Lack of Correlation of Organophosphorus-Induced Delayed Neuropathy with Neuropathy Target Esterase in Hens and Japanese Quails

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Chickens are sensitive to organophosphorus-induced delayed neuropathy (OPIDN), while Japanese quail are less sensitive. Birds were treated with an organophosphate (OP), diisopropylfluorophosphate (DFP) or fenthion, and were examined for the activity of neuropathy target esterase (NTE) in their brains and spinal cords 24 h later, or for clinical signs of OPIDN for 3 weeks. Some birds were treated with OP and then with phenylmethylsulfonyl fluoride (PMSF), a promoter of OPIDN, and some Japanese quail were treated repeatedly with each OP for one week. The activity of NTE in the brains and spinal cords of hens and quail treated with DFP alone was strongly inhibited. Hens showed characteristic hindlimb paralysis, but quail did not. Birds treated with fenthion alone showed neither reduced NTE activity nor clinical signs. PMSF markedly inhibited NTE activity in both types of birds. Hens treated with DFP plus PMSF developed stronger clinical signs than those treated with DFP alone, while fenthion plus PMSF caused no signs. Quail treated with either OP plus PMSF had no signs. Quail treated repeatedly with DFP but not with fenthion had slight clinical signs. Thus, the inhibition of NTE activity was not linked to the onset of OPIDN.

Key words —— organophosphorus-induced delayed neuropathy, neuropathy target esterase, diisopropylfluorophosphate, chicken, Japanese quail

INTRODUCTION

Organophosphorus compounds (OPs) are widely used as insecticides, plasticizers, antioxidants, and intermediates in organic syntheses. These compounds cause acute neurotoxicity *via* the inhibition of acetylcholinesterase (AChE) and cholinesterases. In addition, certain OPs can also induce delayed neurotoxicity, known as organophosphorus-induced delayed neurotoxicity (OPIDN) in humans and other sensitive species, such as chickens, dogs, cats, and cattle, through mechanisms other than AChE inhibition.[1–3] OPIDN is characterized by progressively developing hindlimb ataxia and paralysis, which can be observed in animals approximately 7—14 days after exposure, and by the degeneration of the distal portions of long axons in both the central nervous system (CNS) and peripheral nervous system.[4–6]

Inhibition of the enzyme known as neuropathy target esterase (or neurotoxic esterase; NTE) in the nervous system has been proposed as the initial effect of compounds that induce delayed neurotoxicity.[7] After administration in animals, strong inhibition (>70%) becomes rapidly apparent and seems to be correlated with clinical symptoms of delayed neuropathy. On the other hand, some inhibitors of NTE, such as phenylmethylsulfonyl fluoride (PMSF), do not cause delayed neuropathy in spite of the strong inhibition of NTE. Pre-treatment with these inhibitors protects animals from OPIDN due to administered neuropathic compounds.[7–9] However, when animals are treated with an OP that is followed by one of these compounds, clinical deficits are exacerbated.[10–12]

Abou-Donia and Lapadula[13] divided cases of OPIDN into two classes: Type I and Type II,

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which differ in the length of the delay prior to the onset of symptoms, clinical signs, and the morphology and distribution of neuropathologic lesions. Type I OPIDN compounds, such as diisopropylfluorophosphate (DFP), tri-*ortho*-tholyl phosphate (TOTP), and mipafox, produce the characteristic features mentioned above. However, Type II compounds such as triphenyl phosphite (TPP) produce less inhibition of NTE and have a shorter latent period of OPIDN than Type I compounds.

Fenthion, an insecticide, causes behavioral abnormalities in humans and is suspected of being able to induce OPIDN.[14] This organophosphate is a unique compound, because the neurotoxicity has been identified as an intermediate syndrome (IMS) whose latent period is shorter than that of OPIDN.[15] Additionally, in young chickens, fenthion had prolonged effects on gait, which include a decreased length and increased width of stride, with no inhibition of NTE.[16]

Adult chickens have been used in many experiments with OPs because of their strong sensitivity to compounds that cause OPIDN. However, there are apparent differences in susceptibility to OPIDN among avian species. It has been reported that Japanese quail generally show no clinical signs after exposure to compounds that cause OPIDN in chickens. Japanese quail showed no clinical signs after exposure to TOTP, even though whole-brain NTE activity was inhibited by more than 70%.[17] In contrast, quail exposed to TPP had both clinical signs and axonal degeneration.[18] These studies suggest that NTE may not play a significant role in the pathogenesis of OPIDN.

