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Enhanced Suppressor Cell Activity as a Mechanism of Immunosuppression by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin¹ (41275)

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Abstract. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), a toxic halogenated aromatic hydrocarbon, acts in the body as a cumulative poison. The chronic immunotoxic effects of TCDD were studied in C57BL/6 male mice. Total doses of 100 µg/kg or greater produced cellular depletion in thymus, spleen, and lymph nodes, and the animals appeared sick. No cellular depletion was seen following 0.4 µg/kg, and only thymus was affected by 4 and 40 µg/kg. The antibody response to SRBC and TNP-*Brucella abortus* was impaired following 40 µg/kg TCDD, the delayed hypersensitivity response to oxazalone was impaired by 4 µg/kg and the generation of alloantigen-specific cytotoxic T cells (CTL) was sensitive to as little as 0.004 µg/kg TCDD. *In vitro* analysis of the mechanism of suppression using limiting dilution techniques showed that TCDD did not deplete the precursors of CTL but generated cells capable of suppressing CTL generation *in vitro*.

Halogenated aromatic hydrocarbons (HAHs)² such as polychlorinated biphenyls (PCBs)², polychlorinated naphthalenes, polybrominated biphenyls (PBBs), polychlorinated dibenzofurans, pentachlorophenols (PCP), and polychlorinated dibenzo-*p*-dioxins, pose a serious man-made environmental threat to health (1-9). They are fat soluble, poorly metabolized, and tend to accumulate in the environment and in the body where they act as persistent poisons (1). Polychlorinated dibenzo-*p*-dioxins such as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) formed industrially by pyrolysis of chlorophenols, a particularly toxic subgroup of the halogenated aromatic hydrocarbons, provide a good model system for studying HAH toxicity. Although species vary in the types of

lesions developing after TCDD exposure (8, 10, 11), a consistent observation in all animal species which have been studied is atrophy of the thymus. TCDD-treated rodents show impairment of delayed hypersensitivity responses, reduced ability to mount a graft-versus-host reaction and reduced ability to reject allografts, and show an increased susceptibility to certain infectious agents (8, 10, 12-15). Thus, one of the potentially important aspects of toxicity of TCDD and related HAHs is the effect on the immune system. In order to better understand the mechanism of TCDD immunotoxicity, we have analyzed the effects of different doses of TCDD on several types of immune function of C57BL/6J male mice. Particular attention has been directed to the chronic effects of TCDD since chronicity typifies human exposure. We find that the generation of cytotoxic T lymphocytes (CTL) following alloantigen challenge is particularly susceptible to impairment by low doses of TCDD, and this impairment is caused by a disturbance in the cells which regulate the CTL response. Measurement and characterization of this disturbance may provide a sensitive test for presence of biologically significant amounts of TCDD in the body.

Materials and Methods. Mice. C57BL/6

male mice age 6-8 week from the Jackson Laboratory, Maine, and housed on Teklad chow (6% fat) and water *ad lib*. DBA/2J mice obtained and were used for P-815-X2 (H-2^d) mastocytoma ascites transfer. C3H/He and C57BL/6J × DBA/2J produced in our own colony mice were purchased from University College, Guelph, Ontario.

TCDD treatments. TCI 144-2 purity >99% was obtained from Chemical, Sarnia, Ontario. Hundred and fifty micrograms dissolved in *p*-dioxane and dissolved in corn oil containing specific TCDD and studied 1 week dose.

Cell preparations. The and control mice were killed by dislocation. The spleen, peripheral lymph nodes (axillary and inguinal) were aseptically removed and a single-cell suspension prepared by pressing minced tissue through a stainless-steel screen into medium. Culture medium: α-MEM supplemented with 10% bovine serum (Grand Island, Grand Island, N.Y.), 100 units penicillin and 100 µg streptomycin/ml Hepes buffer, 2-Mercaptoethanol added at a concentration of 10⁻⁵ M for cell culture experiments.

Anti-Thy 1.2 treatment. Serum was purchased from Cytogen Laboratories, Hornby, Ontario. The proportion of thy 1.2-positive cells in peripheral lymph node (PLN) suspensions was determined in a 1:10 suspension initially prepared in phosphate-buffered saline with 0.1% bovine serum albumin by incubation with Na₂⁵¹Cr (Gibco, Grand Island, N.Y.) and washed. 10⁶ cells were incubated with a 1:20 dilution at 4°C for 45 min, washed, and resuspended in a 1:12 dilution of 1% complement (Cedarlane, Ltd., Ontario). After 90 min of incubation at

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² Abbreviations used: HAHs, halogenated aromatic hydrocarbons; PBBs, polybrominated biphenyls; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; SRBC, sheep red blood cells; TNP-BA, trinitrophenylated *Brucella abortus* organisms; PLN, peripheral lymph node (axillary, brachial, inguinal); CTL, cytotoxic T lymphocyte; CTLp, precursor of CTL; PFC, plaque-forming cell; oxazalone, 4-ethoxymethylene-2-phenyl-5-oxazalone.

