Differences in the effects of two hexachlorobiphenyls on superoxide generation by polymorphonuclear leucocytes stimulated by N-formyl-methionyl-leucyl-phenylalanine and phorbol myristate acetate

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Abstract

The effects of hexachlorobiphenyls (HCBs) on superoxide (O_2^-) generation by guinea pig polymorphonuclear leucocytes were examined. 2,3,6,2',3',6'-HCB by itself had only a weak inductive effect on O_2^- generation. This compound, however, enhanced O_2^- generation stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP) about twofold, but not the generation induced by phorbol myristate acetate (PMA). On the other hand, 3,4,5,3',4',5'-HCB suppressed O_2^- generation stimulated by both FMLP and PMA. The inhibitory potency of this compound was far greater with PMA (ID_{50} , 5 μ M) than with FMLP (ID_{50} , 40 μ M).

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Polychlorinated biphenyls (PCBs) are widespread environmental pollutants.^{1,2} As many as 209 isomers and congeners of PCBs are possible according to the number and position of chlorine substitution. The planarity of PCBs is dependent on the position of the chlorine substituent. In the absence of *o*-chlorination the biphenyl molecule can assume a planar conformation, whereas the biphenyl molecule possessing *o*-*o'* chlorine substitution assumes a non-planar conformation because of steric hindrance.³ The relation between molecular conformation and effect has been extensively studied during the past two decades with regard to general toxicity, porphyrin biosynthesis, and induction of many drug metabolising enzymes. Planar PCBs are more generally toxic than non-planar types.^{4,5} Planar PCBs showed greater potency in the induction of porphyrin biosynthesis compared with non-planar types,^{3,6} and in regard to the inductive effect on hepatic drug metabolising enzymes, planar type PCBs induced cytochrome *P*-448 (3-methylcholanthrene-type), whereas non-planar type PCBs induced cytochrome *P*-450 (phenobarbitone type).^{7,8} Thus, the toxic and biological effects of PCBs differ as to whether the molecule assumes a planar or a non-planar structure. It

has also been established that in aromatic hydrocarbon responsive mice (C57BL/6) 3,4,3',4'-tetrachlorobiphenyl (planar) causes immune dysfunction whereas 2,5,2',5'-tetrachlorobiphenyl (non-planar) has no immunosuppressant effect, indicating that immunotoxicity is also dependent on the planarity of the PCB molecule.⁹

Polymorphonuclear leucocytes (PMNs) are one group of phagocytes that play a part in microbicidal activity.¹⁰ They are endowed with complex machinery (NADPH oxidase) located on the cell surface, in the plasma membrane, and inside the cytoplasm, by which they recognise and respond to various signals, activating different functions (ion fluxes, endocytosis, respiration bursts, secretion of enzymes).^{11,12} Among these, O_2^- and hydrogen peroxide (H_2O_2) are essential for microbicidal activity.¹³ Many PCBs are not particularly toxic, but because they are lipophilic and chemically stable, they tend to accumulate in lipid rich parts of cells such as biomembranes.¹⁴ Furthermore, NADPH oxidase is a topic of current interest.¹⁵ It is, therefore, relevant to study whether these activities of PMNs are affected by PCBs. As far as we know, few reports on the effects of PCBs on leucocytes have been published. As a part of a continuing study on PCBs, this paper reports the effects of 2,3,6,2',3',6'-(236-HCB, non-planar type) and 3,4,5,3',4',5'-HCB (345-HCB, planar type) on the O_2^- generation of guinea pig PMNs stimulated by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA). Hexachlorobiphenyls are more resistant to metabolism than the less chlorinated PCBs and 345-HCB is the most toxic of the HCBs.⁴ The toxicity, metabolism, and excretion of 236-HCB has been extensively studied in many animals.¹⁶

Materials and methods

SYNTHESIS OF HCBs

236-HCB was synthesised by Ullman coupling of the corresponding trichloriodobenzene.¹⁷ 345-HCB was synthesised by the Cadgan modification of the Gomberg-Bachmann coupling reaction¹⁸ as described

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Figure 1 Effect of 236-HCB on O_2^- generation. PMNs were suspended at 1×10^6 cells/ml in KRP (pH 7.4, 37°C) containing 1 mM $CaCl_2$, 1 μ M NaN_3 , 10 mM glucose, and 20 μ M Cyt c.

