be determined.

One well-characterized model system for studying HAH immunotoxicity is the in vivo cytotoxic T lymphocyte (CTL) response to allogeneic tumor cells (P815 mastocytoma cells). In this model, 3,4,5,3',4',5'-hexachlorobiphenyl (HxCB) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dose-dependently suppress the induction of CTL activity in C57Bl/6 mice in an Ah receptor-dependent fashion (Clark et al., 1983; Kerkvliet and Baecher-Steppan, 1988; Kerkvliet et al., 1990a; De Krey et al., 1994). In the normal immune response to P815 tumor cells, CTL develop through a pathway which involves signals from antigen-presenting cells (APC) and helper T (Th) cells (Kerkvliet et al., 1996). These signals include cell–cell contact via surface molecules and the production of soluble molecules such as cytokines and eicosanoids (Leung et al., 1982; Marasovsky et al., 1989; Lewis, 1990; Rosenberg and Singer, 1992). A combination of the appropriate signals drives precursor CTL (CTLp) to develop into antigen-specific effector cells capable of killing the P815 tumor cells. Suppression of the CTL response following exposure to TCDD appears to result from an inability of CTLp to proliferate and differentiate into effector CTL. Results from recent studies indicate that decreased interferon-γ (IFN-γ), interleukin (IL)-2, and tumor necrosis factor (TNF)-α production may underlie this suppression (Kerkvliet et al., 1996). IFN-γ and IL-2 are critical cytokines for the proper development of a CTL response (Marasovsky et al., 1989; Alderson and Widmer, 1993). While the lack of

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1996).

While the potential mechanism for this effect remains unclear,
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lipid oxidation and phospholipase (PL)A2 it has been reported that exposure to HAH increases membrane
associated AA, thereby increasing AA metabolite production.
HAH exposure may directly increase the release of membrane-
early target is arachidonic acid (AA) metab-
the appropriate cytokines is likely the explanation for loss of
CTL activity caused by TCDD, experimental evidence sug-
gests that earlier events drive the decreased cytokine produc-
tion in this immunotoxicity model.

One potential early target is arachidonic acid (AA) metab-
olism. Several AA metabolites, or eicosanoids, are potent
immunoregulatory molecules and have been shown to modu-
late lymphocyte activation, cytokine production, and CTL re-
sponses. For example, leukotrienes (LT) are involved in both
inflammatory and immunological responses and can regulate
the production of cytokines. LTB4 enhances IL-1 production
by APC, increases T cell proliferation, and stimulates IFNγ
production by T cells (Goodwin, 1989; Lewis, 1990; Claesson
et al., 1992). Likewise, prostaglandin (PG)E2 has multiple
effects on the immune system, including the inhibition of IL-2
and IFNγ production, enhanced IL-4 production, and inhibition
of T cell proliferation (Garrone et al., 1994; Watanabe et al.,
1994; Hilkens et al., 1996).

As summarized in Fig. 1, several independent pieces of
evidence support the hypothesis that altered AA metabolism
may play a role in HAH-induced immune suppression. First,
HAH exposure may directly increase the release of membrane-
associated AA, thereby increasing AA metabolism production.
While the potential mechanism for this effect remains unclear,
it has been reported that exposure to HAH increases membrane
lipid oxidation and phospholipase (PL)A2 activity, which in
turn could increases the pool of free AA (Mohammadpour et
al., 1988; Alsharif et al., 1990; Al-Bayati and Stohs, 1991;
Tithof et al., 1996).

Alternatively, exposure to HAH may target AA metabolism
downstream of PLA2 by inducing the enzymes which metab-
olize AA (see Fig. 1). The two most likely enzyme pathways
are the cytochrome P450 and cyclooxygenase pathways. Sev-
eral studies have reported that in liver, both in vitro and in vivo
exposure to HAH increases the cytochrome P450-dependent
metabolism of AA (Rifkind et al., 1990; Huang and Gibson,
1991; Yu et al., 1992) and raises the possibility that altered
cytochrome P450-dependent metabolism of AA to immuno-
modulatory metabolites may occur in immune cells as well.
Furthermore, the genes for cyclooxygenases (cox) have been
cloned and sequenced, revealing that both the human and
murine genes contain a dioxin response element (DRE) in their
upstream regulatory region (Kujubu et al., 1991; Kraemer
et al., 1992; Kosaka et al., 1994). Given that cyclooxygenases
convert AA to prostaglandins (DeWitt, 1991) and that PGE2
can suppress CTL development as well as IFNγ and IL-2
production, the direct induction of cox transcription in spleen
cells is a plausible mechanism for HAH-induced immunosup-
pression.