Although NTE has been widely used as a tool for the assessment of OPIDN, the events which occur between the inhibition of NTE and clinical signs have not been precisely defined. In the present study, to identify the relationship between the inhibition of NTE and the pathogenesis of compounds that cause OPIDN, as well as the relationship between motor paralysis induced by fenthion and OPIDN, we examined the effects of DFP and fenthion alone and with subsequent-treatment with PMSF on the induction of OPIDN and inhibition of NTE using two avian species, chickens and Japanese quail.

MATERIALS AND METHODS

Chemicals —— DFP (>98% purity) and fenthion (*O*,*O*-dimethyl-*O*-methyl-4-(methylthio) phenylphosphorothioate, >96% purity) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and PMSF and atropine sulfate were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Phenyl valerate was synthesized and purified by the method of Johnson.[19] *N*,*N*'-Diisopropyl phosphorodiamino-fluoridate (mipafox) was purchased from Oriza Laboratories, Inc. (Newburyport, MA, U.S.A.), and *O*,*O*-diethyl *p*-nitrophenyl phosphate (paraoxon) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.).

Animals and Administration of Compounds —— White Leghorn hens (*Gallus gallus domesticus*) that were more than 12 months old and 3- to 4-month-old female Japanese quail (*Coturnix coturnix japonica*) were used. Birds were allowed food and water *ad libitum* during the experimental period.

DFP, fenthion and PMSF were dissolved in olive oil immediately before administration and injected subcutaneously (s.c.) into the posterior cervical region. In order to reduce the acute cholinergic toxicity of organophosphorus compounds, we injected 20 mg/kg of atropine sulfate, dissolved in saline, s.c. into the posterior cervical region 20 min before treatment with organophosphorus compounds. Birds were given a single dose of olive oil as a vehicle (control), of DFP or fenthion with or without subsequent-treatment with a single dose of PMSF, 24 h later, as indicated by the experimental protocol shown in Table 1. Some quail received DFP or fenthion daily for 7 days (Table 1). These doses had been determined to be acutely toxic but not lethal in preliminary experiments.

Clinical Evaluation —— The birds were examined for body weight and signs of motor dysfunction, such as a gait disturbance, every other day for 3 weeks. A modified graded 9-point scale according to Lotti *et al.*[10] was used to evaluate clinical signs. Clinical signs were recorded as follows: 0=no defects in posture, standing ability, or walking performance; 1=slight changes in walking performance; 2=clear changes in walking performance; 3=walking difficulties, with walking limited to short steps; 4=still able to walk but often remaining sitting; 5=unable to walk, sitting but able to stand when stimulated; 6=unable to stand when stimulated; 7=unable to stand, with dropped wings, and extended hindlimb; and 8=death.

Table 1. Experimental Protocol

Bird	Treatment	OP	PMSF
Hen	OP alone	Olive oil, 1 ml/kg	–
		DFP, 1 mg/kg	–
		Fenthion, 5 mg/kg	–
	PMSF after OP	Olive oil, 1 ml/kg	Olive oil, 1 ml/kg
		Olive oil, 1 ml/kg	PMSF, 30 mg/kg
		DFP, 1 mg/kg	PMSF, 30 mg/kg
		Fenthion, 5 mg/kg	PMSF, 30 mg/kg
Quail	OP alone	Olive oil, 1 ml/kg	–
		DFP, 6 mg/kg	–
		Fenthion, 8 mg/kg	–
	PMSF after OP	Olive oil, 1 ml/kg	Olive oil, 1 ml/kg
		Olive oil, 1 ml/kg	PMSF, 30 mg/kg
		DFP, 6 mg/kg	PMSF, 30 mg/kg
		Fenthion, 8 mg/kg	PMSF, 30 mg/kg
	7-day treatment	Olive oil, 1 ml/kg/day	–
		DFP, 3 mg/kg/day	–
		Fenthion, 5 mg/kg/day	–

OP, organophosphate; PMSF, phenylmethylsulfonyl fluoride. Birds were injected s.c. with olive oil, DFP or fenthion as indicated as OP. For treatment with PMSF, vehicle or PMSF was administered s.c. 24 h after the treatment with DFP, fenthion or olive oil.

