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

Makoto Iwata, Yoshimasa Nishihara, Yoshiya Watanabe, Masanobu Miyahara, Kiyomi Saeki

Abstract
The effects of hexachlorobiphenyls (HCBs) on superoxide (O2) generation by guinea pig polymorphonuclear leucocytes were examined. 2,3,6,2',3',6'-HCB by itself had only a weak inductive effect on O2 generation. This compound, however, enhanced O2 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 O2 generation stimulated by both FMLP and PMA. The inhibitory potency of this compound was far greater with PMA (ID50, 5μM) than with FMLP (ID50, 40μM).

Polychlorinated biphenyls (PCBs) are widespread environmental pollutants. As many as 209 isomers and cogeners 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 α-chlorination the biphenyl molecule can assume a planar conformation, whereas the biphenyl molecule possessing α-α' chlorine substitution assumes a non-planar conformation because of steric hindrance. 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. Planar PCBs showed greater potency in the induction of porphyrin biosynthesis compared with non-planar types, and in regard to the inductive effect on hepatic drug metabolising enzymes, planar type PCBs induced cytochrome P-448 (3-methylcholangthrene-type), whereas non-planar type PCBs induced cytochrome P-450 (phenobarbitone type). 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. Polymorphonuclear leucocytes (PMNs) are one group of phagocytes that play a part in microbialic activity. 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). Among these, O2 and hydrogen peroxide (H2O2) are essential for microbialic activity. 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. Furthermore, NADPH oxidase is a topic of current interest. 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 O2 generation of guinea pig PMNs stimulated by the chemotacetic 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. The toxicity, metabolism, and excretion of 236-HCB has been extensively studied in many animals.

Materials and methods
SYNTHESIS OF HCBS
236-HCB was synthesised by Ullman coupling of the corresponding trichloroiodobenzene. 345-HCB was synthesised by the Cadgan modification of the Gomberg-Bachmann coupling reaction as described.
Five experiments. Percentage on HCB experimental conditions were suspended at a final concentration. Traces increase is calculated by the slope of the trace, and each plot is a mean of three separate experiments.

Figure 2 Effect of 236-HCB on FMLP stimulated \( \text{O}_2 \) 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 \( \text{O}_2 \) 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. 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 \( ^1 \)H-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.

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 Na\(_2\)SO\(_4\), 1 mM CaCl\(_2\), and 20 \( \mu \)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.

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 hydrogenase 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 \( \text{O}_2 \) generation in PMNs. 236-HCB triggered weak \( \text{O}_2 \) generation only at higher concentrations.

Figure 2 shows the effect of 236-HCB on \( \text{O}_2 \) generation induced by FMLP. 236-HCB potentiated FMLP induced \( \text{O}_2 \) generation in a concentration dependent manner. The amount of FMLP induced \( \text{O}_2 \) generation in cells pretreated with 236-HCB (20 \( \mu \)M) was about twice as great as that in control cells. 236-HCB, however, neither potentiated nor
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 \(\mu\)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 \(\mu\)M. The inhibition increased with increases in 345-HCB concentration, with ID\(_{50}\) at 5 \(\mu\)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 phenobarboline type inducer of drug metabolising enzymes. 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 conformation 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. It shows several toxic responses such as teratogenicity, porphyria, cleft palate, thymic atrophy, and immunosuppression. These phenomena are considered to be mediated by the binding of the compound to the aromatic hydrocarbon receptor.

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 who reported that mice treated with a compound related to 345-HCB—namely, 2,3,7,8-tetrachlorobenzop-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 \(\mu\)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.

8 Bandiera S, Safe S, Okey AB. Binding of polychlorobiphenyls classified as either phenobarbin, 3-methylcholanthrene, or mixed type inducers to cytosolic Ah receptor. Chem Biol Interact 1982;39:259-77.


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

M Iwata, Y Nishihara, Y Watanabe, M Miyahara and K Saeki

doi: 10.1136/oem.51.4.271

Updated information and services can be found at:
http://oem.bmj.com/content/51/4/271

**Email alerting service**

These include:

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Notes**

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/