Finally, an HAH-induced increase in IL-1 could indirectly
increase AA metabolism via alterations in PLAr activity or the
amount of cox-2. In other experimental systems, in vitro ex-
posure to TCDD increases the level of IL-1β mRNA and protein
(Sutter et al., 1991; Yin et al., 1994; Gaido and Man-
ess, 1994; our unpublished observations). IL-1 influences AA
metabolism through two different pathways (Jackson et al.,
1993). Cyclooxygenases exist in two forms, a constitutively
expressed form (cox-1) and an inducible form (cox-2) (Mitch-
ell et al., 1993; Crofford et al., 1994; Vane et al., 1994). Cox-2
levels are increased by inflammatory cytokines such as IL-1,
and this induction stems from both increased transcription and
increased mRNA stability (Ristimaki et al., 1994; Riese et al.,
1994). IL-1 is also a potent activator of PLAr, and the release
of AA from membrane phospholipids by PLAr is generally
considered the primary rate-limiting step in the production of
e all eicosanoids (Bravo-Cuellar et al., 1990; Serhan et al.,
1996).

Collectively, these observations from nonimmune cells and
tissues suggest that HAH are capable of modulating AA me-
bolism; however, it remains to be determined whether they
alter AA metabolism in the context of an immunotoxicity
model and whether these changes drive the observed immune
suppression. We present here a detailed examination of AA
metabolism during the cell-mediated immune response to P815
tumor cells, including profiles of two immunomodulatory ei-
cosanoids, changes in cox and IL-1 gene and protein expres-
sion, the release of AA from spleen cell membranes, and the
modulation of these parameters by the most potent HAH con-
gener, TCDD. In addition, using both the response to P815
challenge and the highly TCDD-sensitive antibody response to
sheep red blood cells (SRBC), we have examined the effects of
specific inhibitors of AA metabolism on TCDD-induced immu-

MATERIALS AND METHODS

Reagents. Fetal bovine serum was purchased from Hyclone (Logan, UT). SRBC were obtained from Colorado Serum Co. (Denver, CO). Unless indi-
cated otherwise, all reagents and cell culture supplies were purchased from Sigma Chemical Co. (St. Louis, MO).

**Animals.** Male and female C57Bl/6 mice and female DBA/2 mice (6–8 weeks of age, The Jackson Laboratory, Bar Harbor, ME) were maintained in front of a laminar flow unit and given rodent chow and tap water ad libitum. DBA/2 mice were housed six per cage and were used for weekly in vivo propagation of the P815 mastocytoma cells. Male C57Bl/6 mice were housed singly, whereas female C57Bl/6 mice were housed six per cage.

**TCDD exposure.** TCDD (≥99% purity, Cambridge Isotopes, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil. Mice were given a single immuno-suppressive dose of TCDD by gavage. Control mice received the peanut oil–anisole vehicle. Male C57Bl/6 mice were treated with TCDD (15 μg/kg body weight) or peanut oil vehicle 1 day prior to the ip injection of 1 × 10⁶ viable P815 mastocytoma cells. In one study, female mice were treated with TCDD (5 μg/kg body weight) or vehicle 2 days prior to injection of 2.5 × 10⁴ SRBC.

**Animal treatment.** Animals were killed by CO₂ overdose at various times after antigen treatment. Blood was collected by heart puncture into syringes containing anticoagulant (5.1 mM disodium EDTA) and 0.2 mM indomethacin. Plasma was processed immediately for eicosanoid purification. Peritoneal fluid was collected by lavage with 2 ml phosphate-buffered saline (PBS) containing EDTA and indomethacin. Peritoneal cells were removed by centrifugation and eicosanoids were isolated from the supernatant. Spleens were removed and processed into a single-cell suspension as previously described (Kerkvliet and Oughton, 1993; Kerkvliet et al., 1996).

