Neutrophil Function after Exposure to Polychlorinated Biphenyls in vitro

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Polychlorinated biphenyls (PCBs) are known to be immunotoxic, yet the effects on neutrophil (PMN) function are not well characterized. We incubated PMNs isolated from rat peritoneum with a mixture of PCB congeners, Aroclor 1242, in the absence or presence of either phorbol myristate acetate (PMA) to stimulate generation of superoxide anion (O2-) or N-formyl-methionyl-leucyl-phenylalanine (fMLP) to induce degranulation (measured as release of β-glucuronidase). Aroclor 1242 alone stimulated O2- production at a concentration of 10 μg/ml. Significant cytotoxicity was not observed under these conditions. This concentration of Aroclor 1242 also increased O2- generation in PMNs activated with 20 ng PMA/ml. In the presence of a concentration of PMA (2 ng/ml) that by itself did not stimulate production of O2-, 1 μg Aroclor 1242/ml caused significant generation of O2-, indicating synergy between Aroclor 1242 and PMA. Aroclor 1242 caused release of β-glucuronidase from quiescent PMNs; however, in PMNs stimulated with fMLP to undergo degranulation, Aroclor 1242 inhibited release of β-glucuronidase. The effects of two PCB congeners, one that binds to the Ah receptor (3,3',4,4'-tetrachlorobiphenyl) and one that has little affinity for this receptor (2,2',4,4'-tetrachlorobiphenyl) were examined. 3,3',4,4'-Tetrachlorobiphenyl had no effect on PMN function in vitro, whereas 2,2',4,4'-tetrachlorobiphenyl had effects similar to those observed with Aroclor 1242. These results indicate that PCBs affect PMN function in vitro in a complex manner, stimulating or inhibiting function under different conditions. These effects are apparently not mediated through the Ah receptor. Key words: Ah receptor, degranulation, neutrophils, polychlorinated biphenyls, superoxide anion. Environ Health Perspect 101:430–434 (1993)

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants. Exposure to PCBs is associated with a variety of biological effects including induction of enzymes involved in xenobiotic metabolism, alterations in reproductive function, hepatotoxicity, carcinogenicity, and dermal lesions. Thymic atrophy is a consistent finding in PCB-treated animals, and alterations in immunity after exposure to PCBs have been reported (1). For example, monkeys fed Aroclor mixtures of PCBs for 11 months had lower anti-sheep red blood cell titers and decreased concentrations of γ-globulin compared to controls (2). The splenic plaque-forming cell response to sheep red blood cells was suppressed in mice exposed acutely to Aroclor mixtures of PCBs (3) or to the coplanar PCB congener 3,3',4,4'-tetrachlorobiphenyl (4). In humans accidentally exposed to PCBs, decreases in the total number of T-lymphocytes (4) and in the percentage of peripheral T-lymphocytes (5), as well as in the concentrations of IgM and IgA in serum (5), were reported.

Although effects of PCBs on cell-mediated and humoral immunity have been investigated, little is known about the effects of PCBs on polymorphonuclear leukocytes (PMNs), which contribute to nonspecific immunity. One study reported that 3,3',4,4'-tetrachlorobiphenyl decreased generation of leukotriene B4 from human PMNs stimulated with sodium fluoride (6). Because sodium fluoride activates G-proteins, which results in metabolism of arachidonic acid and subsequent formation of leukotrienes, the authors speculated that the interaction of sodium fluoride and PCBs with PMNs induced synthesis of a set of inhibitory G-proteins that reduced leukotriene formation. Interestingly, in the same study, exposure to 3,3',4,4'-tetrachlorobiphenyl increased leukotriene B4 release from opsonized zymosan-stimulated PMNs and from PMNs pretreated with sodium fluoride, suggesting that PCBs enhance the generation of leukotrienes once production has been initiated. Thus, one function of PMNs is either inhibited or augmented, depending on the experimental conditions. To extrapolate this observation to possible consequences if this happened in vivo, suppression of PMN function by PCBs could lead to increased susceptibility to infection. Alternatively, activation of PMNs has been implicated in tissue injury in a variety of disease models.

