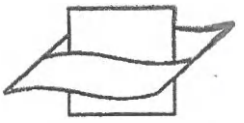
Mutagenesis vol.11 no.4 pp.383–389, 1996

PCBs do not induce DNA breakage *in vitro* in human lymphocytes



K.Belpaeme[1,3], K.Delbeke[2], L.Zhu[2] and M.Kirsch-Volders[1]

Laboratories of [1]Anthropogenetics and [2]Ecology, Free University Brussels (VUB), Pleinlaan 2, 1050 Brussels, Belgium

[3]To whom correspondence should be addressed

Polychlorinated biphenyls (PCBs) are classified by IARC as non-mutagenic *in vivo*. However, despite almost 20 years of research, their mutagenicity *in vitro* is still debatable. In this work the *in vitro* cytochalasin B micronucleus test and the alkaline comet assay applied to human lymphocytes were used to study the genotoxicity of a PCB. PCB77, at concentrations ranging from 0.01 to 100 µg/ml, was used in whole blood or isolated lymphocyte cultures, with final dimethylsulfoxide percentages of 0.5–2%. In the micronucleus test lymphocytes were exposed for 48 h, and in the alkaline comet assay for 30 min, 1 h and 3 h. No increases of single strand breaks or micronucleus frequencies was found, in contrast to previously reported data. Our data indicate that PCB77 has no clastogenic properties in human lymphocytes.

Introduction

Polychlorinated biphenyls (PCBs) used to be widely used industrial compounds. Depending on the number and the position of the chlorine-substituents, 209 different congeners can be distinguished. These congeners are divided in nine isomeric groups and decachlorobiphenyl. Their empirical formula is $C_{12}H_{10-n}Cl_n$ (n = 1–10).

Commercial PCBs were manufactured by several companies and marketed worldwide under a variety of trade names. The best known are Aroclor (Monsanto, USA and UK), Clophen (Bayer, Germany), Phenoclor and Pyralene (Prodelec, France), Kanechlor (Kanegafuchi, Japan), Santotherm (Mitsubishi-Monsanto, Japan), and Fenclor (Caifaro, Italy). Between 1930 and 1970 PCBs were used for many different purposes, due to their useful physical properties (Safe, 1992). Since then applications and production of PCBs have been strictly limited or even forbidden. However, even today PCBs can still be found in almost every component of the global ecosystem, including air, water, sediments, fish, and human adipose tissue, milk and serum (De Voogt *et al.*, 1990). The proven toxicity (developmental and reproductive toxicity, dermal toxicity, endocrine effects, hepatotoxicity, carcinogenesis, induction of diverse phase I and phase II drug metabolizing enzymes) has stimulated many researchers to study these compounds.

There are clear data to indicate that commercial PCBs can be considered non-genotoxic. The position with regard to the planar congener, the 3,3′,4,4′-derivate (PCB77), is, however, still unclear. IARC classified all PCBs as non-mutagenic *in vivo*. A review of the main results on mutagenicity of PCBs is given in Table I. This list shows 23 negative and eight positive results for the mutagenecity of PCBs. Some of the differences may be explained by the fact that some tests were performed with PCB mixtures while others were performed with individual PCB congeners. It is important to note that six of the positive results were obtained using individual congeners. On the other hand, these seemingly contradictory results may also be due to the use of different endpoints and different organisms. Many of the positive data on congeners used poorly defined systems for which limited data were provided and which were difficult to interpret (e.g. the pigeon embryo study).

It is difficult to draw general conclusions from the above reports, but there is also evidence that PCB metabolites are capable of interacting with DNA with the potential to be genotoxic (Butterworth *et al.*, 1995).

Sargent *et al.* (1989) observed a dose-related chromosome breakage in human lymphocytes exposed *in vitro* to 10^{-1}–10^{-4} µg/ml of PCB77 (3,3′,4,4′-tetrachlorobiphenyl). They also noted the synergetic effect of PCB77 with the non-clastogenic PCB congener PCB52 (2,2′,5,5′-tetrachlorobiphenyl). The genetic endpoints used were chromosome aberrations and sister chromatid exchange. This study was aimed at establishing whether the proposed clastogenic potential of PCB77 could also be detected in the micronucleus test and/or the alkaline single cell gel electrophoresis or comet assay.

Materials and methods

Chemicals

Pure PCB77 powder was purchased from Schmidt BV (Amsterdam, The Netherlands). It was dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany). The final concentrations of DMSO in the cultures were 0.5 and 2%.