**In vivo administration of AA metabolism inhibitors.** Indomethacin, an inhibitor of cyclooxygenase-1 and -2, was dissolved in 70% ethanol and diluted in PBS (Smith et al., 1995). Animals were given indomethacin (1.7 mg/kg body weight) daily by gavage for 7 days in a volume of 10 μl/g body weight. Control animals received the PBS vehicle. Male OA (BioMol Research Laboratories, Plymouth Meeting, PA) is an inhibitor of PLA₂ (Mayer et al., 1998; Glaser and Lock, 1995) and BW755c is a dual inhibitor of both cyclooxygenases and lipoxigenases (Myers et al., 1990; Becker et al., 1993, gift from Dr. Gerald De Vries). Both manolaidole and BW755c were dissolved in DMSO and administered ip in a volume of 2.5 μl/g body weight. Animals were given two doses of either manolaidole (1.25 mg/kg body weight) or BW755c (50 mg/kg body weight) 45 min prior to TCDD exposure and again 45 min before SRBC injection. Control animals received DMSO. The efficacy of these doses of each inhibitor was determined in preliminary dose–finding studies in which inhibition of PGE₂ and/or LTB₄ production was measured in preliminary dose–finding studies in which inhibition of PGE₂ and/or LTB₄ production was measured.

**In vivo spleen cell cultures.** Spleen cells were suspended in serum-free Ultraculture medium (BioWittaker, Walkersville, MD) supplemented with 1.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM l-glutamine, 20 mM Hepes, and 5 × 10⁻⁵ M 2-mercaptoethanol. Spleen cells (1 × 10⁷) were cultured with 1 × 10⁶ P815 tumor cells in 2-ml cultures in 24-well plates at 37°C and 5% CO₂ for either 1 h (eicosanoid analysis) or 6 h (cytokine analysis). Cultures of naive spleen cells with P815 tumor cells were included as controls.

**Whole cell lysates.** Whole cell lysates were prepared by incubating equivalent numbers of spleen cells in extraction buffer (10 mM Tris (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, 5 mM NaCl, 1 mM DTT, 3 mM MgCl₂, 0.1 mM PMSF, 10 μg/ml aprotonin, and 10 μg/ml leupeptin) for 45 min at 4°C. Samples were centrifuged at 15,000 rpm in a microcentrifuge, and supernatants were harvested 20 min later and AA release was measured by scintillation counting.

**Ex vivo spleen cell cultures.** Spleen cells were suspended in serum-free Ultraculture medium (BioWittaker, Walkersville, MD) supplemented with 1.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM l-glutamine, 20 mM Hepes, and 5 × 10⁻⁵ M 2-mercaptoethanol. Spleen cells (1 × 10⁷) were cultured with 1 × 10⁶ P815 tumor cells in 2-ml cultures in 24-well plates at 37°C and 5% CO₂ for either 1 h (eicosanoid analysis) or 6 h (cytokine analysis). Cultures of naive spleen cells with P815 tumor cells were included as controls.

**Immunoprecipitation.** Cox-1 and Cox-2 proteins were extracted from whole cell lysates by sequential immunoprecipitation. Lysates containing 15 × 10⁶ cells equivalents from each animal were incubated with 2.5 μl of rabbit anti-murine Cox-2 antibody (Cayman Chemical Co., Ann Arbor, MI) for 2 h at room temperature with continuous end-over-end mixing. To precipitate the protein–antibody complex, protein A-conjugated Sepharose beads (5 mg, Calbiochem, La Jolla, CA) were added and the samples were mixed for another 1.5 h. The protein–antibody–bead complex was pelleted using a microcentrifuge and washed 5× with extraction buffer and 1× with 20 mM Tris, pH 7.4. Beads were then resuspended in 2× SDS-PAGE sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.005% bromophenol blue), and samples were boiled for 5 min and then stored at -70°C. This process was repeated with the supernatants, adding rabbit anti-murine cox-1 (1 μg, Cayman Chemical Co.) to each sample.

**Protein blotting.** Equivalent cellular extracts were subjected to SDS–PAGE by the method of Laemmli (1970). Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol using a Transphor Electrophorblotter (Hoefer Scientific Instruments, San Francisco, CA). Membranes were blocked overnight at 4°C with TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing 5% nonfat dry milk (NFDM). Antibodies were diluted in TBS containing 1% NFDM and the membranes were incubated with primary antibodies for 2 h at room temperature. Polyclonal rabbit anti-murine cox-1 or rabbit anti-murine cox-2 (Cayman Chemicals Co.) were used at 1 μg/ml. HRP-conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL) was used according to the manufacturer's instructions. Following each antibody treatment, blots were washed with TBS containing 1% NFDM and 0.05% Tween 20. Antibody complexes were visualized with chemiluminescent reagents (Amersham) and relative band intensities were quantified by densitometry using ImageQuant software, version 4.1b (PD-PC, Molecular Dynamics, Sunnyvale, CA).

