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Comparative Cholinergic Neurotoxicity of Oral Chlorpyrifos Exposures in Preweanling and Adult Rats

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Chlorpyrifos (CPF) is a common organophosphorus (OP) pesticide. Previous studies have demonstrated that neonatal rats are more sensitive than adults to the acute toxicity of high dosages of CPF. The present study examined lethality and age-related differences in neurochemical indicators and functional signs of neurotoxicity following a broad range of acute and repeated oral CPF exposures. There was about a 9-fold difference in sensitivity to the acute-dose lethality of chlorpyrifos among neonatal (7 days-of-age) and adult (90 days-of-age) rats (LD_{10}: neonates = 15 mg/kg; adults = 136 mg/kg), while juvenile rats (21 days-of-age) exhibited intermediate sensitivity (LD_{10} = 47 mg/kg). Neonatal and adult rats (n = 5–7/treatment/age group/time point) were given CPF (0, 0.15, 0.45, 0.75, 1.5, 4.5, 7.5, or 15 mg/kg/day) for 14 days and sacrificed 4 h after either the first or 14th dose for neurochemical measurements (cholinesterase activity in frontal cortex, plasma and RBC, and muscarinic ([^{3}H]QNB) and nicotinic ([^{3}H]epibatidine) receptor binding in frontal cortex. No overt signs of functional toxicity (involuntary movements, SLUD signs) were noted in either age group by 4 h after the first dose. With repeated CPF exposures, however, signs of cholinergic toxicity were noted in both age groups at the higher dose levels [no observed effect levels (NOELs): neonate = 4.5 mg/kg/day; adult = 7.5 mg/kg/day]. Similar degrees of ChE inhibition were noted in neonatal brain and blood fractions following acute exposure, but substantial ChE inhibition was only noted in adult plasma and RBC 4 h after the first treatment. Following repeated CPF exposures, similar degrees of ChE inhibition were again noted in tissues from immature animals, but a wide range of sensitivity to inhibition was noted in adult tissues. NOELs based on ChE inhibition for adults were about 1–≥10-fold higher than in neonates with acute exposure but only 0.2–2 times higher with repeated dosing. Moreover, dose-related inhibition of brain ChE was similar between age groups, and similar reductions in both QNB and epibatidine binding were noted between the age groups after repeated dosing, even though by the end of the dosing period young animals (juveniles) were still about 3 times more sensitive than adults, based on acute lethality. We conclude that while immature animals can be markedly more sensitive to lethal effects of high doses of CPF, lesser or no age-related differences are apparent, based on non-lethal endpoints, in particular with repeated exposures.

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Chlorpyrifos (CPF) is a widely used (Aspelin, 1994; Marquis, 1986) organophosphorus (OP) pesticide with relatively low acute toxicity compared to many others of this class (Pope, 1999; Pope *et al*., 1991; Richardson, 1995). CPF elicits toxicity by inhibiting acetylcholinesterase (AChE, EC 3.1.1.7) in the central and peripheral nervous systems, allowing accumulation of acetylcholine, excessive stimulation of postsynaptic acetylcholine receptors, and consequent signs of neurotoxicity. Signs of toxicity with extensive AChE inhibition include autonomic dysfunction (e.g., excessive salivation, lacrimation, urination, and defecation, referred to as SLUD signs), involuntary movements (e.g., tremors, fasciculations), respiratory dysfunction, and other symptoms (Costa *et al*., 1982; Ecobichon, 1996; Gallo and Lawryk, 1991).

Infants and children can be exposed to a variety of OP pesticides via different routes, such as by ingestion of residues on food products and by contact with contaminated surfaces after household or lawn applications. OP pesticides are toxic to the nervous system—a critically sensitive and developing organ system in the very young. Earlier reports have suggested that the fetus, infants,and children may be more susceptible than adults to OP pesticide toxicity (Fenner-Crisp, 1995; Goldman, 1995; NAS, 1993; Tilson, 1998). These concerns facilitated the enactment of the Food Quality Protection Act (FQPA) of 1996, passed to protect infants and children from pesticide exposures. FQPA now requires consideration of an additional uncertainty factor to further protect infants and children from pesticide exposures (Goldman, 1998).

Previous animal studies have generally reported that young animals are more sensitive than adults to the acute toxicity of high-level OP pesticide exposures (Benke and Murphy, 1975; Brodeur and DuBois, 1963; Gaines and Linder, 1986; Harbison, 1975). We previously estimated age-related differences in sensitivity to CPF in rats: there was about a 6-fold difference in maximum tolerated dosages between pre-weanling (7-day-old) and adult (90-day-old) rats treated subcutaneously with

CPF (Pope *et al*., 1991). Moser and Padilla (1998) also reported that 17-day-old Long-Evans rats were approximately 5–7 times more sensitive than adult rats to the behavioral and biochemical effects of oral CPF exposure. Several other studies have reported similar age-related differences in sensitivity to CPF (Atterberry *et al*., 1997; Whitney *et al*., 1995). These differences in sensitivity to CPF and other OP pesticides have generally been defined based on differential toxic responses to high acute dosages. Real-world exposures to pesticides normally occur through lower level single (acute) or repeated exposures (e.g., as residues in food products). The present studies were designed to compare neurotoxic responses in immature and adult rats to a broad range of acute and repeated oral CPF exposures, and to determine relative no-observed-effect levels (NOELs) for functional and biochemical endpoints of toxicity. We hypothesized that while immature animals are more sensitive to acute lethality from high dosages of CPF, lesser age-related differences in sensitivity exist based on non-lethal endpoints, in particular with repeated exposures.

