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EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN ON MACROPHAGE AND NATURAL KILLER CELL-MEDIATED CYTOTOXICITY IN MICE.

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IN PRESS

INTRODUCTION

2,3,7,8 Tetrachloro-dibenzo-p-dioxin (TCDD) is one of the most toxic chemicals known, occurring as a contaminant in the production of chlorinated phenols or compounds synthesized from them. One of the major toxic effects of TCDD in rodents is thymic atrophy (12, 15-17). Concomitantly, TCDD-treated animals show impaired cell-mediated immune reactivity (2, 15, 16) and decreased resistance to bacterial infection (14). The effect of TCDD on humoral antibody production has not been thoroughly investigated (2,15) but it appears so far that humoral responses are relatively spared by this agent (2,14-17).
The present investigation, prompted by the severe pollution with TCDD of a densily populated area (Seveso) near Milan (4,18), was designed to elucidate TCDD's effects on natural host defense mechanisms involving natural killer (NK) cells and macrophages. Both these mechanisms are currently taken to represent effective lines of resistance against infection and neoplasia (5,13).

MATERIALS AND METHODS

Mice. Male C57Bl/6 J mice (6 to 8 weeks old), obtained from Charles River Breeding Lab., Calco, Italy were employed throughout.

TCDD. TCDD (1,2, 6 and 30 mg/kg), obtained from Kor Isotopes, Cambridge, Mass., was injected i.p. in a volume of 0.25 ml of an acetone-corn oil mixture (1:6 v:v). Control mice were given the vehicle alone.

Target cells. The YAC-1 lymphoma, mKSATU5 (TU5) kidney line and SL2 lymphoma were obtained through the courtesy of Dr. R. Kiessling (Karolinska Institute, Stockholm, Sweden), Dr. J. Dean (Litton Bionetics, Kensington, Md., USA) and Dr. R.Evans (Chester Beatty Research Institute, Sutton, Surrey, England) respectively. The tumor lines were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (growth medium).

Macrophages. Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with 4 ml of basal medium Eagle (BME) (8,9). After centrifugation and resuspension in BME, differential cell counts were made with Turk's solution and macrophages were seeded in 1 ml BME in the wells of Costar Trays (3524 Costar, Cambridge, Mass.) for cytostasis experiments (10^6 macrophages/well) or in 0.3 ml BME in flat bottomed 6.4 mm culture wells (3596, Costar, Cambridge, Mass.) for cytolysis experiments ($5x10^4$, 10^5 or $2x10^5$ macrophages/well). After 2 h of incubation at 37°C, the wells were thoroughly washed with medium to remove non-adherent cells. More than 90% of the adherent cells were macrophages as assessed by marphology and phagocytosis of latex particles.

Macrophage-mediated cytolytic activity. The cytolytic activity of macrophages was evaluated in terms of ^{3}H-thymidine (^{3}H-TdR) release from prelabelled TU5 target cells, as recently described (10). Briefly, non confluent TU5 cultures in 25 cm^2 tissue culture flasks (Costar, Cambridge, Mass., USA) were incubated overnight with 5 ml of medium containing 0.5 uCi/ml of ^{3}H-TdR (6 Ci/mmole, Amersham Radiochemical Centre, Amersham, England). After exposure to 1 ml trypsin-EDTA for 5 min at 37°C, the cells were washed twice with 50 ml of growth medium. Target cells (10^4/0.3 ml) were incubated for 48 h with a range of attacker to target cell (A:T) ratios in flat bottomed 0.4 mm culture wells (3596, Costar, Cambridge, Mass., USA). At the end of the incubation, 0.1 ml of the supernatant was harvested and counted in a liquid scintillation spectrometer. Total incorporated radioactivity was calculated from tumor cells incubated with 1% sodium dodecyl sulfate (SDS) in water. Percentage isotope release was calculated as A/B x 100, where A is the isotope released in test samples and B is the SDS releasable radioactivity. Specific lysis was determined by subtracting the spontaneous release of tumor cells incubated in the absence of effectors, which under these conditions did not exceed 25% of the total incorporated radioactivity. A semilog plot of the specific cytotoxicity values versus the number of effector cells per sample was obtained and the number of cells required to give 20% specific lysis was arbitrarily defined as one

lytic unit (LU_{20}). This approach permitted a quantitative estimate of the total cytotoxic capacity per peritoneal cavity.