**IL-1 bioassay.** IL-1 bioactivity in spleen cell culture supernatants was measured using the NOB-1 subclone of the murine lymphoma cell line EL-4 (PHLS Cell Repository, Salisbury, UK). NOB-1 cells produce IL-2 in response to IL-1 (α and β), but not in response to other cytokines such as IL-6, IL-4, IFNγ, or TNFα (Gearing et al., 1987; our unpublished observations). Duplicate wells containing 1 × 10⁴ NOB-1 cells were cultured with spleen cell supernatants or dilutions of purified IL-1 standard (Genzyme, Cambridge, MA), and the amount of IL-2 released into the media was determined using an IL-2-specific enzyme-linked immunosorbant assay (ELISA). Antibody pairs and standards for the IL-2 ELISA were purchased from Pharmingen (San Diego, CA), and the assay was performed following the manufacturer's recommended protocol. When necessary, samples were depleted of IL-2 prior to use in the NOB-1 IL-1 assay. IL-2 depletion was performed using 0.1 ng/ml rat anti-murine IL-2-specific monoclonal antibody (Pharmingen, San Diego, CA) and goat anti-rat IgG-conjugated magnetic beads (2 × 10⁶ beads/μl, Dynal A.S., Oslo, Norway).

**Eicosanoid isolation and measurements.** PGE₂ and LTB₄ from peritoneal fluid, plasma, and supernatants from spleen cell cultures were measured using commercially available radioimmuno assay (RIA) kits (Amersham). Eicosanoids were extracted and purified according to the kit manufacturer's instructions.

**AA release.** AA release from spleen cell membranes was measured using the method of Parker et al. (1979). Briefly, spleen cells were resuspended in Ultraculture medium and labeled with [³H]AA (0.5 μCi/ml, sp act 210 Ci/mmol, NEN DuPont, Boston, MA) for 60 min at 37°C. To remove the unincorporated label, cells were washed three times with media and 1 × 10⁷ cells were plated into culture with 1 × 10⁶ unlabeled P815 tumor cells. Culture supernatants were harvested 20 min later and AA release was measured by scintillation counting.

**CTL assay.** The cytolytic activity of spleen cells to P815 tumor cells was measured by 4 h Cr release assay (De Krey and Kerkvliet, 1995). Effector-to-target (E:T) ratios from 200:1 to 12.5:1 were tested in triplicate. The percent cytotoxicity for each E:T ratio was calculated using the equation % cytotoxicity = [(experimental release–naive release)/maximum release–spontaneous release] × 100.

**PFC assay.** The antibody plaque-forming cells (PFC) assay was used to measure the splenic antibody response to SRBC (Kerkvliet and Oughton, 1993) and PFC per 10⁶ spleen cells were determined.

**RT-PCR.** Total cellular RNA was extracted and complimentary DNA (cDNA) libraries were constructed using reverse transcriptase as described by Kerkvliet et al. (1996). Amplification of cDNA was performed by PCR using a DNA Engine thermocycler (MJ Research; 30 cycles: 94°C for 1 min, 60°C
for 1 min, and 72°C for 2 min) using the following IL-1β-specific primers: sense, 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3'; anti-sense, 5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3'. Message integrity was verified using β2-microglobulin-specific primers (data not shown). Products (563 bp) were separated by agarose (2%) gel electrophoresis and visualized by ethidium bromide staining.

