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SHORT COMMUNICATIONS

Role of acid phosphatase in delayed neurotoxicity induced by leptophos in hens

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Leptophos (Phosvel) is an organophosphorous insecticide which has been implicated in the poisoning and paralysis of workers in the Texas factory where it was formerly produced and packaged[1]. This compound produces delayed neurotoxicity in farm animals, hens[2] and ducklings[3], similar to that caused by tri-*o*-cresyl phosphate (TOCP). The clinical condition is preceded by a period of delay. Usually 8–14 days are needed after intoxication to produce ataxia and pathological disruption of axons with subsequent Wallerian degeneration of myelin in the central and peripheral nerves[4–6]. Increased activity of acid hydrolases has been reported in Wallerian degeneration[7, 8] and in lesions of experimental allergic encephalomyelitis and multiple sclerosis[9–11]. Although the mechanism of organophosphorous ester-induced delayed neurotoxicity (OPIDN) is not known, it appears reasonable to assume that lysosomal hydrolytic enzymes, which are believed to function in the cell in the digestion of endocytosed macromolecules, must be involved in this process sooner or later. The present investigation, therefore, was designed to study acid phosphatase levels in the plasma of hens after prolonged oral administration of leptophos. A correlation is made between the level of this enzyme and plasma cholinesterase and the clinical and histopathological changes observed, in an attempt to shed some light on the mechanism of OPIDN. A preliminary account of this work has been presented[12].

MATERIALS AND METHODS

Leptophos [*O*-(4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate], technical grade (94.8% ir, 87.2% gc), was provided by Velsicol Chemical Co., Chicago, IL.

Care and treatment of birds. Laying hens (*Gallus gallus* domesticus), mixed breed, each 18 months old and weighing approximately 1.5 kg (1.4 to 1.6 kg) were used. The hens were placed in individual cages in an air-conditioned room and allowed to adjust to the environment for 1 week before the beginning of the experiment. Six groups of birds (three hens each) were given daily a single oral dose of 0.5, 1, 2.5, 5, 10 and 20 mg/kg of technical leptophos. Three control hens were given a gelatin capsule daily. The oral administration continued until the onset of paralysis, but no longer than 129 days; hens given 0.5 mg/kg were exceptions, with treatment lasting only 73 days (Table 1). The birds were supplied with food and water *ad lib.* All hens were examined daily in order to detect any abnormality in gait or behavior. The body weights of the birds were monitored throughout the experiment, and their eggs collected and weighed. At the end of the observation period after the last dose of each treatment regimen, the hens were anesthetized with pentobarbital, and killed by heart puncture. Blood samples were collected for enzyme studies.

Histological methods. Brains, spinal cords and sciatic nerves of hens were excised and prepared for histological examination as previously described[4].

Enzymatic analysis. Heparinized blood samples were centrifuged at 21,000 *g* for 5 min in a refrigerated Beckman model J-218 centrifuge (Bechman Instruments, Inc., Fullerton, CA) and the resultant blood plasma samples were retained for immediate determination of plasma cholinesterase[4] and acid phosphatase[13] activities. Cholinesterase (EC 3.1.1.8) measurements were made by observing the initial rate of butyryl thiocholine (BuTCH) hydrolysis in a Varian Techtron model 635 UV-VIS spectrophotometer at 412 nm using the color reagent 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). A total of 0.02 ml plasma was added to the reaction mixture which contained 10^{-3} M Tris buffer, pH 7.4, in a final volume of 4.0 ml. The reaction was carried out at 38° for 5 min in a Dubnoff metallic shaker.

Acid phosphatase (EC 3.1.3.2) was estimated by the incubation of 0.25 ml plasma with 8 mM *p*-nitrophenyl-phosphate and 0.3 M acetate buffer (pH 5) in a final volume of 1 ml, in the presence or absence of 10 mM L(+)-sodium tartrate (Sigma Chemical Co., St. Louis, MO). The reaction was carried out at 38° for 60 min in a Dubnoff metabolic shaker. After the reaction was stopped by the addition of 4 ml of 0.5 M NaOH, the *p*-nitrophenol (PNP) released was estimated at 410 nm. The plasma acid phosphatase activity was taken as the tartrate inhibited portion of total activity, the remainder being attributed to the erythrocyte enzyme [14].