Ethidium bromide, Giemsa, EDTA, Tris(hydroxymethyl)–aminomethane, sodium hydroxide, sodium chloride and ethyl methanesulfonate (EMS) were obtained from Merck. Low- and normal-melting-point (LMP and NMP respectively) agarose were purchased from Gibco BRL (MD, USA). *N*-Lauroylsarcosine and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO). Mitomycin C was obtained from Janssen Chemica (Beerse, Belgium).

Lymphocyte cultures

Human peripheral blood was drawn from healthy, non-smoking donors <36 years old, in heparin-containing syringes. Four donors were used: donors 1, 2, 3 or 4.

For isolated lymphocyte cultures the erythrocytes were removed using leukocyte cell separation tubes (Becton Dickinson, Lincoln Park, NJ) and cells were washed twice with phosphate buffer saline (PBS: pH 7); for whole blood, 5 ml cultures were established containing 0.4 ml whole blood; purified lymphocyte cultures were set up as 2 ml cultures in a concentration of 0.5 × 10^6 lymphocytes/ml in glass tubes.

All cultures were set up in duplicate in HAM's F-10 medium (Gibco) supplemented with 15% fetal calf serum (Gibco) and 2% phytohaemagglutinin (Wellcome, Dartford, UK), and were incubated at 37°C for 72 h.

Cells from purified lymphocyte cultures were applied directly to slides using a cytospin centrifuge (Shandon, Pittsburg, PA, USA), according to Vian *et al.* (1993). All slides were fixed in 100% methanol and stained with 5% Giemsa (Merck) in Sorensen buffer (pH 6.8). (Note: in experimental procedures with PCBs only glass materials should be used.)

Treatment

For the micronucleus test the cells were treated with the test chemical, without S9, from 24 h after stimulation until the end of culturing; cytochalasin B was added 44 h starting stimulation at a concentration of 6 µg/ml. For the comet assay cells were treated with the test chemical, without S9, starting 24 h after stimulation, for 30 min, 1 h or 3 h.

Table I. List of main results on mutagenicity of PCBs

Genetic endpoint	Test system	Mutagenicity PCBs (congener or mixture)	Reference
In vitro			
Reversion assay	*Salmonella*	– (mixture)	Silberhorn *et al.* (1990)
= Ames test	*Salmonella*	– (Aroclor 1254)	Schoeny *et al.* (1979)
	Salmonella	– (congener)	Hsia *et al.* (1978)
	Salmonella	+ (congener)	Wyndham *et al.* (1976)
	Salmonella	– (mixture)	Shahin *et al.* (1979)
	bacteria	– (Aroclor 1254)	Natarajan and Obe (1986)
	Salmonella	– (Aroclor 1254)	Dunkel *et al.* (1984)
	Salmonella	– (Aroclor 1254)	Zeiger *et al.* (1988)
	Salmonella	– (congeners)	Schoeny (1982)
Gene mutations	*Salmonella*	– (mixture)	Millisher *et al.* (1979)
	Salmonella	– (mixture)	Haworth *et al.* (1983)
	E.coli	– (congener)	Dunkel *et al.* (1984)
Chromosome aberrations	human lymphocytes	– (mixture)	Hoopengarner *et al.* (1972)
	human lymphocytes	+ (congener)	Sargent *et al.* (1989)
Single stranded breaks	mammalian cell cultures	+ (congener)	Stadnicki *et al.* (1979)
Cell transformation	SHE cells	– (mixture)	Pienta (1980)
UDS, DNA repair	CHO	+ (congener)	Wong *et al.* (1979)
In vivo			
Chromosome aberrations	pigeon embryos	+ (Aroclor 1254)	Peakall *et al.* (1972)
	Drosophila	– (mixture)	Nilsson and Ramel (1974)
	rat bone marrow	– (Aroclors)	Green *et al.* (1975b)
	spermatoid cells	– (Aroclor 1254)	Garthoff *et al.* (1977)
	fish erythrocytes	+ (Aroclor 1254)	Al-Sabti (1985b, 1986b)
Micronuclei	rat bone marrow	– (mixture)	Bruce and Heddle (1979)
	newt larvae	– (Aroclor 1254)	Fernandez *et al.* (1989)
	mouse	– (mixture)	Watanabe *et al.* (1982)
Recombinogenesis	*Drosophila*	+ (congener)	Butterworth *et al.* (1995)
Dominant lethal test	rat	– (Aroclor)	Green *et al.* (1975a)
Chromosome damage	rat bone marrow	+ (congener)	Meisner *et al.* (1992)
^{32}P post labelling	rat liver, kidney, lung	– (Aroclor 1254)	Nath *et al.* (1991)
	rat liver	– (Aroclor 1254)	Chadwick *et al.* (1993)
Clastogenicity	bone marrow	– (mixture)	Odashima (1976)