**Data analysis.** All data were analyzed using Statview (version 4.01, Abacus Concepts, Berkeley, CA) statistical software for Macintosh. An analysis of variance (ANOVA) was used, followed by an unpaired Student's t test to compare means of two independent variables. Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

**Exposure to TCDD Increases the Production of Immunomodulatory AA Metabolites**

While the effects of HAH on AA metabolism have been examined in other cells and tissues, there is little information regarding its impact on AA metabolism in the immune system. Moreover, the role of AA metabolism in the immune response to P815 tumor cells is poorly understood. Therefore, we sought to explore the role of two known immunomodulatory AA metabolites (PGE$_2$ and LTB$_4$) in the response to P815 challenge and to examine whether exposure to TCDD alters their level of production. Eicosanoid levels in the peritoneal cavity and plasma were examined, and metabolite release by spleen cells which were restimulated with P815 cells *ex vivo* for 1 h was also measured. PGE$_2$ and LTB$_4$ levels were compared to naive control animals, which received neither TCDD nor P815 challenge. As shown in Figs. 2 and 3, P815 challenge alone led to several changes in AA metabolism. When compared to naive animals, the most notable alterations were a 30% decrease in peritoneal and plasma LTB$_4$ levels and an approximately twofold increase in PGE$_2$ release from spleen cells. Injection of P815 tumor cells did not affect plasma or peritoneal PGE$_2$ levels nor did it alter splenic LTB$_4$ production.

In mice which were challenged with P815 cells, exposure to TCDD altered AA metabolism in both the peritoneal cavity and the spleen. LTB$_4$ release by spleen cells from TCDD-treated mice was 1.4 times higher than that by vehicle controls on day 6 (Fig. 2C). Similarly, exposure to TCDD caused an increase in PGE$_2$ in both the spleen (1.5-fold higher than control on day 5, Fig. 3C) and the peritoneal cavity. In the TCDD-treated animals, PGE$_2$ levels were 3.5 times higher than those of both vehicle control and naive animals on day 5. By day 6, peritoneal PGE$_2$ levels were 3 times higher than those in vehicle-treated mice and 8-fold higher than those in naive animals (Fig. 3B).

**Exposure to TCDD Causes a Transient Increase in the Release of AA from Spleen Cell Membranes**

To explore the possibility that exposure to TCDD leads to an increased release of AA from membranes, we measured AA release from spleen cell membranes of mice which had been challenged with P815 tumor cells. As summarized in Table 1,
Exposure to TCDD Does Not Alter Cyclooxygenase Transcription or Protein Levels

To determine whether TCDD increases PGE\textsubscript{2} production directly via alterations in cyclooxygenase transcription and translation, splenic cox-1 and cox-2 message and protein levels were examined on days 1 to 6 following P815 challenge. Using a combination of immunoprecipitation and protein blotting, the relative amounts of cox-1 and cox-2 from individual naive and vehicle- and TCDD-treated animals were measured. Spleen cells from naive animals expressed both cox-1 and cox-2, and challenge with P815 tumor cells did not lead to any detectable changes in cox protein. Moreover, exposure to TCDD did not alter splenic cox-1 or cox-2 protein levels (Table 2). The effects of exposure to TCDD on spleen cell cox transcription were examined using reverse transcriptase polymerase chain reaction (RT-PCR). Due to message instability, we were unable to measure cox-2 mRNA directly. In contrast, cox-1 message was detected in spleen cells from naive and vehicle- and TCDD-exposed mice; however, TCDD did not influence cox-1 levels in the spleen (data not shown). The effect of exposure to TCDD on cox gene expression was further examined using four different monocyte and macrophage cell lines derived from Ah-responsive strains of mice. RAW264.7, IC21, Wehi-3, and Wehi-274.1 cells were exposed to 1 nM TCDD with and without 1 \mu g/ml LPS and changes in cox-1 gene expression were examined by RT-PCR. As observed in the spleen cells, cox-1 message was detected under all conditions and treatment with TCDD had no effect on the level of expression (data not shown).

Exposure to TCDD Does Not Alter Splenic IL-1 Message or Protein Levels

We then sought to determine if exposure to TCDD alters IL-1 levels, which could indirectly modulate AA metabolism. As shown in Fig. 4A, challenge with P815 tumor cells resulted in increased spleen cell IL-1 activity on days 5 and 6; however, there was no difference between the vehicle- and TCDD-treated groups. Similar results were obtained using an IL-1\beta-specific ELISA (data not shown). To corroborate these findings, IL-1\beta message in spleen cells was measured using RT-PCR. While IL-1\beta message was present on all days, on no day examined did exposure to TCDD change the amount of splenic IL-1\beta transcript detected (Fig. 4B).

