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Dose-dependent promoting effect of polychlorinated biphenyls on enzyme-altered islands in livers of adult and weanling rats

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The effect of the technical mixture of polychlorinated biphenyls (PCBs) Clophen A 50 on the appearance of enzyme-altered islands initiated by diethylnitrosamine (DEN) in livers of 6 and 3 weeks old female Sprague-Dawley rats was studied. The loss of adenosine-5′-triphosphatase (ATPase), the emergence of gamma-glutamyltranspeptidase (GGTase), and the glycogen storage were used as histochemical markers. Islands were initiated by gastric intubation of 12 x 8 mg DEN/kg body weight/day in adults, or with 1 x 8 mg DEN/kg body weight in weanlings. Clophen A 50 alone initiated only few islands. A dose-dependent enhancement in number and area of islands by an additional treatment with Clophen A 50 of DEN-pretreated animals (2 – 100 mg/kg body weight/weekly, for 7 weeks) was observed in both age groups. In adults, doses between 2 and 100 mg/kg body weight increased number and area of ATPase-deficient islands 2 to 12-fold. In weanlings, application of 10 – 100 mg/kg body weight resulted in an increase of number and area up to 7- and 12-fold, respectively. No promoting effect was found with 2 mg/kg body weight compared to DEN-treated weanlings. The number of islands with coincidence of the three histochemical markers was enhanced dose-dependently in adults, and less marked also in weanlings after the application of the promoter.

Introduction

Polychlorinated biphenyls (PCBs)* are potent promoters in experimental hepatocarcinogenesis. Application of PCBs after treatment with 3′-methyl-4-dimethylaminoazobenzene or diethylnitrosamine (DEN) enhances the incidence of liver tumors in rats (1 – 5). PCBs promote hyperplastic nodules initiated by 2-acetylaminofluorene or DEN (5,6), and enhance the incidence of preneoplastic enzyme-altered islands in rat liver (7,8).

In rodent experimental hepatocarcinogenesis the promoting effect of PCBs and other chlorinated hydrocarbons on the development of enzyme-altered islands has been investigated at high dose levels (e.g., 7). Because of PCBs being ubiquitous and persistent environmental pollutants it is of special interest to gain information on the potential promoting potency of PCBs in low dose ranges and whether a no-effect level exists.

In view of risk evaluation in human carcinogenesis the aim of the present work was to study the dose-dependence of the promoting effect by Clophen A 50. The experiments have been performed with adult and weanling female Sprague-Dawley rats, in order to evaluate the most suitable group for this purpose.

Abbreviations: ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); DEN, diethylnitrosamine; GGTase, gamma-glutamyltranspeptidase (EC 2.3.2.2); PCBs, polychlorinated biphenyls.

Materials and methods

Chemicals

DEN p.a., 99% pure (Serva, Heidelberg, FRG), Clophen A 50, a technical mixture of polychlorinated biphenyls with a mean chlorine content of 54%. The main components are pentachlorobiphenyls (45%), tetrachlorobiphenyls (28%), and hexachlorobiphenyls (16%) (K.Wrabetz, personal communication, 1979). All other chemicals were of analytical grade (Merck, Darmstadt, FRG).

Animals

Female Sprague-Dawley rats (inbred strain, Neuherberg, FRG) 3 and 6 weeks old were used. Twelve groups of 4 rats each were taken from both age groups. Two rats each were housed together in Macrolon cages at 22°C on a 12:12 h light:dark cycle. They received a standard pellet diet (Altromin, Lage, FRG) and drinking water *ad libitum*.

Administration schedules

DEN was dissolved in water (2 mg/ml) immediately before use, and administered between 9:00 a.m. and 11:00 a.m. by gastric intubation. Weanlings received a single dose of 8 mg/kg body weight. Adults were treated with 8 mg/kg body weight/day for 12 consecutive days.

Clophen A 50 was dissolved in olive oil in concentrations of 1 – 50 mg/ml. Of the solutions 2 ml/kg body weight were given p.o. once weekly for 7 consecutive weeks beginning one week after the last application of DEN in the following concentrations: 2 mg/kg body weight (group 2), 10 mg/kg body weight (group 3), 25 mg/kg body weight (group 4), 50 mg/kg body weight (group 5), 100 mg/kg body weight (group 6). Another 5 groups (2a – 6a) received PCBs only, corresponding to the respective DEN-treated groups. Thereafter the animals were kept without further treatment until week 12. Controls were given 2 mg/kg body weight olive oil.