Plasma proteins were determined by the method of Lowry *et al.*[15]. The results are expressed as μmoles BuTCH hydrolyzed/min/mg of protein and nmoles of PNP produced/hr/mg of protein for plasma ChE and plasma acid phosphatase respectively.

RESULTS AND DISCUSSION

The sequence of intoxication, onset of clinical signs, and histological changes in tissues in hens given a daily single dose of leptophos is shown in Table 1. The progress of clinical signs was graded into four stages of ataxia prior to paralysis[12]. T_1, mild ataxia, is characterized by diminished leg movement, a reluctance to walk, and a tendency to slide along the floor or fly. In T_2, gross ataxia, there is a disturbance of the control of leg movement with a change in gait. T_3, severe ataxia, designates the stage in which the legs sprawl out in front and can be neither stretched nor lowered. T_4, ataxia with near paralysis, is marked by difficulty in moving the legs, inability to stand or walk and by a tendency to sit down on the hocks most of the time. At this point, chickens usually show respiratory and swallowing difficulties, with some birds exhibiting a rapid irregular tremor.

Prolonged daily oral administration of small doses of leptophos ranging between 0.5 and 20 mg/kg caused ataxia (T_1) 7–65 days after the beginning of administration. All hens given 20 mg/kg/day showed transient paralysis after 32–37 days of administration. After the discontinuation of treatment in these hens at the onset of paralysis, their clinical conditions improved; paralysis reverted to stages T_4 and T_3 of ataxia. The clinical condition of Hen No. 1 later deteriorated,

Table 1. Sequence of intoxication, onset of clinical signs, and histological changes in tissues from hens given a daily oral single dose of leptophos*

Hen No.	Dose (mg/kg/day)	No. of doses	Days after beginning of administration: Ataxia† T_1	T_2	T_3	Paralysis T_4	Killed	Histological changes‡: Sciatic nerve	Spinal cord
1	20.0	32	10	24	27	30	32	+	++
					79	62			
						107	120 130		
2	20.0	37	14	24	30	35	37	+	++
					90	76	130		
3	20.0	37	7	14	30	34	37	+	++
					90	76	130		
4	10.0	129	10	33	45	72	130	−	+
5	10.0	129	10	33	45	72	130	−	+
6	10.0	129	17	38	62	76	130	−	+
7	5.0	129	33	55	83		130	−	+/−
8	5.0	129	27	58	99		130	−	+/−
9	5.0	129	32	45	99		130	−	+/−
10	2.5	129	40	69	110		130	−	+/−
11	2.5	129	41	58	110		130	−	+/−
12	2.5	129	44	62	110		130	−	+/−
13	1.0	129	60	61	65	66	67	−	−
					69	66	130		
14	1.0	129	60	61	66		130	−	−
15	1.0	129	65	67	69		130	−	−
16	0.5	73	64				74	−	−
17	0.5	73	64				74	−	−
18	0.5	73	62				74	−	−
19	§	129					130	−	−
20	§	129					130	−	−
21	§	129					130	−	−

* Oral administration was continued daily until onset of paralysis or the end of the experiment.

† Clinical grades are: T_1, mild ataxia; T_2, gross ataxia; T_3, severe ataxia; T_4, near paralysis, as described in the text.

‡ The following abbreviations were used: (−) changes absent; (+/−) changes equivocal; (+) changes present; and (++) severe degeneration.

