Pharmacology Biochemistry & Behavior, Vol. 26, pp. 281–286. Copyright © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0091-3057/87 $3.00 + .00

Quantitative Assessment of Tolerance Development to Diisopropylfluorophosphate

DONG-KOO LIM, JOHN C. R. FERNANDO, BETH HOSKINS AND I. K. HO[1]

Department of Pharmacology and Toxicology, University of Mississippi Medical Center Jackson, MS 39216-4505

Received 16 July 1986

LIM, D.-K., J. C. R. FERNANDO, B. HOSKINS AND I. K. HO. *Quantitative assessment of tolerance development to diisopropylfluorophosphate.* PHARMACOL BIOCHEM BEHAV **26**(2) 281–286, 1987.—Rats were treated with diisopropylfluorophosphate (DFP) acutely or daily for 14 days. The quantitative assessment of tolerance development after a challenge dose of DFP, 2 mg/kg, was studied. The subacutely-treated rats developed tolerance to DFP-induced tremors. However, the severity of tremors in DFP-tolerant animals was not significantly different from that of the controls after the challenge dose of DFP was administered. Hind-limb abduction was significantly lower in the subacutely-treated group than in the acutely-treated group. The recovery of body weights in subacutely-treated rats (3.5%/day) was significantly higher than that in acutely-treated rats (2.0%/day). The consummatory behaviors (food and water consumption) recovered faster in subacutely-treated rats than in the acutely-treated group. Body temperatures were decreased to the same extent in both groups, but the subacutely-treated group recovered faster. The total mortality was significantly lower in subacutely-treated rats (10%) than in acutely-treated rats (35%). The results further substantiate the finding that tolerance develops to various DFP-induced signs of toxicity.

DFP Tolerance Behaviors Temperature Mortality

THE acute toxic effects of organophosphates are due to inhibition of the enzyme acetylcholinesterase (AChE) with a resultant accumulation of acetylcholine at neuroeffector sites. However, repeated administration of an organophosphate to animals leads to the development of tolerance to its toxicity, as first shown by Rider *et al.* [17], using octamethyl pyrophosphoramide.

Subsequently, it has been reported that animals which developed tolerance to organophosphates exhibited subsensitivity to cholinergic agonists [1, 3, 5, 13, 21] and supersensitivity to cholinergic antagonists [13,15]. The parameters used for assessing tolerance development to organophosphates included eating and drinking behavior [11, 14, 18–20], thermoregulation [5,16], learning [4,19], and motor function, such as tremor and hind-limb abduction [8].

We previously reported that after rats were exposed subacutely to DFP, the rate of tolerance development to this compound depended upon the parameter used. Our animals failed to show any sign of tolerance development to some symptoms of toxicity, e.g., chewing-movements, even after 28 days of continuous administration of DFP [8]. Furthermore, although the phenomenon of tolerance development to organophosphates is well-established, the quantitative assessment of this tolerance development has not been extensively studied. Therefore, the present study was undertaken to systematically quantify the degree of tolerance development to DFP by assessing various symptoms induced by this compound.

METHOD

Animals and Chemicals

Male, Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) with initial weights of 175–200 g were used. Animals were maintained ad lib on standard laboratory chow and tap water and were housed in a room with automatic 12-hour light and dark cycles and temperature set at 25.5±1.0°C. They were housed two to a cage for one week prior to beginning the experiments. Diisopropylfluorophosphate (DFP), Sigma Co. No. 84F-0248 was used throughout all studies. This preparation of DFP was found to have an IC_{50} value *in vitro* of 7 μg DFP/ml (concentration required to inhibit 50% of the cholinesterase activity in whole rat brain homogenates).