Macrophage-mediated cytostatic activity. Macrophage-mediated cytostatic activity was evaluated using SL2 lymphoma cells as targets as previously described (8,9). Briefly, SL2 lymphoma cells ($5x10^4$ in 1 ml growth medium) were seeded on macrophage monolayers (approximately $1x10^6$ cells) in the wells of Costar trays (3524, Costar, Cambridge, Mass.). Tumor growth was checked daily under an inverted microscope. After 48 h of incubation, lymphoma cells were vigorously resuspended with a Pasteur pipette, transferred to plastic tubes and washed. After resuspension in 1 ml growth medium, SL2 lymphoma cells were pulsed with 0.5 uCi ^{3}H-TdR (specific activity 1.9 Ci/mmole, Schwarz Mann, Orangeburg, N.Y.). Percentage inhibition of ^{3}H-TdR uptake was calculated as $(1-\frac{A}{B}) \times 100$, where A is the isotope uptake in the presence of macrophages and B is the isotope uptake by tumor cells alone.

Spleen NK activity. NK activity was evaluated using splenocytes as effectors and ^{51}Cr-labelled YAC-1 lymphoma cells as targets as previously described (11). Briefly, $5x10^4$ ^{51}Cr-labelled YAC-1 lymphoma cells were cultivated for 12 h with different numbers of splenocytes, the resulting A:T ratios ranging from 10:1 to 50:1. The percentage of specific cytotoxicity was calculated as /(release with effector cells-spontaneous release)/ total releasable radioactivity/ x 100.
Spontaneous release in the absence of effector cells was 0.6-1.5% per hour of incubation and total releasable radioactivity, assessed by osmotic lysis of target cells, was 75% of total isotope incorporated. The total cytotoxic capacity per spleen was calculated in terms of LU 33 as described above for macrophage-mediated lysis.

Statistical analysis. Five mice per experimental group were employed throughout and data obtained with 2-3 replicates per A:T ratio were analysed by Dunnett's test. Results are presented as mean $\pm$ S.E.

RESULTS

Table I shows the effect of TCDD on the number and cellular composition of PEC obtained from C57Bl/6 J mice. Stimulation of the peritoneal cavity with acetone-oil, employed as a vehicle in these studies, resulted in doubling of the number of PEC seven days after treatment, the number of nucleated cells slowly declining thereafter to reach normal values on day 47. Seven days after vehicle administration, greater percentage (34%) of polymorphs was observed than in unstimulated mice (1%). The polymorphonuclear infiltrate had returned to normal values by day 14. TCDD (1.2-30 μg/kg) had no effect on the cellular composition of PEC throughout the observation period (47 days). On the other hand, TCDD-treated mice showed a marked reduction (≃ 75% at the dose of 30 μg/kg) in total nucleated cell numbers on day 14 and 26 after treatment. No significant difference from control values was detected 47 days after treatment with TCDD.

The effect of TCDD on macrophage-mediated cytolytic and cytostatic activity was then investigated. Macrophage-mediated cytolysis was studied using TU5 target cells, which have been shown to be susceptible to the natural cytotoxic capacity of human and murine mononuclear phagocytes (10, Tagliabue et al., in preparation). Under these experimental conditions, TCDD did not significantly modify the spontaneous cytocidal activity of murine peritoneal macrophages tested at A:T ratios from 5:1 to 20:1 (Table II). However, when the total number of recovered LU20/mouse was calculated, TCDD-treated PEC showed a lower cytotoxic potential than controls on day 14 and 26, LU20 values of TCDD-treated macrophages being 6 and 2 compared to 14 and 8 for controls. Results presented in Table II were obtained in a 48 h ^{3}H-TdR release assay, but findings were similar when the incubation time was prolonged to 72 h (results not shown).