MATERIALS AND METHODS

Chemicals

Chlorpyrifos (O,O′-diethyl–3,5,6-trichloro-2-pyridyl phosphorothioate) was purchased from Chem Service (Westchester, PA, >98% pure). [^{3}H]acetylcholine iodide (specific activity 55.2 mCi/mmol), [^{3}H]quinuclidinyl benzilate (QNB, specific activity 49 Ci/mmol) and [^{3}H]epibatidine (specific activity 48.0 Ci/mmol) were purchased from New England Nuclear, Boston, MA. All other chemicals were reagent grade.

Animals

Neonatal (7-day-old), juvenile (21-day-old) and adult (90-day-old) Sprague-Dawley rats were used throughout the experiment. Adult male rats were maintained in community cages until 1 week before treatment, at which time they were transferred to individual steel mesh cages. Pregnant females were housed individually in plastic cages and dates of birth (postnatal day 0) were recorded. Pups were routinely randomized among the dams (no selection by sex) on postnatal day 2 and both sexes were used for neonatal and juvenile treatment groups. Animals were maintained on a 12L:12D illumination cycle.

Treatments

Chlorpyrifos was dissolved in peanut oil (1 ml/kg gavage volume). For acute lethality studies, 7-day-old, 21-day-old, and 90-day-old rats were challenged with one of a series of dosages of CPF, depending on age (neonates, 3–100 mg/kg; juveniles, 10–210 mg/kg; adults, 120–400 mg/kg, n = 6–11/dosage/age group, po). Lethality was recorded over a period of seven days. For neurochemical studies, 7-day-old and 90-day-old rats (n = 5–7/treatment/age group/time point) were treated with either vehicle or CPF (0.15, 0.45, 0.75, 1.5, 4.5, 7.5, 15 mg/kg/day, po) daily for 14 consecutive days and subsets were sacrificed 4 h after either the first or the 14th dose.

Body Weight and Functional Measurements

Body weights and lethality were recorded daily prior to treatment. Functional signs of cholinergic toxicity were measured at 4 h after each exposure, essentially by the method of Moser and co-workers (1988) as described before (Liu and Pope, 1996). Briefly, the rat was held and observed ("blind" regarding treatments) for SLUD signs (1 = normal; 2 = slight; 3 = moderate; 4 = severe), and then placed on a laboratory cart and involuntary movements evaluated (2 = normal; 3 = mild tremors; 4 = whole-body tremors; 5 = myoclonic jerks; 6 = clonic convulsions).

Tissue Collection and Preparation

Four h after the first and 14th treatments, a subset (n = 5–7 per group) was sacrificed and frontal cortexes were collected. Whole blood was collected into 1.5-ml micro-centrifuge tubes containing a small volume (20 μl) of heparin (10,000 units/ml). Heparinized-blood was mixed and immediately centrifuged for separation of plasma and RBC fractions. Packed RBCs (100 μl) were subsequently washed with 1 ml isotonic saline. All samples were frozen at –70°C until the time of assay.

Before assay, cortical samples were thawed at room temperature and suspended in 50 mM Tris–HCl buffer, pH 7.4 (25°C) containing NaCl, 120 mM; KCl, 5 mM; $CaCl_2$, 2 mM; and $MgCl_2$, 1 mM. Tissues were homogenized (1:30, w/v) on ice with a Polytron PT 3000 homogenizer (Brinkman Instruments, Westbury, NY) at 28,000 rpm for 20 s. Homogenates of cortex were assayed for ChE activity and aliquots were centrifuged at 48,000 × g for 10 min at 4°C with a Beckman J2-21 centrifuge. The pellets were washed twice in an equivalent amount of Tris–HCl buffer and then used for receptor binding assays.

Biochemical Measurements

Cholinesterase assay. ChE activity was measured in frontal cortex, plasma and RBC by the radiometric method, using a final concentration of 1 mM [^{3}H]acetylcholine iodide (Johnson and Russell, 1975). Each reaction vial (0.1 ml final volume) contained 0.1% Triton X-100 to aid in tissue disruption. Preliminary experiments delineated conditions of both incubation time and tissue concentration necessary for linear rates of substrate hydrolysis. Plasma and erythrocyte samples were thawed and diluted (1:10, v/v) in the above Tris–HCl buffer before assay.

Muscarinic receptor binding assay. Muscarinic receptor density was determined in the cortex by binding of [^{3}H]quinuclidinyl benzilate, essentially as described by Yamamura and Snyder (1974). Cortical membranes, 100 μl (100–150 μg protein) were incubated for 60 min at 37°C with [^{3}H]QNB (0.75 nM final concentration) in a total volume of 2 ml, then filtered and washed (3 ml, 3×) over Whatman GF/C paper using a receptor binding harvestor (Brandel Model M-24, Gaithersburg, MD). Filters were removed and counted in 4 ml of scintillation fluid (Formula-989, Packard Instrument Co., Meriden, CT) at 43% efficiency in a Beckman LS 3801 scintillation counter. Specificity was determined by inclusion of atropine (10 μM final concentration) in paired samples and calculated as the difference in binding between tissues incubated in the presence and absence of atropine.