Table II. Results of the comet assay (in μm) after *in vitro* treatment of erythrocytes during 30 min, 1 h or 3 h

Number of experiment	Treatment time	Treatment	*n*	Tail length		Tail moment	
				Median	Mean ± SD	Median	Mean ± SD
1	1 h	control	44	24.91	34.17 ± 26.41	3.84	5.36 ± 4.93
(donor 1)		1 mM EMS	50	138.57	137.22 ± 28.54***	75.26	75.16 ± 21.53***
		1 μg PCB77/ml	45	23.91	29.02 ± 16.07	4.49	4.93 ± 3.38
		10 μg PCB77/ml	39	22.01	29.23 ± 23.76*	3.20	4.89 ± 5.92
2	1 h	1 mM EMS	48	63.08	67.59 ± 19.10***	34.00	35.95 ± 15.46***
(donor 1)		10 μg PCB77/ml	50	18.77	28.76 ± 22.31	9.76	14.34 ± 10.64
		25 μg PCB77/ml	48	21.23	30.71 ± 18.85*	11.08	14.21 ± 9.24*
	3 h	control	50	16.84	20.72 ± 12.89	8.85	10.22 ± 6.83
		10 μg PCB77/ml	48	19.42	27.19 ± 18.98	9.76	13.09 ± 8.42
		25 μg PCB77/ml	50	17.81	24.76 ± 16.64	8.82	12.01 ± 8.69
3	30 min	10 μg PCB77/ml	50	18.61	24.14 ± 15.69	6.62	7.78 ± 5.84
(donor 3)		25 μg PCB77/ml	50	17.32	22.70 ± 12.06	6.62	7.26 ± 4.54
	1 h	control	47	18.13	25.40 ± 17.98	7.21	9.79 ± 7.11
		1 mM EMS	48	56.81	57.00 ± 18.20***	21.94	28.32 ± 30.24***
		10 μg PCB77/ml	44	18.61	27.89 ± 19.42	7.40	11.99 ± 11.46
		25 μg PCB77/ml	49	16.19	20.39 ± 13.71	5.11	5.67 ± 3.39*
	3 h	control	48	18.32	28.34 ± 19.11	5.23	8.96 ± 6.71
		10 μg PCB77/ml	49	14.09	25.96 ± 22.13*	6.20	10.17 ± 9.27
		25 μg PCB77/ml	50	14.90	20.22 ± 14.10*	5.01	7.00 ± 5.74

The DMSO percentage in the cultures was 0.5%. Tail length was measured from the centre of the nucleus to the end of the tail. Tail moment = tail length × relative DNA content of the tail. *n* = number of cells measured; EMS = ethyl methanesulfonate.
*$P < 0.01$;*** $P < 0.0001$ compared with the control (Mann–Whitney *U*-test).

PCB77 was dissolved in DMSO before being added to the cultures. In some experiments we used saturation concentrations, i.e. the highest concentration where PCB77 was still soluble in DMSO (= 5 mg/ml DMSO).

Alkaline comet assay

The alkaline comet assay was performed under yellow light as described by Singh *et al.* (1988) with some modifications. LMP and NMP agarose were

Table III. PCB77 concentrations measured in the cultures after treatment

Initial concentration in culture (μg/ml)	Concentration measured in culture after 1 h treatment (μg/ml)	Concentration measured in culture after 3 h treatment (μg/ml)
10	5.1752	2.1024
25	5.0858	2.1350

