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Toxicity of 2,2′,5,5′-Tetrachlorobiphenyl and Its Metabolites, 2,2′,5,5′-Tetrachlorobiphenyl-3,4-oxide and 2,2′,5,5′-Tetrachlorobiphenyl-4-o1 to Cultured Cells *In Vitro*

Stanley S. Stadnicki and James R. Allen

Department of Pathology and Regional Primate Research Center, University of Wisconsin, Madison, Wis. 53706

Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants with detectable levels being found in the soil, air, water, and food chain (CAREY and GOWEN 1976, KUTZ and YANG 1976, DENNIS 1976, JELINEK and CORNELIUSSEN 1976). PCBs have been shown to be present in human tissue samples (RISEBROUGH and BRODINE 1970) and mammalian toxicity of the PCBs has been well documented (KIMBROUGH 1974). PCBs have been shown to be toxic to rats (ALLEN et al. 1976), sub-human primates (ALLEN 1975) and man (KURATSUNE et al. 1976) as well as many other species.

The toxicity of PCB mixtures and specific isomers has also been studied in cell cultures. LITTERST and LICHTENSTEIN (1971) found an ID_{50} of 63 ppm in HeLa cells and 110 ppm in diploid fibroblasts using Aroclor 1254. OHNISHI (1976) and OHNISHI and ARAKAWA (1977) found that Kanechlor 400 was more toxic to epithelial cells than to fibroblasts in primary conjunctival cell cultures. They report an increase in mitotic time (26 min., C to 54 min., 40 µg Kanechlor 400/ml) and an increase in vacuolization and cellular blebs. They found that all cells died at concentrations of Kanechlor 400 > 100 µg/ml. MORI et al. (1975) found Kanechlor to be cytotoxic to HeLa cells at 3-6 µg/ml in the absence of serum proteins.

PCBs have also been shown to be capable of enzyme induction in cultured cells. AHOTUPA et al. (1978) found that 3.6 ppm Clophen A_{50} induced AHH 4-fold and UDPGT 2-fold in primary liver epithelial cell cultures. Primary fibroblast cultures showed no induction. KAWANISHI et al. (1978) studied porphyrin induction by various PCB isomers in primary chick embryo liver cells in culture. They found 3,3',4,4'-tetrachlorobiphenyl to be the most potent inducer. They observed that the amount of induction was dependent on the position of the chlorines, but independent of the degree of chlorination and rate of metabolism.

The role of metabolism in the toxicity of the PCBs is unknown, since few studies have been done with any of the metabolites. HOOPINGARNER et al. (1972) found that lower chlorinated Aroclor mixtures (Aroclor 1016, 1221) which are rapidly metabolized are more toxic than the higher chlorinated mixtures (Arochlor 1254, 1260) which are not rapidly metabolized. This

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effect can be due to metabolite toxicity or to degree of chlorination. YAMAMOTO and YOSHIMURA (1973) found that 5-hydroxy-2,3',4,4'-tetrachlorobiphenyl, the major metabolite of 2,3',4,4'-tetrachlorobiphenyl, was five times more toxic to mice than the parent in an acute four day LD_{50} study.

In addition to the toxic effects in mice seen with 5-hydroxy-2,3',4,4'-tetrachlorobiphenyl, hydroxychlorobiphenyls have been tested for their hemolytic activity against RBCs. The higher chlorinated hydroxybiphenyls (2-OH-2',3',5,5'-pentachlorobiphenyl, 3-OH-2',3',4',5'-tetrachlorobiphenyl, and 4-OH-2',3',4',5'-tetrachlorobiphenyl) were more hemolytic than lower chlorinated hydroxybiphenyls (4-OH-4'-chlorobiphenyl, 3-OH-4,4'-chlorobiphenyl) (MILLER 1978). In addition, the hydroxy chlorobiphenyls were more hemolytic than the corresponding chlorobiphenyl (4-OH-2',3',4',5'-tetrachlorobiphenyl induced 50% hemolysis at 9.0 μM where 2,3,4,5-tetrachlorobiphenyl induced < 37% hemolysis at 800 μM (MILLER 1978).