Administration of DFP

Freshly prepared solutions of DFP in saline were administered subcutaneously (SC) in volumes of 0.1 ml/100 g body

[1]Requests for reprints should be addressed to Dr. I. K. Ho.

weight, daily between 9:00 to 11:00 a.m., for 13 days. In accordance with our previous results [11,24], the dosage schedule for DFP-tolerant animals (subacute) for 13 days was as follows: 1st through 3rd day, 1 mg/kg; 4th through 6th day, 0.5 mg/kg; 7th through 13th day, 1 mg/kg. The non-tolerant (acutely-treated) animals received saline vehicle for 13 days. Both groups of animals received a challenge dose of DFP, 2 mg/kg, SC, 24 hours after the last injection. A group of non-DFP treated (control) animals was also included. These rats received daily injections of saline for 14 days.

Behavioral Analyses

Rats were individually isolated in observation cages. These were transparent plastic rat cages each having a stainless steel grid resting off its floor. Each cage was divided into four compartments with three fitted opaque plastic sheets and was covered with a transparent plastic lid with several ventilation holes. A thin layer of bedding, but neither food nor water, was available; the grid prevented the animals from biting the bedding material or feces.

During the period of subacute administration, the symptoms of tremors and hind-limb abductions were assessed before and 1, 2 and 6 hr after the DFP injections on day 1, 3, 9, and 13. These behaviors were also assessed in both subacutely- and acutely-treated groups at 0, 1, 2, 4, 6 and 24 hr after the challenge dose of DFP, 2 mg/kg, SC. Two behaviors, tremors and hind-limb abduction, were separately scored on each rat, for a duration of 5 min, repeated at 15 min intervals, starting at 15 min after treatment. The scoring scale utilized for hind-limb abduction was: 0=none, 1=slight, 2=moderate and 3=severe. Tremors were scored as 0=none; 1=distinct slow tremor of head; 2=fast tremor (high frequency vibrations) of head, trunk or limbs; 3=intense fast tremor. This scale was comparable to that described previously [25] for oxotremorine-induced tremors. The scores for the intervals were summed and the maximum possible score for a 1 hr period for each behavior was 12 per rat. In general, eight animals per treatment group were used.

Monitoring of Body Weight, Food and Water Consumption

Two hundred grams of food and 250 ml of tap water were available for each cage every day. The body weight of each rat and food as well as water consumption per cage were recorded daily. The daily percent change in body weight of each animal was calculated. The mean body weight change (±S.E.M.) was obtained for all rats in a given group. Daily food and water consumption per 100 g of body weight were determined.

Hypothermia

As an index of body temperature, rectal temperature was measured by a thermistor mounted in a rectal probe connected to a Tele-thermometer (Bailey Instrument Co., Saddle Brook, NJ). The flexible thermistor probe was inserted 60 mm into the rectum. Measurements were taken on each rat prior to each injection of DFP; these were considered to be the initial body temperatures. The temperatures were measured 1, 2, 4, 6 and 24 hr following the last (challenge) DFP injection. The difference at each time point from the initial temperature was recorded for each rat. The mean change in rectal temperature for each group was then calculated.

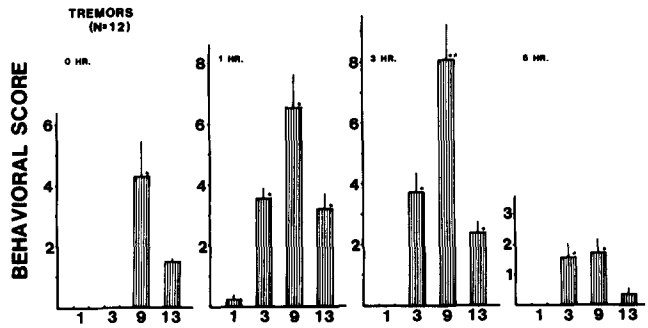

FIG. 1. Tremors occurring during subacute treatment with DFP. Values are means±S.E. of individual rat scores. Rats were monitored before and 1, 3, and 6 hr after administrations of DFP. Values at 0 hr were compared with the control values. Other values were compared with corresponding pre-injection values (0 hr) by using Mann-Whitney U-test and ANOVA. *$p<0.05$ and **$p<0.01$.

