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ORGANOPHOSPHATE POLYNEUROPATHY IN CHICKS*

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Abstract—Young animals are resistant to organophosphate-induced delayed neuropathy (OPIDP), although biochemical changes on Neuropathy Target Esterase (NTE) caused by neuropathic organophosphorus esters (OP) are similar to those observed in the sensitive hen. We report here that the resistance of chicks to single doses of neuropathic OPs is not absolute because ataxia was produced in 40-day-old chicks by 2,2-dichlorovinyl dibutyl phosphate (DBDCVP, 5.0 or 10.0 mg/kg s.c.) and by diisopropyl phosphorofluoridate (DFP, 2.0 mg/kg s.c.). However, the clinical picture was different from that usually seen in hens; spasticity and complete recovery being the main features. α-Tolyl sulphonyl fluoride (PMSF, 300 mg/kg s.c.) promoted both DBDCVP neuropathy (5.0 or 10.0 mg/kg s.c.) and non-neuropathic doses of DFP (1.5 mg/kg s.c.) or DBDCVP (1.0 mg/kg s.c.). The lowest promoting dose of PMSF given 24 hr after 1.5 mg/kg of DFP was 30 mg/kg. Higher doses had a more severe effect but no further increase of OPIDP severity was obtained with doses ranging from 90 to 300 mg/kg. PMSF (30 mg/kg) protected 40-day-old chicks from subsequent doses of neuropathic OPs even when a promoting dose of PMSF followed. At 60 days of age, chicks' resistance to OPIDP decreased because lower doses of neuropathic OPs became effective and, similarly to hens, PMSF did not fully protect from subsequent promotion. In 40-day-old chicks the threshold of NTE inhibition for OPIDP development was 95–97% (DBDCVP 5.0 mg/kg). When promotion followed initiation, the minimal effective inhibition of NTE for initiation by neuropathic OPs was about 90%. In 36-day-old chicks, PMSF (300 mg/kg) promoted OPIDP when given up to 5 days after DFP (1.5 mg/kg) when residual NTE inhibition in brain and sciatic nerve was about 40%. We conclude that chicks' resistance to OPIDP might reflect either a less effective initiation by phosphorylated NTE or a more efficient repair mechanism or both, and also that promotion is likely to involve a target other than NTE.

Organophosphate-induced delayed polyneuropathy (OPIDP§) is a distal central–peripheral axonopathy caused by single doses of some organophosphorus compounds (OPs) [1]. The putative target for OPIDP is a protein called Neuropathy Target Esterase (NTE) which carries an esterase catalytic activity. However, the physiological substrate of NTE is unknown and its purification is only now being achieved [2].

NTE inhibitors might have different toxicological effects. Certain OPs, such as DFP, cause OPIDP when they inhibit at least 70–80% of NTE within hours of dosing. Other inhibitors, such as sulphonyl halides, carbamates and phosphinates, do not cause OPIDP when they inhibit NTE to the same extent. Moreover, when given before a neuropathic OP they protect from OPIDP by occupying NTE [3]. However, when given after a neuropathic OP they promote OPIDP [4, 5]. Only NTE inhibitors have been shown so far to be promoters [1]. Current tentative explanation is that promotion should involve a site other than NTE and that NTE inhibitors initiate OPIDP with different efficacy. Neuropathic OPs would be strong initiators whereas carbamates, phosphinates and sulphonyl halides would be very weak and therefore protective when they occupy NTE [1].

Chicks [6], children [7] and young rats [8] have been reported to be resistant to single doses of neuropathic OPs and only by repeated dosing do chicks become susceptible to mild OPIDP [6]. Reasons for such a resistance are unknown. The nervous system of chicks has higher NTE activity than that of hens, but the biochemical characteristics are similar [9]. Furthermore, single doses of OPs cause NTE inhibition in the chick nervous system similar to that found after effective doses in hens [6, 9], but OPIDP still does not develop. After high inhibition, however, NTE recovery in sciatic nerves of chicks was found to be faster than that in hens, [9] and it was interpreted as the effect of a more efficient repair or of a rapid turnover of growing nerves or both.

This study was undertaken to assess whether single doses of neuropathic OPs, higher than those previously tested, cause OPIDP in 40-day-old chicks and how this effect correlates with inhibition of NTE. The effect of a known promoter, α-tolyl sulphonyl fluoride (PMSF), given after the initiating OP was also assessed.