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male mice age 6-8 weeks were purchased from the Jackson Laboratory, Bar Harbor, Maine, and housed six per cage. Teklad chow (6% fat) and water were provided *ad lib*. DBA/2J mice were similarly obtained and were used for maintenance of P-815-X2 (H-2^d) mastocytoma cells by serial ascities transfer. C3H/HeJ $\times$ DBA/2J F₁ and C57BL/6J $\times$ DBA/2J F₁ hybrids were produced in our own colony. RNC nude mice were purchased from the Ontario Veterinary College, Guelph, Ontario.

TCDD treatments. TCDD Lot No. 851-144-2 purity >99% was obtained from Dow Chemical, Sarnia, Ontario, Canada. Two hundred and fifty micrograms was dissolved in *p*-dioxane and diluted in corn oil. Mice were injected ip weekly with 100 μ l of corn oil containing specified amounts of TCDD and studied 1 week after the fourth dose.

Cell preparations. The TCDD-treated and control mice were killed by cervical dislocation. The spleen, thymus, and peripheral lymph nodes (axillary, brachial, and inguinal) were aseptically removed, and a single-cell suspension was obtained by pressing minced tissue through a 60-mesh stainless-steel screen into cold medium. Culture medium consisted of α -MEM supplemented with 10% v/v fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), 100 units penicillin G and 100 μ g streptomycin/ml and 20 mM Hepes buffer. 2-Mercaptoethanol was added at a concentration of 5×10^{-5} M for cell culture experiments.

Anti-Thy 1.2 treatment. AKR anti-C3H serum was purchased from Cedarlane Laboratories, Hornby, Ontario (Lot 5101). The proportion of thy 1.2-bearing cells in peripheral lymph node (PLN) cell suspensions was determined in two ways. PLN suspension initially prepared in Dulbecco's phosphate-buffered saline with 0.2% Fraction V bovine serum albumin was labeled by incubation with Na₂⁵¹CrO₄ (New England Nuclear) and washed. Labeled cells at 10⁷/ml were incubated with antiserum at a 1:20 dilution at 4 $^{\circ}$ for 45 min and resuspended in a 1:12 dilution of Low-Tox rabbit complement (Cedarlane Laboratories). After 90 min of incubation at 37 $^{\circ}$, the degree

of ⁵¹Cr release was calculated and percentage lysis by anti-thy 1.2 plus complement calculated by subtraction of the complement control. Since TCDD treatment might have altered the sensitivity of the cells to complement, a second method was used. PLN were incubated in a 1/4 dilution of anti-thy 1.2 serum for 45 min at 4 $^{\circ}$, washed, and then incubated in a 40- μ g/ml solution of fluorescein-labeled protein A (Pharmacia). After washing, the proportion of cells showing membrane immunofluorescent staining was determined by counting 200-400 cells under indirect ultraviolet illumination (Leitz).

Antibody response. The antibody response to two different antigens was measured. Mice were injected intraperitoneally with either 5×10^7 washed sheep red blood cells (SRBC) or, 0.1 ml of an 8% suspension of TNP-conjugated *Brucella abortus* (TNP-BA). Spleens were removed from SRBC- or TNP-BA-challenged mice 3-7 days later and tested for antibody-forming cells against SRBC or TNP-SRBC, respectively, using a plaque assay as modified for slides by Dresser and Greaves (16). Freshly frozen guinea pig serum absorbed with 50% packed SRBC was thawed and added at a final dilution of 1/30 as a source of complement to detect "direct" plaque-forming cells. To detect IgG and IgA PFC, direct IgM PFC were first blocked by adding a 1/200 dilution of goat anti-mouse μ during the initial incubation of target and spleen cells. Indirect IgG PFC were then developed by adding rabbit anti-mouse IgG at a predetermined optimal 1/200 dilution, and IgA PFC were developed using a 1/100 dilution of rabbit anti-mouse IgA. The IgG-developing serum was prepared by immunizing rabbits with purified mouse IgG (Miles, Lot 51), and showed only a single line against mouse serum and IgG on Ochterlony plates. The IgA-developing serum was prepared in rabbits using pure mouse myeloma MOPC 315 (IgA λ 2) and was depleted of anti-light-chain activity by passage through a murine IgG-Sepharose-immunoadsorbent column. Pure mouse IgA or IgG added to the PFC assay inhibited only IgA or IgG PFC, respectively, when the developing sera were used.