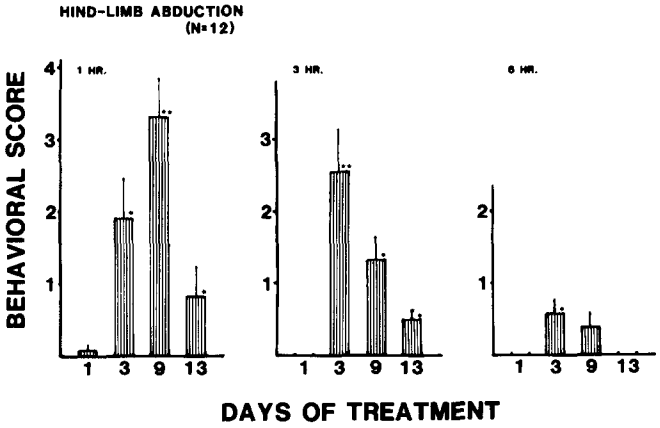

FIG. 2. Hind-limb abductions induced during subacute treatment with DFP. Values are means±S.E. of individual rat scores. Rats were monitored before and 1, 3, and 6 hr after administrations of DFP. Values were compared with corresponding pre-injection values (0 hr) by using analysis of variance. *$p<0.05$ and **$p<0.01$.

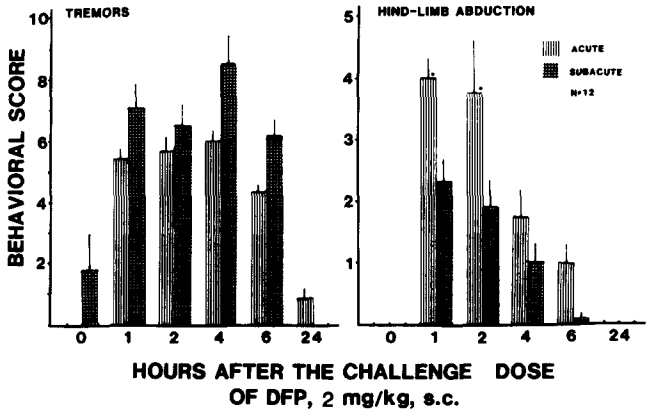

FIG. 3. Effect of the challenge dose of DFP, 2 mg/kg, on the scores of tremors and hind-limb abductions in acutely- and subacutely-treated rats. Mann-Whitney U-test was used for analysis of differences. *$p<0.05$ and **$p<0.01$ between the two treatment groups.

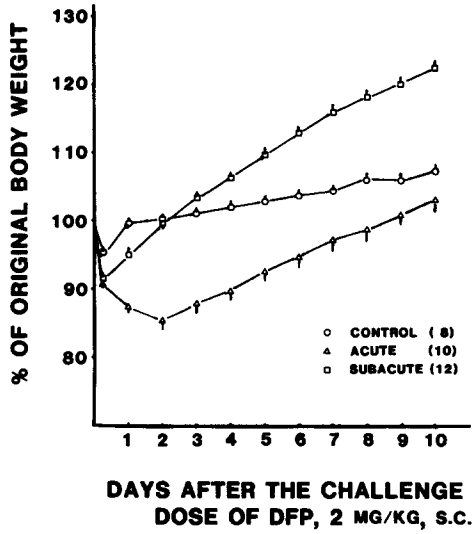

FIG. 4. Percent of original body weight (growth rates) assessed for 10 days after a challenge dose of DFP, 2 mg/kg, in acutely- and subacutely-treated rats. Numbers in parentheses indicate number of rats studied in each treatment group.