These studies indicate that metabolites might play a role in the toxicity of the PCBs. Because commercial PCB mixtures are complex and contain many possible isomers in different amounts, the role of metabolism in the toxicity of the mixture is difficult to study. For this study, we have chosen to work with 2,2',5,5'-tetrachlorobiphenyl (TCB) and its metabolites. The metabolism of TCB has been studied in the rat (VAN MILLER et al. 1975) and monkey (HSU et al. 1975). TCB is metabolized to hydroxylated products with metabolism proposed to occur through an arene oxide (HSU et al., 1975). In this study we looked at the toxicity of TCB, one of its metabolites, TCB-4-ol, and the postulated arene oxide intermediate, TCB-3,4-oxide utilizing L-929 and HeLa cells in culture.

MATERIALS AND METHODS

TCB was prepared from 2,2',5,5'-tetrachlorobenzidine as described by HUTZINGER and SAFE (1972). TCB was purified by recrystalization until > 99.9% purity was seen by gas chromatography. TCB-3,4-oxide was prepared by the method of REICH et al. (1978). TCB-4-ol was prepared by acid hydrolysis of TCB-3,4-oxide. Acid hydrolysis yields a 4:1 mixture of 4-OH:3-OH. The TCB-4-ol and TCB-3-ol were separated by thin layer chromatography after acetylation. The structure of the TCB-4-ol was determined by 270-MHz NMR (REICH et al. 1978) TCB, TCB-4-ol, and TCB-3,4-oxide were dissolved in DMSO for these experiments.

L-929 cells (NCTC clone 929, CCL-1) and HeLa (CCL-2) cells were obtained from American Type Culture Collection, Rockville, MD. Stock L-929 cells were maintained in Eagles minimal essential medium (MEM, Gibco) supplemented with 10% Heat Inactivated Fetal Bovine Serum (HIFBS, Gibco), and antibiotics (penicillin, 100 U/ml and streptomycin, 50 μg/ml, Gibco). Stock HeLa cells

were maintained in MEM supplemented with 10% Calf (Bovine) Serum (CS, Gibco) and antibiotics (as above). Cells were grown in 75 cm^2 plastic tissue culture flasks (Falcon, #3024) in a humidified CO_2/Air (5%/95%) incubator (National, #420).

The HeLa plating assay was performed using the method of HIGGINS et al. (1978). Stock HeLa cells (1×10^3) were plated into 30 cm^2 plastic petri plates (Falcon, #3030, 2 mm grid) in MEM + 10% CS without antibiotics and allowed to attach for 2.0 hrs. After this period, TCB, TCB-4-ol or TCB-3,4-oxide was added in dimethylsulfoxide (DMSO). DMSO volume was kept constant at 0.5%, a level at which no effects were observed from solvent alone. Control cultures received solvent only. Cultures were grown for 7 days, washed, stained, and counted as previously described. The number of colonies formed in the presence of TCB, TCB-4-ol, or TCB-3,4-oxide was compared to the number of colonies formed in the control cultures.

To determine the effect of TCB, TCB-4-ol, and TCB-3,4-oxide on the growth of L-929 cells, 5×10^4 L-929 cells were plated in 25 cm^2 plastic tissue culture flasks (Falcon, #3013) in MEM + 10% HIFBS without antibiotics, and grown for 24 hrs. At this point (T=0), TCB, TCB-4-ol, or TCB-3,4-oxide was added in DMSO (final DMSO concentration 0.5%). Control cultures received DMSO only. Cell number was determined after desired intervals by rinsing flasks with Hanks BSS (without Ca^{2+}, Mg^{2+}), trypsinizing, and counting an aliquot on a coulter counter (Coulter Electronics, model B, 100 µm orifice). Triplicate flasks were done at each time point for each dose, with the exception of the zero time point, for which six flasks were counted.