§ Gelatin capsule controls; hens were given an empty gelatin capsule daily.

however, and ended as paralysis when the experiment was terminated. The clinical conditions of hens given 10.0 mg/kg/day progressed to ataxia with near paralysis (T_4). Hens given 1.0, 2.5 and 5.0 mg/kg showed severe ataxia (T_3). A worsening and subsequent improvement of its clinical condition were also evident in Hen No. 13 given a 1.0 mg/kg/day dosage. The other two hens of this group progressed to the severe ataxia stage (T_3). Hens given 0.5 mg/kg/day for 73 days showed only mild ataxia (T_1).

Figure 1 shows that the number of days of oral administration of small doses of leptophos before the onset of ataxia depended upon and was inversely proportional to the size of the daily single dose. Thus, when a daily oral dose of 0.5 mg/kg was given, an average of 63 days of administration was required to produce ataxia. In contrast, an average of 10 days was needed when the dosage was 20 mg/kg. Figure 1 also shows that the "total administered dose" before the onset of ataxia was proportional to the size of the daily ingested dose. This was not, however, merely a cumulative effect, since at lower daily doses there was a decrease in the total dose required to produce ataxia. It is noteworthy that the dose of 0.5 mg/kg, which showed mild ataxia in this study, caused no abnormality in gait or behavior when it was administered for 60 days[5]. These results indicate that the "no effect" level is influenced by both the duration of administration and the dose level and that continued administration of doses smaller than the threshold dose might result in an obvious state of delayed neurotoxicity.

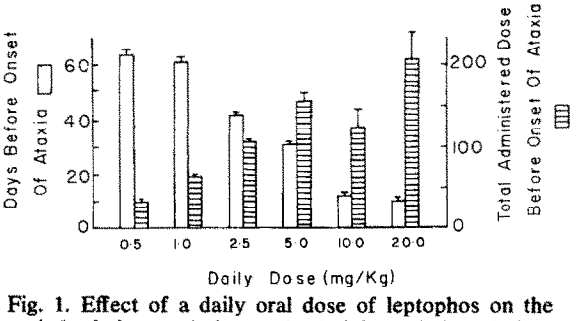

Fig. 1. Effect of a daily oral dose of leptophos on the period of time and the total administered dose before onset of ataxia.

A loss of weight in most of the treated hens was dose dependent and reflected their clinical conditions. Adverse effects on egg weight and production resulted at all dose levels tested.

Histopathologic examination of nerve tissues obtained from treated hens revealed neural lesions in all animals given a daily dosage of 2.5 mg/kg or higher (Table 1). These lesions were present in the spinal cords, and in general the relative severity of tissue damage was dose dependent. Minimal alterations were seen in hens with only ataxia; severe changes were seen in chickens receiving 20 mg/kg. The latter showed swollen and degenerated myelin sheaths and axons from the posterior and lateral columns in the cervical cord and the middle

portion of the anterior columns in the lumbar cord. The process of axonal and myelin sheath disruption was observed in the following sequence: first, axonal swelling; subsequently, axonal degeneration during which vacuolation, clumping and fragmentation of the axon were exhibited; finally, degeneration and loss of the myelin sheath. No histopathological changes were seen in the sciatic nerves of most of the treated hens. The only changes seen were in the severely poisoned hens which had been given a daily dose of 20 mg/kg continually for 129 days. These lesions, moreover, were seen much more easily in the tibial and peroneal nerves, below the division of the sciatic nerve. The changes consisted of numerous foci of swollen myelin sheaths, fragmented myelin and fragmented axons. The present histopathologic observations are in accord with other reports on leptophos[4, 5], and other neurotoxic organophosphorus esters [16].

Daily oral administration of a single dose of leptophos inhibited plasma ChE (Fig. 2). The inhibition was greatest in hens given 20 mg/kg/day which showed paralysis. Daily doses of 0.5 mg/kg which produced mild ataxia caused the least inhibition of plasma ChE. This prolonged effect of small oral doses of leptophos on plasma ChE activity is in accord with earlier studies on the effect of leptophos[5] as well as that of DFP (diisopropyl phosphorofluridate) and Mipafox (N,N'-diisopropyl phosphorodiamidic fluoride) on pseudo ChE in the cervical spinal cords of hens[17]. The experimental results on the inhibition of ChE by neurotoxic organophosphorous compounds are in agreement with the human cases of poisoning by Mipafox, where it was shown that plasma ChE activity remained at low levels for many weeks[18]. The implication of ChE as a possible etiological factor in delayed neurotoxicity[19, 20], however, has been eliminated. There is no consistent correlation between anticholinesterase activity and the delayed neurotoxic syndrome[21].