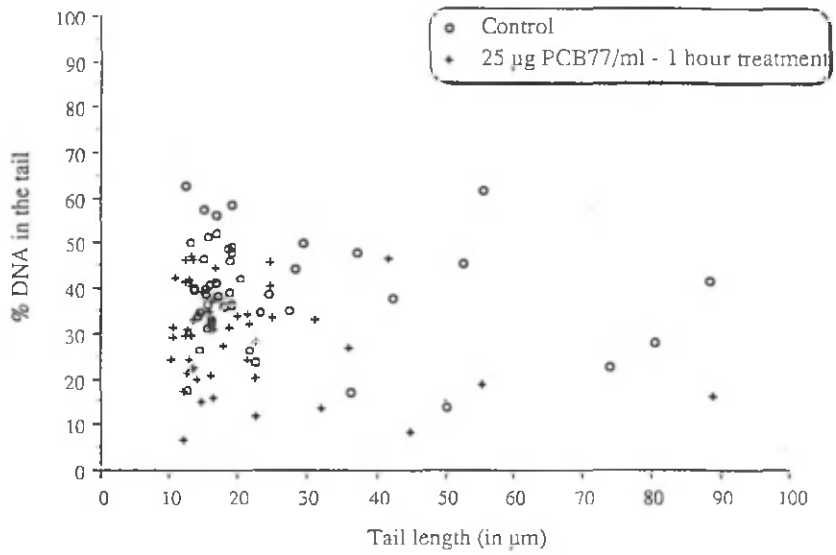


Fig. 1. Percentage DNA in the tail as a function of the tail length. In each cultures ~50 cells were measured. PCB77 = 3,3',4,4'-tetrachlorabiphenyl.

each prepared in calcium- and magnesium-free PBS. Fully frosted slides (Richardson Supply, UK) were covered with 1% NMP agarose and a no. 1 coverslip (Vel, Germany) was added. The agarose was allowed to solidify at room temperature and then removed by scraping with a coverslip. When the slides were completely dry they were covered with 300 μl NMP agarose (0.5%) and a no. 1 coverslip, and placed on ice for a further 10 min to let the agarose solidify. The coverslip was then removed and 10 μl of the cultured blood pellet (5000–50 000 cells) was mixed with 100 μl of 0.6% LMP agarose and carefully layered on top, covered again with a coverslip and put on ice to solidify. Next, a top layer of LMP agarose (100 μl) was added and the agarose allowed to solidify on ice for 10 min. The coverslips were removed and the cells were put in cold, freshly made lysing solution [2.5 M NaCl, 10 mM Tris, 100 mM EDTA disodium salt and 1% (w/v) *N*-lauroylsarcosine, pH 10, supplemented with 10% (v/v) DMSO and 1% (v/v) Triton X-100 before use] for at least 1 h at 4°C. For electrophoresis the slides were placed in a horizontal electrophoresis box filled with freshly made alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >12) for 40 min at 18°C, to allow for the unwinding of DNA. Electrophoresis (300 mA, 0.7 V/cm) was performed in the same buffer for 20 min at 18°C. The slides were removed from the buffer and the excess alkali was neutralized with 1 ml of 0.4 M Tris (pH 7.5) three times during 5 min. The slides were stored with a coverslip in a moist chamber at 4°C until analysis.

Analysis for the comet assay

Before analysis the cells were stained with 100 μl of ethidium bromide (20 μg/ml water) for 10 min, rinsed with distilled water and kept in a moist chamber at 4°C. For analysis, images from a Zeiss fluorescence microscope (magnification ×300) were captured with an air-cooled camera (Photonic Science) type coolview on a frame grabber type DT 2855. Fifty non-overlapping images were selected randomly on the slides. The DNA content of the image was calculated from the sum of light intensities of all pixels in that area. For each separate image the background intensity was subtracted from the image intensity. The comet length was measured by defining manually on the screen the centre of the nucleus, the leading edge of the nucleus and the end of the tail. All the data were transferred to a Macintosh Quadra 650 for analysis. The features extracted from the measurements were essentially the 'DNA content', defined as the total fluorescence associated with an image; the 'tail length', measured from the centre of the nucleus to the end of the tail; and the 'tail moment', defined as the fraction of DNA in the tail (DNA content in tail/DNA content of the image) multiplied by the tail length.

Statistical differences between controls and treated samples were determined with the non-parametric Mann–Whitney *U*-test, which is based on the median and the distribution of the measurements.

Analysis of the micronucleus tests

For each culture, 1000 binucleated lymphocytes (CB) were analysed for the presence of one, two or more MN and expressed per thousand micronucleated CB (MNCB).

Also, the percentage of CB polynucleated cells (polyN), mitotic cells and mononucleated cells containing MN were recorded. As a measure for toxicity or cell cycle delay the relative division index, given by ((CB + 2polyN)/*n* from the treated samples)/((CB + 2polyN)/*n* from the control)×100, was used.

All slides were coded and analysed with a Zeiss or Leitz microscope at magnifications of ×1250 and ×1000 respectively.

Any statistical differences between controls and treated samples were determined with the chi-square test.

Measurement of the PCB77 concentrations in culture

The PCB77 concentration in a 1 ml sample of the cultures was determined by an electron capture gas chromatograph [one column injection, splitting into two capillary columns with different polarities (DB 17–01 and DB 5–02) connected to two electron capture detectors, automatic samplers and automatic integrate systems; Carlo Erba Instruments, GC 8000 series].