Inhibition of Cyclooxygenase Fails to Restore TCDD-Induced CTL Suppression

To test the hypothesis that the TCDD-induced increase in peritoneal and splenic PGE\textsubscript{2} drives the suppression of the CTL response to P815 tumor cells, a metabolic inhibitor of PGE\textsubscript{2} production was used. Mice were treated with indomethacin daily for 7 days, beginning 1 day prior to treatment with TCDD and continuing through 5 days after challenge with P815 tumor cells. When CTL activity was measured on day 9, we found

Compared to naive and vehicle control mice, exposure to TCDD caused a twofold increase in AA release from spleen cell membranes on day 1. This increase was transient, and challenge with P815 tumor cells did not elicit a significant increase in AA release from spleen cells of either vehicle- or TCDD-treated mice on any other day examined.
that the inhibition of PGE₂ production failed to abrogate the suppression caused by TCDD (Fig. 5). Interestingly, treatment with indomethacin caused a small but not statistically significant change in CTL activity in vehicle-treated mice (compare open circles with open squares), suggesting that the CTL response is sensitive to changes in PGE₂.

**Inhibition of Cyclooxygenase and Lipoxygenase or PLA₂ Fails to Restore TCDD-Induced Suppression of the Antibody Response to SRBC**

Due to the fact that P815 tumor cells require lipoxygenase-derived products to proliferate (Ralph and Wojcik, 1990), further analysis of the role of altered AA metabolism in the immunosuppression caused by TCDD required the use of an antigen which was unaffected by inhibitors of AA metabolism. We therefore focused our studies on the antibody response to SRBC, which has been shown previously to be highly sensitive to TCDD (Kerkvliet and Burleson, 1994). Two broad-range inhibitors were used in these studies: BW755c, which inhibits both cyclooxygenases and 5-lipoxygenase (Myers et al., 1990; Becker et al., 1993), and manoalide, which inhibits PLA₂ (Mayer et al., 1988; Glaser and Lock, 1995). As shown in Fig. 6, exposure to TCDD alone caused an 80% reduction in the antibody response to SRBC. However, treatment with neither manoalide nor BW755c, at doses previously determined to be effective, altered this suppression (Fig. 6). Furthermore, treatment with these inhibitors had no effect on TCDD-induced thymic atrophy, decreased spleen weight, and increased liver weight (data not shown).

**DISCUSSION**

Many AA metabolites are potent immunoregulatory molecules, and independent evidence from several laboratories suggests a role for AA metabolites in TCDD immunotoxicity. For example, TCDD and related HAH induce the cytochrome

### TABLE 1

<table>
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<tr>
<th>Spleen cell treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Naive</td>
<td>550.0 (59)*</td>
<td>1096.4 (38)</td>
<td>1064.3 (312)</td>
<td>802.8 (104)</td>
<td>805.9 (28)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>680.8 (29)</td>
<td>989.2 (87)</td>
<td>1030.3 (203)</td>
<td>1002.3 (228)</td>
<td>870.1 (56)</td>
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<td>TCDD</td>
<td>1247.3 (223)*</td>
<td>920.8 (61)</td>
<td>765.1 (130)</td>
<td>765.1 (54)</td>
<td>987.5 (130)</td>
</tr>
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</table>

**Note.** Spleen cells (1 × 10⁷) from either vehicle- or TCDD-treated, P815-challenged mice were labeled ex vivo with [³H]AA and placed into culture. Parallel cultures with spleen cells from naive mice were used as a control. Release of labeled AA from cellular membranes was measured 20 min after the addition of unlabeled P815 tumor cells. Data are expressed as counts per minute (cpm) released into culture supernatants. Numbers in parentheses indicate the standard error of the mean (n = 6 per day for vehicle and TCDD groups, n = 2 per day for naive animals).
* Indicates that the cpm were different from vehicle-treated controls (p ≤ 0.05).

