DELAYED NEUROTOXICITY AND TOXICOKINETICS OF LEPTOPHOS IN HENS GIVEN REPEATEDLY BY LOW-DOSE INTRAVENOUS INJECTIONS

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Abstract.....The repeated intravenous injections (RIVInj) of 5 mg/kg/day leptophos [O-(4-bromo-2, 5-dichlorophenyl) O-methyl phenylphosphonothioate] for 3 consecutive days caused delayed ataxia in 4 out of 9 hens (44.4%). And one out of 9 hens (11.1%) given RIVInj of 3 mg/kg leptophos for 5 days was affected with ataxia. Twenty hens, however, which received a single intravenous injection (SIVInj) of 15 mg/kg leptophos did not exhibit any delayed neuropathic signs at all. Thus, delayed neurotoxicity was increased by the subdividing RIVInj of the critical dose which was shown in the SIVInj of leptophos. The leptophos concentration in plasma and liver decreased very rapidly after finish of either SIVInj or RIVInj. Although no significant differences were observed in the biological half life of leptophos in plasma by different dosages, the mean level of leptophos decreased significantly with frequency of injections. On the contrary, the evident accumulation of leptophos was observed in only sciatic nerve with RIVInj. Leg muscle maintained relatively high level of leptophos after the last injection. These results suggest that leptophos seems to transfer from blood to affinitive tissues such as sciatic nerve or leg muscles and to accumulate there easily in initial stage after repeated iv injections, and that this causes the enhancement of neuropathy with repeated administrations of divided critical dose of leptophos in both iv and oral administration.

Key words : Organophosphate-induced delayed neuropathy (OPIDN), intensified effect, leptophos, repeated iv administration.

INTRODUCTION

Some organophosphates (OPs), such as leptophos (Abou-Donia et al., 1974), TOCP (Smith and Elvove, 1930), EPN (Abou-Donia and Graham, 1978 ; Yamauchi et al., 1985) and cyanofenphos (El-Sebae et al., 1980 ; Kinebuchi et al., 1981), produce "OP induced delayed neuropathy (OPIDN)" in some susceptible species including man. An affected animal exhibits weakness, ataxia or paralysis which develop 8–14 days from the lower limbs, sometimes to the upper limbs. The onset of ataxia is never less than 8 days after the exposure even if the animal receives a large amount of OPs. We have reported previously that OPIDN from leptophos (Kinebuchi and Konno, 1978) or cyanofenphos (Kinebuchi et al., 1981) was enhanced when the critical dose of OPs, which was shown in the case of a single oral dose, was subdivided. It was suggested that, in the case of oral administration, the efficiency of gastrointestinal absorption might be one of the causes of enhanced OPIDN (Yamauchi et al., 1980).

OPIDN was also observed in hens injected leptophos intravenously (iv) (Konno et al., 1986). Although the iv injection of an OP is not always a strict model of the actual exposure, this route of exposure may be useful to eliminate the influence of the gastrointestinal absorption efficiency of an orally dosed OP on the toxic effect.

In the present study, we investigated the mechanism of the enhancement of OPIDN by the repeated administration of the subdivided critical dose of OP, by means of the observation of OPIDN and the toxicokinetics of leptophos which was given repeatedly by the iv route.

MATERIALS AND METHODS

Chemicals : Crystallized leptophos was prepared from Phosvel® emulsifiable concentrate (Nippon Noyaku Co., Tokyo.) containing 34% active leptophos by the procedure described previously (Konno et al., 1977). The purity of the crystal was estimated 96% or more by gas chromatography. The leptophos solution for the iv injection was prepared just before each injection by dissolving crystallised leptophos in dimethyl sulfoxide (DMSO), Tween-80 and then saline so that 1 mg leptophos was available in 2 ml of solution. Acetonitril, *n*-hexane, ethanol, ethyl acetate, DMSO and Tween-80 were purchased from Wako Pure Chemical Industries (Osaka, Japan). These chemicals were of analytical reagents.