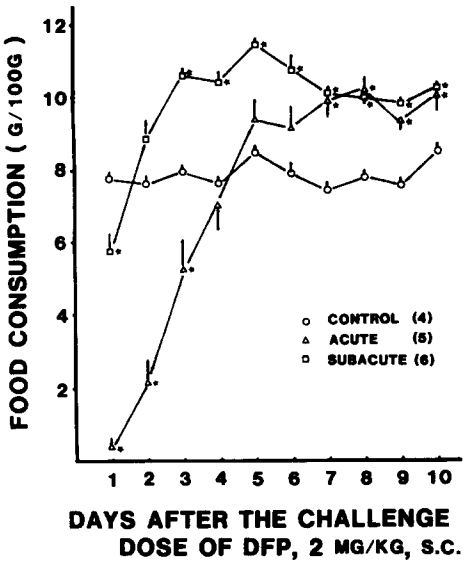

FIG. 5. Food consumption after the challenge dose of DFP, 2 mg/kg, in acutely- and subacutely-treated rats. Numbers in parentheses indicate the original number of rat cages in each treatment group. Stars denote significant ($p<0.05$) difference from the saline-treated control group.

Mortality

The mortality of each group was recorded. The cumulative mortality of 11 separate experiments was tabulated.

Statistical Analysis

Significance of differences in behavioral experiments were analyzed by the Mann-Whitney U-test and the one-way analysis of variance. Growth rate was analyzed by regression analysis. Water and food consumption and temperature data were analyzed by Student's t-test and the χ^2-test was used for assessing mortality.

RESULTS

Tolerance Development to DFP in Terms of Tremors and Hind-Limb Abduction

Neither tremors nor hind-limb abduction were observed in the control group of rats during daily administration of saline, but groups treated acutely with DFP exhibited increases in tremors and hind-limb abduction.

After the first administration of DFP (Figs. 1, 2), both behaviors were present only to a slight extent. Following the ninth injections, these behaviors were maximal. After the thirteenth administration, scores for both behaviors were still significantly higher than those following the first administration (1 mg/kg), but were significantly lower than those following the ninth administration. These results confirmed our previous findings [8] that animals developed tolerance to DFP-induced tremors during subacute administration. Tremors in both acutely- and subacutely-treated rats were apparent and persisted for 6 hr after the challenge dose (2 mg/kg) of DFP; however there was no difference between the two groups (Fig. 3). On the other hand, the incidence of hind-limb abduction in DFP-tolerant animals after the challenge dose of DFP was significantly less than that exhibited by the acutely-treated rats. The duration of hind-limb abduction in tolerant rats was much shorter than that in the non-tolerant animals (Fig. 3).

Tolerance Development to DFP in Terms of Recovery of Body Weight and Consummatory Behaviors

The development of tolerance to DFP in subacutely-treated groups was clearly demonstrated as evidenced by the recovery of body weights after the challenge dose of DFP. As shown in Fig. 4, rats which received a single administration of DFP, 2 mg/kg, lost weight dramatically within the first 48-hour period, as compared with the rats which had developed tolerance to DFP. In the DFP-tolerant animals, the body weight gain during the second day after the challenge dose of DFP was significantly higher than that of the non-tolerant group. On the other hand, the body weight gain of the acutely-treated group did not begin to recover until the third day after the challenge dose of DFP. The percent of body weight gain in the acutely-treated rats did not reach the control level even 10 days after the challenge dose of DFP. The recovery rate of body weights in DFP-tolerant groups was significantly higher than that of the acutely-treated group; being 3.5 and 2%/day in DFP-tolerant and non-tolerant groups, respectively.

The challenge dose of DFP had significantly less effects on food and water consumption by the animals tolerant to DFP than that by non-tolerant animals. As shown in Fig. 5, tolerant rats receiving the challenge dose of DFP consumed significantly less food within 24 hr than did the control rats. Within 3 days after the challenge dose of DFP, the DFP-tolerant animals consumed significantly more food than did control groups. In contrast, when non-tolerant rats were administered the same dose of DFP, the amount of food consumption was markedly reduced when compared with