Histochemical demonstration of enzyme activities and glycogen

The experimental period comprised 12 weeks, beginning with the first application of DEN to adults, or the day of single treatment to weanlings, respectively. All animals were starved for 18 h at the end of the experiment and sacrificed by cervical dislocation. From two lobes of the livers serial cryostat sections of 8 μm thickness were prepared.

The sections were taken from 4 levels, 400 μm apart, for ATPase staining (9). Serial sections to the former were prepared from two levels for the demonstration of GGTase with gamma-glutamyl-alpha-naphthylamide as substrate and Fast Garnet GBC as coupling agent (10), and glycogen with the PAS reaction modified according to Hacker and Bannasch (1979, personal communication).

Eight sections from each animal were stained for ATPase and 4 sections each for GGTase and glycogen. The island number and area were determined using a MOP/AM 01 picture analyzer (Kontron, Eching, FRG). An area of 1 cm^2 per section was evaluated, corresponding to a total area of 8 cm^2 for ATPase and 4 cm^2 for GGTase and glycogen respectively. If not stated otherwise, the results refer to ATPase-deficient islands.

Statistical evaluation was performed by the Students t-test ($p \leq 0.01$).

Results

The body weight was not affected by the PCB-treatment, except in weanlings treated with 100 mg Clophen A 50 (Table I). In this group the body weight amounted to ~80% of that of controls (group 6a). The liver weights and the liver-to-body weight ratios were enhanced after application of Clophen A 50 (Table I). No macroscopical alterations of the livers were observed. The ATPase-activity of normal liver tissue was diminished around the terminal hepatic venole after the

Table I. Body weight (BWT), liver weight (LW), and liver-to-body weight ratio (LW % BWT) in weanling and adult female Sprague-Dawley rats after application of DEN[a] and Clophen A 50[b]

Group	Treatment Clophen A 50 (mg/kg body weight)	Weanlings BWT (g ± S.D.)	Weanlings LW (g ± S.D.)	Weanlings LW % BWT (± S.D.)	Adults BWT (g ± S.D.)	Adults LW (g ± S.D.)	Adults LW % BWT (± S.D.)
1	DEN	247 ± 13	6.9 ± 1	2.8 ± 0.3	260 ± 12	7.1 ± 0.3	2.8 ± 0.2
2	DEN 2	273 ± 13	7.4 ± 0.4	2.8 ± 0.1	261 ± 21	8.5 ± 0.6	3.2 ± 0.2
3	DEN 10	269 ± 6	7.9 ± 0.4	2.9 ± 0.1	268 ± 11	8.8 ± 0.8	3.3 ± 0.2
4	DEN 25	247 ± 5	8.5 ± 0.1	3.5 ± 0.1	261 ± 8	9.1 ± 0.4	3.5 ± 0.2
5	DEN 50	254 ± 15	8.3 ± 0.1	3.3 ± 0.1	264 ± 12	9.4 ± 0.8	3.6 ± 0.2
6	DEN 100	243 ± 12	9.3 ± 0.4	3.8 ± 0.2	251 ± 31	8.9 ± 2.0	3.5 ± 0.3
2a	2	278 ± 10	7.7 ± 0.2	2.8 ± 0.1	251 ± 11	7.7 ± 0.4	3.1 ± 0.3
3a	10	272 ± 8	7.3 ± 0.3	2.7 ± 0.2	250 ± 8	7.9 ± 0.4	3.3 ± 0.1
4a	25	247 ± 8	8.7 ± 0.7	3.5 ± 0.3	264 ± 16	9.5 ± 0.7	3.7 ± 0.2
5a	50	256 ± 12	7.6 ± 0.4	3.0 ± 0.2	278 ± 12	8.2 ± 0.3	3.0 ± 0.1
6a	100	226 ± 12	7.7 ± 0.7	3.4 ± 0.3	266 ± 10	9.5 ± 0.8	3.6 ± 0.2
Control (olive oil)		276 ± 22	7.5 ± 1.4	2.7 ± 0.3	257 ± 16	7.6 ± 0.4	3.0 ± 0.3

[a]Weanlings: 1 x 8 mg/kg body weight; adults: 12 x8 mg/kg body weight; 4 animals per group.
[b]Clophen A 50 was applied for 7 consecutive weeks. Further experimental details are given in Materials and methods.