RESULTS

Figure 1 shows the dose response curve for the HeLa plating assay. This figure shows the mean $\pm$ S.E. for each group (n=6) expressed as a percent of control (c=286 $\pm$ 5.1 colonies/plate, n=15; c=100%). TCB shows inhibition at concentrations greater than 15 µg TCB/ml (10% inhibition at 15 µg/ml, 15% inhibition at 25 µg/ml). TCB-4-ol shows some inhibition at 10 µg/ml and 22% inhibition at 25 µg/ml. TCB-3,4-oxide, which is the most toxic shows significant inhibition at 1 µg/ml (6% inhibition) and shows 68% inhibition with respect to control at 25 µg/ml. TCB-3,4-oxide is significantly more toxic than TCB or TCB-4-ol at all concentrations tested. TCB-4-ol is also more toxic than the parent isomer at cell concentration tested.

Figure 2 shows the growth curve for L-929 cells in the presence of TCB. No effect was observed at 1 or 5 µg/ml, so these points were eliminated for clarity. At 10 and 15 µg/ml, a slight inhibition is observed at 24 hours, but not at subsequent time points. At 20 TCB µg/ml, TCB shows inhibition at all time points.

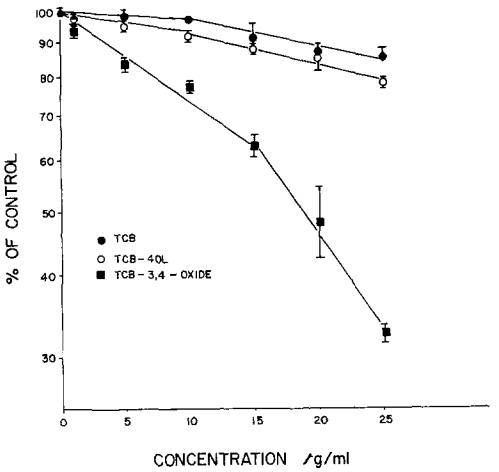

Figure 1. HeLa Plating Assay. Points represent mean ± S.E. (n=6) relative to control.

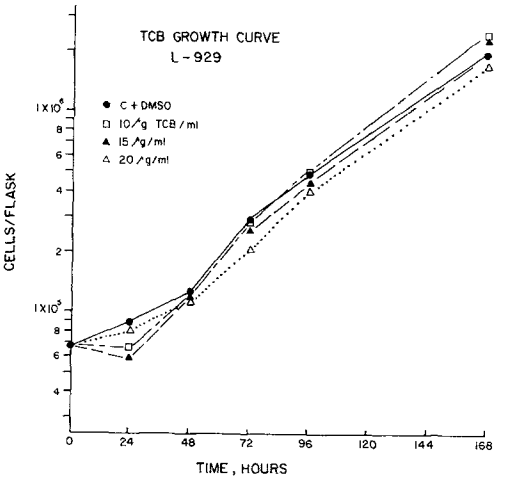

Figure 2. L-929 growth curve for cells exposed to 2,2',5,5'-TCB.

The growth curve for L-929 cells in the presence of TCB-4-ol is shown in Figure 3. No significant inhibition is seen at 1 or 5 µg/ml. At 10 µg/ml, some inhibition is observed at 24 hrs and only slight inhibition is seen at other time points. At 15 and 20 µg/ml, significant inhibition of growth is seen. At 15 µg TCB-4-ol/ml, the number of cells/flask at 168 h is 19% of the control.

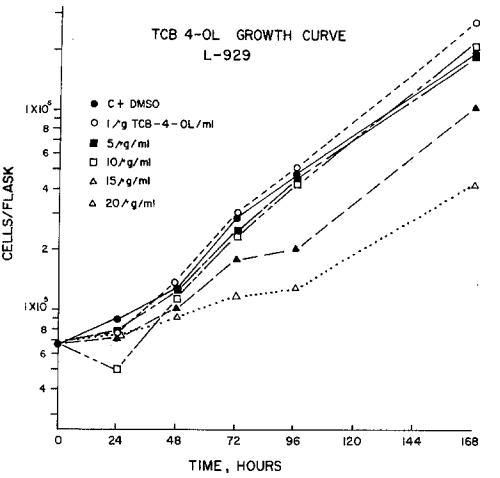

Figure 3. L-929 Growth curve for cells exposed to 2,2',5,5'-TCB-4-ol.