Delayed hypersensitivity response. In the first method used, TCDD-treated and control mice were sensitized by injecting $1-2 \times 10^5$ SRBC iv and 5 days later the mice were tested by injecting 2×10^5 SRBC in 20 μ l into one of the hind footpads (17). Saline (20 μ l) was injected into the other footpad and the degree of swelling was measured after 24 hr. The net amount of swelling observed following SRBC challenge was corrected for swelling produced by medium alone. In a second method, TCDD treated or control mice were sensitized by painting both ears with 3% oxazalone (4-ethoxymethylene-2-phenyl, BDH Chemicals, Poole, England) dissolved in 95% ethanol. Nine to sixteen days later, sensitized and unsensitized control animals were challenged with 1% oxazalone in olive oil (18). Ear thickness was measured prior to challenge and at 24 hr using a constant-pressure micrometer.

Generation and assay of cytotoxic T cells. CTL were generated against the H-2^d alloantigen *in vivo* and *in vitro*. The *in vivo* response to TCDD-treated mice was determined following injection of 2×10^7 P-815 tumor cells (H-2^d) that had been treated with 5000 rad ⁶⁰Co to prevent proliferation. Spleens were removed from the recipients 3-6 days later and the CTL activity was assayed in a 6-hr ⁵¹Cr-release assay against ⁵¹Cr-labeled P-815 targets as described in detail elsewhere (19). The titration curve (percentage specific ⁵¹Cr release vs concentration of sensitized spleen cells) was analyzed by computer for the parameter N_{50} which is proportional to the number of CTL lytic units in the cell suspension. $693 N_{50} \times 10^3$ units is equivalent to 1 lytic unit (the number of cells required to produce 50% lysis in the absence of interfering bystanders (19)).

CTL were generated *in vitro* in two different ways. First, mixed lymphocyte culture (MLC) was performed in 17 $\times$ 100-mm polystyrene tubes (Falcon Plastics, No. 2057) in 3 ml culture medium. Briefly, $1-3 \times 10^6$ spleen or PLN cells from the C57BL/6J mice were mixed with 10^6 C3D2F₁ or B6D2F₁ spleen cells that had been inactivated with 1500 rad. CTL content of the culture was determined after a 5-days incu-

bation period at 37° in a 4-hr ⁵¹Cr-release assay using 2×10^4 P-815 target cells. In some experiments, thymocytes from treated or control mice were added to the cultures. Second, the frequency of precursors of CTL (CTLp) was measured using the limiting dilution microculture method of Teh *et al.* (20). Briefly, small numbers of C57BL/6J spleen or PLN cells were cultured in V-bottom microwells (Linbro 96-MVC-TC) in 0.2 ml culture medium with 2×10^5 irradiated F₁ spleen and 2×10^5 RNC nude spleen cells. Here, the nude spleen cells provide "help" which allows the development of CTL from single precursor cells (20). After 6 days incubation at 37°, 2000 ⁵¹Cr-labeled P-815 target cells were added to each well and the amount of lysis was determined after 5 hr. Those wells showing release exceeding the 95% confidence interval of spontaneous release from negative control wells containing F₁ stimulators and nude spleen alone were scored positive. Under limiting dilution conditions, the number of lymphoid cells yielding 37% negative wells defines the cell number containing, on the average, 1 CTLp according to the Poisson distribution: $P(0) = e^{-u}$, where $P(0)$ represents the fraction of negative wells and u the mean frequency of CTLp per well. The value of u was calculated using a computer program derived from Porter *et al.* (21). The value of u allowed an estimate of the number of CTLp present in the tube MLC cultures and thus, a determination of the yield of CTL per CTLp ($N_{50} \times 10^3$ per 1 CTLp).