Nicotinic receptor binding assay. Nicotinic receptor ([^{3}H]epibatidine) binding was measured essentially by the method of Houghtling and coworkers (1995). Cortical membranes, 100 μl (100–150 μg protein) were incubated for 4 h at room temperature with [^{3}H]epibatidine (1 nM final concentration) in a total volume of 0.25 ml. Filtration, washing and scintillation counting were as described above for the [^{3}H]QNB binding assay. Specificity was determined as the difference in binding between tissues incubated in the presence and absence of nicotine (0.3 mM final concentration). Filters were presoaked in 1% polyethylenimine to reduce nonspecific binding.

Protein content of frontal cortex samples was estimated by the method of Lowry and coworkers (1951) using BSA (bovine serum albumin) as a standard.

Statistical Analysis

Acute lethality, enzyme activities, and receptor-binding data were calculated and plotted as percent lethality, percent inhibition, or percent of control vs. log dosage. Cholinesterase activities were expressed as nmol/min/mg protein (frontal cortex) or nmol/min/μl blood fractions (plasma, RBC). Receptor binding data were expressed as pmol/mg protein (QNB) or fmol/mg protein (epibatidine).

Functional signs of toxicity were analyzed using the Pearson Chi-square test. Body weight data were evaluated using repeated measures analysis of variance

TABLE 1
Combined Control Values for Neurochemical Cholinergic Endpoints in Different Age Groups

Age	Exposure	ChE activity[a]			QNB binding[b]	Epibatidine binding[c]
		Cortex	Plasma	RBC		
Neonate	Acute	77 ± 10	0.25 ± 0.02	0.51 ± 0.03	0.44 ± 0.03	62 ± 7
	Repeated	71 ± 4	0.23 ± 0.03	0.58 ± 0.10	1.35 ± 0.04	84 ± 5
Adult	Acute	196 ± 23	0.29 ± 0.04	0.57 ± 0.03	1.07 ± 0.01	58 ± 3
	Repeated	121 ± 25	0.41 ± 0.03	0.60 ± 0.09	1.28 ± 0.01	46 ± 11

[a] ChE activity in cortex was reported as nmol ACh hydrolyzed/min/mg protein whereas activity in plasma and RBC was expressed as nmol/min/ul blood fractions (mean ± SE, n = 5–7/group).
[b] QNB binding was expressed as pmol/mg protein (mean ± SE, n = 5–7/group).
[c] Epibatidine binding was expressed as fmol/mg protein (mean ± SE, n = 5–7/group).

(ANOVA) followed by linear contrasts using the JMP statistical package (JMP, 1995). Data for ChE activity and QNB and epibatidine binding were tested for significance by ANOVA, followed by Dunnett's method using the JMP program and were reported as the mean ± SE. LD_{10} and ED_{50} values were calculated using the GraphPad Prism® nonlinear regression (fit) program with a 4-parameter logistic equation analogous to the Hill equation (DeLean *et al*., 1978; Motulsky *et al*., 1994–1995). Probability levels of 0.05 were considered significant.

RESULTS

Table 1 shows control values for the biochemical endpoints examined. In general, brain cholinesterase activity and muscarinic receptor binding were lowest in neonates and increased with age. There appeared to be little age-related change in plasma cholinesterase activity, red blood cell cholinesterase activity, or cortical epibatidine binding.

Acute Lethality

Figure 1 shows the acute lethality of oral CPF exposures in neonatal, juvenile, and adult rats. Estimation of LD_{10} values (15 mg/kg, 47 mg/kg, and 136 mg/kg for neonates, juveniles, and adults, respectively) indicated about a 9-fold difference in sensitivity to CPF between neonates and adults. In the repeated dosing studies with the highest dosage (15 mg/kg/day), one neonate died between 4 and 24 h after the first exposure; peak incidence of lethality (3/7) occurred on day 3, and all (7/7) died by day 7. One adult (1/6) died on day 6 in the high-dosage group (15 mg/kg/day). No animals of either age group died in any other treatment groups.

Body Weight Changes

Body weight changes in neonates and adults following repeated CPF exposures are shown in Figure 2. In neonates, body weights in the higher-dosage groups (4.5 mg/kg/day and 7.5 mg/kg/day) were significantly reduced from 12 and 4 days after treatment, respectively, (Fig. 2A). In adults, body weight changes were only noted in the highest dosage group (15 mg/kg/day) from days 10 to 14 of treatment (Fig. 2B). The NOEL based on body weight changes for adults was therefore 5-fold higher than in neonates (7.5 mg/kg/day vs. 1.5 mg/kg/day) (Table 2).

Functional Changes

No overt signs of cholinergic toxicity were noted in either neonates or adults 4 h after the first dose of CPF. With repeated dosing, however, functional signs of toxicity (SLUD, involuntary movements) were observed following 15 mg/kg/day in neonates (7/7, starting between 4 and 24 h after exposure) and adults (2/6, starting at day 3), and with 7.5 mg/kg/day in neonates only (2/6, starting at day 7). The NOEL for adults based on functional signs of toxicity was thus 1.7-fold higher than for neonates, following repeated exposures (Table 2).

