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IMPORTANCE OF ALIESTERASE AS A DETOXIFICATION MECHANISM FOR SOMAN (PINACOLYL METHYLPHOSPHONOFLUORIDATE) IN MICE*

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Abstract—CBDP (2-/*O*-cresyl/4*H*:1:2-benzodioxaphosphorin-2-oxide) pretreatment produced a dramatic increase in the toxicity of soman in mice following the subcutaneous (s.c.) or intraperitoneal (i.p.) route of administration. This increase in soman toxicity was very highly correlated with inhibition of plasma aliesterase activity. Other enzymes (e.g. liver aliesterase and plasma cholinesterase) were inhibited by CBDP pretreatment; however, they did not appear to play a significant role in the potentiation of soman toxicity by CBDP. Liver aliesterase was not inhibited by doses of CBDP which produced significant increases in soman toxicity. Similarly, doses of Iso-OMPA, a selective inhibitor of pseudocholinesterase, which completely inhibited plasma cholinesterase, had no effect on soman toxicity. Pyridostigmine pretreatment which inhibited brain, diaphragm and plasma acetylcholinesterase 27, 57 and 60%, respectively, while not inhibiting plasma aliesterase, did not affect soman toxicity. The results of this study demonstrate that, in mice, plasma aliesterase is an extremely important detoxification route for soman.

Soman (pinacolyl methylphosphonofluoridate) is an extremely toxic organophosphorus poison. There is little doubt that the primary toxic manifestations (miosis, salivation, lacrimation, diarrhea and muscle fasciculations) and lethality of soman are due to inhibition of the enzyme acetylcholinesterase (EC 3.1.1.7) which inactivates the neurotransmitter acetylcholine. However, binding of soman to other tissue sites such as pseudocholinesterase (EC 3.1.1.8), aliesterase (non-specific carboxylesterase; EC 3.1.1.1) [1–4], chymotrypsin (EC 3.4.4.5) [5, 6], trypsin (EC 3.4.4.4) [5] and probably a variety of other serine-containing hydrolase enzymes reduces the concentration of free soman *in vivo*. Binding of soman at other sites which, in the short term, are not life threatening may serve as a means of detoxification *in vivo*.

It has been demonstrated that aliesterase is an important detoxification route for organophosphates *in vivo* [4, 7–9]. Recovery of plasma aliesterase activity was suggested as the major factor in the tolerance of soman [1, 2]. "The ethoxyethyl esters of both malathion and malaxon have been shown to be hydrolysed by carboxylesterase enzymes" [10]; however, it is doubtful that carboxylesterase hydrolyses soman. If soman binding sites on aliesterase were already occupied, the toxicity of soman was potentiated. CBDP (2-/*O*-cresyl/4*H*:3:2-benzodioxaphosphorin-2-oxide), a metabolite of tri-*o*-cresyl phosphate [11, 12], which is an irreversible inhibitor of aliesterase [7, 9], potentiates the toxicity of soman 19.1-fold in rats [13] and 14.9-fold in mice [14]. Similarly, tri-*o*-cresylphosphate (TOCP) or CBDP potentiates the toxicity of sarin (isopropyl methylphosphonofluoridate) [13, 15], tabun (ethyl *N*-dimethylphosphoramidocyanidate) [13], malathion [11] and to a minor degree VX (*O*-ethyl *S*-[2-(diisopropyylamino)ethyl]methylphosphonothioate) [13].

The purpose of this study was to investigate more fully the nature of the potentiation of soman toxicity following CBDP pretreatment in mice.

MATERIALS AND METHODS

Toxicology. Male CD-1 mice (25–30 g) were obtained from Charles River Canada Inc., St. Constant, Quebec. The mice were acclimatized in our animal facility for at least 1 week following their arrival at Defence Research Establishment Suffield prior to use. The mice had access to food and water *ad lib.* before and after drug administration.