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§ Abbreviations: AChE, acetylcholinesterase; NTE, Neuropathy Target Esterase; OPIDP, organophosphate-induced delayed polyneuropathy; OP, organophosphates; DFP, diisopropyl phosphorofluoridate; DBDCVP, 2,2-dichlorovinyl dibutyl phosphate; PMSF, α-tolyl sulphonyl fluoride.

Table 1. OPIDP and enzyme inhibition in 40-day-old chicks after single doses of DBDCVP or DFP

Treatment (mg/kg, s.c.)	% Inhibition* (N = 3) NTE Brain	NTE Spinal cord	NTE Sciatic nerve	AChE Brain	Maximal clinical score (N)	Day of onset–day of recovery†
DBDCVP (1)‡	92 ± 1	87 ± 3	91 ± 2	52 ± 6	0, 0, 0, 0, 0	—
DBDCVP (5)	97 ± 1	96 ± 1	95 ± 2	79 ± 1	2.2 ± 0.4 (15)	9–32
DBDCVP (10)§	ND	ND	ND	ND	3.1 ± 0.6 (7)	6–43
DFP (1)‖	84 ± 1	77 ± 1	90 ± 5	ND	0, 0, 0, 0, 0	—
DFP (2)	ND	ND	ND	ND	0.8 ± 0.2 (6)	6–16

Data are expressed as mean ± SEM or individual values.

* Measured 24 hr after dosing and calculated from the mean activity of controls treated with vehicle only (N = 3).

† When earliest and latest signs of ataxia were detectable in at least one animal.

‡ In hens, this treatment caused 96 ± 1, 86 ± 2 and 83 ± 3% NTE inhibition in brain, spinal cord and sciatic nerve, respectively, and an ataxia score of 7.2 ± 1 [12]. In chicks of 30 days of age the inhibition of NTE by the same treatment was 92 ± 2% in brain and 84 ± 1% in spinal cord (N = 3).

§ PMSF (30 mg/kg, s.c.) protects from this treatment when given 24 hr earlier.

‖ This treatment caused in hens 88 ± 3, 87 ± 2 and 93 ± 1% NTE inhibition, in brain, spinal cord and sciatic nerve, respectively, and an ataxia score of 8.0 [9].

ND, not done.

Table 2. Clinical and biochemical effects of OPIDP promotion by PMSF (300 mg/kg s.c.) in 40-day-old chicks treated with DBDCVP or DFP, 24 hr earlier

Treatment before PMSF (mg/kg, s.c.)	% Inhibition* (N = 3) NTE Brain	NTE Spinal cord	NTE Sciatic nerve	AChE Brain	Maximal clinical score (N)	Day of onset–day of recovery†
DBDCVP (1.0)	94 ± 1	91 ± 1	93 ± 1	58 ± 4	2.0 ± 0.7 (4)	7–12
DBDCVP (5.0)	96 ± 1	94 ± 2	94 ± 1	73 ± 5	5.1 ± 0.5 (10)	7–‡
DBDCVP (10.0)§	ND	ND	ND	ND	6.0 ± 0.6 (3)	5–48
DFP (0.5)‖	95 ± 1	95 ± 1	92 ± 1	ND	0, 0, 0, 0, 0	—
DFP (1.0)	93 ± 1	91 ± 2	92 ± 2	ND	0.4 ± 0.4 (5)	10–16

Data are expressed as mean ± SEM or individual values.

* Measured 24 hr after treatment with PMSF and calculated from the mean activity of controls treated with vehicle only (N = 3).

† When earliest and latest signs of ataxia were detectable in at least one animal.

‡ Chicks were killed 27 days after the last treatment when the clinical score was 2.7 ± 0.7.

§ PMSF (30 mg/kg, s.c.) protects from this treatment when given 24 hr earlier.

‖ DFP (0.5 mg/kg, s.c.) caused 72 ± 3, 71 ± 4 and 71 ± 1% inhibition of NTE in brain, spinal cord and sciatic nerve, respectively (N = 3).

ND, not done.

MATERIALS AND METHODS

Chemicals. Paraoxon (*p*-nitrophenyl-*O,O*-diethyl phosphate) (Sigma Chemical Co., St Louis, MO, U.S.A.) was purified according to Johnson [10]. Mipafox (*N,N*-diisopropylphosphorodiaminic fluoride), phenylvalerate, and 2,2-dichlorovinyl dibutyl phosphate (DBDCVP) were purchased from Lark Enterprise (Webster, MA, U.S.A.). Diisopropyl phosphorofluoridate (DFP) and PMSF were purchased from Fluka AG (Buchs, Switzerland). All other reagents were of the highest analytical grade.