Preparation of Tissues and Assay of NTE Activity —— Animals were killed by decapitation 24 h after treatment. Whole brains and spinal cords (hens, lumbosacral; quail, whole) were removed immediately, freed of blood and meninges, and homogenized in a buffer. The activity of NTE in each homogenate was determined as described by Johnson,[19] with phenyl valerate as the substrate. The inhibition of NTE activity reached a maximum 24 h after dosing, then the NTE activity was recovered quickly; the half-life of NTE is between 4 and 6 days.[20] It has been proposed that assays of NTE should ensure that the peak effect is identified.[21] Therefore, birds which were killed 24 h after treatment were used for the determination of NTE activity. The activity was expressed as nanomoles of phenyl valerate hydrolyzed per minute per milligram of protein under the condition of the assay. The concentration of protein was measured by the method of Lowry *et al.*[22] with bovine serum albumin as the standard.

Statistical Analysis of Data —— The results of assays of NTE activity were compared by Student's *t*-test and the clinical scores were compared by the Mann-Whitney test. Differences were considered significant when probability values were below 5%.

RESULTS

Clinical Signs in Hens

Figure 1 shows the scores for clinical signs in hens treated with an OP or an OP plus PMSF. Birds treated with olive oil alone or in combination with subsequent PMSF did not show any signs of clinical dysfunction. Birds exhibited ataxia within 8 days after the treatment with DFP alone, and their clinical scores increased gradually until complete ataxia was observed 20 days after administration. The first signs of clinical dysfunction in hens treated with DFP followed by PMSF appeared 4 days after the administration of the OP. The clinical scores of hens treated with DFP plus PMSF were significantly higher, between the 12th and 16th day, than those of birds treated with DFP alone. Almost all hens were unable to stand on the 12th day. By contrast, hens treated with fenthion alone or in combination with subsequent PMSF did not show any signs of clinical dysfunction.

Clinical Signs in Japanese Quail

The clinical scores of quail treated repeatedly with OPs are shown in Fig. 2. Quail receiving DFP displayed slightly reduced hindlimb coordination on the 12th—14th day, and this symptom