Animal and Intravenous Injection : Adult laying hens (*Gallus gallus domestics*), 23 months of age and weight ranged 1.3–1.9 kg were used. After one week inspection, hens which showed no abnormal signs were caged individually during the treatment period and allowed to drink and eat *ad libitum*. The leptophos sol. was injected from *V. ulnaris* of hen.

Assessment of Delayed neurotoxicity :

- 1) Four groups of hens received four dosages of leptophos, a single iv injection

(SIVInj) at the rate of 15 mg/kg body weight, daily RIVInj of 5 mg/kg/day for 3 days and 3 mg/kg/day for 5 days. These three groups were to be given equally 15 mg/kg of leptophos in total. The 4th group received 2 ml/kg/day of the vehicle alone for 3 days as control. They were transferred to the indoor paddock after the recovery from acute poisoning, weighed periodically and their behaviour and gait were observed daily for 21 days after each last injection.

2) A grading system was used to note the progress of clinical neuropathic signs, judged mainly from the conditions of the lower limbs, in four stages as we reported earlier (Yamauchi, 1980) ; 'mild ataxia (+)', 'gross ataxia (++)', 'mild paralysis (+++)' and 'severe paralysis (++++)'.

Determination of leptophos concentration in tissues :

1) Heparinized blood samples were collected at 15 and 30 min, 1, 2 and 3 hr after each last OP dose in 5 groups of hens received the iv injection of leptophos of 5 dosages ; SIVInj of 15 mg/kg, daily RIVInj of 5 mg/kg/day for 1, 2, 3 or 4 days. The tissue samples, such as brain (cerebrum), spinal cord (lumbar level), sciatic nerve including tibial and fibular branches, leg muscle (*m. adductor magnus*) and liver, were also removed from both groups of hens, which were killed at 30 min and 3 hr after the last injection, of the respective groups of the same dosages as mentioned above.

Blood samples were also collected daily at 30 min after every iv injections from 3 groups of hens which were given daily RIVInj of leptophos at the rate of 3, 5 mg/kg/day and 2 ml/day of vehicle alone for 4 days.

These collected samples were stored at -20°C prior to assay.

2) The gas-liquid chromatography (GLC) was used for the determination of leptophos concentration in plasma and tissues. The procedures of analysis and the operating parameters of GLC were described in our earlier reports (Konno et al., 1977, 1986). The gas chromatographic analysis was performed with a Hitachi Model 163 (Tokyo, Japan).

Statistic Analysis : Differences in quantitative parameters between groups were examined for statistical significance using the Student's *t* test, Welch's method and Fisher's exact test ; probability values of 5% or less were considered significant.

RESULTS

1. Delayed Neurotoxicity :

No hens showed abnormal behaviour or gait throughout the observation period in both groups where hens received SIVInj of 15 mg/kg leptophos and daily RIVInj of 2 ml/kg vehicle alone for 3 days. On the contrary, 4 out of 9 hens (44.4%) which received daily RIVInj of 5 mg/kg/day leptophos for 3 days were affected with ataxia on the 11–13th observation day after the finish of RIVInj. One of them developed gross ataxia (++) and another died due to neuropathy on the 21st observation day. And out of 9 hens received RIVInj of 3 mg/kg leptophos for 5 days, only one

(11.1%) showed ataxia (+). The results are summarized in Table 1. The incidence rate of neuropathy in the group of hens given RIVInj of 5 mg/kg leptophos for 3 days was significantly higher than that of the group of hens given SIVInj of 15 mg/kg ($P < 0.005$). The body weight of hens at death or on the final observation day decreased significantly in the groups of RIVInj, as comparing with each initial weight ($P < 0.05$). The group of hens given 3 mg/kg/day for 5 days lost significantly ($P < 0.01$) their weight already during the injection period, may be due to loss of appetite by continuing acute poisoning (Table 2).