Animals and dosing. Female chicks of Warren strain were purchased from a local breeder. Animals had free access to standard chicks' diet and water. DBDCVP, DFP and PMSF were dissolved in glycerol formal immediately before use, and given subcutaneously (s.c.) in a maximal volume of 1.5 mL/kg body wt. Animals treated with high doses of DFP (1.5 and 2.0 mg/kg body wt, s.c.) or DBDCVP (5.0 and 10.0 mg/kg body wt, s.c.) were pretreated with atropine sulphate (20 mg/kg body wt, i.p.) in order to avoid acute cholinergic symptoms. Chicks were observed daily for clinical signs of OPIDP, and weighed twice a week until they recovered

Table 3. Dose–response of promotion by PMSF in 40-day-old chicks treated with DFP (1.5 mg/kg, s.c.) 24 hr earlier

PMSF (mg/kg, s.c.)	% NTE inhibition* (N = 3)			Maximal clinical score (N)	Day of onset–day of recovery†
	Brain	Spinal cord	Sciatic nerve		
0	80 ± 3‡	82 ± 3‡	88 ± 2‡	0, 0, 0, 0, 0	—
5	79 ± 3	77 ± 3	76 ± 3	0, 0, 0, 0, 0	—
30	88 ± 2	89 ± 2	89 ± 1	0.2 ± 0.2 (5)	13–16
90	89 ± 3	90 ± 3	91 ± 1	4.2 ± 1.0 (5)§	6–43
120	91 ± 1	89 ± 1	92 ± 1	3.8 ± 0.7 (5)§	6–42
300‖	94 ± 1	94 ± 2	95 ± 1	5.1 ± 0.4 (8)§	6–41

Data are expressed as mean ± SEM or individual values.

* Activity was determined 24 hr after PMSF treatment. PMSF given alone (up to 300 mg/kg/day for 3 days) did not cause ataxia in 40-day-old chicks; 30, 120 and 300 mg/kg PMSF given to naive chicks caused NTE inhibition as follows: 78 ± 1, 78 ± 2 and 78 ± 5%; 90 ± 2, 89 ± 2 and 90 ± 2; 87 ± 2, 86 ± 1 and 93 ± 2%; in brain, spinal cord and sciatic nerve, respectively (N = 3).

† When earliest and latest signs of ataxia were detectable in at least one animal.

‡ NTE activity was measured in a separate experiment, using different controls (N = 3).

§ Clinical score was not different in these groups (Kruskal–Wallis test).

‖ PMSF (30 mg/kg, s.c.) given 24 hr earlier protects from this treatment.

Table 4. Time-course of OPIDP promotion by PMSF (300 mg/kg, s.c.) and NTE inhibition after DFP (1.5 mg/kg, s.c.) in 36-day-old chicks

Time of PMSF administration (days after DFP treatment)	% NTE inhibition* (N = 3)		Maximal clinical score† (N)	Day of onset-day of recovery‡
	Brain	Sciatic nerve		
0.6	88 ± 1	77 ± 2	ND	—
1	72 ± 2	70 ± 6	3.5 ± 0.3 (4)	6–49
3	52 ± 3	55 ± 3	2.0 ± 0.6 (3)	6–28
4	ND	ND	1.6 ± 0.5 (5)	9–27
5	ND	ND	1.3 ± 0.3 (3)	8–19
6	38 ± 1	25 ± 5	0, 0, 0, 0	—
9	26 ± 2	13 ± 6	0, 0, 0, 0	—

Data are expressed as mean ± SEM or individual values.

* Calculated from the mean activity of control animals treated with vehicle only at the time of PMSF dosing (N = 3).

† Maximal clinical scores show a decreasing trend which is correlated with the delay of PMSF treatment (non-parametric Pearson χ^2 test for linear trend, $P < 0.0001$).

‡ When earliest and latest signs of ataxia were detectable in at least one animal.

ND, not done.

completely. Clinical score was assessed according to a 0–8 point scale described previously [5].