Figure 2 Effect of 236-HCB on FMLP stimulated O_2^- generation. Experimental conditions were as in fig 1. Arrows indicate the addition of HCB and FMLP (12.5 nM, final concentration). Traces are representative of at least five experiments. Percentage increase is calculated by the slope of the trace, and each plot is a mean of three separate experiments.

Figure 3 Effect of 345-HCB on FMLP stimulated O_2^- generation. Experimental conditions were as in fig 1. Arrows indicate the addition of HCB and FMLP (12.5 nM, final concentration). Traces are representative of at least five experiments. Percentage inhibition is calculated by the slope of the trace, and each point is a mean of three separate experiments.

by Puttmann *et al.*¹⁹ Both HCBs were purified by alumina or florisil column chromatography or flash chromatography and by recrystallisation in methanol. The structure of 236-HCB (mp 112°C) and 345-HCB (mp 201°C) were confirmed by 1H -nuclear magnetic resonance and mass spectrometry. The purity as determined by gas chromatography was in both cases greater than 98%. HCBs thus synthesised were provided by Dr L W Robertson of the University of Kentucky. The stock solutions of these HCBs were prepared in dimethylformamide.

BIOCHEMICALS

Ferricytochrome c (Cyt c), FMLP, and PMA were purchased from Sigma Chemical Co (St Louis, MO). All other reagents were of the highest purity commercially available.

PREPARATION OF PMNS

PMNs were obtained from male guinea pigs weighing 400–450 g, 16 hours after an intraperitoneal injection of 2% casein containing 0.9% NaCl and 10 mM Tris-HCl (pH 7.4). The cells were washed three times with Ca^{2+} free Krebs-ringer-phosphate buffer (KRP, pH 7.4), separated by centrifugation, resuspended in KRP, and kept on ice until use.²⁰

MEASUREMENT OF GENERATION OF SUPEROXIDES

Superoxide generation was monitored continuously as superoxide dismutase inhibitable Cyt c reduction with a dual wavelength spectrophotometer (Shimadzu UV-300). PMNs suspended in KRP containing 10 mM glucose, 1 mM NaN_3 , 1 mM $CaCl_2$, and 20 μ M Cyt c were incubated in a thermostatically controlled cuvette equipped with a magnetic stirrer. The HCB was added with a microsyringe to the cuvette, and incubated for three minutes. Superoxide generation was then initiated by addition of FMLP or PMA, and measured at 37°C by Cyt c reduction with a wavelength pair of 550–540 nm.²¹

In all experiments, the control contained the same volume of solvent (dimethylformamide), and the final concentration of solvent was less than 0.2%. Release of dehydrogenase lactate, a marker of cell damage, did not differ between untreated and HCB treated cells (and was always less than 5% of the total cell contents).

Results

Figure 1 shows the direct stimulatory effect of 236-HCB on O_2^- generation in PMNs. 236-HCB triggered weak O_2^- generation only at higher concentrations.

Figure 2 shows the effect of 236-HCB on O_2^- generation induced by FMLP. 236-HCB potentiated FMLP induced O_2^- generation in a concentration dependent manner. The amount of FMLP induced O_2^- generation in cells pretreated with 236-HCB (20 μ M) was about twice as great as that in control cells. 236-HCB, however, neither potentiated nor

Figure 4 Effect of 345-HCB on PMA stimulated O_2^- generation. Experimental conditions were as in fig 1. Arrows indicate the addition of HCB and PMA (0.5 nM final concentration). Traces are representative of at least five experiments. Percentage inhibition is calculated by the slope of the trace, and each point is a mean of three separate experiments.

inhibited O_2^- generation induced by PMA (data not shown).