Cholinesterase Inhibition

Figure 3A shows cholinesterase activity in the various tissues in neonatal rats following acute CPF exposure. Cholines-

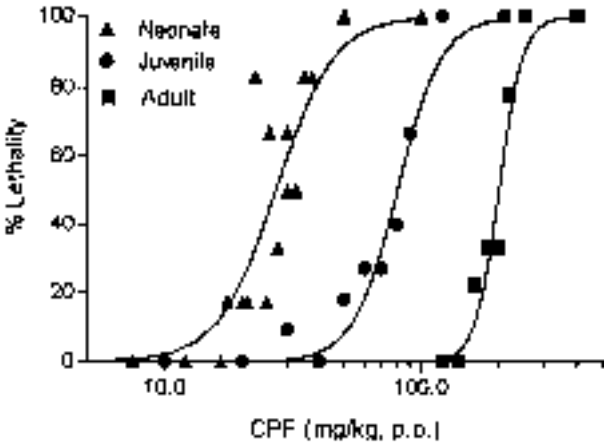

FIG. 1. Acute lethal effects of oral chlorpyrifos in neonatal (7-day-old), juvenile (21-day-old) and adult rats (90-day-old). Neonates, juveniles, and adults (n = 6–11 per dosage group) were dosed with CPF and lethality was recorded daily for 7 days. Dose ranges depended on age (neonatal, 3–100 mg/kg; juvenile, 10–210 mg/kg; adult, 120–400 mg/kg).

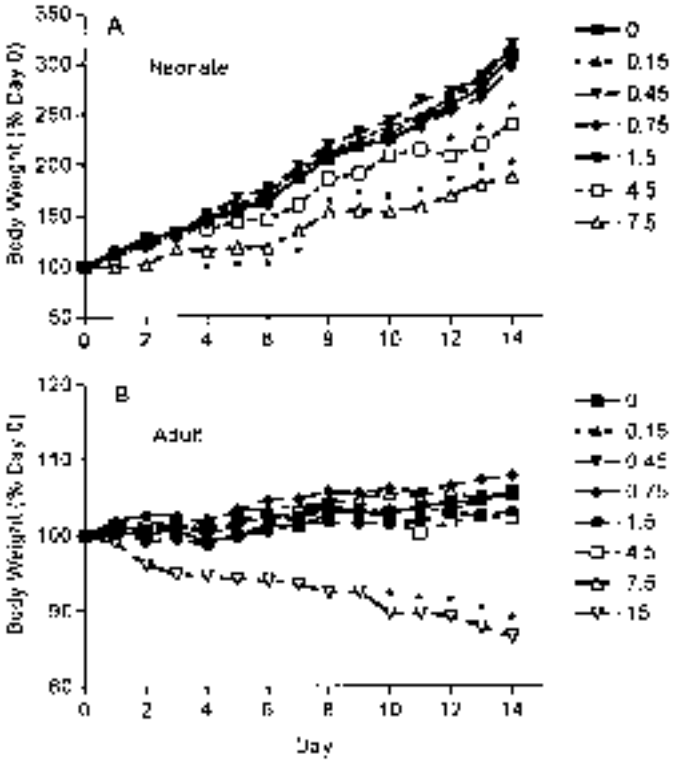

FIG. 2. Mean body weights in neonates and adults after repeated exposures to CPF (0.15, 0.45, 0.75, 1.5, 4.5, 7.5, 15 mg/ kg /day, po). Neonatal (A) and adult (B) rats were treated daily with CPF for 14 days and body weights were recorded daily. Data represent the percent (mean ± SE, n = 5–7/ treatment/time point) of Day 0. Asterisks indicate significant differences from contemporaneous control values ($p < 0.05$). Neonatal group body weights at the beginning of the treatments (Day 0) were: control: 14.8 ± 0.4 g, 0.15-mg/ kg/day: 14.2 ± 0.6 g, 0.45-mg/kg/day: 14.0 ± 1.4 g, 0.75-mg/kg/day: 14.6 ± 1.2 g, 1.5-mg/kg/day: 13.8 ± 0.7 g, 4.5-mg/kg/day: 14.4 ± 1.1 g, and 7.5-mg/kg/day: 14.6 ± 1.3 g. All (7/7) neonatal rats in the 15-mg/kg/day group died by day 7; their body-weight data is not presented. Group average adult body weights at the beginning of the treatments (Day 0) were: control: 332 ± 23 g, 0.15-mg/kg/day: 332 ± 15 g, 0.45-mg/kg/day: 316 ± 17 g, 0.75 mg/kg/day: 311 ± 14 g, 1.5-mg/kg/day: 326 ± 14 g, 4.5-mg/kg/day: 322 ± 14 g, 7.5-mg/kg/day: 327 ± 19 g, and 15-mg/kg/day: 325 ± 15 g.

terase activity was inhibited in a relatively similar manner in neonatal brain and blood fractions in a dose-dependent manner (ED_{50} = 1.5–2.9 mg/kg). Peak inhibition of ChE was 94%, 76%, and 84% in RBC, plasma, and frontal cortex respectively. Figure 3B shows ChE activity in adult rats 4 h after the first dose. Plasma and erythrocyte ChE activity were inhibited in a dose-dependent manner (ED_{50}: plasma = 3.9 mg/kg; RBC = 4.4 mg/kg). Although about 80% inhibition was noted in plasma and RBC at the highest dosage, there was no significant difference in cortical ChE activity between control and treated groups 4 h after acute exposure. Following acute CPF exposure, more extensive ChE inhibition was noted in neonates than in adults (especially in the brain) with NOELs based on ChE inhibition in adult tissues being 1 to ≥10-fold higher than in neonates (Table 2).