To begin to explore possible alterations in PMN function caused by PCBs, the effect of in vitro exposure to Aroclor 1242, a complex mixture of PCB congeners, on PMN function was evaluated. Some biological effects of PCBs, for example, induction of hepatic aryl hydrocarbon hydroxylase activity (7), are mediated through binding to the Ah receptor, a cytosolic receptor that binds to PCBs. The resultant receptor–ligand complex translocates to the nucleus and alters gene expression to initiate toxic and other biologic effects. Binding to the Ah receptor and biological activity depend on the structure of individual congeners, such that coplanar PCB congeners (e.g., 3,3',4,4'-tetrachlorobiphenyl) have the greatest affinity for the receptor (7). To test the potential role of binding to the Ah receptor in initiating alterations in PMN function produced by Aroclor 1242, we examined the effects of two PCB congeners, one which binds with high affinity to the Ah receptor and one that has little affinity for the receptor (8).

Materials and Methods

Aroclor 1242, 2,2',4,4'-tetrachlorobiphenyl (>99% pure), and 3,3',4,4'-tetrachlorobiphenyl (>99% pure) were purchased from ChemService (West Chester, Pennsylvania). Phorbol myristate acetate (PMA) was purchased from LC Services (Woburn, Massachusetts), and superoxide dismutase (SOD) was obtained from Diagnostic Data, Inc. (Mountainview, California). We obtained [3H]-2,3,7,8-tetrachlorodibenzo-p-dioxin ([3H]TClDD) and 2,3,7,8-tetrachlorodibenzo-p-dioxin, used in determining the presence of Ah receptors in rat PMNs, from Stephen Safe (Texas A & M University). All other chemicals were of the highest grade commercially available.

We isolated glycogen-elicited PMNs from the peritoneum of male Sprague-Dawley, retired breeder rats [CD-1CRE:CD (SD)BR VAF/Plus; Charles River Laboratories, Portage, Michigan], as described previously (9). Briefly, 30–40 ml of 1% glycogen in sterile saline was injected into the peritoneum of rats anesthetized with diethyl ether. Four hours later the rats were anesthetized again with diethyl ether and were killed by decapitation. We rinsed the peritoneum with 30 ml of 0.1 M phosphate-buffered saline (PBS) containing 1 U/ml heparin. The rinse solution was filtered through gauze and centrifuged for 7 min at 500g. We lysed contaminating red blood cells in 15 ml of 0.15 M NaCl, and then centrifuged the PMNs for 7 min at 300g. Cells were washed once with PBS,

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then resuspended in Hanks’ balanced salt solution (HBSS). The final concentration of PMNs in the assays was 2 x 10^6 cells/ml. The percentage of PMNs in the cell preparation was routinely >95%, and viability (i.e., ability to exclude trypan blue dye) was ≥95%.

PMNs were stimulated with either N-formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence of cytochalasin B (5 µg/ml) or with PMA. Concentrations of fMLP and PMA were chosen based on previous studies in which these concentrations were shown to stimulate degranulation (9) or superoxide anion production (10), respectively.

For measurement of superoxide anion (O_2^-) generation, Aroclor 1242 was dissolved in methanol, 3,3',4,4'-tetrachlorobiphenyl was dissolved in dimethylformamide, and 2,2',4,4'-tetrachlorobiphenyl was dissolved in 50% acetone/50% dimethylsulfoxide. We added 1 µl of the stock solutions to 1 ml of HBSS containing the suspended PMNs to achieve the desired final concentrations. Control PMNs received an equivalent volume of the appropriate vehicle. Preliminary studies indicated that none of the vehicles affected O_2^- production at the concentrations used.

We incubated PMNs at 37°C with PCBS for 30 min. PMA was then added, and samples were incubated for an additional 10 min. We measured O_2^- generated during this 40-min period spectrophotometrically as the SOD-sensitive reduction of ferricytochrome C (11). For every sample, two tubes were incubated: one to which SOD (85 U/ml) was added before incubation and one to which SOD was added after incubation. We used the difference in absorbance (550 nm) of the cell-free supernatant fluids from these two tubes to estimate the amount of cytochrome C reduced, using an extinction coefficient of 18.5 cm·mM⁻¹.