Figure 3 shows the effect of 345-HCB on O_2^- generation induced by FMLP. By contrast with the stimulatory effect of 236-HCB, this compound inhibited FMLP-induced O_2^- generation in a dose dependent manner, with 50% inhibition (ID_{50}) at $40\text{ }\mu\text{M}$.

Figure 4 shows the effect of 345-HCB on O_2^- generation induced by PMA. 345-HCB began to inhibit the O_2^- generation at $2.5\text{ }\mu\text{M}$. The inhibition increased with increases in 345-HCB concentration, with ID_{50} at $5\text{ }\mu\text{M}$. The inhibition of O_2^- generation by this compound was far more sensitive with PMA than with FMLP as stimulus.

Discussion

236-HCB is classified as a phenobarbitone type inducer of drug metabolising enzymes.⁴ The potency of 236-HCB to trigger O_2^- generation directly in PMNs was very weak. The enhancement of O_2^- generation induced by FMLP, however, indicated a priming effect. The amplification of NADPH oxidase activity by 236-HCB could be beneficial to the bactericidal and tumouricidal activity of PMNs but harmful to the leucocyte and the surrounding tissues. Overproduction of free radicals may cause degeneration and necrosis at sites of acute and chronic inflammation. Because 236-HCB has a rigidly angulated conformation⁶ the intercalation of the compound into the membrane of the cells may increase the number of receptors available for FMLP binding.

345-HCB is classified as a 3-methylcholanthrene type inducer of drug metabolising enzymes and is toxic compared with 236-HCB.⁴ It shows several toxic responses such as teratogenicity, porphyria, cleft palate, thymic atrophy, and immunosuppression.⁴ These phenomena are considered to be

mediated by the binding of the compound to the aromatic hydrocarbon receptor.²²

345-HCB inhibited the O_2^- generation induced by both FMLP and PMA, in a concentration dependent manner, suggesting that the compound decreases the bactericidal and tumouricidal activity of PMNs. This action of 345-HCB may be a direct effect on the cells rather than through the aromatic hydrocarbon receptor. Also it is not a scavenging effect of 345-HCB, as O_2^- generation by the xanthine/xanthine oxidase system is not inhibited by this compound (data not shown). An *in vivo* effect has been described by Thigpen *et al*²³ who reported that mice treated with a compound related to 345-HCB—namely, 2,3,7,8-tetrachlorobenzo-*p*-dioxin—were likely to die from bacterial infections. This may have been the result of the suppression of bactericidal activity of phagocytes including leucocytes.

In summary, 236-HCB and 345-HCB differentially affected O_2^- generation induced by FMLP. The first was stimulatory, whereas the second was inhibitory to O_2^- generation. 236-HCB had almost no effect on O_2^- generation induced by PMA, whereas 345-HCB inhibited significantly with an ID_{50} of $5\text{ }\mu\text{M}$. The stimulatory effect of 236-HCB may be harmful to the leucocyte itself or to the surrounding tissues. The inhibitory effect of 345-HCB, on the other hand, may decrease the bactericidal and tumouricidal activity of the cell. Although further work is needed to confirm this, the difference may be valid for planar and non-planar HCBs in general.