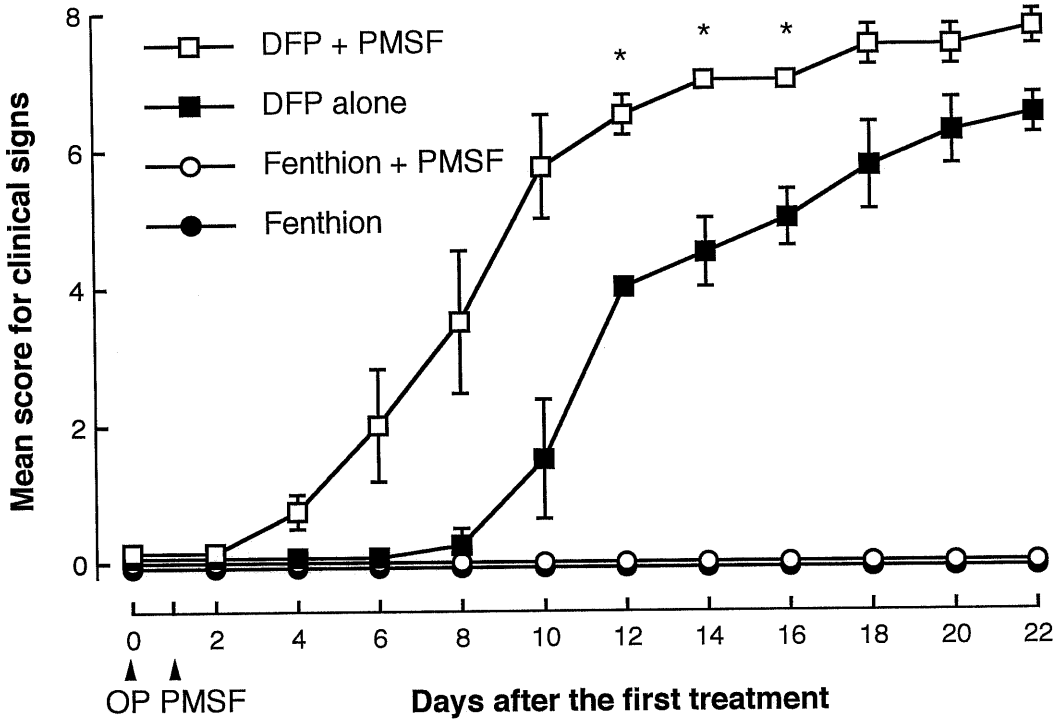

Fig. 1. Clinical Signs of Neurotoxicity in Hens Treated with a Single Injection of DFP or Fenthion or a Single Injection of DFP or Fenthion Followed by PMSF

Hens were scored, as indicated in the text, for signs of lack of motor coordination and ataxia for 3 weeks after treatment (mean± S.E.M., $n=4$). An asterisk indicates a significant difference between results for DFP alone and for DFP+PMSF at $p<0.05$.

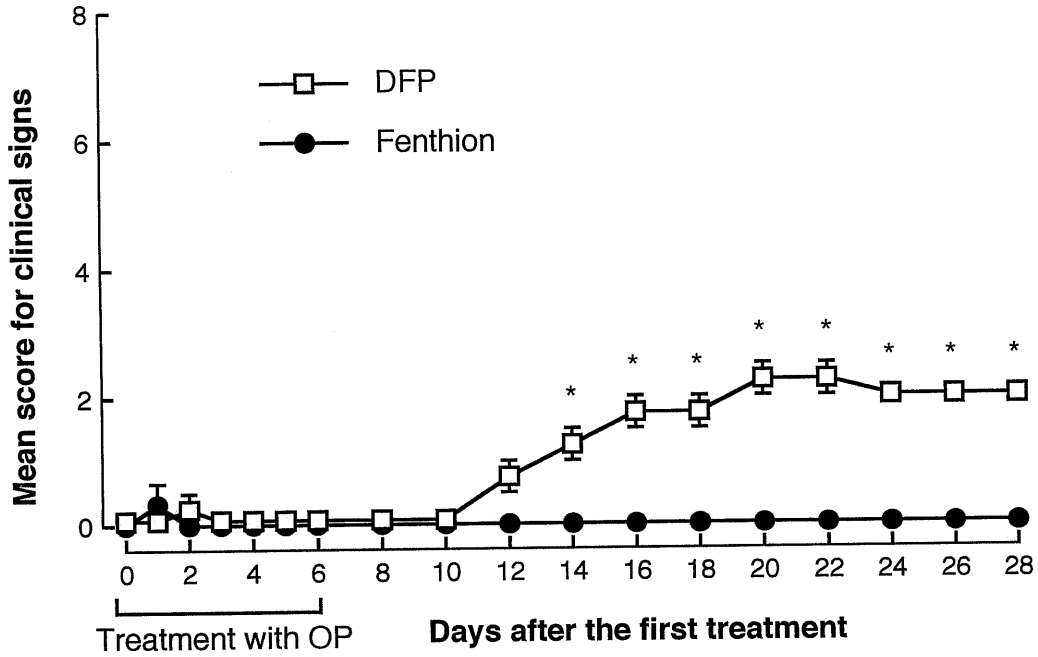

Fig. 2. Clinical Signs of Neurotoxicity in Japanese Quail Treated Repeatedly with DFP or Fenthion

Quail were scored for signs of lack of motor coordination and ataxia for 4 weeks after the first treatment with DFP or fenthion (mean± S.E.M., $n=4$). An asterisk indicates a significant difference from controls at $p<0.05$.