CBDP was administered either by subcutaneous (s.c.) injection in the back of the neck or by intravenous (i.v.) injection in the tail vein. Iso-OMPA (tetraisopropyl pyrophosphoramide) was administered by i.v. injection. CBDP and Iso-OMPA were administered 1 hr prior to soman. Soman was administered by s.c. or intraperitoneal (i.p.) injection. The volume of injection was 1% of body weight in all cases. Ten animals per dose and at least five different doses were used in constructing the LD_{50} curves. Twenty-four hour LD_{50} values were calculated by probit analysis according to the method of Finney [16].

Enzyme determinations. Acetylcholinesterase (AChE), aliesterase and somanase activities were determined as previously described by Clement [17].

With diaphragm tissue, acetylcholinesterase activity was expressed as nmoles acetylcholine (ACh) hydrolysed/mg tissue/min. Duplicate incubations were performed which contained Iso-OMPA

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Table 1. Recovery of the activity of serum and liver aliesterase, blood, brain and diaphragm acetylcholinesterase and liver somanase following pretreatment with CBDP*

Time after CBDP (hr)	Aliesterase†				Acetylcholinesterase										Somanase
								Diaphragm							
	Serum	% Con	Liver	% Con	Brain	% Con	Blood	% Con	Total	% Con	True	% Con	Pseudo	% Con	Liver
Cont	2496 ± 167§	100	86.4 ± 18.5	100	11.6 ± 0.80	100	1072 ± 37	100	1.45 ± 0.11	100	0.70 ± 0.03	100	0.75 ± 0.11	100	739 ± 119
1	45.2 ± 44§	1.8	9.3 ± 2.0§	11	4.8 ± 0.5§	41	287 ± 49§	27	0.57 ± 0.07§	39	0.56 ± 0.06	80	0.04 ± 0.06§	5	790 ± 41
24	280 ± 59§	11	11.3 ± 3.3§	13	5.1 ± 0.7§	44	290 ± 53§	27	0.67 ± 0.06§	46	0.51 ± 0.06§	73	0.15 ± 0.02§	20	
48	1035 ± 145§	41	38.5 ± 3.6§	45	4.5 ± 0.4§	39	385 ± 28§	36	0.94 ± 0.09§	65	0.54 ± 0.04§	77	0.40 ± 0.10§	53	
96	2058 ± 217§	82	75.3 ± 10.3	87	5.0 ± 0.3§	43	491 ± 33§	46	1.32 ± 0.17	91	0.70 ± 0.04	100	0.62 ± 0.14	83	

* Mice were treated with CBDP (50 mg/kg; s.c.) in DMSO at zero time. At various time periods after CBDP administration, animals were killed, and the enzyme activities were determined.

† Units of activity: serum aliesterase = nmoles tributyrin hydrolysed/ml serum/min.
liver aliesterase = nmoles tributryin hydrolysed/mg liver/min.
brain acetylcholinesterase = nmoles ACh hydrolysed/mg brain/min.
blood acetylcholinesterase = nmoles ACh hydrolysed/ml whole blood/min.
diaphragm acetylcholinesterase = nmoles ACh hydrolysed/mg tissue/min.
liver somanase = nmoles soman hydrolysed/g liver/min.

‡ Mean ± S.D. (N = 4–11 observations).

§, Significantly different from control group. § $P \leq 0.001$ and ↑ $P \leq 0.01$.

(19.5 μM), a specific inhibitor of pseudocholinesterase [18–20]. Iso-OMPA was preincubated with diaphragm for 10 min prior to addition of [^{14}C]ACh. The difference in AChE activity with and without Iso-OMPA was considered to be due to true acetylcholinesterase. This procedure was adopted due to the unavailability of a commercial source of radiolabeled butyrylcholine.

Materials. CBDP and soman were prepared by the Organic Chemistry Group, Defence Research Establishment Suffield. Their purity was greater than 97%. Other chemicals were obtained from various commercial sources: Iso-OMPA (ICN-K & K Laboratories); Tributyrin (Fisher); and [^{14}C]ACh (4.0 mCi/mmole; New England Nuclear).