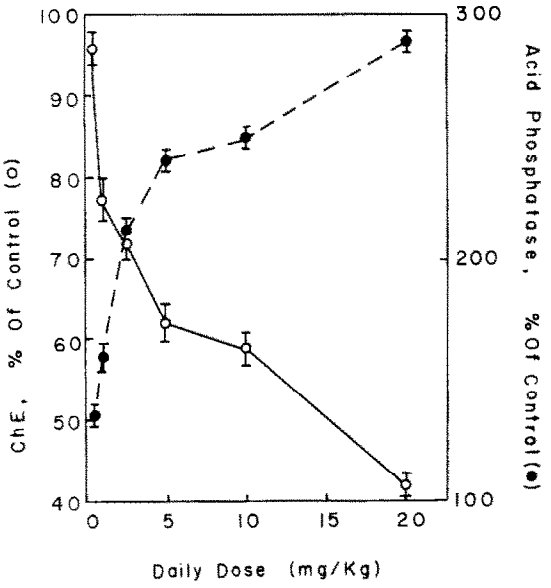

Fig. 2. Effect of a daily single oral dose of leptophos (0.5, 1.0, 2.5, 5.0, 10.0 and 20 mg/kg on plasma ChE (○) and acid phosphatase (●) activity. Results are calculated as the percentage of activity measured in plasma from controls taken at the same time. Each point represents the mean ±S. E. of nine determinations from three birds. The mean ±S. E. of three controls was 1410 ± 320 nmoles BuTCH hydrolyzed/min/mg of protein and 4540 ± 257 nmoles PNP hydrolyzed/hr/mg of protein for plasma ChE and acid phosphatase respectively.

Plasma acid phosphatase activity of all treated birds was significantly increased ($P < 0.001$ to 0.05). The increase over the control ranged between 36 and 190 per cent in hens receiving daily oral doses from 0.5 to 20 mg/kg of leptophos. Again, the effect was dose dependent, i.e. an increase in dose produced an increase in enzymatic activity (Fig. 2). The increased acid phosphatase activity in hen plasma indicates possible *in vivo* lability of lysosomal membranes with the release of this enzyme. Leptophos might cause liver damage, which in turn would lead to the release of acid phosphatase. This mechanism is in agreement with the finding[22] that malathion released arylsulfatase from rat liver lysosomes. On the other hand, it is possible that the source of acid phosphatase is nerve tissue lysosomes. This suggestion is strongly supported[23] by the early increase in acid phosphatase activity found in nerves and neuroglia of hens treated with TOCP.

Although the initial event in delayed neurotoxicity induced by organophosphorous compounds might be inhibition of an esterase[24], it is possible that these compounds cause an interruption of the normal flow of essential metabolic factors (e.g. oxidative enzymes) from the cell body to the distal axon, as has been suggested for Wallerian degeneration[25]. A consequence would be the disruption of the lysosomal membranes, resulting in degeneration of the axis cylinders and ultimately of the enveloping myelin sheath. Of interest is the finding that the membrane permeability of lysosomes of injured cells undergoes changes in Wallerian degeneration[25].

It is suggested that increased acid phosphatase activity, accompanied by decreased plasma ChE activity, may be used as an early warning index of overexposure to leptophos. However, this relationship should be examined for other organophosphorous esters that do not induce the neurotoxic syndrome as well as for ones that do induce delayed neurotoxicity before these enzyme markers are proposed for general use.