Results. Effect of different doses of TCDD on cellularity of lymphoid organs. Figure 1 shows the effects of different doses of TCDD on the cellularity of different organs of the mouse immune system. The reported LD₅₀ in mice for TCDD is approximately 100 μ g/kg (8), although the time of death is quite variable and often delayed. When a total dose exceeding 40 μ g/kg was administered, cellularity of the spleen, thymus, and peripheral lymph nodes was markedly reduced and the animals appeared ill. In contrast, doses of 0.4-40 μ g did not reduce cell numbers in the spleen and peripheral lymph nodes and the animals appeared healthy. Nevertheless, cellular

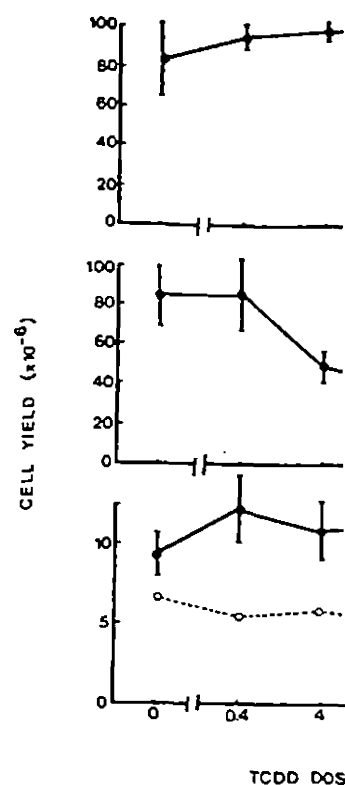


FIG. 1. Effect of TCDD on cellularity of mouse lymphoid organs. Data are SEM for spleen (A), thymus (B), and lymph nodes (C) studies in four to six experiments. 4 μ g/kg represents a dose of 4 parts per billion. In three experiments after the last dose of TCDD, the I.2-bearing cells were determined by complement-dependent lysis (two immunofluorescent staining (one described under Materials and Methods) control mice contained 70.4-74% cells whereas PLN from mice given 43-60%, from mice given 43-60%, and from mice given 40-55%. The dotted line in C shows number of thy I.2-positive cells in

depletion was already apparent at 0.4 μ g/kg dose when the thymus was depleted. Since these data suggest that the effects of TCDD on the thymus were particularly severe compared to other lymphoid organs, we tested whether TCDD affected the peripheral T-cell population. In determining the proportion of I.2-positive cells in PLN suspensions from TCDD-treated and control

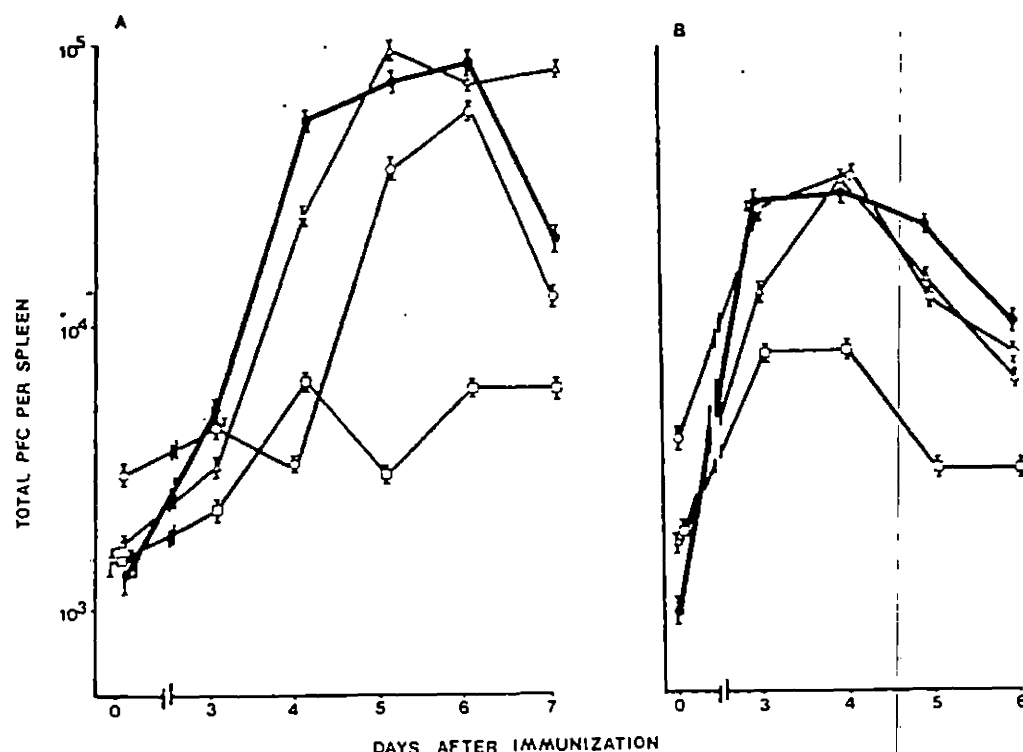


FIG. 2. Effect of TCDD treatment on the antibody response. Control mice (●) or mice treated with a total of 0.4 (△), 4 (○), or 40 (□) µg/kg were given an intraperitoneal immunization with SRBC (A) or TNP-BA (B). The spleens were removed at the times indicated and the number of PFC was measured as described under Materials and Methods. The total IgM + IgG + IgA PFC number $\pm$ SEM is shown, for groups containing three to four pooled mouse spleens.