To study the degranulation response, PMNs were incubated with PCBS at 37°C in the presence of cytochalasin B for 10 min. We then added fMLP and incubated the cells for another 10 min. The activity of β-glucuronidase in the cell-free supernatant fluids was measured as the release of phenolphthalein from its glucuronide at pH 4.5 during incubation at 37°C (12). We determined total β-glucuronidase activity in PMNs lysed with 1% Triton X-100 and sonication, and values are presented as the percentage of total β-glucuronidase released into the buffer. The presence of PCBS did not interfere with determination of the activity of β-glucuronidase.

We estimated cytotoxicity from release of the cytosolic enzyme lactate dehydrogenase (LDH). PMNs were incubated with PCBS in the absence or presence of PMA as described above for measurement of generation of O_2^-.

We determined the activity of LDH in the cell-free supernatant fluids according to the method of Bergmeyer and Bernt (13). A separate aliquot of PMNs was lysed with Triton X-100 and sonication, and total LDH activity was determined in the cell-free supernatant fluids from these lysates. Cytotoxicity is expressed as the percentage of total LDH activity released into the buffer. The presence of PCBS did not interfere with determination of the activity of LDH.

Specific binding of [³H]TCDD to cytosol from rat peritoneal PMNs was determined using the hydroxylapate binding assay (14). Receptor concentrations are presented as femtomoles of [³H]TCDD specifically bound per milligram of protein.

Results

Quiescent PMNs produced no O_2^-; and, with fMLP and Aroclor 1242 up to 1 µg/ml did not stimulate generation of O_2^- (Fig. 1). When PMNs were exposed to Aroclor 1242 at a concentration of 10 µg/ml, a significant amount of O_2^- was produced. Incubation of PMNs with 20 ng PMA/ml, but not 2 ng PMA/ml, caused significant generation of O_2^- compared to quiescent PMNs. A significant increase in O_2^- generation was observed at 1 and 10 µg/ml Aroclor 1242 when PMNs were treated with 2 ng PMA/ml. In PMNs activated with 20 ng PMA/ml, effects of Aroclor 1242 were similar to those seen in the absence of PMA: increased production of O_2^- was only observed at 10 µg Aroclor 1242/ml.

About 7% of the total β-glucuronidase activity appeared in the medium above quiescent PMNs (Fig. 2). Incubation with Aroclor 1242 at concentrations of 1 µg/ml and higher increased release of β-glucuronidase from PMNs. In the absence of Aroclor 1242, a concentration-related increase in the percentage of β-glucuronidase released by control PMNs was observed with fMLP stimulation (Fig. 2). Exposure to Aroclor 1242 did not affect release of β-glucuronidase from PMNs stimulated with 1 nM fMLP. In PMNs stimulated with higher concentrations of fMLP, Aroclor 1242 (10 µg/ml) inhibited degranulation in response to fMLP.

LDH release was increased when PMNs were incubated with 10 µg Aroclor 1242/ml (Fig. 3). These values reached statistical significance only when PMNs were co-incubated with PMA.

Because some effects of PCBS are mediated through the Ah receptor, it was of
interest to determine the presence and concentration of the Ah receptor in rat PMNs. In cytosol from glycogen-elicited PMNs, 14.2 ± 2.5 fmol \(^{3}H\)TCDD/mg protein were specifically bound. This value is consistent with that reported for mouse peritoneal PMNs (18–25 fmol/mg protein) (15).

Exposure to 3,3',4,4'-tetrachlorobiphenyl did not affect generation of O\(_{2}^{-}\) in the absence or in the presence of PMA (Fig. 4A). Release of \(\delta\)-glucuronidase from PMNs was also unaffected by incubation with 3,3',4,4'-tetrachlorobiphenyl (Fig. 4B). This lack of effect was observed in both the absence and the presence of fMLP. LDH release by PMNs was unaffected by exposure to 3,3',4,4'-tetrachlorobiphenyl (Fig. 5).

In the absence of PMA, 2,2',4,4'-tetrachlorobiphenyl did not stimulate production of O\(_{2}^{-}\) (Fig. 6A). In the presence of PMA, concentrations of 2,2',4,4'-tetrachlorobiphenyl of 1 and 10 \(\mu\)g/ml increased generation of O\(_{2}^{-}\).