2. Leptophos Concentration in Plasma :

Fig. 1 (b) shows the relationship between the frequency of RIVInj of 5 mg/kg/day and the leptophos concentration in plasma at selected time intervals (15, 30, 60, 120 and 180 min) after the finish of RIVInj, in comparison with the group of SIVInj of 15 mg/kg (Fig. 1 a). The plasma sample, however, at 3 hr of the group given 5 mg/kg/day for 3 days was not available since the vascular wall of the hens was

Table 1. Delayed neurotoxicity from iv administration of leptophos in hens.

Dose (mg/kg/day×day)	No. of tested hens	No. of neuropathic hens ^{a)} ;						P ^{c)}
		Ataxia		Paralysis		Death ^{b)}	Total	
		+	++	+++	++++			
15×1	20	0	0	0	0	0	0 / 20	—
5×3	9	2	1	0	0	1	4 / 9	0.0053
3×5	9	1	0	0	0	0	1 / 9	n. s.
Vehicle × 3 ^{d)}	9	0	0	0	0	0	0 / 9	n. s.

a) Neurotoxic signs on the final day of the 21days observation period. (Refer to the text).

b) Number of deaths during the observation period.

c) The incidence rate of neuropathy in the 2nd (5 mg/kg ×3 days), 3rd (3 mg/kg ×5 days) and 4th (vehicle) groups were compared with that in the 1st (a single dose of 15 mg/kg) group by using Fisher's exact test and "n. s." means 'not significant'.

d) Hens in this group received iv injections of 2 ml/kg vehicle consisting of DMSO, Tween-80 and saline for 3 days.

Table 2. Change of body weight of hens after iv administration of leptophos^{a)}.

Dose	No. of hens	Body weight (kg, Mean±s.e.)		
		Initial	After last inj.	Final observ. day
15mg/kg	20	1.51±0.036	1.56±0.036	1.56±0.041
5 mg/kg×3	9	1.63±0.106	1.56±0.084	1.44±0.076** ^{b)}
3 mg/kg×5	9	1.60±0.053	1.47±0.059**	1.44±0.070*
Vehicle × 3	9	1.51±0.066	1.53±0.061	1.51±0.039

a) Refer to the foot notes of Table 1.

b) Compared with the initial weight in each group by student's t test : * $P < 0.05$ ** $P < 0.001$.

Fig. 1. Leptophos level in plasma at selected time intervals after finish of a) a single iv injection of 15mg/kg ($n=5$, $\circ$) and b) the repeated iv injections of 5mg/kg/day for one day ($n=5$, $\blacksquare$), 2 days ($n=5$, $\blacktriangle$) and 3 days ($n=6$, $\bullet$). Plots and satellite bars indicate the mean and s. e. (ppm) and the biological half lives were estimated from the data at 15, 30, 60, and 120 min in each group, 41.5 min in 15mg/kg SIVInj group, 28.0, 33.8 and 33.6 min in 5mg/kg RIVInj for 1, 2 and 3 days groups, respectively.

too fragile to needle so frequently, injections and samplings. The semilogarithmic plots of concentration vs time for leptophos showed a monoexponential decline of leptophos concentration in the group of SIVInj, however, declines with 2 (or 3) phases in the groups of RIVInj. The biological half life (BHL) of leptophos in plasma was estimated from the concentration at 15, 30, 60 and 120 min, and that of the SIVInj group was longer than those of RIVInj groups. The BHLs of RIVInj groups were similar to each other in spite of the different frequency of the injections. And the average level of the leptophos concentration became down with the frequency of injections of the same dose (5 mg/kg). Fig. 2 shows the time course of leptophos concentration in plasma which was followed during RIVInj period in 2 groups of hens given 3 or 5 mg/kg/day for 4 days. The level of leptophos in plasma of both groups decreased significantly ($P<0.05$), compared with the level after the 1st injection while RIVInj were still continued.