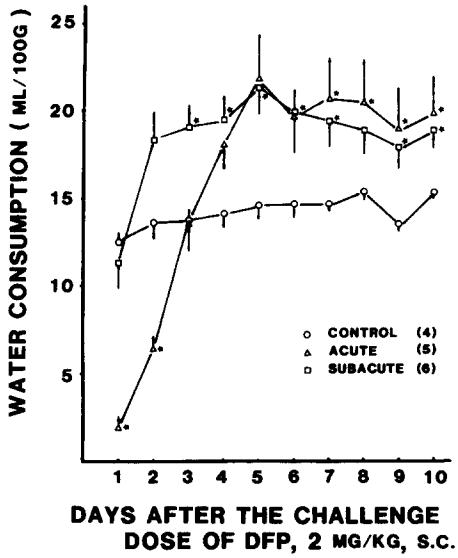

FIG. 6. Water consumption after the challenge dose of DFP, 2 mg/kg, in acutely- and subacutely-treated rats. Numbers in parentheses indicate the original number of rat cages in each treatment group. Stars denote significant ($p<0.05$) difference from the saline-treated control group.

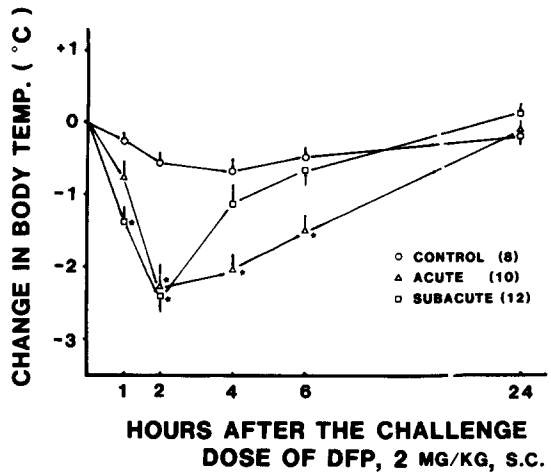

FIG. 7. Changes in body temperature after a challenge dose of DFP, 2 mg/kg, in acutely- and subacutely-treated rats. Numbers in parentheses indicate the number of rats in each treatment group. Stars denote significant ($p<0.05$) difference from the saline-treated control group.

DFP-tolerant and control animals. The acutely-treated group of rats took at least 4 days to return to their normal food consummatory behaviors.

As shown in Fig. 6, water consumption was significantly reduced in acutely-treated animals. On the other hand, the challenge dose of DFP did not change water consumption by DFP-tolerant animals. Interestingly, water consumption in DFP-tolerant animals was significantly higher 2 days after the challenge dose of DFP and the increase lasted for more than 10 days. Although the increase in water consumption was also noticed in acutely-treated group, this phenomenon was not observed until 5 days after the animals received the challenge dose of DFP.

Tolerance Development to DFP in Terms of Body Temperature

The development of tolerance during subacute administration of DFP was also evidenced by the hypothermia produced by the challenge dose of DFP. As shown in Fig. 7, subacute DFP administration shortened the duration of DFP-induced hypothermia. Non-tolerant rats demonstrated a significant reduction in the rectal temperatures 2 hours after the administration of the challenge dose of DFP. Although the DFP-tolerant animals showed the same degree of hypothermia after the same challenge, the body temperatures of this group returned to normal levels within 4 hours. On the other hand, it took more than 6 hours for temperatures of the non-tolerant animals to return to normal.

Tolerance Development to DFP in Terms of Lethality

Subacute administration of DFP reduced DFP-induced lethality. Table 1 summarizes the results of 11 separate experiments on DFP-induced lethality in tolerant and non-tolerant rats. Rats which received 13 daily injections of DFP exhibited a decrease in mortality after the challenge dose of DFP compared to the mortality of the non-tolerant rats. The total mortalities for acutely- and subacutely-treated rats were 35 and 10%, respectively. Therefore, DFP was more than three times as lethal in naive rats as it was in those which had been subacutely-treated with this agent.