When administered i.v., CBDP was dissolved in dimethyl sulfoxide (DMSO) and then diluted with 0.9% saline to produce the required concentration. The solution was constantly stirred during the injection procedure since at higher concentrations of CBDP a turbid solution resulted. Constant stirring ensured that the mixture was homogeneous. Iso-OMPA was dissolved in absolute ethanol and diluted with 0.9% saline to the required concentration. The volume of DMSO or ethanol was 0.2% of the injection volume and, as determined in preliminary studies, did not affect the toxicity of soman. Soman was dissolved in 0.9% saline. All solutions were made up immediately before use.

RESULTS

The results in Table 1 show the effect of CBDP (50 mg/kg; s.c.) at 1 hr after administration on various tissue and serum enzymes. Serum aliesterase activity was inhibited almost completely while liver aliesterase activity was only 11% of control activity. In addition, brain, blood and total diaphragm acetylcholinesterase activities were inhibited to 41, 27 and 39% of control activity respectively. Liver somanase activity was unaffected by CBDP (50 mg/kg) pretreatment. With diaphragm acetylcholinesterase, most of the inhibition produced by CBDP pretreatment was due to inhibition of pseudocholinesterase. Table 1 also shows the time-course of the recovery of the various enzymes back to control activities following exposure to CBDP (50 mg/kg; s.c.). At least 4 days were required for liver aliesterase and diaphragm acetylcholinesterase to return to control activities. Serum aliesterase and brain and erythrocyte acetylcholinesterase activities were only 82, 43 and 46% of control activity, respectively, at 96 hr after receiving CBDP.

The effect of CBDP pretreatment on the toxicity of soman was investigated. Pretreatment with CBDP (50 mg/kg) reduced the soman LD_{50} value to 8.7 μg/kg from a control value of 136 μg/kg (Table 2). CBDP pretreatment potentiated the toxicity of soman approximately 16-fold. As the various enzyme activities returned to control levels over a 4-day period, so did the soman LD_{50} value. The recovery to control of the soman LD_{50} correlated very closely with the recovery of serum ($r = 0.995$; $P \leq 0.01$) and liver ($r = 0.99$; $P \leq 0.01$) aliesterase, diaphragm total acetylcholinesterase ($r = 0.99$; $P \leq 0.01$) and diaphragm pseudocholinesterase ($r = 0.995$;

$P \leq 0.01$) but not diaphragm true acetylcholinesterase ($r = 0.79$; $P \geq 0.1$). Over the 4-day observation period, there was no significant change in brain acetylcholinesterase activity.

In the above experiments, CBDP (50 mg/kg) was administered s.c. in pure DMSO as previously reported by Bošković [13]. Within 1 hr following this injection, the mice were slightly ataxic, had mild tremors, and developed a large s.c. mass. Upon necropsy, this mass was the result of edema as the area surrounding the s.c. injection site appeared to be filled with a straw coloured fluid, probably plasma. Upon decapitation and exsanguination, the mice appeared to be hypovolemic as the blood volume was not as great as that usually found and the blood appeared to be darker in colour. Also, in some animals hematuria was evident. The edema, apparent hypovolemia, mild tremors and ataxia were probably due to the DMSO as they were evident in control animals receiving DMSO only. In light of the adverse effects of administering large doses of CBDP in DMSO, further experiments were performed utilizing smaller CBDP doses which were administered i.v. in saline, with DMSO comprising only 0.2% of the injection volume. This injection mixture (DMSO and saline) appeared to have no observable effect on the mice.

From the previous results (Tables 1 and 2), it appeared that the potentiation of soman toxicity was due to the combined effect of inhibition of liver and serum aliesterase and serum and diaphragm acetylcholinesterase. Further experiments were conducted to inhibit selectively the aliesterases in plasma or liver in order to determine the importance of aliesterase in the potentiation of soman toxicity. This was accomplished by administering small doses of CBDP by the i.v. route. The results in Table 3 demonstrate the effect of various doses of CBDP administered i.v. on the activity of plasma and liver aliesterase and plasma, erythrocyte and diaphragm acetylcholinesterase. CBDP, 5.0 mg/kg, produced marked inhibition of plasma and liver aliesterase, plasma and diaphragm acetylcholinesterase and diaphragm pseudocholinesterase while not significantly inhibiting erythrocyte acetylcholinesterase. As the dose of CBDP was reduced, the inhibition was isolated primarily to the plasma aliesterase and diaphragm total acetylcholinesterase and pseudocholinesterase.