With repeated exposures, degrees of ChE inhibition were again relatively similar among the tissues in neonates (ED_{50} = 1.2–2.2 mg/kg, Fig. 3C). In adults, ChE activities were inhibited in a dose-dependent manner in the various tissues and there was a broad range of sensitivities (ED_{50}: cortex = 3.3 mg/kg/day; plasma = 1.5 mg/kg/day; RBC = 0.5 mg/kg/day) (Fig. 3D). Relatively similar levels of ChE inhibition were noted in frontal cortex in both neonates and adults following repeated dosing, however (ED_{50}: adult = 3.3 mg/kg; neonate = 2.2 mg/kg). With repeated exposures, NOELs based on ChE inhibition in adults were only 0.2–2 fold higher than in neonates (Table 2).

QNB Binding

Figure 4 shows changes in cortical muscarinic receptor ([^{3}H]QNB) binding following acute and repeated CPF. QNB binding was not affected 4 hrs after treatment in either age group (Fig. 4A). Following repeated dosing (7.5 mg/kg/day), however, cortical QNB binding was reduced 23% in adults and 28% in neonates (Fig. 4B).

Epibatidine Binding

Changes in cortical nicotinic receptor ([^{3}H]epibatidine) binding following acute and repeated CPF are shown in Figure 5. As with QNB binding, epibatidine binding did not change significantly in either age group following acute exposure (Fig. 5A). Four h after the 14th dose, however, CPF (7.5 mg/kg/day) reduced cortical epibatidine binding 32% in adults and 25% in neonates (Fig. 5B).

DISCUSSION

We compared functional signs of toxicity, body weight changes, ChE activity, and cholinergic receptor binding in neonatal and adult rats following daily CPF exposure to evaluate the magnitude of age-related differences in toxicity, following acute and repeated oral exposures to this pesticide. Lethality following high, acute exposures was elicited in neonatal rats by approximately 9 times lower dosages compared to adults (LD_{10}: neonate = 15 mg/kg, adult = 136 mg/kg, po). Four h after acute exposure, ChE activity in neonates was inhibited similarly in plasma, RBC, and cortex (ED_{50} = 1.5–2.9 mg/kg) while in adults, significant ChE inhibition was noted only in plasma and RBC. Following repeated dosing, ChE activity in neonatal tissues also showed similar dose-related levels of inhibition while marked tissue differences in sensitivity were noted in adults. While juvenile rats (21 days-of-age) were about 3 times more sensitive than adults to the lethal effects of acute CPF exposure, very similar cholinergic neurochemical changes were induced in adults and neonates (7 days-of-age at the beginning of treatments) treated for 14 consecutive days, and thus evaluated at 21 days-of-age.

As noted above, similar degrees of ChE inhibition were noted in target (brain) and non-target (plasma, RBC) tissues in

TABLE 2
No-observed-effect Levels (NOELs) Based on Different Endpoints following Acute and Repeated Chlorpyrifos Exposures in Neonatal and Adult Rats

			Cholinesterase inhibition				
	Body weight	Signs	Brain	RBC	Plasma	QNB binding	Epibatidine binding
Acute							
Neonate	NA	NA	1.5	0.75	0.15	NA	NA
Adult	NA	NA	≥15	0.75	1.5	NA	NA
Adult/neonate	NA	NA	≥10	1	10	NA	NA
Repeated							
Neonate	1.5	4.5	0.75	0.75	0.75	4.5	4.5
Adult	7.5	7.5	1.5	0.15	0.45	4.5	4.5
Adult/neonate	5	1.7	2	0.2	0.6	1	1

Note. NOELS are defined as the highest dosage that caused no significant change in the endpoint. NA = not applicable.

neonates but marked tissue differences were observed in adults following either acute or repeated dosing. Similar tissue-dependent differences in ChE inhibition between immature and adult rats have been previously reported (Moser and Padilla, 1998). In earlier studies from our laboratory evaluating the anticholinesterase potency of subcutaneously-administered CPF in neonatal and adult rats (Pope *et al.*, 1991; Pope and Chakraborti, 1992), relatively similar degrees of inhibition were noted in blood and brain cholinesterases in both age groups. It therefore appears that blood and brain ChE in adults vary markedly in sensitivity to *in vivo* inhibition following oral but not subcutaneous CPF exposures while tissue differences in response are minimal in neonates regardless of the route of exposure. These age-related differences in tissue response to ChE inhibition are likely due to both substantial differences in hepatic biotransformation capacity between the age groups (Benke and Murphy, 1975; Atterberry, *et al.*, 1997; Mortensen, *et al.*, 1996) and the opportunity for first-pass metabolism with the oral compared to subcutaneous route of exposure.