DISCUSSION

The dosing schedule which we have used to induce tolerance to DFP is somewhat different from that used in our previous studies [8, 11, 24]. It has been previously reported that a dosing schedule of DFP, 1 mg/kg, followed at 3-day intervals with doses of 0.5 mg/kg resulted in tolerance to DFP in experimental animals [14, 18, 19]. In our previous experiments, a daily dosing schedule of 2 mg/kg of DFP for two weeks caused tolerance development in rats. In our later studies [8,24] a daily dose of 1 mg/kg of DFP was used to induce about the same degree of tolerance development to DFP as was previously obtained using 2 mg/kg of DFP. In the present study, it was necessary to reduce the dose of DFP to 0.5 mg/kg between 4th and 6th day of administration. The adjustment of the dose of DFP is often necessary since the potency of DFP obtained from different sources or at different times from the same source will not necessarily be the same [9].

The present study further quantifies the degree of tolerance development to DFP by challenging the animals with an additional dose of DFP. Tolerance development to DFP was evidenced by the attenuation of DFP-induced toxicities such as neurological symptoms, body weight loss, decrease in food and water consumption, hypothermia and lethality. In our previous studies [11], we demonstrated that the tolerance developed to DFP in terms of growth rates and consummatory behaviors during the course of daily DFP

TABLE 1

THE COMPARISON OF THE MORTALITY BETWEEN ACUTELY AND SUBACUTELY TREATED GROUP AFTER THE CHALLENGE DOSE OF DFP, 2 mg/kg

No.	Acutely	Subacutely
1	2/10	1/10
2	2/8	1/4
3	6/13	0/4
4	4/8	1/10
5	4/16	0/7
6	4/15	0/8
7	4/7	1/4
8	7/26	1/11
9	2/5	0/7
10	9/15	4/10
11	3/13	1/13
Total	47/136 (35%)	10/98 (10%)

administration. Furthermore, continued exposure after the tolerance had developed resulted in supra baseline consummatory behaviors while growth rates remained the same as the controls. Additionally, we demonstrated that subacute treatment with DFP for 28 days induced tolerance in terms of tremors and hind-limb abduction [8]. Although the animals treated subacutely with DFP failed to show attenuated tremors after receiving a challenge dose of DFP in the present experiments, the development of tolerance to tremors was evident (Fig. 1) during the course of daily administration of DFP as previously reported [8]. Therefore, we have further assessed tolerance development to DFP by comparing various toxicologic symptoms resulting from administration of a challenge dose of DFP to both DFP-tolerant and non-tolerant animals. These studies further substantiate the phenomena of tolerance development to DFP.

Our results also showed that mortality after a challenge dose of DFP was significantly less in the DFP-tolerant rats than in non-tolerant animals. This is especially interesting because the tolerance development to DFP has not been demonstrated in terms of decreased lethality. Our results show that there was a 10% mortality in animals which had developed tolerance to DFP whereas 35% of naive animals receiving only the challenge dose of DFP died. During the course of our study, we also found that a 95% mortality was obtained when animals received daily administration of DFP for only 7 days prior to the challenge dose. It thus appears that DFP-treated animals are more sensitive to DFP prior to their development of tolerance.

Our results reveal that the effect of a challenge dose of DFP on body weight as well as on consummatory behaviors was much less in tolerant than in non-tolerant rats. Furthermore, the recovery of those parameters in tolerant animals was faster than such recovery in the acutely-treated animals. It has been reported that the decrease of water intake, induced by a challenge dose of scopolamine, was much less severe in DFP-tolerant rats than in controls [2]. It has also been reported that subacute treatment with organophosphates induced subsensitivity to cholinergic agonists [1, 3, 13, 21] and suprasensitivity to cholinergic antagonists [13,15]. The hypothermic effect of organophosphates was also reported to be less after subacute treatment with organophosphates [5,16]. In our studies, although the maximum decrease of body temperature between DFP-tolerant and non-tolerant groups was the same, the recovery from the maximum decrease of body temperature by tolerant rats was much faster than that by acutely-treated rats. The observed dissimilarity in the DFP-induced hypothermia as reported by other investigators [5,16] might be due to the differences in dosing schedules and potencies of DFP used. Furthermore, in the present study a much higher challenge dose of DFP was used for assessing hypothermia in both DFP-tolerant and non-tolerant animals. Clearly, rats treated subacutely with DFP do develop tolerance to its hypothermic effects.