Table II. Effect of Clophen A 50[a] on DEN[b]-initiated, enzyme-altered islands in livers of adult female Sprague-Dawley rats and percentage of coincidence of ATPase-deficiency, emergence of GGTase, and glycogen storage

Group	Treatment Clophen A 50 (mg/kg body weight)	Island number/cm² ATPase (−)	GGTase (+)	Glycogen (+)	Total area (mm²/cm²) ATPase (−)	GGTase (+)	Glycogen (+)	Coincidence
1	DEN −	13 ± 3	10 ± 1	4 ± 2	0.20 ± 0.05	0.08 ± 0.03	0.05 ± 0.02	11%
2	DEN 2	26 ± 11[c,d]	13 ± 2	6 ± 2	0.5 ± 0.2[c,d]	0.22 ± 0.16	0.12 ± 0.06	29%
3	DEN 10	50 ± 7[c,d]	15 ± 5	17 ± 4	1.0 ± 0.2[c,d]	0.45 ± 0.38	0.26 ± 0.09	33%
4	DEN 25	62 ± 9[c,d]	40 ± 5	28 ± 10	1.3 ± 0.2[c,d]	0.45 ± 0.12	0.41 ± 0.18	51%
5	DEN 50	130 ± 24[c,d]	82 ± 26	42 ± 9	1.5 ± 0.3[c,d]	0.63 ± 0.23	0.28 ± 0.11	46%
6	DEN 100	150 ± 28[c,d]	89 ± 19	85 ± 42	2.4 ± 1.4[c,d]	1.72 ± 1.00	1.70 ± 1.64	74%
2a	2	0.2 ± 0.2	2.0 ± 2.2	0.6 ± 0.4	0.003 ± 0.002	0.003 ± 0.003	0.004 ± 0.004	n.d.
3a	10	0.7 ± 0.3	1.5 ± 1.0	1.8 ± 0.4	0.01 ± 0.007	0.002 ± 0.002	0.004 ± 0.003	n.d.
4a	25	1.5 ± 0.3	1.3 ± 0.9	2.0 ± 0.1	0.17 ± 0.002	0.002 ± 0.001	0.008 ± 0.003	n.d.
5a	50	4.1 ± 1.1	1.6 ± 1.1	0.8 ± 0.6	0.10 ± 0.050	0.057 ± 0.060	0.020 ± 0.020	n.d.
6a	100	2.0 ± 0.5	2.3 ± 1.0	n.d.	0.05 ± 0.040	0.064 ± 0.080	n.d.	n.d.
Control (olive oil)		0.06 ± 0.03	0.2 ± 0.1	0.5 ± 0.5	0.008 ± 0.002	0.004 ± 0.001	0.002 ± 0.001	

[a]Adults: 12 x 8 mg/kg body weight; 4 animals per group.
[b]Clophen A 50 was applied for 7 consecutive weeks. Further experimental details are given in Materials and methods.
[c]Significantly different from group 1 ($p \leq 0.01$, t-test).
[d]Significantly different from the respective lower dosed group ($p \leq 0.01$, t-test).
n.d., not determined.

application of 50 and 100 mg Clophen A 50/kg body weight, respectively.

In all groups islands with loss of ATPase occurred more frequently than those with emergence of GGTase or glycogen storage.

Adults

The effects of various doses of Clophen A 50 on DEN-initiated islands in adults are summarized in Table II. The application of 12 x 8 mg DEN/kg body weight initiated about 13, 10 and 4 ATPase-deficient, GGT-positive and glycogen storing islands per cm², respectively (group 1). Clophen A 50 alone produced few enzyme-altered and glycogen storing islands (0.2 − 4.1 per cm², groups 2a − 6a).