3. Leptophos in Tissues :

The time trends of leptophos levels in several tissues during the RIVInj, leptophos were measured at 30 min and 3 hr after every iv injection of 5 mg/kg leptophos for 1 to 4 RIVInj (Fig. 3). As for the brain, the concentration of leptophos decreased significantly from 30 min to 3 hr ($P<0.05$), but no significant differences were observed among the 4 groups. In the sciatic nerve, however, the leptophos concentration rather showed somewhat increasing trends from 30 min to 3 hr after each RIVInj and seemed to accumulate gradually with the repeated

Fig. 2. Leptophos level in plasma of 2 groups of hens (n=5, in each) at 30 min after every iv injections of 3 and 5mg/kg/day for 4 days. Columns and bars indicate the mean and s. e. (ppm). * (P<0.05) and ** (P<0.01) indicate significant difference from each initial value (after 1st injection) by using Welch's method.

injections. The similar trend was observed slightly in the spinal cord, as well. The leg muscle kept relatively high level of leptophos after each RIVnj. On the other hand, the leptophos concentration in liver decreased sharply from 30 min to 3 hr after each RIVInj and the mean level also reduced gradually with the frequency of iv injections.

DISCUSSION

It is well known that toxic effect of a certain dose of a chemical, including organophosphate (OP) inhibitors of cholinesterase, is generally reduced by the repeated oral (po) administration of subdivided smaller doses over some days, to take some instances OPs, TOCP (Henschler, 1958 ; Barnes, 1975), cyanofenphos (Konno et al., 1983) and MOCP (mono-2-cresyl diphenyl phosphate) (Lotti and Jonson, 1980).

There have been, however, few reports of the 'enhanced' toxic effect which is shown when a subeffective dose of a compound is given by subdividing small doses

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Fig. 3. Leptophos level in several tissues at 30 min and 3hr after finish of the repeated iv injections of leptophos at the rate of 5mg/kg/day for one day in the 1st group (n=5, 6), 2 days in the 2nd group (n=3, 6), 3 days in the 3rd group (n=3, 5) and 4 days in the 4th group (n=3, 5). Columns and satellite bars indicate the mean and s. e. (ppm) and significant difference by Welch's Method (* : $P < 0.05$) was observed between the values at 30 min and 3hr.

for consecutive days. We have previously reported that the highest incidence and severity of OPIDN was observed in hens given 5 po doses of 50mg/kg leptophos among several dosages, such as a single dose of 250mg/kg, 2 doses of 125mg/kg, 3 of 83mg/kg and so on, of the critical dose (250mg/kg) (Kinebuchi and Konno, 1978 ; Yamauchi et al., 1980). Furthermore, the group of hens which received totally 200–600mg/kg of cyanofenphos by 4 subdivided po doses on every 2 days showed higher incidence of OPIDN than those given the same amount by a single po dose (Kinebuchi et al., 1981). Although Smith et al. (1932) reported that 12 doses of 10mg/kg/day (totally, 120mg/kg) or 26 doses of 5mg/kg/day (130mg/kg) of TOCP produced severe ataxia and 76 doses of 2.5mg/kg/day (about 190mg/kg) did not, OPIDN of TOCP in their result also should be interpreted to be intensified by subdividing into small doses, because a single po dose of 250mg/kg could not produce OPIDN in hens (Yamauchi, unpublished). However, we also found at the same time that OPIDN decreased again in hens given po 250mg/kg of leptophos subdivision into more frequent and smaller amounts of doses. These results seemed to suggest that these alternations of OPIDN by po administration may depend on the efficiency of gastrointestinal absorption of OP. Therefore, we employed iv injection as a route of exposure to eliminate the influence of the gastrointestinal absorption rate of OP from po administration.