Results

Alkaline comet assay

Three experiments (donors 1, 1 and 3 respectively) were conducted with PCB77 concentrations of 1, 10 and 25 μg/ml culture, and treatment times of 30 min, 1 h and 3 h. The tail lengths (median, mean ± SD) and tail moments (median, mean ± SD) are presented in Table II.

For a positive control 1 mM EMS was used in the comet assay and the final DMSO percentage in the cultures was 0.5%. It was obvious that the positive control induced a highly statistically significant increase in tail lengths and tail moments ($P < 0.0001$), but that the PCB77 treatment led to only slightly positive results ($P < 0.01$) in four cases for tail length or tail

Table IV. Number of binucleated cells (nCB), percentage of binucleated cells (% CB) and frequency of induced micronuclei (nMNCB) in human lymphocytes *in vitro* exposed to 3,3',4,4'-tetrachlorobiphenyl (PCB77)

Treatment	nCB			% CB			nMNCB			% MNCB		
	Exp. 1 (donor 1)	Exp. 2 (donor 2)	Exp. 3 (donor 2)	Exp. 1 (donor 1)	Exp. 2 (donor 2)	Exp. 3 (donor 2)	Exp. 1 (donor 1)	Exp2 (donor 2)	Exp. 3 (donor 2)	Exp. 1 (donor 1)	Exp. 2 (donor 2)	Exp. 3 (donor 2)
Control	–	2000	2000	–	60.70	59.00	–	11	12	–	5.50	6.00
0.1 µg MMC/ml	1000	2000	2000	25.70	46.80	54.55	25	36	36	25.00*	18.00*	18.00*
0.5% DMSO												
µg PCB77/ml												
0	1000	2000	2000	49.00	56.05	47.50	3	6	13	3.00	3.00	6.50
0.01	–	2000	–	–	59.15	–	–	13	–	–	6.50	–
0.1	–	2000	2000	–	62.25	54.60	–	10	10	–	5.00	5.00
10	681	2000	2000	38.90	62.60	69.40	8	8	9	11.75	4.00	4.50
25	1000	2000	2000	47.60	51.50	63.70	1	12	16	1.00	6.00	8.00
0.5 mg saturation	–	2000	2000	–	58.65	46.10	–	24	13	–	12.00*	6.50
1 mg saturation	–	–	2000	–	–	68.25	–	–	7	–	–	3.50
2 mg saturation	–	–	2000	–	–	70.75	–	–	7	–	–	3.50
2% DMSO												
µg PCB77/ml												
0	1599	2000	–	35.45	46.45	–	4	37	–	2.50	18.50	–
0.01	–	2000	–	–	50.55	–	–	23	–	–	11.50	–
0.1	–	2000	–	–	54.20	–	–	22	–	–	11.00	–
10	246	2000	–	39.42	52.35	–	0	28	–	0.00	14.00	–
25	875	2000	–	37.00	49.00	–	11	19	–	12.75*	9.50	–
100	1090	2000	–	29.80	40.20	–	11	37	–	10.09	18.50	–
0.5 mg saturation	–	2000	–	–	47.80	–	–	11	–	–	5.50	–
1 mg saturation	–	–	–	–	–	–	–	–	–	–	–	–
2 mg saturation	–	–	–	–	–	–	–	–	–	–	–	–

Saturation = highest concentration where PCB77 was soluble (5 mg/ml DMSO) with extra PCB77 powder added. The given frequencies result from duplicate cultures. The final DMSO percentage in the cultures was 0.5 or 2%.
*$P < 0.05$ (chi-square test).

Table V. Percentage of polynucleated cells (% polyN), and relative mitotic index in human lymphocytes *in vitro* exposed to 3,3',4,4'-tetrachlorobiphenyl (PCB77)