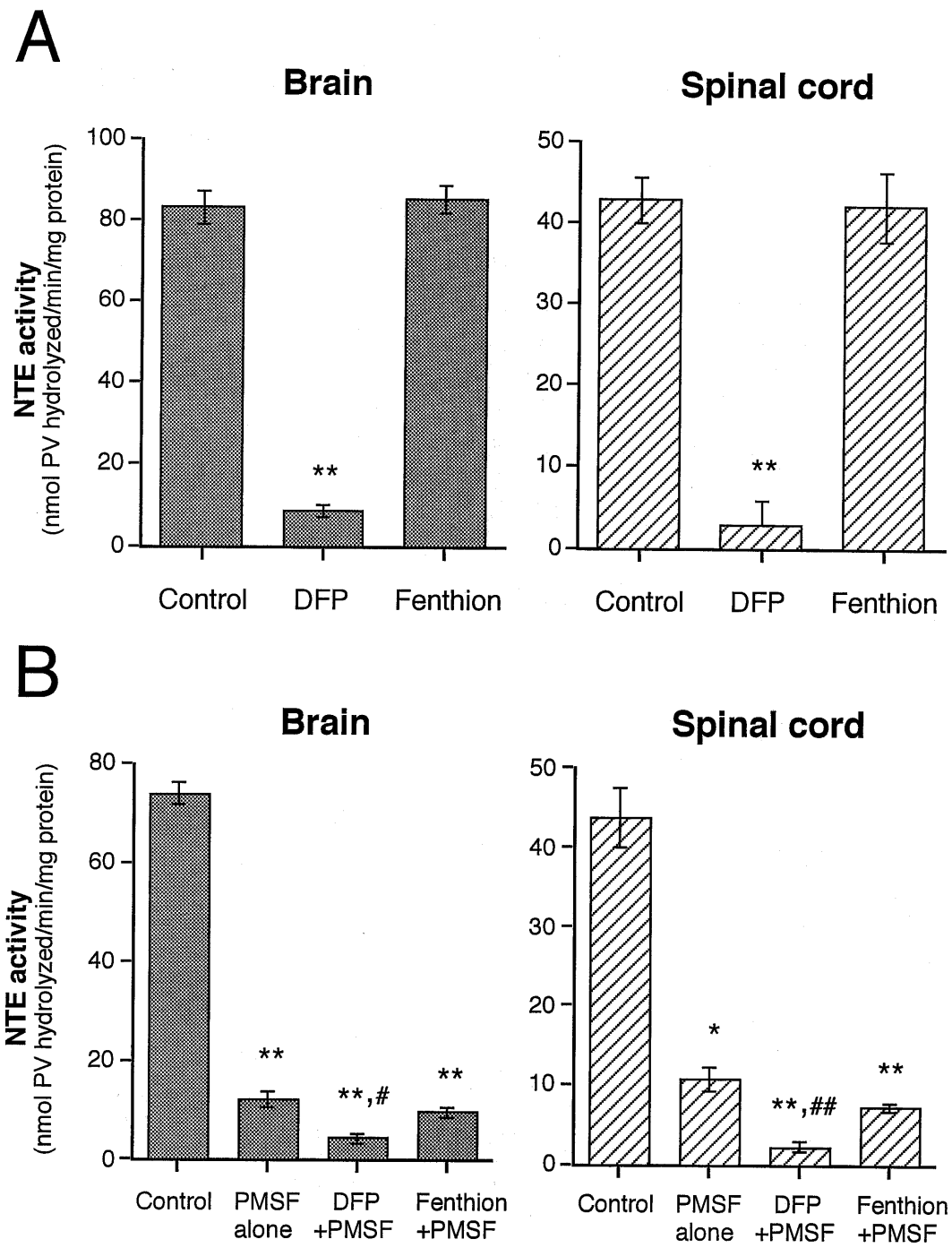

Fig. 3. The Activity of NTE in Tissues of the CNS 24 h after a Single Injection of DFP or Fenthion (A) or a Single Injection of DFP or Fenthion Followed by PMSF (B) in Hens

