Carcinogenic and co-carcinogenic potential of 2,3,7,8 - tetrachlorodibenzodioxin in a host-mediated in vivo/in vitro assay

Th. Massa[1], B. Schlatterer[2] and P. Chandra[1]*

[1]Laboratorium für Molekularbiologie (ZBC), Klinikum der Johann-Wolfgang Goethe Universität, D-6000 Frankfurt/Main

[2]Umweltbundesamt, D-1000 Berlin

* Correspondence address: Prof. P. Chandra, Head of Molecular Biology, University Medical School, Theodor-Stern-kai 7, D-6000 Frankfurt 71

Abstract:

In an in vivo/in vitro assay system (Massa et al., 1990) we have detected the carcinogenic activity of 2,3,7,8-tetrachlorodibenzodioxin (TCDD). The carcinogenic potential measured in this system is concentration-dependent. Experiments with other carcinogenic compounds have revealed that TCDD at low doses can act as co-carcinogen.

Introduction:

We have developed a host-mediated assay system for the detection of the transforming action of chemical carcinogens on peritoneal macrophages. Directly as well as indirectly acting carcinogenic substances administered intraperitoneally to NMRI mice could be examined in this way. Resident macrophages were recovered by peritoneal lavage from treated and untreated mice and were cultured in soft agar. After 5-6 days normal and transformed cells could be distinguished. Statistical analysis comparing cells from 2,3,7,8-tetrachlorodibenzodioxin(TCDD)-treated animals with those from control mice proved that the test is positive at least on a significance level of 5% using the *t*-test. TCDD revealed a cell-transforming potential that showed a dose-dependent response in this host-mediated assay. The co-carcinogenic activity of TCDD was established in experiments with diphenylhydantoin. Low doses of diphenylhydantoin which did not exhibit any transforming potential in our system gained a high oncogenic potential by the simultaneous administration of low doses of TCDD which, as well had no transforming potential. Using monospecific antibodies to tumor necrosis factor α (TNF-α) we have found that TCDD suppresses the secretion of TNF-α. The experimental data reported here lead to the conclusion that TCDD has a carcinogenic as well as co-carcinogenic activity which, probably, involve the regulation of α-TNF secretion.

Material and methods:

Host mediated in vivo/in vitro assay

All animals were 8 week-old male mice of the inbred NMRI strain weighing approximately 30g. They were obtained from the central Breeding Laboratories of the University of Frankfurt and were maintained under specific pathogen free conditions. They had free access to standard dict (Altromin) and water. All chemicals were reagent grade and were dissolved for each experiment immediately before use. At day 0, 125µg lipopolysaccharid (Sigma, LPS, E. coli, serotype no. 0127: B8), dissolved in 1ml phosphate buffered saline (PBS), was asceptically administered to each mouse intraperitoneally. Substances to be examined were dissolved or emulsified in 1ml PBS containing 100ng 12-O-tetradecanoylphorbol-13-acetate, (TPA, Sigma, P-8139) or were dissolved in

0.2ml emulsion of 30% DMSO and 70% peanutoil emulsified in 0.8ml PBS . This cocktail was then administered at day 4 intraperitoneally. Control animals were given either PBS containing 100ng TPA, or PBS alone or 0.2ml of 30% DMSO/70% peanutoil in 0.8ml PBS.

Macrophages were collected by repeated peritoneal lavage four days later. The approximate yield of macrophages per mouse was 2-4 x 10^6. The suspended peritoneal macrophages were centrifuged at 600 x g for 10 min., resuspended and washed two times using 5ml of a cell culture medium (up-medium), containing 2/3 Hank's 199 (Seromed) with 10% foetal calf serum (FCS) and 1% penicilline/streptomycine and 1/3 conditioned Hank's medium (CSF). The production of conditioned Hank's medium is described below.

One half of the resuspended cells (2,5ml/mouse) was plated into a sterile culture bottle (one bottle/mouse, 50ml, 25cm^2, Nunc, No. 163371), supplemented by the same volume of up-medium. The bottles were incubated at 37°C in an atmosphere containing 5% CO_2. Nonadhering cells were removed 24 hours later by changing the medium. Metabolically acidified medium was replaced by conditioned medium within the first week. Then acidified medium was replaced by Hank's 199 *medium containing 10% FCS and 1% penicilline/streptomycine (1/1)*.