Treatment	% polyN			Relative mitotic index		
	Exp. 1 (donor 1)	Exp. 2 (donor 2)	Exp. 3 (donor 2)	Exp. 1 (donor 1)	Exp. 2 (donor 2)	Exp. 3 (donor 2)
Control	–	9.15	7.55	–	100	100
0.1 µg MMC/ml	0.80	5.70	3.25	45	74	82
0.5% DMSO						
µg PCB77/ml						
0	5.90	9.70	5.00	100	100	100
0.01	–	11.90	–	–	109	–
0.1	–	17.70	10.20	–	129	102
10	5.60	21.60	14.75	82	140	134
25	6.60	7.10	9.85	100	87	112
0.5 mg saturation	–	11.05	7.45	–	107	82
1 mg saturation	–	–	12.65	–	–	126
2 mg saturation	–	–	7.30	–	–	115
2% DMSO						
µg PCB77/ml						
0	0.65	0.65	–	100	100	–
0.01	–	0.95	–	–	110	–
0.1	–	1.50	–	–	120	–
10	0.16	1.70	–	–	117	–
25	1.60	1.55	–	109	109	–
100	0.20	0.80	–	82	88	–
0.5 mg saturation	–	0.70	–	–	103	–
1 mg saturation	–	–	–	–	–	–
2 mg saturation	–	–	–	–	–	–

Saturation = highest concentration where PCB77 was soluble (5 mg/ml DMSO) with extra PCB77 powder added. The given frequencies result from duplicate cultures. The final DMSO percentage in the cultures was 0.5 or 2%.
*$P < 0.05$ (chi-square test).

moment. This was probably due to outliers, and was not biologically significant.

In the second experiment PCB77 concentrations were measured in the blood cultures by an electron capture gas chromatograph, to check whether the PCB77 was still present after treatment and not completely lost by absorption to the glass of the culture tubes or evaporated. The results of the measurements are given in Table III. The measured concentrations were lower than the initial concentrations (10 and 25 μg/ml), but still within the range expected. This indicates that the blood was exposed to concentrations in the same range as predicted.

In Figure 1 the % DNA in the tail is analysed as a function of the tail length. It is clear that the lymphocytes treated with 25 μg PCB77/ml for 1 h do not have a significantly higher DNA content in the tail than the control cells. This indicates that no breaks occurred. The same results were found for all the other treated cultures.

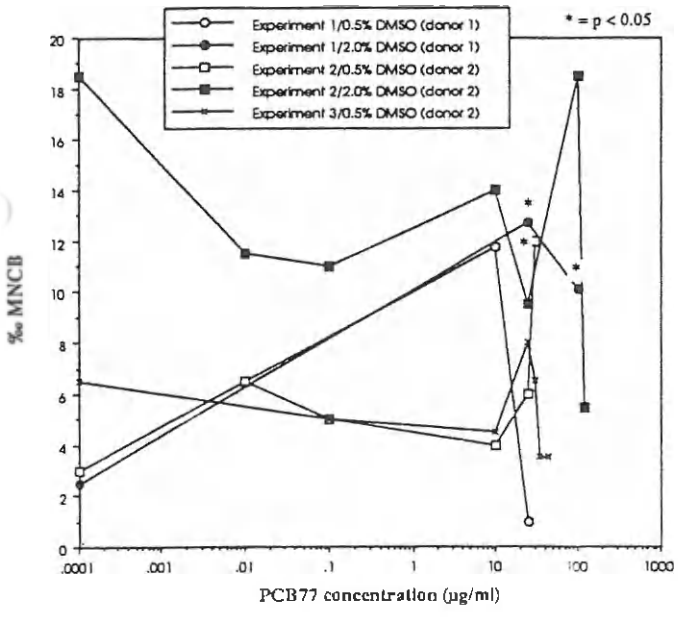


Fig. 2. Frequency of binucleated lymphocytes with micronuclei (‰ MNCB) from human lymphocyte cultures treated with 3,3',4,4'-tetrachlorobiphenyl (PCB77). Each point represents the mean of two parallel cultures. DMSO = dimethylsulfoxide.

Micronucleus test

Three experiments, with two different donors, were performed to analyse the induction of micronuclei by PCB77 in isolated human lymphocytes. The results are presented in Tables IV and V.

With a final concentration of 0.5% DMSO in the culture, no increase in MN frequency was observed with doses of 10–25 μg PCB77/ml culture (experiment 1). However, if the final DMSO concentration was increased to 2% (as used in Sargent's experiments) a significant increase in MN frequency was found (experiment 1). The 2% DMSO control was, however, very low (2.5‰ MNCB).

With a broader range of concentrations (experiment 2), starting from 0.01 μg/ml up to the saturation concentration, and 0.5 and 2% of DMSO in culture, only with the saturation concentration was a statistically significant increase in MN frequency was observed with 0.5% DMSO in culture. However, no significant mitotic delay was seen.

Finally, to check whether different increasing saturation concentrations would give an increased MN induction, we tested three saturation concentrations (experiment 3). We used 0.5% DMSO in culture to eliminate possible negative effects of a high percentage of DMSO. No increase in MN frequency was obtained, and accordingly no decrease in mitotic index.