The effect of pretreatment with various doses of

Table 2. Effect of CBDP administered s.c. on soman toxicity*

Time after CBDP (hr)	Soman LD_{50} (μg/kg)	95% limits	% Control
	136	(129–142)	100
1	8.7	(7.5–9.7)	6.4
24	24.6	(19.2–30.4)	18
48	89.9	(83.2–96.5)	66
96	148	(141–182)	109

* Mice were pretreated with CBDP (50 mg/kg; s.c.) dissolved in DMSO. Soman was injected s.c. in the sacral region of the back at various times after CBDP.

Table 3. Effect of various doses of CBDP administered i.v. on mouse aliesterase and acetylcholinesterase activities*

CBDP (mg/kg)	Aliesterase†				Acetylcholinesterase								
								Diaphragm					
	Plasma	% Con	Liver	% Con	Plasma	% Con	Erythrocyte	% Con	Total	% Con	True	% Con	Pseudo	% Con
0	1750 ± 344‡	100	86.4 ± 18.5	100	1150 ± 180	100	333 ± 70	100	1.39 ± 0.11	100	0.80 ± 0.14	100	0.60 ± 0.2	100
0.05	1158 ± 214§	66	87.2 ± 12.6	101	1026 ± 267	89								
0.10	1053 ± 134	60	102 ± 16.6	118	945 ± 136	82			1.53 ± 0.18	110	0.95 ± 0.11	119	0.57 ± 0.11	97
0.25	798 ± 77§	46	85.1 ± 13	98	798 ± 71	63			1.39 ± 0.14	100	0.93 ± 0.10	116	0.46 ± 0.14	78
0.50	438 ± 94§	25	77.4 ± 7.3	90	372 ± 44¶	32	267 ± 15	80	0.94 ± 0.08¶	68	0.65 ± 0.05	81	0.29 ± 0.09§	49
1.0	207 ± 12¶	12	61.0 ± 7.0§	71	359 ± 36¶	31	292 ± 43	88	1.04 ± 0.09**	75	0.82 ± 0.07	103	0.22 ± 0.07	37
2.5	124 ± 34¶	7	36.6 ± 20¶	42	337 ± 39¶	29	295 ± 18	89	1.01 ± 0.06¶	73	0.74 ± 0.04	93	0.27 ± 0.06**	46
5.0	90 ± 32¶	5	10.7 ± 1.4¶	16	148 ± 10¶	13	287 ± 55	86	1.08 ± 0.05¶	78	0.78 ± 0.02	98	0.30 ± 0.05§	51

* Mice were injected i.v. with various doses of CBDP. One hour later the mice were killed and enzyme activities were determined.
† Units of activity are the same as those in Table 1 except that plasma aliesterase = nmoles tributyrin hydrolysed/ml plasma/min.
‡ Mean ± S.D. (N = 3–11 observations).
§–** Significantly different from control group: § $P \leq 0.05$, ¶ $P \leq 0.001$, and ** $P \leq 0.02$.

Table 4. Effect of various doses of CBDP administered i.v. on the toxicity of soman administered either s.c. or i.p.*

CBDP dose (mg/kg)	Route of administration of soman			
	s.c.		i.p.	
	LD_{50}	% Control	LD_{50}	% Control
0	136 (129–142)†	100	393 (366–417)	100
0.10	75.5 (68–81)	56	277 (267–301)	70
0.25	66.1 (64–69)	49	208 (196–221)	53
0.50	37.5 (35–41)	28	132.5 (123–140)	34
1.0	17.7 (16.8–19)	13	71.4 (66.6–76.7)	18
2.5	10.1 (9.7–10.5)	7	35.2 (32.9–37.8)	9
5.0	6.95 (6.8–7.2)	5		

* CBDP was administered i.v. 1 hr prior to administration of soman.
† LD_{50} Value of soman (μg/kg) with 95% confidence limits in parentheses.