The second half of the suspension was transfered into soft agar as follows: One 24-well plate (Greiner, No. 662160) was used for each mouse. First 0.2ml underlayer was pipetted into each well. After its solidification 0.2ml upperlayer with the peritoneal macrophages at a concentration 2-4 x 10^5 cells/ml were added. The plates were then incubated at 37°C, in a water satured atmosphere containing 5% CO_2. 24 hours later 0.2ml conditioned medium was added per well. 5 to 6 days later the growth of cell colonies was evaluated. Underlayer: 0.6% agar (Difco, No. 0140-01); 59.4% Hank's 199 (1% streptomycine/penicilline); 20.0% fetal calf serum; 20.0% conditioned medium (contains CSF). Upperlayer: 50.0% up-medium (containing peritoneal macrophages); 29.4% Hank's 199 (1% streptomycine/penicilline); 0.6% agar (Difco, No. 0140-01); 20.0% fetal calf serum.

Production of conditioned medium

Mouse fibroblasts L-929 cells were grown in Hank's 199 medium containing 10% FCS and 1% streptomycine/penicilline. 2 x 10^6 cells (of 1 x 10^5 cell/ml) were given into a culture bottle (Nunc, 260ml, 80cm^2). They were cultured at 37°C after being equilibrated with a 5% CO_2 in air two days beyond the time reaching confluency. Supernatants of these cultures were collected. After centrifugation at 2000 x g for 10 mimutes the supernatant was filtered through a membrane filter (Millipore, No. SLGV 025BS). This filtrate was used as conditioned medium containing CSF.

Evaluation of the test:

The transforming potential of substances was characterized as described elsewhere (Massa et al., 1990); briefly, microscopically distinguishable clone sizes were divided into 10 classes (C0-C9). The frequencies of clone sizes of a defined class were determined for each 24-well plate. They were related to the cell number and were represented as indicated in Table 1-4. The microscopically determined frequency of the clone size of each class was multiplied with a factor considering the significance of the clone size. The resulting products of classes C0-C9 were summed up for each 24-well plate (result of one animal) separately. The median of each experimental group consisting of 5 to 6 animals and representing 5 to 6 24-well plates designates the transfroming potency of the respective substance.

Results and discussion:

In the host-mediated in vivo/in vitro assay with peritoneal macrophages, TCDD revealed a cell-transforming potential that showed a dose-dependent response (Table 1). The highest concentration of TCDD used was 10% of LD50 (LD50 of TCDD: 125µg/kg in mice) to decrease the acute toxicity as much as possible. We determined the lowest doses of TCDD and phenytoin, which were positive in the host-mediated assay. A co-administeration of a low-dose of TCDD with a low-dose diphenylhydantoin revealed a higher cell-transformation potential than the sum of the cell-transforming potentials when both substances are tested alone (Table 2). This suggests that the co-administration of low doses of TCDD and diphenylhydantoin has a higher carcinogenic potential than each substance itself.

It is interesting that the co-carcinogenic activity of TCDD is dependent on the time and schedule of application. Intraperitoneal application of TCDD two days before intraperitoneal injection of

Table 1: Transforming potential of various concentrations of TCDD.