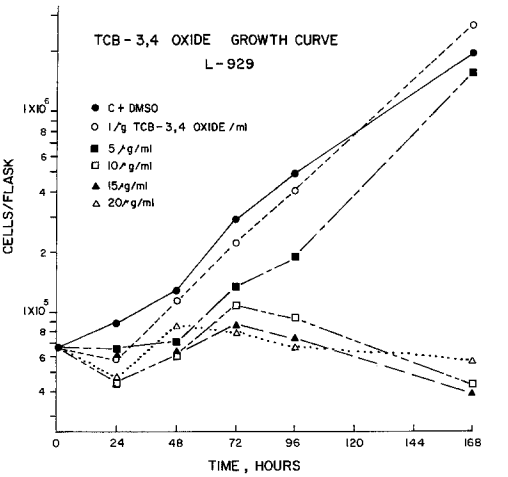

Figure 4. Growth curve for L-929 cells exposed to 2,2',5,5'-TCB-3,4-oxide.

TCB-3,4-oxide shows the most inhibition to L-929 growth as can be seen in Figure 4. Slight inhibition of growth is observed for the first 96 hrs at 1 µg TCB-3,4-oxide/ml. At 5 µg/ml, significant inhibition of growth is observed at all time points. At concentrations of TCB-3,4-oxide of 10 µg/ml or greater, growth of L-929 cells is completely inhibited.

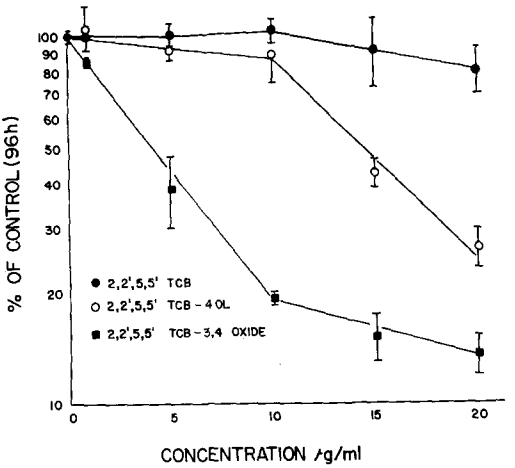

Figure 5. Dose response for L-929 cells exposed to 2,2',5,5'-TCB, 2,2',5,5'-TCB-4-ol, or 2,2',5,5'-TCB-3,4-oxide.

Figure 5 shows the dose response curve for L-929 cells after 96 hours growth in the presence of TCB, TCB-4-ol, or TCB-3,4-oxide. This figure shows the mean $\pm$ S.E. for each group (n=3) plotted as a percentage of the number of cells/flask in the control (c=4.91 $\pm$ 0.11 x 10^5 cells/flask, n=9; c=100%). As can be seen from this curve, TCB-3,4-oxide shows significant inhibition with respect to the control at all concentrations tested. TCB-4-ol shows slight inhibition at 5 and 10 µg/ml and significant inhibition at 15 and 20 µg/ml. TCB shows inhibition only at 20 µg/ml. TCB-3,4-oxide is significantly more toxic than TCB-4-ol or TCB at all concentrations tested. TCB-4-ol shows more toxicity than TCB at concentrations > 5 µg/ml.

DISCUSSION

The contribution of the phenols and their postulated arene oxide intermediates to the overall toxicity of PCB mixtures is unclear. YAMAMOTO and YOSHIMURA (1973) found that 5-hydroxy 2,3',4,4'-tetrachlorobiphenyl, the major metabolite of 2,3',4,4'-tetrachlorobiphenyl was 5 times more toxic to mice (LD_{50} 0.43 g/kg vs. 2.15 g/kg) than the parent compound. Hydroxychlorobiphenyls have also been shown to be more toxic than the parent isomers when measured by erythrocyte lysis (MILLER 1978). However, in Miller's study, the various PCBs studied were hydroxylated on one ring with the chlorines on the other, which is not representative of the PCBs in the commercial mixtures.