Biochemistry. Animals were killed by decapitation. Brain, lumbosacral spinal cord and sciatic nerve were dissected immediately, and washed with ice-cold buffer (50 mM Tris–HCl pH 8.0, containing 0.2 mM EDTA). Tissues were then cleaned, weighed and kept at −80° until used. NTE activity in brain and spinal cord was measured according to Johnson [10] and in sciatic nerve according to Caroldi and Lotti [11] using 250 μM paraoxon instead of 40 μM, as described previously [9]. Control values of NTE activity (mean ± SEM, N = 9–10) in brain, spinal cord and sciatic nerve in 40-day-old chicks were 1941 ± 39,708 ± 42 and 43 ± 1 nmol/min/g tissue, respectively. At 30 days of age NTE activities (N = 3) in brain and spinal cord were found to be 2505 ± 171 and 663 ± 45 nmol/min/g tissue, respectively.

RESULTS

Table 1 shows that chicks treated with doses of DFP or DBDCVP which cause OPIDP in hens (1 mg/kg), did not develop OPIDP despite high NTE inhibition (about 90%). However, chicks treated with higher doses of DFP (2.0 mg/kg) or DBDCVP (5.0 mg/kg) became mildly ataxic. This dose of DBDCVP was also effective in younger chicks (30 days) but not in those of 10 days of age (data not shown). The maximal clinical score lasted for approximately 5 days and animals recovered completely, the time of recovery being dependent on the severity of symptoms. The clinical picture

Table 5. PMSF promotion of OPIDP in 60-day-old animals previously protected with PMSF and challenged with DFP

Treatment (mg/kg, s.c.)*				
PMSF (30)	DFP (1.5)	PMSF (300)	Maximal clinical score† (N)	Day of onset–day of recovery‡
−	+	−	2.6 ± 0.9 (5)	6–32
+	+	−	0, 0, 0, 0, 0	—
−	+	+	6.6 ± 0.5 (5)	6–64
+	+	+	0.7 ± 0.2 (4)	7–14

* Doses were given 24 hr apart.
† Mean ± SEM (N) of maximal clinical score.
‡ When earliest and latest signs of ataxia were detectable in at least one animal.

was characterized by spasticity instead of the flaccid ataxia which is usually seen in hens.

Table 2 shows that PMSF (300 mg/kg) promoted to OPIDP ineffective doses of DBDCVP or DFP (see Table 1). Promotion occurred when inhibition of NTE caused by the initiating OP was >90% in sciatic nerve. This is shown by lack of promotion after 0.5 mg/kg s.c. DFP. The speed of recovery from OPIDP in promoted chicks was also inversely related to the severity of symptoms as that in unpromoted OPIDP.

The dose–response relationship of PMSF promotion after the initiation by DFP (1.5 mg/kg) is shown in Table 3. The lowest dose of PMSF which caused mild signs of ataxia (in one chick out of five) was 30 mg/kg. The severity of ataxia increased at the dose of 90 mg/kg but then it levelled off. NTE inhibition in chicks treated with DFP only, as shown in this table, seems somewhat higher than that in birds also treated with PMSF (5.0 mg/kg), and it is likely to be due to small differences in control values.

The time-course of promotion by PMSF (300 mg/kg) after DFP (1.5 mg/kg) was studied in 36-day-old chicks and results are shown in Table 4. The maximal effect was observed 1 day after DFP and it eventually disappeared 6 days thereafter. Residual 35–50% NTE inhibition in both brain and sciatic nerve correlates with a promotable condition.

Forty-day-old chicks were fully protected by PMSF (30 mg/kg) from induction or promotion of OPIDP (see notes on Tables 1–3). However, older animals (60 days) approaching full sensitivity to OPIDP were only partially protected by this dose of PMSF when promotion was given (Table 5), as it is known to occur in hens [5].

DISCUSSION

Chicks are resistant to single doses of OPs which cause OPIDP in hens. However, either by increasing the dose or by giving PMSF as a promoter, chicks become ataxic. Spasticity which is seen in chicks is similar to that observed in hens when a critical NTE inhibition was obtained in spinal cord and not in sciatic nerve [12, 13]. A spastic syndrome was observed also in late stages of human OPIDP when the lesions of central axons became evident because of regeneration of peripheral nerve axons [14]. However, the clinical effects observed in chicks, even if suggesting a predominant lesion of spinal cord axons, are not consistent with the clinical recovery, because spinal cord lesions do not fully recover. It will therefore be essential to perform a detailed histopathological study in chicks in order to understand the morphological correlate with these clinical findings.