In the absence of fMLP, 2,2',4,4'-tetrachlorobiphenyl did not significantly affect release of \(\delta\)-glucuronidase from PMNs (Fig. 6B). Beta-glucuronidase release from PMNs exposed to 2,2',4,4'-tetrachlorobiphenyl was not affected by addition of fMLP at a concentration of 1 nM. When PMNs were activated with higher concentrations of fMLP, exposure to 2,2',4,4'-tetrachlorobiphenyl (1 or 10 \(\mu\)g/ml) inhibited the fMLP-stimulated release of \(\delta\)-glucuronidase.

Exposure to 10 \(\mu\)g 2,2',4,4'-tetrachlorobiphenyl/ml increased release of LDH, and this was only statistically significant when PMNs were also exposed to PMA (Fig. 7).

### Discussion

In the present study, the function of rat PMNs was affected by exposure to PCBs in vitro. Aroclor 1242 stimulated O\(_{2}^{-}\) generation in a dose-related manner (Fig. 1). Production of O\(_{2}^{-}\) by PMA-activated PMNs was also altered. Two concentrations of PMA were used in these studies: one that does not cause significant production of O\(_{2}^{-}\) (2 ng/ml), and one that does (20 ng/ml). The purpose of using the smaller concentration of PMA was to allow detection of synergy between PCBs and a known activator of PMNs. Such a synergistic effect was observed with Aroclor 1242: the concentration–response curve to Aroclor 1242 shifted to the left in the presence of 2 ng PMA/ml. This effect on O\(_{2}^{-}\) production occurred in the absence of significant cytotoxicity to the PMNs, decreasing the likelihood that the increased generation of oxygen free-radicals occurred in response to cell injury. The mechanism by which PCBs enhance O\(_{2}^{-}\) generation by PMNs is unknown. PCBs might increase O\(_{2}^{-}\) generation through effects on protein kinase C (PKC). Activation of PKC is associated with generation of O\(_{2}^{-}\) in PMNs stimulated with PMA (16), and PCBs have been reported to activate PKC directly (17–19). Alternatively, PCBs may increase the rate of generation of O\(_{2}^{-}\).

Several reports suggest that PCBs have effects on immunity. For example, exposure to PCBs in vivo caused suppression of humoral immunity in mice and monkeys (1–3,20). In mice this effect was related to the chlorine content of PCB mixtures: those mixtures with a higher percentage of chlorine were more potent immunosuppressants (3). Many of the biological effects of PCBs and other halogenated aromatic hydrocarbons correlate with binding affinity for the Ah receptor (8), with TCDD having the highest affinity for the receptor. Accordingly, several of the immunotoxic effects of PCBs appear to be mediated through the Ah receptor. Treatment of mice with a coplanar congener (3,3',4,4'-tetrachlorobiphenyl) that binds to the Ah receptor with high affinity resulted in inhibition of the primary direct splenic antibody response, whereas treatment with a congener with lower affinity for the Ah receptor (2,2',5,5'-tetrachlorobiphenyl) did not alter this response (4). In addition, 3,3',4,4'-tetrachlorobiphenyl was immunotoxic in C57BL/6J mice, which are sensitive to TCDD and have high affinity Ah receptors, but not in DBA/2J mice, which have defective Ah receptors. These results suggest that the Ah receptor may play a role in producing immunotoxic effects.

This hypothesis is supported by the observation that TCDD itself is immunotoxic. Cell-mediated immunity was sup-
Figure 6. Superoxide anion generation (A) and release of β-glucuronidase (B) by PMNs exposed to 2,2',4,4'-tetrachlorobiphenyl. Experiments were performed as described in the legends to Figures 1 and 2. The vehicle used for controls was 50% acetone/50% dimethylsulfoxide. Total β-glucuronidase activity was 1.35 ± 0.16 U/10^6 PMNs. (A) n = 5; (B) n = 4. *Significantly different from respective value with 0 µg 2,2',4,4'-tetrachlorobiphenyl/ml; **significantly different from respective value without PMN stimulus.