Many studies have provided evidence that tolerance to organophosphates is due primarily to a subsensitivity to ACh via reduction in the number of muscarinic [3–7, 10, 24, 26, 27] and nicotinic receptors [6, 22, 23]. Although tolerance development to organophosphates may be due primarily to down-regulation or subsensitivity of cholinergic receptors, down-regulation of cholinergic receptors does not explain some of the phenomena observed in subacutely-treated animals. For example, our binding experiments revealed that the maximum decrease of muscarinic receptors occurred after 7 days of administration of DFP [12] using the same dosing schedule as in the present experiments. However, there was no evidence of tolerance development after 7 days. It therefore appears that down-regulation of cholinergic receptors precedes the production of tolerance as assessed by toxicological parameters. We suggest that other biochemical events may also be involved in the development of tolerance during the course of DFP administration. Work is in progress to study the involvement of noncholinergic mechanisms in organophosphate tolerance.

ACKNOWLEDGEMENT

This work was supported by a contract, DAMD17-85-C-5036, from U.S. Army Medical Research and Developmental Command.

REFERENCES

1. Brodeur, J. and K. P. Dubois. Studies on the mechanisms of acquired tolerance by rats O,O-diethyl-s-2-(ethyl thio)ethyl phosphorodithioate (Di-syston). *Arch Int Pharmacodyn* **149:** 560–570, 1964.
2. Chippendale, T. T., G. A. Zawolkow, R. W. Russell and D. H. Overstreet. Tolerance to low acetylcholinesterase: modification of behavior without acute behavioral change. *Psychopharmacologia* **26:** 127–139, 1972.
3. Costa, L. G., B. W. Schwab, H. Hand and S. D. Murphy. Reduced ^{3}H-quinuclidinyl benzylate binding to muscarinic receptors in disulfoton-tolerant mice. *Toxicol Appl Pharmacol* **60:** 441–450, 1981.
4. Costa, L. G. and S. D. Murphy. Passive avoidance retention in mice tolerant to the organophosphorus insecticide disulfoton. *Toxicol Appl Pharmacol* **65:** 451–458, 1982.