The approximate median effective dose (ED_{50}) for OPIDN and LD_{50} of leptophos by iv administration were estimated to be 21.6mg/kg and 37.6mg/kg, respectively (Konno et al., 1986). We employed mainly a total amount of 15mg/kg leptophos as a subneurotoxic dose in this study. Evidently the divided small doses (5mg/kg/day) of leptophos by RIVInj for 3 days produced severer neuropathy than SIVInj of 15mg/kg although smaller doses (3mg/kg/day) for a few more days (for 5 days) could not (Table 1). These were similar to the results from the po dose (Kinebuchi and Konno, 1978 ; Yamauchi et al., 1980). It suggests that the enhancement of OPIDN by subdividing dose of OP might be independent from the route of administration. Therefore, the time courses of leptophos in blood and tissues had to be determined after some different RIVInjs.

The chemical behaviour of leptophos *in vivo* after iv injection may demonstrate the model of the po dosed chemical after being absorbed into circulated blood. The leptophos concentration decreased exponentially in plasma of all groups (Fig. 1). BHL of leptophos in plasma ranged from 28 to 42 min in this study. BHLs for other amount of iv injections were as follow ; 36 min for SIVInj of 10mg/kg (Konno et al., 1986) and 30 min for SIVInj of 30mg/kg (Yamaguchi et al., 1987). These results suggest that the BHL of leptophos in plasma may be around 30–40 min independently of the amount and frequency of administration. The leptophos levels in plasma after finish of these RIVInjs of 5mg/kg/day decreased with frequency of iv injections. The reason why the OP level in plasma dropped down after more frequent RIVInj is very interesting. Hence, the change of the leptophos concentration in plasma was followed under the period (4 days) of RIVInj of small doses (3 or

5mg/kg/day). The leptophos levels in both groups dropped down after every injection day by day (Fig. 2). It may suggest that leptophos which is injected into blood stream become easy to disappear rapidly and to transfer to some tissues.

On the other hand, the time course of the leptophos concentration was followed in early stage after every RIVInjs in several tissues (Fig. 3). In liver, the very rapid decline of leptophos from 30 min to 3 hr after every iv injection and the reduction of the mean levels of the OP by RIVInj were observed. These trends were similar to those in plasma. On the contrary, the leptophos concentration in sciatic nerve rose up somewhat from 30 min to 3 hr after every RIVInj and accumulated gradually by RIVInj. Leg muscle kept relatively high concentration of the OP and indicated the trend rather like 'sciatic nerve type' than 'plasma-liver type'. Brain and spinal cord showed the intermediate trend between these 2 types. These results suggest that the neurotoxic OP, such as leptophos, may transfer from circulated blood into some affinitive tissues to the OP, such as sciatic nerve, leg muscle or adipose tissue, in early stage after iv injection, and that the OP will accumulate gradually in these tissues with the repeated administration. These findings coincide with our previous prediction (Yamauchi, 1980). Abou-Donia described, however, that sciatic nerve had the lowest amount of ^{14}C at 12 hr after the topical application (Abou-Donia, 1979) or the po administration (Abou-Donia, 1980) of ^{14}C -leptophos. The difference between our results and his may partially be due to each method; we measured unchanged leptophos itself in early stage after administration while he counted ^{14}C of both leptophos and its metabolites at 12 hr or more after dosing. If the rapid redistribution of the OP from blood to tissues in the case of po administration is caused by the lipophilia of the OP (Abou-Donia, 1980), much more concentration of leptophos should be detected in the nervous tissues which have much lipid.

The results of the present study also suggest that leptophos may transfer from blood to these affinitive tissues more efficiently by RIVInj and too small dose may not be able to accumulate above the threshold level of clinical neuropathy, even following repeated dosing. Clinical neurotoxic signs may appear after accumulating neurotoxic OP over the critical level and affecting the target tissues. The severity of tissue injury may depend on not only the leptophos level in the tissue, but also the relation between the threshold level and time interval which the OP is able to remain above the threshold in the target tissue.

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