Figure 7. Release of lactate dehydrogenase (LDH) from PMNs exposed to 2,2',4,4'-tetrachlorobiphenyl. PMNs were incubated as described in the legend to Figure 1, and LDH activity released by the PMNs was determined as described in Materials and Methods. Total LDH activity was 58 ± 7 U/10^6 PMNs (n = 5). *Significantly different from respective value with 0 µg 2,2',4,4'-tetrachlorobiphenyl/ml.

enzymes, whereas 3,3',4,4'-tetrachlorobiphenyl binds to the Ah receptor with high affinity and causes induction of drug-metabolizing enzymes similar to that seen with TCDD (8). Effects on PMN function similar to those seen with Aroclor 1242 were observed when PMNs were exposed to 2,2',4,4'-tetrachlorobiphenyl (Fig. 6A), but 3,3',4,4'-tetrachlorobiphenyl did not alter O2- production by PMNs (Fig. 4A).

The difference between results presented here and those observed in mouse PMNs after exposure in vivo may be due to the difference in exposure regimens (i.e., in vivo versus in vitro) or the choice of species. In addition, although [3H]TCDD bound specifically to cytosol from rat PMNs, indicating the presence of Ah receptors that bind ligand, it is not known whether these receptors are functional.

It is unlikely that 2,2',4,4'-tetrachlorobiphenyl in the Aroclor mixture accounted entirely for the effects observed with Aroclor 1242 because 2,2',4,4'-tetrachlorobiphenyl was no more potent than the mixture. It is probable that alterations in PMN function after exposure to Aroclor 1242 are at least partly due to non-coplanar PCB congeners present in the mixture and also to additive, cooperative, or synergistic effects of congeners.

Although exposure to PCBs enhanced the respiratory burst of PMNs in vitro, the effects on degranulation were more complex. In unstimulated PMNs and in PMNs treated with a subthreshold concentration of fMLP, exposure to Aroclor 1242 caused release of β-glucuronidase from the cells. However, Aroclor 1242 attenuated fMLP-induced enzyme release. fMLP binds to a specific receptor to stimulate degranulation, and one possible explanation for the effects seen with Aroclor 1242 is that it acts as a weak or partial agonist at the fMLP receptor. Alternatively, the Aroclor 1242-induced stimulation of degranulation in quiescent PMNs and the inhibition of fMLP-induced activation may occur by two different mechanisms. These effects occurred only at a concentration of Aroclor 1242 at which cytotoxicity was observed, and it cannot be ruled out that cytotoxicity to the PMNs contributed to inhibition of degranulation.

As with effects on generation of O2-, effects of PCBs on degranulation do not appear to be mediated through the Ah receptor, because 3,3',4,4'-tetrachlorobiphenyl was ineffective but 2,2',4,4'-tetrachlorobiphenyl produced effects similar to Aroclor 1242. 2,2',4,4'-Tetrachlorobiphenyl may contribute to the effects observed with Aroclor 1242, as 2,2',4,4'-tetrachlorobiphenyl was more potent than Aroclor 1242 in inhibiting degranulation. The possibility that other congeners in the mixture or additive or synergistic effects among congeners contribute to the responses in PMNs cannot be ruled out.

In summary, exposure to PCBs in vitro alters PMN function in a complex manner. Aroclor 1242 enhances generation of O2- in quiescent and activated PMNs and stimulates degranulation in quiescent cells, but it attenuates fMLP-induced degranulation. 2,2',4,4'-Tetrachlorobiphenyl produces the same pattern of effects as Aroclor 1242, but the coplanar congener 3,3',4,4'-tetrachlorobiphenyl does not affect PMN function in vitro. These results suggest that the observed effects of PCBs on rat PMN function in vitro are not Ah receptor dependent.

Similar changes in PMN function after exposure to PCBs in vivo could contribute to altered response to infection. Recent findings with bacterial endotoxin-induced toxicity support this hypothesis. PMNs play a central role in tissue injury due to endotoxin in liver (25) and lung (26), and treatment with PCBs (2,27) or TCDD (28) increases sensitivity to endotoxin. It is not known whether the increased sensitivity to endotoxin is mediated through PMNs, but the possibility remains that alterations in PMN function caused by PCBs in vivo could affect the response to subsequent exposure to pathogens.

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