Control/ carcinogenic substance	No of plate	Frequency of clon size								Transforming potential of various concentrations of TCDD
		Unspecific		Specific						
		5-9	10-14	15-19	20-24	25-29	30-49	50-69	70-99	>100
0.2ml DMSO/P.oil	1	10	4.0	1.3	1.3	–	–	–	–	–
+0.8ml PBS	2	3.3	–	–	–	–	–	–	–	–
+100ng TPA	3	6.0	–	–	–	–	–	–	–	–
i.p. per NMRI-mouse	4	–	–	–	–	–	–	–	–	–
	5	4.2	–	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	–	–	–
0.2ml DMSO/P.oil	1	6.0	0.5	0.5	0.5	–	–	–	–	–
+250ng TCDD	2	22	6.0	3.0	1.5	2.0	–	–	–	–
+0.8ml PBS	3	76	19	3.3	1.3	1.0	1.0	0.5	–	–
+100ng TPA	4	8.8	3.0	0.3	–	–	–	–	–	–
i.p. per NMRI-mouse	5	47	15	6.3	2.3	–	–	–	–	–
	6	25	9.0	5.0	1.8	0.5	0.3	0.8	–	–
0.2ml DMSO/P.oil	1	63	18	8.0	1.7	0.8	0.8	–	–	–
+15.6ng TCDD	2	10	4.3	2.3	–	–	–	–	–	–
+0.8ml PBS	3	125	32	5.8	–	–	–	–	–	–
+100ng TPA	4	110	54	25	2.0	1.0	1.0	–	–	–
i.p. per NMRI-mouse	5	33	19	17	1.0	–	–	–	–	–
	6	–	–	–	–	–	–	–	–	–
0.2ml DMSO/P.oil	1	140	32	1.3	1.0	–	–	–	–	–
+7.8ng TCDD	2	90	4.2	–	–	–	–	–	–	–
+0.8ml PBS	3	100	74	2.7	–	–	–	–	–	–
+100ng TPA	4	150	6.0	–	–	–	–	–	–	–
i.p per NMRI-mouse	5	75	4.0	1.2	–	–	–	–	–	–
	6	85	16	1.6	–	–	–	–	–	–

Table 2: Co-carcinogen effects of TCDD with phenytoin.

Control/ carcinogenic substance	No.of plate	Frequency of clon size								Transforming potential of TCDD and phenytoin
		Unspecific		Specific						
		5-9	10-14	15-19	20-24	25-29	30-49	50-69	70-99	>100
0.2ml DMSO/P.oil	1	200	4.0	0.5	–	–	–	–	–	–
+7.8ng TCDD	2	140	4.0	–	–	–	–	–	–	–
+0.8ml PBS	3	150	25	1.0	0.3	–	–	–	–	–
i.p. per NMRI-mouse	4	66	60	2.7	–	–	–	–	–	–
	5	83	4.2	–	–	–	–	–	–	–
0.2ml DMSO/P.oil	1	166	3.3	–	–	–	–	–	–	–
+0.8ml PBS	2	133	2.3	–	–	–	–	–	–	–
+100µg phenytoin	3	160	33	2.0	0.7	–	–	–	–	–
i.p. per NMRI-mouse	4	150	40	0.7	–	–	–	–	–	–
	5	30	–	–	–	–	–	–	–	–
0.2ml DMSO/P.oil	1	60	12	1.4	1.2	0.8	0.4	0.6	0.2	–
+7.8ng TCDD	2	66	6.6	0.7	0.3	–	–	–	–	–
+0.8ml PBS	3	70	13	1.0	–	0.3	–	–	–	–
+100µg phenytoin	4	50	13	1.0	0.3	–	–	–	0.3	–
i.p per NMRI-mouse	5	170	50	1.0	0.3	0.3	–	0.3	0.3	–

diphenylhydantoin and vice versa result in a clear lower cell-transformation potential than a co-administration of both carcinogens together (Table 3). This suggests that TCDD acts as a co-carcinogen and not as tumor promotor (Table 3 and 4) like TPA.The comparison of 7.8ng TCDD, equivalent with 24pmol TCDD (M_{TCDD}= 321.69) and 100ng TPA, equivalent with 270pmol TPA (M_{TPA}= 364.44) indicates TCDD as a very strong co-carcinogen.

Table 3: Cocarcinogen effects of TCDD/phenytoin, variation of injection-time.