Each column and vertical bar indicates the mean and S.E.M. ($n=3$). Asterisks and sharps indicate significant differences from controls ($^{*}p<0.005$, $^{**}p<0.001$) and from values for PMSF alone ($^{\#}p<0.05$, $^{\#\#}p<0.01$), respectively. PV, phenyl valerate.

was observed until the end of the observation period. By contrast, quail treated repeatedly with fenthion did not show any signs of clinical dysfunction during the observation period. Neither of the OPs caused signs of clinical dysfunction in quail, and PMSF did not cause or potentiate any signs of clinical dysfunction in quail that had been treated previously with either of the OPs (data not shown). Birds treated with olive oil alone or in combination with subsequent PMSF did not show any signs of clinical dysfunction.

Assay of NTE Activity

The activity of NTE in brains and spinal

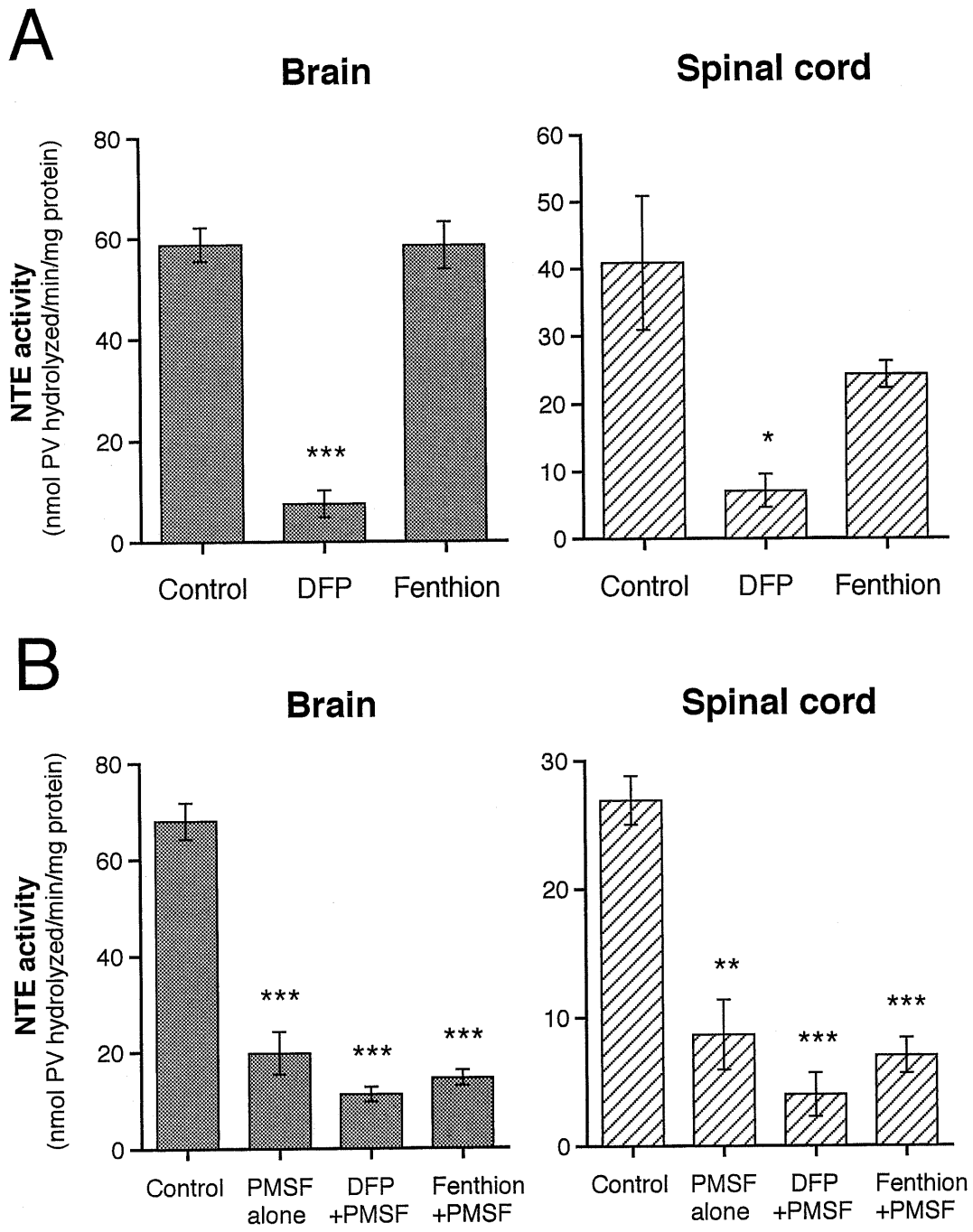

Fig. 4. The Activity of NTE in Tissues of the CNS 24 h after a Single Injection of DFP or Fenthion (A) or a Single Injection of DFP or Fenthion Followed by PMSF (B) in Japanese Quail

Each column and vertical bar indicate the mean and S.E.M. (A, $n=4$; B, $n=5$). Asterisks indicate significant differences from controls (*$p<0.05$, **$p<0.005$, ***$p<0.001$). PV, phenyl valerate.

cords is shown in Figs. 3A and 3B for hens, and in Figs. 4A and 4B for quail. In both birds (Figs. 3A, 4A), treatment with DFP alone severely inhibited the NTE activity in the brain (hen, 89% ; quail, 87%) and the spinal cord (hen, 93% ; quail, 82%). By contrast, treatment with fenthion alone hardly inhibited the activity of NTE in the brains and spinal cords of either hens or quail (Figs. 3A, 4A).

The treatment with PMSF alone severely reduced the NTE activity in brains (hen, 83% ; quail, 70%) and spinal cords (hen, 75% ; quail 67%) (Figs. 3B, 4B). In comparison with the effect of PMSF alone, DFP followed by PMSF had a more significant inhibitory effect on the activity of NTE than PMSF alone in both the brain and spinal cord from hens. By contrast, treatment