In this report, we show that the metabolites of 2,2',5,5'-tetrachlorobiphenyl are more toxic than the parent isomer. TCB

did not show toxicity at concentrations below 15 µg/ml to HeLa cells or 20 µg/ml to L-929 cells. TCB-4-ol is more toxic than TCB to both HeLa and L-929 cells, and shows significant inhibition at concentrations greater than 10 µg/ml. TCB-3,4-oxide is significantly more toxic to HeLa and L-929 cells than either TCB or TCB-4-ol. TCB-3,4-oxide shows significant inhibition with respect to controls at all concentrations tested. Comparing figures 1 and 5, it appears that TCB-4-ol and TCB-3,4-oxide are more toxic to L-929 cells than to HeLa cells. The inhibition seen for TCB is at higher concentrations than that reported by MORI et al. (1975) who report an effect at 3-6 µg/ml, but they used Kanechlor 400 as opposed to a specific isomer and work was done in absence of serum. LITTERST and LICHTENSTEIN (1971) found Aroclor 1254 had an ID_{50} to HeLa cells of 63 ppm, but their work was done in 15% serum. It appears from these results that serum has a protective effect against PCB induced toxicity in cell culture.

Our finding that TCB-3,4-oxide is more toxic than TCB-4-ol in mammalian cells contrasts that of HSIA et al. (1978) who found that TCB phenols were more toxic to Salmonella typhimunium than TCB or TCB-3,4-oxide. HSIA et al. (1978) also found TCB-phenols were more inhibitory than TCB to Mg^{++} ATPase in isolated hepatocyte plasma membranes.

The reasons behind the increased toxicity of the metabolites with respect to the parent compound are unknown. For the phenolic metabolite, increased toxicity could be due to the amphipathic nature of the phenols which would allow for stronger interaction with the membrane or due to increased solubility of the phenols. LIN et al. (1979) found a high concentration of unconjugated monohydroxy-2,2',5,5'-TCB associated with liver cell membranes from rats fed an acutely toxic dose (1.7 g/kg) of 2,2',-5,5'-TCB, and postulated that the interaction of the phenolic metabolite with the membrane was responsible for the decrease in Mg^{++}/Ca^{++} ATPase and for the acute toxicity. However, TCB-3,4-oxide is less soluble and less amphipathic than the TCB-4-ol, but it is more toxic and more potent in induction of DNA SS breaks. Since many known heptotoxins induce lesions via covalent interactions with macromolecules in the liver (GILLETTE et al. 1974) PCB toxicity may also occur through this route. GARDNER et al. (1973) proposed that PCBs are metabolized via liver microsomal enzymes to arene oxide intermediates which are capable of producing damage by nucleophilic reaction with macromolecules. SEYMOUR et al. (1976) and SHIMADU (1976) reported irreversible association of PCBs with macromolecules in rat and monkey microsomes in vitro, postulating that binding occurred via the arene oxide intermediate. Although the arene oxide intermediate has been postulated to be an in vivo metabolic intermediate, PCB-arene oxide intermediates have not yet been isolated in vivo or in vitro.

The metabolism of aromatic compounds to phenolic metabolites has long been considered to be a mechanism of detoxification for a variety of foreign substances and drugs, including many environmental pollutants. However, at times the metabolic products formed during this process may turn out to be more hazardous to the animal than the parent compound. This is seen with the formation of carcinogenic epoxides from benzo(a)pyrene and may also be the case for the PCBs. In this study we have shown that TCB metabolites, TCB-3,4-oxide and TCB-4-ol, are more toxic than the parent, and thus the metabolites of TCB may be involved in the toxicity of TCB.

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