A low number of islands was found in controls treated with olive oil only (0.06 − 0.5 per cm²). The promoting effect of Clophen A 50 was dose-dependent, and resulted in a significant increase of number and area of ATPase-deficient islands with all doses used as compared to the effect of DEN alone ($p \leq 0.01$, group 1). The application of 2 mg/kg body weight

Table III. Effect of Clophen A 50[a] on DEN[b]-initiated, enzyme-altered islands in livers of weanling female Sprague-Dawley rats and percentage of coincidence of ATPase-deficiency, emergence of GGTase, and glycogen storage

Group	Treatment	Island number/cm²			Total area (mm²/cm²)			Coincidence
	Clophen A 50 (mg/kg body weight)	ATPase (−)	GGTase (+)	Glycogen (+)	ATPase (−)	GGTase (+)	Glycogen (+)	
1	DEN –	12 ± 4	5 ± 2	5 ± 2	0.2 ± 0.01	0.03 ± 0.002	0.03 ± 0.01	18%
2	DEN 2	13 ± 2	4 ± 1	4 ± 1	0.1 ± 0.04	0.02 ± 0.007	0.02 ± 0.01	15%
3	DEN 10	29 ± 5[c,d]	7 ± 1	8 ± 3	0.4 ± 0.09[c,d]	0.07 ± 0.03	0.06 ± 0.04	13%
4	DEN 25	22 ± 4[c]	15 ± 2	9 ± 1	0.4 ± 0.05[c]	0.16 ± 0.02	0.08 ± 0.03	44%
5	DEN 50	87 ± 13[c,d]	39 ± 7	33 ± 6	1.5 ± 0.60[c,d]	0.97 ± 0.30	0.53 ± 0.17	36%
6	DEN 100	89 ± 10[c]	60 ± 13	31 ± 3	2.3 ± 0.50[c]	0.90 ± 0.30	0.40 ± 0.07	67%
2a	2	0.4 ± 0.3	0	0	0.004 ± 0.004	0	0	n.d
3a	10	0.4 ± 0.3	0	0	0.003 ± 0.003	0	0	n.d.
4a	25	0.7 ± 0.5	1.6 ± 1.3	2.5 ± 0.6	0.005 ± 0.005	0.001 ± 0.001	0.010 ± 0.004	n.d.
5a	50	2.2 ± 0.4	0.6 ± 0.4	0.9 ± 0.3	0.02 ± 0.007	0.006 ± 0.004	0.005 ± 0.002	n.d.
6a	100	2.1 ± 0.9	1.7 ± 0.4	0.8 ± 0.5	0.02 ± 0.010	0.030 ± 0.010	0.012 ± 0.009	n.d.
Control (olive oil)		0.3 ± 0.1	0	0	0.005 ± 0.001	0	0	

[a]Weanlings: 1 x 8 mg/kg body weight; 4 animals per group.
[b]Clophen A 50 was applied for 7 consecutive weeks. Further experimental details are given in Materials and methods.
[c]Significantly different from group 1 ($p \leq 0.01$, t-test).
[d]Significantly different from the respective lower dosed group ($p \leq 0.01$, t-test).
n.d., not determined.

once a week for 7 consecutive weeks caused an ~2-fold increase in number and area (group 2), 7 x 10 mg raised number 4-fold, and area 5-fold (group 3), 7 x 25 mg increased number 5-fold, and area 7-fold (group 4). 7 x 50 and 7 x 100 mg enhanced number 10- and 12-fold, and area 8- and 12-fold, respectively (groups 5, 6).

There was a dose-dependent increase in number and total area of GGTase-positive and glycogen storing islands in the dose range from 2 to 100 mg Clophen A 50/kg (group 1–6). An ~20-fold increase in the number of glycogen storing islands and a 20- to 30-fold enhancement of total area of GGTase-positive and glycogen storing islands was observed at the highest dose of 100 mg/kg (group 6). The percentage of islands with coincidence of the three markers augmented with increasing doses of Clophen A 50 from 11% in DEN treated animals up to 74% after additional application of 100 mg Clophen A 50/kg body weight (groups 1–6).

Weanlings

The results for weanlings are shown in Table III. The application of DEN (1 x 8 mg/kg body weight) or Clophen A 50 initiated ATPase-deficient islands in the same range as in adults (12 islands per cm², group 1, and 0.4–2.2 islands per cm², groups 2a–6a). The number of GGTase-positive and glycogen storing islands with DEN alone amounted to ~5 per cm² (group 1). In controls treated with olive oil, few ATPase-deficient islands only emerged (0.3 ± 0.1 per cm²).

The application of 2 mg/kg body weight Clophen A 50 to DEN-pretreated rats was ineffective. Administration of higher doses resulted in a significant and dose-dependent enhancement of number and area of ATPase-deficient islands compared to groups 1 and 2. Ten and 25 mg enhanced number and area about two-fold (groups 3 and 4), 50 and 100 mg caused a 7-fold increase in number and augmented area 8-fold and 12-fold, respectively (groups 5 and 6). The increase in number of GGTase-positive and glycogen storing islands was in the same range as that of ATPase-deficient ones. The total area was enhanced up to 30-fold in the animals treated with 50 or 100 mg/kg.