with fenthion followed by PMSF did not potentiate the inhibitory effect of PMSF on the activity of NTE in brains and spinal cords. In Japanese quail, the inhibitory effect of DFP followed by PMSF on the activity of NTE in both tissues was greater than that of PMSF alone, although the difference was not significant. By contrast, fenthion followed by PMSF did not potentiate the inhibitory effect of PMSF on the activity of NTE in either tissue.

DISCUSSION

Clothier and Johnson[23] predicted that NTE needs not only to be inhibited using neuropathic OP compounds, but also to undergo a further reaction, called "aging," for the induction of OPIDN. However, it is not known how NTE that was inhibited and underwent the "aging" reaction causes delayed neuropathy. If the inhibition of NTE as enzymes is essential to the pathogenesis of OPIDN, animals exposed to some compound should have clinical symptoms whenever the activity of NTE is strongly inhibited.

In the present experiments, adult hens administered DFP developed clinical signs characteristic of OPIDN, and the activity of NTE in brains and spinal cords was markedly inhibited. Additionally, hens treated with DFP followed by PMSF exhibited stronger clinical signs and inhibition of NTE than in hens given DFP alone. These results are in accordance with those in previous studies.[10-12] However, in the adult Japanese quail, although treatment with DFP alone inhibited NTE activity in the same manner as in the hens, no signs of delayed neuropathy were noted during the observation period. Francis *et al.*[24] also reported that Japanese quail showed no clinical signs after exposure to several different compounds that induce OPIDN in animals. Bursian *et al.*[17] and Varghese *et al.*[18] administered TOTP orally to Japanese quail. Whole-brain NTE activity was inhibited by more than 76% and 90%, respectively, within 24 h, but no clinical signs were observed. Additionally, the present experiments showed that quail treated with DFP followed by PMSF exhibited strongly inhibited NTE activity, as did hens, but they developed no clinical signs characteristic of OPIDN. It has been reported that NTE undergoes an aging effect after exposure to DFP,[23] so these results indicate that the inhibition and aging of NTE by OPIDN compounds might not be significantly correlated with the symptoms of OPIDN.