Control/ carcinogenic substance	No of plate	Frequency of clon size									Transforming potential of TCDD/ phenytoin
		Unspecific		Specific							
		5-9	10-14	15-19	20-24	25-29	30-49	50-69	70-99	>100	
4.Tag	1	100	50	35	5.0	3.5	2.5	–	–	–	3.9
0.2ml DMSO/P.oil	2	30	2.0	0.8	–	0.4	–	–	–	–	
+7.8ng TCDD	3	5.0	–	–	–	–	–	–	–	–	
+0.8ml PBS	4	50	5.0	2.0	1.0	–	–	–	–	–	
+100µg phenytoin	5	100	33	6.7	5.0	2.3	1.7	1.7	1.0	1.0	
2.Tag											
0.2ml DMSO/P.oil	1	60	3.0	3.0	1.0	–	–	–	–	–	0.3
+0.8ml PBS											
+100µg phenytoin	2	3.0	1.0	–	–	–	–	–	–	–	
4.Tag	3	8.0	1.0	0.3	–	–	–	–	–	–	
0.2ml DMSO/P.oil											
+7.8ng TCDD	4	–	–	–	–	–	–	–	–	–	
+0.8ml PBS	5	125	25	2.0	–	–	–	–	–	–	
2.Tag											
0.2ml DMSO/P.oil	1	200	6.0	0.7	–	–	–	–	–	–	0.5
+7.8ng TCDD											
+0.8ml PBS	2	–	–	–	–	–	–	–	–	–	
4.Tag	3	300	–	–	–	–	–	–	–	–	
0.2ml DMSO/P.oil											
+0.8ml PBS	4	25	1.7	0.8	–	–	–	–	–	–	
+100µg phenytoin	5	22	1.5	0.5	–	–	–	–	–	–	

Table 4: Co-carcinogenic effect of TCDD compared with tumor promotor TPA.

Control/ carcinogenic substance	No.of plate	Frequency of clon size									Transforming potential of phenytoin,TPA ph./TPA, ph./TCDD
		Unspecific		Specific							
		5-9	10-14	15-19	20-24	25-29	30-49	50-69	70-99	>100	
1ml PBS	1	–	–	–	–	–	–	–	–	–	0.0
i.p. per NMRI-mouse	2	–	–	–	–	–	–	–	–	–	
	3	–	–	–	–	–	–	–	–	–	
	4	5.0	–	–	–	–	–	–	–	–	
	5	–	–	–	–	–	–	–	–	–	
1ml PBS	1	–	–	–	–	–	–	–	–	–	0.0
+100ng TPA	2	25	2.5	–	–	–	–	–	2.5	–	
i.p. per NMRI-mouse	3	–	–	–	–	–	–	–	–	–	
	4	4.0	–	–	–	–	–	–	–	–	
	5	10	–	–	–	–	–	–	–	–	
0.2ml DMSO/P.oil	1	21	2.9	0.3	0.3	0.3	–	–	–	–	0.0
+0.8ml PBS	2	2.0	0.7	–	–	–	–	–	–	–	
+100µg phenytoin	3	3.3	0.7	–	–	–	–	–	–	–	
i.p. per NMRI-mouse	4	5.5	–	–	–	–	–	–	–	–	
	5	–	–	–	–	–	–	–	–	–	
0.2ml DMSO/P.oil	1	233	17	3.3	–	–	–	–	–	–	1.0
+0.8ml PBS	2	111	18	2.7	0.6	–	–	–	–	–	
+100ng TPA	3	25	6.3	–	–	–	–	–	–	–	
+100µg phenytoin	4	150	12	1.0	–	–	–	–	–	–	
i.p per NMRI-mouse	5	56	2.8	–	0.6	–	–	–	–	–	
0.2ml DMSO/P.oil	1	100	50	35	5.0	3.5	2.5	–	–	–	3.9
+7.8ng TCDD	2	30	2.0	0.8	–	0.4	–	–	–	–	
+0.8ml PBS	3	5.0		–	–	–	–	–	–	–	
+100µg phenytoin	4	50	5.0	2.0	1.0	–	–	–	–	–	
i.p per NMRI-mouse	5	100	33	6.7	5.0	2.3	1.7	1.7	1.0	1.0	

References:
Massa Th, Gerber T, Pfaffenholz V, Chandra A, Schlatterer B, Chandra P A host mediated in vivo/in vitro assay with peritoneal murine macrophages for the detection of carcinogenic chemicals. J. Cancer Res. Clin. Oncol. (1990) Volume 116, in Press