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>Cox-1</td>
<td>172.5 (34)*</td>
<td>151.4 (45)</td>
<td>209.7 (44)</td>
<td>231.1 (35)</td>
<td>241.6 (41)</td>
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<td>263.0 (52)</td>
<td>267.8 (70)</td>
<td>204.5 (31)</td>
<td>158.0 (33)</td>
<td>362.5 (64)</td>
<td>272.0 (54)</td>
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<td>TCDD</td>
<td>275.7</td>
<td>206.4 (15.5)</td>
<td>237.7 (101)</td>
<td>256.1 (59)</td>
<td>244.3 (27)</td>
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<td>Naive</td>
<td>952.2 (86)</td>
<td>498.7 (55)</td>
<td>867.2 (44)</td>
<td>772.3 (43)</td>
<td>330.7 (104)</td>
<td>790.4 (103)</td>
</tr>
<tr>
<td>Cox-2</td>
<td>975.6 (94)</td>
<td>352.4 (36)</td>
<td>778.3 (39)</td>
<td>739.0 (52)</td>
<td>268.3 (39)</td>
<td>779.7 (71)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>883.9</td>
<td>365.1 (67)</td>
<td>695.9 (6.6)</td>
<td>463.6 (279)</td>
<td>227.6 (92)</td>
<td>778.2 (205)</td>
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**Note.** Cox-1 and Cox-2 levels were measured by sequential immunoprecipitation, electrophoresis, and immunoblotting of spleen cell lysates from naive and vehicle- or TCDD-treated, P815-challenged mice. Immunoblotted proteins were visualized using cox-1 and cox-2 antibodies (Cayman Chemical Co.) coupled with a chemiluminescent detection system (Amersham). Relative band intensities were quantified by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). There was no significant difference between any treatment groups on any day.
* The data represent the average band intensity ±SEM. (n = 6 per day for vehicle and TCDD groups, n = 2 per day for naive animals).
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P450-dependent metabolism of AA in liver (Rifkind et al., 1990; Huang and Gibson, 1991) and increase membrane lipid oxidation in hepatocytes and macrophages (Mohammadpour et al., 1988; Alsharif et al., 1990, 1994), either of which could lead to increased release of AA from cellular membranes. TCDD has also been shown to increase IL-1 expression (Sutter et al., 1991; Yin et al., 1994; Gaido and Maness, 1994; Fan et al., 1997). Since IL-1 is a potent regulator of PLA₂ and cox (Jackson et al., 1993; Serhan et al., 1996), this suggests another pathway through which exposure to TCDD could alter AA metabolism. Finally, perhaps the strongest evidence that exposure to TCDD may alter the production of immunomodulatory AA metabolites is the discovery of a DRE in the upstream regulatory region of the cox genes (Kraemer et al., 1992). This finding raises the question of whether TCDD, via the Ah receptor, directly induces cox transcription in immune cells, leading to increased PGE₂ production.

PGE₂ has been shown to suppress IL-2 and IFNγ production, T cell activation and the development of cell-mediated immune responses (Garrone et al., 1994; Watanabe et al., 1994; Hilkens et al., 1996). This scenario of suppression parallels our previous observations in mice challenged with P815 tumor cells in the presence of TCDD. TCDD-treated animals exhibit decreased IL-2 and IFNγ production, reduced T cell activation and differentiation, and an overall suppression of the response to challenge with the tumor cells (Kerkvliet et al., 1996). Initial characterization of changes in two potent immunomodulatory AA metabolites following P815 challenge revealed that, compared to vehicle control mice, TCDD treatment increased PGE₂ levels in the peritoneal cavity and increased LTB₄ and PGE₂ production by spleen cells. Exposure to TCDD also caused a twofold increase in AA release from spleen cell membranes 1 day after challenge with P815 tumor cells. These findings were intriguing because they support the theory that exposure to TCDD increases the production of immunomodulatory AA metabolites such as PGE₂.

However, when we examined the levels of splenic cox protein and gene expression, we found no effect of TCDD. Furthermore, in vitro exposure to 1 nM TCDD did not alter cox levels in several different monocyte/macrophage cell lines (our unpublished observations). These findings are in contrast to several reports in the literature in which in vitro treatment with

**FIG. 4.** Exposure to a single immunosuppressive dose of TCDD does not alter the amount or time course of splenic IL-1 message or protein. [A (and inset)] In two separate experiments, spleens from naive, vehicle-treated, or TCDD-treated mice were cultured for 6 h with P815 cells and the release of IL-1 into culture supernatants was measured using the NOB-1 bioassay, as described under Materials and Methods. Data points represent the mean ±SEM (n = 6 animals per treatment group). *Indicates a significant difference from naive animals (p ≤ 0.05) (B) IL-1β gene expression was measured by RT-PCR, as described under Materials and Methods. The data represent spleen cell IL-1β mRNA levels on days 1 through 5 following in vivo challenge with P815 cells and 1 h restimulation in vitro. P815 cells cultured alone produced no detectable IL-1. Titration of individual samples confirmed that the message detected was within the linear range (not shown).