is shown in Fig. 3. Even the 0.4 µg/kg dose of TCDD impaired the ability to generate a maximal response on Days 6 and 7. We concluded that the CTL response was particularly sensitive to functional impairment

by TCDD. We therefore decided to direct our subsequent investigations toward elucidation of the mechanism of impairment of CTL generation by TCDD.

In vitro analysis of effect of TCDD on

TABLE 1. EFFECT OF TCDD TREATMENT ON DELAYED HYPERSENSITIVITY REACTIONS

Treatment given	Increment in footpad swelling 24 hr after oxazalone challenge ^a ($\times 10^{-2}$ mM, mean $\pm$ SEM)	Increment in ear thickness 24 hr after oxazalone challenge ^b ($\times 10^{-2}$ mM, mean $\pm$ SEM)	
		Immune recipient	Control recipient
Control (corn oil)	23.7 $\pm$ 5.2 (10)	10.9 $\pm$ 1.9 (8)	0.17 $\pm$ 0.87
0.4 µg TCDD	24.5 $\pm$ 5.1 (10)	16.8 $\pm$ 4.1 (8)	-0.83 $\pm$ 1.35
4 µg TCDD	34.5 $\pm$ 4.1 (10)	6.8 $\pm$ 1.4 (8)*	-0.83 $\pm$ 1.35
40 µg TCDD	14.7 $\pm$ 4.9 (10)	7.4 $\pm$ 0.7 (8)*	0 $\pm$ 1.29

^aThe mice were sensitized by intravenous injection of $1-2 \times 10^5$ SRBC and tested 5 days later by intracutaneous injection of 2×10^5 SRBC into the left hind footpad. A similar volume of saline was injected into the right hind footpad as control for the nonspecific effects of the fluid injection. Parentheses enclose the number of measurements.

^bThe mice were sensitized to oxazalone in ethanol and ear swelling in sensitized and non-sensitized animals was measured 24 hr after application of oxazalone in saline oil.

*Significantly lower than control by Student's *t* and Mann-Whitney *U* tests, $P < 0.05$.

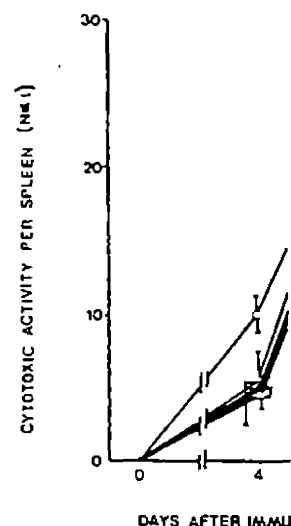


FIG. 3. Effect of TCDD treatment on CTL activity *in vivo* following alloantigen immunization. Mice (●) or mice given 0.4 (△), 4 (○) µg/kg were immunized with irradiated tumor cells as described under Materials and Methods. CTL activity was measured in spleen from groups of three to four mice.

CTL generation. The suppressive effect of TCDD treatment on the generation of CTL was analyzed *in vitro* using techniques that have been employed to elucidate the mechanism of immunosuppression in pregnant mice (24). Experiment II shows that TCDD treatment impaired the ability of spleen and peritoneal exudate cells from TCDD-treated mice to kill H-2^d alloantigen target cells in culture. The frequency of CTL generation was normal or slightly reduced in TCDD-treated animals as determined by limiting dilution microculture. This indicated that TCDD had a direct toxic effect on CTL generation. With this conclusion in mind, we increased the frequency of CTL generation in spleens of animals given a 4 µg/kg dose of TCDD that produced gross splenomegaly in this organ. It can also be seen from the experiment that the cytotoxic activity per unit volume of spleen was usually subnormal when compared to control animals from TCDD-treated animals. Experiment 2 shows that CTL activity was affected by doses of 1

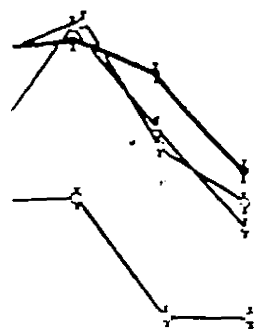


FIG. 3. Effect of TCDD treatment on the generation of CTL *in vivo* following alloantigen challenge. Control mice (●) or mice given 0.4 (Δ), 4 (○), or 40 (□) $\mu\text{g/kg}$ were immunized with irradiated allogeneic P-815 tumor cells as described under Materials and Methods. CTL activity was measured in spleen cell suspensions from groups of three to four mice at the times indicated.