Varghese *et al.*[18] reported that doses of 62.5 and 125 mg/kg body weight of TPP, proposed as a Type II OPIDN compound,[13] produced delayed clinical signs of OPIDN and axonal and/or terminal degeneration in the cerebellum and brainstem in quail, with an accompanying small reduction in NTE activity. The reduction was, however, below the threshold level (about 70%) for the inhibition of NTE for OPIDN. Doses of TPP that caused hind-leg ataxia, followed by paralysis, caused less than 40% (subthreshold) inhibition of brain NTE in the rat.[25,26] It has been proposed that TPP might be a neurotoxic agent that is distinct from traditional compounds which induce OPIDN because of the rapid and severe onset of clinical signs, accompanied by widespread and severe degeneration of the CNS.[18] However, TPP contains a reactive phosphorus atom and a hydrolyzable ester bond, which should induce the inhibition and aging of NTE. Therefore, if TPP-induced delayed neuropathy is a form of OPIDN, these earlier studies also cast doubt on NTE as the molecular target in OPIDN.

Some recent reports on OPIDN have suggested putative target(s) other than NTE. Abou-Donia and Lapadula[13] suggested that a neuronal cytoskeleton component such as microtubules, neurofilaments, or microfilaments are involved in the pathogenesis of OPIDN and toxic neuropathies. The single oral administration of TOTP to chickens was shown to enhance the *in vitro* phosphorylation of cytoskeletal proteins such as alpha- and beta-tubulin, microtubule-associated protein-2, and neurofilament triplet proteins. The authors concluded that the effects were related to altered Ca^{2+}-calmodulin kinase II activity. Konno *et al.*[27] reported that there are high-affinity binding sites for [^{3}H]DFP, different from the active sites of AChE, butyrylcholine esterase and NTE, and from acetylcholine receptors. If the target site(s) for OPIDN is an enzyme, whose activity was altered quickly and strongly, like NTE or Ca^{2+}-calmodulin kinase II, the symptoms of OPIDN should appear more immediately. In the present experiments with Japanese quail, although neither a single dose of DFP, nor treatment with DFP followed by PMSF caused any evidence of delayed neuropathy or promotion of neuropathy, daily doses of DFP for

one week caused slight clinical signs. The daily application of small doses of Type I compounds are more efficient than a single dose in causing OPIDN in hens, too.[13] Therefore, the real target site might be protein or a functional molecule, such as tubulin, a microtubule-associated protein or neurofilament, which tends to accumulate in the axon, and the accumulated targets might be involved in neurologic dysfunction.

In the hen and the Japanese quail, a single dose of fenthion caused neither delayed clinical signs nor inhibition of NTE, while fenthion followed by PMSF inhibited NTE in excess of 70% without delayed or promoted clinical signs. In addition, daily doses of fenthion for one week did not cause clinical signs of delayed neuropathy. Farage-Elawar *et al.*[16,28] reported that a single oral dose of fenthion had prolonged effects on gait, which were, however, different from signs of OPIDN, but it did not inhibit NTE in young chicks. Recently, it was reported that Wistar rats poisoned with fenthion had clinical signs such as muscle weakness or fasciculations and muscle fiber necrosis, which are not typically caused by OPIDN compounds, but might be related to the persistent inhibition of AChE.[29] Therefore, it is unlikely that fenthion causes OPIDN.

In conclusion, we demonstrated here that the Japanese quail, "a non-susceptible" animal, shows clinical signs of OPIDN after repeated exposure to DFP, an indication that there is a target site in the Japanese quail. Our data suggest that the inhibition of NTE might not play a significant role in the neurotoxic action of compounds that cause OPIDN. In addition, the target site(s) might be a functional molecule(s) other than NTE. These results support the hypothesis that the inhibition of NTE may not be involved in the pathogenesis of OPIDN.

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