Neuropathic effects in 40-day-old chicks correlate with NTE inhibition in sciatic nerve higher than 90% and if promotion is given the minimal NTE inhibition in sciatic nerve is also about 90%. These levels of NTE inhibition are strikingly different from those which correlate with OPIDP and promotion of OPIDP in the hen: about 70% NTE inhibition by DFP or DBDCVP correlates with OPIDP whereas about 40% inhibition is promotable by PMSF [5, 13, 15]. These results suggest that NTE modifications induced by neuropathic OPs are "weaker" in chicks than in the hen. This might be due either to "intrinsically weak" modifications of juvenile NTE

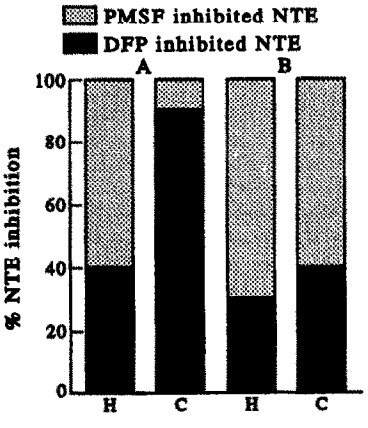

Fig. 1. NTE inhibition and OPIDP promotion by PMSF in hens (H) and chicks (C). (A) PMSF was given 24 hr after the minimal initiating dose of DFP. Hen data are from Ref. 5; the dose of DFP was 0.3 mg/kg s.c., that of PMSF was 120 mg/kg s.c. Chick data are from Table 3. (B) PMSF was given either 11 (H) or 5 (C) days after DFP. Hen data are from Ref. 5; the dose of DFP was 0.5 mg/kg s.c. and caused about 80% NTE inhibition after 24 hr. Chick data are from Table 4.

or to more efficient repair mechanisms in developing birds, or both. The first explanation is derived by analogy with data in hens where different levels of NTE inhibition correlate with OPIDP and with promoted OPIDP, depending on the chemistry of NTE inhibitor [1]. Therefore, the "intrinsic" activity of inhibited NTE to initiate OPIDP might depend both on the chemistry of the residue attached to NTE and on the developmental stage of NTE itself. The second explanation is based on the observation that recovery of NTE activity in sciatic nerve of chicks after high inhibition is quicker than in the hen [9] and that the neuropathy fully recovers.

PMSF promotes both neuropathic and non-neuropathic doses of OPs in chicks. However, the extent of promotion is greater after initiation by DFP than after DBDCVP because the ataxia score increased from 0 to 5.1 and from 2.2 to 5.1, respectively. Similarly, dose–response studies indicated that the clinical score reaches a plateau, despite further increase of PMSF doses. These results would imply either that promotion is somehow limited to a fixed degree or that the maximal clinical effect cannot be achieved in chicks. In this respect, however, very high doses of initiators followed by PMSF were able to paralyse chicks (data not shown).

Promotion by PMSF was obtained in chicks up to 5 days after DFP, while in hens this period of "sensitivity" lasted for 11 days [5]. These different periods correlated with the speeds of recovery of NTE activity in sciatic nerve of chicks and hens [9]. Promotable NTE inhibition is 40% 5 days after DFP and 90% 24 hr after single doses. Diagram representation of these findings is given in Fig. 1. Therefore, it seems that promotion in chicks is more effective when less NTE is available and that 90% of NTE inhibition has or had to be reached for promotion to occur. Furthermore, it can be concluded that a target other than NTE is likely to be involved in promotion.

PMSF pretreatment protects 40-day-old chicks from OPIDP and subsequent promotion, whereas protection is not complete in 60-day-old chicks if promotion follows. This is similar to previous results in hens [5] and in general, it suggests that chicks approach full sensitivity to OPIDP at 60 days of age.

In conclusion, these experiments indicate that the resistance of chicks to OPIDP caused by single doses of OPs is only a matter of dose and that nearly complete inhibition of NTE correlates with clinical neuropathy. Promotion of OPIDP also occurs in chicks, but very high NTE inhibition by the initiating OP is required. These quantitative differences in NTE inhibition between chicks and hens correlating with OPIDP and promotion, may underline some qualitative ones between juvenile and adult NTE once modified by the OP. However, it can also be hypothesized that a more severe lesion (i.e. higher NTE inhibition) is required to overcome the more efficient chicks' repair mechanism(s). Promotion is likely to involve a site other than NTE, and seems limited to a certain degree of clinical severity which is in turn probably determined by the potency of the initiating OP.

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