Figure 2 gives a summary of the ‰ MNCB of the three experiments. Figure 3 gives the relative mitotic indices. In none of the three experiments could a statistically significant decrease of mitotic index be seen.

Discussion

PCBs are omnipresent chemicals. The major problem with PCBs is that they are very stable and will therefore stay in the environment for many years. Unfortunately, the mechanism

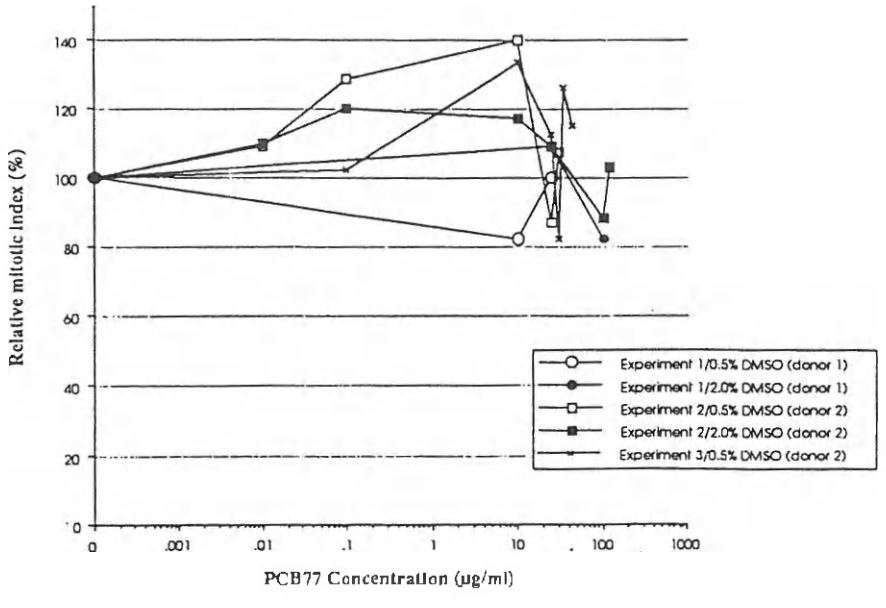


Fig. 3. Relative mitotic index from isolated lymphocyte cultures treated with 3,3',4,4'-tetrachlorobiphenyl (PCB77). Each point represents the mean of two parallel cultures. DMSO = dimethylsulfoxide.

leading to thier toxicity is not yet known, which renders us unable to compensate for the problems they cause. Accumulated PCBs (e.g. in oils of condensers and transformers) can be destroyed by burning the oils at temperatures >2200°C, but there is no mechanism for 'cleaning up' PCBs already spread in the ecosystem. Therefore it is still necessary and desirable to study the possible damage caused by these chemicals and its mechanism of action, in order to try and find mitigating measures.

Our aim was to assess whether the clastogenic effects of the planar PCB77 (Sargent *et al.*, 1989) could be confirmed with two relatively simple genetic techniques (micronucleus test and the comet assay).

The comet assay has not previously been used to study the effect of PCBs on human lymphocytes in culture. This technique has already proved its usefulness in other mutagenicity studies (e.g. Vijayalaxmi *et al.*, 1992; Olive and Durand, 1992; Tice, 1995). The results of the three experiments here are highly comparable, although in experiment 1 the tail length and tail moment of the EMS treatment were higher than in the other experiments. EMS treatment in all cases led to highly significant results ($P < 0.0001$).

In all experiments the SD of tail length and tail moment were rather high. This reflects the high variability of response between the different cells within one culture. In the cultures treated with PCB77 concentrations of 1, 10 and 25 µg/ml for 30 min, 1 h and 3 h, only in 6 of the 24 PCB treated cultures were slightly significant results obtained for tail length or tail moment. These positive results are probably due to outliers which stretch the distribution of the results, but without real biological significance. In experiments 1 and 3 the tail lengths and tail moments are even slightly lower than the control. The fact that there is no difference in response between 30 min and 60 min of treatment with PCB77 indicates that no repair had occurred after 60 min. However, in the future it might be interesting to test even shorter treatment times (5, 10 and 15 min) since repair may occur very quickly (McKelvey-Martin *et al.*, 1993).

When using the comet assay, there are some uncertainties with respect to the endpoint being measured. In addition, there are concerns at distinguishing cytotoxicity and apoptosis from DNA damage. Therefore, the results of the comet assay have to be interpreted with care.