While both plasma and RBC activities were inhibited in adults in a dose-related manner by acute CPF, cortical ChE activity was not significantly inhibited. The dosages used in these studies were based on neonatal sensitivity and were thus low relative to acute lethal dosages in adults (LD_{10} = 136 mg/kg). It is therefore not surprising that target enzyme inhibition following acute exposure in adults was not noted. Another reason for the lack of significant cortical ChE inhibition in adults after acute exposure is the time of assessment, i.e., other studies suggest that the time of peak brain ChE inhibition is different in neonates (4 h) from that time in adults (between 4 and 24 h) (Moser and Padilla, 1998; Won *et al.*, 1999).

Although there was substantial ChE inhibition in all 3 tissues in neonates and in plasma and RBC of adults with higher dosages, no overt signs of toxicity were noted in any treatment groups 4 h after acute exposures. The lack of signs of toxicity in neonates four h after acute exposure (even though the highest dosage was the LD_{10}) is related to the fact that signs of toxicity (and lethality) generally developed later (>4 h after CPF administration).

It is apparent from a number of studies that neonatal rats are more sensitive to acute toxicity following either oral or subcutaneous acute high dosages of CPF (Atterberry *et al*., 1997; Moser and Padilla, 1998; Pope and Chakraborti, 1992; Pope *et al*., 1991). Age-related differences in sensitivity appear lesser in magnitude with lower-level exposures, however. For example, while there was about a 6-fold higher sensitivity of neonatal, compared to adult, rats to acute subcutaneous CPF exposures based on lethality, differences in sensitivity based on brain ChE inhibition (ED_{50}s) were only 2–3 fold (Pope and Chakraborti, 1992; Pope and Liu, 1997). In the present study, neonatal rats were about 9 times more sensitive than adults to oral CPF based on lethality. The much higher sensitivity of the young animal, when comparing lethal exposures, may be partly due to maturational differences in feedback inhibition of acetylcholine release (Disko *et al*., 1998; Pedata *et al*., 1983), i.e., autoreceptor-mediated inhibition of transmitter release in rat brain is essentially absent at postnatal day 7 but develops over the next few weeks. Age-related differences in this adaptive mechanism would most likely only be important with very high levels of anticholinesterase exposures causing extensive AChE inhibition. On the basis of body weight changes following repeated dosing, the NOEL for adults was 5-fold higher than for neonates (7.5 vs. 1.5 mg/kg/day). Using functional signs (SLUD signs, involuntary movements) following repeated dosing as the endpoint of toxicity, even lesser age-related differences were noted (NOELs: adults = 7.5 mg/kg/day; neonates = 4.5 mg/kg/day). Thus, age-related differences in sensitivity to CPF appear greater in magnitude when based on lethality compared to non-lethal functional or biochemical endpoints.

While the blood ChE level (either plasma or RBC) is generally considered a sensitive marker of anticholinesterase exposure, brain ChE inhibition can be the most sensitive indicator of neurotoxicity following OP pesticide exposures. In previous