CTL generation. The suppressive effect of TCDD treatment on the generation of CTL was analyzed *in vitro* using the same methods that have been employed to characterize the mechanism of immunosuppression in pregnant mice (24). Experiment 1 in Table II shows that TCDD treatment impaired the ability of spleen and PLN cells from TCDD-treated mice to generate CTL against the H-2^d alloantigen *in vitro* in tube cultures. The frequency of CTLp, however, was normal or slightly increased in TCDD-treated animals as determined by limiting dilution microcultures. This observation indicated that TCDD did not have a direct toxic effect on CTLp. In agreement with this conclusion was the marked increase in CTLp frequency seen in the spleens of animals given a 400 $\mu\text{g/kg}$ dose of TCDD that produced gross cellular depletion in this organ. It can also be appreciated from the experiment that the yield of cytotoxic activity per unit precursor was usually subnormal when lymphoid cells from TCDD-treated animals were studied. Experiment 2 shows that CTL generation was affected by doses of TCDD as low as

0.004 $\mu\text{g/kg}$. Furthermore, mixing cells from TCDD-treated mice with those of normal mice appeared to suppress the CTL response of the untreated cells.

Demonstration of suppressor cells in the thymus of TCDD-treated mice. The above observations suggest that TCDD may enhance suppressor cell activity. We examined this possibility by adding thymocytes from TCDD-treated or control mice to MLC cultures containing normal PLN cells and quantitated the number of CTL generated *in vitro*. The effect of different numbers of thymocytes on the generation of CTL from normal PLN is shown in Fig. 4. Dramatic suppression of CTL generation was observed when $4-8 \times 10^6$ thymocytes from mice given 0.4 $\mu\text{g/kg}$ and 0.04 $\mu\text{g/kg}$ TCDD were tested. Less suppression was seen when thymocytes from mice given 0.004 $\mu\text{g/kg}$ were used. Direct addition of TCDD up to doses of 0.025 μg to the cultures produced no suppression (data not shown). If the entire dose of TCDD given to a mouse was concentrated in the thymus (containing on the average 8×10^7 cells) then 4×10^6 thymocytes from a mouse given 0.4 $\mu\text{g/kg}$ would add 0.00050 μg TCDD to the culture. These data suggest that the suppression of CTL generation in TCDD-treated mice represented suppressor cell activity and was not a direct toxic effect of TCDD.

Discussion. The data in this paper show that the type of immunotoxicity produced by chronic exposure to TCDD depends on the dose. In agreement with the results of Faith *et al.* (25), we found reduced delayed hypersensitivity reactions as assessed by ear swelling at a 4 $\mu\text{g/kg}$ dose of TCDD that had little effect on the antibody response. The most interesting finding, however, was the exquisite sensitivity of the CTL response to TCDD. CTL generation was impaired *in vivo* at the 0.4 $\mu\text{g/kg}$ TCDD dose which produced no thymic atrophy or detectable impairment of antibody or delayed hypersensitivity responses. Impairment of CTL generation could be demonstrated *in vitro* with TCDD doses as low as 0.004 $\mu\text{g/kg}$ when washed spleen or lymph node cells from TCDD-treated animals were stimulated in tube cultures with alloantigen. Some insight into the cellular basis for

●) or mice treated with a solution with SRBC (A) or or of PFC was measured (number = SEM) is shown.

fore decided to direct investigations toward mechanism of impairment of TCDD.

Effect of TCDD on

VITRE REACTIONS

in ear thickness
azalone challenge^b
mean = SEM)

Control recipient

0.17 = 0.87
-0.83 = 1.35
-0.83 = 1.35
0 = 1.29

15 days later by injection
the right hind footpad to
number of measurements,
and nonsensitized animals