CBDP administered i.v. on the s.c. and i.p. toxicity of soman was evaluated. The s.c. and i.p. LD_{50} values of soman were reduced to a similar degree (% of control) following CBDP pretreatment (Table 4). Pretreatment with CBDP (5 mg/kg) reduced the soman LD_{50} value from 136 to 6.95 μg/kg. This dose of CBDP administered i.v. caused extensive inhibition of plasma and liver aliesterase and plasma acetylcholinesterase, whereas CBDP (0.1 mg/kg), which significantly inhibited only plasma aliesterase (60% of control activity), reduced the s.c. and i.p. soman LD_{50} value to 75.5 μg/kg (56% of control) and 277 μg/kg (70% of control) respectively.

In further experiments, the effect of inhibition of plasma acetylcholinesterase only on the toxicity of soman was investigated. Iso-OMPA, a specific inhibitor of pseudocholinesterase [18–20], was administered i.v. 1 hr prior to soman injection. Iso-OMPA produced ≥84% inhibition of plasma cholinesterase (composed primarily of pseudocholinesterase; [21]); however, this did not affect significantly soman toxicity (Table 5).

The residual plasma acetylcholinesterase activity (Table 5) which was not inhibited by Iso-OMPA (5 mg/kg; i.v.) was inhibited completely by physostigmine (1 μM), indicating the presence of true acetylcholinesterase in mouse plasma.

Further experiments were performed to determine the role of inhibition of diaphragm acetylcholinesterase in the potentiation of soman toxicity by CBDP. Pyridostigmine, a quaternary carbamate anticholinesterase, was used as a pretreatment in some experiments. Carbamate anticholinesterases do not inhibit aliesterase [22, 23]. Pyridostigmine (1.2 mg/kg; i.v.), administered 10 min before sacrifice, did not inhibit plasma aliesterase but inhibited diaphragm, plasma and brain acetylcholinesterase 57, 60 and 27% respectively. However, pyridostigmine pretreatment did not potentiate the toxicity of soman. The resultant s.c. soman LD_{50} value in pyridostigmine (1.2 mg/kg, i.v., 10 min before soman) pretreated mice was 138 (128–156; 95% limits) μg/kg which is not significantly different from that obtained in control animals (136 μg/kg).

DISCUSSION

CBDP pretreatment (50 mg/kg, s.c., and 5 mg/kg, i.v.) produced a dramatic increase (15.6-fold and 19.6-fold respectively) in the toxicity of soman in mice (Tables 2 and 4) similar to that reported by other investigators [13, 15]. However, it was evident (Tables 1 and 3) that CBDP pretreatment inhibited

Table 5. Effect of Iso-OMPA on aliesterase and cholinesterase activity and soman toxicity in mice*

Dose (mg/kg)	Aliesterase†				Cholinesterase			Soman toxicity		
	Plasma	% Control	Liver	% Control	Plasma	% Control		LD_{50}‡	95% Limits	% Control
Control	1750 ± 344§	100	86.4 ± 18.5	100	1119 ± 184			136	129–142	100
0.25	1449 ± 56	83	85.6 ± 11	99	174 ± 83‖	15		138	132–146	101
0.5	1533 ± 145	88	48.7 ± 10‖	56	183 ± 26‖	16		133	125–148	98
1.0	1190 ± 131¶	68	29.0 ± 1.7‖	34	128 ± 34‖	11		94	88–99	69
2.5	859 ± 167‖	49	21.0 ± 1.7‖	24	117 ± 32‖	10		88	83–96	65
5.0	535 ± 78‖	31	20.4 ± 0.9‖	24	127 ± 48‖	11		61	58–66	45
10.0	281 ± 68‖	16	18.3 ± 0.2‖	21	104 ± 42‖	9		31	29–33	23