Without DEN, GGTase-positive and glycogen storing islands were found only in animals treated with 25, 50 or 100 mg/kg body weight Clophen A 50, respectively (groups 4a–6a). The percentage of islands with coincidence of the three markers amounted to 18% in DEN-treated rats and 67% in those treated with DEN and 100 mg Clophen A 50 (groups 1–6).

Discussion

A number of compounds has been identified as promoters in experimental hepatocarcinogenesis (e.g., 11). In these experiments preferentially high doses of the respective promoting agents have been used, which do not reflect the actual exposure of man due to environmental contamination. Little information on the role of promoters in human tumorigenesis is available until now. The evaluation of dose-response relationships of suspected promoters, especially in low dose ranges, may be an important contribution to human risk assessment. Recently a positive dose-effect relationship has been demonstrated in rat liver for the tumor promoter phenobarbital as indicated by the enhancement of the number of preneoplastic islands, and likewise in mouse liver with dieldrin, which enhanced dose-dependently the tumor incidence (12,13).

In our experiments the evaluation of the dose-dependence of the promoting capacity of PCBs on preneoplastic islands revealed that doses of 2–100 mg/kg body weight, applied once weekly, are effective in both age groups, with one exception: in weanlings a non-effect-level was observed at a dose of 2 mg Clophen A 50, which failed to cause a significant enhancement of island number and area compared to DEN-treated animals within the experimental period of 12 weeks.

Treatment with Clophen A 50 enhanced mainly the number of preneoplastic lesions as demonstrated earlier for phenobarbital (14,15). This can be explained by the existence of initiated dormant cells which proliferate only after an additional promoting stimulus (16).

Although the present results may well be explained in terms of a promoting activity of PCBs, the possibility of a weak initiating potency of PCBs cannot be excluded definitely, with regard to the results obtained with Clophen A 50 alone (Tables II, III, groups 2a – 6a). On the other hand, a very small number of islands was observed in the livers of olive oil-treated animals too, indicating the existence of spontaneously initiated cells. The dose-dependent increase in the groups treated with PCBs only might be explained in analogy to the DEN-treated animals as a dose-dependent promoting effect on such initiated but dormant cells.

The PCB-treatment causes a dose-dependent increase in the simultaneous occurrence of the three histochemical markers in adults, and less marked also in weanlings, indicating a qualitative and quantitative influence on marker expression.

The evaluation of a dose-response relationship and of a no-effect level is important since PCBs are ubiquitous environmental contaminants. Under normal conditions the amounts ingested by humans are generally much lower than those applied in the present experiments. An overall estimate in the order of 0.005 – 0.010 mg/day PCB dietary intake for the general public seems reasonable (17). In the case of the Yusho mass food poisoning however in 1968, the daily PCB intake of the patients amounted to ~0.03 – 0.15 mg/kg body weight of Kanechlor 400 (mean chlorine content ~48%) for 71 days (18). This exposure is in the range of the lowest dose in our experiments (average daily intake of 0.29 mg/kg body weight), which is still effective in adult rats. The Yusho patients suffered from severe toxic effects mainly in the skin. Their livers did not show any abnormalities in gross appearance or in routine function tests. An epidemiologic study in 1979 showed that neoplasms account for the largest number of deaths in Yusho patients although the data presently available are not sufficient for establishing a causal nexus (19).

Considering the data presented, under normal conditions a promoting effect in humans due to PCB exposure seems unlikely. However, the highly lipophilic PCBs accumulate in the adipose tissue, amounting to 1 – 2 mg/kg adipose tissue in humans (17). This source may be a factor of hazard in periods of rapid emaciation.

Various experimental procedures have been proposed as test systems for promoters in hepatocarcinogenesis (6,20 – 24). Our experimental model seems to be especially suitable for a screening test system for suspected promoters, because of the high sensitivity of the rats of both age groups, and a minimum in experimental manipulation. The use of weanlings offers the advantage, that a single and low dose of DEN is sufficient for the induction of a considerable number of islands, thus a promoting effect by the initiator can be excluded.

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