0.05

TABLE II. EFFECT OF TCDD ON THE FREQUENCY OF CTLp AND GENERATION OF CTL IN *IN VITRO* MASS MLC CULTURES

Treatment of mice	CTLp frequency ^a	Number of cells per culture ^b ($\times 10^6$)	Cytotoxic activity per culture ^c ($\text{Nod} \times 10^3$)	Estimated CTLp per culture	Yield CTL/CTLp
1 Control PLN	1/292	1	4635 $\pm$ 394	3425	1.35
0.4 μg PLN	1/211	1	2505 $\pm$ 317	4739	0.53
4 μg PLN	1/255	1	2617 $\pm$ 233	3922	0.67
40 μg PLN	1/150	1	4590 $\pm$ 197	6667	0.69
400 μg PLN	NC	NC	NC	NC	—
Control spleen	1/1660	2	4156 $\pm$ 351	1205	3.45
0.4 μg spleen	1/1561	2	1408 $\pm$ 97	1282	1.10
4 μg spleen	1/1469	2	2347 $\pm$ 170	1361	1.72
40 μg spleen	1/1607	2	1745 $\pm$ 104	1245	1.40
400 μg spleen	1/206	2	2007 $\pm$ 100	9709	0.21
2 Control PLN	1/1463	3	326 $\pm$ 18	2051	0.159
0.004 μg PLN	1/1006	3	184 $\pm$ 20	2982	0.062
0.04 μg PLN	1/1296	3	116 $\pm$ 17	2315	0.050
0.4 μg PLN	1/1229	3	130 $\pm$ 43	2441	0.053
Mixture	—	1.5 + 1.5	131 $\pm$ 21	2246	0.058

^a Frequency of precursors of CTL was determined using limiting dilution microcultures as described under Materials and Methods. NC = insufficient cells to perform culture.

^b The test lymph node or spleen cells from the treated mice were cultured with irradiated F₁ stimulator cells in MLC tube cultures and the resulting cytotoxic activity was measured after 5 days incubation. The number of initial CTLp per culture was calculated by multiplying cell number by the CTLp frequency.

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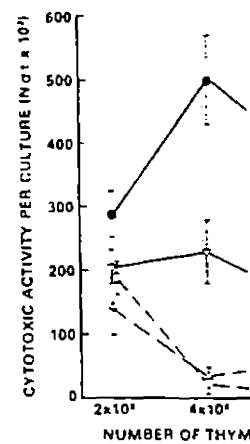


FIG. 4. Thymocytes from TCDD press CTL generation *in vitro*. The numbers of thymocytes from control mice given 0.004 (▽), 0.04 kg (□), and 0.04 kg (○) on the generation of CTL from 2 $\times$ 10⁴ shown. Five to six thymus donor group.

this impairment was obtained the frequency of CTLp in 1 microcultures. It was found that the frequency of CTLp was not reduced with TCDD. Thus, the generation *in vivo* and in tube represented a defect in the conversion of CTLp into CTL. This suggested that TCDD treated those cell types which regulate the generation of CTL in tube MLC. For example, TCDD could either reduce the amount of "help" available from T cells and accessory cells, or suppress T-cell activity that could be generated under *in vitro* conditions allowing cell-cell interaction (24). In thymus 1.2-positive cells from TCDD-treated mice (Fig. 1) with a reduction in T helper cell activity. On the other hand, studies have suggested that suppressor cells may be generated. Figure 3 shows the generation of CTL *in vitro* by P-815 tumor cells that had been needed to activate T helper cells. In TCDD-treated mice, the early times but seemed to be

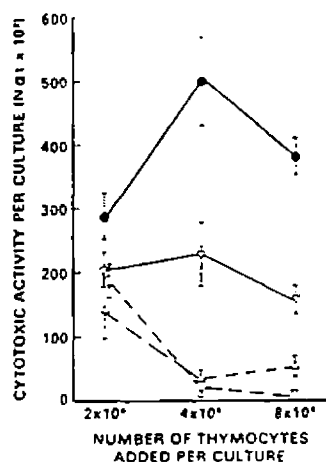


FIG. 4. Thymocytes from TCDD-treated mice suppress CTL generation *in vitro*. The effect of different numbers of thymocytes from control mice (●) or from mice given 0.004 (▽), 0.04 (○), or 0.4 (Δ) µg per kg on the generation of CTL from 2×10^4 normal PLN is shown. Five to six thymus donors were used per group.