- 1 Parkinson A, Safe S. Aryl hydrocarbon hydroxylase induction and its relationship to the toxicity of halogenated aryl hydrocarbons. *Toxicol Environ Chem Rev* 1981;4:1-46.
- 2 Safe S. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology and mechanism of action *Crit Rev Toxicol* 1984;13:319-95.
- 3 Kawanishi S, Mizutani T, Sano S. Induction of porphyrin synthesis in chick embryo liver cell culture by synthetic polychlorobiphenyl isomers. *Biochim Biophys Acta* 1978;540:83-92.
- 4 Yoshimura H, Yoshihara S, Ozawa N, Miki M. Possible correlation between induction modes of hepatic enzymes by PCB and their toxicity in rats. *N Y Acad Sci* 1979;320:179-92.
- 5 McConnell EE. In: Kimbrough RD, ed. Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins, and related products. Amsterdam: Elsevier North-Holland Biomedical Press, 1980;109-50.
- 6 Sassa S, Sugita O, Ohnuma N, Imajo S, Okumura T, Noguchi T, Kappas A. Studies of the influence of chloro-substituent sites and conformational energy in polychlorinated biphenyls on uroporphyrin. *Biochem J* 1986;235:291-6.
- 7 Parkinson A, Robertson L, Safe L, Safe S. Polychlorinated biphenyls as inducers of hepatic microsomal enzymes: structure-activity rules. *Chem Biol Interact* 1980;30:271-85.
- 8 Bandiera S, Safe S, Okey AB. Binding of polychlorinated biphenyls classified as either phenobarbitone, 3-methylcholanthrene-, or mixed type inducers to cytosolic Ah receptor. *Chem Biol Interact* 1982;39:259-77.
- 9 Silkworth JB, Grabstein EM. Polychlorinated biphenyl immunotoxicity: dependence on isomer planarity and the Ah gene complex. *Toxicol Appl Pharmacol* 1982;65:109-15.
- 10 Babior BM. Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* 1978;298:659-68.
- 11 Badwey JA, Karnovsky ML. Active oxygen species and the functions of phagocytic leukocytes. *Annu Rev Biochem* 1980;49:695-724.
- 12 Rossi F. The O_2^- forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. *Biochim Biophys Acta* 1983;853:65-89.
- 13 Hume DA, Gordon S, Thornally PJ, Bannister JV. The production of oxygen-centered radicals by Bacillus-Calmette-Guérin-activated macrophages. *Biochim Biophys Acta* 1983;763:245-50.
- 14 Lin FS, Hsia MT, Allen JR. Acute hepatotoxicity of a

- tetrachlorobiphenyl. *Arch Environ Contam Toxicol* 1979;8:321-33.
- 15 Cross AR, Jones OTG. Enzymatic mechanisms of superoxide production. *Biochim Biophys Acta* 1991;1057:281-98.
 - 16 Sipes IG, Slocumb ML, Chen H-SG, Carter DE. 2,3,6,2',3',6'-Hexachlorobiphenyl: distribution, metabolism and excretion in the dog and the monkey. *Toxicol Appl Pharmacol* 1982;62:317-24.
 - 17 Hutzinger O, Safe S, Zitko V. Polychlorinated biphenyls: synthesis of some individual chlorobiphenyls. *Bull Environ Contam Toxicol* 1971;6:209-19.
 - 18 Cadgan JIG. A convenient new method of aromatic arylation. *J Chem Soc* 1962;4257-8.
 - 19 Puttmann M, Mannschreck A, Oesch F, Robertson LW. Chiral effects in the induction of drug-metabolizing enzymes using synthetic atropisomers of polychlorinated biphenyls (PCBs). *Biochem Pharmacol* 1989;38:1345-52.
 - 20 Kuroki M, Kamo N, Kobatake Y, Okimasu E, Utsumi K. Measurement of membrane potential in polymorphonuclear leukocytes and its changes during surface stimulation. *Biochem Biophys Acta* 1982;693:326-34.
 - 21 Babior BM, Kippes RS, Curnutte JT. Biological defense mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest* 1973;52:741-4.
 - 22 Poland A, Glover E. 2,3,7,8-Tetrachlorodibenzo-p-dioxin: segregation of toxicity with the Ah locus. *Mol Pharmacol* 1980;17:86-94.
 - 23 Thigpen JE, Faith RE, McConnell EE, Moore JA. Increased susceptibility to bacterial infection as a sequela of exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Infect Immunol* 1975;12:1319-24.