5. Costa, L. G., B. W. Schwab and S. D. Murphy. Differential alterations of cholinergic muscarinic receptors during chronic and acute tolerance to organophosphorus insecticides. *Biochem Pharmacol* **31:** 3407–3413, 1982.
6. Costa, L. G. and S. D. Murphy. ^{3}H-Nicotine binding in rat brain: Alteration after chronic acetylcholinesterase inhibition. *J Pharmacol Exp Ther* **226:** 392–397, 1983.
7. Ehlert, F. J. and N. Kokka. Decrease in ^{3}H-quinuclidinyl benzilate binding to muscarinic cholinergic receptor in the longitudinal muscle of the rat ileum following chronic administration of diisopropyl fluorophosphate. *Proc West Pharmacol Soc* **20:** 1–7, 1977.
8. Fernando, J. C. R., B. Hoskins and I. K. Ho. Effect on striatal dopamine metabolism and differential motor behavioral tolerance following chronic cholinesterase inhibition with diisopropylfluorophosphate. *Pharmacol Biochem Behav* **20:** 951–957, 1984.
9. Ho, I. K. and B. Hoskins. Variation of commercial diisopropylfluorophosphate preparations in toxicological studies. *Drug Chem Toxicol* **6:** 421–427, 1983.
10. Levy, A. The effect of cholinesterase inhibition on the ontogenesis of central muscarinic receptors. *Life Sci* **29:** 1065–1070, 1981.
11. Lim, D. K., B. Hoskins and I. K. Ho. Assessment of diisopropylfluorophosphate (DFP) toxicity and tolerance in rats. *Res Commun Chem Pathol Pharmacol* **39:** 399–418, 1983.
12. Lim, D. K., B. Hoskins and I. K. Ho. Evidence for the involvement of presynaptic cholinergic functions in tolerance to DFP. Submitted for publication, 1987.
13. McPhillips, J. J. Altered sensitivity to drug following repeated injections of a cholinesterase inhibitor to rats. *Toxicol Appl Pharmacol* **14:** 67–73, 1969.
14. Overstreet, D. H. The effects of pilocarpine on the drinking behavior of rats following acute and chronic treatment with diisopropylfluorophosphate and during withdrawal. *Behav Biol* **9:** 257–263, 1973.
15. Overstreet, D. H. Reduced behavioral effects of pilocarpine during chronic treatment with DFP. *Behav Biol* **11:** 49–58, 1973.
16. Overstreet, D. H., M. D. Kozar and G. S. Lynch. Reduced hypothermic effects of cholinomimetic agents following chronic anticholinesterase treatment. *Neuropharmacology* **12:** 1017–1032, 1973.
17. Rider, J. A., L. Z. Ellonwood and J. M. Coon. Production of tolerance in the rat to octamethyl pyrophosphoramide (OMPA). *Proc Soc Exp Biol Med* **81:** 455–459, 1952.
18. Russell, R. W., B. J. Vasquez, D. H. Overstreet and F. W. Dalglish. Effects of cholimolytic agents on behavior following development of tolerance to low cholinesterase activity. *Psychopharmacologia* **20:** 32–41, 1971.
19. Russell, R. W., B. J. Vasquez, D. H. Overstreet and F. W. Dalglish. Consummatory behavior during tolerance to and withdrawal from chronic depression of cholinesterase activity. *Physiol Behav* **7:** 523–528, 1971.
20. Russell, R. W., D. H. Overstreet, C. W. Cotman, V. G. Carson, L. Churchill, F. W. Dalglish and B. J. Vasquez. Experimental tests of hypotheses about neurochemical mechanisms underlying behavioral tolerance to the anticholinesterase diisopropyl fluorophosphate. *J Pharmacol Exp Ther* **192:** 73–85, 1975.
21. Schwab, B. W. and S. D. Murphy. Induction of anticholinesterase tolerance in rats with doses of disulfoton that produce no cholinergic signs. *J Toxicol Environ Health* **8:** 199–204, 1981.
22. Schwartz, R. D. and K. J. Kellar. Nicotinic cholinergic receptors binding sites in the brain: regulation in vivo. *Science* **220:** 214–216, 1983.
23. Schwartz, R. D. and K. J. Kellar. In vivo regulation of [^{3}H] acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J Neurochem* **45:** 427–433, 1985.
24. Sivam, S. P., J. C. Norris, D. K. Lim, B. Hoskins and I. K. Ho. Effects of acute and chronic cholinesterase inhibition with diisopropylfluorophosphate on muscarinic, dopamine and GABA receptors of the rat striatum. *J Neurochem* **40:** 1414–1422, 1983.
25. Weinstock, M., A. P. Zavadil and I. J. Kopin. Peripheral catecholamine mediate certain responses to central cholinergic receptor stimulation by oxotremorine. In: *Monographs in Neural Sciences*, edited by M. M. Cohen. Basel: Karger, 1980, pp. 138–145.
26. Yamada, S., M. Isogai, H. Okudaira and E. Hayashi. Regional adaptation of muscarinic receptors and choline uptake in brain following repeated administration of diisopropylfluorophosphate and atropine. *Brain Res* **268:** 315–320, 1983.
27. Yamada, S., M. Isogai, H. Okudaira and E. Hayashi. Correlation between cholinesterase inhibition and reduction in muscarinic receptors and choline uptake by repeated diisopropylfluorophosphate administration: antagonism by physostigmine and atropine. *J Pharmacol Exp Ther* **226:** 519–525, 1983.