this impairment was obtained by measuring the frequency of CTLp in limiting dilution microcultures. It was found that the frequency of CTLp was not reduced by treatment with TCDD. Thus, the impaired CTL generation *in vivo* and in tube MLC cultures represented a defect in the development of CTLp into CTL. This observation suggested that TCDD treatment affects those cell types which regulate the generation of CTL in tube MLC cultures. For example, TCDD could either reduce the amount of "help" available from T helper cells and accessory cells, or increase suppressor T-cell activity that can be detected under *in vitro* conditions allowing sufficient cell-cell interaction (24). The reduction in thy 1.2-positive cells in the PLN of TCDD-treated mice (Fig. 1) is consistent with a reduction in T helper subpopulations. On the other hand, several observations have suggested that suppressor cells may be generated. Figure 3 shows that the generation of CTL *in vitro* to allogeneic P-815 tumor cells that lack Ia antigens needed to activate T helper cells (26, 27) was normal in TCDD-treated animals at early times but seemed to "shut off" as the

response continued. Secondly, mixing lymph node cells from TCDD-treated animals with those from normal mice produced suppression (experiment 2, Table II). Thirdly, thymocytes obtained from mice treated with low doses of TCDD (0.004-0.4 µg per kg) suppressed the generation of CTL *in vitro*. This suppression could not be explained by the amount of TCDD that would be present in the thymocytes. Additional experiments have shown that the presence of thymic suppressor activity parallels the impairment of CTL generation in PLN and that both alterations ultimately disappear as the dose of TCDD is progressively reduced to 0.00004 µg/kg. ((31), manuscript in preparation). Taken together, these data indicate that TCDD may impair the generation of CTL through the generation of suppressor T cells.

The mechanism by which TCDD alters the thymocyte population to produce suppression is unclear. It is unlikely that the effects of low dose TCDD on thymus can be attributed to malnutrition or to an increase in cortisone levels in the body. Malnutrition tends to deplete cells in the murine spleen and thymus and to increase the cellular and humoral immune response (28). Our mice showed normal antibody responses and DTH but impaired CTL generation at TCDD doses that caused neither lymphoid organ atrophy (Fig. 1) nor significant weight loss (data not shown). Serum cortisol levels in TCDD-treated animals have been found to be normal by Vos *et al.* (10). TCDD is known to interact with a cytoplasmic binding protein or "receptor" and this "receptor" is present in the thymus as well as liver (29, 30). Indeed, in the rat, the highest concentration of "receptor" is found in thymus (30). Thus, it is possible that TCDD may act directly on thymus to alter T-cell differentiation or perhaps the life span of certain T-cell subpopulations thereby promoting the generation of suppressor T cells. Alternatively, the alteration in thymus could be mediated indirectly through effects of TCDD on hepatic enzyme activity (5, 31).

It can be seen from Table I that there was a slight (but not statistically significant) increase in oxazolone-induced ear swelling

after 0.4 $\mu\text{g/kg}$ TCDD and a similar effect on footpad swelling after 4 $\mu\text{g/kg}$ TCDD. The stimulatory effect of 0.4 and 4 $\mu\text{g/kg}$ TCDD on the background level of PFC has already been noted. We also found that these doses of TCDD increased the number of spleen colony-forming cells in bone marrow by 25–39% (data not shown) and thus it is possible that the 0.4 and 4 $\mu\text{g/kg}$ doses of TCDD have a small nonspecific stimulatory effect on a variety of different cell types in addition to suppressor cells that influence the immune system.

Our data showing that low doses of TCDD in mice impairs the generation of CTL may have some relevance to the effects of exposure to other HAHs. We have recently found that PCBs also impair CTL generation in mice (31), manuscript preparation). Bekesi *et al.* (32) have reported that Michigan residents exposed to PBBs in their diet show a reduced number of T cells in blood and impaired mitogen and MLC responses *in vitro*. The effects of TCDD on the murine immune system may therefore have some generality.

The biologic significance of the immunotoxicity detected *in vitro* following exposure to low doses of TCDD and HAHs is not yet known. Our data show that a sufficiently high dose of TCDD produces impairment of the antibody response to SRBC in mice. Cattle heavily exposed to PBBs develop mastitis and furunculosis due to bacterial infections (33), and TCDD-treated mice are reported to be more susceptible to *Salmonella* infection (14). However, we and others (25) find that lower doses of TCDD impair primarily the cellular arm of the immune response, and as shown in this paper, the generation of CTL appears to be particularly sensitive. One would predict from these observations that low doses of TCDD and related HAHs should increase susceptibility to only certain types of infectious agents. For example, CTL appear to play an important role in the defense against certain types of virus infection (34, 35). Susceptibility to duck hepatitis virus (15) and susceptibility to herpesvirus infection in mice (36) is increased by PCB exposure. Experiments are currently in progress

in our laboratory to determine if the increased thymic suppressor cell activity produced by very low TCDD doses in mice confers an increased susceptibility to lethal viral infections.

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