In the three micronucleus experiments conducted with PCB77 concentrations ranging from 0.01 to 100 µg/ml and three saturation concentrations, and 0.5 or 2% DMSO in culture, in only three cases (i.e. with 2% DMSO in culture and one of the saturation concentrations) was a significant ($P < 0.05$) induction of micronuclei seen. This indicates that almost no acentric breaks were formed and/or no chromosomes were lost. The lack of decrease of mitotic index proves that the cell division was normal and that the number of binucleated cells was as high in the treated cultures as in the control cultures.

The reason for the application of the very low PCB77 concentrations is that Sargent *et al.* (1989) stated that especially low, non-toxic concentrations of certain PCBs may interact to produce superadditive genotoxic effects. They emphasized the importance of studying mutagenic effects of chemical contaminants at doses well below toxicity since cell death or mitotic arrest at higher doses can obscure significant genotoxic effects occurring due to chronic low-level exposures.

In theory the micronucleus test should give the same type of response as the chromosome aberration test (Heddle *et al.*, 1991). However, the chromosome aberration test will give more detailed information, whereas the micronucleus test can also detect effects on the spindle apparatus. Our micronucleus results are in contradiction with those of Sargent *et al.* (1989) on chromosome aberrations. The latter found that, in the presence of a final DMSO percentage of 2%, PCB77 does induce chromosome aberrations *in vitro* in human lymphocytes. The frequency of chromosome aberrations in the control culture (2% DMSO) was very low: 5.46 ± 2.0 chromatid breaks in 2211 cells. For a concentration of 0.01 µg PCB77/ml, dissolved in 2% DMSO, Sargent's group found 16.0 ± 5.0 chromatid breaks. They conclude that PCBs are mutagenic and that there was a synergistic effect with PCB52 (2,2′,5,5′-tetrachlorobiphenyl). However, as this group found chromatid breaks, we would expect to find micronuclei resulting from those breaks.

It is generally accepted that the maximum DMSO percentage in culture should not exceed 0.8%, because DMSO is known to have mutagenic effects itself when applied in high concentrations. Sargent, however, used 2% DMSO. The experiments conducted here suggest that the effects observed by Sargent *et al.* (1989) were due to the DMSO itself or to the synergetic effect of DMSO and the PCBs rather than to PCBs alone.

As far as the mechanisms of action are concerned, Sargent *et al.* (1989) proposed that the clastogenic effects of PCB77 may be due to direct intercalation on the DNA (Wassom *et al.*, 1978). After intercalation the DNA structure may be disturbed and mutations may occur. The clastogenic effect may also result from a cascade of toxic effects associated with binding to the Ah receptor (Poland and Glover, 1980). Other proposed mechanisms are that PCB77 might act through free radical generation or induction.

The best known effect of planar PCBs so far is the induction of the P-450 enzyme system (Ariyoshi *et al.*, 1990). The group of Safe has already performed much research in this field (Safe, 1984, 1989, 1992; Safe *et al.*, 1985). Data from the majority of the studies suggest that the oxidative metabolism of PCBs results in the production of arene oxide intermediates, which are strong electrophiles and alkylating agents. The latter could then alkylate macromolecules, e.g. proteins, DNA and RNA (Wyndham and Safe, 1978). Thus the metabolism of PCBs could exert the toxic action, but no proof has yet been found that PCB77 is metabolized to a reactive intermediate (Yoshimura and Yamamoto, 1973; Darnerd and Brandt, 1986; Murk *et al.*, 1994). Only less chlorinated, less toxic PCBs would be metabolized and excreted. This suggests that metabolization of PCBs would lead to detoxification; hence it is not the metabolite that is toxic but the parental molecule (Safe, 1992). In the future it might be interesting to conduct experiments with planar PCBs present with a promutagen, to see whether PCB77 can activate the promutagen by inducing enzyme systems.

In conclusion, it is clear that the majority of research has pointed in the direction of a non-genotoxic mechanism of action for PCBs (see Table I). Our results confirm this assumption. One of the most toxic planar PCBs, 3,3′,4,4′-tetrachlorobiphenyl, did not cause DNA breaks or chromosome loss detectable by the micronucleus test and the comet assay. We consider that the earlier effect mentioned by Sargent *et al.* (1989) was essentially due to the high percentage of DMSO (2%) in the culture.

Acknowledgements

The authors are very grateful to Kris Cooreman and Patriek Roosse (Rijkstation voor Zeevisserij in Ostend) for their help.

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Received on December 12, 1995; accepted on February 12, 1996