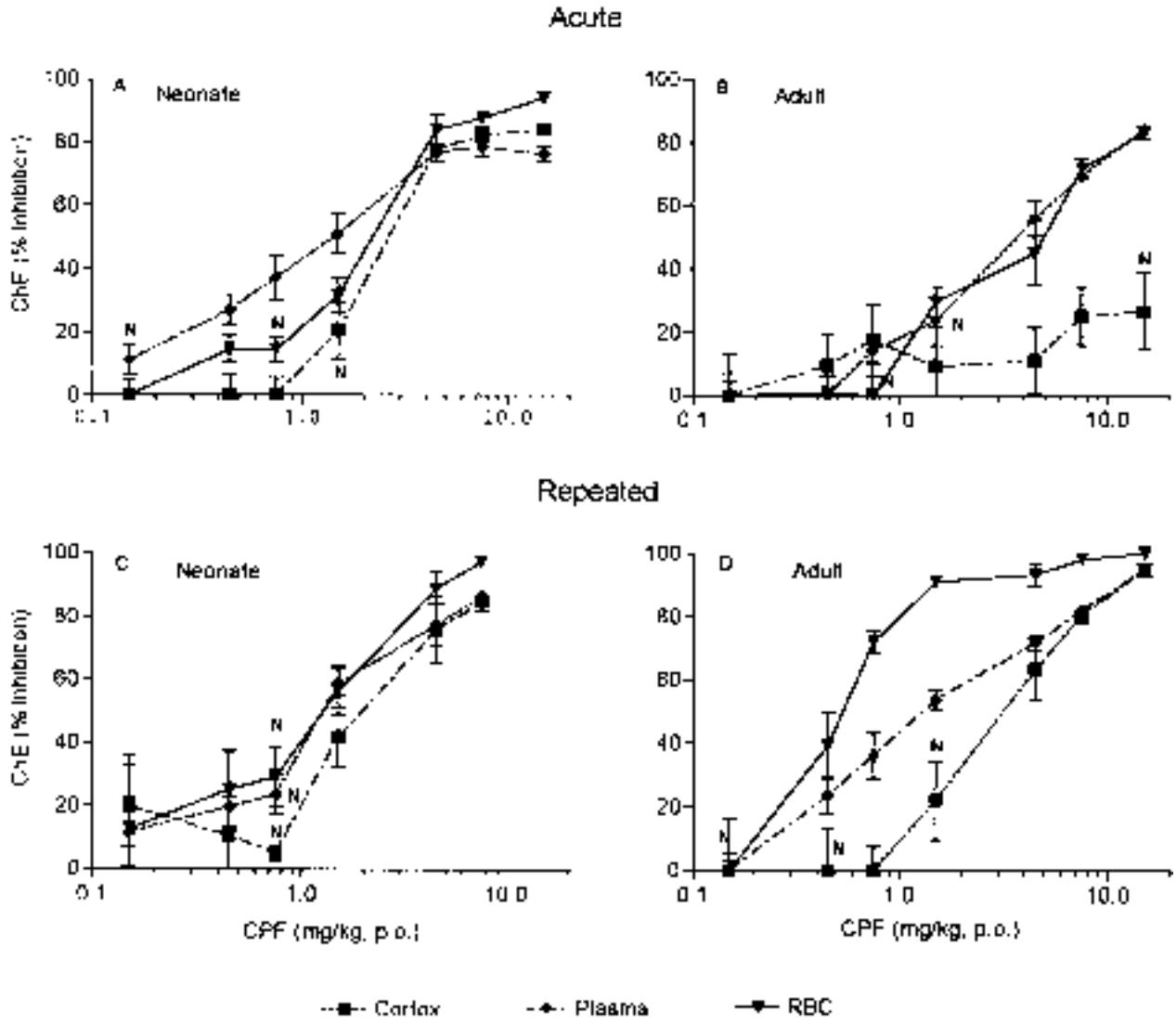

FIG. 3. Effects of chlorpyrifos (CPF: 0.15, 0.45, 0.75, 1.5, 4.5, 7.5, and 15 mg/kg/day, po) on cholinesterase (ChE) activity in cortex, plasma, and RBC. Neonatal and adult rats were treated daily with CPF for 14 days and sacrificed 4 h after either the first (A, B) or 14th (C, D) doses. Data represent the percent (mean ± SE, n = 5–7/treatment/time point) of inhibition. Control values are listed in Table 1. An "N" indicates the highest dosage without significant change (NOEL). NOELs for different endpoints are listed in Table 2.

studies comparing neurochemical responses of neonatal and adult rats to daily subcutaneous chlorpyrifos exposures (5- or 10 mg/kg/day, Liu *et al*., 1999), very similar changes in ChE activity, total muscarinic receptor ([^{3}H]QNB), and M2-preferential muscarinic receptor ([^{3}H]AF-DX 384) binding were noted throughout the 14-day exposure period. In the present studies, neonatal brain ChE activity was inhibited at lower acute dosages compared to adult brain ChE (Fig. 3A). Likewise, similar cholinergic neurochemical changes (ChE activity, QNB binding, and epibatidine binding) were also noted in frontal cortex in both age groups, following repeated exposures in the present studies (Fig. 3B).

Collectively, NOELs based on ChE inhibition for adults were 1 to ≥10-fold higher than in neonates with acute exposures but only 0.2–2 fold higher with repeated dosing (Table 2). Age-related differences in sensitivity to CPF therefore appear greater with acute compared to repeated exposures. One contributing factor in the relative "resistance" of neonates to repeated exposures is the more robust recovery of acetylcholinesterase activity following each exposure. More rapid recovery of ChE activity in neonatal compared to adult tissues has been reported previously (Liu *et al.*, 1999; Moser and Padilla, 1998; Pope *et al.*, 1991). The more rapid recovery of cholinesterase is probably due to higher levels of basal protein synthesis in the developing animal (Lajtha and Dunlop, 1981; Michalek, 1982), but could also be due to adaptive responses involving specific changes in acetylcholinesterase synthesis (Chiappa *et al*., 1995; Kaufer *et al.*, 1998). Thus, with repeated

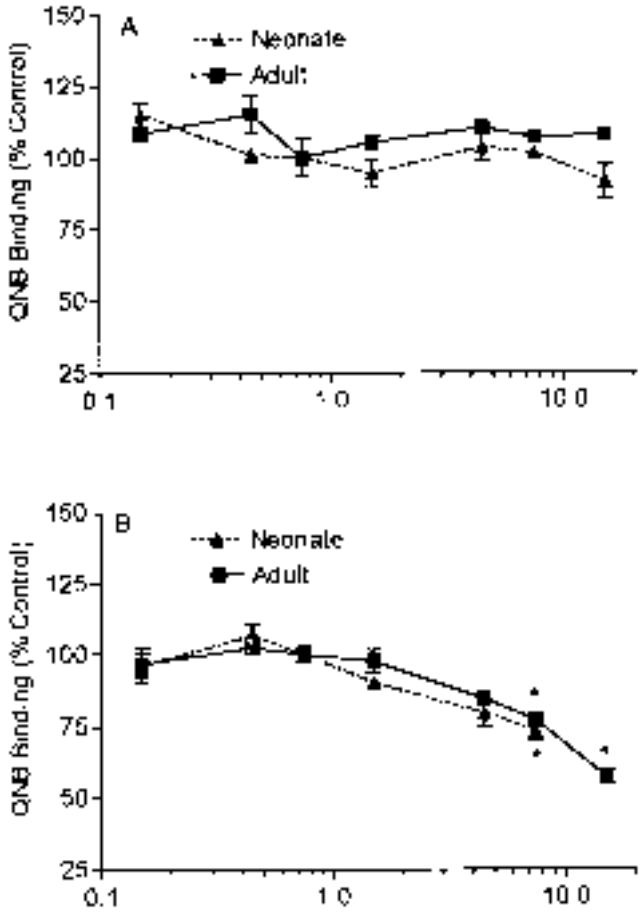

FIG. 4. Changes in cortical [^{3}H]QNB binding following acute and repeated CPF exposure to 0.15, 0.45, 0.75, 1.5, 4.5, 7.5, or 15 mg/ kg/day, po). QNB binding was measured as described in Materials and Methods. Animals were treated with CPF daily for 14 days and sacrificed 4 h after either first (A) or 14th doses (B). Data represent the percent (mean ± SE, n = 5–7/treatment/ time point) of vehicle control values. Control values are listed in Table 1. Asterisks indicate significant differences from corresponding control values ($p < 0.05$).