**FIG. 5.** Indomethacin treatment does not alter TCDD induced suppression of CTL activity. C57Bl/6 mice were given a single dose of either TCDD (closed symbols) or peanut oil vehicle (open symbols) 1 day prior to the ip injection of 1 × 10⁷ P815 tumor cells. PBS control (circles) or indomethacin (1.7 mg/kg body weight, squares) was administered daily by gavage for 7 days (-1 to 5). All animals were killed on day 9 and CTL activity was measured by ⁵¹Cr release assay, as described under Materials and Methods. Data points represent the mean ±SEM (6 animals per group). *Indicates a significant difference from vehicle-treated animals (p ≤ 0.05). There was no statistically significant difference between the double vehicle group and the peanut oil vehicle plus indomethacin group. Testing of basal and antigen-induced peritoneal PGE₂ levels in separate animals verified the efficacy the indomethacin treatment.
0.01–10 nM TCDD induced the expression of Cox in a variety of cell types, including thymocytes, kidney cells, hepatoma cells, and seminal vesicle cells (Kraemer et al., 1996; Olness et al., 1996; Schuhmacher et al., 1996; Liu et al., 1997; Puga et al., 1997). These differences suggest that the effects of TCDD on Cox induction may be cell type-specific. An examination of TCDD-induced metabolism of AA in the liver and spleen further supports the idea that the effects of TCDD are cell-specific (Lee et al., submitted for publication). In this study, in vivo treatment of C57Bl/6 mice with TCDD (20 µg/kg) induced NADPH-dependent metabolism of AA in liver homogenates, leading to increased production of HETEs and ω-hydroxy and vicinal diols. However, when AA metabolism in spleen homogenates from the same animals was examined, there was no difference between the levels of splenic AA metabolism in vehicle- and TCDD-exposed mice.

The lack of effect of TCDD on splenic AA metabolizing enzymes led us to directly examine the role AA metabolism in TCDD-induced immune suppression using metabolic inhibitors. Blocking cyclooxygenase activity with indomethacin during the early stage of the response to P815 cells did not prevent the suppression of CTL activity caused by TCDD. A previous study supports these findings (De Krey et al., 1994). In this study, exposure of mice to an immunosuppressive dose of HxCB increased PGE2 production on days 7 through 9, yet indomethacin treatment on days 4 through 8 failed to alter HxCB-induced splenic CTL suppression. Together, these findings indicate that while exposure to HAH increases splenic and peritoneal PGE2, this increase is neither necessary nor sufficient to suppress the CTL response to P815 tumor cells. Similarly, inhibition of PLA2 with manoalide or dual inhibition of cyclooxygenase and lipoxygenase activity using BW755c failed to reverse the TCDD-induced suppression of the antibody response to SRBC challenge. Interestingly, blocking these pathways of AA metabolism had no deleterious effects on the CTL response to P815 tumor cells or antibody formation in response to SRBC, suggesting that AA metabolism is not an essential component of either of these immune responses.

Finally, with regard to the ability of TCDD to influence AA metabolism indirectly via increased IL-1 production, we found that, in the context of the response to P815 tumor cells, exposure to TCDD did not alter splenic IL-1β gene expression or IL-1 protein levels. Thus, as with TCDD-induced AA metabolism, the induction of IL-1β by TCDD also appears to be tissue-specific. Several reports have documented the induction of IL-1β following both in vivo and in vitro exposure to TCDD; however, the model systems used for these experiments were human keratinocyte cell lines, keratinocyte primary cultures, or rat liver (Sutter et al., 1991; Gaido and Maness, 1994; Yin et al., 1994; Fan et al., 1997).

Collectively, our findings provide strong evidence that, in the context of the models tested, exposure to TCDD and related HAH may alter the level of some immunomodulatory AA metabolites. However, these changes appear to be indirect and not relevant to the immune suppression caused by this class of compounds. Nevertheless, these experiments do not address the role of other AA metabolites, namely, those produced by cytochrome P450-dependent AA metabolism in the liver. The precise identity and biological function of these metabolites is unknown, making it difficult to speculate whether these AA metabolites may underlie some aspects of TCDD toxicity.

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