* Mice were injected with various concentrations of Iso-OMPA, i.v. One hour later the animals were killed and various enzyme activities were determined.
† Units of activity were the same as those in Table 3.
‡ Soman LD_{50} (μg/kg; s.c.).
§ Mean ± S.D. (N = 3–11 observations).
‖,¶ Significantly different from control group: ‖ $P \leq 0.001$ and ¶ $P \leq 0.01$.

a number of different enzymes to varying degrees, making it difficult to determine inhibition of which enzyme(s) was responsible for the potentiation of soman toxicity. By administering various doses of CBDP intravenously it was possible to selectively inhibit one particular enzyme more than the others. For example, CBDP (0.5 mg/kg; i.v.; Table 3) pretreatment, which inhibited significantly plasma but not liver aliesterase, increased the toxicity of soman (Table 4). This result suggested that inhibition of liver aliesterase did not play a significant role in the potentiation of soman poisoning by CBDP. This was confirmed by experiments with Iso-OMPA (0.5 mg/kg; i.v.; Table 5). Liver but not plasma aliesterase was inhibited significantly; however, this had no effect on the soman LD_{50} value (Table 5).

The ineffectiveness of Iso-OMPA pretreatment (at doses which completely inhibited plasma pseudocholinesterase) to potentiate the toxicity of soman illustrated that inhibition of pseudocholinesterase was not a factor in the potentiation of soman toxicity by CBDP. Pyridostigmine pretreatment produced marked inhibition of acetylcholinesterase in blood, brain and diaphragm without inhibiting plasma aliesterase activity. However, pyridostigmine pretreatment did not potentiate soman toxicity, suggesting that inhibition of acetylcholinesterase was not a locus for the marked potentiation of soman toxicity by CBDP pretreatment.

In the experiments utilizing Iso-OMPA, the residual acetylcholinesterase activity was inhibited by physostigmine which suggested the presence of a true acetylcholinesterase in mouse plasma. However, most of the plasma cholinesterase activity was composed of pseudocholinesterase [21]. Plasma acetylcholinesterase has been detected in bird [24], dog [8], rat [25] and human plasma [26]. Alternatively, this *apparent* plasma acetylcholinesterase could be due to erythrocyte ghosts contaminating the plasma.

Somanase, a phosphorylphosphatase which hydrolyses soman to pinacolyl methylphosphonic acid [27], is situated primarily in the liver. Soman consists of a mixture of four stereo isomers [28], two of which are extremely toxic. Somanase, like sarinase, appears to hydrolyse preferentially the less toxic isomers of soman. Thus, unless there is some isomerization process which is functional *in vivo* (one has been proposed for sarin), it is doubtful whether this enzymatic degradation is important *in vivo*. CBDP (50 mg/kg; s.c.; Table 1) had no effect on somanase activity in the liver; thus, it can be concluded that somanase does not play a role in the CBDP potentiation of soman toxicity. In addition, Clement [17] found that following phenobarbital pretreatment, which reduced the toxicity of soman *in vivo*, somanase activity was not altered significantly. Previous authors have questioned the importance of phosphorylphosphatases as a major detoxification route due to their relatively low activity *in vivo* [29, 30]. Somanase may be important in explaining the differences in LD_{50} value between the s.c. and i.p. toxicity of soman, i.e. in the first pass through the liver following i.p. administration free soman may be detoxified by somanase and/or aliesterase present in this organ. Alternatively, the difference in s.c. and i.p. toxicity could be due to the rate of absorption from the injection site to achieve a lethal concentration of free soman or it could be a combination of the above. Sterri and Fonnum [31] concluded that, in the liver, somanase was more important than aliesterase in detoxifying soman; however, they perfused the liver with a salt solution, not whole blood.

Thus, it appears that the CBDP potentiation of soman toxicity is primarily due to inhibition of plasma aliesterase as demonstrated by the high correlation between inhibition of plasma aliesterase and potentiation of soman toxicity. Additional studies from this laboratory have demonstrated the role and importance of aliesterase in soman poisoning [3, 17, 32].

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