exposures, though ChE inhibition may be greater in the neonate, greater synthesis of new ChE molecules may allow the neonate to tolerate relatively higher exposures. Similarly, accelerated turnover of RBCs in immature animals (see review of Landaw, 1988) may contribute to the apparent lower sensitivity of RBC cholinesterase in younger animals following repeated CPF exposures (Fig. 3, Table 2). With identical intermittent exposures (every 4 days), ChE inhibition in adult tissues can surpass levels of inhibition noted in neonatal tissues (Chakraborti *et al.*, 1993), apparently because of this age-related difference in the rate of ChE recovery. Obviously, the recovery of ChE activity is an important factor that can contribute to age-related differences in sensitivity with repeated exposures (Lassiter *et al.*, 1998).

Historically, OP pesticides have often been regulated on the basis of blood ChE inhibition, but the use of such data for regulatory decisions has been debated for years (Chen *et al.*, 1999; U.S. EPA, 1998). It has been argued by some that blood ChE inhibition is a marker of exposure while brain ChE inhibition is a marker of neurotoxicity. It is worth noting, however, that in the present studies with both acute and repeated exposures, blood (plasma or RBC) cholinesterase was similar or higher in sensitivity to CPF when compared to brain ChE or the other toxic endpoints, in either age group. Moreover, NOELs for blood cholinesterase inhibition in adults were the same or lower than those based on brain ChE inhibition (the most sensitive generally accepted marker of neurotoxicity) in neonates. These data provide evidence that use of blood cholinesterase inhibition in risk assessments of OP pesticides, while possibly being a conservative marker of neurotoxicity in adults, may be adequately protective when considering sensitive subpopulations such as infants and children.

In summary, while neonates can be markedly more sensitive to the lethal effects of high dosages of CPF, lesser age-related

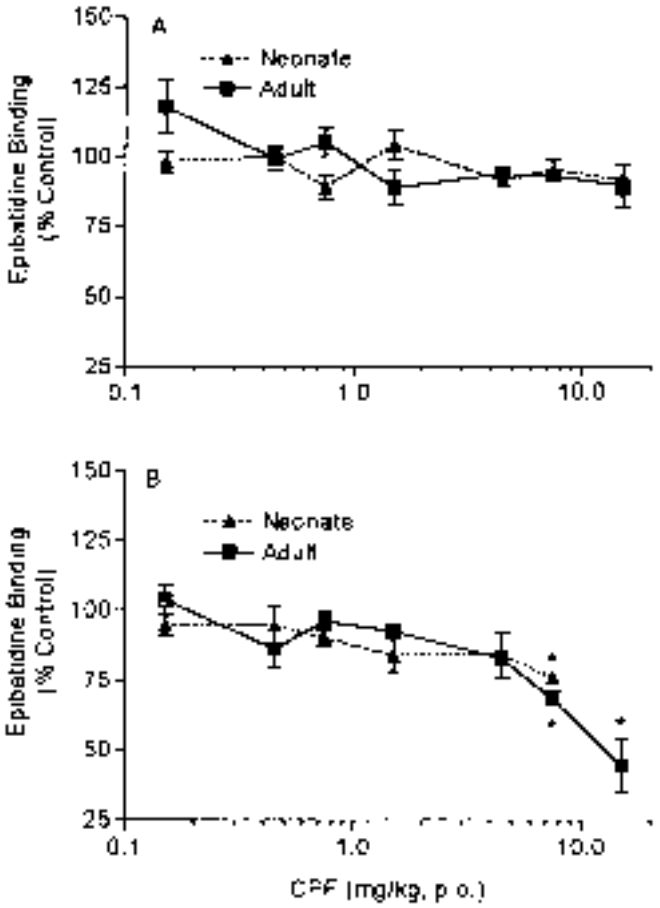

FIG. 5. Changes in cortical [^{3}H]epibatidine binding following acute and repeated CPF exposures to 0.15, 0.45, 0.75, 1.5, 4.5, 7.5, or 15 mg/kg/day, po). Epibatidine binding was measured as described in Materials and Methods. Animals were treated daily for 14 days and sacrificed 4 h after either first (A) or 14th doses (B). Data represent the percent (mean ± SE, n = 5–7/treatment/ time point) of vehicle control values. Control values are listed in Table 1. Asterisks indicate significant differences from corresponding control values ($p < 0.05$).

differences are apparent with non-lethal endpoints, in particular following repeated exposures. Age-related differences in sensitivity to CPF appear markedly influenced by dosing magnitude and frequency. Finally, the use of adult blood-cholinesterase data may be adequate to protect the young from any quantitative differences in sensitivity to the cholinergic neurotoxicity of CPF.

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