In a series of experiments the fucntional capacity of macrophages was evaluated by measuring their response to the activating stimulus endotoxin (13). As shown in Table III, in the presence of endotoxin macrophages showed increased cytocidal activity on tumor cells and treatment with

TCDD did not alter their capacity to respond to this activating stimulus.

The relationship between the cytostatic and cytolytic effects of macrophages on tumor cells is still not clear (10). Therefore it was considered of interest to evaluate the cytostatic activity of endotoxin-treated macrophages after administration of TCDD. As shown in Table IV, the growth inhibitory capacity of TCDD-treated macrophages, in terms of inhibition of ^{3}H-TdR uptake by SL2 lymphoma cells, was the same as controls.

In an effort to elucidate better the interaction of TCDD with natural cell-mediated host defense mechanisms, we studied the effect of this chemical (30 µg/kg i.p.) on NK cell activity, a cell-mediated reaction possibly representing an *in vitro* correlate of *in vivo* natural resistance to tumors and infections (5,13). Spleen NK activity per unit number of lymphoid cells was similar in TCDD-treated and control mice (Table V). However, since in agreement with previous data (12, 15-17), total spleen cell numbers were significantly reduced 7 and 14 days after TCDD, total spleen cytotoxic capacity expressed as LU 33/organ was less in mice exposed to this chemical. Table V shows cytotoxicity data obtained with YAC-1 tumor target cells 7 and 14 days after TCDD. Similar results were obtained on day 23 in one experiment using the RLO1 lymphoma (5) as target.

DISCUSSION

The results presented here indicate that TCDD does not affect the functional status of murine macrophages and NK cells as judged from their cytotoxic activity per unit number of effector cells. However, although macrophage-mediated and NK cell-mediated cytotoxicity on tumor cells was not modified on a per cell basis, fewer macrophages and spleen cells were recovered from TCDD-treated mice. Therefore the total cytotoxic capacity expressed as LU/organ was significantly reduced after exposure to TCDD but recovery was complete by day 47. This effect of TCDD on NK activity is similar ot that of irradiation (5,6) and of cytotoxic agents such as Adriamycin (11). In contrast, hydrocortisone, cyclophosphamide and azathioprine markedly suppress NK activity on a per cell basis too (5,6,11).

TCDD has been shown to result in thymic atrophy and in cell depletion of thymus dependent areas of lymphoid organs in rodents (2,15-17). In fact, thymus weight was consistently lower in the TCDD-treated mice (results not presented). The nature of NK cells has not been completely elucidated but recent evidence suggests that they may belong to the T cell lineage (5). Therefore the observed lack of inhibition of NK activity per unit number of lymphoid cells is somewhat suprising and suggests a relative resistance to this agent of the T cell subset (prethymic T cells) which has been proposed as effectors of NK activity (5).

Macrophages and NK cells derive from bone marrow precursors (3,13). Microscopic examination of the bone marrow of TCDD-treated animals revealed marked hypocellularity (12). Thus the bone marrow toxicity of TCDD might at least partially account for the smaller number of LU recovered from the spleen and peritoneal cavity of mice exposed to this agent.

There is evidence that macrophages and NK cells represent important mechanisms of in vivo resistance against infection and neoplasia (5,13).

Thus impairment of these effector mechanisms by TCDD might play a role in the decreased resistance to infection of mice exposed to this chemical (14) and in its carcinogenic and cocarcinogenic activity (1,7).

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Table I - Effect of TCDD on mouse peritoneal exudate cells (PEC).