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MOHAMED B. ABOU-DONIA
Department of Pharmacology,
Duke University Medical Center,
Durham, NC 27710, U.S.A.

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A unique *in vivo* stimulation of labeled amino acid incorporation into protein by fusidic acid in the rat*

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Fusidic acid, an antibacterial steroidal antibiotic, produced by *Fusidum coccineum*, was first isolated by Godtfredsen *et al.* [1] and tentatively characterized by Godtfredsen and Vangedal [2]. The three antibacterial steroid antibiotics, helvolic acid, fusidic acid and cephalosporin P_1, are chemically related.

Preliminary work by Harvey *et al.* [3] and Yamaki [4] showed that fusidic acid inhibits protein synthesis in whole cells and synthesis directed by both polyuridylic acid and endogenous messenger in cell-free extracts of various bacteria. Further work by Harvey *et al.* [5] has shown that fusidic acid affects the final polymerization of amino acids after formation of the ternary complex (polyribosomes with bound phenylalanyl S-RNA). Fusidic acid stops the movement of aminoacyl- or peptidyl-tRNA from the acceptor site to the donor site even if the donor site is empty [6]. Fusidic acid also stabilizes both prokaryotic [7] and eukaryotic [8] ribosome—translocation factor—GDP complexes while allowing a single round of GTP hydrolysis and translocation. Okura *et al.* [9] and Willie *et al.* [10] have reported that sodium fusidate and the sodium salt of 24,25-dihydrofusidic acid, respectively, inhibit polypeptide chain elongation by binding to the ribosome—elongation factor-G—GDP complex, thereby preventing its dissociation.

Active cation transport across the cell membrane is a function of Na^+, K^+-ATPase. This complex enzyme system can also be inhibited by fusidic acid [11]. Furthermore, steroids of the fusidane family structurally resemble the bile salts, which act as alimentary biodetergents [12, 13]. Several derivatives of fusidic acid are similar in chemical and biophysical properties to bile salts [14, 15]. This surface activity and micelle formation are similar to those found for the interaction of a drug with receptor sites, serum proteins or membrane components *in vivo* [16].

Similarities in the structure of some steroid anabolic hormones (testosterone, estrogen, etc.) to fusidic acid suggest that the mode of action of these hormones on protein synthesis in eukaryotes might be elucidated through the use of fusidic acid *in vivo* and *in vitro*.

MATERIALS AND METHODS

Male and female Sprague–Dawley strain and female Germ-Free (Axenic) Sprague–Dawley rats of various weights, fed or fasted, were injected i.p. with saline, sodium fusidate, deacetylated cephalosporin P_1, cephalosporin P_1, cephalothin P_1, or one of the six fusidic acid analogues and a radioactive amino acid at arbitrarily determined concentrations and times as noted in the table, figure or results.

Bilateral orchidectomy, ovariectomy, adrenalectomy or thyroidectomy and hypophysectomy were performed under ether anesthesia. After thyroidectomy, rats that gained little or no weight during a 30-day period were used. Hypophysectomy was at least 1 month prior to experimental use and all other surgically altered rats were used 1 week later. Adrenalectomized rats were given access to 1% (w/v) NaCl solution rather than water. Hypophysectomized rats were fed ground Purina Lab Chow containing 30% sucrose (w/w) moistened with evaporated milk. Sodium fusidate, 16-epideacetylfusidic acid-potassium salt (WG-551 K), tetrahydrofusidic acid-sodium salt (WG-553 Na), 24,25-dihydrofusidic acid-sodium salt (WG-559 Na), 3-*O*-acetyl-24,25-dihydrofusidic acid-sodium salt (WG-593 Na), 3-*O*-acetyl-16-epifusidic acid-sodium salt (WG-598 Na) and 3-*O*-acetyl-16-epi-24,25-dihydro-fusidic acid-sodium salt (VD-1163 Na) were a gift of Dr. W. O. Godtfredsen and Dr. W. von Daehne, Leo Pharmaceutical Products, Ballerup,

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