Day after TCDD	TCDD (μg/kg)	PEC ($x10^6$)	% Macrophages	% Lymphocytes	% Polymorphs
-	-	2.1 ± 0.3	45	54	1
7	Vehicle	4.9 ± 0.3	37	29	34
	1.2	4.9 ± 0.2	35	32	33
	6	4.8 ± 0.5	34	25	41
	30	5.8 ± 0.9	39	26	35
14	Vehicle	4.1 ± 0.2	58	40	2
	1.2	2.0 ± 0.6	NT	NT	NT
	6	2.7 ± 0.7	NT	NT	NT
	30	1.3 ± 0.2*	54	45	1
26	Vehicle	3.1 ± 0.4	49	50	1
	1.2	1.3 ± 0.2*	NT	NT	NT
	6	1.3 ± 0.01*	NT	NT	NT
	30	1.0 ± 0.07*	52	45	3
47	Vehicle	2.7 ± 0.2	52	48	0
	30	2.0 ± 0.1	55	44	1

*$p < 0.05$ versus mice given vehicle

NT = not tested

Table II - Effect of TCDD on macrophage-mediated cytolytic activity.

ay after TCDD	TCDD (μg/kg)	Macrophages (x10^6)	% specific lysis 20:1	10:1	5:1	L U20/mouse
7	-	1.9	32.4 ± 2.3	24.5 ± 1.5	15.4 ± 0.3	24
	1.2	1.7	31.5 ± 0.5	20.3 ± 1.8	12.9 ± 1.9	19
	6	1.6	30.2 ± 2.4	27.6 ± 0.3	14.7 ± 0.5	21
	30	2.2	34.0 ± 1.5	28.4 ± 1.2	16.8 ± 1.4	36
14	-	2.4	20.5 ± 1.4	18.4 ± 2.8	14.8 ± 1.1	14
	30	0.7	22.4 ± 1.9	19.1 ± 0.3	16.7 ± 0.9	6
26	-	1.5	19.8 ± 0.3	17.4 ± 1.2	12.5 ± 0.8	8
	30	0.5	17.4 ± 2.4	16.9 ± 0.2	14.1 ± 1.6	2
47	-	1.4	22.7 ± 4.5	18.0 ± 3.5	NT	12
	30	1.1	37.9 ± 4.3	27.8 ± 2.9	NT	14

Table III - Effect of TCDD on in vitro stimulation of macrophage cytolytic activity by endotoxin.

Day after TCDD	Endotoxin [+] (10 µg/ml)	TCDD (µg/kg) -	1.2	6	30
7	-	32.4 ± 2.3	31.5 ± 0.5	30.2 ± 2.4	34.0 ± 1.5
	+	45.0 ± 1.6*	44.4 ± 2.7*	46.2 ± 1.2*	45.1 ± 4.5*
14	-	20.5 ± 1.4	NT	NT	22.4 ± 1.9
	+	31.4 ± 2.5*	NT	NT	34.3 ± 2.9*
26	-	19.8 ± 0.3	NT	NT	17.4 ± 2.4
	+	33.5 ± 1.4*	NT	NT	31.6 ± 1.6*

Results are % specific ^{3}H-TdR release from prelabelled mKSA TU5 target cells (mean ± S.D.). The A:T ratio was 20:1

[+]Salmonella typhosa 0901 (Difco)

*$p < 0.05$ versus samples without endotoxin

Table IV - Effect of TCDD on macrophage-mediated cytostatic activity

Day after TCDD	Endotoxin (10 µg/ml)	TCDD (µg/kg)+ -	30	6	1.2
7	-	30.5 ± 3.4	31.4 ± 0.9	25.3 ± 4.3	34.3 ± 2.1
	+	58.0 ± 0.4*	64.0 ± 3.2*	63.0 ± 3.1*	59.4 ± 3.4*
14	-	15.0 ± 1.2	19.2 ± 2.3	16.4 ± 0.6	13.9 ± 0.4
	+	66.3 ± 3.2*	73.4 ± 4.5*	75.3 ± 4.2*	64.6 ± 1.4*
21	-	24.3 ± 0.9	24 ± 1.5	18.3 ± 3.6	22.7 ± 3.2
	+	56.8 ± 2.3*	52.3 ± 1.3*	59.4 ± 1.9*	56.1 ± 1.7*

+Results are presented as % inhibition of ^{3}H-TdR uptake by SL2 lymphoma cells (mean ± S.E.)

*$p < 0.05$ versus samples without endotoxin

Table V - Effect of TCDD on spleen NK activity against YAC-1 lymphoma cells.

Day after TCDD	TCDD (30 µg/kg)	Spleen cells ($x10^6$)	% specific ^{51}Cr release 50:1	20:1	10:1	LU33/spleen
7	-	79.2 ± 11	42.9 ± 6.9	38.7 ± 3.5	24.2 ± 5.9	105
	+	52.6 ± 9*	43.6 ± 4.7	40.0 ± 5.6	31.3 ± 3.9	87
14	-	75.3 ± 5.5	39.2 ± 1.8	26.9 ± 0.6	20.4 ± 1.1	50
	+	48.1 ± 3*	36.2 ± 2.8	30.3 ± 4.3	20.0 ± 1.2	32

*$p < 0.05$ versus controls.

SUMMARY

C57Bl/6 J mice (6-8 weeks old) were given single i.p. doses (1.2, 6 and 30 µg/kg) of 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) and macrophage-mediated and natural killer (NK) cell-mediated cytotoxicity was evaluated at different times after treatment. Peritoneal macrophage cytolytic activity was measured as ^{3}H-thymidine release from prelabelled mKSATU5 target cells in a 48 h assay; macrophage-mediated cytostasis was assessed in terms of inhibition of ^{3}H-thymidine uptake by SL2 lymphoma cells. Spleen NK activity was measured using ^{51}Cr-labelled YAC-1 lymphoma cells as targets. TCDD did not modify spontaneous macrophage-mediated and NK cell-mediated cytotoxicity per unit number of effector cells nor did it affect the macrophages' capacity to express increased cytolytic and cytostatic activity in the presence of endotoxin. Lower numbers of peritoneal macrophages and splenocytes were recovered from TCDD treated mice. Thus the total numbers of lytic units recovered from animals exposed to TCDD were lower than controls. Impairment of these cellular effector mechanisms, due to cell loss rather than inhibition of function, might play a role in the lowered resistance to bacterial infection of mice given TCDD and in the carcinogenic and cocarcinogenic activity of this chemical.

RESUME

A des souris C57B1/6 (âgées de 6-8 semaines) on a administré des doses uniques en i.p. (1,2,6 et 30 ug/kg) de 2,3,7,8 tetrachloro-dibenzo-p-dioxine (TCDD), l'activité cytolytique des macrophages et des cellules natural killer a été est evaluée à différents moments aprés le traitement.

La libération de ^{3}H thymidine par des cellules cibles marquées m kSATU5, permet, aprés 48 h de meaurer l'activité cytolytique des macrophages. L'inhibition de la capture de ^{3}H thymidine par les cellules du lymphome SL2 permet de measurer la cytostase due aux macrophages. L'activité destructice de la rate est meaurée en utilisant des cellules du lymphome YAC-1 marquées par ^{51}Cr.

L'activité cytotoxique spontané, exprimée par unité de cellule effectrice, n'est pas modifieé par le TCDD, il n'y a pas inhibition de l'augmentation de l'activité cytolytique et de la cytostase en presence d'endotoxine. Un nombre plus fiable de macrophages peritonéaux et de splenocytes fut denombré sur des souris traitées au TCDD. Ainsi l'activité cytotoxique total était plus faible çhez animaux traité que chez les temoins. L'affaiblissement de ces mécanismes cellulaires serait du à une diminution de cellules plutot qu'à une inhibition de fonction, et pourrait jouer un role as la diminution de resistance aux affections bactériennes des souris ayant reçu du TCDD ainsi que dans l'activité carcinogene et cocarcinogene de la substance.

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